Makara Journal of Science

Volume 27
Issue 1 *March*

Article 5

3-25-2023

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Recommended Citation

Khoirunnisa, Nur Syafira; Anwar, Syaiful; Sudadi, Untung; and Santosa, Dwi Andreas (2023) "Enrichment Media Selection and Co-Culture Potential among Exoelectrogen Bacteria Vary with Ecological Factors," *Makara Journal of Science*: Vol. 27: Iss. 1, Article 5.
DOI: 10.7454/mss.v27i1.1452
Available at: https://scholarhub.ui.ac.id/science/vol27/iss1/5

Makara Journal of Science, 27/1 (2023), 31–41
doi: 10.7454/mss.v27i1.1452

Enrichment Media Selection and Co-Culture Potential among Exoelectrogen Bacteria Vary with Ecological Factors

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Received October 30, 2022 | Accepted February 10, 2023

Abstract

Staphylococcus saprophyticus ICBB 9554 and *Citrobacter freundii* ICBB 9763 are exoelectrogen bacteria applied as microbial fuel cells (MFC). We selected enrichment media for both these exoelectrogen bacteria, characterized their synergistic traits, and evaluated the growth conditions under different ecological factors. In this study, different enrichment media, such as those containing sugar, molasses, and palm sugar (2, 4, and 6% w/v) were tested for *S. saprophyticus* ICBB 9554. Meanwhile, technical sodium acetate (NaAc), commercial vinegar 25%, and cider vinegar (5, 10, and 15 mM acetate concentration) were tested for *C. freundii* ICBB 9763. Both the exoelectrogen bacteria were tested for the synergistic trait by inhibitory test, metabolic activity, and electricity generation performance in MFC. Different ecological factors, including salinity (2, 3, and 4 dS/m), culture media pH (5.8, 6.5, and 6.9), and temperature (20 °C, 27 °C, and 31 °C) were investigated for bacterial growth. The selective enrichment medium ingredient for *S. saprophyticus* ICBB 9554 was 2% molasses while that for *C. freundii* ICBB 9763 was 0.05 M technical sodium acetate. Both bacteria were suspected to grow synergistically and could be inoculated simultaneously as a co-culture in MFC. The effect of different ecological factors indicated that co-culture could grow better than pure culture. This study's findings provide important data on potential enrichment media for exoelectrogen bacteria that are beneficial for scale-up to reduce the operational cost.

Keywords: bacterial growth, co-culture, ecological parameters, potential medium, synergize

Introduction

Microbial fuel cells (MFCs) constitute a device that can convert chemical energy into electrical energy through organic and inorganic substance oxidation via microbial metabolism [1]. Research on MFC has been conducted on both the laboratory and a large scale. There are several challenges and requirements to be prepared for scaling up, such as the microbes used in the set-up, especially bacteria. Past studies have successfully explored and obtained exoelectrogenic bacteria, that is, *Staphylococcus saprophyticus* ICBB 9554 and *Citrobacter freundii* ICBB 9763. These two bacteria are currently being studied using highly concentrated substrates. *S. saprophyticus* ICBB 9554 was isolated using a glucose substrate, while *C. freundii* ICBB 9763 was isolated using acetate and fumarate. Thus, glucose derivate medium was selected for *S. saprophyticus* ICBB 9554, while acetate derivate was selected for *C. freundii* ICBB 9763. Pure substrates incur a high cost for large scale implementation. Therefore, this research was conducted as a preliminary study to select the enrichment media for exoelectrogenic bacteria propagation.

S. saprophyticus ICBB 9554 is a strain of exoelectrogen bacterium isolated from the paddy soil ecosystem in Banten, Indonesia, and potentially used in the system of MFC [2]. Exoelectrogen bacteria can oxidize completely organic matter to CO_2, while electrons are transferred to electrodes outside their cells [3]. The MFC is a device that converts chemical energy in organic compounds and into electrical energy through microbial catalysis at the anode under anaerobic conditions and the reduction of terminal electron acceptors, especially oxygen, at the cathode [4].

Staphylococcus is a coagulase-positive, non-hemolytic, gram-positive bacteria that is a common cause of uncomplicated urinary tract infections (UTI), especially in sexually active young women [5]. *S. saprophyticus* is resistant to drugs most often used for the empirical treatment of UTIs [6]. The genus *Staphylococcus* was

reported to be a potential exoelectrogen bacterium, for example, *S. equoruma* [7], *S. capitis*, *S. epidermidis* [8], and *S. saprophyticus* [9]. Several studies have demonstrated electrogenic gram-positive bacteria that expressed genes encoding proteins responsible for the extracellular electron transfer (EET) process, which enhances bacterial growth.

C. freundii ICBB 9763 was also reported as an exoelectrogen in MFC isolated from sediment-contaminated hydrocarbon [10]. *Citrobacter* sp. is a petrophilic, iron-reducing bacteria (FRB), a facultative anaerobe, and an electrochemically active bacteria [11]. *Citrobacter* sp. is a gram-negative bacteria with thin cell walls. Therefore, electrons can be easily transferred through their cell membrane. C-type cytochrome proteins can achieve EET in the outer membrane of FRB with heme as the active center [12].

The synergism test can evaluate these bacteria's ability to work simultaneously as a co-culture. Several reports of *Staphylococcus* antagonism assay have been studied, including research antagonists between *Bacillus* and *S. aureus* [13], between *S. aureus* and *Escherichia coli* [14–15], and between *S. epidermidis* and *Propionibacterium acnes* [16]. The synergism test was conducted by inhibitory test using disk assay and performance test of pure culture and co-culture to generate electricity in MFC.

We also analyzed the growth of these two bacteria in different ecological environments. The following ecological parameters were also tested: salinity, pH, and temperature. Salinity was tested on bacterial growth because the isolates were used in a saline environment for further analyses. Salt affects bacteria growth by increasing the osmotic pressure. A high osmotic pressure can inhibit or even cause plasmolysis that kills microorganisms. However, microorganisms can adapt to low osmotic pressure by producing osmolytes that require significant energy, therefore their growth and activity become slower [17]. The pH homeostasis in metabolism is essential as it affects the structure/function of biological macromolecules, chemical reaction kinetics, and thermodynamic force [18]. Meanwhile, the temperature affects chemical and biological reactions (for example, enzymatic) and then affects bacterial growth [19].

Materials and Methods

Exoelectrogen bacteria viability test in enrichment media. The viability cell of exoelectrogen bacteria was evaluated by viable plate count in nutrient agar [20]. Three types of enrichment media were used as exoelectrogen bacteria substrates, each consisting of three concentration levels. The enrichment media for *S. saprophyticus* ICBB 9554 contained a solution of sugar, molasses, and palm sugar (2%, 4%, and 6% w/v, respectively). The enrichment media for *C. freundii* ICBB 9763 consisted of technical sodium acetate (NaAc), commercial vinegar 25%, and cider vinegar for acetate concentrations of 5, 10, and 15 mM, respectively. The commercial vinegar 25% and cider vinegar concentration were first calibrated using the acid-base titration method with a hydrogen oxide (NaOH) titer and phenolphthalein indicator. The concentration of acetate (CH3COOH) was calculated using the following equation 1:

$$M_a \times V_a \times a = M_b \times V_b \times b \quad (1)$$

where, M_a was acid molarity (CH_3COOH), "V_a" was the acid volume (CH_3COOH), "a" was acid valence (CH_3COOH), "M_b" was base molarity (NaOH), "V_b" was the base volume (NaOH), and b was base valence (NaOH). One loop of exoelectrogens bacteria was grown in different pre-culture media, that is, *S. Saprophyticus* ICBB 9554 in Thioglycollate Broth media and *C. freundii* ICBB 9763 in nutrient broth (NB) for overnight (ON). Then, 500 µL of the bacterial suspension was added to 10 mL of the enrichment media. The test was conducted by analyzing the colony density using the total plate count (TPC) method every 24 h for 3 days. The best enrichment media was the medium that maintained the highest cell viability.

Synergism test. *S. saprophyticus* ICBB 9554 and *C. freundii* ICBB 9763 were tested for synergistic properties to determine whether the two bacteria could work simultaneously. Three tests were conducted in the synergism test, that is, the inhibitory test using the disk diffusion method [21], microbial metabolic activity [22] using the triphenyl tetrazolium chloride (TTC) method [23], and performance test on electricity generation in MFC using selected enrichment media from previous test [24].

The inhibitory test was conducted in two ways, that is, *S. saprophyticus* ICBB 9554 as a testing bacteria against *C. freundii* ICBB 9763 targeting bacteria and *vice versa*. The testing bacteria were grown on NB medium by taking one loop and incubating overnight targeting bacteria were grown in nutrient agar (NA) medium using the spread and pour plate methods. Then, a 6-mm sterile disk was dipped into a suspension of testing bacteria grown in an NB medium. The disks immersed in isolate suspension were placed on the NA medium containing the targeting bacteria. The inoculant was incubated for 24 h until the isolates grew. The absence of an inhibition zone indicated that both bacteria could grow simultaneously.

The metabolic activity of bacterial cultures was measured by using the 2,3,5-TTC assay. Colorless TTC is enzymatically reduced to red 1,3,5-triphenyl formazan

(TPF) by metabolically active bacteria. The isolated bacteria were fermented in 10 mL of the basal culture medium (composition: NH_4SO_4-0.5 g, KH_2PO_4-3 g, $NaNO_3$-1.5 g, $MgSO_4.7H_2O$-0.02 g, and several carbon sources) in 20-mL flasks. The cultures were incubated at an ambient temperature for 72 h. The culture broth was centrifuged (10,000 × *g* for 10 min), and the supernatant was collected for enzymatic assay. The wells are prepared aseptically using a cook borer in Petri dishes containing the basal culture medium. The culture broth and supernatant (100 µL) were loaded into wells and incubated overnight at room temperature. Staining was performed by spotting the TTC reagent on an agar plate and incubating it for 20 min in the dark. The appearance of red zones around the wells indicated that the bacteria could metabolize specific substrate-degrading enzymes.

Electricity generation performance was performed in an MFC system using an enriched medium as the substrate for extracellular electrogenic bacteria. The MFC chamber was a dual-chamber design. The anode compartment was filled with a sterile substrate solution, phosphate buffered saline (pH of ± 7), and 3 mL of exoelectrogen bacteria (neat or co-culture) cultured overnight (ON). The substrates used in this study were sugar, molasses, and palm sugar at three concentrations (2, 4, and 6% w/v, respectively) as a carbon source and 1 g/L urea as a nitrogen source. Meanwhile, the cathode compartment was filled with 0.1 M $KMnO_4$ and 0.01 M KH_2PO_4 and sterilized in an autoclave. MFC performance was monitored by measuring current (I) and voltage (V) using a multimeter every 8 h during the 3-day incubation period. We then calculated the power (P) using the formula $P = I \times V$. The power density was normalized by dividing the power value by the area of the anode.

Exoelectrogen bacteria viability test at various salinity, pH, and temperature levels. Both pure and co-culture, if both the bacteria were synergists, were tested under different environmental conditions for selected enrichment media. The first factor was salinity (2, 3, and 4 dS/m), the second factor was pH (pH1 = 5.8, pH2 = 6.5, and pH3 = 6.9), and the third factor was the temperature (T1 = 20, T2 = 27, and T3 = 31 °C).

First, the exoelectrogen bacteria, *S. saprophyticus* ICBB 9554 and *C. freundii* ICBB 9763, were grown on NB pre-culture media by taking one loop and incubated One Night (ON). Second, 250 µL of the bacterial suspension was inoculated in 10 mL of the selected enrichment media, with added salt based on the defined concentration. The test was conducted by analyzing the value of colony density using the TPC method [24] every 24 h for 3 days.

The results of the salinity test formed the basis for the next stage. The highest salinity concentration was combined with the temperature and pH parameters. The test was designed using a randomized factorial design with two factors: temperature and pH. Both bacteria, *S. saprophyticus* ICBB 9554 and *C. freundii* ICBB 9763, were tested sequentially for each of the combination factors.

The temperature and pH conditions test were kept similar to the salinity test. The exoelectrogen bacteria, *S. saprophyticus* ICBB 9554 and *C. freundii* ICBB 9763, were cultured in NB pre-culture medium by taking one loop and incubating ON. As much as 250 µL of the two bacterial suspensions were inoculated in 10 mL of the selected enrichment medium containing the maximum salt concentration. A mixture of bacterial culture and salt media was placed under conditions according to the experimental design. The assessment was conducted in the same way, that is, by analyzing the colony density value using the TPC method every 24 h for 3 days.

Data analysis. First, the data were processed on Microsoft Excel. Second, the data were analyzed by using the analysis of variance (ANOVA) with the SPSS 26 software. In case of significant differences among the treatments, the data was analyzed with Duncan Multiple Range Test (DMRT) at a 95% confidence interval.

Results and Discussions

Exoelectrogen bacteria viability at enrichment media. Bacterial colony density was observed every 24 h for 3 days of incubation. Generally, the trend of *S. saprophyticus* ICBB 9554 colony density was almost similar in every experiment, showing an increase from 24 to 48 h and then decreasing at 72 h onward (Figure 1). In contrast, the 6% (w/v) molasses medium showed a decrease at 48 h and then an increase at 72 h. *S. saprophyticus* ICBB 9554 optimally grew at 48 h. However, creating a growth curve with a narrower observation time interval was necessary to understand the optimal growth point. The *S. saprophyticus* ICBB 9554 was low density in the sugar medium compared to the others, ranging from $1.00 \pm 0.85 \times 10^4$ CFU/mL to $1.03 \pm 0.85 \times 10^6$ CFU/mL (Figure 1a). The highest colony density of *S. saprophyticus* ICBB 9554 was observed on a 2% molasses medium, reaching $1.62 \pm 0.36 \times 10^8$ CFU/mL. Meanwhile, palm sugar gave a bacterial density similar to that of molasses, which ranged from $8.35 \pm 0.00 \times 10^6$ CFU/mL to $9.16 \pm 0.16 \times 10^7$ CFU/mL.

The trend of *C. freundii* ICBB 9763 growth is illustrated in Figure 1b. It demonstrates that commercial vinegar inhibited *C. freundii* ICBB 9763 growth with a decreasing pattern at 48 h of incubation, with an increase at 72 h. The density values ranged from $1.00 \pm 0.23 \times 10^4$ CFU/mL to $1.03 \pm 0.21 \times 10^6$ CFU/mL. The medium growth occurred on a cider vinegar medium with a density value ranging from $9.75 \pm 0.25 \times 10^4$ CFU/mL to

1.51 ± 0.90 × 10^7 CFU/mL. Meanwhile, the highest colony density was detected in the technical sodium acetate media, which tended to be stable from 24 h to 72 h. The density in the technical sodium acetate media ranged from 5.80 ± 0.60 × 10^6 CFU/mL to 2.07 ± 0.85 × 10^7 CFU/mL.

The results of DMRT test (Table 1) suggest that effect of media type and concentration affected ($p < 0.05$) the growth of the two bacteria. In *S. saprophyticus* ICBB 9554 bacteria, molasses and palm sugar were potentially determined as the selected enrichment media. However, as molasses demonstrated a higher cell density, it was selected for the subsequent test. Whereas, in *C. freundii* ICBB 9763, only the technical sodium acetate component showed the potential for selection in the enrichment medium. Because the media concentration did not have a significant effect, the lowest medium concentration was selected for the subsequent test to minimize the cost incurred.

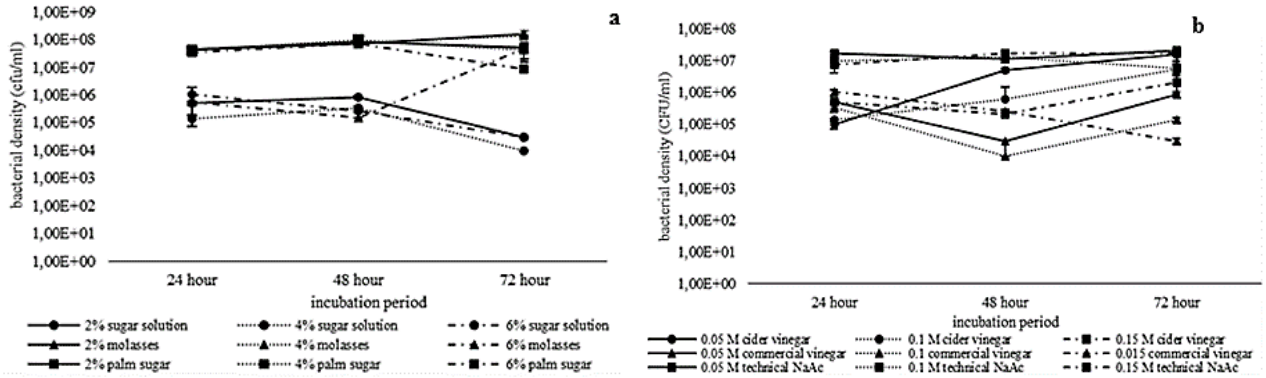

Figure 1. *Staphylococcus saprophyticus* ICBB 9554 (a) and *Citrobacter freundii* ICBB 9763 Growth in Different Enrichment Media

Table 1. The Influence of a Single Factor Enrichment Media Type and Media Concentration as Well as the Interaction of Factors on the Density of Bacteria

***Staphylococcus saprophyticus* ICBB 9554**		***Citrobacter freundii* ICBB 9763**	
Code[a]	**Bacterial density (cfu/ml)[b]**	**Code[a]**	**Bacterial density (cfu/ml)[b]**
A1C1	(4.61 ± 0.41) × 10^5 a	B1D1	(6.69 ± 0.77) × 10^6 ab
A1C2	(1.62 ± 0.17) × 10^5 a	B1D2	(2.01 ± 0.28) × 10^6 a
A1C3	(4.39 ± 0.52) × 10^5 a	B1D3	(9.45 ± 0.10) × 10^5 a
A2C1	(9.25 ± 0.62) × 10^7 b	B2D1	(4.61 ± 0.41) × 10^5 a
A2C2	(8.61 ± 0.48) × 10^7 b	B2D2	(1.62 ± 0.17) × 10^5 a
A2C3	(1.65 ± 0.28) × 10^7 a	B2D3	(4.39 ± 0.52) × 10^5 a
A3C1	(5.96 ± 0.20) × 10^7 ab	B3D1	(1.61 ± 0.48) × 10^7 c
A3C2	(5.95 ± 0.32) × 10^7 ab	B3D2	(9.51 ± 0.36) × 10^6 bc
A3C3	(3.82 ± 0.32) × 10^7 ab	B3D3	(1.37 ± 0.56) × 10^7 c

[a] A1 (sugar solution), A2 (molasses), A3 (palm sugar), B1 (cider vinegar), B2 (commercial vinegar), B3 (technical sodium acetate), C1 (2%), C2 (4%), C3 (6%), D1 (5 mM), D2 (10 mM), D3 (15 mM), n = 3.
[b] Numbers in the same column followed by the same letter indicate that the DMRT test results are not significantly different (α = 0.05).

Table 2. Synergism Test Results Between *Staphylococcus saprophyticus* ICBB 9554 and *Citrobacter freundii* ICBB 9763

Testing vs. targeting bacteria	**Method**	
	Pour plate	**Spread plate**
9554 vs. 9763	No inhibitory zone	No inhibitory zone
9763 vs. 9554	No inhibitory zone	No inhibitory zone

Table 3. Metabolic Activity Test of Pure Culture and Co-culture to Test Substrate Utilization

Testing Material	Glucose	Sucrose	Molasses	Sodium Acetate
9554 culture broth	Red zone	No red zone	Red zone	Red zone
9554 supernatant	Red zone	No red zone	Red zone	Red zone
9763 culture broth	Red zone	Red zone	Red zone	Red zone
9763 supernatant	Red zone	Red zone	Red zone	Red zone
Co-culture culture broth	Red zone	Red zone	Red zone	Red zone
Co-culture supernatant	Red zone	Red zone	Red zone	Red zone

The low density of bacteria in the sugar medium can be attributed t the fact that sucrose is not a compatible medium for *S. saprophyticus* ICBB 9554. Several studies have reported that *Staphylococcus* can ferment sucrose [25–29]. Meanwhile, the inability of *Staphylococcus* to consume sucrose has already been reported [30], where 70% sucrose has been reported to inhibit 90% biofilm formation. The ability to ferment sugar in the genus *Staphylococcus* impacts methicillin resistance. On the contrary, the methicillin-sensitive group experienced a decrease in both the type and ability to ferment sugar [27]. The addition of sugars, including glucose, fructose, and sucrose, in the bacterial growth media, was reported to cause a decrease in the *Staphylococcus* growth [31] as a result of damage to the unique cell wall structure called the Pentaglycine Bridge. The appearance of *Staphylococcus* cells on the media containing sugar was observed to result in thinner cell walls. Another reason for the addition of sucrose causing the inhibition of bacterial growth is that increasing sucrose concentration causes an increase in the osmotic pressure, which affects biofilm formation and physiological activities such as acid production [32].

The tested enrichment media for *C. freundii* ICBB 9763 in this study includes acetate derivatives due to the bacterial use of sodium acetate as a carbon source at the isolation stage [9]. Some research has also been reported on MFC with *Citrobacter* sp. as a biocatalyst, suggesting that acetate acts as an electron donor or carbon source, which is then oxidized in microbial metabolism [33, 34, 11].

Synergism test. The synergism test was conducted to determine synergistic growth and the ability to generate electricity in MFC. The inhibitory test (Table 2) result showed that the pour and spread plate methods for testing bacteria and targeting bacteria do not challenge each other. Therefore, both these bacteria can be inoculated simultaneously (co-culture).

A metabolic activity test for several substrates continued the synergistic test. The results shown in Table 3 indicated that *C. freundii* ICBB 9763 and co-culture could use all tested enrichment media. On the other hand, *S. saprophyticus* ICBB 9554 showed a reduced capability to metabolize sucrose, supporting that *S. saprophyticus* ICBB 9554 requires a low sugar level for growth (Figure 1). Thus, the selected enrichment medium, that is, molasses and sodium acetate, could be potentially used in the subsequent experiment.

The species of the genus *Staphylococcus*, such as *Staphylococcus aureus*, can reportedly catabolize glucose and excreted acetate into the culture medium. However, some strains fail to catabolize acetate during the post-exponential growth phase, resulting in significantly lower growth yields than strains that catabolize acetate [35]. In contrast, a previous study [36] compared the proteomic analyses of *Citrobacter* sp. metabolism under glucose-fed and acetate-fed conditions. These results suggested that 64 proteins were selectively expressed in the glucose medium, and 124 were detected only in the acetate medium. The acetate medium showed elevated levels of fatty acid biosynthesis, ATP biosynthesis, and energy. According to the UNIPROT classification and KEGG regulatory pathways, most of the upregulated proteins associated with acetate nourishing were involved in acetate assimilation, glycerol metabolism, glyoxylate cycle, energy production, and lipid metabolism.

Microbial species in symbiotic communities evolve chemical or physical interaction patterns such as metabolite-mediated cooperation/conflict and space competition. These interactions can be beneficial, neutral, or harmful to the fitness of individuals in a community. In the case of nutrient utilization, interactions between synergistic microbes in colonies result in the degradation of organic compounds by a certain type of microbe that can act as a substrate for other microbes [37]. However, the ecological parameter also affects synergistic behavior. Bacteria preferring a particular pH in their environment indirectly inhibit or support the growth of other types of bacteria by changing the pH to its optimum value. Bacteria change the pH of their environment to optimum values through the production of acid or alkaline compounds [38].

We analyzed the cooperative interaction between *S. Saprophyticus* ICBB 9554 and *C. Freundii* ICBB 9763 to generate electricity in MFC. The results indicated that *C. freundii* ICBB 9763 in the molasses medium gave the highest voltage (Figure 2a). Statistical analysis revealed

that co-culture in molasses produced the highest value of 0.27 ± 0.039 mA, which was significantly ($p < 0.05$) different from that in other treatments (Table 4). Co-culture bacteria in the molasses medium have the highest power density during the initial incubation period, although, in the middle of incubation, *C. Freundii* ICBB 9554 becomes the highest (Figure 2c). Although both the treatments were not significantly different ($p > 0.05$) (Table 4), co-culture yielded the highest value of 28.90 ± 6.05 mW/m^2 compared to that of *C. freundii* ICBB 9763 (25.86 ± 5.19 mW/m^2). According to this description, *S. Saprophyticus* ICBB 9554 and *C. Freundii* ICBB 9763, as co-cultures, simultaneously generated higher electricity than pure culture.

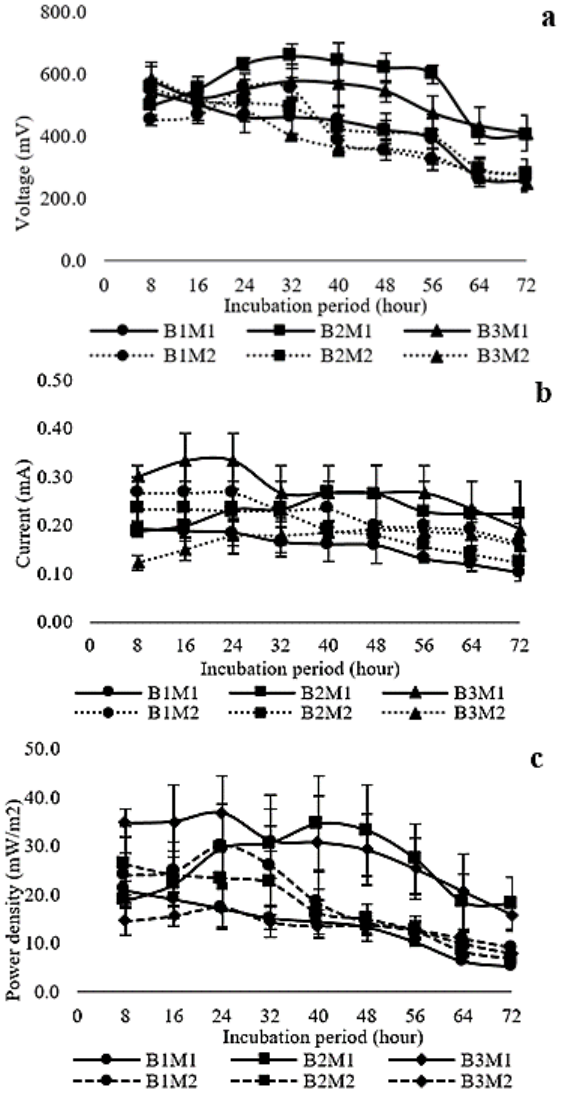

Figure 2. Electricity Generation During Incubation Under the Following Parameters: (a) Voltage, (b) Current, and (c) Power Density of *Staphylococcus saprophyticus* ICBB 9554 (B1), *Citrobacter freundii* ICBB 9763 (B2), and Co-culture (B3) in Molasses Medium (M1) and Technical Sodium Acetate Medium (M2)

Exoelectrogen bacteria viability at different salinity, pH, and temperature levels. The growth of *S. saprophyticus* ICBB 9554 and *C. freundii* ICBB 9763 on different ecological factors was conducted to determine whether these bacteria could grow on all tested conditions that could be used later in the MFC system. The viability of *S. saprophyticus* ICBB 9554 was better in molasses media than in sodium acetate medium under different salinity levels (Figure 3a). The growth trend increased from 24 to 48 h, but decreased from 72 h onward. Only 3 dS/m molasses media showed a different trend, increasing from 24 h to 72 h. The density in molasses media ranged from 1.95 × 10^5 CFU/mL to 2.13 × 10^6 CFU/mL. Meanwhile, the sodium acetate media produced a lower density than molasses for *S. saprophyticus* ICBB 9554 growth, which ranged from 8.03 × 10^4 CFU/mL to 9.30 × 10^5 CFU/mL. The growth of *S. saprophyticus* ICBB 9554 at higher salinity levels or a salt concentration of 4 dS/m on molasses medium was quite good, reaching 10^6 CFU/mL. This resistance at high salt concentrations further testes the different pHs and temperatures.

The density of *C. freundii* ICBB 9763 on the effect of different salinity levels on 2% molasses media and 0.05 M NaAc is shown in Figure 3b. When compared to the growth of *S. saprophyticus* ICBB 9554, *C. Freundii* ICBB 9763 showed greater growth at all treatments, and in the following sequence in terms of medium component: sodium acetate > molasses > sodium acetate. The density ranged from 1.14 × 10^6 CFU/mL to 2.52 × 10^7 CFU/mL, showing a continuous increase from 24 to 72 h. The density of *C. freundii* ICBB 9763 under different salinity conditions was reviewed and was good up to 4 dS/m salt content, which is approximately 10^7 CFU/mL.

The trend of co-culture growth (Figure 3c) was similar to that of *C. freundii* ICBB 9763. The co-culture population ranged between 1.80 × 10^6 CFU/mL and 4.12 × 10^7 CFU/mL. This value was higher than that for pure culture. Thus, we assumed that co-culture inoculation simultaneously resulted in much better bacterial growth than that of pure culture.

The medium type and concentration factor demonstrated a significant effect ($p < 0.05$) for all bacterial growth. The results suggested that *S. saprophyticus* ICBB 9554 had a preference for molasses medium, with a bacterial density of 1 (1.15 ± 0.81) × 10^6 CFU/mL, while *C. freundii* showed a preference for technical sodium acetate, with a bacterial density of 1.29 ± 0.49 × 10^7 CFU/mL. The co-culture showed a preference for technical sodium acetate, with a bacterial density of 2.55 ± 0.12 × 10^7 CFU/mL (Table 5).

Table 4. The Effect of Bacterial and Enrichment Medium Type on Electricity Generation Potential

Code[a]	Voltage (mV)[b]	Current (mA)[b]	Power density (mW/m^2)[b]
B1M1	418.48 ± 9.88 a	0.16 ± 0.019 a	13.58 ± 1.80 a
B2M1	557.74 ± 30.13 b	0.23 ± 0.044 bc	25.86 ± 5.19 b
B3M1	519.41 ± 39.87 b	0.27 ± 0.039 c	28.90 ± 6.05 b
B1M2	407.15 ± 19.30 a	0.22 ± 0.031 abc	18.93 ± 3.49 a
B2M2	432.70 ± 19.20 a	0.19 ± 0.035 ab	17.25 ± 3.45 a
B3M2	398.93 ± 22.27 a	0.17 ± 0.024 ab	13.38 ± 2.39 a

[a] B1 (*Staphylococcus saprophyticus* ICBB 9554), B2 (*Citrobacter freundii* ICBB 9763), B3 (co-culture), M1 (molasses), and M2 (technical sodium acetate), n= 3

[b] Numbers in the same column followed by the same letter indicate that the DMRT test results are not significantly different (α = 0.05).

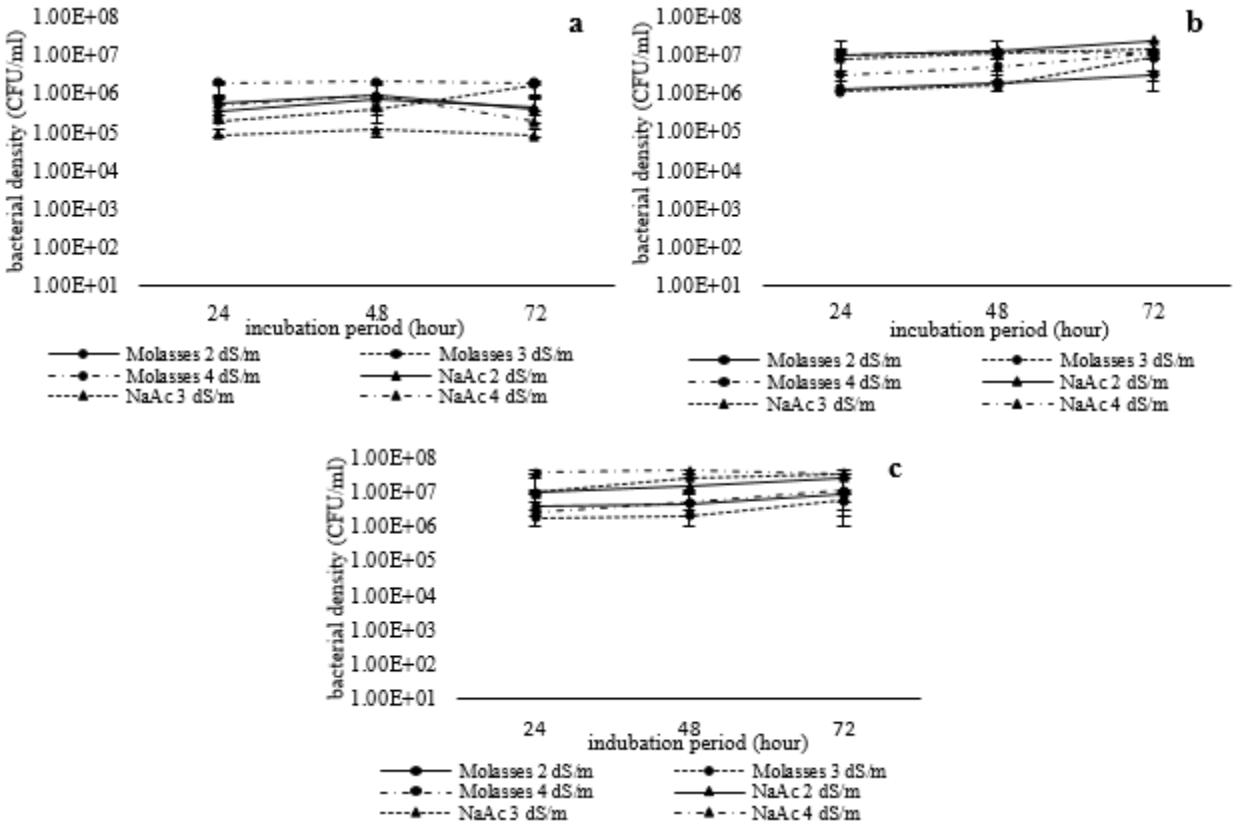

Figure 3. Bacterial Growth (a) *Staphylococcus saprophyticus* ICBB 9554, (b) *Citrobacter freundii* ICBB 9763, (c) Co-culture) at Different Salinity Levels

Table 5. Effect of Enrichment Medium Type and Salt Concentration on Bacterial Growth

Code[a]	*Staphylococcus saprophyticus* ICBB 9554[b]	*Citrobacter freundii* ICBB 9763[b]	Co-Culture[b]
M1S1	(6.23 ± 0.25) × 10^5 a	(2.12 ± 0.95) × 10^6 a	(5.65 ± 0.26) × 10^6 ab
M1S2	(8.02 ± 0.86) × 10^5 a	(3.82 ± 0.42) × 10^6 ab	(3.10 ± 0.21) × 10^6 a
M1S3	(2.04 ± 0.93) × 10^6 b	(6.74 ± 0.45) × 10^6 ab	(6.04 ± 0.41) × 10^6 ab
M2S1	(5.25 ± 0.22) × 10^4 a	(1.60 ± 0.81) × 10^7 c	(1.68 ± 0.84) × 10^7 bc
M2S2	(9.79 ± 0.23) × 10^5 a	(1.13 ± 0.30) × 10^7 bc	(2.20 ± 0.11) × 10^7 c
M2S3	(5.52 ± 0.37) × 10^5 a	(1.15 ± 0.16) × 10^7 bc	(3.76 ± 0.32) × 10^7 d

[a] M1 (molasses), and M2 (technical sodium acetate), S1 (2 dS/m), S2 (3 dS/m), and S3 (4 dS/m), n= 3

[b] Numbers in the same column followed by the same letter indicate that the DMRT test results are not significantly different (α = 0.05).

At a low temperature of 20 °C, the fluctuation *of S. saprophyticus* ICBB 9554 growth was sharper (Figure 4a). At higher temperatures, both 27 °C and 31 °C, were more stable. The density of *S. saprophyticus* ICBB 9554 on pH and temperature testing ranged from 1.37×10^5 CFU/mL to 7.32×10^6 CFU/mL. Good viability was treated at 27 °C and pH 5.8 because the viability increased slightly, but tended to be stable for 3 days with a high value of 2.65×10^6 CFU/mL; 3.41×10^6 CFU/mL, and 3.72×10^6 CFU/mL, respectively. Meanwhile, poor viability was observed in the treatment at 31 °C and pH 6.9, wherein the pattern was stable, but the value was low (i.e., 4.58×10^5 CFU/mL, 5.90×10^5 CFU/mL, and 6.35×10^5 CFU/mL, respectively). The estimates of bacterial species per gram of soil varied between 2,000 and 8.3 million [39]. According to this data, there was still a chance for *S. saprophyticus* ICBB 9554 to survive in the soil during rice cultivation.

The density of *C. freundii* ICBB 9763 at different pH and temperature (Figure 4b) revealed that the *C. freundii* ICBB 9763 density at every treatment and the observation time were almost constant. The cell density ranged from 6.85×10^6 CFU/mL to 4.18×10^7 CFU/mL. This value was higher when compared to that after treatment on *S. saprophyticus* ICBB 9554. These results were similar to those of the salinity test in that the density of *C. freundii* ICBB 9554 was higher than that of *S. saprophyticus* ICBB 9554. The average trend continued to increase from 24 h to 72 h, with the highest recorded at 27 °C and pH 6.9 (cell density: 4.18×10^7 CFU/mL). In contrast, the lowest density was recorded at 20 °C and pH 6.5.

The density of co-culture bacteria on pH and temperature testing (Figure 4c) revealed greater results than that for pure cultures with a pattern similar to the growth pattern of *C. freundii* ICBB 9763. The co-culture colony density values ranged from 7.80×10^6 CFU/mL and 4.87×10^7 CFU/mL. The temperature and pH exposure imposes environmental stress indicating that the bacterium must adapt to maintain homeostasis [40]. Then, low temperatures can induce changes in membrane fluidity [41]. Gram-negative bacteria have cell walls under lipopolysaccharide layers when compared to gram-positive bacteria [42].

The difference in the growth patterns between a pure culture and a co-culture can be explained by the fact that a pure culture may behave much differently than when combined with other species. A mixed culture is a subsample from a complex natural community with two or more microbial strains that can provide a simple community to rationally and robustly analyze and describe individual community members [43]. Within a community, it is expected that different species interact with each other with a resultant effect that may differ from the effect of individual component species [44].

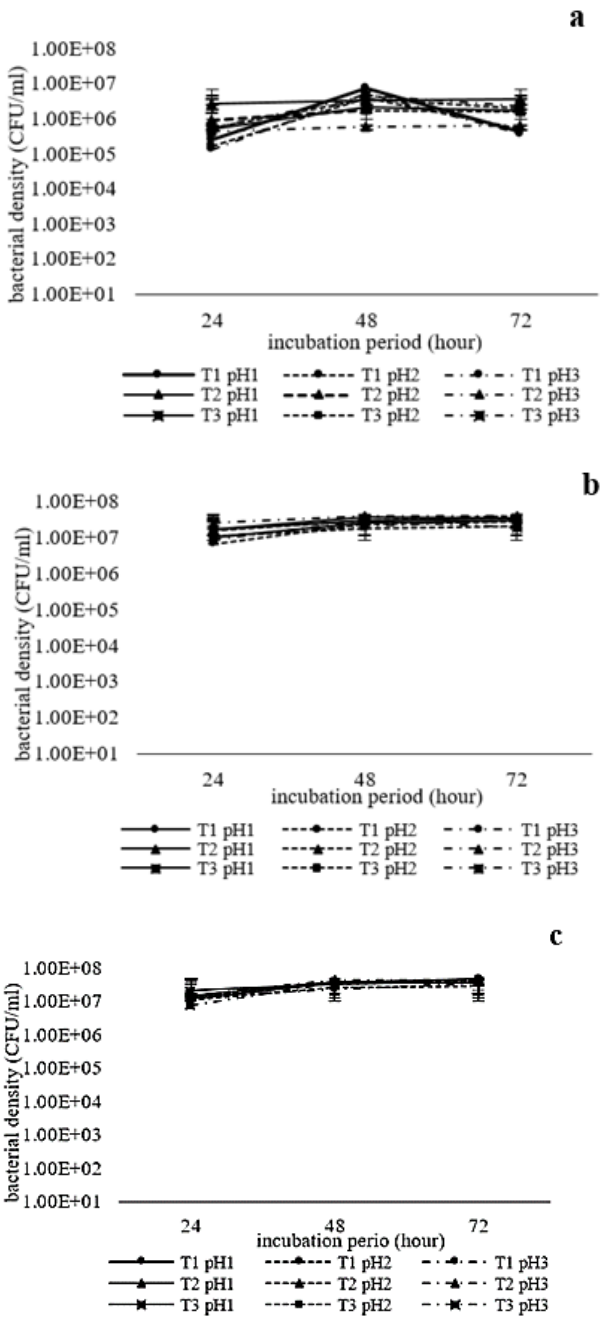

Figure 4. Bacterial Growth ((a) *Staphylococcus saprophyticus* ICBB 9554, (b) *Citrobacter freundii* ICBB 9763, and (c) Co-culture) at Different pH and Temperatures

Statistical results demonstrated that tested temperature and pH did not significantly affect ($p > 0.05$) bacterial growth (Table 6). This result implied that the bacterial growth was not interrupted by ecological factors. These results were beneficial for the subsequent experiments, especially for the scale-up study, because the environmental parameters were selected in accordance with the natural condition.

Table 6. Effect of Temperature and pH on the Bacterial Growth

Code[a]	*Staphylococcus saprophyticus* ICBB 9554[b]	*Citrobacter freundii* ICBB 9763[b]	Co-Culture[b]
T1pH1	$(2.67 \pm 0.40) \times 10^6$	$(2.28 \pm 0.11) \times 10^7$	$(3.19 \pm 0.18) \times 10^7$
T1pH2	$(1.52 \pm 0.21) \times 10^6$	$(1.96 \pm 0.11) \times 10^7$	$(2.25 \pm 0.97) \times 10^7$
T1pH3	$(1.91 \pm 0.29) \times 10^6$	$(2.06 \pm 0.93) \times 10^7$	$(2.40 \pm 0.10) \times 10^7$
T2pH1	$(3.26 \pm 0.55) \times 10^6$	$(2.97 \pm 0.11) \times 10^7$	$(3.03 \pm 0.14) \times 10^7$
T2pH2	$(1.42 \pm 0.42) \times 10^6$	$(2.92 \pm 0.12) \times 10^7$	$(3.08 \pm 0.15) \times 10^7$
T2pH3	$(5.61 \pm 0.92) \times 10^5$	$(3.61 \pm 0.92) \times 10^7$	$(3.41 \pm 0.17) \times 10^7$
T3pH1	$(1.49 \pm 0.87) \times 10^6$	$(2.70 \pm 0.86) \times 10^7$	$(3.05 \pm 0.80) \times 10^7$
T3pH2	$(2.04 \pm 0.15) \times 10^6$	$(1.70 \pm 0.51) \times 10^7$	$(3.23 \pm 0.15) \times 10^7$
T3pH3	$(2.44 \pm 0.19) \times 10^6$	$(1.97 \pm 0.11) \times 10^7$	$(2.72 \pm 0.17) \times 10^7$

[a] T1= 20 °C, T2= 27 °C, dan T3= 31 °C, pH1= 5.8; pH2= 6.5; pH3= 6.9, n= 3

[b] Numbers in the same column followed by the same letter indicate that the DMRT test results are not significantly different ($\alpha = 0.05$).

Conclusion

Our research findings suggested that 2% molasses is a potential enrichment medium for *S. saprophyticus* ICBB 9554 and that technical sodium acetate is a potential enrichment medium for *C. freundii* ICBB 9763. The bacteria's growth was stable in every sodium acetate concentration; therefore, 0.05 M was selected as it provides a bacterial density of $1.61 \pm 0.48 \times 10^7$ CFU/mL. Both bacteria could simultaneously grow as co-culture as they did not inhibit the growth of each other and simultaneously generated greater electricity than that from a pure culture. Nevertheless, the lower temperature incubation negatively affected *S. saprophyticus* ICBB 9554 growth, which reduced the bacterial growth, while, *C. freundii* ICBB 9763 showed better growth at a lower temperature. The co-culture resulted in better growth than the pure culture at every salinity, pH, and temperature level tested. Eventually, the tested ecological parameters demonstrated interference with bacterial growth.

Acknowledgments

We would like to thank the Ministry of Research and Technology of the Republic of Indonesia through the Program Mahasiswa Menuju Doktor untuk Sarjana Unggul scholarship (2668/IT3.L1/PN/2020) for providing the funding for this research. We also thank to Biology Laboratory of Soil Science and Land Resource Department of IPB University and the Indonesian Center for Biodiversity and Biotechnology (ICBB) which facilitated for place and some equipment.

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