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OPTIMIZATION OF LACCASE ACTIVITY MEASUREMENT IN A CRUDE OIL POLLUTED SOIL

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Abstract

The effect of substrate concentration, soil incubation buffer volume and substrate incubation duration on laccase activity in a crude oil polluted soil were evaluated in this study. These parameters were studied over a specified range of values; substrate concentrations: 1, 5, 10, 15, 20 and 25 mM; soil incubation buffer volume: 25, 50, 75, 100 and 125 mL; and incubation duration: 30, 60, 90, 120 and 150 min. Laccase activity was evaluated by spectrophotometric method using pyrogallol as substrate. The optimum values of laccase activity in micromole per hour per gram soil [µmol/h/g] obtained were 2.80, 2.16 and 2.12 for the corresponding optimum substrate concentration (5 mM), soil incubation buffer volume (50 mL) and incubation duration (60 min) respectively. The use of these optimum values in laccase assay will provide a very useful indication of microbial metabolic activities during the process of bioremediation of the polluted soil.

Introduction

Crude oil exploration and other related activities globally over the years have greatly impacted on the environment with its attendant negative effect on the ecosystem (ITE et al. 2013). The exposure of microbial communities in the environment to petroleum hydrocarbon pollution leads to selective enrichment and genetic changes resulting in increased proportion of hydrocarbon degrading microbes including bacteria and fungi (primary degraders) encoding hydrocarbon catabolic genes (LEAHY and

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COLWELL 1990, SAFDARI et al. 2018). These catabolic genes mediate the degradation of petroleum hydrocarbon pollutants in the soil using their respective enzyme systems (SILES and MARGESIN 2018). Most organic pollutants including crude oil are transformed aerobically (ROJO 2009) through several metabolic processes catalysed by various microbial enzymes including oxygenases, reductases, hydroxylases and dehydrogenases (VARJANI and UPASANI 2017, POLYAK et al. 2018). The various hydrocarbon fractions of crude oil have different and diverse degradation pathways controlled by different catabolic genes (WANG and SHAO 2013). The initial attack incorporates oxygen into the organic pollutant followed by stepwise peripheral degradation pathways that convert the petroleum hydrocarbon to tricarboxylic acid cycle (TCA) intermediates (WANG and SHAO 2013). The metabolites formed such as acetyl – CoA, succinate and pyruvate are then used by the microbes to synthesize cell biomass (CHANDRA et al. 2015).

The oxidative enzymes expressed by soil microorganisms have been broadly classified based on the electron acceptor involved in their oxidative process (SINSABAUGH 2010, BACH et al. 2013). The first group utilizes oxygen as electron acceptor while the second uses hydrogen peroxide. In assays of environmental samples, SINSABAUGH (2010) used the generic term phenol oxidase to refer to the activity of several enzymes that require oxygen as electron acceptor including monooxygenases, dioxygenases, tyrosinase, catechol oxidase, laccase etc. Similarly, all hydrogen peroxide requiring oxidative enzymes are assayed as peroxidase. Laccases (benzediol: oxygen oxidoreductase EC 1.10.3.2) are the largest class of phenol oxidase present in the soil (BALDRIAN 2006, SINSABAUGH 2010). They are a group of multicopper enzyme family involved in phenolic compounds oxidation with oxygen as electron acceptor (STREK and TELESINSKI 2015). Extracellular laccases are produced by microorganisms in response to organic pollutants in the soil; plants also produce both intracellular and extracellular laccases used in the synthesis of lignin and other secondary compounds (BALDRIAN 2006, SINSABAUGH 2010). Although, laccases are primarily taken to be fungal enzymes, some bacteria are known to also secrete laccase – like enzymes (BALDRIAN 2006, SINSABAUGH 2010). These bacterial laccases exist as components of larger protein complexes and they exhibit much greater activity in the soil than fungal laccases (BACH et al. 2013). Laccases are more active than peroxidases at beginning of organic pollutant transformation due to their low redox potential (MARTINEZ et al. 2005).

The estimation of the potential oxidative enzymes activity in the soil is done by measuring oxidation rate of a model substrate (BACH et al. 2013,

BURNS et al. 2013). Phenol oxidase (laccase) activity is assayed with the substrate alone in contrast to the addition of hydrogen peroxide for peroxidase activity (BACH et al. 2013). Pyrogallol, L-3,4-dihydroxyphenylalanine (L-DOPA) and 2,2'-azinobis-3-ethylbenzothiazoline-6-sofonic acid (ABTS) are some commonly used model substrates for oxidative enzymes assay in the soil (SINSABAUGH 2010, BACH et al. 2013). Pyrogallol is the most easily oxidized and is also less susceptible to interference by soil minerals (HEWINS et al. 2016). Soil enzyme activities and microbial indices are useful indicators of the recovery of the soil during bioremediation (ALRUMMAN et al. 2015). These indicators provide evidence of the metabolic activities and viability of the microorganisms in the soil (MARGESIN et al. 2000). Laccase was chosen as an indicator of soil microbial activity in this study due to its involvement in the degradation of complex molecules including recalcitrant soil organic matter and carbon cycling (BALDRIAN 2006).

The development of appropriate protocol for the estimation of laccase activity in a crude oil polluted soil is integral to the deployment of an effective bioremediation strategy as it provides a basis to monitor the soil attenuation process. Such protocol must take into consideration factors including substrate concentration, soil incubation buffer volume and duration of substrate incubation that are involved in the enzyme activity measurement. Hence, this work was designed to determine optimum levels of these experimental factors in the estimation of laccase activity in a crude oil contaminated soil.

Materials and Methods

Reagents and equipment

Pyrogallol was purchased from Kermel Chemicals, China. All other chemicals and reagents used were of analytical grade. The following equipment were used in the study: analytical balance (BL – 200S, Setra Systems, USA); centrifuge (800D, Phoenix, USA); constant temperature magnetic stirrer (78 HW – 1, China); pH meter (PHS – 3C, Phillips Scientific, USA); top – loading precision balance (PA512, Ohaus – Pioneer, USA); spectrophotometer (Model 6305, Jenway, England).

Sample collection and preparation

Soil sample was collected from a fallow area without any history of petroleum hydrocarbon pollution in Samuel Adegboyega University, Ogwa, Edo State, Nigeria. The sample was artificially contaminated in the laboratory with crude oil at a concentration of 1% (w/v) in an open container and kept for two weeks. During this period, the contaminated soil was replenished with water and mixed once a week. Thereafter, the soil samples were air-dried for five days and sieved with a 2 mm sieve. Baseline properties of both the contaminated and uncontaminated soil samples were determined using standard analytical procedures (data not included).

Laccase activity assay

Laccase activity was estimated using pyrogallol as substrate (ALLISON and JASTROW 2006). One gram (1 g) crude oil contaminated soil was weighed into a 250 mL conical flask and 50 mL acetate buffer (50 mM, pH 5) was added. The flask was incubated at room temperature for 1 h with vigorous shaking every 20 min. Thereafter the volume of the buffer was increased to 125 mL and the flask shaken vigorously. An aliquot of the soil suspension (10 mL) was centrifuged at 4000 rpm for 10 min to obtain the supernatant used for the enzyme assay. The test experiment contained 2 mL of the supernatant (soil suspension) and l mL substrate (25 mM). This was incubated in the dark at room temperature for 1 h. A sample control containing 2 mL supernatant and 1 mL buffer and also substrate control containing 1 mL substrate and 2 mL buffer were treated as the test experiment. The absorbance was measured at 460 nm using a spectrophotometer. Buffer solution was used as blank. Laccase activity was calculated using Eq. (1).

$$\text{Laccase activity [µmol/h/g]} = \frac{A \cdot \text{V1}}{E \cdot \text{V2} \cdot T \cdot W}$$

where:
A – net absorbance [test – sample control – substrate control]
V1 – volume of buffer used [mL]
E – molar extinction coefficient for pyrogallol – 4.2 per µmol
V2 – volume of soil suspension [mL]
T – substrate incubation time [h]
W – mass of soil sample [g]

Effect of buffer incubation volume on laccase activity

One gram (1 g) contaminated soil sample was weighed into five different 250 mL conical flasks and 25, 50, 75, 100 and 125 mL of 50 mM acetate buffer pH 5 was added to each flask respectively. The flasks were incubated at room temperature for 1 h with vigorous shaking every 20 min. Thereafter the volume of the buffer in each flask was increased to 125 mL by the addition of 100, 75, 50, 25 and 0 mL of 50 mM acetate buffer pH 5 respectively. Laccase activity was then determined after centrifugation of the soil suspension as already described above.

Effect of substrate incubation time on lacccase activity

One gram (1 g) contaminated soil sample was weighed into 250 mL conical flasks and 50 mL of 50 mM acetate buffer (pH 5) was added. The flask was incubated at room temperature for 1 h with vigorous shaking every 20 min. Thereafter the volume of the buffer was increased to 125 mL by the addition of 75 mL of 50 mM acetate buffer (pH 5). Aliquot (10 mL) of the soil suspension was centrifuged at 4000 rpm for 10 minutes. The supernatant was used for laccase activity assay as already described using different substrate incubation duration of 30, 60, 90, 120 and 150 min respectively.

Effect of substrate concentration on laccase activity

One gram (1 g) contaminated soil sample was weighed into 250 mL conical flasks and 50 mL of 50 mM acetate buffer (pH 5) was added. The flask was incubated at room temperature for 1 h with vigorous shaking every 20 min. Thereafter the volume of the buffer was increased to 125 mL by the addition of 75 mL of 50 mM acetate buffer (pH 5). An aliquot (10 mL) of the soil suspension was centrifuged at 4000 rpm for 10 minutes. The supernatant was used for laccase activity assay as already described using different substrate concentrations of 1, 5, 10, 15 and 25 mM respectively.

Data analysis and presentation

All experiments were performed in triplicates. The values were presented as means ± standard error (SE).

Results and Discusion

Laccase catalyse the oxidation of pyrogallol (a phenolic substrate) to quinone. This is autooxidised to dark brown pigment whose absorbance was measured with a spectrophotometer at 460 nm. The colour intensity is proportional to the enzyme activity. Experimental conditions including soil incubation buffer volume, substrate incubation duration and substrate concentration in the enzyme activity assay were studied. The result obtained for the effect of soil incubation buffer volume is presented in Figure 1. The optimum buffer incubation volume was 50 mL with laccase activity of 2.16 µmol/h/g. Microbial oxidative enzymes in soil are usually measured in extracts using appropriate buffer (BALDRIAN 2009). The buffer controls pH and dilute sample (GERMAN et al. 2011). Acetate buffer (50 mM) used in this study is reported in literature to support high recovery of lignolytic enzymes from the soil (BALDRIAN 2009). The use of high buffer volume before sample incubation in the substrate is to reduce the possibility of interference from organic matter present in the soil (BALDRIAN 2009). ALLISON and JASTROW (2006), BACH et al. (2013) and SAIYA-CORK et al. (2002) all reported use of 125 mL acetate buffer (50 mM, pH 5) in assay for laccase in forest soil.

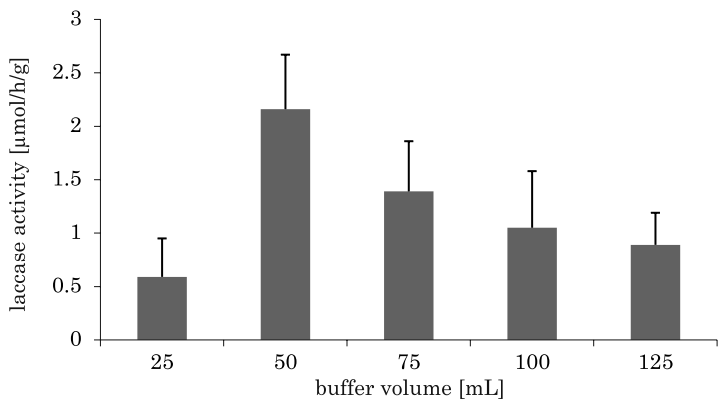

Fig. 1. Effect of buffer incubation volume on laccase activity in crude oil contaminated soil

In the determination of the effect of substrate incubation duration, the various enzyme activities obtained in the study were linear up to 60 min incubation (Figure 2). This time was therefore taken as the optimum incubation duration. Since soil enzyme activity is usually calculated as a rate with assumption of zero order kinetics (GERMAN et al. 2011), it is important to use substrate incubation duration that will produce linear response with time (SAIYA-CORK et al. 2002). ALLISON and JASTROW (2006) used

incubation time of 1 h to improve detection limit; this is in agreement with the optimum substrate incubation time obtained in this study. In contrast, BACH et al. (2013) reported that reaction rates for pyrogallol remained linear over 3–4 h, they however noted that longer incubation times yielded inconsistent results.

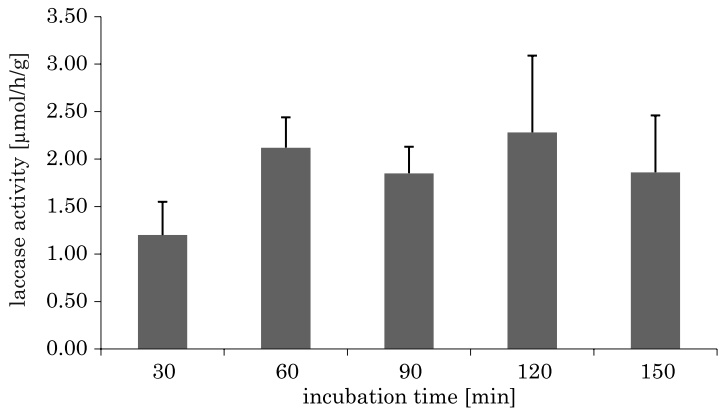

Fig. 2. Effect of substrate incubation time on laccase activity in a crude oil contaminated soil

In this study, the enzyme activity was assayed by monitoring the rate of oxidation of the model substrate pyrogallol over a range of concentrations. Substrate concentration is an important consideration in soil enzyme assays (BALDRIAN 2009, SINSABAUGH 2010).

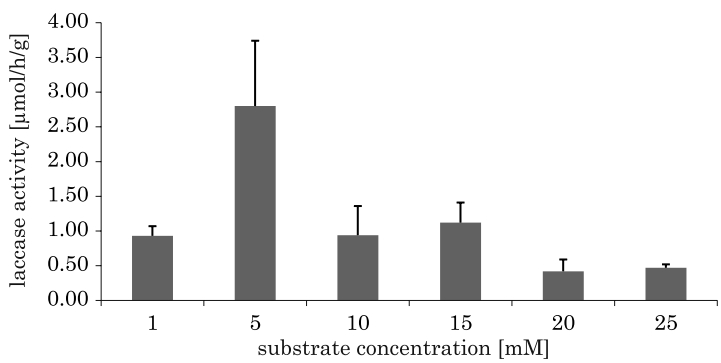

Fig. 3. Effect of substrate concentration on laccase activity in a crude oil contaminated soil

Assays for oxidases that may not follow Michaelis-Menten kinetics may require excess concentration of substrate in the reaction system to allow the enzymatic reaction to proceed at a maximal rate (BALDRIAN 2009). This explains the substrate concentration (25 mM) used in this study. However, the results obtained revealed that the optimum substrate

concentration for maximum enzyme activity during this study was 5 mM (Figure 3). This result is in agreement with the 5 mM Pyrogallol reported by BACH et al. (2013).

Conclusion

Laccase activity was measured in a crude oil contaminated soil using pyrogallol as a model substrate. Experimental conditions including incubation buffer volume, substrate incubation duration and substrate concentration were optimized. The use of these optimum values of the various parameters studied in laccase assay will ensure optimal assay conditions for efficient evaluation of laccase activity in the soil. This will provide a very useful indication of microbial metabolic activities and the viability of the microorganism during the process of bioremediation of crude oil polluted soil.

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