The oxygen reduction reaction is one of the most studied reactions in electrocatalysis, particularly for bio-fuel cell cathodes. Laccase is a blue multi-copper oxidase that catalyzes the reduction of oxygen during the oxidation of phenolic compounds. Several studies have discussed using laccase as an electrocatalyst for oxygen biosensors during the oxidation of phenolic compounds. Several studies have shown very interesting behavior and functionality, particularly in biofuel cell applications, their practical use is currently limited by short lifetimes due to enzyme degradation and loss of activity on the electrode surface, electrons are received from the electrode by the T1 copper center of the active site, which then transfers the electrons via the amino acid conjugates to the tri-nuclear cluster (T2 and T3 copper centers), where O₂ will get reduced to water. Most of the laccases studied for the oxygen reduction reaction are of fungal origin, including Trametes versicolor (TVL), Aspergillus niger, or Pleurotus ostreatus. As a high potential laccase, TVL is characterized by a high T1 copper redox potential and high catalytic activity between pH 4 and 5. TVL has been widely studied as an enzyme bio-electrocatalyst on a variety of immobilization supports for the electrochemical reduction of oxygen. One of the challenges in enzyme electrocatalysis is to obtain an efficient electrical communication between enzyme redox sites and the electrode surface. Several approaches have been tried to maximize the electro-catalytic activity of the laccase modified electrodes. Among the various nanomaterials, multiwall carbon nanotubes (MWCNT), have been extensively studied as enzyme immobilization support by our group. MWCNT provide high surface area and conductivity, which helps to increase the number of enzyme active sites on the electrode (enzyme loading). In previous work from our laboratory, it has been demonstrated that Trametes versicolor laccase (TVL) possesses high electro-catalytic activity for bio-electrochemical reduction of oxygen via direct electron transfer. In that work, TVL was immobilized onto MWCNT-modified electrode surface through molecular tethering agent 1-pyrenebutanoic acid succinimidyl ester (PBSE). This method of immobilization using molecular tethering agent has been proven to dramatically improve the overall electro-catalytic activity of the laccase-modified electrodes.

Though TVL-based enzyme catalysts possess high electro-catalytic activity for O₂ reduction reaction and could be used in biofuel cell applications, their practical use is currently limited by short lifetimes due to enzyme degradation and loss of activity on a bioelectrode. Thermophilic bacterial laccases, on the other hand, have shown very interesting behavior and functionality, particularly in high temperature environments. Thermophilic laccases are generally more resistant to denaturing and loss of activity than their mesophilic counterparts. Bacillus sp. FNT is a thermophilic bacterium with optimal growth conditions at pH 8 and 50°C. It was isolated from a hot spring and first characterized at Fundación Biociencia, Chile. The native enzyme was found to have a spore coat with laccase activity and was found to possess a maximum activity at 70°C and at pH 7 to 8. Bacillus sp. FNT gene associated with the laccase activity was cloned and introduced to E. coli BL21 for overexpression and purified to homogeneity. The recombinant thermophilic Bacillus sp. FNT laccase (FNTL) was studied in our previous work. Bacillus sp. FNT laccase was tethered onto MWCNT surface using PBSE as molecular tethering agent. As depicted in Figure 1, PBSE could non-covalently bind onto MWCNT through π-π stacking and covalently bind onto the laccase enzyme. FNTL-MWCNT conjugates also exhibited superior stability compared to TVL-MWCNT over a period of 2 weeks. Few studies have reported key kinetic parameters of oxygen reduction reaction catalyzed by laccase. In this work, a comprehensive electro-kinetic investigation of thermophilic Bacillus sp. FNT laccase using hydrodynamic voltammetry measurements obtained using a rotating ring-disk electrode (RRDE) is presented. The key electro-kinetic and mechanistic parameters for the Bacillus sp. FNT laccase catalyzed oxygen reduction were determined for the first time. These studies provide a quantitative method of analysis to evaluate the electro-catalytic activity of the catalysis reaction using laccase functionalized nan conjugates.
Electrochemical measurements.—A glassy carbon disk and platinum ring electrodes were mounted on a rotating shaft controlled by a RPM controller. The glassy carbon disk electrode was polished using an electrode polishing kit consisting 0.3 μm aluminum powder. A Ag/AgCl reference electrode and platinum wire auxiliary electrode (both from CH Instrument Inc.) were used for electrochemical measurements. All the electrochemical measurements were carried out at 25 ± 2 °C in a 125 mL voltammetry cell unless otherwise stated. All potentials reported are with respect to the Ag/AgCl reference.

**FNTL-MWCNT conjugates preparation.—**Multiwall carbon nanotube (MWCNT) was used as the immobilization support for FNTL. The glassy carbon disk was coated with MWCNT suspension and dried at 70 °C. 10 mM PBSE in DMF was drop-casted on the surface of electrode and incubated for 15 min. Followed by washing the electrode with DMF and phosphate buffer (pH = 7), laccase enzyme solution was added on the surface of the electrode by drop casting and incubating for 30 min on ice. The cross linker PBSE is tethered onto MWNT using pyrenebutanoic acid succinimidyl ester (PBSE). The tethered laccase reduces oxygen to water via direct electron transfer. (b) Rotating ring disk electrode set up for oxygen reduction reaction.

Figure 1. (a) Schematic of the FNTL-MWCNT bioconjugates, where FNTL is tethered onto MWNT using pyrenbutanoic acid succinimidyl ester (PBSE). The tethered laccase reduces oxygen to water via direct electron transfer. (b) Rotating ring disk electrode set up for oxygen reduction reaction.

Figure 2. Comparison of CVs of the complete (FNTL, PBSE, MWCNT) and incomplete (without PBSE or MWCNT) bio-conjugates using glassy carbon electrode obtained in the presence and absence of O2.

Results and Discussion

**Role of molecular tethering agent.—**Figure 2 compares the cyclic voltammograms (CVs) of the complete *Bacillus sp. FNTL-MWCNT* nanoconjugate catalysts (FNTL, PBSE, MWCNT) with that of the control (catalyst without PBSE or MWCNT). The CVs showed that the molecular tethering agent PBSE is essential for enhancing the electron transfer capability, resulting in significantly better electro-catalytic activity for oxygen reduction compared to the catalyst that lacked PBSE. This can be seen in the cathodic wave of the CV obtained in O2 saturated electrolyte, which clearly deviates from the CV obtained in the O2 deprived electrolyte (N2 saturated electrolyte) at and below 0.37 V. The onset potential for oxygen reduction is 0.37 V and the open circuit potential was 0.46 V. In the absence of MWCNT, the electrode possesses a low surface area that results in low capacitance and low non-faradaic currents as shown in the CVs.

A notable difference between *Bacillus* bacteria-based FNTL-MWCNT conjugates and the previously reported fungal-based TVL-MWCNT conjugates was the difference in the open circuit and onset potentials. Bacterial laccases are known to exhibit lower redox potentials for the T1 copper center compared to their fungal counter parts. The stability of the bio-conjugates were also monitored and compared in a 16-day time range at room temperature in our previous study. FNTL-MWCNT showed consistent electrochemical catalytic activity for oxygen reduction compared to the catalyst that lacked PBSE. This can be seen in the cathodic wave of the CV obtained in O2 saturated electrolyte, which clearly deviates from the CV obtained in the O2 deprived electrolyte (N2 saturated electrolyte) at and below 0.37 V. The onset potential for oxygen reduction is 0.37 V and the open circuit potential was 0.46 V. In the absence of MWCNT, the electrode possesses a low surface area that results in low capacitance and low non-faradaic currents as shown in the CVs.

Figure 2. Comparison of CVs of the complete (FNTL, PBSE, MWCNT) and incomplete (without PBSE or MWCNT) bio-conjugates using glassy carbon electrode obtained in the presence and absence of O2.

Electrochemical characterization.—The electrochemical measurements were conducted in a 125 mL electrochemical cell using 100 mM phosphate buffer as electrolyte (pH 7). A bi-potentiostat was used to perform linear sweep voltammetry (LSV) on the disk electrode and at a fixed applied potential on the ring electrode at different disc rotational speeds. For LSV, the disk potential was swept from +0.5 V to −0.9 V at a scan rate of 20 mV/s, while the ring potential was kept constant at +1.0 V. The Tafel plot measurements were obtained on the disk electrode in the potential range of 0.5 V to +0.1 V versus the onset potential for oxygen reduction. The kinetic parameters such as Tafel slope, number of electrons, and rate constants were calculated using the data obtained in voltammogram and Tafel plot measurement.

Electrochemical characterization.—The electrochemical measurements were conducted in a 125 mL electrochemical cell using 100 mM phosphate buffer as electrolyte (pH 7). A bi-potentiostat was used to perform linear sweep voltammetry (LSV) on the disk electrode and at a fixed applied potential on the ring electrode at different disc rotational speeds. For LSV, the disk potential was swept from +0.5 V to −0.9 V at a scan rate of 20 mV/s, while the ring potential was kept constant at +1.0 V. The Tafel plot measurements were obtained on the disk electrode in the potential range of 0.5 V to +0.1 V versus the onset potential for oxygen reduction. The kinetic parameters such as Tafel slope, number of electrons, and rate constants were calculated using the data obtained in voltammogram and Tafel plot measurement.
activity, whereas the catalytic activity of TVL-MWCNT dramatically decreased over 16 days.

Hydrodynamic study.—The electrochemical reduction of oxygen using FNTL-MWCNT modified electrodes were studied using the RRDE. The RRDE hydrodynamic scheme is shown in Figure 1b, where a RRDE electrode is immersed in electrolyte containing O2. On the surface of disk, O2 is reduced and the products are transferred to the ring electrode immediately due to rotation. At the ring a potential of +1.0 V vs Ag/AgCl was applied to oxidize the reaction product H2O2 to O2. The magnitude of the ring current allows for a direct measurement of the amount of H2O2 generated at the disk. High ring currents generally indicate a low electro-catalytic activity of laccase-MWCNT conjugates for a direct 4-electron reduction of O2 to water.

Due to the high potential applied on the ring electrode, hydrogen peroxide that was formed at the disk electrode as a result of incomplete oxygen reduction would be oxidized to oxygen at the ring electrode resulting in an oxidation current at the ring. Linear sweep voltammetry (LSV) was conducted using RRDE electrode modified with FNTL-MWCNT conjugates. Disk current (I_D) and ring current (I_K) was monitored at different rotation speed (0 – 1500 rpm). The resulting voltammograms are given in Figure 3. The onset potential of oxygen reduction is 0.35 V regardless of the rotation rate. The LSV obtained at 0 rpm clearly showed a significant cathodic wave in O2 saturated electrolyte at and below 0.35 V with a steep slope. Below 0.15 V, the cathodic disk current started to decrease indicating the mass transfer limitations of O2 in the solution. With increasing disk rotation speeds, the mass transfer limitation was significantly lowered resulting in high limiting currents (below −0.8 V). It is noteworthy that the slopes of oxygen reduction remain the same (1.18 ± 0.22 mV/dec) with increasing rotation speeds, indicating a stable electro-catalytic activity of FNTL-MWCNT bioconjugates at all rotation speeds.

Effect of rotation speed.—The voltammograms shown in Figure 3a were used to understand the relation between the limiting current and O2 concentration, which can be described by Levich equation

$$I_L = 0.62nFAC_0D^{1/2}v^{-1/6}ω^{1/2}$$

where $I_L$ is the limiting current, $n$ is the number of electrons transferred per oxygen molecule, $F$ is the Faraday’s constant, $A$ is the electrode surface area (0.196 cm²), $C_0$ is the electrode concentration, $D$ is the diffusion coefficient of O2 in the bulk (1.22 mM), $v$ is the angular rotation rate, $ω$ is the apparent diffusion coefficient of oxygen in the bulk (1.22 mM). The $I_k$ values were obtained from the LSV of the disk electrode at −0.9 V at which the rate of O2 reduction equals the rate of O2 diffusion. The Levich plot of limiting current versus the angular rotation rate of RRDE was plotted in Figure 4.

Koutecky – Levich analysis.—The Koutecky-Levich voltammograms, Figure 3a, were used to understand the relation between the limiting current and O2 concentration, which can be described by the Koutecky-Levich equation

$$I_{kL} = I_D + I_k = \frac{1}{I_k} + \frac{1}{I_D} = \frac{1}{I_k} + \frac{1}{0.62nFAC_0D^{1/2}v^{-1/6}ω^{1/2}}$$

where $I_k$ is the kinetic current of the disk electrode given by $I_{kL} = (I_k I_k-I_D)/I_D$, $I_D$ is the diffusion-limited current, $n$ is the number of electrons transferred, $F$ is the Faraday’s constant, $C_0$ is the concentration of oxygen in the electrolyte, $D$ is the diffusion coefficient of O2 in the bulk, $v$ is the kinematic viscosity (cm²/s) and $ω$ is the angular rotation rate. The slope yielded values were obtained from the LSV of the disk electrode at +0.9 V at which the rate of O2 reduction equals the rate of O2 diffusion. The Koutecky-Levich voltammogram of the disk electrode is shown in Figure 4.

For RRDE measurements, the limiting current at any rotation rate can be expressed as

$$I_{L(i)} = I_{kL(i)} + I_{kR}$$

where $I_{kL(i)}$ is the kinetic current of the disk electrode given by $I_{kL} = (I_k I_k-I_D)/I_D$, $I_{kR}$ is the diffusion-limited current, $n$ is the number of electrons transferred, $F$ is the Faraday’s constant, $C_0$ is the concentration of oxygen in the electrolyte, $D$ is the diffusion coefficient of O2 in the bulk, $v$ is the kinematic viscosity (cm²/s) and $ω$ is the angular rotation rate.

In order to investigate the number of electrons transferred in the reaction, the RRDE was used to understand the relation between the limiting current and O2 concentration, which can be described by the Koutecky-Levich equation

$$I_{kL} = I_D + I_k = \frac{1}{I_k} + \frac{1}{I_D} = \frac{1}{I_k} + \frac{1}{0.62nFAC_0D^{1/2}v^{-1/6}ω^{1/2}}$$

where $I_k$ is the kinetic current of the disk electrode given by $I_{kL} = (I_k I_k-I_D)/I_D$, $I_D$ is the diffusion-limited current, $n$ is the number of electrons transferred, $F$ is the Faraday’s constant, $C_0$ is the concentration of oxygen in the electrolyte, $D$ is the diffusion coefficient of O2 in the bulk, $v$ is the kinematic viscosity (cm²/s) and $ω$ is the angular rotation rate. The slope yielded values were obtained from the LSV of the disk electrode at +0.9 V at which the rate of O2 reduction equals the rate of O2 diffusion. The Levich plot of limiting current versus the angular rotation rate of RRDE was plotted in Figure 4.

The electrochemical reduction of oxygen using FNTL-MWCNT modified electrodes were studied using the RRDE. The RRDE hydrodynamic scheme is shown in Figure 1b, where a RRDE electrode is immersed in electrolyte containing O2. On the surface of disk, O2 is reduced and the products are transferred to the ring electrode immediately due to rotation. At the ring a potential of +1.0 V vs Ag/AgCl was applied to oxidize the reaction product H2O2 to O2. The magnitude of the ring current allows for a direct measurement of the amount of H2O2 generated at the disk. High ring currents generally indicate a low electro-catalytic activity of laccase-MWCNT conjugates for a direct 4-electron reduction of O2 to water as it measures the quantity of H2O2 intermediate from the reaction.

Due to the high potential applied on the ring electrode, hydrogen peroxide that was formed at the disk electrode as a result of incomplete oxygen reduction would be oxidized to oxygen at the ring electrode resulting in an oxidation current at the ring. Linear sweep voltammetry (LSV) was conducted using RRDE electrode modified with FNTL-MWCNT conjugates. Disk current (I_D) and ring current (I_K) was monitored at different rotation speed (0 – 1500 rpm). The resulting voltammograms are given in Figure 3. The onset potential of oxygen reduction is 0.35 V regardless of the rotation rate. The LSV obtained at 0 rpm clearly showed a significant cathodic wave in O2 saturated electrolyte at and below 0.35 V with a steep slope. Below 0.15 V, the cathodic disk current started to decrease indicating the mass transfer limitations of O2 in the solution. With increasing disk rotation speeds, the mass transfer limitation was significantly lowered resulting in high limiting currents (below −0.8 V). It is noteworthy that the slopes of oxygen reduction remain the same (1.18 ± 0.22 mV/dec) with increasing rotation speeds, indicating a stable electro-catalytic activity of FNTL-MWCNT bioconjugates at all rotation speeds.

**Figure 3.** Rotating ring disk electrode measurements using linear sweep voltammetry at various disk rotation speeds from 0.6 to −1.0 V at the scan rate of 20 mV s⁻¹. (a) Plot of disk current (I_D) vs disk potential; (b) Plot of ring current (I_K) vs disk potential.

**Figure 4.** Plot showing the Levich regions for ORR by bio-nanoconjugates modified disk electrode. Rotation speeds ranged from 0 to 1500 RPM.
lacase-MWCNT catalyzed oxygen reduction reaction, the B value in Equation 2 was substituted by the slope value obtained in Figure 5, and n value was calculated from the slope. The resulting number of electrons transferred per oxygen molecule was between 3.2 and 3.35. These calculated n values were then validated using ring current data in the following analysis.

**Ring current analysis.**—As shown in Figure 3b, the ring current indicated a variation in \( \text{H}_2\text{O}_2 \) oxidation at the ring electrode at various rotational speed and disk potentials. The \( I_R \) obtained at low rotation rate was lower in the diffusion-limited region compared to the region above the onset potential. This behavior is understandable as the ring current measures the oxidation of \( \text{H}_2\text{O}_2 \) which does not happen at low potentials. \( I_R \) also increased with increasing rotation rates and became constant at very high rotation rate. A detailed analysis was conducted to understand the amount of \( \text{H}_2\text{O}_2 \) formation at the disk. The average number of electrons transferred per oxygen molecules could be an indication of the ability of a catalyst to preferentially catalyze a 4-electron reduction of \( \text{O}_2 \) to \( \text{H}_2\text{O} \) over a 2-electron reduction to \( \text{H}_2\text{O}_2 \). The number of electrons was calculated by the equation:

\[
n = \frac{4}{1 + \frac{I_R}{N I}}
\]  

where \( I_R \) is the ring current and \( I \) is the measured disk current, and \( N \) is the collection efficiency, which refers to the percentage of \( \text{H}_2\text{O}_2 \) that was collected at the ring electrode. It is a geometric parameter that was experimentally determined to be 0.256 by the manufacturer for the RRDE set up. A potential range of 0.145 V to −0.9 V was used for the calculation of the number of electrons involved in oxygen reduction reaction using the above equation. The calculated values of n at different rotation rates were plotted against disk potential in Figure 6. Figure 6 showed high number of electrons involved in the reduction reaction at high rotation rates varying from 3.5 to 3.7, indicating that the catalyst predominantly catalyzes 4-electron reduction of \( \text{O}_2 \) to \( \text{H}_2\text{O} \) with minimal \( \text{H}_2\text{O}_2 \) formation as intermediate. The results also suggest that FNTL-MWCNT bioconjugates possess high electro-catalytic activity for oxygen reduction reaction.

**Electro-catalytic activity.**—The electro-catalytic activity of FNTL-MWCNT bioconjugates for oxygen reduction was also evaluated using Tafel slopes, a key metric used for kinetic valuations. The Tafel slopes were obtained from the voltammograms in the kinetic region of oxygen reduction (0.15–0.35 V). The Tafel slope in this study is the slope of the linear region in a potential vs \( I_k \) plot as shown in Figure 7, between \( 1 \times 10^{-3} \) and \( 1 \times 10^{-2} \) mA. The obtained Tafel slope from the above region was 45 mV per decade change of kinetic current and was constant at high rotation rates within Levich region. The theoretical value of Tafel slope for a four-electron reduction of oxygen is 15mV per decade change in \( I_k \). The closer the experimental value to the theoretical value, the better the electro-catalytic behavior of the catalyst.

**Electrochemical rate constant.**—We previously proposed a reaction mechanism for oxygen reduction on the bioconjugates-modified electrode, as shown in Figure 8a, which is similar to the one reported by Damjanovic. In this model, \( \text{O}_2 \) in bulk solution first diffuses to disk electrode functionalized with FNTL-MWCNT bioconjugates and get reduced on the electrode surface in two ways: a four-electron \( \text{O}_2 \) reduction, or a two-electron \( \text{O}_2 \) reduction. \( k_1, k_2 \) and \( k_3 \) are the reaction rate constants for each of the reactions on the RRDE surface as depicted in Figure 8a. \( k_1 \) represents the rate constant of the complete reduction (four-electron reduction) of oxygen to water. \( k_2 \) represents the rate constant of the incomplete reduction of oxygen to hydrogen peroxide (two-electron reduction). The intermediate hydrogen peroxide that formed on disk electrode partially diffuses to ring electrode, and the remaining hydrogen peroxide diffuses to bulk solution. \( k_3 \) represents the rate constant of the oxidation of hydrogen peroxide to oxygen on ring electrode. All the rate constants were calculated by

![Figure 5. Koutecky-Levich plots at various disk potentials (−0.25–−0.4 V) outside the kinetically limited region.](image)

![Figure 6. Number of electrons as a function of disk potential.](image)

![Figure 7. Tafel plot obtained from the calculated kinetic current values at different RPM in the kinetic region.](image)
higher than that of the oxygen reduction on metallic surfaces, indicating that FNtL-MWCNT bioconjugates have higher electro-catalytic activity than metallic catalysts on a per catalytic site basis.33

**Electron transfer rate.**—The electron transfer rate of oxygen reduction reaction could be influenced by several factors, such as the conductivity of the electrode, the distance between the catalyst and the electrode, and the intrinsic electro-catalytic activity of the catalyst. The electron transfer rate constant of FNtL-MWCNT bioconjugates catalyzed oxygen reduction reaction could be calculated using the following equation:

\[ I_0 = nFk_{\text{act}} \]

Where \( I_0 \) is the exchange current obtained from Tafel plot, \( n \) is the number of electrons, \( F \) is the Faraday's constant, \( k_{\text{act}} \) is the electron transfer rate constant, \( c \) is the concentration of the anolyte in the bulk solution. The exchange current density can be calculated using a current vs overpotential plot also known as Tafel plots. Figure 8b shows the log \( I_0 \) vs overpotential of the chemical oxygen reduction reaction, which can be expressed using the equation

\[ I = a + b \exp(\eta) \]

Where \( a \) and \( b \) are empirical fitting constants and \( \eta \) is the overpotential. For this, the disk electrode was swept between 0.1 to 0.5 V (−0.2 to +0.2 V vs onset potential) at 0 RPM. Ideally this plot should be linear. However, it appears nonlinear because of the large voltage window used for sweeping the voltage. From the plot in Figure 8b, \( I_0 \) was calculated by intersecting the tangents at nonlinear regions of the Tafel curve. The obtained values of equilibrium exchange current density \( (I_0) \) and the electron transfer rate \( (k_{\text{act}}) \) were \( 7.54 \times 10^{-6} \pm 1.24 \times 10^{-7} \) A cm\(^{-2} \) and \( 1.60 \times 10^{-5} \pm 2.83 \times 10^{-7} \) cm s\(^{-1} \), respectively.

**Conclusions**

This work presents a comprehensive electrochemical kinetic study of oxygen reduction reaction using a thermophilic laccase-based bioconjugate. The electro-kinetic parameters for FNtL catalyzed oxygen reduction reaction such as order of reaction, number of electrons transferred per \( \text{O}_2 \), Tafel slopes, kinetic rate constant of reactions at the disk electrode, and the electron transfer rate constant are being reported for the first time. The results demonstrated that FNtL not only possesses high stability, but also exhibits excellent bio-electrocatalytic activity. The kinetic properties reported in this work could be used as a benchmark of the potential usefulness of thermophilic FNT laccase in the future development of bioelectrocatalysis for bio-fuel cell applications.

**ORCID**

Yan Zhou https://orcid.org/0000-0003-1900-5277
Ramaraja P. Ramasamy https://orcid.org/0000-0002-5004-1754

**References**


Downloaded on 2018-10-26 to IP 200.89.68.74 address. Redistribution subject to ECS terms of use (see ecsdl.org/site/terms_use) unless CC License in place (see abstract).