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Microbial Electrochemical Treatment of Methyl Red Dye Degradation Using Co-Culture Method

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Abstract: Methyl red, a synthetic azo dye, was reported for not only being mutagenic but also its persistence has severe consequences on human health, such as cancer, alongside detrimental environmental effects. In the present study, the *Pseudomonas putida* OsEnB_HZB_G20 strain was isolated from the soil sample to study the catalytic activity for the degradation of methyl red dye. Another isolated strain, the *Pseudomonas aeruginosa* PA 1_NCHU strain was used as an electroactive anodophile and mixed with the *Pseudomonas putida* OsEnB_HZB_G20 strain to see the effect of co-culturing on the power generation in single-chambered microbial fuel cells (MFCs). The *Pseudomonas putida* OsEnB_HZB_G20 and *Pseudomonas aeruginosa* PA 1_NCHU strains were used as co-culture inoculum in a 1:1 ratio in MFCs. This work uses isolated bacterial strains in a co-culture to treat wastewater with varying methyl red dye concentrations and anolyte pH to investigate its effect on power output in MFCs. This co-culture produced up to 7.3 W/m^3 of power density with a 250 mgL^{-1} of dye concentration, along with 95% decolorization, indicating that the symbiotic relationship between these bacteria resulted in improved MFC performance simultaneous to dye degradation. Furthermore, the co-culture of *Pseudomonas putida* and *Pseudomonas aeruginosa* in a 1:1 ratio demonstrated improved power generation in MFCs at an optimized pH of 7.

Keywords: methyl red; biological treatment; dye degradation; *Pseudomonas putida*; microbial fuel cells; power density

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1. Introduction

Dyes (e.g. methyl red) are widely used in various goods produced by sectors, including textiles, food, and pharmaceuticals [1]. Because they are simple to use, consume less energy, have a wide range of structural variations (Figure 1), and come in a wide range of color hues, dyes are widely employed [2]. India's textile industries positively impact the economy, employment, and large-scale activities [3]. High salt concentrations, 50–2500 Pt/Co units of high color, and an alkaline pH (7–10) are all present in textile effluents. These effluents have 80–6000 mg/L of biological oxygen demand (BOD) and 150–12,000 mg/L of chemical oxygen demand (COD) [4]. Due to their chemical background and sophisticated, robust architecture, the dyes resist conventional sewage remediation techniques [5].

Aquatic life could be damaged and light penetration activity is decreased due to inefficient azo dye industrial effluent discharge. A system incapable of effective dye removal negatively impacts the environment and inhibits the growth of aquatic phototrophs [6]. Azo dyes can cause various harmful side effects in humans, including cancer, chromosomal abnormalities in cells, splenic carcinomas, eye irritation, hepato-carcinoma, and nuclear

abnormalities [7]. These dyes have the potential to cause fever, kidney damage, and cramping since they are highly soluble in water and can enter humans through the food chain [8]. These dyes have three main effects; teratogenic, mutagenic, and carcinogenic. The pigment methyl red is also mutagenic [9]. It has the mono-azo bond but lacks sulfonated groups. Researchers have employed various ways to remove harmful and resistant azo dyes from wastewater.

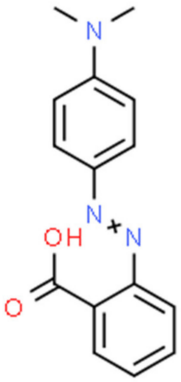


Figure 1. Chemical structure of Methyl red.

Physical, chemical, and biological approaches are frequently used to address the breakdown of leftover azo dyes in wastewater. The physical and chemical processes include adsorption, flocculation, electro-coagulation, precipitation, ozonation, and irradiation. The dyes break down using physical processes such as adsorption, ion exchange, and membrane filtering. These physical approaches had two major drawbacks: first, they were only effective when there was a small volume of wastewater, and second, they did not actually remove the colors but rather transported them to other phases. By pumping wastewater from the dyeing process over beds of ion exchange resins, some unwanted cations and anions in the polluted water can be exchanged for sodium and hydrogen ions, respectively. A synthetic process that uses the synergistic impact of multiple chemical oxidations is called advanced oxidation. It is a remarkable decolorization method because it can eliminate harmful chemicals and colors even in abnormal conditions. Regrettably, this is a pricey procedure with unfavorable metabolic outcomes. It also has a narrow range of usable environments because it is pH-dependent. Dye degradation was not achieved by these methods; consequently, dye leaching could occur in the long run. The Activated sludge process and anaerobic digestion can produce a huge quantity of sludge. This is another big issue that causes secondary pollution. These technologies are not only challenging to implement, in addition, the ASP technology is also energy intensive. Dye removal can be achieved by microbes or isolated enzyme molecules, and aerobic/anaerobic techniques rely on the use of bacteria to break down the colors. Moreover, ozone molecules can be used in extremely potent chemical oxidation processes, causing even dye molecules with double bonds and complicated aromatic rings to break down. These medications are effective but have undesirable side effects and are costly to acquire. Last but not least, associative enhanced oxidation methods exist [10], which can be broken up into several smaller parts. To remove discoloration artificially, scientists developed a technique called advanced oxidation. In unusual situations, it can remove dangerous compounds and colors, making it an ideal decolorization method. In addition to being expensive, this procedure also has unfavorable metabolic effects. It also requires specific pH levels and operating conditions, making it pH-dependent. Studies have not extensively looked into the electrochemical reduction approach because of its low yield in contaminant breakdown compared to direct and indirect electro-oxidation methods. Using reactive dyes in pad-batch color baths is one example of a highly colorful effluent that this technique is ideally suited to clean up. Dye reduction yields a mixture of hydrazine and amino chemicals. They emphasize the importance of a separate cell when using chloride-based dye baths;

doing so is necessary to avoid the production of chlorine and chlorinated byproducts. A major perk of this method is that it requires no chemical treatments and generates no sludge at any stage. Although it is an effective strategy for disposing of both soluble and insoluble dyes, the operation is more costly and less efficient than alternatives due to greater flow rates, as it produces dangerous compounds and requires energy. Fenton's reagent, a catalyst, and a hydrogen peroxide mixture is used in the Fenton process for color removal from wastewater. Using biological techniques for the degradation of dye with biological phenomena such as bioremediation is a sustainable method for removing dye from textile waste with the lowest possible cost and the shortest possible working period. Success was achieved in treating textile wastewater using only the most fundamental biological technique. When compared to alternative processes, biological degradation is more cost-effective, environmentally safe, and produces less sludge. By severing the bond, specifically the chromophoric group, dyes can be decolorized and broken down into less harmful inorganic chemicals. Inexpensive and efficient, adsorption can remove the color from water. In the adsorption process, activated carbon is the main component used. However, its high price tag and lack of sustainability make it impractical for general use. On the other hand, adsorption by biomaterial is an alternative method for bio-adsorptive color removal.

Bioremediation, unlike the more conventional methods, is a preferable choice for dealing with textile waste since it is less hazardous, costs less money, has less of a negative impact on the environment, and is more often than not successful. Since the process of anaerobic dye degradation of effluent is relatively simple, it could be a viable option since it is an unspecific azo-dye degradation method. Reduced azo dyes are produced through direct enzymatic processes, catalyzed by bacteria called azo-reductase. Most bacteria that degrade colors in aerobic environments require an additional carbon source since they cannot use the dye itself as a carbon source. Few bacteria can survive on azo compounds alone. *Pigmentiphaga kullae* K24 and *Xenophilus azovorans* KF 46 are two examples of bacteria that can hydrolyze -N=N- bonds and grow on amines. Bioaccumulation refers to the active uptake of toxicants by living cells, while biosorption describes the passive uptake of such substances by nonliving biological matter. Biosorption has many benefits over bioaccumulation, the most prominent being that it does not require the use of living organisms for the indefinite treatment of especially hazardous effluents [11].

The idea of electricity generation by microorganisms has been studied for over two decades and extensively researched. Microbial fuel cells (MFCs) are bio-electrochemical systems (BES) that oxidize organic materials for current production using bacteria as catalysts. These systems use electroactive bacterial oxidation of the substrate or fuel to produce electrons [12–14]. The electrons are delivered to the anode through various extracellular electron transfer mechanisms, travel through an external load, and combine with a cathodic electron acceptor. The most crucial element of MFCs is electrochemically active bacteria (EAB) since their capacity to produce oxygen is essential to generating electricity [15]. Wastewater, soil sediment, and anaerobic sludge are often utilized as a source of inoculum in MFCs because they naturally contain electroactive bacteria like dissimilatory metal or sulfate-reducing bacteria that may be enriched during MFC operation. Studies on the microbial community in MFCs have identified numerous microorganisms that depend on one another to carry out specific tasks, such as the breakdown of complex organic substrates [16]. Nevertheless, it is difficult to understand the dynamics of the microbial community and the roles that different species play in substrate oxidation and electron transport due to the variety of the microbial community [17].

MFCs use bacteria as catalysts to convert chemical energy into electricity. Microorganisms oxidize the organic substrate, releasing electrons in this process [18]. These inocula include live, electroactive microorganisms along with other hydrolyzing microbes in combination as inoculum at the anode chamber. Studies on the structure of the microbial community in MFCs show that many different types of bacteria work together, encouraging one another to complete decomposing complex organic substrates without causing

'product inhibition' [19]. The advantages of MFCs include high thermodynamic efficiency, less sludge production, and single-step bioelectricity production [20]. They are suitable for the environment because they drastically cut down on emissions of carbon dioxide and other hazardous pollutants. Because of their reduced bulk, fuel cells represent a viable alternative to traditional fuel sources [21].

Co-culturing was found as an effective strategy to treat complex organic substances for bioenergy production. In addition to providing chances to enhance technological applications, co-culture also supports the symbiotic interactions between microbial species that are the driving force for the anodic oxidation in the MFC operation [22]. Some microbial cultures do better and provide more of the desired functional metabolic activities when co-cultured with another species rather than in a monoculture population [23]. However, there are not many reports available on simultaneous methyl red dye degradation with concomitant electricity production. The goal of this research is to use isolated bacterial strains as inoculum in a co-culture to treat wastewater containing varying concentrations of methyl red dye under optimal conditions while simultaneously producing electricity by using MFCs.

In the current investigation, the degradation of methyl red dye by microorganisms was studied and the present study reports the isolation and screening of bacteria for the dye degradation, which was degraded in the MFC's anode chamber using the co-culture approach. Bacteria were isolated, identified, and put to work in the anode chamber as biocatalysts. Additionally, different concentrations of methyl red (50, 100, 150, 200, 250 mg/L) were used in MFCs to find the influence of initial dye concentration on volumetric power density, dye removal (in percentage), and coulombic efficiency in a batch mode reactor. Presently, a co-culture (blend of the isolated microorganisms *Pseudomonas putida* OsEnB_HZB_G20 strain and *Pseudomonas aeruginosa* PA1 NCHU strain) was used as inoculum to enumerate the power output and cod removal. Dye removal efficiency and power output were measured as a function of variables, including dye concentration and anolyte pH.

2. Materials and Methods

2.1. Soil Sample Collection

The contaminated soil sample was collected from the surroundings of a local fabric dyeing shop located next to Sharda University, UP, India. The soil from the depths ensured the existence of a significant amount of moisture necessary for microbe survival.

2.2. Microbial Screening and Isolation

The soil sample was collected in a test tube and transferred to the research laboratory. A total of 1 g of soil sample was suspended in 10 mL of sterile sodium chloride solution at 0.85% (w/v) and mixed well by stirring. This dilution technique was repeated thrice and the sample was inoculated onto the minimal salt medium (MSM) containing agar plates containing 250 mgL^{-1} of the methyl red dye concentration at 37 °C for 12–24 h in the incubator [24]. The isolated colonies grew on the culture plate and showed prominent decolorization zones. A colony of bacteria surrounded by prominent zones of decolorization was chosen because the decolorization zones imply that the dye in the surrounding regions was degraded [25]. Further, for the utilization of EAB, *Pseudomonas* sp. was used, where the same serial dilution techniques under the same conditions were used. Later, both microbes were grown using an LB medium. The pH of both growth mediums was adjusted to 7.0 for the pure culture of both the microorganisms, i.e., the methyl red degrading bacteria and *Pseudomonas* sp. The bacterial colonies are then picked and streaked on a fresh nutrient agar plate to culture pure isolates. Those isolates are then preserved in 60% glycerol at −80 °C. Isolation was followed by molecular characterization and biochemical analysis of the isolated strains. Gram Staining was performed to study the morphology of the bacteria [26]. 16S rRNA sequencing was carried out by Sanders Lifesciences Pvt, Hyderabad, India. The PCR was carried out in the same lab and the results were submitted

to GenBank. The *Pseudomonas putida* and *Pseudomonas aeruginosa* isolate were named as B1 and B2 respectively.

2.3. Decolorization Study

One colony of *Pseudomonas putida* bacteria was cultured in 10 mL of broth with 0.85% sodium chloride and yeast extract at 37 °C and kept on a shaker for 24 h to form a pre-culture or isolate. The culture was kept under the same conditions, including incubation at 37 °C for one full day [27]. The flask contained 200 mL of minimal salt media (MSM) with 250 mgL^{-1} of methyl red dye, into which 5 mL of pre-culture was inoculated. Using a UV/Visible spectrophotometer, the cell densities of bacterial cultures of 10 mL were measured at 600 nm at various time points to determine the rate of growth. Next, the bacteria were centrifuged at 15,000 rpm for 20 min to separate the cells, and the supernatant was collected and analyzed with a spectrophotometer to determine the dye concentration. Methyl red dye's absorbance at 425 nm was measured [28]. A blank was represented by a media free from methyl red dye and inoculum, while a control was represented by a medium containing dye but no inoculum. The efficiency (in percent) of dye decolorization was determined using the following formula:

$$Decolorization(\%) = \frac{A0 - A}{A0} \times 100$$

where $A0$ is the initial absorbance of the medium before the addition of the inoculum and A is the absorbance of the medium after it was decolorized [29].

2.4. Microbial Fuel Cell Design and Bacterial Inoculation

A total of five 300 mL volume air-cathode single-chamber MFCs were used for dye degradation studies, with carbon felt anodes measuring 6 cm × 6 cm, cathodes left open to the air, and stainless steel wiring throughout the circuit [30]. There were two openings on top of the anode chamber, one for the electrode terminal and the other for the reference electrode (Ag/AgCl, saturated KCl; +197 mV, Equiptronics, India) and the sample. Next, we used an MFC containing a 1:1 combination of the respective isolated bacterial cultures B1 and B2 to test whether or not these microbes were capable of degrading the Methyl red dye and producing current at the same time. After the OCV was stable, polarization and power density curves were obtained by performing an electrochemical examination of the MFC with a variable resistor (10–10000Ω). The stainless-steel mesh employed in this research was of the SS-304 variety and included 50 squares per inch in its holes. The SS mesh was made with wire that was 0.17 mm in diameter. A hidden copper wire served as the cathode terminal for the connection. As a result, the external resistance was connected via the disguised copper wires. All tests had an inter-electrode spacing of roughly 2.5 cm. Equal distances from the MCA were used when positioning the anodes. Extra ports were hermetically sealed with clamped tubes to create an oxygen-free zone [31].

The digital multimeter was used to measure the current flow (HTC 830L). The polarization experiment utilized a data recorder and a changeable external resistance stage. The volumetric power density was measured like in earlier studies.

$$P_d = EI/V$$

The P_d (W/m^3), volumetric power density is calculated by normalizing to the effective anolyte volume where E and I are voltage and current, respectively, coupled to precisely imposed loads, and V_{and} is the volume of the anolyte containing methyl red dye [32]. The MFC's internal resistance was calculated by finding the slope of the linear region of the voltage versus current graph. The effect of dye concentration and pH was investigated on volumetric power output by varying the different concentrations of MR dye and different anolyte pH. It was investigated utilizing electrochemical methods, such as polarization studies, cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) [33]. Each MFC's platinum rod and anode functioned as the counter and working electrodes,

respectively, in the 3-electrode arrangement used in the EC lab's electrochemical interrogations. The voltage and current vs. the Ag/AgCl counter electrode (+197 mV vs. SHE) were measured [34]. The EIS was achieved by applying alternating current (AC) at a frequency between 100 kHz and 100 MHz with a sinusoidal perturbation of 5 mV. The EIS spectra were modeled as an analogous network to determine charge transfer resistance (R_{ct}) and solution resistance (R_s) [35]. CV was recorded in the potential window of 0.0–0.6 V at a scan rate of 2 mV/s [36].

2.5. Anolyte pH and Substrate Concentration Optimization

The anolyte pH and dye concentration play important roles in determining microbial dye catabolism and simultaneous bioelectricity generation [37].

2.5.1. Effect of Anolyte pH

To find out the optimum pH conditions, the anolyte pH of the growth media was adjusted to different anolyte pH using HCl or NaOH before the inoculation, where it was incubated at 37 °C. After each batch study, the results were analyzed to find out the optimum pH where maximum power output can be obtained.

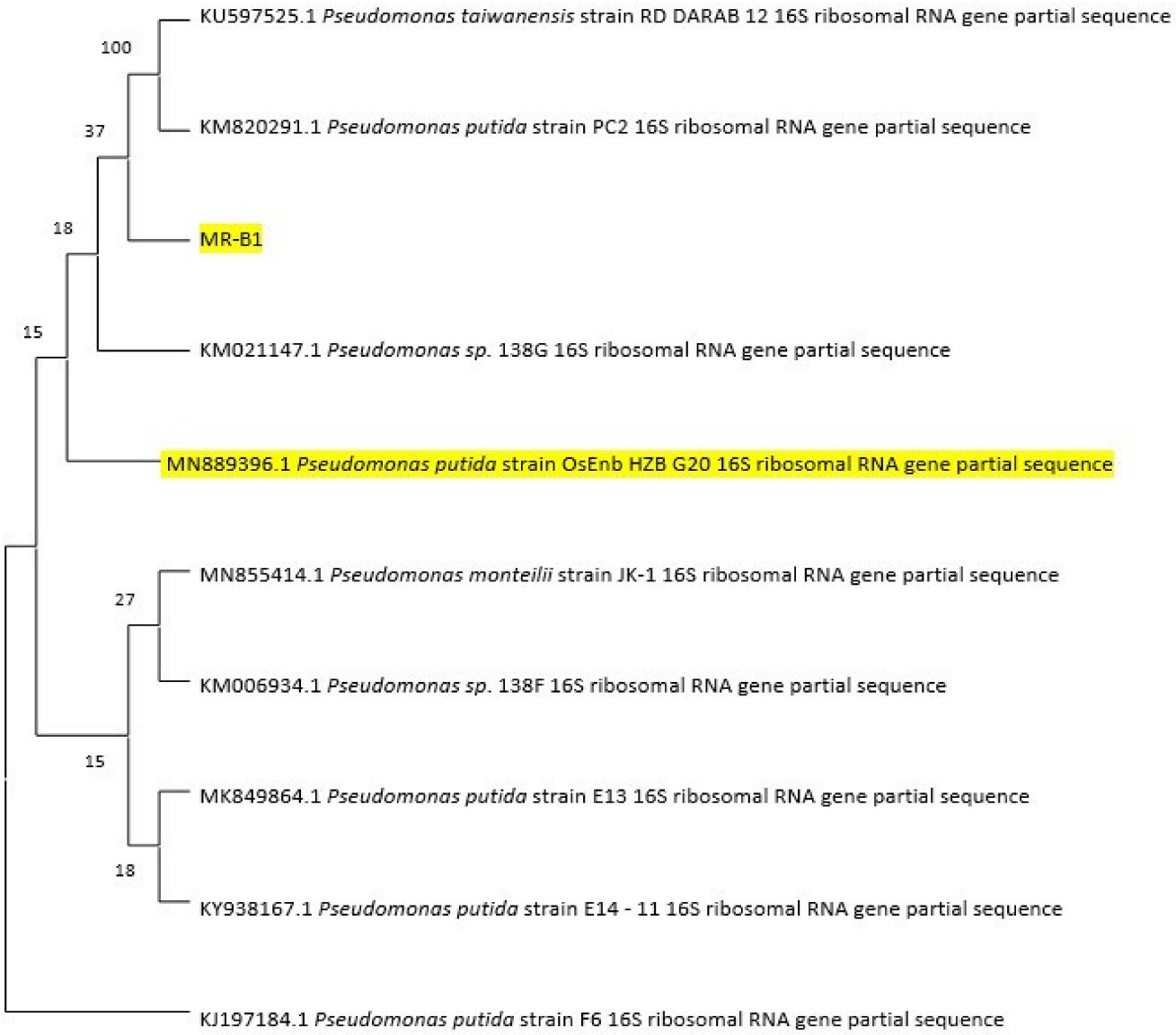
2.5.2. Effect of Substrate Concentration

After completing the optimization of the pH, the growth medium was used to examine the effect of substrate concentrations. The substrate concentration was varied as 50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L, and 250 mg/L. The inoculated cultures were maintained at the optimized pH of 7 and incubated in the shaking condition on an orbital shaker at 37 °C and 150 rpm, respectively, for 24 h to obtain relevant results. It was reported that growth was inhibited at 300 mg/L Methyl red concentration. The MFCs were run in batch mode with a 36 h cycle time (approximately 37 °C) [38]. After the initial anolyte pH was adjusted using an acid or base solution, the pH was optimized before the addition of the inoculum. In total, 10% v/v of inoculum was used per MFC, and an optical density of 0.8 in our tests with the isolated microorganisms (B1 and B2). Methyl red dyes of varying concentrations were utilized to make an anolyte, which was mixed with low salt media. MSM broth had the following ingredients (in g/L): dye, peptone 5, Na_2HPO_4, K_2HPO_4, NH_4NO_3, $MgSO_4$, and $CaCl_2$. The pH was maintained at 7.0. To optimize the anolyte pH, moderate HCl and NaOH were used [39].

3. Results

3.1. Isolation and Screening of B1 and B2

Streak plating on MSM agar plates for five to ten generations was used to enrich and isolate colonies. The B1 strain from a soil sample was found to be the *Pseudomonas putida* OsEnB_HZB_G20 strain (Figure 2a), which was capable of the biodegradation of methyl red and B2 identified as *Pseudomonas aeruginosa* PA1_NCHU and used for co-culturing. These strains were identified by 16S ribosomal RNA gene analysis, performed by Sandors Lifesciences Pvt. Ltd. Hyderabad, India. Their phylogenetic tree is reported in Figure 2a,b.



(**a**)

Figure 2. *Cont.*

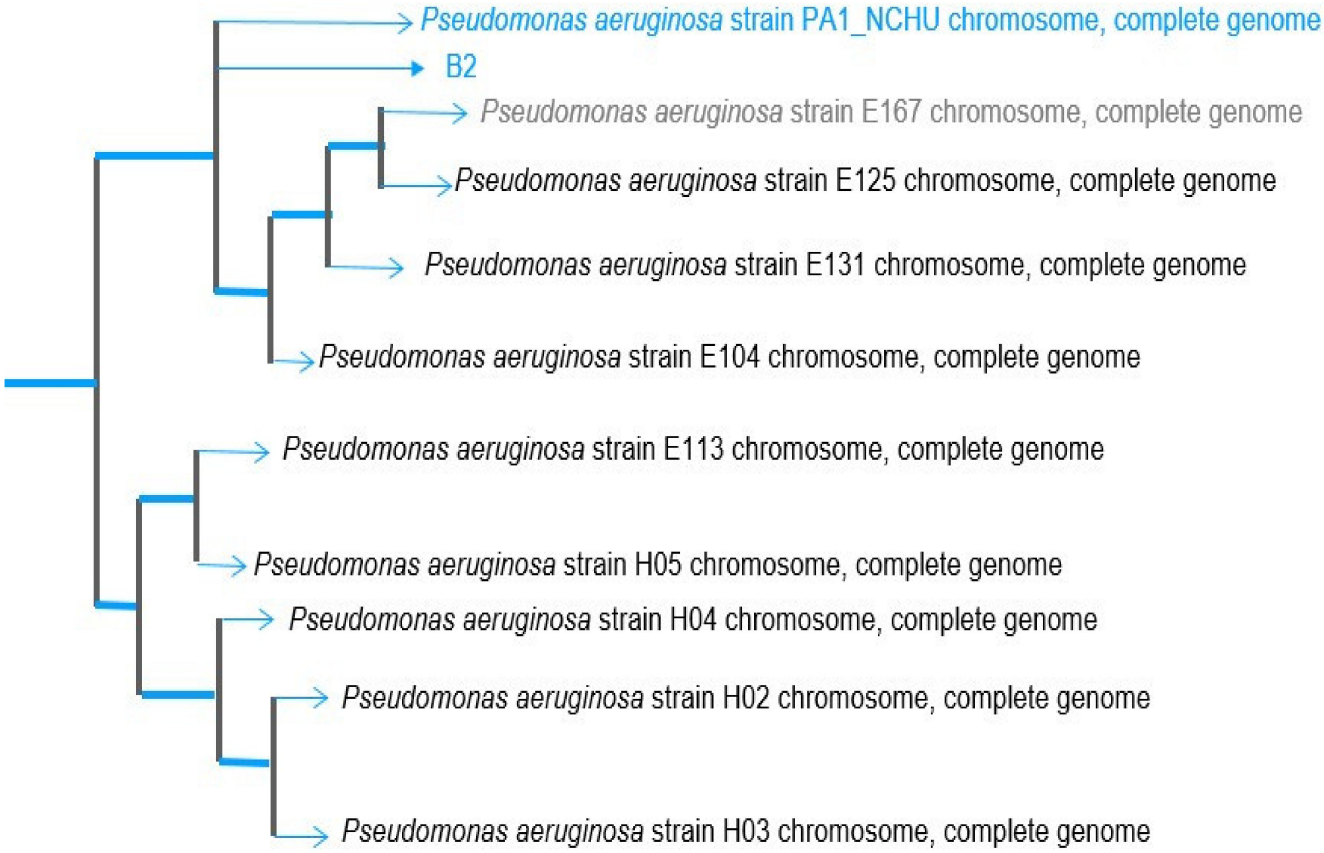


Figure 2. Phylogenetic tree of (**a**) *Pseudomonas putida* OsEnB_HZB_G20 strain; (**b**) *Pseudomonas aeruginosa* PA1_NCHU (Adapted from [33]) (Highlight and different colors of font represent microbial species of interest for this study).

3.2. Genome Sequence of Psudomonas putida OsEnB_HZB_G20 and Pseudomonas aeruginosa PA1_NCHU

An in-house bacterial DNA extraction toolkit and Nanodrop 2000 spectrophotometer were utilized to collect genomic DNA for 16s rRNA sequencing. This 1.5 kbp DNA fragment was amplified using high-fidelity PCR-polymerase. For PCR amplification, 135 ng of extracted DNA was utilized with enzyme buffer, 10 pM of primers, 0.5 mM of dNTPs, and 3.2 $MgCl_2$. The forward and reverse primers were optimized to melt at 57 degrees Celsius; the forward primer containing 72.22 percent G.C. and the reverse primer containing 68.42 percent G.C. Bacterial DNA sequences were compared using the BLAST software to find the most closely related species in the database.

3.3. Dye Concentration Optimization

Dye dosage was analyzed as an essential parameter throughout MFC's performance in sync with the degradation of the dye. MFC's efficiency and methyl red decolorization was highly reliant on anodic conditions, including neutral pH, anode compartment anaerobic circumstances, catholyte content, and so on. The co-culture survived 250 mg/L methyl red concentrations with no discernible effect on decolorization or power generation [40].

3.4. Methyl Red Dye Degradation in sMFCs

MFCs were operated using a methyl red-containing MSM medium as an anolyte that supported the growth of microorganisms and the degradation of the methyl red azo dye as the sole carbon source. Dye decolorization was not as efficient with 50 mg/L as compared

to a 250 mg/L dye concentration (Table 1). The low dye content as the sole carbon source might produce less biofilm and consequently less power output. The dye was utilized as a carbon source for microbial metabolism; in fact, it was observed that power generation was limited at low concentrations of dye. Accordingly, an effort was made to find the optimal dye concentration and 200 mg/L was discovered to be the most effective as there was no significant difference between the earlier one compared with 250 mg/L. Voltage generation and data were recorded only after the potential was fixed, and the device showed no fluctuations. Figure 3 shows the efficiency of the MFC in terms of methyl red decolorization and incubation time. Figure 3 shows that within 24 h of inoculation, methyl red had already begun to degrade. This decolorization process sped up with longer incubation times. After 36 h, a high 95% and 93% dye removal were achieved with 250 mg/L and 200 mg/L MR, respectively [41].

Table 1. The examined set of the decolorization variables selected for the optimization using co-culture (*Pseudomonas putida* strain OsEnB_HZB_G20 + *Pseudomonas aeruginosa* PA1_NCHU).

Factors	Range Investigated	Operational Range	Optimized Factor
Methyl red concentration	50–300 mg	50–250 mg	250 mg
pH	5–8	5–8	7.0

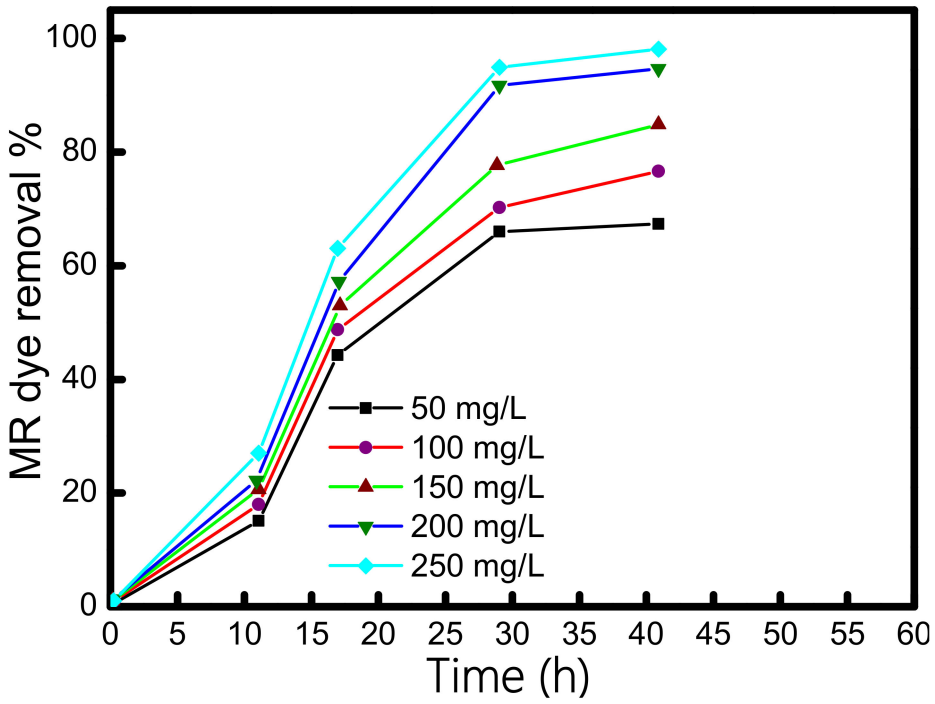


Figure 3. Methyl red dye decolorization in percentage by an incubation period in sMFC at 7 pH using co-culture.

3.5. Effect of anolyte pH

Changes in anolyte pH between 5.0 and 8.0 were shown to affect the efficiency with which the target bacteria degrade methyl red dye (Table 1). Almost 95% dye degradation was achieved at pH 7 with a dye concentration of 250 mg/L. After 36 h of incubation at 37 °C, its maximum decolorization effectiveness was 95% at pH 7, on a contrary, only 81.5% at pH 8. While at pH 5 and 6, decolorization was only 66% and 73.3%, respectively. These findings are consistent with those of Pearce et al., Chan and Kuo, and Shah (2014), who all found that neutral pH is optimal for azo dye decolorization. Having the co-cultures grow in methyl red-containing media at pH 5, 6, 7, and 8 revealed that the growth medium's pH

affected the isolates' development. The pH 7 and 8 showed rather effective dye degradation compared to pH 5 and 6.

3.6. Polarization Study of MFCs

The half-cell polarization results of MFCs using different dye concentration at anode chambers was plotted, where the current density vs. the anode and cathode half-cell potential is shown in Figure 4. The figure depicts a drop in anode half-cell potential with respect to the current density for all examined anodes, resulting in a parallel drop in the anode half-cell potential [42]. The variation in the anodic half-cell potential was observed owing to the difference in the of the build-up of electrons generated by the exoelectrogenic microbe using dye as a carbon source. The dye concentration at the anolyte is responsible for the drop in anode potentials. The formation of inefficient growth of the co-culture biofilm due to the availability of low dye concentration (as sole carbon source) in the anolyte might be the reason for the subsequent drop in anode voltage. The anodic half-cell potential of the MFC showed substantial variance with different concentrations of methyl red dye as the substrate. The anode half-cell potential of −280 mV, −282 mV, −300 mV, −370 mV, and −340 mV was recorded at 50, 100, 150, 200, and 250 mg/L concentrations, respectively, against 1 kΩ external resistance. The anodic half-cell was much improved with the increase in dye concentration [43]. It is obvious as higher substrate concentration ensured high electrogenesis at the anode surface. Nevertheless, no significant difference in anode half-cell potential was observed with a dye concentration of 200 and 250 mg/L indicated 200 mg/L is the optimum concentration of dye for this study.

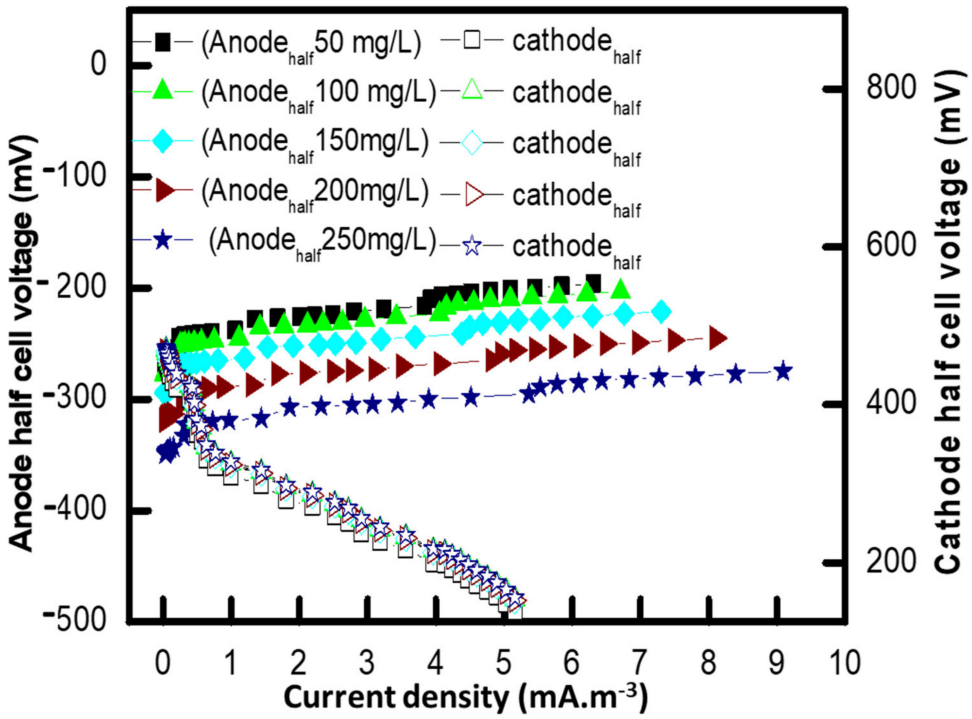


Figure 4. Analysis of anode and cathode half-cell with respect to volumetric current density. The anode and cathode half voltage data points are presented as solid and open symbols, respectively.

The polarization investigation was performed using a co-culture of *Pseudomonas putida* and *Pseudomonas aeruginosa* in a 1:1 (*v*/*v*) ratio with the different concentrations of MR dye in an anode chamber. It was observed that the volumetric power densities of MFCs depend significantly on the different dye concentrations in the anode, as shown in Figure 5. With regard to the different methyl red concentrations (50 mg/L, 100 mg/L, 150 mg/L, 200 mg/L, 250 mg/L) in the anode chamber, the highest generated power was 7.3 W/m^3 in the case of the 250 mg/L dye concentration. At dye concentrations of 50 mg/L,

100 mg/L, 150 mg/L, and 200 mg/L, power densities of 3.5 W/m^3, 4.1 W/m^3, 4.8 W/m^3 and 7.2 W/m^3 were observed, respectively. These results supported the earlier investigation. Table 2 described earlier reports where different dye was used as carbon source in the anolyte for simultaneous bioelectricity generation with concomitant dye removal in MFCs. The recent finding suggests improvement in volumetric power output.

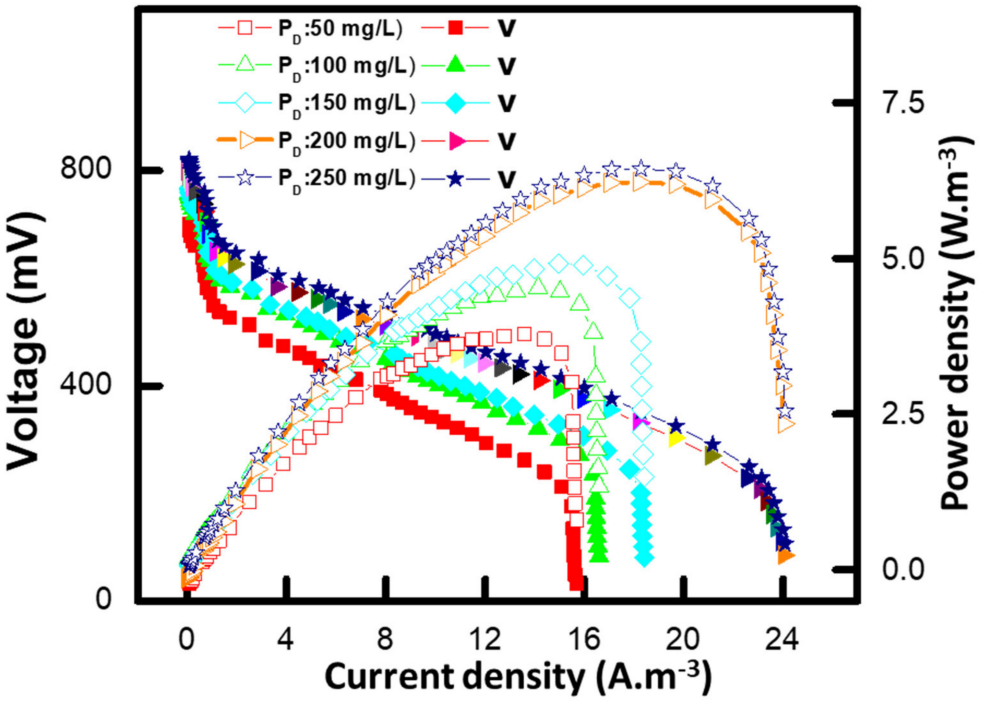


Figure 5. Polarization plots for MFCs (power density and DC voltage as a function of current density) with MFCs with different dye (Methyl red) concentrations as anolyte. The voltage and volumetric power density data points are presented as solid and open symbols.

Table 2. Showing microbial dye degradation efficiency with power density.

Dye	Bacteria	Decolorization Efficiency	Power Density	Resistor	References
Reactive Blue	*Pseudomonas aeruginosa*	90%	2004 μW/m^2	220 Ω	[44]
Reactive red	*Pseudomonas aeruginosa*	74%	4100 μW/m^2	220 Ω	[44]
Congo red	*Pseudomonas aeruginosa*	80%	586 μW/m^2	220 Ω	[44]
Methyl orange	*Pseudomonas aeruginosa*	94%	4100 μW/m^3	220 Ω	[44]
Acid orange	*Shewanella oeidensis*	93%	-	-	[45]
Acid black 172	*Trichoderma*	85%	129 mA/m^2	NA	[46]
Acid black 172	*Aspergillus*	88%	133 mA/m^2	NA	[46]
Remazol brilliant blue R	*Pleurotus ostreatus*	80–90%	180.5 mW/m^2	NA	[47]
Methyl red	*Pseudomonasputida* + *Pseudomonas aeruginosa*	95%	7.3 W/m^3	50 Ω	This study

Table 2 shows some previous research work with references relevant to this work where microbial dye degradation was achieved with simultaneous production of power.

3.7. Effect of Different Methyl Red Concentrations on the Internal Resistance of MFC

EIS was used to find out charge transfer and solution resistance of MFCs having different dye concentration at the anode chamber. EIS is a potent approach that enables independent investigation of charge transfer stages with various AC frequency responses. Utilizing the method's ability, the charge transfer resistance value was calculated, which describes the rate of charge exchange at the electrode-solution interface. With respect to

different methyl red concentrations (50 mg/L, 150 mg/L, 200 mg/L, 250 mg/L) in the anode chamber, the internal resistance produced was 345 Ω in the case of 50 mg/L dye concentration. At dye concentrations of 150 mg/L, 200 mg/L, and 250 mg/L, the internal resistance observed was 231.2 Ω, 202.5 Ω, and 162.7 Ω, respectively (Figure 6). The finding corroborated earlier results.

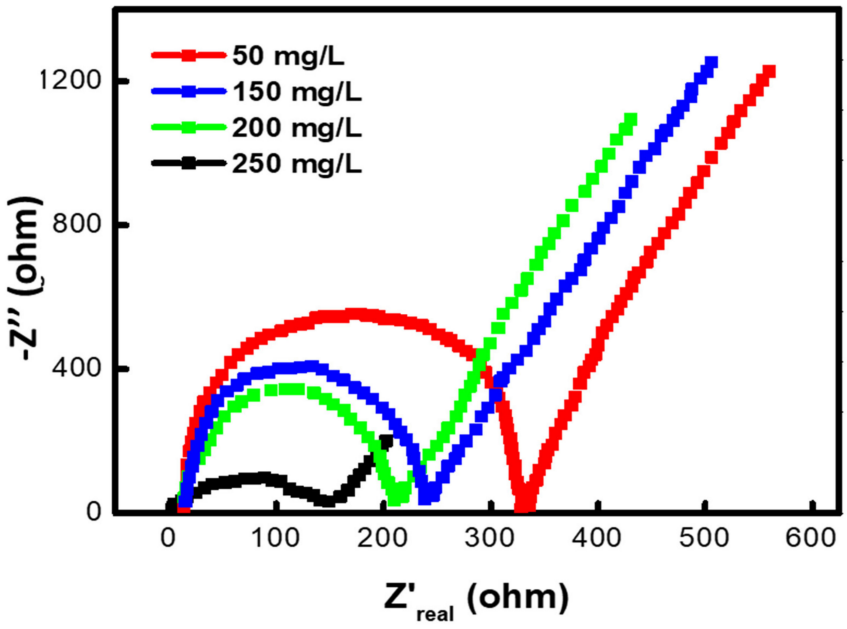


Figure 6. Analysis of the Nyquist plot of MFCs with different concentrations of MR dye on an anode chamber.

4. Cyclic Voltammetry Analyses

The CV curve is seen in the accompanying Figure 7 where the Ag/AgCl reference electrode was used. The scanning rate was 2 mV s^{-1} and used a potential window ranging from 0.1 V to 0.6 V. An oxidation peak at 0.45 V indicated substrate degradation on the anode surface. Higher capacitance was found with a 200 mg/L dye concentration. It was observed that the capacitance was comparable to 250 mg/L, on the contrary, the lowest at 50 mg/L indicated a lack of substrate oxidation at the anode. This result supports past outcome.

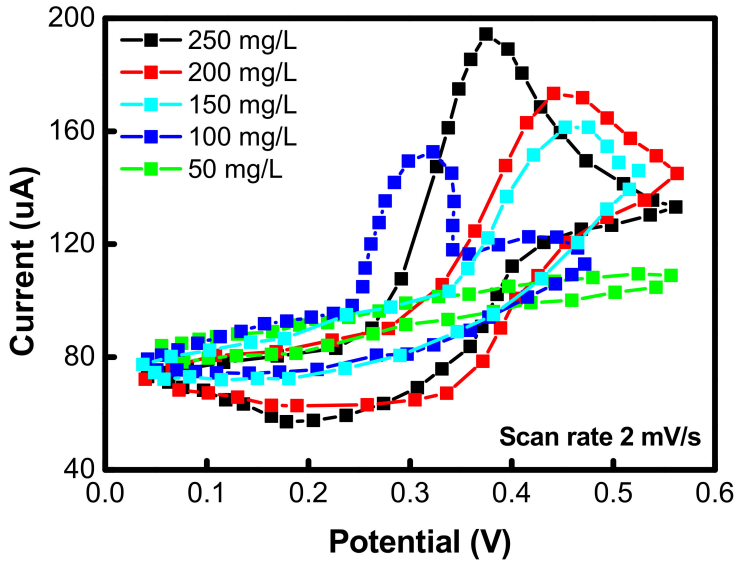


Figure 7. Cyclic voltammetry was recorded at a scan rate of 2 mV/s with different concentrations of methyl red dye on the anode.

5. Discussion

The *Pseudomonas putida* strain has showcased its ability to degrade various chemical compounds that threaten the environment and human health [48]. In this study, *Pseudomonas putida*, capable of methyl red dye degrading bacteria was screened, isolated and used as biocatalyst for inoculum preparation in MFCs. Inoculum was prepared by blending *Pseudomonas putida* with *Pseudomonas aeruginosa*, an electrogenic bacteria. Earlier finding also suggest that *Pseudomonas* could be an effective microbe for MR degradation. Table 3 enlisted different microbes capable of degrading MR. The researchers set out to see how well the *Pseudomonas putida* OsEnB_HZB_G20 strain could break down methyl red in the lab. The selected bacterial strain was superior in breaking down the azo dye methyl red and the following conditions (36 h of incubation at 37 °C, at a dye concentration of 250 mg/L, and a pH of 7) were found to be ideal. The maximum electrical output was noted when co-cultured with the isolated *Pseudomonas aeruginosa* PA1_NCHU species. Methyl red dye degradation was analyzed through UV–vis spectroscopy. Isolated bacteria (*Pseudomonas aeruginosa* PA1_NCHU *and Pseudomonas putida* strain OsEnB_HZB_G20) were co-cultured to increase their overall power output. The application of electricigens is the focus of future research on MFCs. Better performance from an MFC may be achieved by carefully selecting and cultivating high-quality electricigens. This research work has achieved the most efficient power production using the co-cultivation of *Pseudomonas aeruginosa* and *Pseudomonas putida*. The synergistic relationship between isolated strains and their application, such as in MFCs, might make their usage in wastewater treatment a practical alternative for reducing the cost of existing treating systems with the simultaneous production of electricity. It concludes that MFCs may be utilized to create high-quality commodities (i.e., H_2) simultaneously. In real-time, environmental factors may be tracked using biosensor-based MFC technology. It is interesting to consider MFC-based bioremediation, which was proposed to eliminate many different contaminants, such as aromatic or substituted organic compounds and heavy metals. Producing electricity while performing bioremediation helps to make this process an energy-intensive process, unlike other energy-extensive processes.

To the best of our knowledge, there are no research articles and reports available on the inhibitory effect between *P. putida* and *P. aeruginosa* strains. It was already reported that both *P. putida* and *P. aeruginosa* can be used for co-culturing during microbial remediation and suggested that these microbes can coexist with other microbes without significantly hampering their metabolic activity. Park et al., 1999 demonstrated the biodegradation of chloronitrobenzenes by coculture of *Pseudomonas putida* and *Rhodococcus* sp. Maulianawati et al., 2021 performed biodegradation of DDT using a consortium of *Pleurotus eryngii* and *P. aeruginosa*. In the present study, a continuous bioelectricity production with a concomitant degradation of methyl red dye was observed while using the co-culture of *Pseudomonas putida OsEnB_HZB_G20* and *Pseudomonas aeruginosa PA 1_NCHU*. This observation indicated there might be a synergistic effect between *Pseudomonas putida OsEnB_HZB_G20* and *Pseudomonas aeruginosa PA 1_NCHU*, and no antagonist behavior was found between the two. *Pseudomonas putida* degrades the methyl red dye that produces benzoic acid and o-xylene as an end product of methyl red dye degradation and this was analyzed by Fourier transform infrared (FTIR), GC–MS, and carbon 13 Nuclear Magnetic Resonance (NMR) [49]. Further, it was reported in another study that benzoic acid and xylene degraded and utilized by *Pseudomonas aeruginosa* as an energy source [50,51]. It was found in previous studies that mixed cultures are more efficient than monocultures in the degradation of textile effluents such as azo dyes. Bacterial consortia, therefore, became the focus of research, which proved degrading capabilities more efficiently (Table 3). Because of their synergistic behavior in the microbial population metabolism, they have shown a significantly higher level of biodegradation and mineralization by the consortium of microbes in breaking down the synthetic dyes compared to a monoculture of microbes. The bacterial strains in the mixed culture treat various positions of the aromatic ring to degrade the dye's molecular structure, and the produced metabolites are further destroyed by the remaining strains [47–59]. Hence, there is symbiotic effect instead of an antagonism effect as both species

use different carbon sources and there might be no product inhibition [60–62] because the dye as a substrate was degrading continuously with the simultaneous production of electricity.

Table 3. Previously isolated microorganisms for degradation of Methyl red.

Degrading Microorganism	Reference
Saccharomyces cerevisiae ATCC9763	[53]
Galactomyces geotrichum MTCC 1360	[54]
Sphingomonas paucimobilis	[55]
Rhodococcus strain UCC 0016	[9]
Pseudomonas aeruginosa	[56]
Bacillus sp. strain UN2	[57]
Enterobacter asburiae strain JCM6051	[58]
Klebsiella sp. strain Y3	[59]
Bacillus stratosphericus SCA1007	[25]
Enterobacter agglomerans	[60]

6. Conclusions

The goal of this research study was to treat wastewater with different concentrations of methyl red dye under optimum conditions by using isolated bacterial strains in a co-culture with the simultaneous production of electricity, taking advantage of the extracellular electron transfer mechanism of bacteria to make it an energy-intensive process. The pure culture of both isolated bacteria was targeted for co-culturing in a 1:1 ratio to enhance power output by using an electrochemical system. This was performed to see if the microbes could help each other. The co-culture inoculum of the *Pseudomonas putida* strain OsEnB_HZB_G20 and *Pseudomonas aeruginosa* PA1_NCHU showed a synergistic interaction between the microbes, which caused the MFC to produce more effective power and achieve the most efficient power production using the co-cultivation of isolated microbes. This study supports that "inter-species ecological communication" can activate MFCs electrochemically and play a significant role in power enhancement. However, a more in-depth understanding of the mutualistic interaction of bacteria in co-culture or mixed culture-injected MFCs needs a systematic examination.

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