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Electricity generation and ethanol production using iron-reducing, haloalkaliphilic bacteria

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ELECTRICITY GENERATION AND ETHANOL PRODUCTION USING IRON-REDUCING, HALOALKALIPHILIC BACTERIA

by

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ABSTRACT

Microbial life in extreme environments has been studied primarily for their metabolic activities. Very few commercial or industrial applications have been known from these systems. In this study, the metabolic pathways and properties of bacteria from a haloalkaliphilic environment of Soap Lake, Washington were employed in two research applications related to energy production. In the first study, the bacterial cultures that were known to reduce iron (III) were used in a Microbial Fuel Cell (MFC) reactor. Iron reducing bacteria have shown to shunt their electrons on to the surface of a carbon electrode of an MFC, and the electrons can be harvested to produce current. The research showed that haloalkaliphilic bacteria colonized the electrode surface efficiently and produced current densities up to 12.5 mA/m^2. The open circuit voltage was constant over several days reaching up to ~1V. Molecular characterization of 16S rRNA gene and RFLP analysis showed that the bacterium responsible was a single species with 97% 16S rRNA gene similarity to *Halanaerobium sp*.

The second study focused on applying the fermentative mechanism of the bacterial cultures from Soap Lake. Bacteria were found to use glycerol as the substrate and ferment it to ethanol. Glycerol is a major by-product of biodiesel industry and therefore bacterial fermentation of this glycerol to ethanol would help to manage the waste as well as produce a value-added product. Analysis of glycerol and ethanol concentrations showed a conversion rate of about 2.83%. Several strategies were adopted to identify the bacterial species involved in the fermentation process. Results from RFLP and 16S rRNA gene analysis indicated that this bacterium showed 97% gene similarity to *Halanaerobium sp.*

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1. INTRODUCTION

1.1. ENERGY AND BIOLOGY

With the rapid depletion of fossil fuels and the insatiable need for energy, much research is now focused on renewable, clean, and efficient means of extracting energy. Among the other energy sources currently available, bioenergy in the form of fuels has a definite edge. For example, scientists are now interested in using waste from human activities to meet a portion of the world's energy demands. A US Department of Energy survey estimated that the energy available from agricultural, animal, industrial, and human waste could eventually meet approximately 7% of the total energy demand of the United States (Chynoweth et al., 2001). Furthermore, the commercial scale production of biodiesel and bioethanol from a variety of sources is a promising means to provide for the high energy demands of the future. Many bacterial species such as *Escherichia coli*, *Klebsiella oxytoca*, and *Zymomonas mobilis* (Dien et al., 2003) are naturally able or have been genetically engineered to produce ethanol from various sugars, though only the latter is currently used in an industrial scale. Glycerol is a major byproduct of both biodiesel and bioethanol generation (Yazdani and Gonzalez, 2007; Ito et al., 2005). With the current scale of production of these two fuels, glycerol is now considered waste, whereas it was once viewed as a high-value commodity. The biological fermentation of glycerol to ethanol has been shown to occur in certain genetically modified strains of *Enterobacter* (Ito et al., 2005) and a few other bacterial species; the possibility of developing this process for mass production of ethanol is promising.

In the context of energy gleaned from biomass, it is important to understand that energy is ultimately in electrons found in the organic materials. Most bioenergy output

consists of high-density electron carriers. Some of the best electron and energy output are hydrogen, methane gas, and electricity (Rittmann, 2008). The production of electricity using microorganisms in a Microbial Fuel Cell (MFC) has been suggested as a promising approach to obtain current from organic wastes and renewable biomass (Lovley, 2008). An MFC is a device that converts chemical energy to electrical energy by the catalytic reaction of microorganisms.

1.2. EXTREMOPHILES IN ENERGY PRODUCTION

Studies of glycerol production from biodiesel have suggested that glycerol-rich effluent has a high salt content due to the transesterification process involved in the conversion of oil to biodiesel. Various strategies have been employed to convert glycerol into value added products. Fermentation has been suggested as an excellent approach, and products like 1, 3- propanediol, 2, 3-butanediol (Homann et al., 1990), and ethanol have already been shown to be produced by various groups of microorganisms (Yazdani and Gonzalez, 2007; Ito et al., 2005). Ethanol production, in particular, is now receiving considerable attention. However, the rates of production of ethanol and hydrogen by certain bacteria used in the fermentation process have dropped considerably. This diminution has been attributed to a high salt concentration (Ito et al., 2005) in the glycerol waste streams. The use of an alkaline treatment has been proposed as an alternative to steam blasting in the separation of lignin from cellulose in bioethanol production. This process would generate a highly alkaline waste stream. Thus, the fermentation of waste products in each of these cases requires bacteria that can thrive under more saline and alkaline environments.

Experiments performed on MFCs suggest that the power density obtained with an ionic strength of 400 mM is 85% greater than that obtained using the same medium at an ionic strength of 100 mM (Liu et al., 2005). However, not all bacteria can survive these saline conditions. In addition, industries, including agro-based, petroleum, and leather generate waste water streams that are highly saline and contain high loads of organics that are sometimes difficult to treat by normal wastewater treatment techniques (Lefebvre and Moletta, 2006). The use of halophiles that can thrive in such high salt conditions in an MFC reactor, would likely help to treat the wastes as well as generate electricity. Furthermore, although many have reported the use of other extremophilic microorganisms like thermophiles (Milliken and May, 2007; Jong et al., 2006; Choi et al., 2004) and acidophiles (Malki et al., 2008) in MFCs, fuel cells operated with halophiles have received little attention (Miller & Oremland, 2008).

This study investigates the energy-related applications of haloalkaliphilic strains isolated from Soap Lake, Washington, a meromictic (mixing of water layers does not occur for years) lake with an alkaline pH (9-11) and a salinity of about 14%. The application of these bacterial cultures in an MFC to monitor the current and power production is described as well as attempts to characterize the species that produce current, by molecular techniques. Efforts to use these haloalkaliphilic bacteria in the conversion of glycerol to ethanol by culturing, and the identification of the bacteria by using molecular characterization are also described.

1.3. GOALS AND OBJECTIVES

Hypothesis 1

Microbial Fuel Cells generate electricity directly from microorganisms grown on an electrode surface. An increase in ionic strength may enhance current density; however, such an increase would require microbes that can survive high salinity. **The use of haloalkaliphilic microbes in MFCs would encourage consistent production of power because of the hypersaline environment and the low solubility of oxygen in that environment (Oren, 2002)**. Efficient current output would depend on determination of the possible electron donors and electron acceptors and on the use of the most productive cathodic and anodic media in the fuel.

Objective 1

1.1. A double-chambered fuel cell was constructed and operated with the haloalkaliphilic cultures. Current, voltage, and other electrical variables were measured over time to determine the maximum amount of power that could be extracted from the system.

1.2. Molecular characterization was performed to shed light on the microorganisms associated with the electrode and thus with current production.

1.3. Microphotographs were acquired to confirm the presence of microorganisms on the electrode surface and to inform the choice among the various electron acceptors (i.e. electrode materials) available.

Hypothesis 2

Haloalkaliphilic bacteria can be used to ferment glycerol to ethanol. The biodiesel industry produces an alkaline, salty glycerol waste stream. Bacteria are able to utilize this waste and produce ethanol. However, these microorganisms have to be tolerant of alkaline and hypersaline conditions that match the glycerol waste stream conditions. Bacterial samples from Soap Lake that can grow optimally at both high pH and salt conditions are ideal for use in this fermentation process.

Objective 2

2.1. The rate of glycerol consumption and ethanol production in the bacterial cultures enriched from Soap Lake sediments were measured.

2.2. As the enrichment culture was suspected to be mixed, antibiotic amended media were used to select against the microbes that were not responsible for the fermentation. The bacteria involved in glycerol fermentation activity could be identified. Molecular analysis was performed to determine these bacteria.

2. REVIEW OF LITERATURE

2.1. HALOALKALIPHILIC MICROORGANISMS

Extremophiles are organisms that thrive in extreme environments such as high temperature (thermophiles), high salt (halophiles), acidic (acidophiles), or alkaline (alkalophiles) conditions. Although difficult to culture and study, potentially useful extremophiles have recently been discovered (Pennisi, 1997, Eichler, 2001). Halophiles, in particular, are interesting as they have a large range of metabolic potentials and possess many ways to cope with the osmotic stress caused by the high salt concentrations in their environment (Oren, 2002).

Halophiles show great variability in tolerances to ionic composition, total salt concentration, and pH of their environments. Depending on their salt requirements (Larsen, 1962), halophiles may be classified as (i) slight (most rapid growth at 2% to 5% NaCl), (ii) moderate (most rapid growth at 5% to 20% NaCl), or (iii) extreme (most rapid growth at 20% to 30% NaCl). Microorganisms surviving in a hypersaline environment with a high pH of 8.5-11 are referred to as haloalkaliphiles. Many haloalkaliphilic bacteria have been isolated from various soda lakes throughout the world (Horikoshi, 1999). Several lakes (Big Soda Lake, Mono Lake, Soap Lake) in the great basin of the western United States with salinity ranging from 8.9% to 10% (wt/vol) are highly alkaline, with pH values of 9 to 10 (Oremland and King, 1989). Halophilic microorganisms efficiently colonize many hypersaline environments and may attain high population densities (Oren, 1999).

2.1.1. Diversity. Recent studies on ecology, physiology, and taxonomy of haloalkaliphiles have revealed an impressive diversity in highly saline and alkaline lakes (Dodia et al., 2006). Halophilic environments possess microorganisms that exhibit diverse biochemical activities. For example, strictly anaerobic halophiles have been studied for their metabolic activities, including fermentation, sulfate-reduction, iron-reduction, homoacetogenesis, methanogenesis, and oxidization of organic carbon and energy sources (Ollivier et al., 1994).

2.1.2. Metabolic Activities. Dissimilatory reduction of certain metals and metalloids by anaerobic bacteria has geochemical, ecological, microbiological, and environmental significance in that the process has been said to have had the most important influence in the chemical changes of anaerobic sediments and soil (Lovley, 1993; Lovley, 1991). Iron (III) and Mn (IV) appear to be the most common metals to function as terminal electron acceptors in the biosphere. Bacterial respiration using selenium and arsenic has also been reported in many haloalkaliphilic environments (Blum et al., 1998).

Studies have found that a wide variety of fermentative microorganisms reduce Fe (III) or Mn (IV) during anaerobic growth (Bromfield, 1954). Fermentation of various carbohydrates has been reported in many halophilic species. A few of them, like *Halobacteroides* species are characterized by acetate, ethanol, and H_2-CO_2 production from glucose (Rengipat et al., 1988), whereas *Haloanaerobium* species (Zeikus et al., 1983) produce acetate, propionate, butyrate, and H_2-CO_2. Lately, a novel halotolerant, alkaliphilic, dissimilatory Fe (III)-reducing bacterium was isolated from salt flat sediments collected from Soap Lake, Washington (Pollock et al., 2007). Complete

oxidation of organic compounds such as acetate and formate (Lovley, 1991) to carbon dioxide is possible with Fe (III) as an electron acceptor (equations 2.1.1, 2.1.2), whereas incomplete oxidation has been shown in the case of lactate and pyruvate where the end products are acetate and carbon dioxide (equations 2.1.3, 2.1.4).

$$Acetate^- [CH_3COO^-] + 8Fe\ (III) + 4H_2O \rightarrow 2HCO_3^- + 8Fe\ (II) + 9H^+ \quad (2.1.1)$$

$$Formate^- [HCOO^-] + 2Fe\ (III) + H_2O \rightarrow HCO_3^- + 2Fe\ (II) + 2H^+ \quad (2.1.2)$$

$$Lactate^- [CH_3CH\ (OH)\ COO^-] + 4Fe\ (III) + 2H_2O \rightarrow Acetate^- [CH_3COO]^- + HCO_3^- + 4Fe\ (II) + 5H^+ \quad (2.1.3)$$

$$Pyruvate^- [CH_3COCOO^-] + 2Fe\ (III) + 2H_2O \rightarrow Acetate^- [CH_3COO]^- + HCO_3^- + 2Fe\ (II) + 3H^+ \quad (2.1.4)$$

Most representatives of the *Haloanaerobiales* ferment simple sugars such as glucose to products such as ethanol, acetate, H_2, and CO_2 (Oren, 1999).

2.1.3. Soap Lake Environment. One example of a haloalkaline environment is found at Soap Lake, Washington. Soap Lake is a meromictic, stratified lake consisting of two separate water layers that have not mixed for at least two thousand years. Its salinity ranges from ~13 g/l in the mixolimnion and ~70 g/l in the monimolimnion. It also has high alkaline condition with the pH of the water column averaging 9.9 and the sediment pH averaging 10.6 (Dimitriu et al., 2008). The high alkalinity is attributed to the abundance of bicarbonate (5209 mg/L) and carbonate (6870 mg/L). Various biochemical activities occurring in the Soap Lake have been identified and studied. Methanogenesis is minimal compared to other such activities (Anderson, 1958). Iron reduction has been shown to take place in Soap Lake along with fermentation and oxidation of organic

materials. The lake has very low dissolved oxygen, making it a perfect site for the study of anaerobic activities (Dimitriu et al., 2008).

2.2. MICROBIAL FUEL CELLS

In a Microbial Fuel Cell, bacteria catalyze the oxidization of organic substrates and transfer the resulting electrons to an anode electrode. These electrons then move through an electrical circuit to the cathode where they are transferred to an electron acceptor like oxygen (Rittmann, 2008). Extensive research has been performed throughout the world on bioelectricity production from a range of fermentation products and waste organic materials (Regan and Logan, 2006). Studies of MFCs have focused on the attachment and growth of the biofilm of anode respiring bacteria (ARB) and the means of transferring electrons to the surface of anode.

2.2.1. Characteristics of Anode Respiring Bacteria. Investigators in the field of biological electricity production have taken up the task of identifying and isolating those bacteria that have the capability of transferring the electrons to an electrode. Several studies indicate that the closest metabolic activity most similar to electron transfer in an MFC is dissimilatory metal reduction (Bretschger et al., 2007; Bond et al., 2002; Chaudhuri and Lovley, 2003). In the absence of oxygen, dissimilatory metal reducing bacteria (DMRB) transfer their electrons to a metal such as iron or manganese, which acts as the terminal electron acceptor (Lovley, 1993). In an MFC, the iron or manganese is replaced by the anode, to which the bacteria shunts away electrons generated due to the oxidation of organic compounds. However, not all DMRBs are capable of transferring electrons to the anode of an MFC (Miller & Oremland, 2008). Among the types of metal

reduction possible, iron reduction seems to resemble most closely the process occurring in an MFC; hence much investigation has sought to enrich cultures for dissimilatory iron reduction activity (Lovley, 2008; Chaudhuri and Lovley, 2003; Holmes et al., 2004). Among the microorganisms first reported to possess the electrode reducing activity were *Clostridium*, *Geobacter* , *Aeromonas*, *Rhodoferax*, *Desulfobulbus* and *Shewanella*, all of which have been shown to exhibit dissimilatory metal (especially iron) reduction activity (Bretschger et al., 2007).

Electrons can be transferred to the anode in any of the following ways: direct transfer by means of bacterial structures called nanowires, indirect transfer using intermediate electron shuttles, conduction through the biofilm matrix; or a combination of these mechanisms (Lovley, 2008; Rittmann, 2008; Logan et al., 2006). Significant research on *Shewanella putrefaciens* indicated that specific cytochromes outside of the cell make the bacteria electrochemically active when grown under anaerobic conditions (Kim et al., 2002). Recent studies have found a predominance of *Gamma proteobacteria, Beta proteobacteria, Rhizobiales*, and *Clostridia* on the anode surface (Lovley, 2008). Mixed-culture MFCs generate higher power densities than pure cultures, perhaps due to synergistic interactions within the anode communities and the participation of currently unknown strains and mechanisms (Jung and Regan, 2007). For example, some species may act fermentatively, degrading the more complex organic material and providing the breakdown products to the anode respiring bacteria, which then oxidize the organic compound completely.

A recent study used MFCs to understand the electron transfer abilities and terminal electron accepting processes in a highly alkaline and salty environment (Miller

& Oremland, 2008). In addition to exhibiting dissimilatory reduction of arsenate and selenium, the bacteria in these experiments produced electricity when inoculated in a fuel cell apparatus. A few other bacteria from extreme environments have been reported to generate electricity. For example, thermophilic bacteria cultured in an MFC produced large power densities (Choi et al., 2004).

2.2.2. Design of Microbial Fuel Cell. Researchers have proposed a variety of scalable designs for constructing an MFC. In most studies, the configuration commonly adopted was the traditional, dual chambered (H-shaped) MFC, in which two bottles or chambers are connected by means of a tube containing a separator membrane (Logan et al., 2006). Initially, reactors used a salt bridge (Min et al., 2005) as the ion exchange channel between the anode and the cathode chamber, but these were later replaced by cation/proton exchange membranes. Single chambered fuel cells that use air or aqueous cathodes result in a higher power density than the conventional two-chambered model (Logan et al., 2006). Many modifications of the existing models have recently been implemented to increase the power densities and to maintain a consistent current production. Some of the best known designs include an upflow tubular type MFC (Rabaey et al., 2005), a flat plate design (Min and Logan, 2004), a stacked MFC (Aelterman et al., 2006), and a U-tube MFC (Zuo et al., 2008). A sediment-type MFC, that uses sediments and the overlying water as the anode and cathode respectively, has recorded power densities as high as 55 mW/m^2 with sea water (Scott et al., 2008). Bio-electrochemically assisted microbial reactors (BEAMRs) are an improvement on MFCs. In BEAMRs, a small potential applied to the anode generates hydrogen at the cathode (Liu et al., 2005).

2.2.3. Materials and Operating Conditions of Microbial Fuel Cells. The anode and the cathode materials for the reactor are selected based on several properties, including high surface area, chemical stability, biocompatibility (anode), and good conductivity. As an anode material, carbon is preferable to metals such as copper because the latter is toxic to the bacteria (Tanisho et al., 1989). A higher anode surface area produces higher current densities (Chaudhuri and Lovley, 2003). Thus, carbon felt, carbon foam, and graphite felt have proved very effective. The most commonly used testing materials, however, include graphite rods, carbon paper and cloth, and reticulated carbon (Logan et al., 2006). The cathode electrode is usually the same material as the anode, although researchers have experimented with various combinations. In some trials, the cathode chamber worked under the principle of oxygen reduction, and platinum catalysts were used to increase the rate of reduction (Reimers et al., 2001). The ion exchange membrane plays an important role, not only for its intended purpose of transferring protons from the anode to cathode chamber, but by preventing oxygen intrusion in the reverse direction. Membranes used for this purpose include materials such as Nafion (Bond et al., 2002) or Ultrex (Rabaey et al., 2003).

A major application of MFCs is in the treatment of wastewater. Hence, most experiments have used sewage samples in the anodic chamber (Angenent et al., 2004; Zuo et al., 2008; Min and Logan, 2004). However, a variety of media ranging from simple to complex has been developed for the anode from numerous sources to study the features of any anode respiring bacteria that may be present. The cathode chamber, on the other hand, requires a solution that can accept the incoming protons from the anode. Among the most common cathode solutions are water (containing a platinum catalyst)

with air pumped in (Reimers et al., 2001), potassium ferricyanide (Zuo et al., 2008; Oh and Logan, 2006), and potassium dichromate (You et al., 2006). The choice of cathodic solution is important because experimental results have shown that some have advantages over the others. For example, MFCs with ferricyanide have been shown to produce 1.5-1.8 times more power densities than those using air with platinum (Oh and Logan, 2006), and potassium permanganate has produced 4.5 and 11.3 times more power density than ferricyanide and oxygen respectively (You et al., 2006).

Various other factors contribute to efficient extraction of maximum power from an MFC system. These include the distance between the two electrodes, ionic strength, and temperature (Liu et al., 2005). A power increase of up to 85% has been observed when ionic strength (NaCl concentration) is varied. Since MFCs are primarily applicable in waste water treatment, increasing the salt concentration of waste water would seem impractical. However, results of studies on the effects of ionic strength suggest that when used with bacteria that can grow on salt water, MFCs may be highly effective for use with saline industrial wastewaters or in conjunction with wastewater treatment systems (Bond et al., 2002). Industries such as agro-based, petroleum, and leather generate highly saline wastewaters with large amount of organics that are sometimes difficult to treat by using a conventional wastewater treatment facility (Lefebvre and Moletta, 2006). This difficulty would be primarily due to the fact that the anaerobic bacteria normally used in the digesters cannot tolerate such high salt concentrations.

2.3. GLYCEROL TO ETHANOL CONVERSION

The recent interest in biofuels has been driven by high energy prices, increasing energy imports, concerns about petroleum supplies, and greater recognition of the environmental consequences of fossil fuel use (Hill et al., 2006). Biodiesel is the foremost alternative, and it has attracted much interest due to its many promising benefits. To make biodiesel production economically feasible, many strategies have been adopted by various industry sectors. One such strategy is to use the byproducts or waste streams generated during the production of biofuel (Yazdani and Gonzalez., 2007). Following is an analysis of recent studies intended to investigate potential use of glycerol, a primary byproduct of the biodiesel industry.

2.3.1. Biodiesel Production Process. Biodiesel has been produced from a number of sources. Esters from vegetable oils are the best substitutes for diesel because they demand no modification of the diesel engine and have a high energy yield (Haas, 2005). Some of the best sources of biodiesel have been agricultural: oil from soybeans (Haas, 2005), castor, sunflower, and sorghum (Ugarte and Ray, 2000). Another natural but nonagricultural source of biodiesel that has received recent attention is microalgae (Scragg et al., 2003; Chisti, 2007). Biodiesel is produced by the transesterification of triglycerides (i.e., oil obtained from sources such as those listed as above) with short chain alcohols in the presence of a suitable catalyst (Pinto et al., 2005). The transesterification reaction is shown in Figure 2.1

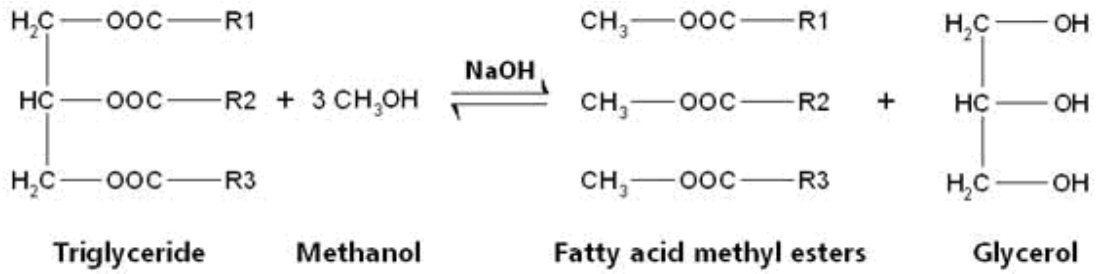


Figure 2.1. Transesterification reaction involving the conversion of triglycerides into biodiesel with the simultaneous production of glycerol.

The transesterification reaction can be initiated by both acid and alkaline catalysts using a homogeneous or heterogeneous catalytic process. Sodium and potassium hydroxide are commonly used as industrial catalysts (Ma and Hanna., 1999). Transesterification using conventional alkali catalysis quickly imparts high conversion levels of triglycerides to their corresponding methyl esters. In most commercial production processes, glycerol is the most common byproduct, and some alcohol is produced that can be reused. Before the expansion of biodiesel production, glycerol was a high-value commodity used in the production of cosmetics. With the current scale of biodiesel production, however, glycerol is widely available, and the excess must be disposed of properly. Every 9 kg of biodiesel is estimated to produce about 1 kg of a crude glycerol by-product (Dasari et al., 2005). A few manufacturers outside the biodiesel industry generate glycerol in their waste stream. Among these is the oleochemical industry, whose waste streams contain around 55–90% glycerol (Hazimah et al., 2003).

2.3.2. Strategies for Useful Application of Glycerol. Crude glycerol derived from biodiesel production has a very low value because of its impurities, and further refinement of crude glycerol will depend on the scale of production and the availability of a glycerol purification facility (Pachauri and He, 2006). As mentioned above transesterification through alkali catalysis produces the desired reaction but the process creates problems with the recovery and treatment of wastes (Fukudu et al., 2001). In light of recent developments, scientists are now contemplating ways to convert glycerol into value-added products for use in the fuel, chemical, automotive, pharmaceutical, detergent, and building industries (Pagliaro et al., 2007).

Among the fields in which glycerol might be used, the fuel sector has probably received the most attention. Biological transformation has advantages over chemical techniques because it circumvents problems related to low product specificity, use of high pressure and/or temperatures, and high contaminate levels in crude glycerol (Yazdani and Gonzalez, 2007). The fermentation process, especially with glycerol, is profitable due to several advantages such as cost effectiveness, higher fuel yields, and reduced chemical waste. This process also generates more reducing equivalents than are obtained from common sugars such as glucose or xylose (Dworkin and Falkow, 2006).

2.3.3. Glycerol Fermentation Mechanism in Bacteria. Glycerol is particularly important in the osmoregulation of algae, yeasts, and fungi, but only a little is known about the role of glycerol in the metabolic or regulatory activities of prokaryotes (Galinski, 1993). Studies of the metabolism involved in fermentation of glycerol have been observed in many species of Enterobacteriaceae family including *Citrobacter freundii* and *Klebsiella pneumoniae* (Homann et al., 1990). One of the most widely

studied products of glycerol fermentation, 1, 3-propanediol, is synthesized by two parallel pathways, one oxidative and another reductive (Dworkin and Falkow, 2006). The latter pathway produces the main fermentation product 1, 3-propanediol by means of reduction through a coenzyme, B12-dependent glycerol dehydratase.

A considerable number of reducing equivalents is generated when the reduced carbon of glycerol is incorporated in the cell biomass (Booth, 2005). However, only few studies have investigated the mechanisms and pathways mediating the dissimilation of glycerol in glycerol-fermenting organisms like *Propionibacterium* and *Anaerobiospirillum* (Lee et al., 2001). Complete oxidation of glycerol has also been reported previously, and some have suggested that the process could be improved through interspecies hydrogen transfer when the glycerol fermenters are grown in co-culture with hydrogen-consuming, sulfate-reducing bacteria (Tsai et al., 1995). In two marine *Desulfovibrio* strains, glycerol has been shown to be degraded to acetate through glycerol-3-phosphate and dihydroxyacetonephosphate as a part of the glycolytic pathway (Kremer and Hansen, 1987).

When grown at suitable conditions (e.g., acidic pH, specific medium composition and prevention of hydrogen gas accumulation), *E. coli* has been found to ferment glycerol (Gonzalez et al., 2008). Owing to its abilities to grow over a wide range of conditions, strains of *E. coli* have been genetically engineered to efficiently produce ethanol (Ingram et al., 1987). Yeasts and the bacterium Z*ymomonas mobilis* are capable of converting sugars directly into ethanol alone through the use of pyruvate decarboxylase. An attempt to engineer *E.coli* to express in them such a yeast-like pathway was unsuccessful (Dien et al., 2003). However, efforts aimed at modifying and expressing certain desired genes are

proving effective with the formation of hydrogen, formate, etc., when glycerol is used as the carbon source (Ito et al., 2005; Sakai and Yagishita, 2007). Other organisms shown to be able to ferment organic compounds to produce ethanol include *Klebsiella planticola* (Jarvis et al., 1997) and *Enterobacter aerogenes* HU-101 (Ito et al., 2005). The latter species was tested to determine if it could use crude glycerol from the biodiesel industry as a carbon source. This work demonstrated that the strain HU-101 ferments glycerol to ethanol and formate. The production of ethanol, however, was minimal due to the high salinity of the waste stream. It was suggested that the use of bacteria that can be genetically modified or naturally capable to survive these saline conditions would be suitable for the treatment process.

PAPER

I. ELECTRICITY GENERATION IN A MICROBIAL FUEL CELL USING BACTERIA FROM A HALOALKALIPHILIC LAKE

1. ABSTRACT

Anaerobic, metal-reducing bacteria have been shown to attach and transfer electrons obtained from organic compound oxidation to the surface of electrodes in a microbial fuel cell (MFC), thereby producing current. Such electron shunting ability has been identified in several species of bacteria. The present study used bacteria isolated from a saline, alkaline environment, Soap Lake, Washington, in an MFC. These samples were initially enriched for iron (III) reduction in batch cultures and the enrichments were used to inoculate the anode chamber of the MFC. Scanning electron microscopy showed the bacteria attached to a carbon cloth electrode. Current densities up to 12.5 mA/m2 were shown to be produced. The open circuit voltage was constant over several days, reaching up to ~1V. The MFC that was run with uninoculated media showed very little current which dropped within one day. Cyclic voltammetry experiments demonstrated that the bacteria made use of an electron mediator, methylene blue to enhance the transfer of electrons to the anode. Molecular characterization of the partial 16S rRNA gene and RFLP analysis showed that the bacterium responsible was a single species with a 97% similarity to the 16S rRNA gene of *Halanaerobium sp*.

2. INTRODUCTION

In a microbial fuel cell (MFC), bacteria produce current by oxidizing organic compounds and shunting the resulting electrons on to the anode electrode surface. The anode electrode, acting like a terminal electron acceptor, receives and transfers the electrons obtained from the bacteria over to the cathode to form an electrical circuit. In the cathode chamber, the electrons are passed on to an electron acceptor such as oxygen or potassium dichromate (Rittmann, 2008). A wide range of fermentation products and organic compounds are currently used in MFCs for current extraction (Regan and Logan, 2006). The primary intended application of MFCs is in waste water treatment; therefore several researchers have used waste water from sewage plants in the anodic chamber of the fuel cell (Angenent et al., 2004; Zuo et al., 2008; Min and Logan, 2004).

Bacteria capable of transferring electrons to an electrode are referred to as anode respiring bacteria (ARB) or exoelectrogens (Lovley, 2008). This electron transfer is performed by the bacteria either through a direct transfer or by using an electron shuttling compound or mediator (Dogan et al., 2009). Obligate and facultative anaerobic bacteria use certain metals or inorganic oxidized compounds as their terminal electron acceptors (Lovley, 1993). Iron (III) is one of the most common metals used by bacteria under anaerobic conditions. In an MFC, this iron (III) is replaced with the anode electrode (usually a carbon material). In many investigations, microbes were initially enriched for iron (III) reduction activity before being employed in an MFC. Some of the first reported ARB include *Clostridium, Geobacter, Aeromonas, Rhodoferax, Desulfobulbus,* and *Shewanella* (Bretschger et al., 2007). *Gamma proteobacteria, Beta proteobacteria, Rhizobiales*, and *Clostridia* are predominantly found on the anode surface (Lovley,

2008). Jung and Regan (2007) have suggested that a synergistic interaction exists in an MFC that would account for the higher power densities obtained by using a mixed rather than a pure culture. For example, some species in a mixed culture may act fermentatively, degrading the more complex organic material and providing the breakdown products to the ARB, which would then oxidize the organic compound completely.

Several modifications in the design of MFCs have been adopted to improve current and power densities. A dual chambered configuration is the most common one applied in research (Logan et al., 2006). The anode and cathode materials for the reactor are selected based on several properties, including high surface area, improved chemical stability, biocompatibility (anode), and good conductivity. The choice of materials and conditions, and the understanding of the mechanism by which the bacteria attach and grow on these electrodes are important to obtain better yields of current. In one study, for example, the power production in an MFC showed an increase of up to 85% when the ionic strength (i.e., sodium chloride concentration) was raised from 100 mM to 400 mM (Liu et al., 2005).

Extremophilic microorganisms in MFCs have been applied extensively, particularly thermophiles (Milliken and May, 2007; Jong et al., 2006; Choi et al., 2004) and acidophiles (Malki et al., 2008). However, the application of halophilic or alkaliphilic bacteria in MFCs has not been investigated extensively (Miller & Oremland, 2008). This study investigates the use of haloalkaliphilic bacteria from Soap Lake in an MFC to extract electricity. Soap Lake, located in Washington, has salinity ranging from ~13 g/l in the mixolimnion to ~70 g/l in the monimolimnion. It also has high alkalinity, with the pH of the water column averaging 9.9 and the sediment averaging 10.6 (Dimitriu

et al., 2008). The bacteria from this lake have been shown to reduce iron (III) (Pollock et al., 2007; Dimitriu et al., 2008). Employing halophiles would help to substantiate the proposition mentioned earlier that higher ionic strength could increase the power values. The mechanism by which the bacteria donate their electrons to the anode was also studied by using cyclic voltammetry. Molecular diagnostic techniques were used to predict the bacterial species involved in the production of electricity.

3. METHODS

3.1. Bacterial Enrichments and Optimization. The sediment samples from Soap Lake were earlier enriched for iron reduction activity (Patel, 2006). The present study used bacterial cultures from these iron reducing enrichments as the initial cultures. These cultures were then further sub-cultured by the following techniques: The bacteria were grown in a slightly modified Soap Lake basal medium (SLBM) called SL3 medium (Begemann et al., 2008). This medium is an anaerobic, synthetic medium mimicking the chemical conditions present in Soap Lake (Mormile et al., 1999). The media has the following components (per liter of de-ionized water): K_2HPO_4, 13.5g; Yeast Extract, 1g; NaCl, 70g; Na_2MoO_4, 4.84g; Cysteine-HCl (10%), 1.5 ml; Na_2CO_3, 40g; $CaCl_2$. $2H_2O$, 0.42g; $FeSO_4.7H_20$, 0.18g; SiO_2. $2H_2O$, 0.75g; $MgCl_2$. $2H_2O$, 0.852g; $MnSO_4$, 0.448g; NH_4NO_3, 5g; Methylene blue, 0.4g; Trace metal solution, 10ml. The trace metal solution consists of the following in 1 liter of de-ionized water: Nitrilotriacetic acid, 1.63 g; $MgSO_4$. $7H_2O$, 3 g; $MnCl_2$. $4H_2O$, 0.5 g; FeSO4. $7H_2O$, 0.1 g; $CaCl_2$, 0.1 g; $CoCl_2$. $2H_2O$, 0.1 g; $ZnCl_2$, 0.13 g; $CuCl_2$. $2H_2O$, 0.007 g; AlK $(SO_4)_2.12H_2O$, 0.01 g; H_3BO_3, 0.01 g; Na_2MoO_4, 0.025 g; $NiSO_4$. $6H_2O$, 0.03g; Na_2WO_4. $2H_2O$, 0.025g and NaCl, 1g.

Glycerol and other substrates, acetate, formate, butyrate, and propionate, were also separately used as electron donors (at a concentration of 30mM for each). The media were used to enrich for iron reduction activity by the addition of 20mM iron (III) citrate.

The medium was made anaerobic by boiling and cooling under an atmosphere of N_2:CO_2 (80:20) and subsequent placement in an anaerobic glove bag. The glove bag was maintained under a constant N_2:H_2 (90:10) atmosphere. Cysteine-hydrochloride was added to the media in the glove bag, and the pH was brought to ~10.5-11 with 10N NaOH. The reduced medium was then distributed in serum bottles capped with butyl-rubber stoppers and sealed by using aluminum crimps. The serum bottles containing the medium were then placed in an autoclave and sterilized for 30 minutes at 121ºC. The change in the methylene blue reagent from blue to colorless was used as an indicator of complete anaerobic conditions in the medium. Both Na_2CO_3 and iron (III) citrate were added after autoclaving. An inoculum volume of 10% was used for all inoculations. All analyses were performed on cultures that had been subcultured at least three times at an approximate interval of two to three weeks or until dense, black precipitation was observed, indicating ferric iron reduction. Optimal salinity and pH were determined by growing bacteria in SL3 medium with varying amounts of sodium chloride and at various pH levels. The growth of the bacteria was linked to the rate of production of iron (II).

3.2. Analysis of Iron Reduction. Iron reduction was initially confirmed by the formation of black precipitates of ferrous (II) iron on the bottom of the serum bottles. To quantitatively determine the amount of reduced iron [Fe (II)] present in the sample, the ferrozine technique was used (Lovley, 1986).

3.3. Microbial Fuel Cell. A dual chambered MFC was constructed with 250 ml Pyrex glass bottles (Corning Inc, Corning, NY). Both chambers possessed an arm ending in a joint (Figure 1). A proton exchange membrane (Nafion N 115, Fuel Cell Store, San Diego, CA) was sealed between the joints and clamped with a pinch clamp. The top opening of each chamber was closed with a rubber stopper. Prior to inoculating the MFC, the bacterial cultures were grown with four different electrode materials to determine the material to which the bacteria can bind most efficiently. The four electrode materials included carbon cloth (Clean Fuel Cell Energy, LLC, Clearwater, FL), carbon paper, reticulated carbon, and graphite rods. Scanning electron micrographs were acquired to confirm the most suitable electrode material to be used as anode. Electrodes cut into 5cm X 5cm dimensions were soaked in distilled water for one day before use in the MFC. The cathode and anode materials were connected through a titanium wire (California Fine Wire Company, Grover Beach, CA) to complete the circuit. The entire apparatus was wrapped with aluminum foil and autoclaved for 15 minutes before placement in the anaerobic glove bag overnight.

A sterilized solution of the modified SL3 media, excluding iron (III) citrate, was used as an anode solution. Electron donors, glycerol, acetate, and formate, were employed in separate trials. The cathode chamber contained either potassium ferricyanide (50mM) in a phosphate buffer or potassium dichromate (10mM). The anode chamber was continuously mixed by using a magnetic stirrer. A 10% inoculum of bacterial culture from the enrichments was added to the anode chamber. All exchange of medium components and inoculations was performed under anaerobic conditions. An MFC

experiment was run with all components of the media except the bacterial cultures to determine whether current was produced without the presence of bacteria.

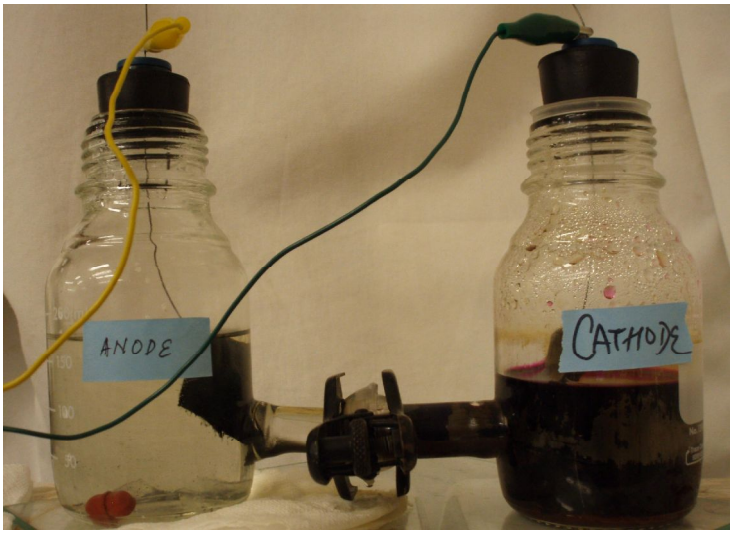


Figure 1. Dual-chambered MFC showing the anode and cathode chambers.

3.4. Electrical Measurements. The voltage (V) and resistance (R) were measured by using a digital multimeter (HP). Current (I) and power (P) were calculated by applying Ohm's law (V=IR) and power law (P=IV) respectively. Current and power densities were calculated by dividing the obtained current and power values by the surface area of the electrode (i.e., 25cm^2). Polarization and power curves were derived by applying resistances from 500 to 1 kΩ using a variable resistor box, allowing sufficient time for voltage stabilization at each applied resistance (Logan et al., 2006). Cyclic voltammetry (CV) experiments were performed by using a Princeton Potentiostat Model 273A at a scanning rate of 50mV/s. The experiments were performed under various conditions to understand the catalytic activity occurring on the surface of the carbon cloth

electrodes. Previously, methylene blue was shown to act as a potential electron mediator enhancing the amount of current produced (Sund et al., 2007). To determine the role of this compound in electron transport, CV experiments were run with bacteria grown in separate media in the presence and absence of methylene blue. Experiments were also conducted by using only the bacteria with no carbon substrate in the media. Changes in the cyclic voltammograms that occurred when formate was added were then recorded.

3.5. Scanning Electron Microscopy (SEM). Imaging using SEM was required to determine the most suitable anode material for use in the MFC, and also to confirm the presence of bacteria in MFCs that produced current. The anode material was carefully removed from the serum bottles (or anode chamber) and a small piece (1cm X 1cm) was cut and used for imaging. The samples were prepared for SEM by the technique mentioned in Milliken and May, 2007. Before imaging with a field emission SEM Hitachi S4700 at Missouri S&T, the samples were sputter coated with gold and palladium for 4 minutes. The images were edited and analyzed with ImageJ software. In addition, SEM images of the bacteria from the media were obtained. Bacterial samples of 2 ml were filtered through a 0.22-µm filter membrane. The membrane was then prepared for SEM analysis as above and images were obtained. In other cases, the bacterial cultures were centrifuged, resuspended in a 2.5% glutaraldehyde fixative, and sent to the University of Missouri's Electron Microscopy Core Facility for imaging by using SEM.

3.6. DNA Isolation and Polymerase Chain Reaction (PCR). A small portion of anode material was cut, and the electrode was scraped by using a sterile spatula. Bacteria present in the anode media (in which current production was observed) was collected separately along with the anode material. From both collections, DNA was isolated by

using an UltraClean Soil DNA isolation kit (MoBio Laboratories, Solana Beach, CA). The isolated DNA was stored at -20ºC. Following DNA isolation, PCR was performed to amplify bacterial 16S rRNA gene fragments. Each reaction mixture consisted of the following components in a PCR tube: 2X GoTaq Green Master mix (Promega, Madison, WI), 12.5µl; forward primer 27F (10 pmol), 2.5µl; reverse primer 1392R (10 pmol), 2.5µl; nuclease free water, 5.5µl, and DNA template, 2µl. Primers were synthesized by MWG Biotech (High Point, NC). The primer sequence was as follows: 27F, 5'-TTCCGGTTGATCCYGSCR-3' (Lane, 1991); 1392R, 5'-ACGGGTGTGTRC-3' (Stahl and Amann, 1991). The PCR conditions for amplifying DNA included an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds**,** 50°C for 1 minute, and 72°C for 1 minute and 30 seconds. The reaction was completed with a final extension step at 72°C for 10 minutes. PCR was also performed with archaeal primers, 21F (5′-TTCCGGTTGATCCYG CCGGA-3′) and 1392r (5′-GGTTACCTTGTTACGACTT-3′) (Lane 1991), to verify if any members of the archaeal group were present. The PCR conditions for amplifying archaeal 16S rRNA gene included an initial denaturation at 95°C for 2 minutes, followed by 35 cycles of 95°C for 45 seconds**,** 50°C for 45 seconds, and 72°C 45 seconds. The reaction was completed with a final extension step at 72°C for 7 minutes. The components for the reaction were similar to those used in amplifying bacterial 16S rRNA gene except for the primers. Following PCR amplification, the products were run on a 1% agarose gel. After staining with 0.5µg/ml ethidium bromide solution, the gel was observed under a UV lamp to confirm visually the presence of the DNA fragment (based upon molecular weight). The

PCR amplification products were purified by using a QIAquick PCR purification kit (Qiagen, Valencia, CA).

3.7. Cloning and Restriction Fragment Length Polymorphism. The amplified 16S rRNA genes were then ligated into suitable vectors provided in the p-GEM T-Easy Vector system kit (Promega, Madison, WI, USA). The manufacturer's protocol was followed throughout the cloning process, including transformation. The transformation reaction was examined by using the blue/white screening method. In this method, transformed *E. coli* cells containing the plasmid vectors were grown on X-gal-IPTG-LB-ampicillin agar plates overnight at 37°C. White colonies (containing the inserts) were each grown in 5ml LB-ampicillin broth overnight. The plasmid DNA was then isolated from the cultures by using a Wizard Plus SV Miniprep kit (Promega, Madison, WI, USA). To test for the presence of multiple strains in the cultures, another PCR reaction was performed with primers 27F and 1392R on the plasmids under the conditions described above. The PCR products were then double digested with restriction enzymes, Eco R1 and Bam H1 (Promega, Madison, WI) according to the manufacturer's instructions. The digestion patterns were viewed on a 2% agarose gel.

3.8. Sequencing and Phylogenetic Analysis. The 16S rRNA gene was sequenced on a 3130 Applied Biosystem (Foster City, CA) with primers SP6: 5'-CATTTAGGTGACACTATAG-3' and T7: 5'-TAATACGACTCACTATAGGG-3' (Nag et al., 1988) at the Missouri S&T cDNA Resource Center (Rolla, Missouri). The acquired sequences were edited and aligned by using MEGA 4 clustal alignment software (Tamura et al., 2007). The sequences were then compared for related bacterial species by running them on NCBI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) (Zhang et al., 2000) and

EZ-Taxon (Chun et al., 2007). A neighbor-joining phylogenetic tree was constructed by using the MEGA 4 software.

4. RESULTS

4.1. Iron (III) reduction. Reduction of iron (III) was visually confirmed by the presence of black precipitates (Figure 2) of iron (II) on the bottom of the serum bottles. Results from quantification of reduced iron in media containing various electron donors showed that all substrates were used (as shown by the rate of iron (II) production) at almost equal rates (Figure 3). Formate, which had the highest iron reduction rate among the substrates used, was chosen for use in the MFC.

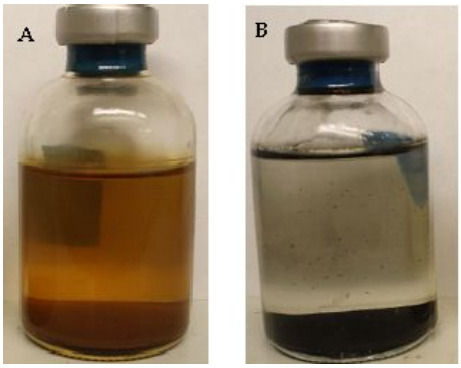


Figure 2. Iron (III) reduction. A. Before inoculation. B. Formation of black precipitate of Fe (II) after inoculation.

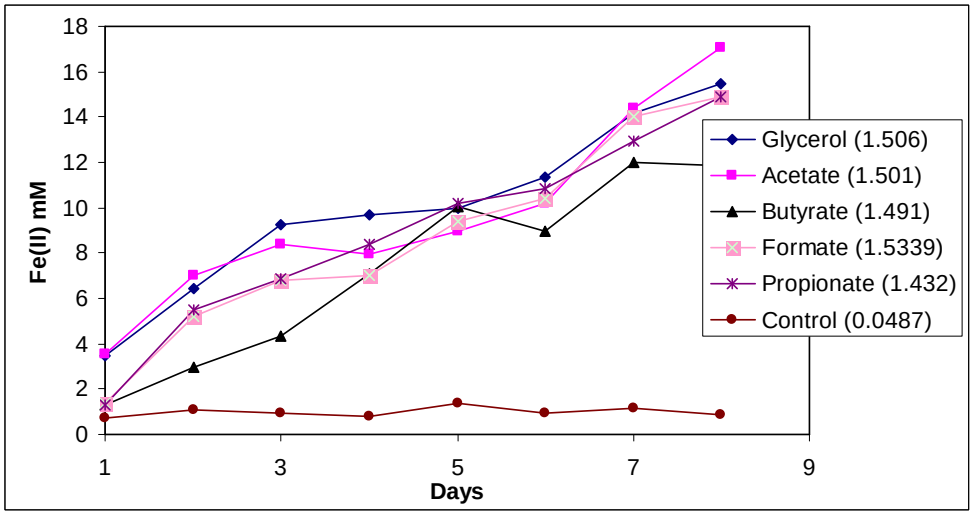


Figure 3. Substrate utilization linked to production of iron (II). The values in the parentheses indicate the rate of iron (II) production in mM/day.

The optimization results for pH and salinity are shown in Figures 4 and 5 respectively. The figures demonstrate that maximum production of iron (II) occurred at a pH of 11 and salinity of 7%. These salinity and pH value can thus be considered optimum for the growth of this haloalkaliphilic bacterial culture under iron-reducing conditions.

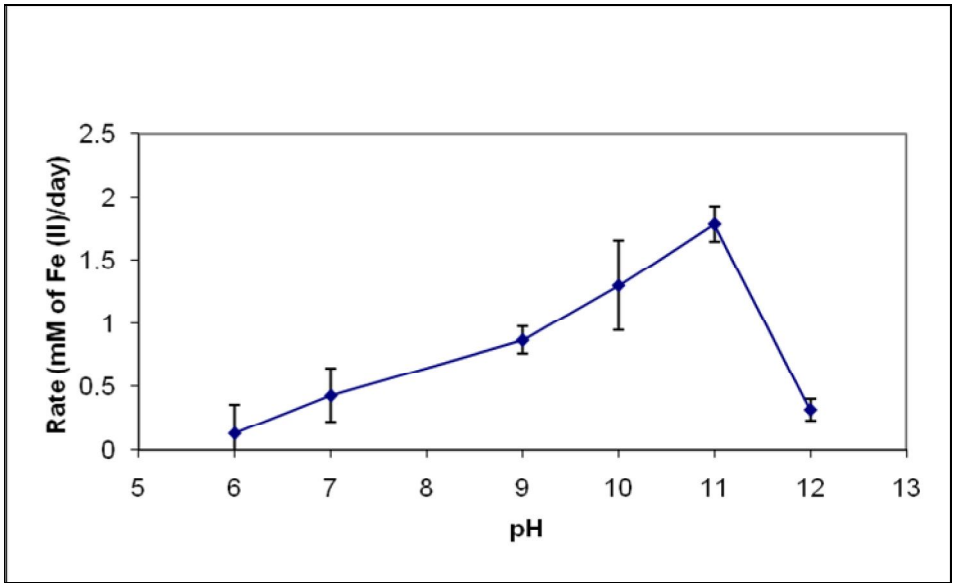


Figure 4. Optimal pH related to the rate of iron (II) production.

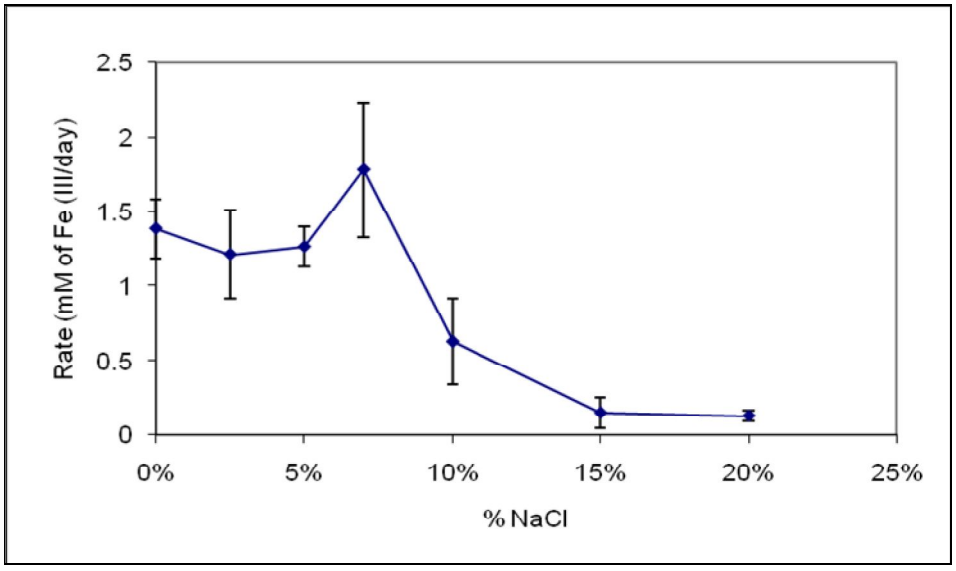


Figure 5. Optimal salinity related to the rate of iron (II) production.

4.2. Electrode Biocompatibility. The choice of the suitable anode material to be used in MFC was determined by using SEM imaging. Carbon paper and carbon cloth were effectively bound by bacterial colonies, as shown by the SEM images in Figure 6. Reticulated carbon and graphite rods did not show the presence of bacterial colonies (Figure 7).

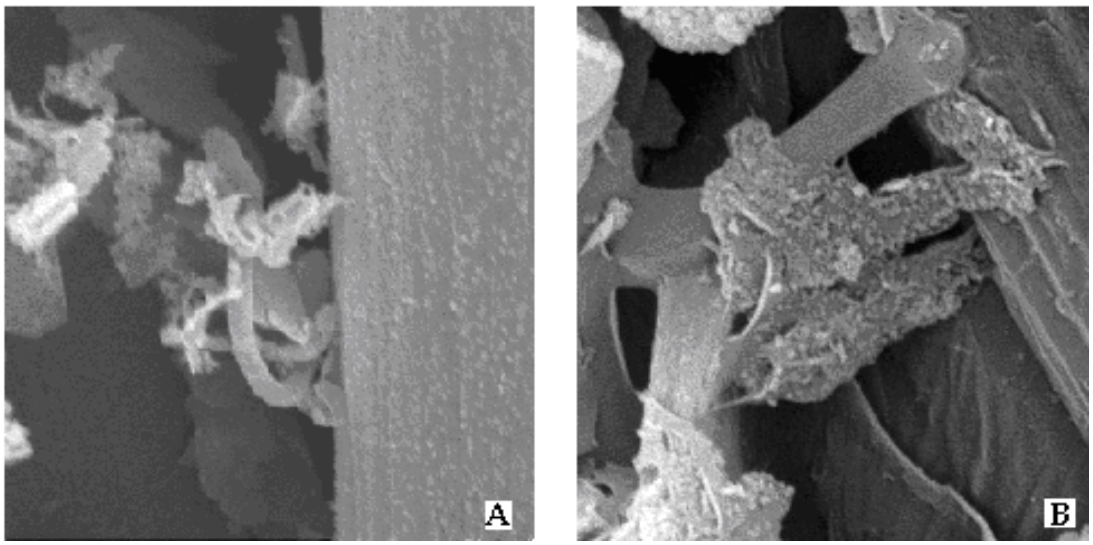


Figure 6. SEM image of bacteria attached to carbon cloth (A) and carbon paper (B) (Original magnification: 5980X (A), 25000X (B)).

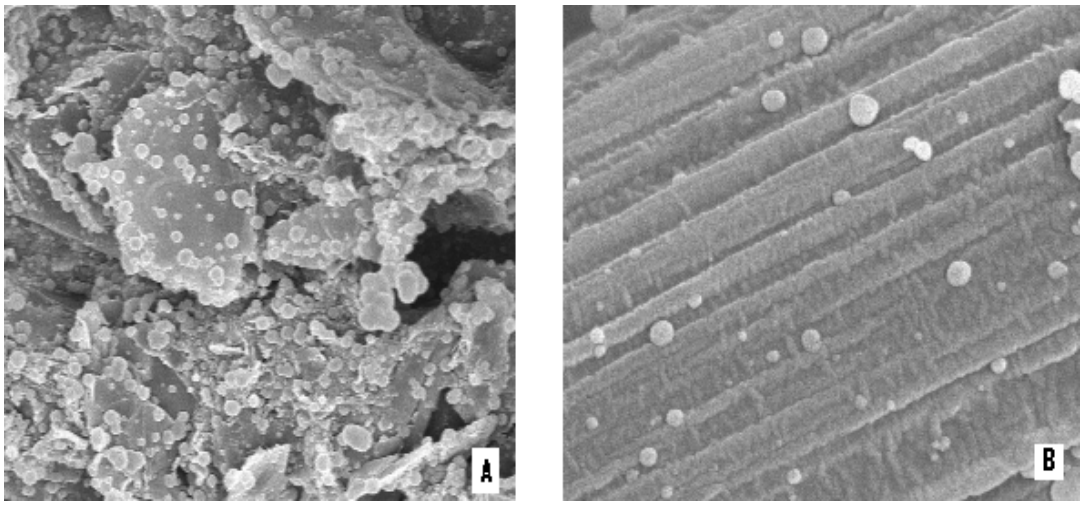


Figure 7. SEM image of reticulated carbon (A) and graphite carbon (B) materials (Original magnification: 2500X (A), 11000X (B)).

4.3. Electrical Measurements. The maximum current density obtained in the normal run with formate as the substrate was 12.52 mA/m^2, as shown in Figure 8. Figure 9 represents the open circuit voltage calculated with no resistance applied, reaching a maximum of 1 V. Current and power densities with the uninoculated run in the MFC was much less and dropped to negligible amounts within 24 hours (Figure 10). The maximum power density obtained with formate was 1.66 mW/m^2 at a current density of 4.7 mA/m^2, as shown by the power curves in Figure 11. The internal resistance of the MFC was calculated by measuring the slope of the polarization curve, which gave a value of 61 Ω.

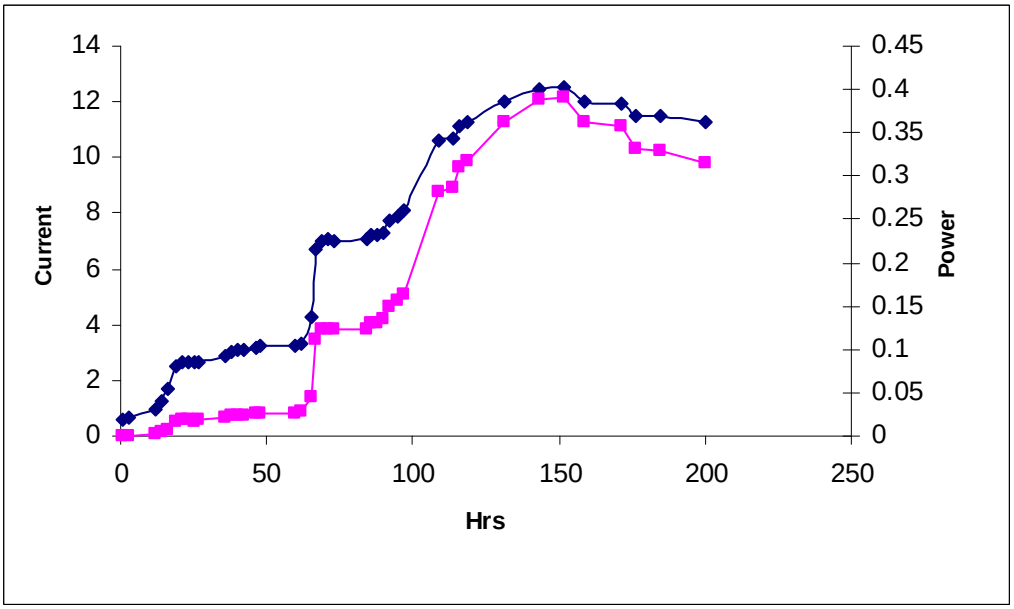


Figure 8. Current density (diamonds) in mA/m^2 and power density (squares) in mW/m^2 by using formate (30mM) as substrate.

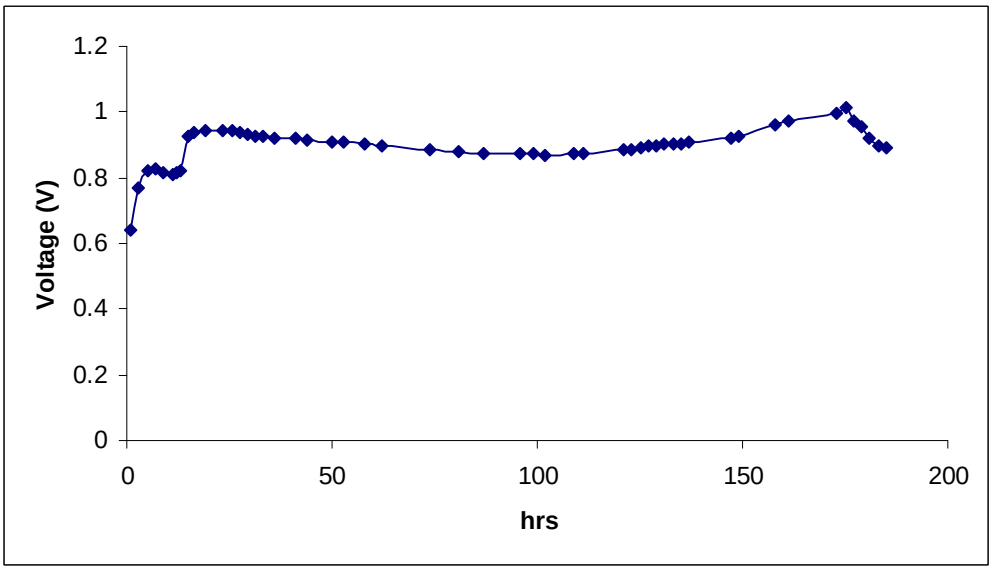


Figure 9. Open circuit voltage (in Volts) by using formate (30mM).

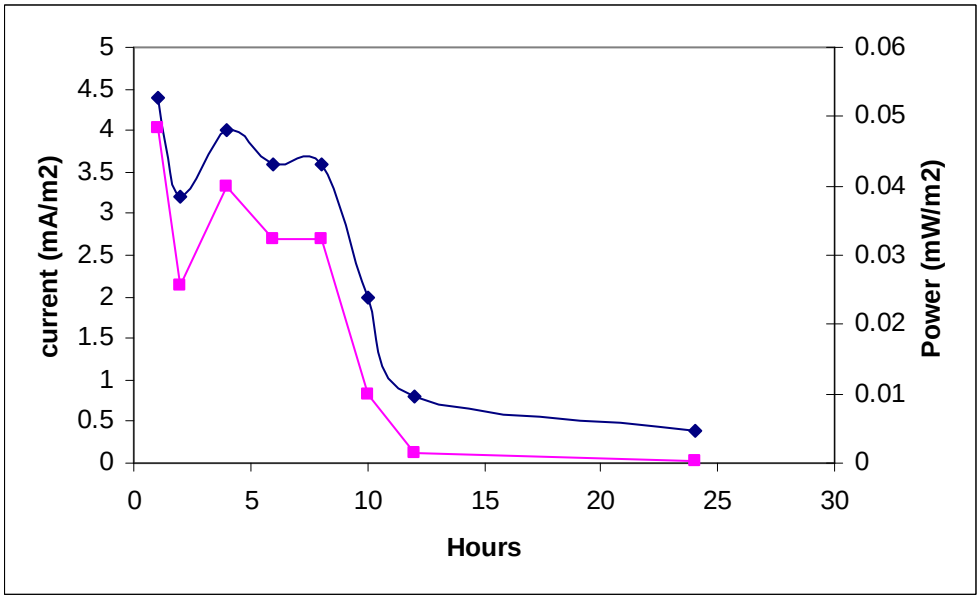


Figure 10. Current and power densities in MFC with no bacterial inoculation.

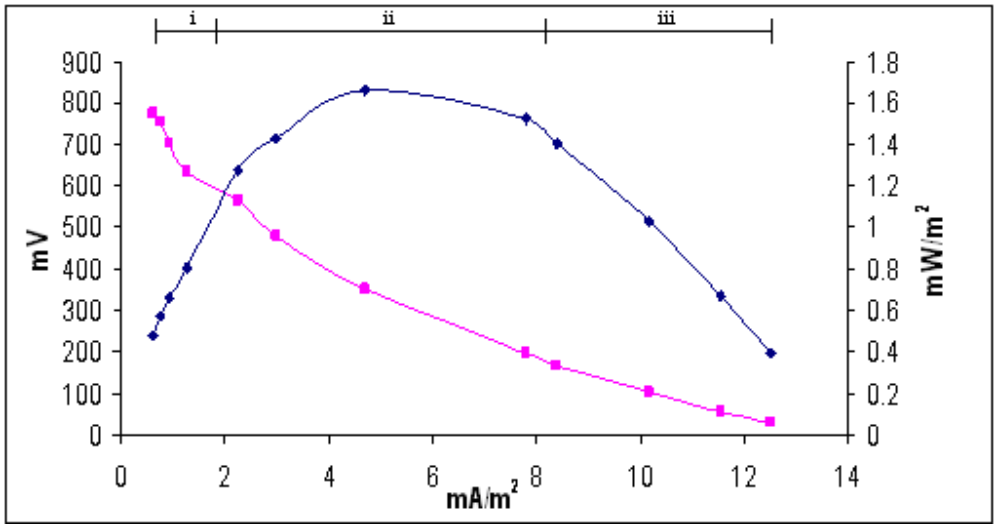


Figure 11. Polarization curve (squares) plot between current density and voltage and power curve (diamonds) plot between current density and power density. Sections i, ii, and iii represent the regions where different losses occur on the polarization curve.

The results from cyclic voltammetry experiments (Figure 12 and 13) indicate the influence of methylene blue in the catalysis reaction. In the absence of methylene blue and formate, the maximum peak current (green) obtained was very less. The peak current with bacterial media containing methylene blue alone (red) showed a slight increase than the one with only formate (blue). A considerably higher value of peak current was observed when the media containing methylene blue and formate (black) was tested. These results suggest that the bacteria make use of the electron mediator compound, methylene blue to enhance the transfer of electrons to the anode.

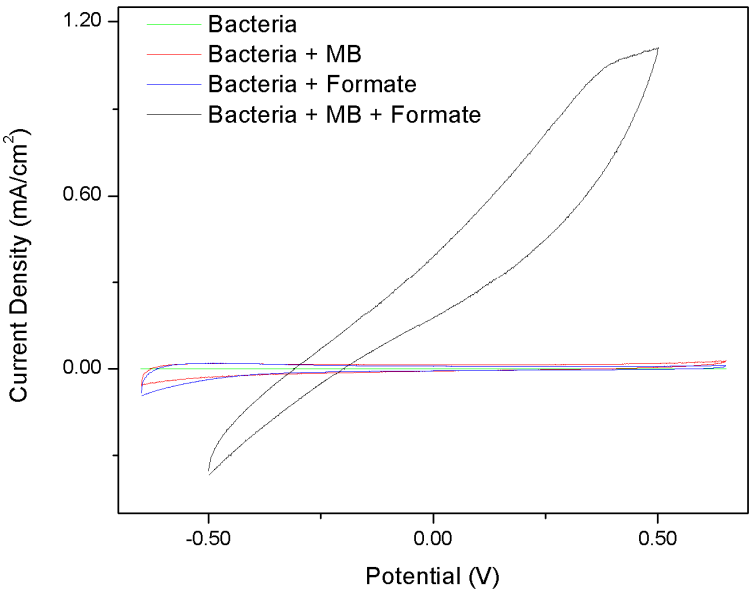


Figure 12. Cyclic voltammogram results obtained with different media components.

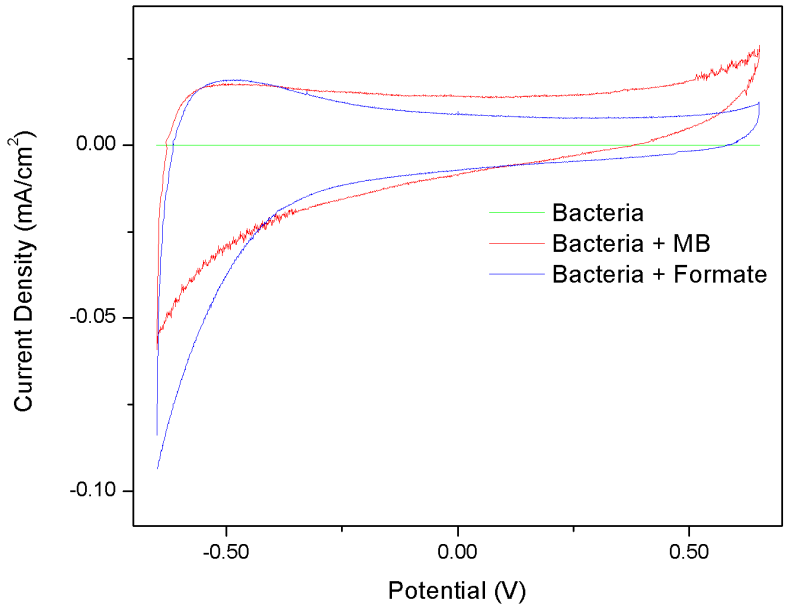
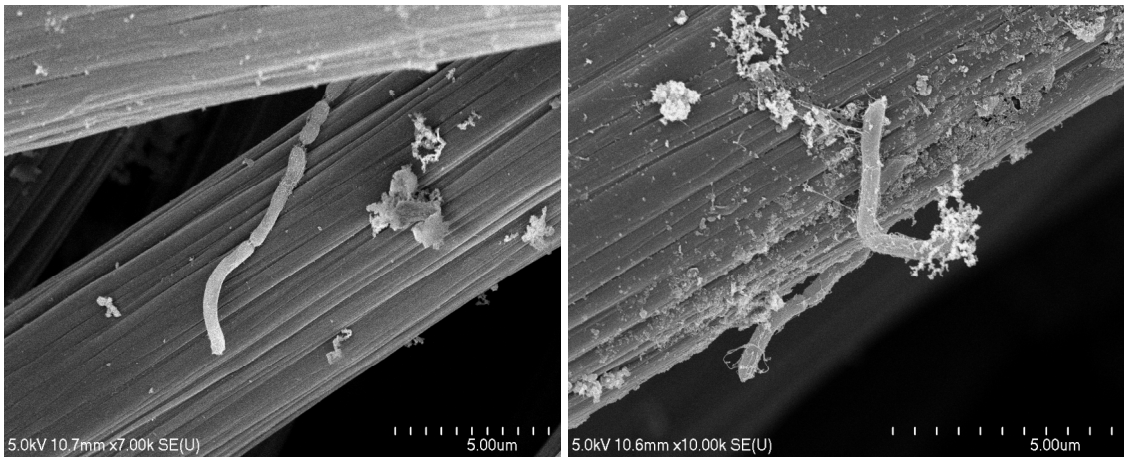


Figure 13. Cyclic voltammograms (enlarged results of bacteria, bacteria+MB and bacteria+formate).

4.4. Scanning Electron Microscopy. Images obtained from the carbon cloth anodes used in the MFC show growth of bacteria on the surface of the electrode (Figure 14, A and B).



(A) (B)

Figure 14 (A and B). Bacteria attached to the surface of a single carbon cloth strand.

4.5. Molecular Characterization. As shown in Figure 15, RFLP of the clones from the bacteria attached to the anode material and from the anode media showed similar digestion patterns.

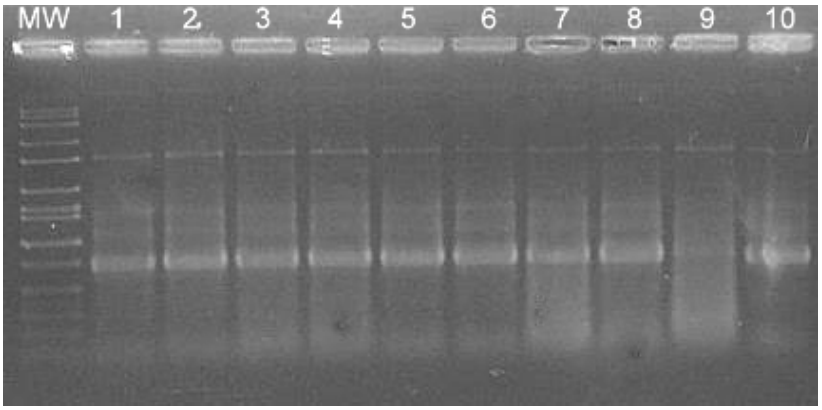


Figure 15. Restriction Fragment Length Polymorphism pattern of ten of the transformed clones from the MFC anode.

Analysis of the 16S rRNA sequences of the clones showed the presence of a single species of bacteria, confirming the RFLP results. Both the bacterial DNA collections (i.e., from the electrode alone and the media) revealed the same RFLP patterns and similar relatedness profile with other bacteria in the database, concluding that the haloalkaliphilic bacteria in the MFC is a pure culture. PCR performed with the archaeal primers did not reveal any products. Hence it was concluded that no archaeal species was present in the culture. The closest match based on results from NCBI-BLAST gave ~97% gene similarity to *Halanaerobium sp*. and a number of uncultured clones. Results from EZ-taxon gave a 96.4% gene similarity with *Halanaerobium praevalens*. The phylogenetic tree by using neighbor-joining method was constructed with the 16S rRNA gene (Figure 16). Bootstrap analysis for 100 replicates was performed. The haloalkaliphilic species in the phylogenetic tree did not cluster closely with any of the other bacterial strains and therefore could possibly represent a novel species.

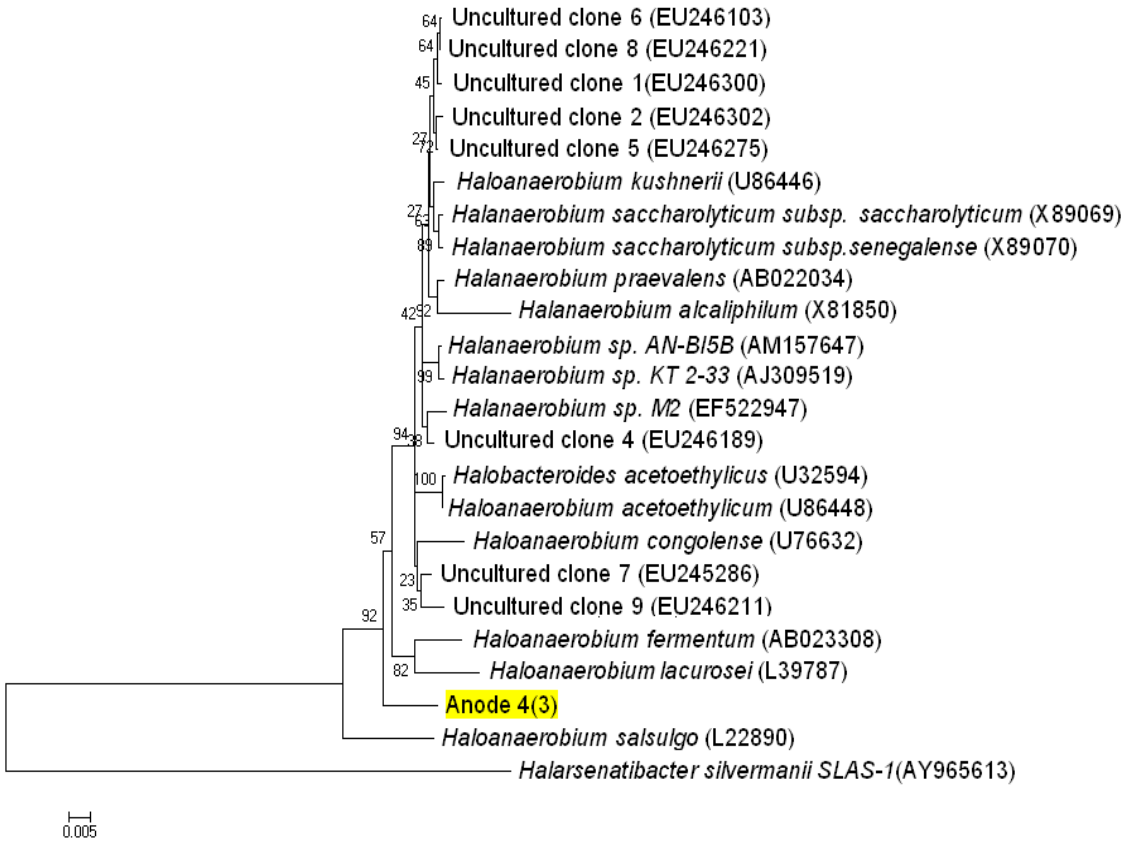


Figure 16. Phylogenetic tree constructed by using neighbor-joining analysis with 100 bootstrap replicates. Bar represents 0.02 substitutions per nucleotide position. The electricity generating culture is colored. NCBI accession numbers are provided in parentheses.

5. DISCUSSION

The Microbial Fuel Cell using haloalkaliphilic bacteria produced current and voltage for a consistent amount of time. The bacterial enrichment for iron (III) reduction prior to inoculation in the MFC could have helped in reducing the lag time for the development of current. Formate showed the fastest rate of iron (II) production and was thus chosen as the substrate in the anode chamber. Other experiments (not shown here) were run with potassium ferricyanide as the cathode solution. However, the voltage and

current drop in these cases was rapid owing to the minimal stability time of the chemical. Hence, potassium permanganate was applied in the other experiments, providing a more stable oxidizing environment.

Analysis of the polarization curves permits prediction of the actual resistances to the reaction (sections (i), (ii), and (iii) in Figure 11). Moving down the curve, the initial steep drop (i) in voltage was primarily due to the activation losses that occur during the transfer of electrons at the anode surface. The following steadier decrease in voltage (ii) with an increase in current is attributable to ohmic losses, i.e., the resistance to the flow of electrons through the carbon cloth material. The final decrease in voltage (iii), due to concentration losses (Logan et al., 2006), is very limited; therefore, the reaction was not affected by the mass transport effects of the various components of the media. Residual iron from the iron-enriched bacterial inoculation may have competed with the carbon cloth electrode to act as a terminal electron acceptor. However, such an effect would not have significantly hindered the shunting of electrons to the electrode because the cultures used for inoculating the MFC would already have exhausted iron (III) leaving a significant portion of iron (II), which has not been shown to act as an electron acceptor. A study of the power curve (Figure 11) reveals that the maximum power density that could be obtained with the system was 1.6mW/m^2. The power and current values, though low, were consistent in a single batch-type run. The low values can be attributed to several factors, including the various losses mentioned above. The high percentage of sodium chloride in the media could have either increased the current (Liu et al., 2005) or hindered efficient electron transfer, although no evidence exists for the latter possibility. The haloalkaliphiles and most halophilic microorganisms have a slow growth (Oren,

1999) and metabolic rate and hence, using these organisms in an MFC may not contribute to better power values as expected. However the bacteria do produce consistent amount of power during the batch cycle.

Images with SEM revealed the presence of bacteria attached to the anode electrode; however, there was no heavy colonization of the bacteria on the electrode surface. This effect may have arisen for either or both of the following: the anode sample might have been disturbed during transportation resulting in dislodged cells from the electrode before imaging, or the bacteria may not have had direct contact with the electrode. That is, the bacteria could have been using an electron mediator such as methylene blue to transfer electrons to the electrode (Kim et al., 2002). The results from cyclic voltammetry confirmed the role of methylene blue in the transfer of electrons to the anode. The bacteria, along with the medium supplemented with formate, alone did not show high peak current. However, with the medium containing methylene blue, the peak value was very high, demonstrating the influence of the compound on the performance of the haloalkaliphilic MFC. Moreover, the methylene blue did not show peak current values when present alone with the bacteria. This showed that formate oxidation by the bacteria is important and the electrons released during the process were been carried by methylene blue to the anode, which served as the terminal electron acceptor. The experiments also suggest the reason for the lack of heavy colonization on the electrode surface when viewed under SEM. The bacteria did not need to physically attach themselves to the electrode as they used the electron shuttling compound to aid in the process.

The presence of a single bacterial species revealed by RFLP analysis was confirmed with the results from the 16S rRNA sequencing and BLAST. Previously, it was found that a pure culture produced current densities lower than or equal those of to mixed cultures (Nevin et al., 2008). In this study, a single haloalkaliphilic bacterial species was responsible for current production in a MFC. Since the closest match had a 97% similarity to the 16S rRNA sequences identified by BLAST and EzTaxon, the bacteria could be a novel species. Further biochemical and physiological characterization will be necessary, however, to confirm this hypothesis.

The effort reported here to use haloalkaliphiles in an MFC to produce current was successful with the extraction of a consistent amount of current and power. The bacteria seemed to mimic efficiently the iron (III) reduction activity in the MFC, although factors such as resistances and design could have lowered the current and power values. The bacteria showed growth on the surface of the carbon cloth electrode. A single species was determined to be involved in the electron transfer to the electrode, and this determination was confirmed by several molecular techniques. Better design and experimentation with other MFC configurations would enhance current knowledge of electricity production using haloalkaliphiles. A larger-scale version of the project would be helpful in applying the research to industrial use. Several parameters and conditions however must be optimized and analyzed for efficient application.

II. FERMENTATION OF GLYCEROL TO ETHANOL USING HALOALKALIPHILIC BACTERIA

1. ABSTRACT

Glycerol from biodiesel industry was a high value commodity that is currently being considered as an excess waste due to the large amount being produced. Microbial fermentation of glycerol to ethanol is an excellent strategy to consume the excess glycerol and, in turn, produce a value added product. The glycerol waste stream from biodiesel industry is characterized as a highly alkaline and saline solution. In the current research, bacteria from a haloalkaliphilic environment, Soap Lake, Washington, were studied for their fermentative activity on glycerol due to their ability to grow optimally under such extreme conditions. Glycerol was consumed at a slow rate and the process depended on the presence of ferric iron. The concentration of ethanol formed was low and did not show a particular rate of production. The maximum conversion rate obtained was around 2.83%. Molecular characterization by using RFLP and sequencing of the partial 16S rRNA gene revealed the presence of single bacterial species possessing 97% gene similarity to *Halanaerobium sp*.

2. INTRODUCTION

Biodiesel is developing into an excellent alternative for fossil fuels. With the rapid rise in the production of commercial biodiesel, this new industry requires that the entire operation be performed in an economically feasible and efficient manner. A prospective strategy to meet the above requirement would be to convert the wastes from biodiesel production into useful commodities. Glycerol, an inevitable by-product in biodiesel manufacturing, is produced in surplus amounts. It is estimated that for every 9 kg of biodiesel produced, about 1 kg of a crude glycerol is being generated (Dasari et al., 2005). Microbial fermentation of glycerol to ethanol has been shown to occur in several bacteria including members of the family Enterobacteriaceae such as *Citrobacter freundii* and *Klebsiella pneumoniae* (Homann et al., 1990).

1, 3-propanediol is a primary fuel product produced by the fermentation of glycerol in organisms such as *Klebsiella*, *Propionibacterium* and *Anaerobiospirillum* (Lee et al., 2001). Currently, ethanol can be produced with glycerol as the substrate in genetically modified species of *E. coli* (Gonzalez et al., 2008), *Klebsiella planticola* (Jarvis et al., 1997), and *Enterobacter aerogenes* HU-101 (Ito et al., 2005). Research on using the crude glycerol from biodiesel industry showed that the production of ethanol was minimal due to the presence of high salt in the glycerol waste stream (Ito et al., 2005). Furthermore, the transesterification process, which is the primary reaction involved in biodiesel production, requires use of alkali catalysts to initiate and quicken the process. This alkali catalyzed reaction generates a waste stream with glycerol that is highly alkaline. To ferment the glycerol efficiently, it would therefore be beneficial if the bacteria that are used for the process are able to grow optimally at such salty, alkaline

environments. The goal of this research was to use haloalkaliphilic bacteria from Soap Lake, Washington in converting glycerol to ethanol and to determine the bacterial species involved in the fermentation process.

3. METHODOLOGY

3.1. Enrichment and Isolation. Bacterial samples that were enriched earlier for iron reduction were used as starting cultures for this experiment (Patel, 2006). The bacteria were grown in a slightly modified Soap Lake Basal Medium (SLBM) called as SL3 medium (Begemann et al., 2008). SLBM is an anaerobic, synthetic medium mimicking the chemical conditions present in Soap Lake (Mormile et al., 1999). The media has the following components (per liter of de-ionized water): K_2HPO_4, 13.5g; Yeast Extract, 1g; NaCl, 70g; Na_2MoO_4, 4.84g; Cysteine-HCl (10%), 1.5 ml; Na_2CO_3, 40g; $CaCl_2$. $2H_2O$, 0.42g; $FeSO_4.7H_20$, 0.18g; SiO_2. $2H_2O$, 0.75g; $MgCl_2$. $2H_2O$, 0.852g; $MnSO_4$, 0.448g; NH_4NO_3, 5g; Methylene blue, 0.4g; Trace metal solution, 10ml. The trace metal solution consists of the following in 1 liter of de-ionized water: Nitrilotriacetic acid, 1.63 g; $MgSO_4$. $7H_2O$, 3 g; $MnCl_2$. $4H_2O$, 0.5 g; FeSO4. $7H_2O$, 0.1 g; $CaCl_2$, 0.1 g; $CoCl_2$. $2H_2O$, 0.1 g; $ZnCl_2$, 0.13 g; $CuCl_2$. $2H_2O$, 0.007 g; AlK $(SO_4)_2.12H_2O$, 0.01 g; H_3BO_3, 0.01 g; Na_2MoO_4, 0.025 g; $NiSO_4$. $6H_2O$, 0.03g; Na_2WO_4. $2H_2O$, 0.025g and NaCl, 1g.

The media was enriched for iron reduction activity by the addition of iron (III) citrate at a concentration of 20mM. The glycerol conversion to ethanol in the absence of iron was studied and iron (III) citrate was excluded from the medium for this purpose. In other cases, medium was supplemented with sulfide in the form of sodium sulfide and the

rate of glycerol consumption and ethanol production were monitored. In separate trials, the medium was supplemented with antibiotics streptomycin (1000 µg/ml) and cycloserine (400 µg/ml) to identify the bacterial species that were present in the media. The optimal conditions for growth were determined by measuring the amount of iron (II) produced at different pH and salinity. The technique and results for optimization are as described in Chapter 3.

3.2. Physiological Characterization. Gram staining of the bacteria was performed by using a Gram staining kit (BBL Microbiology systems, Sparks, MD). A simple staining using crystal violet was performed to view the bacterial morphology under a light microscope. The bacterial samples were prepared for Scanning Electron Microscope (SEM) imaging similar to the technique used in Chapter 3. Before imaging with a Field Emission SEM Hitachi S4700, the samples were sputter coated with gold and palladium for 4 minutes. The morphological structure of the bacteria was analyzed by using Transmission Electron Microscopy. For this purpose, the bacterial culture was centrifuged following which the supernatant was added to 2.5% glutaraldehyde and sent for imaging at University of Missouri's Electron Microscopy Core Facility.

3.3. Quantization of Ethanol and Glycerol. The concentration of ethanol was analyzed using a 6890 Gas Chromatography (Agilent Technologies, Santa Clara, CA) fitted with a flame ionization detector. A DB-FFAP column (Agilent Technologies, Santa Clara, CA) was operated with nitrogen as the carrier gas (40 mL/min) at a temperature of 250°C. The temperatures of the inlet and the detector were maintained at 220°C and 250°C respectively. A constant flow of hydrogen and air was supplied to the detector. An

injection volume of 1 µl was used. In parallel, the concentration of glycerol was quantified by using a Free Glycerol reagent kit (Sigma-Aldrich, St. Louis, MO).

3.4. DNA Isolation and PCR Amplification. DNA was isolated from bacterial cultures from the enrichments that were suspected to ferment glycerol into ethanol and from bacterial enrichments grown with the antibiotics streptomycin and cycloserine. DNA was isolated by using UltraClean Soil DNA isolation kit (MoBio Laboratories, Solana Beach, CA). The isolated DNA was stored at -20ºC. Following DNA isolation, PCR was performed to amplify bacterial 16s rRNA gene fragments. Each reaction mixture consisted of the following components in a PCR tube: 2X GoTaq Green Master mix (Promega, Madison, WI), 12.5µl; forward primer 27F (10 pmol), 2.5µl; reverse primer 1392R (10 pmol), 2.5µl; nuclease free water, 5.5µl and DNA template, 2µl. Primers were synthesized by MWG Biotech (High Point, NC). The primer sequence was as follows: 27F, 5'-TTCCGGTTGATCCYGSCR-3' (Lane, 1991); 1392R, 5'-ACGGGTGTGTRC-3' (Stahl and Amann, 1991)

The PCR conditions for amplifying DNA was an initial denaturation at 94°C for 5 min followed by 35 cycles with each cycle consisting of 94°C for 30 seconds**,** 50°C for 1 minute and 72°C for 1 min 30 sec. The reaction was completed with a final extension step at 72°C for 10 minutes. Following PCR amplification, the products were run on a 1% agarose gel. After staining with 0.5µg/ml ethidium bromide solution, the gel was observed under a UV lamp to visualize the presence of desired DNA fragment (based upon molecular weight). PCR amplification products were purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA).

3.5. Cloning and RFLP. The amplified 16S rRNA genes from the ethanol producing cultures and the antibiotic amended media were then ligated in separate reactions into a suitable vector provided in the p-GEM T-Easy Vector system kit (Promega, Madison, WI, USA). The manufacturer's protocol was followed for the steps of the cloning process including transformation. The transformation reaction was examined by using the blue/white screening method. In this method, transformed *E. coli* cells containing the plasmid were grown on X-gal-IPTG-LB-ampicillin agar plates overnight at 37°C. White colonies (containing the inserts) were grown in 5ml LB-ampicillin broth overnight after which the plasmid DNA was isolated from each of the cultures by using a Wizard Plus SV Miniprep kit (Promega, Madison, WI, USA). To test the purity of the cultures, another PCR reaction was performed with the primers 27F and 1392R on the plasmid clones obtained, the conditions being the same as mentioned earlier. The PCR products were then double digested with restriction enzymes, Eco R1 and Bam H1 (Promega, Madison, WI) according to the manufacturer's instructions. The digestion patterns were viewed on a 2% agarose gel.

3.6. Sequencing and Phylogenetic Analysis. Sequencing of the 16s rRNA gene was done on a 3130 Applied Biosystem (Foster City, CA) with primers SP6: 5'-CATTTAGGTGACACTATAG-3' and T7: 5'-TAATACGACTCACTATAGGG-3' (Nag et al., 1988) at the Missouri S&T cDNA Resource Center (Rolla, MO). The acquired sequences were edited and aligned by using MEGA 4 Clustal alignment software (Tamura et al., 2007). The compiled sequences were then compared for related bacterial species by running the sequences on NCBI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) (Zhang et al., 2000). A neighbor-joining

phylogenetic tree was constructed using MEGA 4 software. Since the cultures used in electricity production in Chapter 3 and in the glycerol fermentation studies were the same, a pairwise sequence comparison was performed by using Jalview 2.4 (Clamp et al., 2004) to check the percentage identity of the 16S rRNA gene sequences. To further analyze the similarity of both the sequences, a phylogenetic tree was constructed which included the 16S rRNA gene sequences of the electricity producing cultures and the glycerol fermenting cultures.

4. RESULTS

4.1. Enrichments. The bacterial enrichments were found positive for growth by the formation of black precipitates of iron (II). As described in Chapter 3, the optimal pH for growth was at 11 and the optimal salinity was 7%, determined by monitoring the production of Fe (II). The antibiotic amended media and enrichments in the absence of iron (III) citrate showed growth of bacteria when observed microscopically.

4.2. Glycerol and Ethanol Analysis. The concentration of glycerol (Figure 1) decreased steadily over time in cultures that were grown with iron (III), but very less glycerol was consumed in the media that did not have iron (III). The cultures that were grown in the presence of sulfide and iron (III) had a similar pattern of glycerol degradation with a slightly lower rate than the one with iron (III) alone.

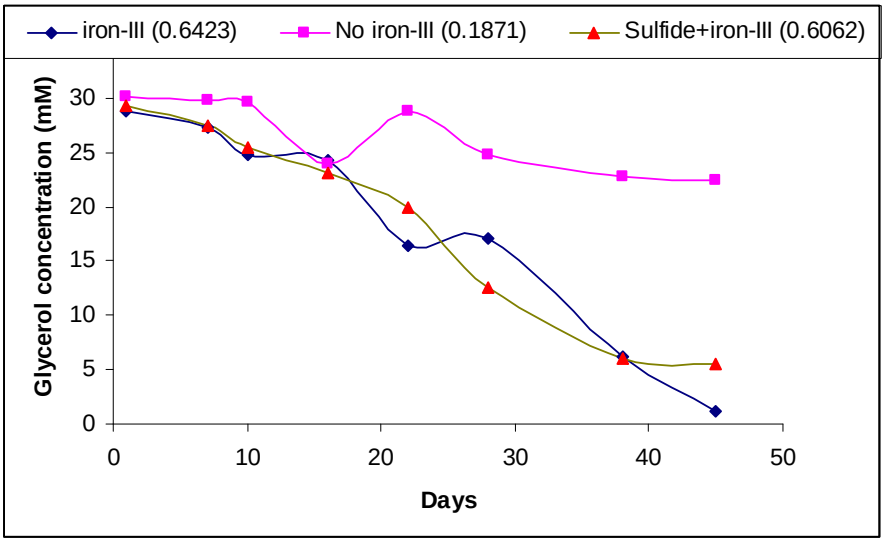


Figure 1. Concentration of glycerol. The values in the parentheses indicate the rate of glycerol consumption in mM/day.

The ethanol concentration did not reveal any specific production rate and all the three different conditions of growth produced very little ethanol as can be seen in Figure 2.

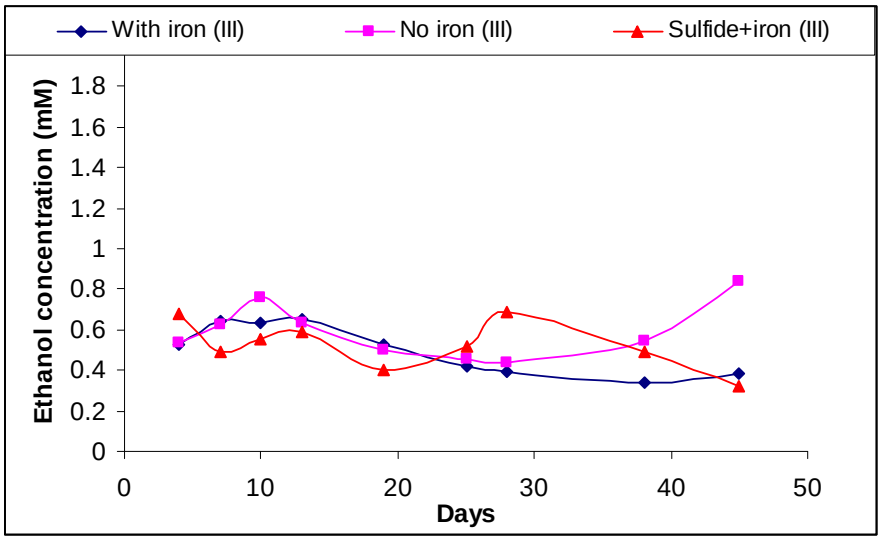


Figure 2. Concentration of ethanol produced in cultures.

4.3. Physiological Characterization. Gram staining of the bacterial cultures revealed that the bacteria were Gram-negative rods. In both Gram staining (Figure 3) and simple staining (Figure 4), bacterial cells that were ovoid or irregular in shape were observed, in addition to the normal, rod-shaped cells. SEM (Figure 5) and TEM (Figure 6) analysis revealed bacterial cells that were mostly rod shaped, with a length of approximately from 1.4 to 2.4 µm.

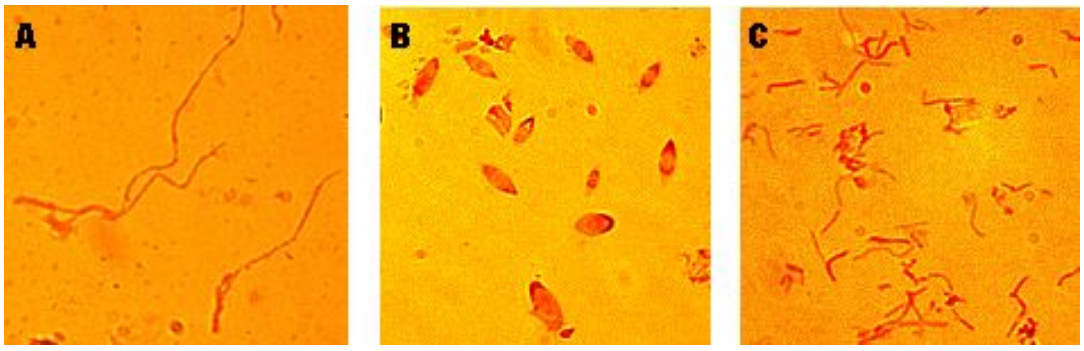


Figure 3. Gram staining images of the different morphologies exhibited by the bacterial species: A. elongated, B. ovoid, and C. rod shaped.

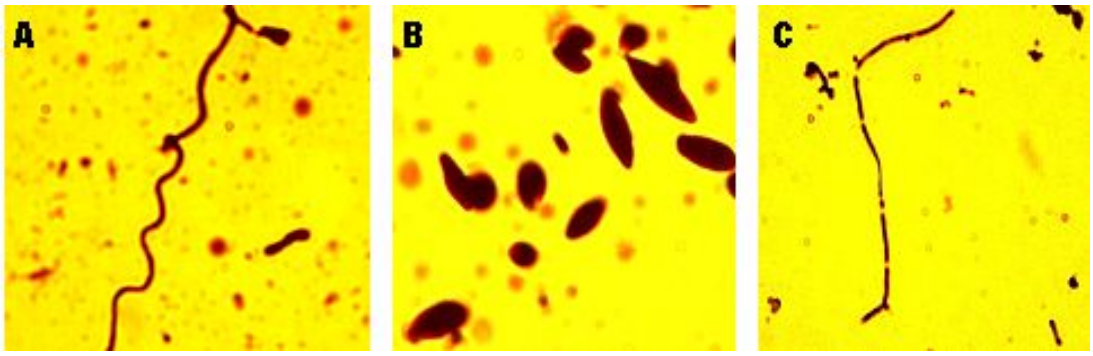


Figure 4. Simple staining images of bacterial cultures showing different morphologies: A. elongated, B. ovoid, and C. rod shaped.

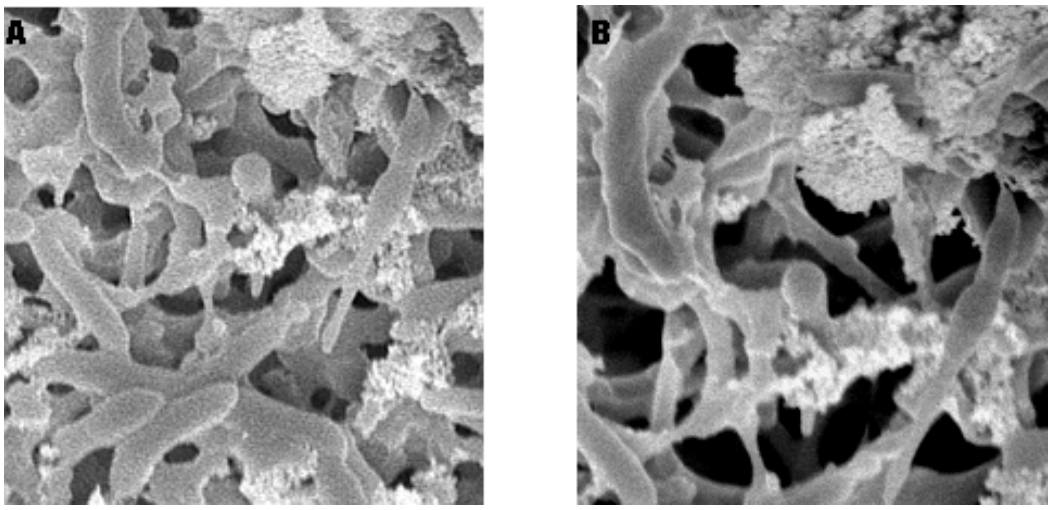


Figure 5. SEM image of bacterial colonies growing on iron taken at original magnifications of 11000 X (A) and 20000 X (B).

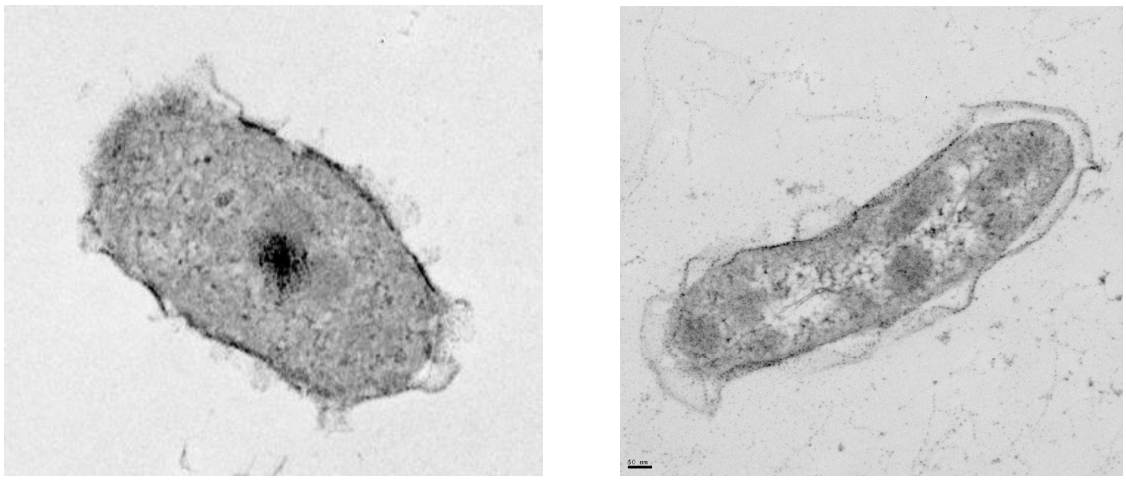


Figure 6. TEM images of bacteria from the glycerol consuming cultures grown with iron (III).

4.4. Molecular analysis. Bacteria from the antibiotic amended media and the normal SL3 media were isolated and subjected to molecular analysis. Though bacteria in the streptomycin containing media showed good amplification products with PCR, the

cycloserine cultures did not reveal any PCR products. RFLP of all the clones obtained from bacteria grown in the normal SL3 media which produced ethanol and from the streptomycin cultures showed a similar digestion pattern (Figure 7). Sequencing of the 16S rRNA gene of the selected clones and comparison with NCBI-BLAST revealed that all the clones showed analogous relationship with other bacterial species in the databases, further suggesting that the culture could a single species of bacteria. The closest match based on results from NCBI-BLAST gave ~97% gene similarity to *Halaanerobium sp*. and a number of uncultured clones. Results from EzTaxon gave a 96.4% gene similarity with *Halanaerobium praevalens.*

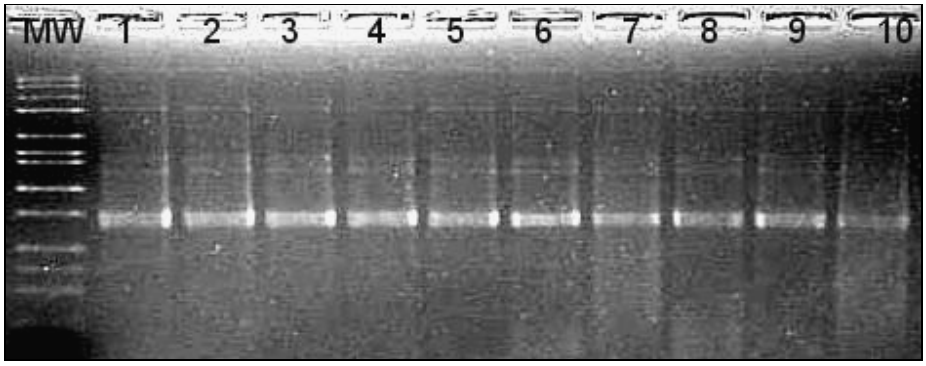


Figure 7. RFLP of ten of the transformed clones from the ethanol producing bacterial cultures.

The phylogenetic tree was constructed by using neighbor-joining method with the 16S rRNA gene (Figure 8). Bootstrap analysis for 100 replicates was performed. The haloalkaliphilic species in the phylogenetic tree did not cluster closely with any of the other bacterial strains and therefore could possibly represent a novel species. The

pairwise sequence comparison of the 16S rRNA gene between the electricity producing cultures in Chapter 3 and the glycerol fermenting cultures showed a 98.89% identity, confirming that both the species are the same. The phylogenetic tree containing the 16S rRNA gene sequences of both the electricity producing cultures and the glycerol fermenting cultures (Figure 9) revealed that the species grouped together very closely, thus confirming that the same species is involved in both experiments.

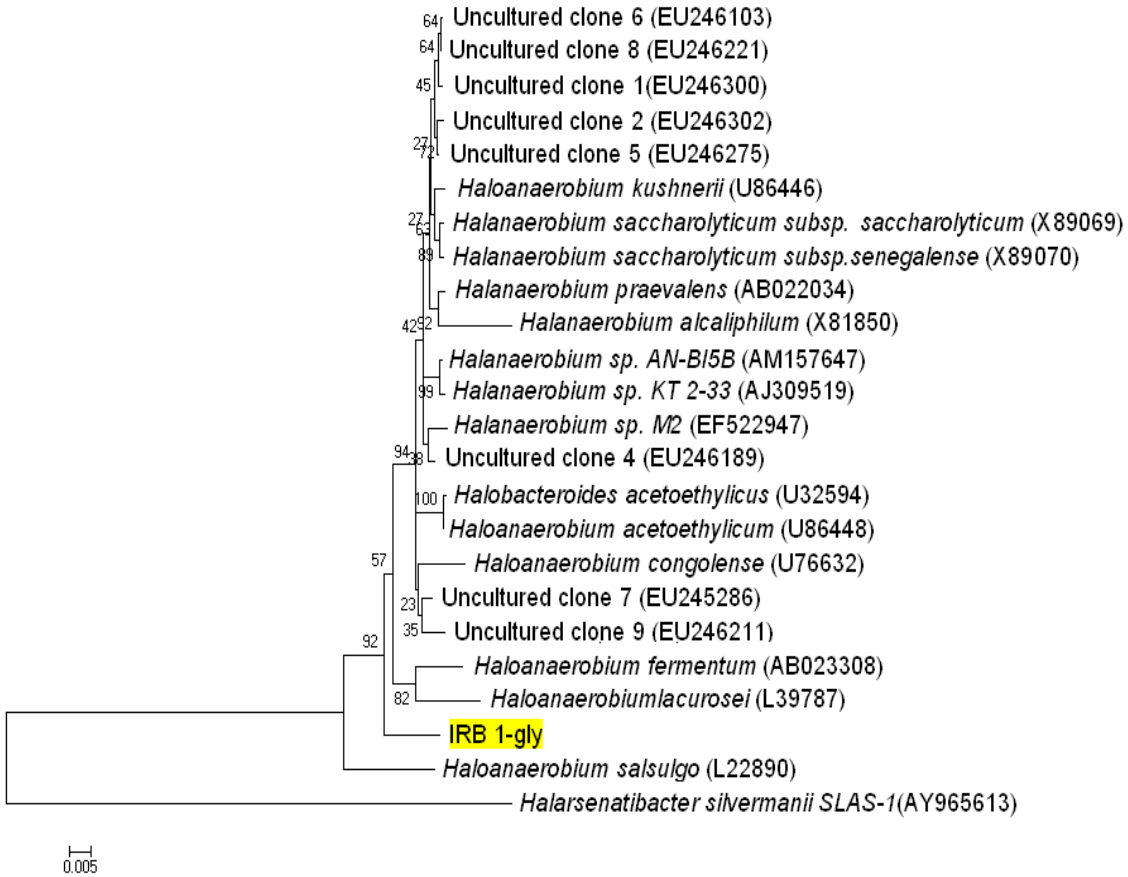


Figure 8. Phylogenetic tree constructed by using neighbor-joining analysis with 100 bootstrap replicates. Bar represents 0.02 substitutions per nucleotide position. The glycerol consuming culture is colored. NCBI accession numbers for the uncultured clones are provided in parentheses.

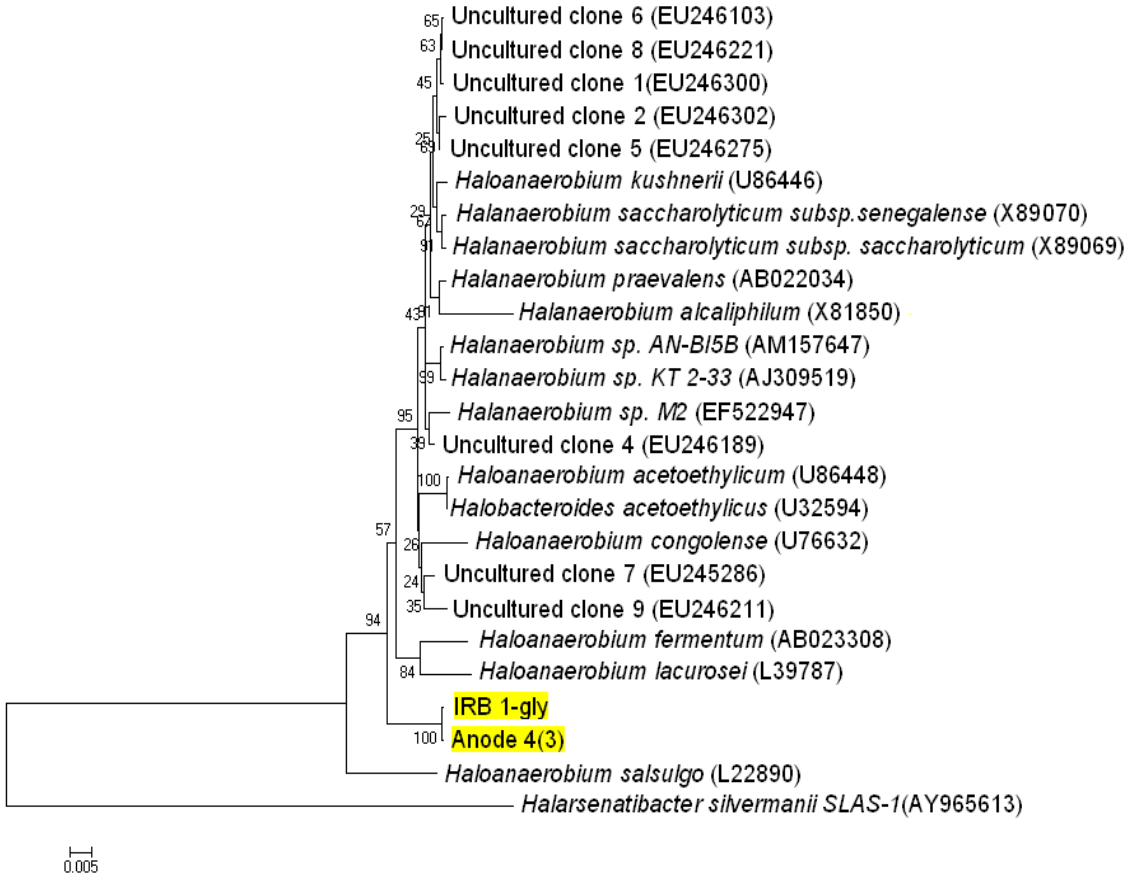


Figure.9. Phylogenetic tree constructed by using neighbor-joining analysis with 100 bootstrap replicates, revealing the similarity between the electricity generating and glycerol fermenting cultures (colored). Bar represents 0.002 substitutions per nucleotide position.

5. DISCUSSION

The bacterial cultures did not show glycerol consumption in the absence of iron. Earlier research suggests that organic compounds are oxidized under fermenting conditions even in the presence of iron (III). Iron is reduced to an extent in such processes (Lovley D.R., 1991). However, the absence of iron (III) did not favor the consumption of glycerol to produce ethanol in the current study. According to thermodynamic calculations, using iron (III) as a minor electron sink aids the bacteria

with a slightly higher energy gain than relying solely on fermentation (Lovley et al., 1989). The ethanol production process using haloalkaliphiles agrees to a certain extent to the above statement though the larger iron (III) concentration in this case could act as a major electron sink. It could therefore be said that the fermentation of the organic compound (glycerol) depends on the presence of iron. The addition of sulfide to provide a more reducing environment did not enhance the efficient production of ethanol. The consumption of glycerol occurred at a slow rate. This can be attributed to the low growth rate of halophilic organisms (Oren, 1999). It is also possible that the bacteria were using another component of the media as their initial substrate and then switched to glycerol.

The ethanol production was not high in any of the cases owing to several reasons such as the slow growth rate of the bacteria; loss during storage and sample preparation due to its volatility; and production of other fermentation products other than ethanol. It is interesting to note that the bacterial species that consumed glycerol was also involved in the production of electricity. The bacteria seemed to have the ability to switch between fermentation and dissimilatory metal reduction, if repeated sub-culturing and suitable conditions were maintained.

Further optimization aimed specifically for efficient rate of ethanol production would give better yields of desirable product. Various modifications can be experimented with the bacterial cultures such as using crude glycerol from biodiesel wastes and supplying higher volumes of glycerol to see conversion rate and maximum tolerance of glycerol concentration. Hydrogen analysis would help to thermodynamically verify the reaction based on previous results. Validation of the single species involved in glycerol consumption can be attained with further biochemical and physiological characterization.

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VITA

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