

Electricity Production and Phenol Removal of Winery Wastewater by Constructed Wetland – Microbial Fuel Cell Integrated With Ethanol Tolerant Yeast

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Abstract: Winery wastewater is one of the most concerning wastewater because it contains a high concentration of ethanol and phenolic compound that can inhibit the wastewater treatment potential of microorganisms. In this study, the ethanol tolerant yeast with laccase activity was integrated with constructed wetland -microbial fuel cell (CW-MFC) to remove ethanol contamination wastewater treatment limitation and simultaneously generate electrical energy. For the experiment, ethanol tolerant yeast with laccase activity was selected and grown in the CW-MFC filled with winery wastewater. The wastewater treatment (chemical oxygen demand (COD) and phenol removal) potential and electrochemical properties were monitored. The results indicated that the maximal laccase activity of 158.12 ± 0.52 U/mL was gained from the yeast strain ET-KK. The maximal current density and power density of 139.17 ± 1.44 mA/m² and 38.74 ± 0.80 mW/m² were generated where the maximal COD and phenol removal of $79.14 \pm 0.92\%$ and $85.04 \pm 0.07\%$ were obtained. This study used the CW-MFC integrated with laccase-producing ethanol tolerant yeast for winery wastewater treatment and electricity generation.

Keywords: phenol; winery wastewater; laccase; ethanol tolerant yeast; constructed wetland; microbial fuel cell.

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1. Introduction

The winery wastewater is an important high ethanol content wastewater that has high chemical oxygen demand (COD) and contains insufficient inorganic nitrogen (N) and phosphorus [1]. It holds 2145 mg/L of COD, 400 mg/L of total organic carbon, 22.6 mg/L of total polyphenols (gallic acid), and 3639 mg/L of ethanol [1-2]. Various processes such as adsorption, photocatalytic process, coagulation, flocculation, decantation, ozonation, constructed wetland (CW), and microbial fuel cell (MFC) [2-5]. For microbial cells, ethanol can provide negative effects such as cell membrane damage, growth inhibition, endocytosis inhibition, transport processes inhibition, and increased membrane fluidity [6]. Thus, the ethanol tolerant microbe has been interested in using ethanol contaminated treatment.

Ethanol tolerant yeast has been isolated and applied in various advantages. In Tikka *et al.*, the ethanol tolerant yeast isolated from sugar-rich fruit, *Saccharomyces cerevisiae*, with a 12% ethanol tolerance level, has been applied in fermentation [7]. The bacterium *Acetobacter malorum* isolated from cactus fruit has been used as a vinegar starter [8]. Although, the ethanol tolerant yeast *S. cerevisiae* and *Saccharomyces boulardii* isolated from food supplements can enhance the beverage flavor and nutrition of functional beer [9]. No previous study used an ethanol tolerant yeast for wastewater treatment and electricity generation.

The constructed wetland coupling with microbial fuel cell (CW-MFC) allows the potential to achieve bioelectricity and an unpolluted ecosystem [10]. The CW-MFC can improve the limitation of the constructed wetland (CW) and microbial fuel cell (MFC) in scaling up and treatment performance. However, the previous works have focused on more environmental treatment applications than electricity generation [11].

In Patel *et al.*, the CW-MFC has been used in textile dye wastewater treatment. The maximal power output of 197.94 mW/m² was achieved [12]. On the other hand, the CW-MFC planted with *Phragmites australis* was used for greywater treatment. The results showed that the maximal COD and phosphate removal efficiencies of 91.70% and 61.50% were achieved, respectively. At the same time, the maximal power output of 67.67 mW/m³ was reached. The result recommended that combining the MFC system with CW allows energy recovery and provides effective wastewater treatment [13]. In Xie *et al.*, the CW-MFC was used to biodegrade nitrobenzene and electricity generation. The maximal power output of 1.53 mW/m² and nitrobenzene removal of 92.89% was gained [14]. According to previous studies, this study aims to use ethanol tolerant yeast for ethanol contaminated wastewater treatment and electrical energy recovery using the CW-MFC. In this study, the ethanol tolerant yeast with laccase activity was used as an anode biocatalyst in the CW-MFC for electricity generation and wastewater treatment (COD and phenol removal) from the ethanol contaminated winery wastewater.

2. Materials and Methods

2.1. Microbes.

The five strains of ethanol tolerant yeast (*Pichia* sp. ET-SS, *Pichia* sp. ET-AM, *Pichia* sp. ET-KK, *Saccharomyces* sp. ET-PW, *Pichia* sp. ET-PM) were isolated from the honey mead, mulberry, pineapple, traditional beverage starter, and palm vinegar (Figure 1) for ethanol contaminated wastewater treatment in the next section. They were kept at Microbial fuel cell & Bioremediation Laboratory, Thaksin University, Thailand. The cultures were maintained in the sucrose-yeast broth (10% (w/v) sucrose and 0.1% (w/v) Brewer's yeast) [15] and kept under 4 °C until being used.

2.2. Laccase activity.

The ethanol tolerant yeasts were cultured in 50 mL of modified basal media (20 g/L glucose, 1 g/L yeast extract, 1 g/L KH₂PO₄, 0.5 g/L MgSO₄ · 7H₂O, and 0.5 g/L KCl) [16] at 30 °C for 3 days. All reactions were centrifuged at 12,000 rpm for 10 mins at 4 °C. The 10 µL of supernatants were used as a crude enzyme for laccase activity determination. Laccase activity was monitored spectrophotometrically (Shimadzu, Japan), using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) substrate according to a previous study of Palmieri *et al.* [17].

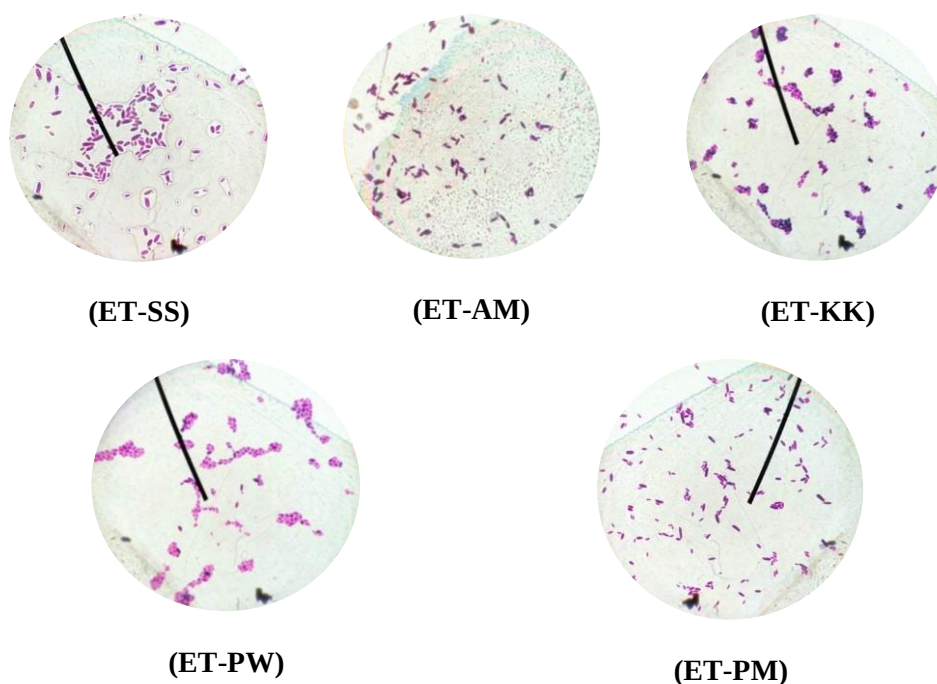


Figure 1. The ethanol tolerant yeast strains were used in this study.

2.3. Synthetic winery wastewater.

The winery wastewater was prepared according to a modified study of Welz and Roes-Hill with 2027 mgCOD/L of final concentration (127 mgCOD/L glucose, 100 mgCOD/L gallic acids, 1000 mgCOD/L ethanol, and 800 mgCOD/L acetic acids) [18]. The distilled water was sterilized at 121 °C for 15 mins before being used for synthetic wastewater preparation.

2.4. CW-MFC design and operation.

The diagram of CW-MFC is shown in Figure 2; the 20 cm² of the microwave-expanded graphite plates were used as electrodes. The 1.7 L of the plastic box was used as the CW-MFC reactor. The 500 g of volcanic rock was used as a system matrix. The macrophyte *Epipremnum aureum* was planted on the cathodic electrode for enhancing electron reduction [19]. The copper wire was used to connect between electrodes. The 10% (v/v) ethanol tolerant yeast (1×10^8 cell/mL) and the 90% (v/v) of synthetic winery wastewater were added into the CW-MFC reactor and incubated at room temperature for 3 days for inoculation of yeast on an anodic surface. The anolyte was fed out; the fresh synthetic winery wastewater was fed in. The electrochemical properties were studied.

The closed-circuit voltage (CCV) at 1 K Ω was monitored every 60 mins. The current and power were calculated according to Ohm's law. The current density and power density based on electrode area (m²) and working volume (m³) were calculated.

$$I = V / R \quad \text{----} \quad (1)$$

$$P = IV \quad \text{----} \quad (2)$$

$$CD = I / A \quad \text{----} \quad (3)$$

$$PD = P / A \quad \text{----} \quad (4)$$

where I is the current (A), V is the CCV at 1 K Ω (V), R is the external resistance (Ω), P is the power (W), CD is the current density (A/m² or A/m³), A is the electrode area (m²) or working volume (m³), and PD is the power density (W/m² or W/m³).

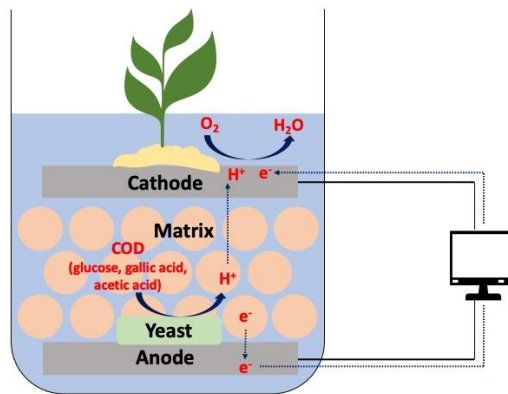


Figure 2. The diagram of CW-MFC was used in this experiment.

2.5. COD and phenol removal.

The COD removal was studied using COD Digestion Vials, High Range Plus (Hach, United States). The experiment was carried out according to the reactor digestion method. The influent and effluent were centrifuged at 12,000 rpm for 10 mins at 4 °C before being used. The 0.2 mL of supernatants were added into the COD vial and inverted several times gently and heated in a DRB200 reactor (Hach, United States) for 2 hr. All reactors were cooled down to room temperature and inverted, the vials were inserted into the COD meter to determine COD concentration.

The phenol removal was measured according to Chaijak *et al.* [20]. Briefly, the 0.5 mL of wastewater was mixed with 4.5 mL of deionized water and 5 mL of Folin-Ciocalteu reagent. It was incubated for 5 mins, then the 5 mL of 10% (w/v) Na₂CO₃ solution was added. The absorbance was monitored at the wavelength of 760 nm

3. Results and Discussion

3.1. Laccase activity.

Laccase is the multi-copper oxidoreductase qualified to catalyze the oxidation of organic and inorganic compounds, such as phenol, aromatic amine, ketone, lignin, and others [21-23]. In this study, the ethanol tolerant yeast strains were cultured in basal media. Then, the supernatants were collected to monitor laccase activity. The maximal laccase activity of 158.12 ± 0.52 U/mL was achieved from the ET-KK isolated from the pineapple peel (Figure 3). In Bazanella *et al.* [24], the laccase producing fungi *Pleurotus pulmonarius* was isolated from pineapple peel. The 2.8 U/mL of laccase was gained.

Wisniewska *et al.*, the laccase activity of 0.22 U/mL was gained from *Kabatiella bupleuri* G3 IBMiP isolated from soil [21]. The yeast *Candida zeylanoides* generates extracellular laccase with approximately 0.03 U/mL [25]. In Aung *et al.*, the halotolerant yeast *Aureobasidium melanogenum* isolated from the mangrove environment has laccase activity of 3.12±0.17 U/mL [23].

3.2. Electrochemical properties.

The ethanol tolerant yeast strains were added onto an anodic electrode of the CW-MFC with 1,000 mL (0.001 m³) of working volume. In comparison, the graphite plate electrode with 0.002 m² of surface area has been used. The CCV at 1000 Ω was collected every 60 mins (Figure 4). The electrochemical properties of CW-MFC are shown in Table 1. The results

indicated that the CW-MFC with ethanol tolerant yeast ET-KK could generate the highest electricity. The maximal power output of $38.74 \pm 0.80 \text{ mW/m}^2$ was gained. The power output (mW/m^2) of CW-MFC with ET-KK based on operating time (min) is displayed in Figure 5.

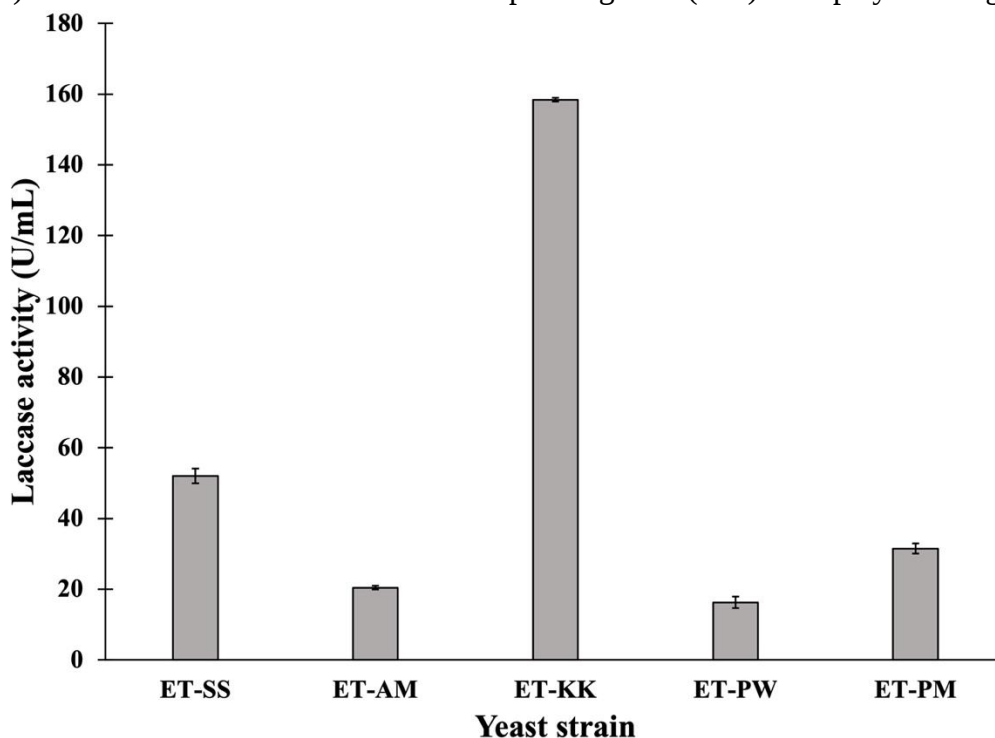


Figure 3. The laccase activity of ethanol tolerant yeast.

Table 1. Electrochemical properties of CW-MFC with ethanol tolerant yeast strain.

Parameter	ET-SS	ET-AM	ET-KK	ET-PW	ET-PM
CCV (mV)	219.33±6.43	80.00±1.00	278.33±2.89	103.33±5.77	96.67±1.53
Current (mA)	0.11±0.01	0.08±0.00	0.28±0.00	0.10±0.01	0.10±0.00
Current density (mA/m^2)	53.67±3.21	40.00±0.50	139.17±1.44	51.67±2.89	48.33±0.76
Current density (mA/m^3)	107.33±6.43	80.00±1.00	278.33±2.89	103.33±5.77	96.67±1.53
Power (mW)	0.01±0.00	0.01±0.00	0.08±0.00	0.01±0.00	0.01±0.00
Power density (mW/m^2)	5.77±0.68	3.20±0.08	38.74±0.80	5.35±0.61	4.67±0.15
Power density (mW/m^3)	11.55±1.36	6.40±0.16	77.48±1.60	10.70±1.21	9.35±0.29

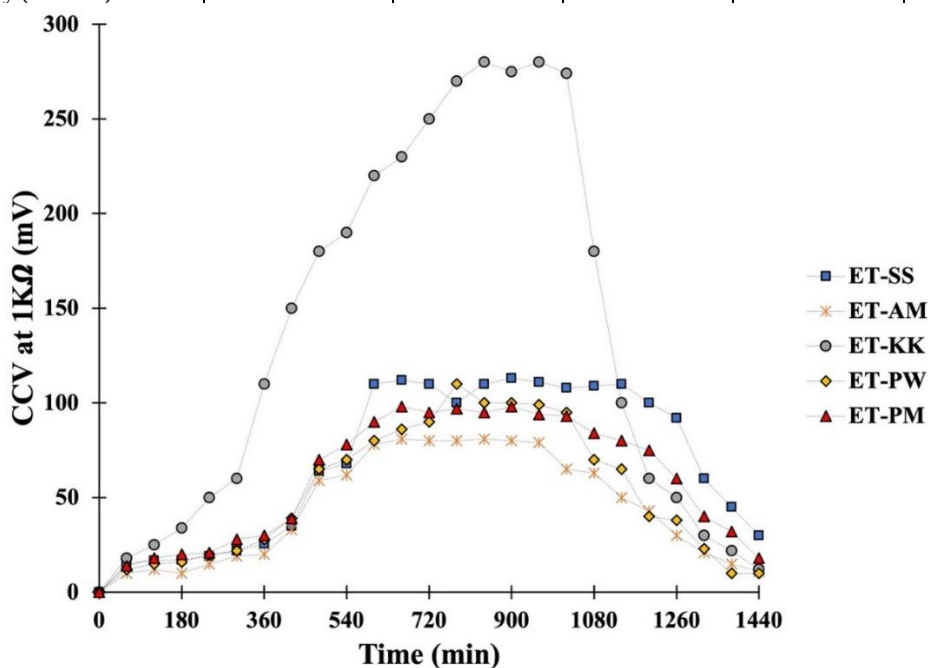


Figure 4. The CCV of CW-MFC at 1000 Ω external resistance.

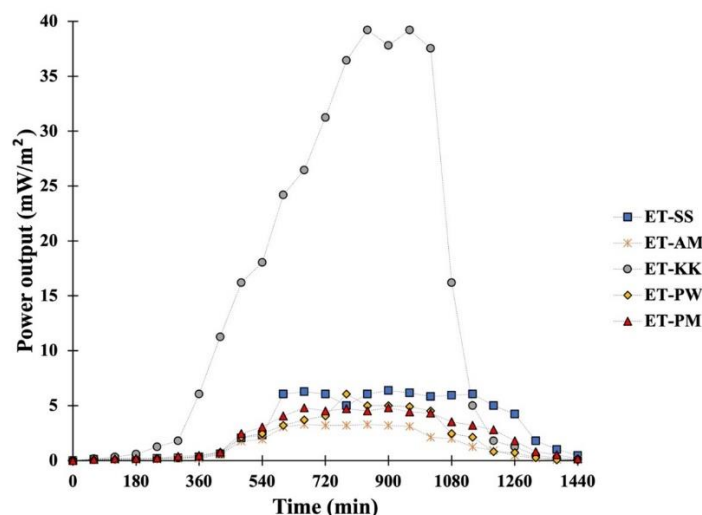


Figure 5. The power output of CW-MFC with ethanol tolerant yeast.

Table 2. Review of electricity generation of MFC using alcohol contaminated wastewater as a substrate.

MFC type	Wastewater	Power output (mW/m ²)	Reference
CW-MFC	Winery wastewater	38.74±0.80	This study
Membrane-less MFC	Winery wastewater	274.90	[27]
Dual-chamber MFC	Winery wastewater	8.37 – 420.00	[28]
Dual-chamber MFC	Brewery wastewater	1.68 – 38.34	[29]
Dual-chamber MFC	Winery wastewater	0.80	[30]
Dual-chamber MFC	Brewery wastewater	10.69 – 80.01	[31]

According to Table 2, the maximal power output of CW-MFC with *Pichia* sp. ET-KK, where the synthetic winery wastewater was as a substrate, is 38.74±0.80 mW/m². The power output of this study is higher than the previous studies that use the dual-chamber MFC. However, it still provided a lower power output than using membrane-less MFC. In Sonawane *et al.*, the distillery wastewater has been treated by the high surface MFC. The results showed the maximal electricity output of 621 mW/m² was generated. However, this system still needs to be constructed the air-cathode for achieving electrical energy [32].

3.3. COD and phenol removal.

The COD and phenol removal were studied after the CW-MFC operation; the results presented the maximal COD and phenol removal of 79.14±0.92% and 85.04±0.07%, respectively, were using 24 hr of operation without energy supply.

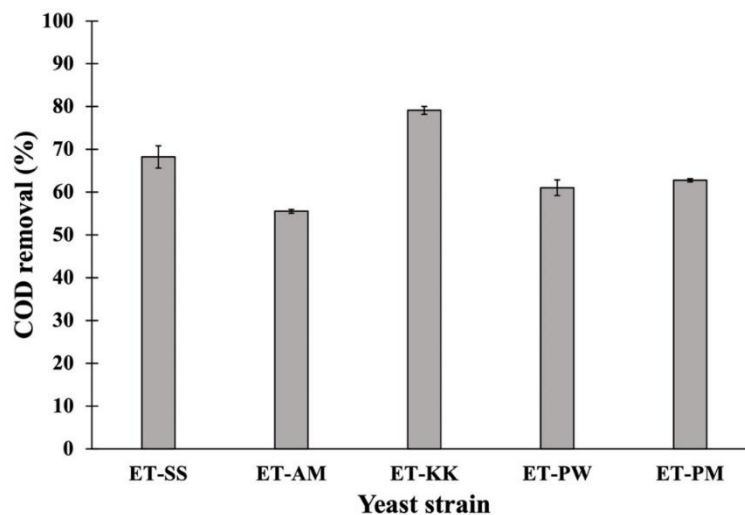


Figure 6. The COD removal of CW-MFC.

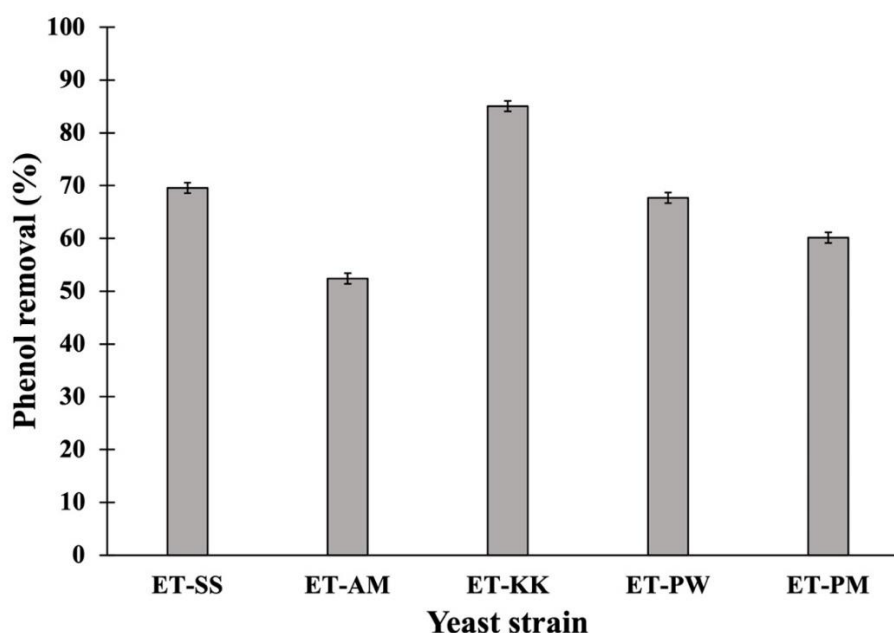


Figure 7. The Phenol removal of CW-MFC.

On the other hand, phenol removal has been investigated with various processes, including chemical, physical and biological processes. In Gholipour and Hosseini, the phenol removal of 91.87% was achieved by using the catalytic wet peroxide oxidation (CWPO) process [33]. It also gained approximately 60% removal potential from bio-adsorption by low-cost polyacrylamide/starch hydrogels [34].

In terms of using microbial enzymes, the extracellular laccase of white-rot fungi *Trametes versicolor* was immobilized on glycidyl methacrylate – polyacrylamide alginate gel has been used for phenol removal from olive mill wastewater. The 70.00% of phenol removal was achieved [35]. At the same time, the combination of peroxidase and polyphenol oxidase enzyme achieved about 98.10% phenol removal. However, this process can be stabilized using only 7-time of phenol removal [36]. A review of using microbial enzymes for phenol removal from wastewater is shown in Table 3.

Table 3. Reviews of using microbial enzymes for phenol removal from wastewater.

System	Wastewater	Enzyme	Removal (%)	Power output (mW/m ²)	Reference
CW-MFC	Winery	Laccase	79.14±0.92	38.74±0.80	This study
Biodegradation	Synthetic	Peroxidase	92.00	None	[37]
Biodegradation	Synthetic	Horseradish peroxidase	96.00	None	[38]
Biodegradation	Textile	Peroxidase	94.95	None	[39]
Biodegradation	Leather	Peroxidase	91.49	None	[39]

4. Conclusions

The ethanol tolerant yeast ET-MFC isolated from pineapple peel provides the highest laccase activity with 158.12±0.52 U/mL. The CW-MFC treating winery wastewater can act as a combination of MFC with a CW system for wastewater treatment and simultaneously electricity generation. The ethanol tolerant yeast strains with laccase activity have been used as an anodic whole-cell biocatalyst. The performance of CW-MFC has a high potential in both terms of wastewater treatment (COD and phenol removal) and electricity generation. The maximal power output, COD removal, and phenol removal of 38.74±0.80 mW/m²,

79.14±0.92%, and 85.04±0.07% were achieved. This study gained the novelty of using ethanol tolerant yeast to treat the toxic ethanol contaminated wastewater by the CW-MFC.

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Conflicts of Interest

The authors declare no conflict of interest.

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