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Electricity production from *Rhizobium sp.* Biofertilizer enriched soil using microbial fuel cells

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Abstract
The aim of present study is to cultivate *Rhizobium* bio fertilizer enriched soil and to harvest electricity production. *Rhizobium* bio fertilizer production was done by using selective and optimized media to increase crop yields by reducing use of chemical fertilizers to maintain ecosystem. It was usually prepared as carrier-based (vermicast) inoculants containing effective microorganisms. Furthermore, the efficiency of inoculation of *Rhizobium* bio fertilizer on green leafy vegetables was investigated. In our research, the microbial fuel cell is constructed using anode and cathode electrodes and the production of electricity is measured by using power measurement. The maximum voltage obtained from *Rhizobium* enriched soil is 350 mV. Finally, we concluded that the *Rhizobium* enriched soil act as an alternate source of energy.

Keywords: *Rhizobium*, vermi cast, microbial fuel cell, bio fertilizer

1. Introduction
In India, Nitrogen and Ammonia fertilizers are used for agriculture field. By providing artificial fertilizers (Nitrogen) to the plant it uses 50%, 2-20% get evaporated and 15-20% get reacted with clay and remaining 2-10% come in contact with surface and ground water normally nitrogen fertilizers interfere with both underground and surface water [1]. 22% cultivated area in Europe get affected by nitrogen fertilizers. High concentration of these elements causes disease in infant's methemoglobinemia. Stomach acid produced in less than 6 months baby. Nitrate reacts with Hemoglobin and human get struggled to death. This all effects are stopped by use of bio-fertilizers. Bio fertilizers are products masking living cells of distinct types of microorganisms, which have an ability to change nutritionally significant elements from unattainable to offered form through biological processes. Some bacteria are capable to fix the atmospheric nitrogen. Among various types of bio fertilizers, rhizobacteria, plant growth-promoting rhizobacteria, phosphate-solubilizing bacteria, and so on. *Rhizobium sp*. has beneficial effects on plant yield due to the increase in fixed nitrogen content in soil. The microbial secretion of stimulation hormones, gibberellin, auxin and cytokine established the ability of *Rhizobium sp.*to solubilizing phosphate. Bio fertilizers are defined as preparations containing, living cells or latent cells of efficient strains of microorganisms that help crop plants' uptake of nutrients by their interactions in the rhizosphere when applied through seed or to soil. Bio fertilizers are known as microbial inoculants, which are artificially multiplied cultures of certain soil microbes that can improve soil fertility and crop productivity [2].

The presence of *Rhizobium sp.* in soils has useful effects on plants, but the wealth of these bacteria is related to many factors, soil physico-chemical (e.g. organic matter, pH, temperature, soil moisture) and microbiological assets. There are some microorganisms, which stimulate the *Rhizobium* population in soil thereby increasing the nitrogen fixation by *Rhizobium* [3]. Besides nitrogen fixation, *Rhizobium* also produces Thiamine, Riboflavin, Nicotine, In dole Acetic Acid and Gibberellin. The seed germination is improved to a considerable extent, when the *Rhizobium* is applied to seeds. Bacteria of the genus *Rhizobium* synthesize auxin, cytokinin and GA-like substances. These growth materials are the primary substance controlling the enhanced growth of plant. These hormonal substances, which patent from the rhizosphere or root surface, affect the growth of the closely allied higher plants.

Microbial fuel cell (MFC) is an emerging technology in recent years and has fascinated a lot of attention from researchers in the fields of wastewater treatment and bioenergy production. As an advanced technology, microbial fuel cell is able to utilize the microorganisms to degrade organic waste in the wastewater and turn it into electricity. MFC (Microbial Fuel Cell) has major role in power consumption. And also has many applications like powering LED, recharging batteries, and many medical devices etc [4]. MFC convert the organic substrate into electricity by using the microorganism as catalyst[5] Generally, MFC can be separated into two important parts: anode and cathode. Microbes living in the anodic chamber are capable of degrading organic matters and transferring the electrons generated during the oxidation process to cathode via metal wires. During the break-down process, carbon dioxide is generated as an oxidation product. However, there is no carbon dioxide emission due to the statistic that the carbon from biomass is initially from the atmosphere. With proton exchange membrane (PEM), between anode and cathode, a proton is able to transfer from anodic chamber to cathode chamber. In the cathode chamber, electrons can be used by electron acceptors for example, oxygen associations with protons transferred from anode to cathode to yield water [6]. Oxygen present in the anodic chamber may lead to the failure of the whole system; therefore, it is essential to guarantee that the anodic chamber is under anaerobic condition [6]. For example, if using

sodium acetate in the anodic chamber,
An anodic reaction could occur as follows:

$$2HCO_3^- + 9H^+ + 8e^- \rightarrow CH_3COO^- + 4H_2$$

For cathodic reaction:

$$O_2 + 4H^+ + 4e^- \; 2H_2O$$

MFC might be best characterized as a bioreactor where microorganisms act as catalyst in metabolizing the natural substance containing the organic carbon to produce electricity. The microbial fuel cell is a system in which enzyme catalytic energy is transformed into electrical energy by electrochemical process [7]. Electrons are yield by the oxidation of organic material in which microbes act as catalyst. The electrons thus produced are transferred to a terminal electron acceptor such as oxygen nitrate and sulphate. Now these terminal electron acceptors are get compact by these electrons. A new product is made which can leave the cells when fatal electron acceptors are diffused into the cells. However there are some microorganisms specifically bacteria that can transfer their electrons in the outer space adjoining the cells which are established by the pending terminal electron acceptors. These bacteria are called exogenic and can be used to produce power within microbial fuel cells [8]. In bacterial metabolism, the anaerobic bacteria which get die in the presence of oxygen are used as a catalyst and their extra cellular electrons are utilized by microbial fuel cell. To complete the electron allocation to the electrodes the fermented product must syndicate with other constituents such as aromatic compounds through effective and anaerobic oxidation [9]

Sediment microbial fuel cell
Sediment microbial fuel cells (SMFCs), also referred to as benthic MFCs in some cases, is a special application of MFC to generate electricity by degrading organic matter in sediment as well as by utilizing the electron acceptors in the cathode area. An anode of such SMFC is embedded in the sediment and the cathode is positioned in overlying water. SMFCs follow a certain mechanism: microorganisms degrade organic compounds present in sediment, generating electrons and protons. Electrons are relocated from anode to cathode through an external circuit, and protons flow from sediment side to the cathode side and syndicate with oxygen on the cathode side to produce electricity.
The aim of this study is to isolate and identify the *Rhizobium sp.* for integrated production of bio fertilizer and generate the electricity.

2. Materials and methods

2.1 Sample collection
The soil samples were collected from the rhizosphere of tomato plant at Maanojpatti in Tanjore district using sterile polythene covers and brought to the laboratory for analysis.

2.2 Soil testing
The soil sample was send to laboratory to calculate the availability of nutrients and to know the physical and chemical properties including pH, organic carbon and available nitrogen, phosphorus, potassium, sulphur, zinc and boron.

2.3 Carrier preparation
Vermi cast was used as a carrier material. The raw materials were ground and dried in a hot air oven at 60°C for 2 days. The materials were autoclave at 121ºCof 15lb for 30minutes.

2.4 Isolation of *Rhizobium sp.*
Rhizobium was isolated from the soil sample by serial dilution method. 1g of air-dried soil sample was dissolved in 10ml of sterile distilled water and considered as a stock solution. The soil suspension was diluted up to 10-5 level. Each diluted soil suspension was spread on the surface of the appropriate petri plate containing Yeast Extract Mannitol (YEM) agar medium which is the selective medium used for isolating *Rhizobium*. The pH of the medium was adjusted to 7.0 by using 1N HCl/1N NaOH. The plates were incubated at 28ºC for 2-5days. The growth on the medium was presumed to be *Rhizobium* and these isolated colonies were purified by repeated streaking on YEM Agar medium and were preserved as slant culture for confirmation.

2.5 Identification of isolates
The isolate was identified on the basis of morphological and biochemical characterization. In biochemical characterization, IM ViC test, Urea, Starch hydrolysis, Sugar fermentation were performed by using standard method. Morphological characteristics of the isolate viz. shape, size, color, elevation, surface, margin and gram's nature were observed.

2.6 Mass cultivation of rhizobium
A loopful of *Rhizobium* pure culture was transferred into 250ml conical flask containing 100ml of YEM broth and kept in the rotary shaker on 120rpm for 3 days. The selective broth used for the mass culturing of *Rhizobium* is as follows:

Table 1: Selective yem broth

Components	Quantity (g/L)
Yeast extract	1g
Mannitol	10g
Di potassium phosphate	0.5g
Magnesium sulphate	0.2g
Sodium chloride	0.1g
Calcium carbonate	1g
Distilled water	1L

After incubation, 10ml of the inoculum was transferred to 1000ml of the YEM broth for mass multiplication.

2.7 Biofertilizer production using *rhizobium sp.*
In our study, 750ml of the broth containing *Rhizobium* was mixed with 1000g of sterile carrier (Vermicast). After proper mixing, carrier containing inoculant was packed in sterile polyethylene bags, sealed and incubated under room temperature for 7 days and above formulated microbial inoculants used as Bio fertilizer.

2.8 Soil application
The bio fertilizer was mixed with soil in the ratio 1:4. The water was sprinkled above the mixture to balance the

moisture content. It was broadcast into the tomato plant at the time of sowing.

2.9 Construction of MFC

MFC was constructed for the production of electricity from the *Rhizobium* Bio fertilizer enriched soil sample. Soil was patted down in MFC to make a smooth surface and anode was placed on the bottom of the soil and the soil sample was added finally. The cathode was placed at the top of MFC and the setup was closed using a lid. The digital millimeter was used to measure the electricity production.

3. Results and discussion

3.1 Soil sample collection

The soil samples were collected from the rhizosphere of tomato plant at Maanojpatti in Tanjore district using sterile polythene covers and brought to the laboratory for analysis.

3.2 Soil testing

The tomato plant soil sample(sample 1) and bio fertilizers enriched tomato plant soil sample(sample 2) were send to Tamil Nadu Veterinary and Animal Sciences University, Namakkal and the result were shown in the table. It includes pH, EC, organic carbon, N, P, K etc.

Table 2: Soil testing report of experimental soils

Particulars	Sample 1	Sample 2	Normal range
Soil Ph	6.52	7.64	6.5-7.5
EC dSm-1	0.044	0.02	<4 dSm-1
Organic carbon (%)	0.63	0.83	0.50-0.75 %
Available Nitrogen (kg ha-1)	226	570	280-450 kg ha-1
Available Phosphorus (kg ha-1)	28.3	19.8	11-22 kg ha-1
Available Potassium (kg ha-1)	453	235	118-280 kg ha-1

3.3 Carrier preparation

Vermicast was dried in a hot air oven at 60ºC for 2 days. The materials were autoclave at 121ºC of 15lb for 30minutes. After sterilization, this sterile carrier was used for bio fertilizer production.

3.5 Isolation of *rhizobium*

Rhizobium was isolated from the rhizosphere of tomato plant by serial dilution method and obtained the pure culture by repeated streaking on Yeast Extract Mannitol (YEM) Agar medium. This media is very specific and optimized media for the isolation of *Rhizobium*.

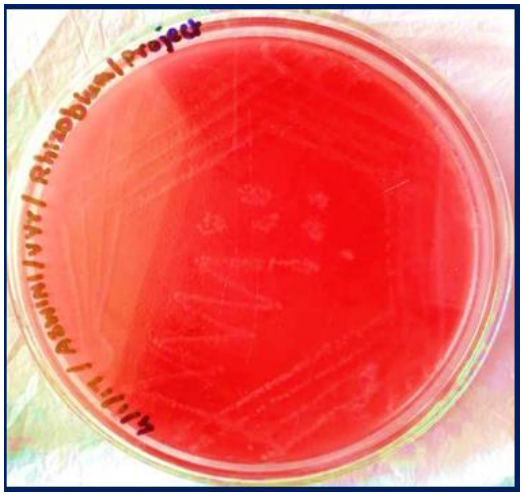

Fig 1: Isolation of rhizobium

3.6 Morphological characterization of *Rhizobium*

The isolated colonies were gram negative, motile rods. In general, the colonies were small, circular, convex, whitish pink and glistering with entire margin.

Table 3: Morphological characterization of *Rhizobium*

S. No	Colony Characteristics	*Rhizobium* Characteristics
1.	Shape	Circular
2.	Size	Small
3.	Color	White
4.	Margin	Entire
5.	Elevation	Convex
6.	Consistency	Mucoid
7.	Opacity	Translucent
8.	Gram staining	Gram-negative

3.7 Biochemical characterization of *rhizobium*

The confirmation of Rhizobium was done through various biochemical tests. Isolates was showed positive results to Indole, Citrate, Methyl Red, Glucose, Sucrose, Xylose and Mannitol test where they expressed negative results to Voges-Proskauer, Nitrate Reduction, Urease, Lactose and Fructose.

Table 4: Biochemical characterization of *rhizobium*

S. No	Biochemical tests	Results
1.	Indole test	Positive
2.	Citrate test	Positive
3.	Voges Proskauer test	Negative
4.	Methyl Red test	Positive
5.	Nitrate reduction test	Negative
6.	Urease test	Negative

Table 5: Carbohydrate utilization test of *rhizobium*

S. No	Carbohydrate Utilization tests	Results
1.	Glucose	Positive
2.	Sucrose	Positive
3.	Lactose	Negative
4.	Fructose	Negative
5.	Xylose	Positive
6.	Mannitol	Positive

3.8 Mass cultivation of *rhizobium*

Rhizobium were cultivated in the selective YEM broth and kept in the shaker for 3-5 days.

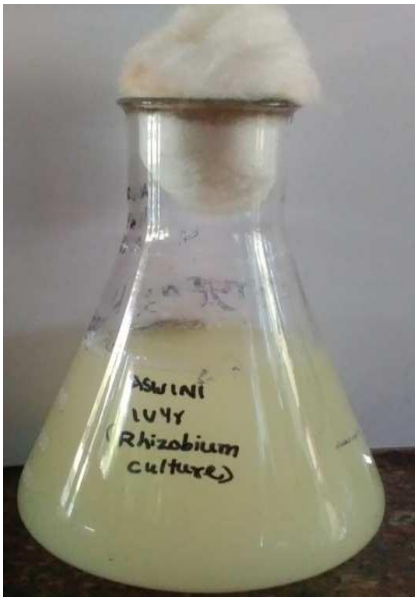

Fig 2: Mass Cultivation of *Rhizobium sp.*

3.9 Production of biofertilizer

The organism thus identified was inoculated in YEM broth for mass cultivation of *Rhizobium*. After incubation time, the broth was used as inoculants for the production of bio fertilizer. The inoculants were mixed with a carrier material (Vermicast) and used as a bio fertilizer. Bio fertilizer applied as soil application where it function to mobilize the availability of nutrients especially NPK by their biological activity and help to enhance soil health by building up the micro flora to supply different kind of nutrients in the soil. The bio fertilizer was mixed with the soil and sprinkle the water to the mixer. Finally, this mixture was broadcast at the time of sowing.

3.10 Construction of microbial fuel cell and electricity production

The microbial fuel cell was constructed based on the procedure and the electricity was produced. The microorganisms using in MFC are those who can directly transfer electrons to anode using anode as terminal electron acceptors, those who can't directly but use mediators to transfer electrons to anode and those who can accepts electron from cathode. Initially, there was no electricity production in Soil MFC when the soil sample was inoculated. MFCs directly change substrate energy into electricity which is helpful in every feature. In short, the emergence of MFC technology has plethora of applications in the daily lives as it is ecofriendly and green technology. After incubation, we noticed that the increase in electricity production continuously. The Microbial Fuel Cell was constructed finally based on the procedure and the electricity was produced. Voltage reading in the digital millimeter was recorded. Microbial fuel cell is built upon the basic principle in which biochemical energy is renewed into electrical energy. Consumption of organic substrate (e.g. sucrose) by microorganism in aerobic condition produces CO2 and H2O. If the incurable electron acceptor oxygen is negotiated by moderator then the electrons will be surrounded by mediator, which will get abstracted and transporter to the electrode at anodic chamber. However when oxygen is not present they yield carbon dioxide, protons and electrons as defined below:

$$C_{12}H_{22}O_{11} + 13H_2O \rightarrow 12CO_2 + 48H^+ + 48e^-$$

It was first reported by M.C. Potter in the year 1910 with *E. coli*. For present study 500mL bottle was used for making the anodic and cathodic chamber. Microbial fuel cell is able to consume the microorganisms to vitiate organic waste in the wastewater and chance it into electricity. The maximum voltage of 300Mv with *E. coli* was obtained by M.C.Potter. The silver nanoparticles incorporated salt bridge was also tested for its efficacy to transport H+ ions and it was observed that initially the voltage rise rapidly but soon the voltage starts falling down. The optimum concentration was establish to be 7g/l for all the four isolated strains. An early paper by [10] describes much of the initial work focused towards developing glucose powered fuel cells for use within heart pacemakers. The favored organisms for MFC operations are metal reducing, anodophilic and flagellated microorganisms. *Geobacter* species are of interest because of their novel electron transfer capabilities, the ability to transfer electrons outside the cell and passage these electrons over long distances via conductive filaments recognized as microbial nanowires [11]. Several organisms that are known to produce fermentation products and belong to the genus *Clostridium, Alcaligenes, Enterococcus*, have been used for MFCs operations [12]. Four different bacterial isolates were tested for efficacy in producing potential difference. The four marine bacterial isolates belonging to genus *Pen bacillus*, *Pseudomonas*, *Stenotrophomonas* and *Alcaligens* were use as electron donor. They were readings as single cell MFCs and in all possible combination. Marine bacteria grow biofilms on the MFC electrodes, allowing substantial conversion volume and occasions for extracellular electron transfer (EET) [12]. There is fewer information about bacteria belong to genus *Pen bacillus* and its application in MFC which gave the maximum potential difference i.e. voltage generation of 1033 mV monitored by *Pseudomonas* which produce the voltage of 1010 mV. The consortium of *Pen bacillus* and *Pseudomonas* produced maximum voltage. The maximum potential difference reported by Cahyani and Gerard in the year 2008 for *Pseudomonas* is 0.2 V but it is with nafion membrane which is very expensive and comparatively the result obtained with simple salt bridge. MFC have wide application in bio hydrogen production via bio electrolysis, wastewater treatment and cathodic de nitrification, bioremediation, biosensors, in-situ power source for remote areas [13].

4. Conclusion

Rhizobium forms large, flat, soft, gummy colonies. *Rhizobium* species are free living, motile, feeding on dead organisms. They found regular in structure appearing in straight rods while nitrogen fixing exists as irregular one. These bacteria are regarded as Plant Growth Promoting Rhizobacteria (PGPR) which synthesize growth substance that enhances plant growth and development and inhibit phyto pathogenic growth by secreting inhibitors. It also helps in nutrient Up take and produces some biochemical substances such as protein, amino acids etc. *Rhizobium* improves seed germination and has beneficiary response on

Crop Growth Rate (CGR). It helps to increase nutrient availability and to restore soil fertility and nutrient management system due to its significant role in soil sustainability. More research is necessary in future to explore the potentiality of *Rhizobium* in soil fertility. Overall our study was showed the isolation and identification of *Rhizobium* that has the potential to be used as a bio fertilizer. These attributes of the isolate will be of great advantage in agriculture field next trial play a vital role in plant growth promotion, disease suppression and subsequent enhancement of yield. Soil MFC has been constructed from the *Rhizobium* enriched bio fertilizers for electricity production with low cost without salt agar bridge and costly PEM. The constructed MFC provides an enough voltage to power the LED bulbs in the *Rhizobium* bio fertilizers influenced soil. MFC produced from the *Rhizobium* bio fertilizers influenced soil are more efficient in producing electricity.

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