Evaluation of bioelectricity productivity using alkaliphilic *Bacillus alkalogaya* BW2(1) as a possible exoelectrogens for improvement of microbial fuel cell performance

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ABSTRACT

The present study put forth with the fundamental objective to the exploration of exoelectrogens from the extremophilic environment and to investigate the electricity generation from them. A total of 20 bacterial cultures were isolated, from which BW2(1) was selected for the further investigation of the microbial fuel cell (MFC). The experimental results performed that the strain *Bacillus alkalogaya* BW2(1) was capable of utilizing organic acids and sugars as electron donors to generate electricity. The MFC was constructed and the electricity generation was measured after various intervals using various parameters and substrates, 937 mV electricity was generated after 1 hour, but after 48 hours the electricity generation dramatically decreases to 570 mV. The effect of pH on MFC was also studied, pH enhanced electricity, indicating the requirement of pH for bacterium BW2(1). This is a valuable information for bioelectricity production and optimization from *B. alkalogaya* BW2(1) has bright future toward the improvement and production of bioelectricity for entirely new areas of industrial and biotechnological applications.

1. INTRODUCTION

Microbial fuel cells technology is an advantageous method for electricity generation and to overcome the issues of power generation [1]. The microbial fuel cell (MFC) can be used as a promising technology for bioelectricity generation and it is attracting researchers worldwide. Microbial fuel cells are bioreactors that can degrade organic or inorganic matter and simultaneously produce electricity. Microbial-mediated electron release at the anode and succeeding electron utilization at the cathode is the main couple of process in ideal MFC [2] (Fig. 1). Herein, chemical energy is converted into electrical energy [3]. In MFC, bacteria are used as a catalyst and can generate electricity. Kim et al. [4] reported the first mediatorless MFC by using the bacteria *Shewanella putrefaciens* as biocatalyst. These bacteria oxidize organic or inorganic matter [5]. The bacteria used in MFC are electrochemically active; these are mostly found in aquatic environment. These bacteria usually convert the carbon substrate to chemical energy. In MFC, two electrodes are used—anode and cathode. In MFC, there are two chambers connected by the proton exchanger membrane. In one chamber, there is anode and in another one there is the cathode. The anode is the biological site where microorganisms are present and the cathode is the abiotic site [6]. In cathode compartment, electron acceptors play a vital role in improving the performance of MFC by using different substrates like glucose, mannitol, etc., at different concentrations can enhance the performance and give high results, these are the key factors that affects both electrochemical as well as biochemical processes [5]. Electrons attraction and biomass growth varies with different environment and nutrients [7]. MFC is a simple, reliable, rapid, and noninvasive [8]. The pH and salt also play an important role in MFC, they control and maintain optimum conditions for bacterial growth and generation of electricity. Haloalkaliphiles, representing the most diverse group of halophiles could grow optimally at high pH

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salt concentration along with growing optimally at pH values at or above 10. In this regards, the stability of haloalkaliphilic organism toward the bioelectricity generation; however, only few of them have been explored for their properties and industrial application. The search for bacteria that function optimally at higher pH and thereby would have higher catalytic rates of bioelectricity generation which were halophilic bacterial strain isolated from Lonar lake was the aim of the present study. The alkaline Lonar lake (Latitude 19°58ʹ, Longitude 76°36ʹ) is a unique basaltic rock meteorite impact crater, ranking third in the world. Lonar crater is filled with saline water. The uniqueness of the lake water is its salinity and high alkalinity. The lake water is alkaline having an average pH of 9.5–10. Due to the uniqueness, the lake has evoked much scientific value among researchers. The presence of brackish water inside the crater having pH 10 is distinctive feature of the ecosystem along with the concentration of chlorides, calcium carbonate, and water over a long period of time [9]. Alkaliphilic microorganisms have attracted much interest because of their ability to produce bioelectricity that are active and stable at high pH values. The unusual properties of these power offer a potential opportunity for their utilization in processes demanding such extreme conditions. The isolation and characterization of these bacterial strains could provide knowledge on the power generation from bacteria strains in this unexplored haloalkaliphilic Lonar lake environment. The objectives of this study were to examine the possibility of generating electricity in an MFC with alkaliphilic microorganisms as biocatalysts. This is the first attempt of exploiting alkaline Lonar soda lake microbial communities for bioelectrical energy generation in MFC.

2. MATERIALS AND METHODS

2.1. Enrichment and Isolation of Microorganisms

Lonar lake water and sediment sample were collected in sterile bottles and polythene bags, respectively, from the defined sampling site. Enrichment of water samples and sediment samples was carried out in Horikoshi I, Horikoshi II, and nutrient agar at pH 10, nutrient agar at pH 10.0 with 30 g l−1 sodium chloride. All flasks were incubated at 37°C on a rotary shaker (100 rpm) for 48 hours. After enrichment, the organisms were isolated on respective media agar plates and incubated at 37°C for 24 hours. Well isolated and morphologically distinct colonies from these plates were transferred on the respective medium slants and maintained as stocks. Bacterial cultures were examined for their cultural, morphological character. Standard biochemical test was also performed according to Bergey’s Manual of determinative bacteriology.

2.1.1. 16S rRNA gene sequences and phylogenetic analysis

DNA was extracted from bacilli culture using standard phenol-chloroform protocol. The partial sequence of the 16S rRNA gene was amplified by using polymerase chain reaction and universal primer Eubacteria specific primers (Table 1). The polymerase chain reaction (PCR) condition used was an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute and extension at 72°C for 1 minute and final extension at 72°C for 10 minutes. The amplified 16S rRNA gene PCR products from these isolates were directly sequenced after purification with polyethylene glycol and NaCl procedure and directly sequenced on the Applied Biosystems Model 3730 DNA sequence (Foster, CA). The 16S rRNA gene sequence was analyzed using the basic local alignment search tool program Multiple Sequence Alignment of approximately 900 bp sequences that were performed using CLUSTAL W, version 1.8. A phylogenetic tree was constructed from evolutionary distances using the neighbor-joining method of the Molecular Evolutionary Genetics Analysis 4 program package.

2.2. Cultural Condition

Culture was retrieved by streaking on Horikoshii II (soluble starch 10.0, peptone 5.0, yeast extract 5.0, KH₂PO₄, 1.0, MgSO₄·7H₂O 0.2, Na₂CO₃ 10.0, agar 20.0.) agar plates and incubating at 37°C. For MFC operation, two–three isolated colonies were inoculated in 100 ml of Horikoshii II Broth and incubated for 48 hours at 37°C (160 rpm) in shaking conditions.

2.2.1. Enrichment of culture

Culture was inoculated in 100 ml Horikoshii II media and incubated for 48 hours at 37°C. Incubation was done at room

| Table 1. List of primers. | Primer length | Aniline temperature | Reference |
|---------------------------|--------------|---------------------|-----------|
| Primer Sequence | Primer length | Aniline temperature | Reference |
| 16F 27 5′ CGAGAATTGATCMTGCGTCCT-3′ | 21 | 55°C | Present study |
| 16R 1525 5′ GTCTGCAGTCTAGAAGGAGGTG-3′ | 30 | 55°C | Present study |
2.3. MFC Assembly Design and Component

2.3.1. Electrode
Carbon electrode (aluminum) of dimension 15 cm × 0.2 cm was used as cathode and anode. These electrodes were tightly fixed with the containers containing medium, culture, and distilled water.

2.3.2. Cathodic chamber
The cathodic chamber of the MFC was made up of 100 ml plastic bottles which were surface sterilized and then filled with distilled water. Distilled water works as a catholyte.

2.3.3. Anodic chamber
In the cathodic chamber, 100 ml plastic bottles were used. The bottles were surface sterilized, by washing it with 70% ethyl alcohol; followed by ultraviolet (UV) exposure for 15 minutes. Then 100 ml of the previously enriched culture of bacteria was added in sterile soil. This soil with bacteria was filled in the bottle.

2.3.4. Salt bridge
The 1% agarose was dissolved in distilled water. This mixture was boiled for 2 minutes and poured in the polyvinyl chloride pipes (dimension 10 cm × 3 cm) under aseptic condition. The salt bridge was sealed pack and kept in the refrigerator for proper settling. Two holes were made in the lower side of bottles for the insertion of the salt bridge containing agarose gel.

2.3.5. Circuit assembly
Circuit assembly was made by connecting two chambers internally and externally. Internally chambers were connected by a salt bridge and externally the circuit was connected with aluminum wires which were joined by the multimeter by two ends.

2.3.6. Measurement of potential difference and current
The potential difference generated by the fuel cell was measured by using multimeter from HAOFYUE-DT830D.

2.4. MFC Operations
In MFC, all components are connected internally with the help of the salt bridge and externally with wires to the multimeter. The pure colony was aseptically transferred in 100 ml specific broth and incubated at 37°C at 160 rpm for 48 hours. Foregoing MFC operation, the bottles used were surface sterilized by 70% alcohol and exposed to UV radiation for 15 minutes. Then under aseptic conditions, the salt bridge was sealed inside the holes of bottles. First sterile soil was placed in and then culture broth of BS1(2) was poured to prepare liquid suspension in the anodic chamber. Sterile distilled water was poured in the cathodic chamber. MFC set up was kept at static conditions and its operation was carried out at room temperature. To check the isolates ability to generate potential difference, these isolates were tested by varying carbon source, pH, organic acids, and salts concentration was one by one. The MFCs were run up to 48 hours and in every 2 hours interval voltage was recorded in all cases [10].

2.5. Statistical Analysis
Statistical analysis of MFC and correlation with each other data was analyzed by the Statistical Package for Social Sciences (SPSS) and MATLAB (Figs. 8 and 9).

3. RESULTS AND DISCUSSION
Morphological characteristics, optimum pH and salt tolerance of all the strains were studied. The isolate BW2(1) was identified on the basis of biochemical characteristics as described earlier and further confirmed by 16S rDNA sequencing (Table 2). Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain BW2(1) was affiliated with the genus Bacillus (Fig. 2). The 16S rRNA gene sequences, strain BW2(1) shown a high level of similarity with the type strain of Bacillus and showed a substantial degree of relatedness to references 16S rRNA sequences of

| Table 2. Morphological and physiological analysis of bacilli isolated from Lonar crater. |
|---------------------------------------------------------------|
| Gram character | + | Growth at 3% NaCl | + |
| Shape of bacteria | Long rod | Growth at 4% NaCl | + |
| Size of bacteria (Length (µm)) | 4 | Growth at 5% NaCl | + |
| Size of bacteria (Width (µm)) | 0.4 | Growth at 6% NaCl | + |
| Arrangements of cell | Single | Growth at 7% NaCl | + |
| Spore bearing | + | Catalase | + |
| Position of spore | Central | Oxidase | + |
| Shape of spore | Cylindrical | Indol | – |
| Swollen sporangia | + | Methyl red | – |
| Capsule | + | Voges–Proskauer | – |
| Motility | Motile | Citrate | – |
| Type of motility | Highly motile | Urease | – |
| Flagella | + | Nitrile | – |
| Size of colony | 2 | Glucose | – |
| Pigment | White | Arabinose | – |
| Colony shape | Circular | Mannitol | – |
| Colony elevation | Convex | Xylose | – |
| Colony edge | Entire | Lactose | – |
| Internal structure of colony | Wavy interlaced | Trehalose | – |
| Colony on slant | Eschimulate | Sucrose | – |
| Growth at 37°C | + | Cellobiose | – |
| Growth at 45°C | + | Galactose | – |
| Growth at 50°C | – | Maltose | – |
| Growth at 55°C | – | Fructose | – |
| Growth at pH 7 | + | Salcin | – |
| Growth at pH 8 | + | Sorbitol | – |
| Growth at pH 9 | + | Raffinose | – |
| Growth at pH 10 | + | Starch hydrolysis (mm) | + |
| Growth at pH 12 | + | Lipid hydrolysis (mm) | – |
| Growth at 1% NaCl | + | Casein hydrolysis (mm) | – |
Bacillus in the database. The 16S rRNA sequences of the BW2(1) isolated strains have been deposited in the National Center for Biotechnology Information GenBank under accession numbers JQ319530.

The highest similarity values with the sequences of obligately alkaliphilic and alkali tolerant, aerobic endospore-forming bacteria. Lonar lake strain BW2(1) was shown high similarity to Bacillus alkalogaya. The traditional cultivation based methods have great importance in research, providing the chance in investigations of biotechnologically significant bacterial isolates under *in vitro*. Biological processes are eco-friendly and they can be used as a promising alternative. Microbial fuel cells (MFCs) are newly developed processes that help to generate electricity using microorganisms. In the present study, 20 bacterial cultures were isolated by using different enrichment media. Out of them, four bacterial strains: BW4(3), BS1(1), BW4(1), and BW2(1) were selected for the electricity generation and screened on the Horikoshi II enrichment medium.

Here in this study, the culture was aerobically prepared at alkaline pH (10) and incubated for 24 hours and then aseptically poured into the anodic chamber. After this preparation, MFC was constructed and electricity generation was measured at various intervals continuously from 24 hours to 58 hours after incubation. The bioelectricity generated was 224 mV and 316 mV by BW4(1) and BS1(1) after 24 hours, respectively. The 245 mV bioelectricity was generated by BW2(1) at 24 hours that gradually increased to the electricity generation 921 mV after 48 hours. In present investigation, the BW2(1) was found prominent for the bioelectricity generation. BW2(1) strain was screened and selected for the further bioelectricity generation and characterization. At 24 hours, the 245 mV electricity was generated that was continuously increased with a peak of 927 mV and then went down to decrease (Fig. 3). Velasquez-Orta et al. [11] evaluated the performance of MFCs using two different types of algae as substrates; Chlorella vulgaris (a microalgae) and Ulvalactuca (a macroalgae). One of the first pure cultures to be studied as an oxidation catalyst in MFC systems was *Shewanella oneidensis* [12]. An MFC can be a very robust device when it is subjected to short-term changes in operating parameters such as sugar, NaCl, acetate, lactate, and pH.

3.1. Effect of Sugar on Bioelectricity Generation

The most extensive studies have been reported on the bioelectricity generation by using carbohydrate-rich wastes such as food processing wastewater, starch processing wastewater, and chocolate-based wastewater [13]. Electricity generation by direct oxidation of glucose in mediatorless microbial fuel cells was reported by Chaudhuri and Lovley [14]. In the present studies, the anodic chamber was supplemented with various sugar for the bioelectricity generation. Tarte et al. [15] effect of NaCl and glucose on the generation of electricity was also studied. MFC was prepared from waste water and different sugars were mixed with it to see the effect of sugar on electricity generation. The bacterium BW2(1) was supplemented with the various sugars like in glucose, sucrose, lactose, maltose, mannitol, and starch. After the addition of various sugars, the electricity generation was found to be increased up to 460 mV by glucose and after supplement with sucrose (550 mV), maltose (296 mV), mannitol (451 mV), starch (310 mV), but the optimum electricity generation was found after addition of lactose 657 mV (Fig. 4). Microbial fuel cells (MFCs) are most fascinating bioelectrochemical devices that use living catalysts to produce electric energy from organic matter present naturally in the environment or in waste. Kumar et al. [16] investigate bioelectricity generation and treatment of sugar mill effluent using a microbial fuel cell. MFCs can also aid in waste water treatment.
3.2. Responses of Voltage Output to pH Variations

In recent years, many researchers are keen interested and studying MFC technology. Generally, different parameters both operational and designing factors affect the MFC performance. MFC can be a very robust device when it is subjected to short-term changes in operating parameters such as pH. To check the effect of pH on electricity generation, an MFC inoculated with a BW2(1) was constructed. Gonzalez del Campo et al. [17] revealed that in the case of the effects of medium pH on the power generation of an MFC, it was found that acidification of the anode affected. In the present investigation, 48-hours-old culture generated the bioelectricity of 245 mV at neutral pH while at increased alkaline pH 12, 921 mV optimum electricity was generated (Fig. 5). Initial pH influenced the performance of MFCs and the types and abundance of anodic microbes was studied by Zhang et al. [18] and revealed that under acidic conditions, voltage outputs and power generation were lower, while chemical oxygen demand removal was faster.

3.2.1. Effect of NaCl on bioelectricity generation

Salinity effect on the microbial fuel cell performance was investigated. The 1.5% and 2% of NaCl was added into the MFC, after the addition of NaCl, the electricity generation that was found to be increased was 55 mV and 50 mV. After 57 hours incubation, the electricity generation was found optimum for 1% NaCl 187 mV while after 50 hours incubation bioelectricity generation was optimum for 2% NaCl (90 mV) (Fig. 6) Muralidharan et al. [19] studied on the impact of salt concentration on electricity production in microbial hydrogen-based salt bridge fuel cells. These MFCs are better option for electricity generation. Parkash et al. [20] studied the impact of various salt and concentration on the electricity generation based on dual-chambered MFC. They revealed that the KCl salt bridge was efficient in electricity generating than the NaCl. Dhundale et al. [10] studied the effect of salt on MFC, NaCl was the prominent electricity generation than the KCl, indicating a bacterium ARS4 was required NaCl for the MFC.

3.2.2. Effect of lactate and acetate on bioelectricity generation

Biochemical routes that lead to acetate produce more hydrogen than those that lead to butyrate production [21]. Bioelectricity generation from acetate in two-chambered MFCs is well-known. Here we demonstrate that electricity can be generated from lactate and acetate in a double-chambered MFC, and we compare the power densities obtained from acetate and lactate with those previously obtained in the same system using sugar. In the present investigation, optimum of 188 mV and 166 mV bioelectricity were generated in the presence of lactate and acetate, respectively (Fig. 7).

3.2.3. Relation of energy generation with pH, carbohydrate, hydrogen donor species, and salt

Electricity generation (mV) by MFC constructed with the B. alkologaya BW2(1) was analyzed statistically. pH value showed a strong positive correlation with lactose in terms of electricity generation (mV) among all tested carbohydrate sources. In case of salt concentration, 2% NaCl shows a positive correlation with pH but 1.5% NaCl concentration dramatically showed a negative correlation with said pH. In present construction of MFC, we used two hydrogen producer substrates; lactate and acetate; among that acetate is showed a positive correlation with pH in their electricity generation (mV) values. The surprising thing in this investigation is that every positively correlated individual is also showed positive correlation with each other (Fig. 8) In short, the present investigation strongly suggested that for MFC-based electricity generation by using B. alkologaya BW2(1) will give the best output at the optimum composition of lactose, acetate, and 2% NaCl as a source of carbohydrate, hydrogen donor species, and salt, respectively, at specific pH (Fig. 9)
Figure 8: 8(1)–(10): Correlation analysis: Relationship between bioelectricity generation at different experimental parameters.
4. CONCLUSION
Among 20 bacterial isolates, *B. alkalogaya* BW2(1) was selected for the further investigation of MFC. The MFC was constructed in presence of *B. alkalogaya* BW2(1) and electricity generation was measured at various intervals, 937 mV of electricity was generated after 48 hours at pH 12, which was dramatically decreased up to 570 mV after 48 hours. The pH enhanced electricity generation indicating a bacterium BW2(1) require alkaline pH for better production of electricity. The entire study came with a conclusion that the MFC is a trusted technology to the development of sustainable future of electricity generation. Now time came to not only explore microbial diversity but also judicious utilization of them required for overcoming on needs and challenges in sustainable energy development. Now time came to not only explore microbial diversity but also judicious utilization of them required for overcoming on needs and challenges in sustainable energy development.

AUTHOR’S CONTRIBUTIONS
Pinky Khemchandani, Gayatri Aher, Parthsarathi Dikonda performed all the parameters under the guidance of Vishal Dhundale. Vijayashree Hemke, Vishal Dhundale and Dhananjay Desai took part in designing experimental setup. Dhananjay Desai and Vishal Dhundale prepared entire manuscript.

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How to cite this article:
Dhundale V, Hemke V, Desai D, Khemchandani P, Aher G, Dikonda P. Evaluation of bioelectricity productivity using alkaliphilic *Bacillus alkalogaya* BW2(1) as a possible exoelectrogens for improvement of microbial fuel cell performance. J Appl Biol Biotech 2020;8(01):69–75. DOI: 10.7324/JABB.2020.80112