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Enhancing Biodegradation of Pyridine with Trehalose Lipid in *Rhodococcus pyridinivorans* sp. Strain HR-1-Inoculated Microbial Fuel Cell

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Abstract: A Gram-positive exoelectrogen *Rhodococcus pyridinivorans* sp. strain HR-1 was cultivated from leachate-fed microbial fuel cell (MFC) to study the biodegradation effect of pyridine. In the comparison with mixed cultured MFC, HR-1 presented a remarkable electrical capacity with a maximum output of 4.33 W/m^3 under 30 °C in neutral anolyte with 1 g/L acetate as a substrate. Further, HR-1 demonstrated the environmental resistance as a Gram-positive strain. Microbial metabolism was evident at pH between 5–9 and temperature in the range of 20–40 °C, whereas optimal condition for pyridine degradation was observed at 30 °C. This is the first study reporting the degradation of pyridine in the bio-electrochemical system that achieved a 42% ± 5% degradation rate in a full operation cycle at 2 g/L of the concentration. Considering the nonnegligible internal resistance of HR-1-inoculated MFC, trehalose lipid was also introduced as a bio-surfactant to reduce the charge transfer obstacle between the microbes and the electrodes. The surface morphology illustrated that the strain had a plump shape with a high specific area. Accordingly, bio-surfactant addition promoted the anode biomass (1.2 ± 0.1 mg/cm^2 to 1.7 ± 0.2 mg/cm^2) and achieved a higher degradation rate (68% ± 4%). The feasibility of electrochemical disposal on pyridine and eminent adaptability of strain sp. HR-1 as a Gram-positive exoelectrogen makes MFC a practical approach for real application.

Keywords: microbial fuel cells; gram-positive exoelectrogen; pyridine; trehalose lipid; *Rhodococcus pyridinivorans*

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1. Introduction

Treatment of leachate carrying high concentration of pyridine is an emerging but challenging research area in environmental biotechnology [1,2]. Albeit several strategies are tested, a cost-effective and nature-based solution is timely to reclaim the leachate without environmental footprints. Microbial fuel cell (MFCs) is such a technique that employ electrochemically active microbes as anode biocatalysts to transform the chemical energy of contaminants into electricity and decontaminate the source water [3]. Compared to general fuel cells, MFCs have the advantage of mild operating conditions and a wild availability of organic matter, including intractable oilfield-contaminated wastewater, municipal sewage, and industrial pollutants [4,5]. Electrode materials, chamber configuration [6,7], and exoelectrogens are hot topics of MFCs, which have accumulated numerous research experiences.

Exoelectrogen can transfer electrons to extracellular form from the degrading substrates [8]. Currently, numerous exoelectrogens are separated from MFCs based on sub-

strates of activated sludge or humus, which are mostly distributed in the phylum of *proteobacteria*, *firmicutes*, *acidobacteria*, and *actinobacteria* [9–11], including *Alpha-proteobacteria*, *Beta-proteobacteria*, *Gamma-proteobacteria*, *Deltapro-teobacteria*, *Epsilon-proteobacteria* [12,13], *Clostridia* [14,15], *Acidobacteria* [8,13], and *Actinobacteria* [15,16]. There are four well-known methods for moving the electrons from an exoelectrogen to an electrode: (1) a direct connection with an electrode allows transfer of electrons via cytochrome (Cyt c) and a membrane-bound protein [17]; (2) via electrically conductive flagellin-like nanowires [18]; (3) using electron shutters, a group of electrochemical active substances [19]; and (4) electrokinesis, transfer of electrons from an electrode surface by a rapid wave of flagellin [20]. Electron transfer mechanisms vary with each strain and operation condition, to ensure how electrons are transferred to further promote the power production. The electron transfer mechanisms vary with the environment. Nanowires are conductive flagellins which can mediate inaccessible electron transfer, meanwhile, as secondary metabolites, electron shuttles are available for long distance transfer from cell membrane to electrode surface. Biofilm, as the basis of direct contact mechanism, widely existed in the single chamber air-cathode MFC and able to convert electrons from intracellular to the electrode.

Recently, most MFCs are operated under mild conditions, at neutral pH and at room temperature [21,22]. However, when applied in effluent treatment such as leachate, a high pH or temperature will distinctly affect the exoelectrogen's bioactivity, especially those with weak resistance to the operating condition changes. Thus, to find an adaptable exoelectrogen is becoming urgent. Although most of the exoelectrogen found at present are gram-negative bacteria, the cell membrane structure of the positive bacteria is more compact, which has more advantages in the tolerance to extreme environments and severe influent fluctuation. The dense cell membrane could hinder the transmembrane transmission of electrons. However, the introduction of glycolipid surfactants is capable of reducing the surface tension, thus enhancing the transmission effect of electrons.

Compared to domestic sewage, leachate is more concentrated in organic matter and the quality parameters are more unstable, which makes it difficult to dispose of; the untreated leachate will severely harm the environment. Considering the adaptability of MFC, some research has been done to prove the feasibility of generating electricity with a leachate substrate. Specifically, Damiano et al. [23] reported that a maximum power density of 653 mW/m^3 was generated with 16% COD removal rate from landfill leachate in a single-chamber air cathode MFC, and in a membrane-less bio-electrochemical system, Zhang et al. [24] revealed that MFC could generate 260 mW/m^3 with a 84% COD removal rate. However, recent research only focuses on the degradation of organic matter in substrates but bacterium in leachate fed MFC have not been cultivated yet. In addition, the degradation of organic matter in leachate is not specific to a certain component, which could lead to the inability of extending the experience of electrochemical treatment on leachate to other fields and explore other potential substrates for bio-electrochemical systems. The current findings illuminate the existence of exoelectrogen in leachate based MFC which spontaneously have the ability to produce electricity in extreme conditions.

According to the component analysis of leachate, pyridine is a common small molecular organic pollutant with strong carcinogenic effects [25,26]. Fenton and microwave-coupled systems are traditional and effective methods for pyridine treatment [27–29]. However, considering that the Fenton method has additional strong oxidizing chemical reagents and considering the high energy consumption of the microwave method, how to achieve eco-friendly protection while ensuring the treatment effect is urgent. MFC is capable of disposing of the various organic substrates, such as typical organic dyes [30], due to the presence of anodic microbial catalysts. Pyridine has been demonstrated to be degradable by *Rhodococcus pyridinivorans* HR-1, which inhibited the potential of degradation feasibility in HR-1-fed MFC.

In this study, a newly isolated strain of *Rhodococcus pyridinivorans* is separated from Guangzhou landfill refuse. The cultivation was operated on the anode carbon cloth of a leachate fed MFC in a benchtop with sterilized scissors. The single colony obtained

by multiple lines is further inoculated into MFC after sequencing to verify the power generation ability. Strain *Rhodococcus pyridinivorans* HR-1 is finally selected as the target strain through comparison. HR-1 is employed as a biocatalyst in a single-chamber air cathode MFC to evaluate the degradation of pyridine under different conditions. Cyclic voltammetry (CV) analyses are conducted to elucidate the existence of an electron shutter, scanning electron microscope (SEM) is operated to observe how bacteria adhere to an electrode surface, and gas liquid chromatographic (GLC) is conducted to evaluate the degradation effect. The aim of this study is to verify that the available substrates of microorganisms do not differ in the natural state and in the bio-electrochemical system. The results could promote the production of MFCs towards practical application.

2. Materials and Methods

2.1. Separation and Cultivation of Exoelectrogen

The leachate-fed MFC was operated with diluted leachate (1: 10, v/v) sampled from Xingfeng municipal landfill, Guangzhou. After stable operation over 60 days, the anode was screened in a mineral salt solution (component (g/L): NaCl 10 g, $MgSO_4.7H_2O$ 0.7 g, $(NH_4)_2SO_3$ 1 g, KCl 0.7 g, KH_2PO_4 2 g, $K_2HPO_4.12H_2O$ 3 g, $CaCl_2$ 2 mg, $FeCl_3$.6H2O 0.05 g, $MnCl_2.4H_2O$ 0.5 mg, $CuSO_4$ 0.5 mg, $ZnSO_4.7H_2O$ 10 mg; and pH 7.5). The suspension was then diluted in a 10× gradient concentration four times and finally inoculated into a LB agar medium for single-strain cultivation.

The initial anolyte medium comprised PBS and substrate (1.0 g/L). PBS constituents were as follows (per liter): 2.93 g of KH_2PO_4, 0.3 g of $FeSO_4$, 5.87 g of K_2HPO_4, 2 g of NaCl, 5 g of $(NH_4)2SO_4$, 10 mL of trace mineral solution, and 10 mL of trace vitamin. While testing in CV, the vitamin was wiped out to eliminate its electrochemical active interference. LB medium comprised the following components (per liter): 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl. The media were sterilized at 121 °C for 20 min in an automatic autoclave (Zealway GR60DA, Shanghai, China) to avoid any microbial contamination. Substrate generation in MFC was under anaerobic respiration, whereby filtered N_2 was used for 20 min to purge all inoculated medium into MFC.

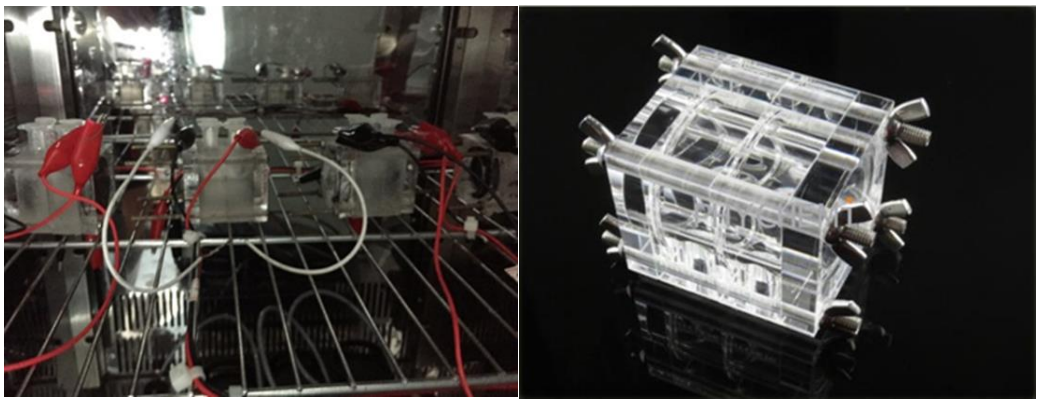
2.2. Identification of Strain

The 16S r RNA gene of Strain HR-1 was amplified on a thermocycler by using universal primers: 27F (5′-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [31,32]. The whole PCR amplification program contained the following procedures: final extension for ten min at 72 °C comes after thirty-five cycles of initial denaturation at 98 °C for five min, denaturation at 95 °C for forty-five sec, annealing at 55 °C for forty-five sec, and extension at 72 °C for ninety sec. The 25 µL PCR reaction system contained the following components: template 0.5 µL, ddH_2O 10 µL, forward primer 1 µL, reverse primer 1 µL, DNA cleavage ribozyme (mix buffer) 12.5 µL. Agarose gel electrophoresis (AGE) is conducted to detect the amplicon. Sequencing work is delivered to The Beijing Genomics Institute (BGI, Guangzhou, China) and the sequencing result is submitted to EZbiocloud (http://www.ezbiocloud.net/, accessed on 15 August 2022) to compare homology by BLAST. Mega7 was used to construct the phylogenetic tree.

2.3. MFC Construction and Operation

In this study, plexiglas (L= 5 cm, W = 5 cm, H= 5 cm, and r = 2 cm with 50 mL working volume) was used to construct a single-chamber air cathode MFC (Figure 1). The electrode, which served as the anode, was filled with carbon fabric. The carbon cloth was soaked in acetone for 6 h before use and then rinsed with deionized water. Platinum carbon powder was placed in a membrane cathode and mixed by Nafion (Hesen, Shanghai, China) [33]. To conduct electricity, a wire of titanium was employed between the anode and cathode. In order to ensure the tightness to prevent leakage of anolyte, the chamber was sealed with rubber rings in interfaces and tightened with screws. At 1k Ω of external resistance, MFC was operated at 30 °C (Boxun BSC250, Shanghai, China). As the output voltage was

dropped < 20 mV after each cycle, anolyte was replaced with a fresh medium containing 0.1 g/L of acetate.



(**A**) (**B**)

Figure 1. Single-chamber air cathode of microbial fuel cell (MFC). (**A**) presented the real operation condition in a constant incubator; (**B**) illuminated the Plexiglas structure of a single chamber air cathode before operation.

2.4. Optimal Operation of Substrate Degradation

The activated microbes were suspended in the PBS medium before inoculation. In the substrate availability experiment, the concentration of acetate, glucose, sucrose, and pyridine were 1 g/L, analytically pure (Aladdin, Shanghai, China). The initial pH was set in the range from 5–9 using 1 M NaOH or 1 M HCl. The operation temperature was regulated from 20 °C to 40 °C by the constant incubator.

2.5. Analytical Methods

A Keithley multichannel (2750, Solon, USA) was connected to the external resistance to achieve data collection every 30 min by real-time online monitoring with sample accuracy of 0.001 V. Electricity was determined with I = U/Rext. Prior to that, current (I) and power (P) density data was stabilized with the working area of the electrode. The external resistor was changed in the range of 10–10,000 Ω via a slide rheostat to attain a polarization curve, while the voltage was recorded at each resistance level after the value was stable for at least 2 min.

The electrochemical performances of the MFCs anode were measured through cyclic voltammetry (CV) by using the CHI-1010E analytical system (ChenHua Instruments Co. Ltd., Shanghai, China). Here, the scan rate was 50 mV/s from −0.8 V to 0.8 V. In order to avoid any interference, vitamin was wiped out from the last-fed anolyte for MFC. Pure N_2 was purged for 15 min into the chamber to eliminate any residual oxygen. For the experiment, the MFC anode served as an electrode, MFC cathode as a counter electrode, and an Ag/AgCl electrode (MF-2052, BAS) (assumed +197 mV vs. standard hydrogen electrode) as a reference electrode. The experiments were performed at the same temperature (30 °C), and the scanning was repeated thrice.

Before SEM, samples should be preprocessed [34]. Since the bacteria are single-cell organisms with a soft structure, samples were pretreated as per the critical point of the drying method. The anode membrane was fixed with 2.5% glutaraldehyde for 4 h, followed by washing of the sample using 0.1 M PBS 4–6 times and dehydration by various concentrations of alcohol (30 to 100%). Lastly, dehydrated anode membrane was changed by tert butyl alcohol (TBA).

Gas-liquid chromatography (GLC) was used to measure the pyridine degradation rate. Precisely, organic substrate was quantified using a gas chromatograph with a flame ionization detector (GC-FID) equipped with a capillary column (30 m × 250 μm × 0.25 μm) (19091S-433-Agilent HP-5ms). The injector and detector temperatures were kept at 250 °C. Oven temperature was initially set at 40 °C (held for 5 min), then increased to 150 °C at

a speed of 20 °C/min with a temperature retention period of 10 min. From there, the temperature was increased again to 250 °C at a rate of 10 °C min, which was then held for another 10 min. The flow rate was 50 mL/ min, and high-purity helium was used as carrier gas.

3. Results and Discussion

3.1. Isolation, Taxonomy, and Characterization of HR-1 from Leachate-Fed MFC

The exoelectrogen was separated from a MFC anode membrane based on leachate from landfill refuse Xingfeng which had been operated stably for over 60 days and named HR-1. After several episodes of scribing and purification, a single bacteria colony was obtained with a diameter of about 2 mm. The strain was rod shape, non-spore forming, non-motile, Gram-positive, and the colonies were light orange and round wrinkles.

After PCR, the whole strain sequence length was 1476 bp; the sequence got an accession number of AF173005 in EZbiocloud. The comparison results in Figure 2 showed that strain HR-1 had a homology of 98.82% with *Rhodococcus pyridinivorans* PDB9. The phylogenetic tree revealed that HR-1 and PDB9 were in the same branch of a phylogenetic tree. Combined with bacteria colony traits, HR-1 was authenticated as *Rhodococcus pyridinivorans*.

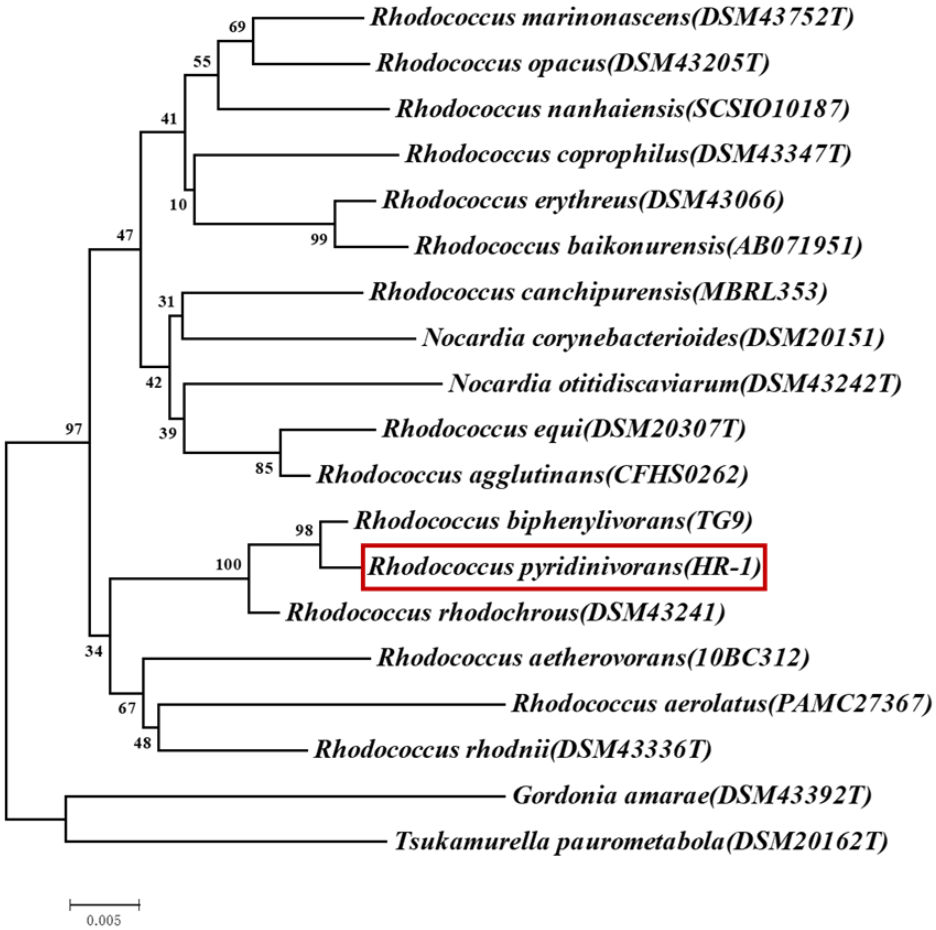


Figure 2. Phylogenetic tree using the 16s rDNA gene sequence to show the links between strain HR-1 and similar species. As an outgroup sequence, the strain *Rhodococcus* pyridinivorans PDB9 was used. To build the tree, the neighbor-joining technique was used. 1000 replicates were used to obtain the bootstrap values at the nodes (only values greater than 50% are shown).

3.2. Electrical Performance Comparison between Leachate-Fed and HR-1 Inoculated MFC

The performance of MFC inoculated with HR-1 was monitored under the same condition with the leachate-fed MFC to evaluate the electrical capacity. The electrical perfor-

mances were presented in Figure 3 including generation cycles and polarization curves. In Figure 3A, it was obvious that these MFCs were close in cycle length and maximum output, which the leachate-fed MFC could recover from the substrate exchange earlier. The curves revealed that HR-1 was electrochemically capable in MFC of metabolizing and acted as the dominant microbes in the mixed colony of leachate-fed MFC. Nevertheless, when the polarization curves were compared in Figure 3B,C, the performance presented a gap. The maximum power density of leachate-fed MFC was 6.79 W/m^3, which was 1.57 times that of the HR-1-inoculated MFC. Two MFCs were close in maximum voltage but quite different in power density; the results can be explained by Ohm law, since the mixed colony of leachate-fed MFC contributed to a lower internal resistance. Under the synergetic effect of various microorganisms, it was easier to transfer electrons across membranes and to the electrodes [35].

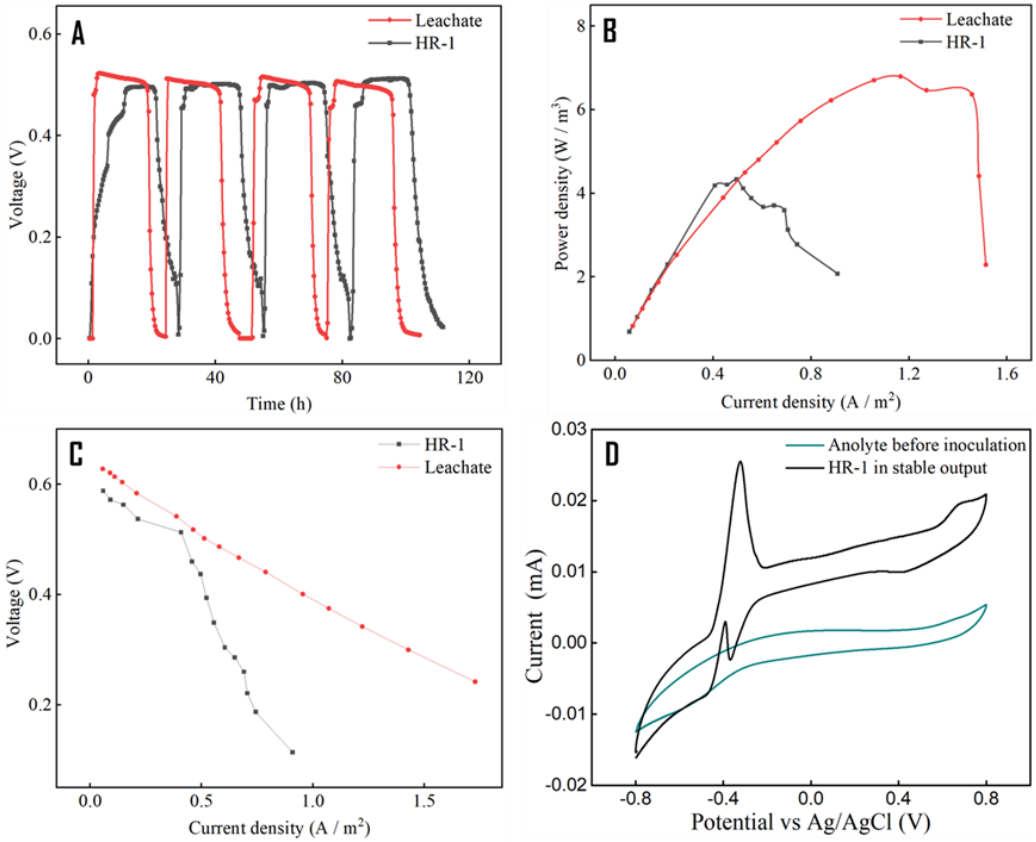


Figure 3. (**A**) Operation cycles of MFCs; (**B**,**C**) Polarization curves of leachate-fed MFC and HR-1-inoculated MFC; (**D**) CV curves of anolyte before Inoculation and HR-1-inoculated MFC in the stable phase.

To investigate the existence of electron shutters in extracellular electron transfer, CV was conducted at the initial inoculation and stable operation phase (Figure 3D). When MFC was initially inoculated with anolyte wiped free of vitamins before inoculation, there were no redox peaks observed. After operation with HR-1 inoculation for several cycles, redox peaks appeared at about −0.4 and −0.3 V (vs. Ag/AgCl). These findings are in accordance with the previous studies in which exoelectrogen self-excreted electrochemical active substances to transfer electrons between bacteria and electrodes [36–38]. The oxidation and reduction peaks were symmetrical, which implied that the redox reaction of strain HR-1 in MFC might be a reversible reaction. Besides, the PBS solution with organic substrate was refreshed after each cycle, and MFC exhibited a quick strong recovery, suggesting that strain HR-1 in MFC adhered to the anode.

3.3. Optimization of Substrate Degradation Conditions

Figure 4A presented the power output performance in HR-1-inoculated MFCs with different substrates in anolyte under a fixed external resistance of 10^3 Ω. It was obvious that strain HR-1 could produce electricity from various substrates, especially small-molecule carbohydrates. Acetate was the metabolite of most saccharides; strain HR-1 could output a maximum voltage of 513 mV, which revealed that acetate might be the direct electron donor for the electricity production. Glucose and sucrose were monosaccharides and output a maximum voltage of 493.3 and 473.7 mV, which were a little lower than acetate. *Rhodococcus pyridinivorans* was named for its ability to degrade pyridine [39]. Although pyridine was a small organic molecule, the electrical performance seemed unobtrusive, and the maximum voltage was 205 mV. However, considering the low solubility of pyridine in PBS solution and the clear anodic solution after degradation, the result was promising if the solubility was promoted by a surfactant addition or orbital incubator.

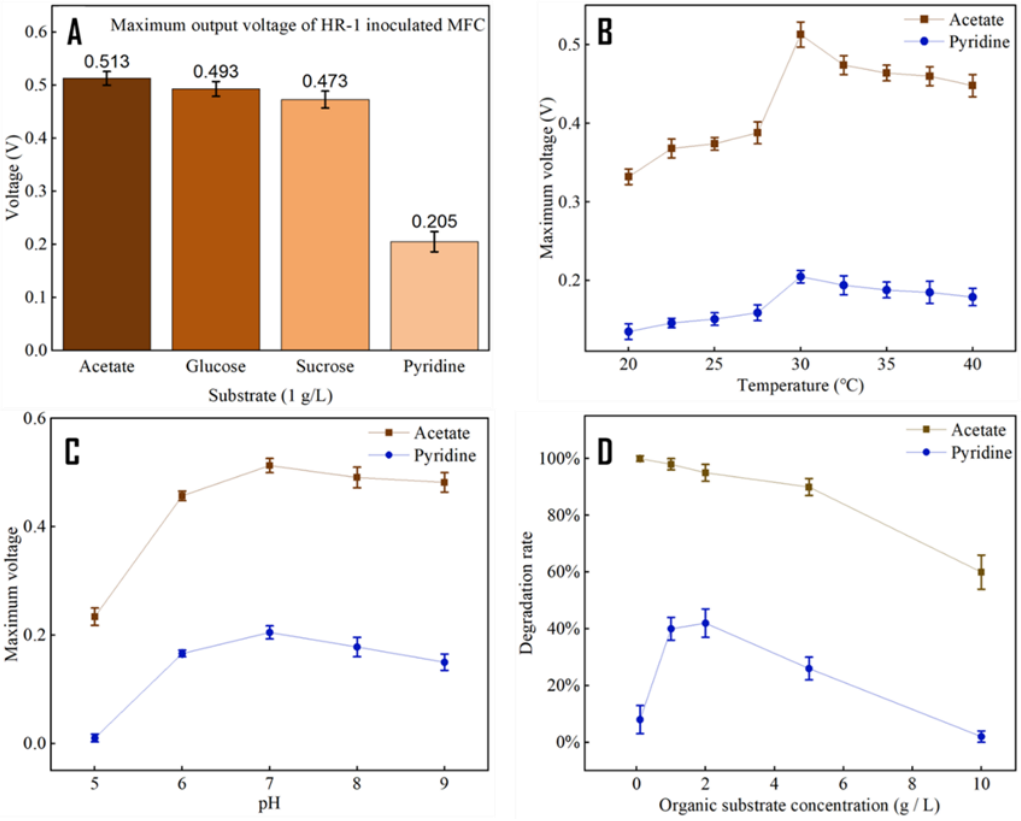


Figure 4. (**A**) Substrate availability of HR-1-inoculated MFC, the concentration was 1 g/L; (**B**) Maximum voltage of HR-1-inoculated MFC with acetate and pyrimidine under different temperature, the range was from 20–40 °C, the concentration was 1 g/L; (**C**) Maximum voltage under different pH, the range was from 5–9, with 1 g/L concentration; (**D**) Relationship between initial concentration and degradation rate, the operation condition was temperature at 30 °C and neutral.

Optimal temperature and pH were tested with acetate and pyridine as the substrate, respectively. In 1 g/L initially, HR-1-inoculated MFC generated electricity in a wide range of temperature from 20 °C to 40 °C (Figure 4B). In the acetate-fed MFC, when temperature was higher than 30 °C, the output was above 400 mV (445, 435.7, 432.6, and 421 mV, respectively); when temperature was below 30 °C, the output was above 300 mV (332.4, 368.4, 374.4 and 377.5 mV, respectively), which were much lower than at the temperatures higher than 30 °C. With the rapid rise of temperature from 20 °C to 27.5 °C, the output grew slowly. In contrast, when the temperature was over 30 °C, the output voltage declined gradually with the rapid rise of temperature. Since the activity of enzymes determines

the metabolism degree of the microorganism, a too high or too low temperature, such as 20 °C or 40 °C, could impact enzyme activity. MFC was a sealed acrylic container full of anolyte; a high temperature could accelerate the velocity of ionic flows in the MFC chamber to transfer the electrons. Based on the above factors, MFC could perform better in higher temperatures. The operation temperature of leachate-based MFC was 30 °C, at which strain HR-1 separated; it was also the optimal temperature for strain HR-1 to generate electricity. The behavior of pyridine under a variety of temperatures was similar to that of acetate. The electrical performance of pyridine as the substrate was weaker than that of acetate and the fluctuation was milder. The comparison revealed that temperature dominated the MFC output by affecting relative enzyme activity.

Figure 4C compared the electrical performance of HR-1-inoculated MFC under different pH with acetate and pyridine as the substrate, respectively. *Rhodococcus pyridinivorans* in HR-1 could grow under a wide pH range from 6–9 and the optimal pH was 7.5–8.5 [40], while the strain HR-1-based MFC performed favorably as well and output a maximum voltage of 456.5, 513, 491 and 482 mV, respectively. It was obvious that the neutral condition was the most suitable environment to metabolize the HR-1 strain. It can be speculated that HR-1 was more durable to alkalinity, for the pyridine-fed MFC could only output 10 ± 7 mV voltage under a pH of 5. Electricity generation with Gram-positive exoelectrogens under an alkaline environment had been proved in previous studies [41–43]. As a novel Gram-positive microbe with electrochemical activity, HR-1 inherited alkalinity endurance while maintaining practical output.

The initial concentration was adjusted as 0.5, 1, 2, 5 and 10 g/L, respectively. Figure 4D presented the downtrend of the acetate degradation rate; it was observed that acetate was the direct electron donor to the extracellular space in anaerobic respiration. At the concentration of 0.5 g/L, the degradation rate of acetate was 100%. Meanwhile, the maximum degradation rate of pyridine occurred at the concentration of 2 g/L, which revealed that the solubility coefficient impeded the biodegradation of pyridine [44].

3.4. Introduction of Trehalose Lipid to Enhance Pyridine Degradation Rate

Although pyridine proved to be biodegradable in HR-1-inoculated MFC, compared to common substrates such as acetate, the degradation rate was unsatisfactory in a full generation cycle. At the hydraulic retention time of 30 h, the maximum degradation rate was only 42%, which presented a greater scientific significance rather than practical value. Due to the dense cell membrane structure of Gram-positive bacteria, the transmembrane transmission of electrons is hindered by non-conductive peptidoglycan and phosphoteichoic acid. Meanwhile, the surface of the cell membrane is a water-soluble phospholipid molecule, while the substrate pyridine is a non-polar substance, the solubility is low when it is transferred into the membrane and metabolized by microorganisms. Surfactants like glycolipid are biocompatible when reducing surface tension, which can effectively increase solubility and promote degradation effect. Trehalose lipid was a glycolipid surfactant with remarkable biocompatibility and had been proved efficient in MFCs [45]. The introduction of biosurfactants could effectively reduce the interfacial tension, enhance the transmembrane exchange of substrates and the firm attachment of microbes on the anode. Figure 5 revealed that adding 20 mg/L of trehalose lipid dramatically promoted the biomass, which reflected the increment of aerobic bacterial count. Besides, the surfactant increased the solubility of pyridine and resulted in the promotion of the degradation rate ($42\% \pm 5\%$ to $68\% \pm 4\%$). The treatment is simple, and the enhancement is prominent.

To further explore how trehalose lipid affected the metabolism of HR-1, surface morphology was screened by SEM. Figure 6A illustrated the adhesion of microbes on the anode surface, which demonstrated the existence of a biofilm mechanism [46]. Strain HR-1 was rod-shaped without flagellum and arranged in a single layer due to the surface contact suppression. When treated with trehalose lipid, the cells were plump in Figure 6B, which was higher in specific surface area. The effectively reduced surface tension and better metabolic performance increased the degradation rate of pyridine by 61.9% at length.

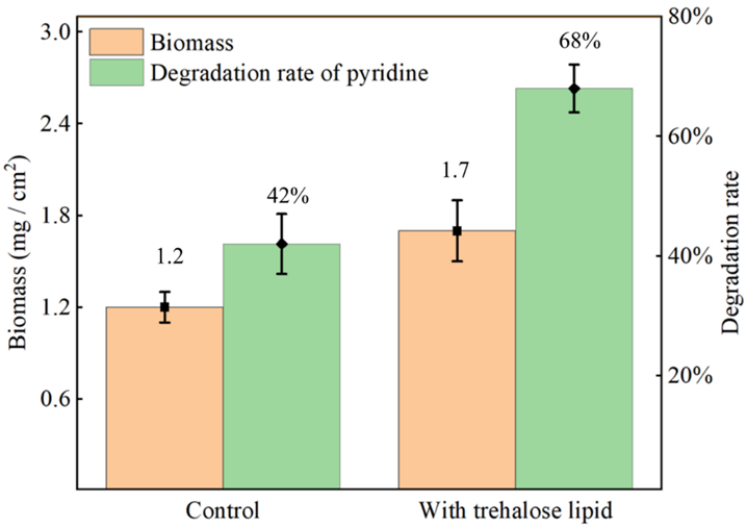


Figure 5. Biomass and degradation rate comparison with and without trehalose lipid; the operation condition was set at 30 °C, neutral and the pyridine concentration was 2 g/L. Biomass was evaluated by protein content; the addition of trehalose lipid was 20 mg/L.

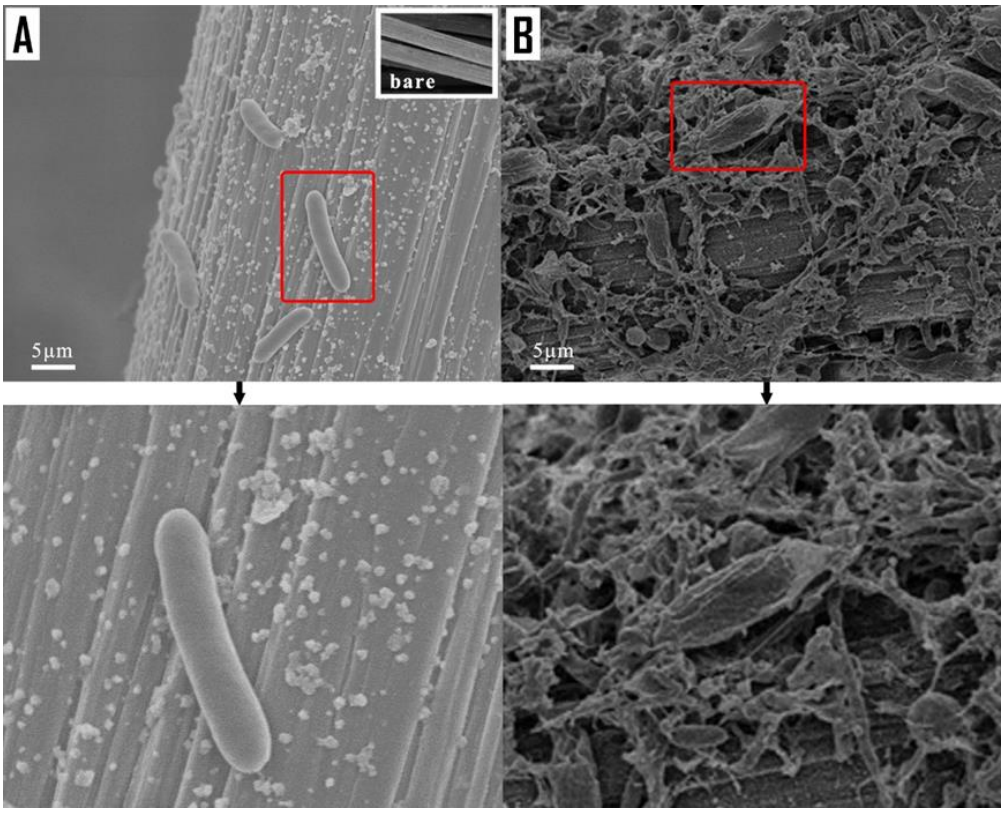


Figure 6. (**A**) Surface morphology of HR-1-inoculated MFC and the bare anode; (**B**) Plump cells on the anode with trehalose lipid. The plotting scale was 5 μm on anode carbon cloth.

4. Conclusions

This study for the first time proved the biodegradation of pyridine in a *Rhodococcus pyridinivorans* HR-1-inoculated MFC. The initial degradation rate of 2 g/L pyridine was 42% ± 5%; after adding 20 mg/L trehalose lipid, a glycolipid biosurfactant, the degradation rate increased to 68% ± 4%. The evaluation of optimal conditions confirmed the practical application potentials of HR-1 when applied in fluctuated real wastewater. Meanwhile,

the degradation of pyridine in MFC expanded the availability of substrates in the bioelectrochemical systems. The results broadened the range of electrochemical active microbes especially in Gram-positive bacteria and provided an alternative way of disposing of the pyridine-containing wastewater.

Author Contributions: P.C.: Conceptualization; Data curation; Formal analysis; Writing–original draft. M.U.: Data curation; Formal analysis; Writing–original draft. M.A.: Formal analysis; Investigation; Methodology. H.S.: Formal analysis; Investigation: Validation. L.Z.: Conceptualization; Data curation; Funding acquisition; Writing–review & editing. M.G.E.-D.: Supervision; Funding acquisition; Project administration; Review & editing. All authors have read and agreed to the published version of the manuscript.

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