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Comprehensive report

永續發展目標下以微重力下細菌適應性於微生物燃料電池開發應用

SDG-driven bacteria microgravity biological adaptation applied in microbial fuel cell

顏子恆

Tzu-Heng Yen

指導教授: 李財坤 博士

Advisor: Tsai-Kun Li, Ph.D.

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謝誌

在這兩年的碩士生涯中，從第一學期因為新冠肺炎而被迫取消前往日本學習的計畫，到第四學期順利在法國取得實習機會，一路走來受到很多貴人的相助與提拔讓我順利完成學業。首先要感謝我的指導教授李財坤老師在碩士兩年的時間給我自由發揮獨立思考的空間以及無數次的研究科學討論。謝謝宜蘭大學王金燦老師、邱信霖老師與Raymond學長參與微重力裝置的設計與討論以及同為研究生的Manas協助我進行微重力裝置實驗，讓太空生物學計畫能順利進行。

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中文摘要

21世紀，人類面臨來自社會以及自然氣候的全球化挑戰。在此其中，全球可利用能源的短缺問題吸引了越來越多科學家的關注。

細菌的生長取決於多種因素，例如溫度、濕度和氧氣含量。雖然科學家尚未完全理解微重力條件如何影響生物體的生存能力，但細菌的代謝過程對化學能轉化為電能至關重要，也在驅動微生物燃料電池（MFCs）中扮演著關鍵角色。透過微生物燃料電池（MFCs)、微重力應用和綠色能源的應用，人類得以建立可持續能源來源，同時減少溫室氣體排放並減少對化石燃料的依賴。這些措施在應對氣候變化方面扮演著關鍵的角色，有助於改善空氣品質和人類整體健康。此外，將MFCs與微重力應用整合於太空和地球上，還有助於資源保護和廢物處理。這些成果與聯合國永續發展目標相一致：推動為所有人提供經濟、可靠、可持續和現代化的能源，從而提升人類生活品質。基於我目前在法國的實習專注於微流體技術在釀酒過程中的創新和使用優質酵母菌株，我相信MFCs、微重力應用、釀酒處理和綠色能源的研究合作擁有巨大的未來潛力。

我的研究探討微重力對微生物生長和微生物燃料電池電力生產效率的影響。我們與國立宜蘭大學機械與電機工程系合作，創建了一個隨機定位裝置來模擬微重力環境。透過在微重力條件下培養 *E. coli* 和 *Shewanella Oneidensis* MR-1，得以測量固定在微重力裝置上的微生物燃料電池不同的電壓值。我們假設在微重力條件下， *E. coli* 和 *Shewanella Oneidensis* MR-1的生長速度比正常重力環境下更快。實驗結果顯示，大腸桿菌的生長速率和MFCs的電力生產確實受到抑制。這是因為氧氣不足或是細胞無法均勻吸收營養物質導致。

關鍵字: 微生物燃料電池、微重力適應、隨機定位儀、*E. coli*、*Shewanella Oneidensis* MR-1

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Abstract

21th century is a special period that the human beings meet the global challenge from both social problems and natural climate. Among them, the shortage of accessible energy attracts a lot of attentions from researchers.

Bacteria growth depends on several factors such as temperature, humidity, and oxygen levels. Although scientists have yet to fully comprehend how microgravity conditions affect organisms' ability to thrive. It is also important to understand that bacteria's metabolic processes are necessary in converting chemical energy into electrical energy, which plays a crucial role in powering microbial fuel cells (MFCs).

By collaborating microbial fuel cells (MFCs), microgravity applications, and green energy, significant outcomes can arise for human beings. Through collaboration, sustainable energy sources are linked while diminishing greenhouse gas emissions and reducing dependence on fossil fuels. This crucial role in tackling climate change leads to improved air quality and overall human health. Moreover, resource conservation and waste management are facilitated by integrating MFCs with microgravity applications both in space and on Earth. These consequences align with the Sustainable Development Goal of ensuring access to affordable, reliable, sustainable, and modern energy for all, ultimately advancing the quality of life for humanity. Given my current internship in France focusing on Microfluid sorting innovation for winemaking and the use of superior yeast strains, the research collaboration among MFCs, microgravity applications, winemaking treatment, and green energy holds great potential as a future direction of work.

This study investigates the effect of microgravity on the growth of microorganisms and microbial fuel cells' electricity production efficiency. Cooperating with Department

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of Mechanical and Electro-Mechanical Engineering at National Yilan University, we created a Random positioning machine to mimic microgravity condition. Demonstrating culture of *E. coli* and *Shewanella Oneidensis* that were shown to produce electricity in microgravity conditions and measurement of different voltage in MFC which is fixed at microgravity device. We hypothesis that the growth of *E. coli* and *Shewanella Oneidensis* is quicker than in normal gravity. The experimental results showed that the growth rate of *E. coli* and the electricity production of MFCs were indeed inhibited. This is caused by insufficient oxygen or the inability of cells to absorb nutrients evenly.

Key words: Microbial fuel cells, Microgravity adaption, Random positioning machine, *E. coli, Shewanella Oneidensis* MR-1

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Background

1. Sustainable development goal (SDG) and Microbial fuel cell (MFC)

Depends on World bank data. Electricity shortage is a global energy crise which result from population growth, extreme weather, peak demand and unstable global political situation [1, 2]. In emerging and developing economies, where a significant portion of each household's budget is already allocated to food and energy [3, 4], the situation has become even more severe. Furthermore, depends on International Energy Agency, 770 million people don't have access to electricity globally [5]. Therefore, it is important to support the 7th UN SDG goal, ensuring the access to affordable, reliable, sustainable and modern energy for all. The use of sustainable and clean energy and related technologies can reduce fuel costs for marginalized communities. [6, 7] These renewable sources of energy include solar, wind, geothermal, hydroelectric, and biomass. Solar and wind energy are the most widely used types due to advancements in technology and decreasing costs. Geothermal and hydroelectric are important sources particularly in areas with high resources. [8-10] Biomass is less commonly used but has great potential as a future source of significant energy production. [11]

In conclusion, the primary driving forces behind the development of microbial fuel cells are twofold: the growing demand for clean and renewable energy sources and the continual need for power during extended space missions [14, 15]. It is evident that these rationales align with the United Nations' Sustainable Development Goal "Providing universal access to affordable,

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reliable, sustainable, and modern energy for all."

2. Microbial fuel cells (MFCs)

Microbial fuel cell (MFC) is a type of device that utilize organic compounds as a source of fuel to generate electricity [16, 17]. Typically, a MFC is composed of an anaerobic anode chamber and an aerobic cathode. [20]. In the anode of an MFC, microorganisms oxidize the organic compounds, leading to the release of protons and electrons. The electrons produced during the oxidation process transfer through an external electric circuit to the cathode and combine with electron acceptors. Afterward, the reduced compound will be form. [18, 19] Among them, the most common cathodic reactions is the reduction of oxygen. Wastewater generated from various industrial sources that contain organic substrates can be used as a critical source for producing bioenergy using MFCs [20]. Interestingly, National Aeronautics and Space Administration (NASA) is preparing for long-duration Mars space missions, accommodating larger crews so that importance of wastewater reuse and recycling on the International Space Station (ISS) is increasing [21]. As a result, by using MFC, it will become an innovative way to treat wastewater in space and on the earth and also produce electricity in same time.

3. Microbial fuel cells (MFCs) model bacteria

3.1 *Escherichia coli* K12 MG1655

E. coli is a non-spore-forming, Gram-negative bacterium and also a classic model organism for various study like bacterial adaptation, bacterial

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genetic, bacterial metabolism etc. [22] Among *E. coli* strains, *Escherichia coli* K12 MG1655 is one of well characterized strains and can produce electron by utilizing extracellular electron transport (EET) pathway [23]. The transportation of electrons from redox-active electron donors to acceptors is a crucial mechanism known as extracellular electron transfer (EET). This process serves essential functions in various redox processes such as *E. coli* metabolism transferring organisms to electrons acceptors which can applied in microbial fuel cells [24].

3.2 Shewanella Oneidensis MR-1

This Gram-negative, facultative anaerobic, heterotrophic bacterium is one of most studied bacteria strain that is able to use extracellular electron transfer to transfer electrons extracellularly to solid electron acceptors [25-27]. A lot of research has been conducted to explore its capacity to respire a wide range of electron acceptors, including oxygen, nitrate, sulfur compounds, metals, and organic substances. Furthermore, investigations have been dedicated to examining its capability as an electrochemically active bacterium, which can release electrons to electrodes and accept electrons from electrodes in bio electrochemical systems (BESs) [28, 29].

4. Space mission

Sixty-six years ago, the initial artificial satellite was launched to undertake the primary objective of unraveling the enigmas of the heavens. Space exploration has since become one of the most significant accomplishments in human history, with spacecraft being utilized for a variety of purposes, including

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meteorology, navigation, space colonization, and transportation of people and cargo. An important consideration is the issue of waste management during space missions [30, 31]. Currently, solid waste is gathered in a canister as a part of the space station's toilet hygiene system, and these canisters are disposed of during destructive reentry of cargo spacecraft [32]. Meanwhile, urine is collected separately and processed through the station's Water Recovery System's distillation assembly to turn it into potable water [33]. Although astronaut waste is currently transported back to Earth, recycling it would be more advantageous for longer space expeditions because it contains valuable resources necessary for astronaut survival. Through recycling, waste can be transformed into drinkable water, fertilizer, and even electricity with the help of a recently discovered microbe. The second issue to consider is the shortage of electricity when traveling through the darkness of space. Solar power is currently the best source of energy for spacecraft, and engineers have created efficient technologies to convert solar energy into electrical power. The solar arrays on the spacecraft generate more electricity than the station requires at any given time, with approximately 60% of the electricity used to charge the station's batteries while it is in sunlight [34-36]. However, if the spacecraft is in darkness for an extended period due to planetary alignment or solar system malfunction, alternative resources will be needed to provide electricity. [37]

5. Microgravity device and Random positioning machine (RPM)

To mimic the condition in space, random positioning machine is the most efficient device to approach this goal. A Random Positioning Machine (RPM) is designed to perform continuous random alterations in orientation with respect

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to the gravitational force experienced by a biological experiment. When the changes in direction occur at a faster rate comparing to the object's response time to gravity, the RPM can produce effects similar to those observed in true microgravity. Therefore, organisms that exhibit relatively quick responses to gravity, such as plants and other systems, are ideal subjects for investigation using RPMs. Through the controlled rotation of two axes, this instrument reduces the overall cumulative gravitational force at its center [38-40, 50]. (Figure. 1) In the Random Positioning Machine (RPM), when set to random mode, the speed of individual frames and the direction of rotation are varied in a random manner. This leads to an unpredictable and symmetrical trajectory. Placing an experiment precisely at the center of the RPM is crucial because the rotation of the sample also induces centripetal acceleration.

Specific Aim

Due to the increasing demand for alternative energy and the issue of electricity shortage, the development of microbial fuel cells has become more crucial. These fuel cells, which combine microbial model systems with electricity production, have the potential to provide renewable energy for space missions and human use. However, more research is necessary to fully understand how bacteria respond to microgravity and to discover sustainable ways of using this technology. The purpose of this experiment is to elucidate how microgravity will influence the power generation efficiency of a fuel cell containing *E. coli* K12 MG1655 and *Shewanella oneidensis* MR-1. The experiment started by measuring the growth curve of *E. coli* K12 MG1655 and *Shewanella oneidensis* MR-1 in normal gravity in aerobic condition. Later on, trying to make a growth curve of *E. coli* K12 MG1655 and *Shewanella oneidensis MR-*

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1 in normal gravity and then compare it to the growth curve in microgravity condition. Afterward, compare the electricity production of microbial fuel cells under normal gravity and microgravity. We found that the growth of bacteria is slower in microgravity device.

Material and Method

Bacterial Strains and Growth Conditions

The stock resource of *E. coli* K12 MG1655 *i*s provided by the DNA Topoisomerase Laboratory at National Taiwan University led by Prof. Tsai-Kun LI. Besides, *Shewanella oneidensis* MR-1 was bought from the Bioresource Collection and Research Center in Taiwan (BCRC). To culture these bacteria, *Shewanella oneidensis* MR-1 and *E. coli* K12 MG1655 are grown separately in LB broth at 30°C and 37°C under shaking at 150 rpm overnight. [41, 42] The bacteria that have been cultured overnight are then adjusted to a specific concentration as inoculum for the microbial fuel cell (MFC) or bacteria suspension.

Measurement of the growth curve of *E. coli* K12 MG1655 and *Shewanella oneidensis* MR-1 in normal gravity in aerobic condition

50 ml centrifuge tube were used as the container of bacteria suspension. The bacteria suspension was mixed with LB broth or artificial wastewater to achieve a final concentration of an O.D (600nm) value of 0.1 which is usually used as start concentration [43]. Two strains are growed at 25°C under shaking at 150 rpm over-night.

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Comparison of bacterial growth curve in microgravity and normal gravity

1. **The growth of *E. coli* K12 MG1655 and *Shewanella oneidensis* MR-1**

We use 50 ml centrifuge tube as the container of bacteria suspension and then take the cultured bacteria suspension as mentioned above in order to match the structure design on random positioning machine (RPM). The bacteria suspension will be mixed with LB to achieve a final concentration of an O.D (600nm) value of 0.1. The gap between top and tube is covered by parafilm two times in order to mimic the anaerobic environment. All incubations in the RPM (with a rotation rate of 12 rpm) and in normal gravity condition will be done at 26°C because the RDM cannot put into incubator.

2. Investigation of bacterial growth curve by measuring O.D and CFU/ml

2.1 O.D

Take 1 ml of suspension from 50 ml centrifuge tube and measuring absorbance at 600 nm at the beginning of the experiment and after one overnight culture. [44]

2.2 CFU/ml

Take 1 ml of suspension from 50 ml centrifuge tube at the beginning of the experiment and one day later. The number of bacteria was counted in LB agar plates by using the serial dilution method and shown as log CFU/ml.

Microbial fuel cells

1. Formation and pretreatment

The MFC (Microbial Fuel Cell) consists of two chambers - the anode, which is an anaerobic chamber, and the cathode, which is an aerobic chamber. Both chambers have a diameter of 5 x 5 x 2.5 and contain up to 62.5 ml of electrolyte solution. Square-shaped graphite felts with dimensions of 5x5 cm are

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used in anode and anaerobic chamber which are pretreated by washing with 10% hydrogen peroxide at 85°C for three hours. [45] The graphite felts are then stored in deionized, distilled water for using. Nafion 117 proton exchange membranes are utilized and undergo a pretreatment process involving immersion in various solutions. Firstly, they are soaked in a 3% hydrogen peroxide solution for a duration of one hour. Following this, the membranes are immersed in deionized or distilled water for a period of two hours. Lastly, they undergo treatment in a 0.5M sulfuric acid solution for an additional hour. All of these steps are carried out at an approximate temperature of 80ºC.The Nafion is rinsed in deionized water between the steps and the membrane is then stored in ddH_2O. [46]

2. Electrolyte solution

a. Anodic artificial wastewater for single-strain MFC (g/L):

CH_3COONa (1), K_2HPO_4 (6.065), KH_2PO_4 (3), NaCl (0.5), NH_4CL (0.1), $MgSO_4$ · 7H2O (0.1), Trace metal solution (Sigma Aldrich, USA) 1 ml. [47]

b. Cathodic solution (g/L):

$K_3[Fe(CN)_6]$ (16.4), KH_2PO_4 (3.53), K_2HPO_4 (25.46)

3. Random positioning machine (RPM)

The microgravity device we used is the cooperation product with Thermofluid Bio-Energy Lab at National Ilan University led by Prof. Chin-Tsan Wang. The RPM (Figure. 2) can go up to 30 rpm, minimum 10 rpm. It includes 2 motors, for each frame, both powered through a 24V controller in series with the actuator, the control program is feeded through the Arduino.

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Results

Growth curve of *E. coli* k12 mg1655 and *Shewanella oneidensis* MR-1 under normal gravity using LB medium or artificial wastewater under Aerobic environment

First step of this experiment is to confirm the growth speed of two strains in LB medium and artificial wastewater. From the Figure. 3, we found that *E. coli* K12 MG1655 and *Shewanella oneidensis* MR-1 have similar growth trend in LB or artificial wastewater. When the both bacteria were cultured in LB medium, the growth rate is much quicker than in artificial wastewater.

The growth curve comparison of OD between normal gravity and microgravity in anaerobic environment

After culturing *E. coli* K12 MG1655 and *Shewanella oneidensis* MR-1 for one day in LB medium under normal gravity and microgravity conditions, a comparison of their growth rates revealed no significant difference between the two environments (Figure. 4). However, when the bacteria were cultured in artificial wastewater, their growth rate decreased significantly compared to when cultured in LB medium. LB after 1 day culture show that these two strains don't have better growth condition under microgravity.

The growth curve comparison of CFU/ml between normal gravity and microgravity in anaerobic environment

The results reveal that for *Shewanella oneidensis* MR-1, between different culture medium, waste water will let bacteria concentration increase in microgravity condition whereas the concentration didn't change in LB group in microgravity growth condition (Figure. 5).

The voltage of MFC in microgravity powered by *E. coli* in LB

The voltage measurement that I have done is only MFC in microgravity

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powered by *E. coli* in LB. The figure shows that the voltage keeps in almost zero during the first 56 hours and then go up to 0.04 MV until 140 hours (Figure. 6).

The operation of random positioning machine

In the figure. we can see that under the rotation rate of 12 rpm. The gravity is decrease from 1 G to approximately 0.1 G after 1000 seconds and then work smoothly for keeping gravity at 0.1 G (Figure. 7).

Discussion and conclusion

The growth curve in normal gravity and microgravity

The results show that the bacteria growth is downregulated under microgravity growth condition which is opposite to our purpose to find a way to improve electricity production by MFCs. However, it is reasonable because *E. coli* K12 MG1655 and *Shewanella oneidensis* MR-1 can survive between aerobic (outside a host) and anaerobic condition as part of their lifestyle. [48, 49] *E. coli* is a bacterium with versatile metabolism, capable of thriving in oxygen-rich and oxygen-depleted environments. It utilizes a flexible biochemical approach, prioritizing aerobic respiration over anaerobic respiration, and anaerobic respiration over fermentation. [50]. For *Shewanella oneidensis* MR-1, the reasonable consequence is the metabolism influenced by the rotation of random positioning machine, gene regulation which need further exploration. [51]

Figure 5. shows the growth curves of *E. coli* K12 MG1655 and *Shewanella oneidensis* MR-1 in both 1G (normal gravity) and microgravity conditions, measured by colony-forming units (CFUs). None of the experimental groups exhibited a doubling

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of their population throughout the duration of the experiment. This could be attributed to different exponential growth phases of the two strains or the lack of liquid movement in the microbial fuel cell (MFC) setup. When considering the growth phases of the two strains, it can be observed that after one day of culturing, a significant portion of the cells entered the death phase. [52] Consequently, although there may be a high number of cells present, their ability to form colonies on agar plates decreased substantially. The growth curve presented in Figure 5 does not demonstrate any noticeable significant growth. Another influence of gravity on bacteria is linked to two indirect processes: (a) settling of cells and nutrients, and (b) buoyant convection. [53] It was found that the absence of convection decreasing phosphate or oxygen availability in microgravity and further modifying microbial behavior in *Pseudomonas aeruginosa*. [54] The diminished convection resulting in substrate concentration gradients has been suggested as a broad explanation for the microbial alterations observed in microgravity.

Furthermore, the growth curve analysis of *E. coli* K12 MG1655 and *Shewanella oneidensis* MR-1 in normal gravity is inadequate due to the absence of data points between 6 and 25 hours, making it difficult to perform a thorough analysis on exponential phase. Therefore, it is crucial to establish a system that can continuously monitor the growth conditions within the microgravity device. In previous study, fluorescent proteins such as green fluorescent protein (GFP) has shown its advantage in noninvasive methods counting GFP-positive cells with flow cytometry which can be a way of continuous monitor and future research direction. [55]

The voltage of MFC in microgravity powered by *E. coli* in LB

The measurement of voltage shows that there are approximately 0.05 V after 56 hours culture. One of the reasons is that the bacteria was trying to adapt the new growth condition.

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Previous study has showed that the distance of electrode and material of electrode will influence the electricity production. [56] I utilized square-shaped graphite as electrode in my experiment. One effective way to elevate the electricity is to use other electrode made by other material. Interestingly, new material electrode such as Ti-TiO_2 has been reported that current densities were 476.6 mA/m^2 while graphite electrodes has only 31 mA/m^2. [57] In the future work, I think it is an efficient way to use different material as electrode.

Future work

Based on previous studies, microgravity has been shown to elicit varied responses in bacteria through different mechanisms. These include gene regulation, increased resistance to environmental factors, formation of thicker biofilms, and changes in electron transport chains (ETC) [58-63]. In future research, it is crucial to ascertain the overall trend and determine whether microbial fuel cells exhibit enhanced power generation capabilities in a microgravity environment, backed by comprehensive data analysis. Even if we observe improved or diminished power generation efficiency, it remains uncertain whether it is attributed to an increase in colony numbers, enhanced power generation efficiency at an individual bacteria level, or improved efficiency despite an unchanged colony count. Therefore, it is necessary to investigate whether changes in power generation efficiency under microgravity conditions are linked to alterations in colony numbers or other factors. This investigation will yield consequential insights into the underlying mechanisms:

- The growth rate of bacteria is faster, resulting in an increase in the number of colonies and improved power generation efficiency.
- The growth rate of bacteria remains unchanged, but the power generation efficiency per unit of bacteria improves.

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- The growth rate of bacteria slows down during power generation, but power generation efficiency improves.
- The downregulation or upregulation of gene that lead to the change of bacteria growth

Determining which scenario is more likely requires further investigation and experimentation under microgravity conditions. In my opinion, the scenario where the growth rate of bacteria remains constant, but the power generation efficiency per unit of bacteria improves is highly plausible and represents an ideal condition. This condition is advantageous as it leads to an upregulation of electricity density while maintaining a consistent bacterial growth rate. This means that the same rate of nutrition consumption in the anode can be sustained, resulting in improved power generation efficiency without compromising the growth of the bacteria. Another important point is to considering the operation of MFCs fixed in random positioning machine. The factors including the temperature, PH value, the way to tackle bubble in MFCs during operation, the way to take bacteria from MFCs. All of the points above will be crucial to elucidate the mechanism behind MFCs electricity production.

Biofilm status in MFC

According to prior research, a lower external resistance typically results in a greater rate of electron transfer from the biofilm to the electrode, leading to a greater proton production rate within the biofilm. This can cause a decrease in pH, creating extreme environment for the electrochemically active bacteria strains [64]. In my experiment, I did not assess the biofilm status, which will be a focus of my future work.

In conclusion, if I have sufficient resources to pursue further research, I intend to

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undertake the following steps:

1. Replicate the growth curve analysis of *E. coli* k12 mg1655 and *Shewanella oneidensis* MR-1 in both normal gravity and microgravity conditions. This time, measurements of O.D or CFU/ml will be taken every 2 hours to capture a more detailed picture of bacterial growth dynamics.
2. Develop a microgravity device that can be accommodated within an incubation box, allowing for controlled experimentation in a microgravity environment.
3. Assess the status of the biofilm formed on the electrode to gain insights into its composition and development.
4. Explore the influence of different combinations of artificial wastewater to determine the bacterial preferences and their impact on power generation efficiency.
5. In order to apply the microbial fuel cell (MFC) system to a cubic satellite, miniaturization is being pursued. This approach is being adopted due to limitations on the Taiwanese side, where the capacity to develop a spaceship is not available. By miniaturizing the MFC system and integrating it into a cubic satellite, it becomes feasible to investigate and comprehend the biochemical adaptations under actual space conditions.

By implementing these strategies, a more comprehensive understanding of bacterial behavior in microgravity and its implications for power generation efficiency can be achieved.

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Figures

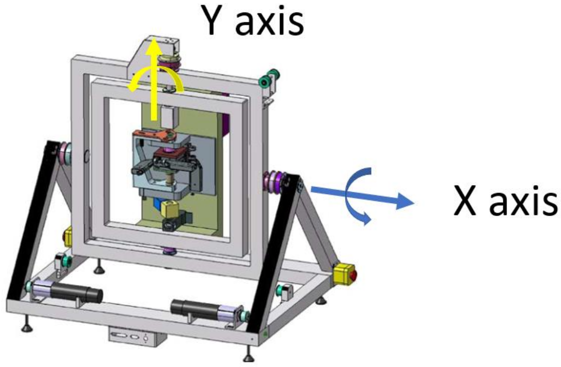

Figure. 1 Design diagram of RPM (A.G. Borst. Et al., 2014)

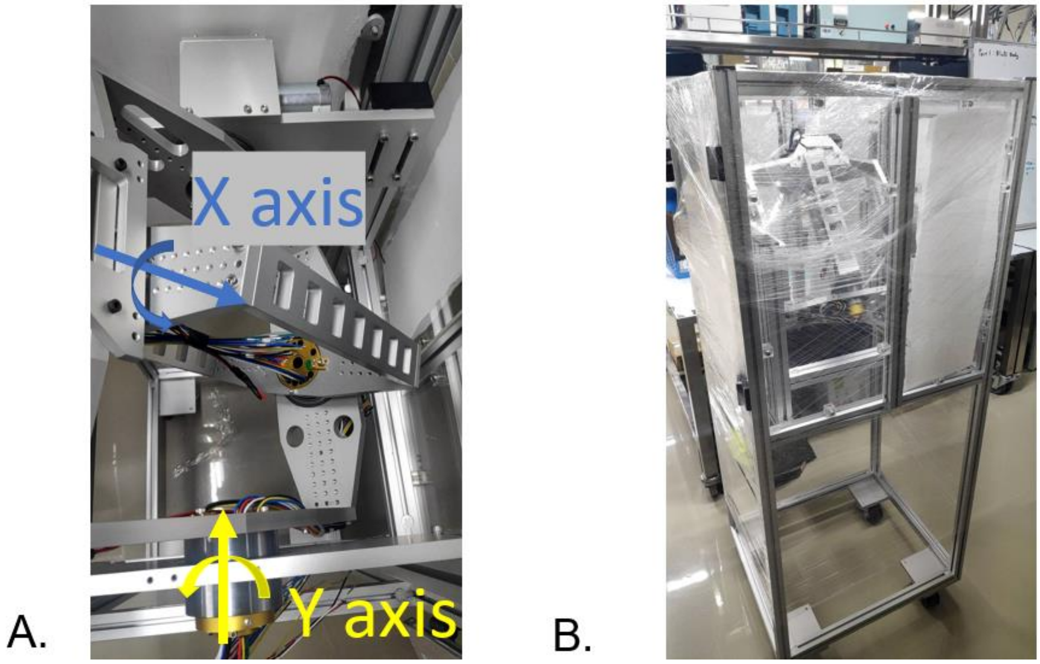

Figure. 2 RPM made by our team

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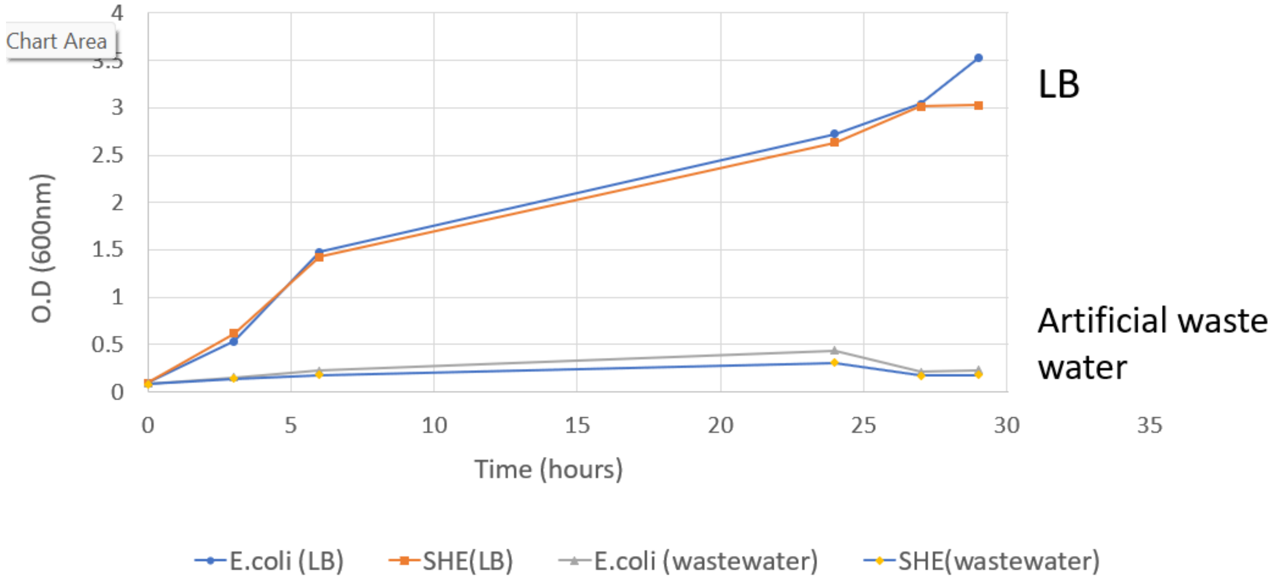

Figure. 3 Growth curve of *E. coli* K12 MG1655 and *Shewanella oneidensis* MR-1 in normal gravity

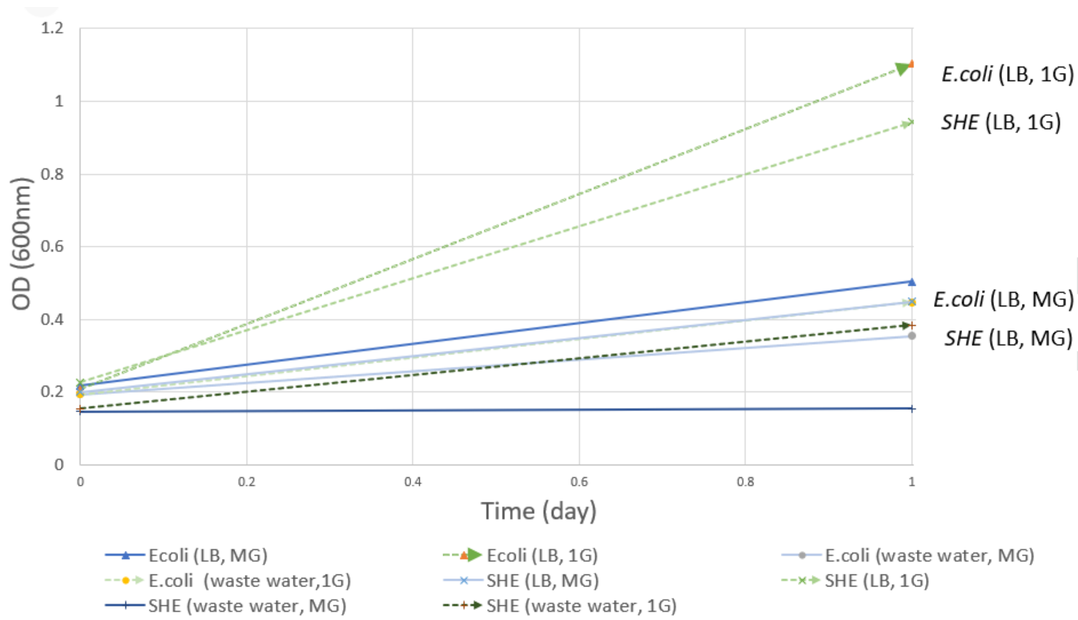

Figure. 4 Growth curve of E. coli K12 MG1655 and Shewanella oneidensis MR-1 in 1G and microgravity by OD (600nm)

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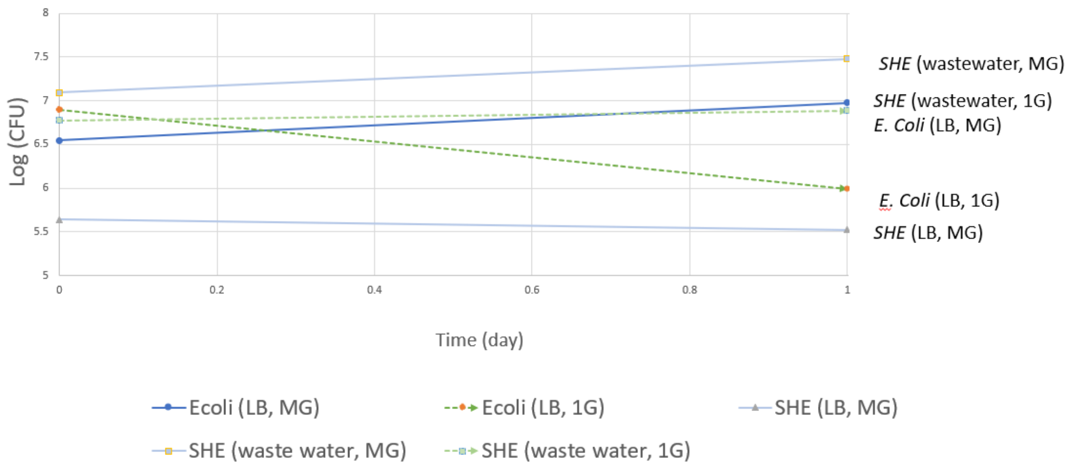

Figure. 5 Growth curve of E. coli K12 MG1655 and Shewanella oneidensis MR-1 in 1G and microgravity by CFU

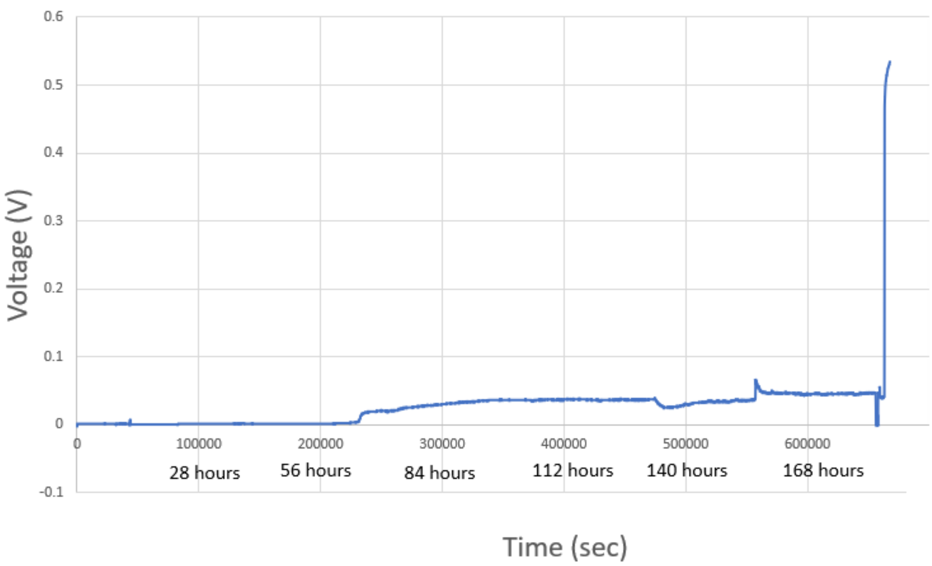

Figure. 6 The voltage of MFC in microgravity (E.coli in LB)

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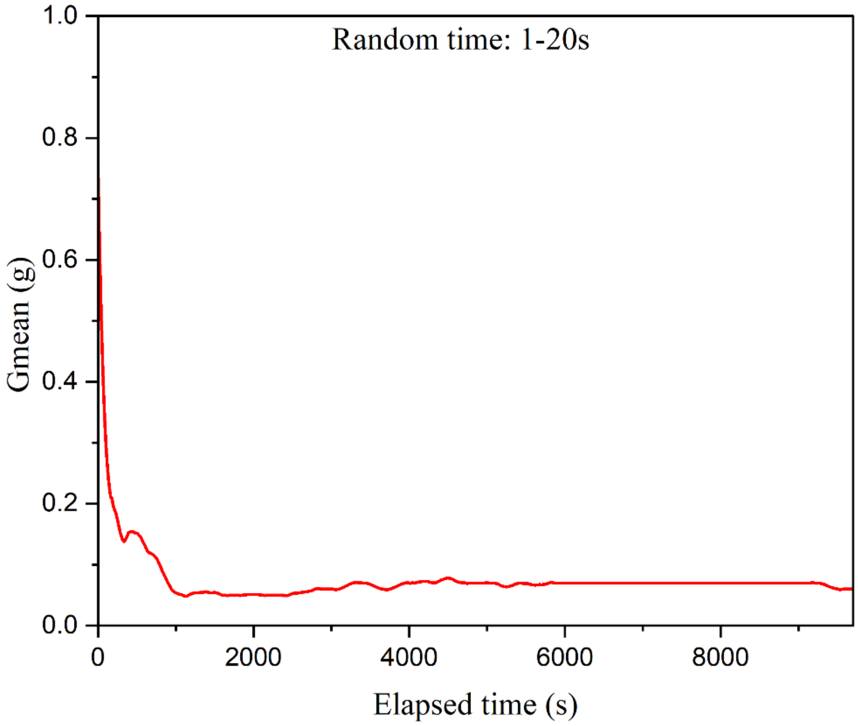

Figure. 7 The microgravity effect measurement by time

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