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Exploring the Untapped Possibilities of Wastewater Bacteria in Microbial Electrochemical Technology

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ABSTRACT
Energy is essential to modern civilization, yet using conventional energy sources like coal and petroleum puts the environment at danger. As a result, the need for sustainable energy alternatives is urgent. This research primarily investigates the potential of microbial fuel cells (MFCs) as an alternative energy source, with a specific focus on their ability to generate power using wastewater from the treatment plant at Ahmadu Bello University in Zaria, Nigeria. The study screens a total of 50 isolates against a 200 mV benchmark in order to evaluate the bacteria's ability to produce energy from wastewater. In the experiment, Bacillus cereus turned out to be the top producer. Significantly, isolates with the labels 2, 3, A, and C showed encouraging outcomes; they reached peak voltages of 0.879, 0.841, 0.840, and 0.827 volts, in that order.
KEYWORDS*: Electrogenic potential, Microbial Electrochemical Technologies, Microbial Fuel Cell, Proton Exchange Membrane and Wastewater*

INTRODUCTION:

In today's world of sustainable energy solutions, alternative technologies are becoming more and more significant, and Microbial Fuel Cells (MFCs) are turning into indispensable instruments in this revolutionary attempt. In contrast to conventional fuel cells, MFCs possess the exceptional ability to convert chemical energy directly into electrical power by means of the catalytic properties of microorganisms. Because of this feature, a large range of organic resources, including proteins, fatty acids, and carbohydrates, can be utilised as sustainable fuels, giving MFCs a distinct advantage. MFCs' adaptability allows them to be utilised as versatile and eco-friendly energy converters, greatly aiding global attempts to achieve sustainable energy(Roy *et al*., 2023).

Because the electrochemical inert nature of microbial cell surfaces is inherently linked to the successful electron transfer in MFCs, mediator intervention is necessary. This, together with the large variety of appropriate organic materials, sets MFCs apart from more traditional cells such as methanol and hydrogen fuel cells. MFCs can be built utilizing diverse materials and configurations and are subject to a range of operating circumstances, such as temperature, pH, and reactor size. Recent findings show how specific iron-reducing bacteria may be able to assist mixed-culture MFCs in reaching higher power, illuminating the unexplored potential in this field of microbial electrochemical technology(Santoro *et al*., 2020).

METHODOLOGY

Research Location and Sample Acquisition:

The research's subject was the Ahmadu Bello University wastewater treatment plant in Zaria, Nigeria. Wastewater samples were collected from the facility and transported to the laboratory in sterile containers. So that the samples' organic content wouldn't be destroyed by bacteria, they were stored at 4°C until further analysis. The organic component of wastewater serves as the target substrate for microbial oxidation in the Microbial Fuel Cell (MFC) setup.

Media Preparation

Various media were employed in this research:

Luria-Bertani (LB) Agar Plate: Ten grammes of tryptone, ten grammes of NaCl, twenty grammes of agar, and five grammes of yeast extract were diluted in one thousand millilitre of distilled water to make LB agar plates. The medium's pH was adjusted to neutral using 5N NaOH, and then it was autoclaved and put into sterile Petri dishes (Adebule Ap et al., 2018).

Luria-Bertani (LB) Broth: LB broth was made in the same way as LB agar plates, but without the agar component. Test tubes were then filled with the broth before it was autoclaved to ensure sterilisation.

Biochemicals Media: A range of biochemical media, including H_2S, Indole, Methyl Red, Voges-Proskauer, Citrate starch Fermentation, and Amylase test media, were prepared in compliance with the manufacturer's instructions and autoclaved for sterilisation.

Isolation of Bacteria: Wastewater samples were serially diluted, and from each dilution, 0.1 mL was spread on LB agar plates and incubated at 37˚C for 24 hours. Morphologically distinct colonies were purified, and Gram staining and other properties were determined (Naureen *et al*., 2015).

Screening of bacteria for electricity-producing potentials: A system for cultivating single strains in MFCs and analyzing their electrochemical activity was adapted from the tube MFC method described by Clauwaert *et al*, (2007). In this system, tube of equal volume (10 mL), length and diameter were sterilized with the use of ethanol (70%) by submersion on three occasions for an hour each before it was assembled. The tube was seal off at the bottom with 2mL 2% agar-agar and 1% sodium chloride make the salt bridge. Sterilized graphite rods connected each to copper wire was inserted inside each tube as the anode electrode.7mL of LB broth was added to each tube and each colony of a pure culture of its cell suspension were injected into each tube to screen for their electricity producing potentials. The top of the tube was insulated to prevent air from getting in, while the bottom end where the salt bridge was positioned was submerged into a solution of a hexacyanoferrate catholyte (100 mM phosphate-buffered 50 mM potassium hexacyanoferrate solution) and a graphite-plate electrode as the cathode. This operation spanned a duration of 5 days to ascertain the electricity production potential of the isolated colony. The electrical voltage produced by each tube MFC was used as an indicator for assessing electrochemical activity and was consistently measured using a multi-meter. Throughout this research endeavor, unless explicitly specified, the bacterial isolate demonstrating notable electricity-producing capabilities was earmarked for subsequent identification processes.

Plate I: Tube microbial fuel cell (TMFC)

Identification: Isolates were characterized based on morphological, microscopic, and biochemical features and cross-matched with existing profiles in laboratory manuals and literature(Clauwaert, Van Der Ha, *et al*., 2007).

Confirmatory Identification of the isolates by 16s rRNA-PCR assay

The PCR conditions followed the protocol described by Ben-Dov *et al*, (1997) with the modification of using bacterial cell lysate as the template DNA. (Khojand S. et al., 2013). A loopful of the short-term culture of electrogenic microorganisms grown on an LB agar plate was transferred to a tube containing 100 µL of water, heated at 100ºC for 5 minutes, and then cooled. The resulting cell lysate was briefly centrifuged for 10 seconds at 10,000 rpm. Subsequently, 5 µL of the supernatant, containing the genomic DNA, was used for PCR. The PCR reaction was performed in a 20 µL mixture comprising 5 µL template DNA, 150 mM dNTPs, 20 pM of each of the four primers (Table 1), and 0.5 U of Taq DNA polymerase. The amplification protocol in a DNA thermocycler included an initial denaturation at 94ºC for 4 minutes, followed by 35 cycles of denaturation at 94ºC for 45 seconds, annealing at 48-55ºC for 45 seconds, and extension at 72ºC for 1 minute. The reaction concluded with a final extension at 72ºC for 4 minutes. The 16S rRNA gene banding patterns were visualized via agarose gel electrophoresis. A 15 µL aliquot of each amplification product was loaded onto a 1.2% agarose gel and run in TAE buffer (40 mM Tris-acetate, 1 mM EDTA) at 100 volts for 60 minutes. The gels were stained with ethidium bromide and documented using a 100 bp molecular weight marker.

The obtained 16s rDNA amplicons were sent for sequencing analysis. The bioinformatics analysis was performed by BLAST software (Katara et al., 2016)

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Table 1 Primer sets to be used in the amplification of 16s rDNA

16s rRNA	Primer Sequence (5'-3')	Size (bp)	Reference
27F	5'-AGAGTTTGATCCTGGCTCAG-3'	1522	(Abellan-Schneyder et al., 2021)
1492R	5'-GGTTACCTTGTTACGACTT-3'	1522	(Abellan-Schneyder et al., 2021)

Key: **F** = forward primer, **R** = reverse primer

RESULT

Isolation, screening and identification of bacteria with electricity-producing potentials.

Isolation of bacteria from wastewater

The electricity generating bacteria were isolated from wastewater sample from the wastewater treatment facility of Ahmadu Bello University Zaria Nigeria, after 24 hours incubation at 37°c on Luria-Bertani agar plate and morphologically distinct colonies were counted as shown on Table 1.

Screening of electricity producing potential of isolates

In accordance with the methodology outlined by Clauwaert *et al*. (2007), the tube microbial fuel cell (MFC) technique was employed to assess the electricity-generating capabilities of 50 bacterial isolates. Among these, 14 isolates demonstrated noteworthy potential for electricity production. Table 2 showcases the individual performance of these 14 isolates in terms of their electricity production. Notably, isolates designated as 2, 3, A, and C exhibited the most promising results, attaining peak voltages of 0.879, 0.841, 0.840, and 0.827 volts, respectively.

Identification and characterization of bacteria with electricity-producing potentials

Both morphological and biochemical characteristics were used to characterize the bacterial isolates. Interestingly, the isolates had smooth edges and a white cream color with a smooth surface. Microscopic analysis of the resulting characterisation using Gram staining revealed rod-shaped gram-positive bacteria. Biochemical examinations showed that the MR, VP, catalase, oxidase, urease, and citrate tests all showed positive results, however the indole test showed negative results. Moreover, Table 3 illustrates the results of the triple sugar iron (TSI) test, which showed an acidic/acidic (A/A) pattern without gas production and infrequently without hydrogen sulfide (H_2S) creation. Bacillus species were suggested to be the bacterial isolates.

The chosen bacterial isolates were also subjected to molecular identification. After extracting genomic DNA and observing it under UV light, a portion of the 16S rDNA gene was amplified using 27F and 1492R primers. As shown in Plate II, a single discrete PCR amplicon band of about 1500 bp was found by subsequent electrophoresis on an Agarose gel. Using this molecular method, the isolate was further determined to be the B. cereus strain. Using BLAST against the NCBI GenBank database, the 16S rDNA gene was sequenced for each of the four isolates, with the top two sequences chosen based on maximum identity score. These sequences were aligned using the Clustal W multiple alignment program, which produced a distance matrix that was then used with MEGA11 to create phylogenetic trees, as illustrated in Figure 1.

Table 1: The colony forming unit of isolate of bacteria from wastewater

Replica	Bacteria colony (cfu/ml)
1st	$4.0 * 10^{-6}$
2nd	$7.0 * 10^{-6}$
3rd	$6.0 * 10^{-6}$
Average	$5.67 * 10^{-6}$

Key: **CFU/ml** = Coliform forming unit per milliliters

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Table 2: Open circuit voltage (OCV) measurement during the screening in millivolts.

Isolate codes	Voltage(mV) 24hrs	Voltage(mV) 48hrs	Voltage(mV) 72hrs	Voltage(mV) 96hrs	Voltage(mV) 120hrs
1	0.166	0.400	0.586	0.440	0.459
2	0.225	0.333	**0.840**	**0.814**	**0.879**
3	0.190	0.351	**0.823**	**0.823**	**0.841**
4	0.200	0.293	0.653	0.523	0.200
5	0.150	0.296	0.527	0.570	0.409
6	0.175	0.312	0.764	0.523	0.436
7	0.250	0.295	0.304	0.428	0.243
8	0.200	0.293	0.653	0.523	0.200
9	0.144	0.275	0.653	0.523	0.632
10	0.250	0.295	0.304	0.428	0.243
A	0.193	0.327	**0.840**	**0.823**	**0.749**
B	0.156	0.430	0.506	0.433	0.307
C	0.230	0.315	**0.827**	**0.796**	**0.708**
D	0.240	0.285	0.304	0.400	0.343

Key: mV = millivolt

Table 3: Cultural, Microscopic and Biochemical Characteristics of Selected Electrogenic Bacterial Isolates.

Isolates code	Growth	H_2S	Indole	Motility	Glucose	Lactose	Sucrose	Citrate	Starch hydrolysis	Amylase	MR	VP	urease	Gram reaction	Gas	Inference
2	WC	_	_	_	+	_	_	_	+	+	+	+	+	+rods	_	*Bacillus sp*
3	WC	+	_	+	+	_	_	+	+	+	+	+	+	+rods	_	*Bacillus sp*
A	WC	+	_	+	+	_	_	+	+	+	+	+	+	+rods	_	*Bacillus sp*
C	WC	_	_	_	+	_	_	_	+	_	+	_	+	+rods	_	*Bacillus sp*

Key: **WC**: white cream hue with a smooth surface texture and edges, **2, 3, A, C**: electrogenic bacterial isolates, +: positive test, -: negative test, H_2S: hydrogen sulfide production.

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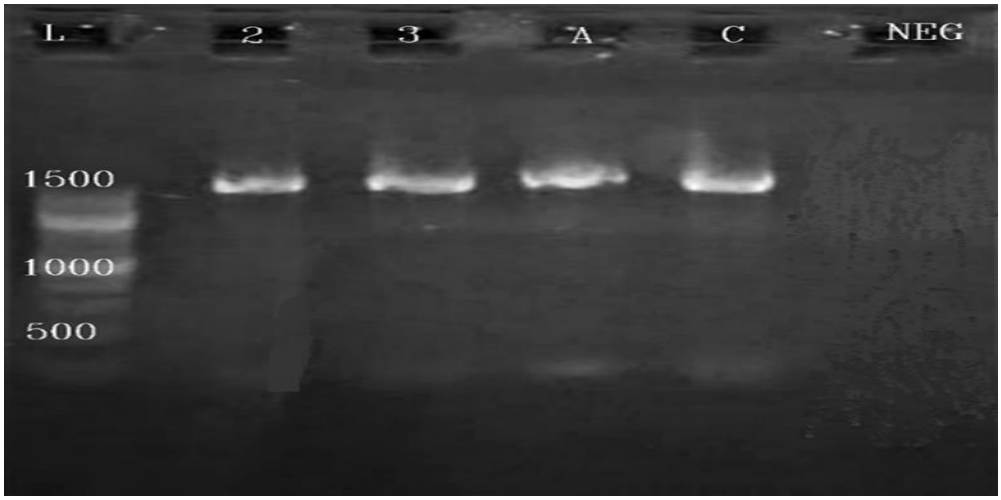

Plate II: Electrophoresis gel of 16s rRNA gene banding pattern of selected bacteria isolates
Key: L: ladder, **2, 3, A, C**: electrogenic bacterial isolates, **Neg:** negative control

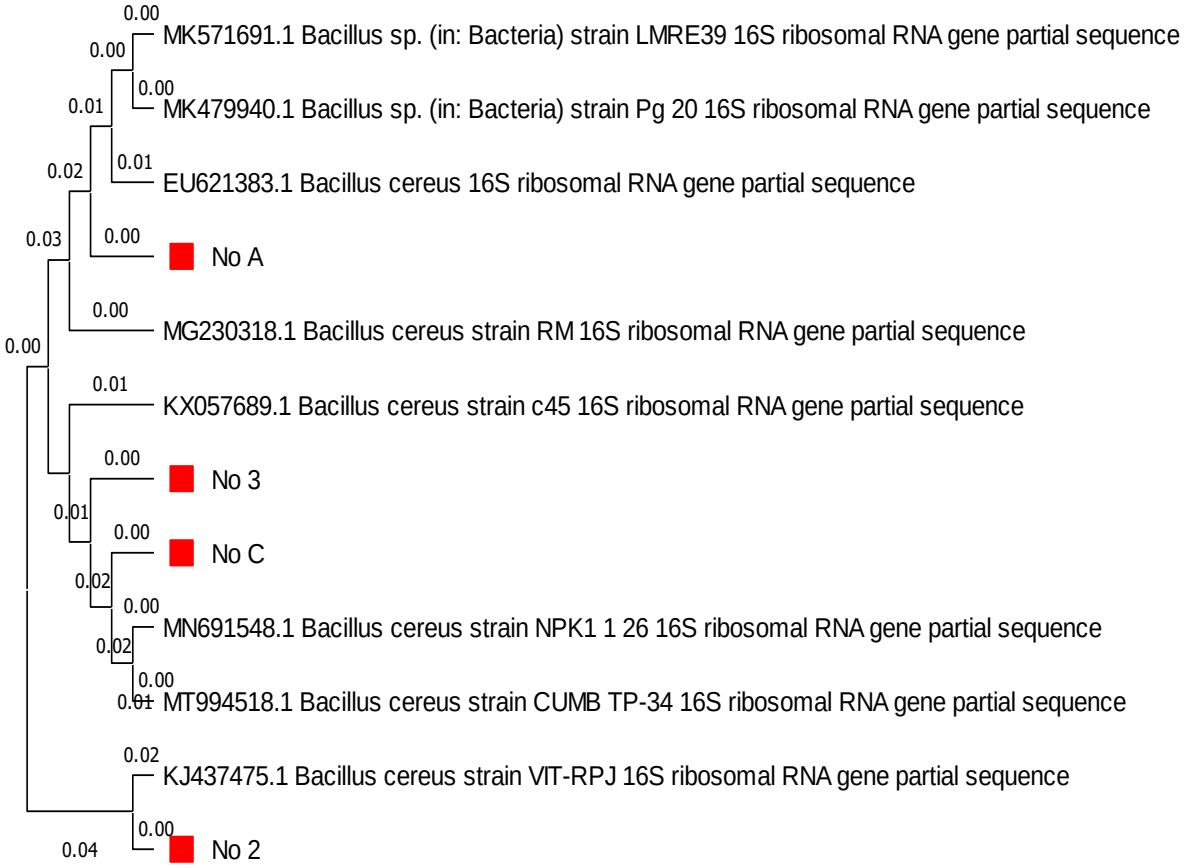

Figure 1: Phylogenetic tree of electrogenic bacterial isolates with closely related strains based on 16s rRNA gene Evolutionary analysis
Key: No 2, 3, A, C; electrogenic isolates used

DISCUSSION

By using a composite sample that was obtained from several collection points, it was possible to get a representative wastewater sample that accurately captured the spatial changes in the microbial composition throughout the sampling area. This methodology recognizes the intrinsic variability of wastewater and

guarantees a more thorough evaluation of the microbial communities existing in the system.(Lancaster & Keller-mcnulty, 1998)

By counting individual colonies on agar plates, the dilution and plating procedure made it possible to quantify the microbial population density. The average count of 5.67×10^6 CFU/mL indicates a significant microbial burden in the wastewater sample. Given the presence of nutrients and organic matter in wastewater habitats, culturable bacteria are expected to flourish there, and the observed microbial density is consistent with that expectation(Barrow *et al.*, 2003).

The high microbial load found emphasizes how important it is to treat wastewater effectively in order to reduce the risks to human health and the environment that could arise from microbial contamination. Furthermore, measuring microbial density is a useful way to evaluate the effectiveness of wastewater treatment methods and track alterations in microbial populations over time(Ryu *et al.*, 2021).

There is no specific benchmark for categorizing a bacterial isolate as electrogenic. However, electrogenic bacteria are typically defined as bacteria capable of generating an electrical current through their metabolic activity, often through electron transfer mechanisms. These bacteria are often studied for their potential use in microbial fuel cells and their biotechnological applications. Researchers typically confirm the electrogenic capabilities of bacteria isolate though various experimental approaches, such as measuring electrical current production or studying the genes and pathways involved in electron transfer (Schneider *et al.*, 2023).

Electrogenic bacteria have emerged as pivotal entities in the realm of microbial electrochemistry, exhibiting the ability to transfer electrons to electrode surfaces. This research investigated the selection criteria for electrogenic bacteria, particularly focusing on their application in wastewater treatment. The establishment of a 200mV threshold voltage serves as a practical measure to assess the electrogenic potential of bacterial strains. Those capable of meeting or exceeding this benchmark demonstrate promise for electricity generation applications, such as microbial fuel cells (MFCs) and bioelectrochemical systems (Schneider *et al.*, 2023).

In microbial fuel cells, the efficacy of energy conversion from organic matter to electricity is paramount. Bacterial strains achieving higher voltages exhibit enhanced energy extraction capabilities, thereby contributing to greater efficiency in power generation processes. Wastewater environments exhibit considerable variability in terms of composition and physicochemical parameters. The adoption of a 200mV benchmark allows for standardized assessments across diverse conditions, ensuring the selection of adaptable and effective bacterial strains for wastewater treatment applications (Garbini *et al.*, 2023).

Electrogenic bacteria not only hold promise for electricity generation but also exhibit potential in bioremediation and nutrient removal processes. Strains capable of achieving the 200mV criterion offer dual functionality, thereby enhancing both wastewater treatment efficiency and energy production. The utilization of a common benchmark facilitates comparability and collaboration among researchers in the field of electrogenic bacteria. By adhering to the 200mV criterion, consistency in findings and methodologies is ensured across different laboratories and research groups (Garbini *et al.*, 2023).

the adoption of a 200mV voltage benchmark serves as a pragmatic approach for selecting electrogenic bacteria from wastewater. These microorganisms not only represent promising candidates for sustainable energy production but also offer significant potential for improving environmental quality through enhanced wastewater treatment processes.

The results obtained strongly suggest that the isolates can likely be identified as members of the Bacillus genus. *Bacillus spp*. represent a well-documented genus of gram-positive, rod-shaped bacteria renowned for their diverse metabolic capabilities. These bacteria are notably recognized for their ability to produce acid, oxidase, urease, and catalase enzymes, indicative of their metabolic versatility. Moreover, they exhibit the capacity to utilize citrate as a carbon source, further highlighting their metabolic adaptability (Barrow *et al.*, 2003).

Bacillus spp. is ubiquitous in various environments, including wastewater, underscoring their resilience and widespread distribution. Previous research has extensively documented their involvement in microbial fuel cell studies due to their inherent metabolic versatility and proficiency in electricity generation through microbial metabolism. The inclusion of *Bacillus spp*. in such investigations is grounded in their ability to thrive in diverse conditions, making them promising candidates for applications in microbial electrochemistry, including wastewater treatment and energy production (Sreelekshmy *et al.*, 2022).

The PCR approach is commonly used for bacterial identification and classification. The 16S rDNA gene is a conserved region in the bacterial genome, and its sequence is unique to each bacterial species. PCR amplification of the 16S rDNA gene, followed by sequence analysis and comparison with a database, is a reliable method for bacterial identification. The use of phylogenetic analysis provides information on the evolutionary relationship between the isolated strain and other bacteria. The constructed phylogenetic tree can help identify the closest relatives of the isolate and provide insights into its evolutionary history. Overall, the results suggest that the bacterial isolate is a strain of *Bacillus cereus* and provide information on its evolutionary relationship to other bacteria. The use of molecular techniques for bacterial identification and classification is a

powerful tool for microbiologists and is becoming increasingly important in clinical and environmental settings (Kumar *et al*., 2018).

CONCLUSIONS

The process of isolating, screening, and identifying bacteria with electricity-producing potentials was a critical step in our research. By employing techniques such as enrichment cultures, biochemical assays, and molecular biology tools, we identified *Bacillus cereus* strains capable of electron transfer and electricity generation without mediators or electron shuttles. This highlights the importance of microbial diversity in bio-electrochemical systems and lays the groundwork for further exploration and optimization of electricity-producing bacteria.

Exploration of Microbial Diversity: Continuation of efforts to isolate and screen bacteria with electricity-producing potentials should be encouraged. Exploration of diverse environments and ecosystems may uncover novel bacterial strains with superior electrochemical properties.

RECOMMENDATION

Continuation of efforts to isolate and screen bacteria with electricity-producing potentials should be encouraged. Exploration of diverse environments and ecosystems may uncover novel bacterial strains with superior electrochemical properties.

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