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Utilization of *Trametes versicolor* for the production of laccase and its application in oxytetracycline degradation from wastewater

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ABSTRACT

The performance of *Trametes versicolor* following its growth in different lignocellulosic substrates was evaluated. Results showed that the maximum laccase activity was observed in the OPEFB medium, 2051.189 U/L, which is 3.23 folds higher than basal mediums like malt and glucose. The optimum concentration of OPEFB and pH for the submerged culture of *T. versicolor* were obtained at 30 g/L and pH 5.5, respectively. OPEFB can be fully utilized as a medium for the growth of *T. versicolor* thus, the wasteland can be minimized. The performance of *T. versicolor* in basal medium and OPEFB was evaluated using antibiotic oxytetracycline as model emerging micropollutant that contained in wastewater. It was observed that OTC degradation performance (percentage degradation %) of the *T. versicolor* in OPEFB medium with mycelium from *T. versicolor* culture degraded almost 99% within 50 h at T48 compared to basal medium with *T. versicolor*'s mycelium. These results demonstrated that mycelium *T. versicolor* could offer better OTC degradation performance since the mycelia of *T. versicolor* absorbed the OTC and then underwent a degradation process.

Keywords: Trametes versicolor; Lacasse; Oxytetracycline; Degradation; Agro

1. Introduction

Antibiotics are commonly and widely used for medical purposes and become an emerging water contaminant in recent years. Nowadays, even in agriculture, many farmers are using various types of antibiotics contaminant in large amounts to grow their products rapidly and get economic benefits but it has some adverse effects to our environment greatly [1–3]. Variety of antibiotics such as penicillin, rifampicin, gentamicin, nitrofurantoin, doxycycline, etc. were detected in different types of aqueous medium such as industrial water, lakes water, surface water, hospital wastewater and drinking water [4,5] and have harmful effects on the aquatic system and human beings. Tetracycline groups of antibiotics such as tetracycline (TC) and oxytetracycline (OTC) are mainly used in various bacterial infections and were first time reported in the 1940s [6]. In the last few years, many researchers reported that it was widely found in waste resources due to the discharge of pharmaceutical and hospital wastewater that directly enters the water bodies [7–9]. These antibiotics are persistent in nature and can exist in water for a long time. Various chemical, physical and biological methods have been used to degrade these contaminants from wastewater to make pure and drinkable

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water [10–13]. Conventional water treatment methods cannot easily remove these antibiotics. Thus, many researchers have developed an alternative method like a biological method for the biodegradation of antibiotics [5]. Nowadays, the application of specific enzymes has a great demand to remove these types of water contaminants and become an effective and promising alternative method to secure the environmental life. Laccases are a kind of enzyme mainly found in wood-decaying (white-rot) fungi and have the potential to degrade antibiotics [14]. *Trametes versicolor* is a white-rot fungus that contains laccase enzymes with lignin-degrading ability has got much attention from researchers and deploys the highest redox potential compared to other laccases [15].

Laccases (EC1.10.3.2) are multicopper oxidoreductase enzymes that can oxidize a wide range of phenolic and aromatic compounds with the simultaneous reduction of molecular oxygen to water as a by-product [16]. Laccases can be isolated from plants, fungi, insects, bacteria and actinomyces [17]. Laccases are considered as a proficient and environmentally friendly catalysts and have been applied in many industrial and environmental field such as biotechnology application, food-processing industry, biopharmaceuticals, and biodegradations [16,18]. However, their use in a biotechnological process or industrial application has been limited because of high production costs. Yet, many researchers are trying to develop new methods to increase laccase production [19].

One of the considerable and effective method for improving laccase production is by using lignocellulosic wastes or substrates as a medium culture for fungus. Previous work has shown that lignocellulosic residues can stimulate laccase production since the residues contain carbon, nitrogen and various mineral that are essential elements for microorganism's growth, enzyme production and metabolite synthesis [20]. Lignocellulosic wastes mainly contain three components namely cellulose, hemicellulose and lignin and can be directly depolymerized by laccase as natural substrates [16]. Lignocellulose, the major source of agricultural waste, is considered as a low-cost nutrient substitute for laccase production since 200 billion tons of agricultural waste are generated every year [21,22]. The most plentiful and cheap lignocellulose agricultural wastes are crop straw (e.g., from rice, wheat, or corn). Another abundant source of lignocellulosic waste (80–95 million tonnes in Malaysia and Indonesia) is oil palm biomass [23]. The ultimate goal to fully utilize the agricultural waste is achieved by efficient bioconversion of lignocellulose while major agricultural wastes are burned or left to rot and contribute to environmental pollution [16].

This paper presents the laccase activity of *T. versicolor* by adding several types of lignocellulosic substrates into submerged cultures. The objective of this study is to select the most potent laccase inducer from different types of lignocellulosic substrates tested: oil palm empty fruit bunch, oil palm trunk, coconut fiber, and rice straw by optimizing the culture conditions, including concentration and pH. At last, this laccase enzyme was applied to determine the degradation ability of extracellular liquid obtained in the cultures towards the OTC.

2. Materials and methods

2.1. Materials

The Oil palm trunk (OPT), oil palm empty fruit bunch (OPEFB), and coconut fiber (CF) were obtained from T&H Coconut Fibre Sdn. Bhd. (Malaysia). The rice straw (RS) was collected from a local paddy field located in Semangor, Perak, Malaysia. Malt extract powder, glucose, 2, 2′ azino-bis-(3-ethylbenzthia-zoline sulfonic acid) (ABTS, 98.0%) and oxytetracycline (OTC) were purchased from Sigma-Aldrich (U.S.A.). Sodium acetate trihydrate ($CH_3COONa{\cdot}3H_2O$, 99.0%), peptone from casein, and methanol (CH_3OH, 99.9%) were obtained from Merck (Germany). The deionized water (DI water) was produced using the Purite Water System model Analyst HP 40 purchased from Purite Ltd. (England).

2.2. Microorganism

T. versicolor strain, ATCC 42530, was purchased from the American Type Culture Collection (U.S.A.). It was cultured and maintained on 2% malt agar slants at 4°C. Sub-cultures were monthly prepared as per routine from the mother culture.

2.3. Biomass and medium preparation

All types of lignocellulosic substrates (OPT, OPEFB, RS, and CP) were ground and sieved into the particle size range of 100 μm and 200 μm. The powdered form of all the selected biomass was washed with double distilled water to remove dirt and impurities. After then, these materials were dried in an oven at 50°C. *T. versicolor* was grown on liquid-state cultures using Blakelee's formula to determine the laccase activity. The basal of fermentation's medium contained (g/L): malt 30; glucose; peptone 1. 100 mL of culture medium was prepared in 250 mL Erlenmeyer Flask and autoclaved. The malt was replaced by OPT, OPEFB, RH, RS, CP, and CH for parametric studies on laccase production.

2.4. Inoculum preparation and culture conditions

T. versicolor was cultured on a plate containing malt agar by streaking the *T. versicolor* from slant using a loop. It was incubated for 7 d. Control cultures without biomass were prepared by transferring four discs (1 mm) of *T. versicolor* to 250 mL Erlenmeyer flasks containing 100 mL of basal growth medium. The flasks were incubated in an orbital shaker (150 rpm) at 30°C. The enzyme activity was monitored for 7 d and the laccase activity was measured every 24 h. The mycelium and medium were separated by filtration and the dry weight of the mycelium was weighed for 7 d to evaluate the growth of *T. versicolor*. The experiment was repeated three times.

The experimental procedure was repeated for different culture conditions by replacing malt with 30 g/L of OPT, OPEFB, RS, and CF and tested individually to check the effect of different types of biomass on laccase production yield. For the most effective biomass listed, the glucose is then replaced with biomass plus malt in culture media to determine the best pair of biomass in the culture medium that produce a high laccase yield.

The concentrations of selective biomass were varied from 10, 30 and 50 g/L to optimize the best concentration to give high laccase production. The effect of pH on laccase activity was studied by growing the *T. versicolor* at pH 4.5, 5.5 and 6.5 for biomass medium culture. All the experiments were repeated three times.

2.5. Degradation of antibiotic (oxytetracycline)

The produced laccase from *T. versicolor* has been applied to degrade the oxytetracycline experiment. The experiment was carried out to degrade OTC at different period at T_0 and T_{96}. For every experiment, four batches were selected (1) culture medium (glucose, peptone, EFB); (2) culture medium + 100 μg/mL OTC; (3) culture medium + *T. versicolor*; (4) culture medium + *T. versicolor* + 100 μg/mL OTC. Batches 1, 2 and 3 are control experiments while 4 is test experiment. All the flasks were covered with aluminium foil to avoid photodegradation of OTC. The degradation activity of OTC was performed by taking different concentration in the flask and laccase activity for OTC degradation were noted daily. The entire experiment has been repeated for three times.

1,000 μg/mL stock solution was prepared by dissolving OTC in methanol solvent. After then, deionized water was added to dilute the OTC solution to achieve the desired concentration. Few discs of active fungus were transferred to the other flask which contained the medium and the 50 mL of OTC solution. All flasks were incubated at 30°C ± 1°C in shaker incubator with 150 rpm [24].

2.6. Analytical procedure

2.6.1. Laccase activity

The laccase activity was measured using UV-Vis Shimadzu Spectrophotometer at wavelength 420 nm. 3 mL of substrate solution was prepared by mixing 5 mM ABTS and 0.1 M pH 5 sodium acetate buffer and placed in a quartz cuvette. The analysis started when adding 0.3 mL supernatant of the sample that was obtained from a centrifugal process (2,800 rpm for 3 min) of the medium. The oxidation of ABTS occurred, followed by the increase of wavelength for 120 s. 1 μm/L of substrate is oxidized by one unit of enzyme per minute, which can be calculated using Eq. (1).

$$UL^{-1} = \frac{(\Delta A)(V_t)(D_f)(10^6)}{(t)(\varepsilon)(d)(V_s)} \quad (1)$$

where U is enzyme activity (μmol/min mL), ΔA is the difference between final absorbance and initial absorbance, V_t is total volume of the reaction, D_f is dilution factor, t is reaction time, ε is molar extinction coefficient (M/cm or L/mol cm), d is optical path (1 cm), and V_s is volume of sample (mL). 3 replicates samples were done for every laccase activity analysis [25].

2.6.2. OTC concentration

1 mL of culture medium from the control flask and experimental flask were taken and centrifuged at 2,800 rpm for 3 min. The OTC concentration was determined using UV/Vis Shimadzu spectrophotometer at a maximum wavelength of 275 nm. Quartz cuvette was used and the sample was diluted with dilution factor 50.

3. Results and discussions

3.1. Laccase production of T. versicolor

The growth of *T. versicolor* was determined by the amount of laccase production in the basal medium and the cell's dry weight for 168 h as presented in Fig. 1. Growth started to begin after 24 h as the laccase activity increased while the dry weight also slightly increased up to 0.084 g. The exponential phase started to occur as there was a sudden increase in the laccase activity and dry cell weight. The mycelium adapted to the new environment and rapidly multiplied until the 4th day of incubation (Fig. 1b), where the laccase activity and dry cell weight also reached the maximum up to 700 U/L and 0.34 g, respectively. Zawawi et al. also found that the optimum laccase activity for *T. versicolor* was on the 4th day [26]. The laccase activity started to deplete after 96 h followed by the dry cell weight. This is probably because the nutrients inside the medium started to reduce, the changing in pH medium, and the accumulation of toxic by-products [22]. The size of pellet and volume of mycelium affect the laccase activity itself as the pellet size growth and mycelium increased, the laccase activity decreased. These results may be interpreted in terms of mass transfer (oxygen) limitations that affect the decrease of metabolic rate and the death of cells. Besides that, when the size of the pellet and volume of mycelium increased, the production of extracellular protein decreased as the nutrient limitation was delayed [27].

3.2. Laccase production using lignocellulosic substrates

The laccase production of *T. versicolor* is highly influenced by some factors such as culture condition including type of medium, composition of medium, pH, presence of

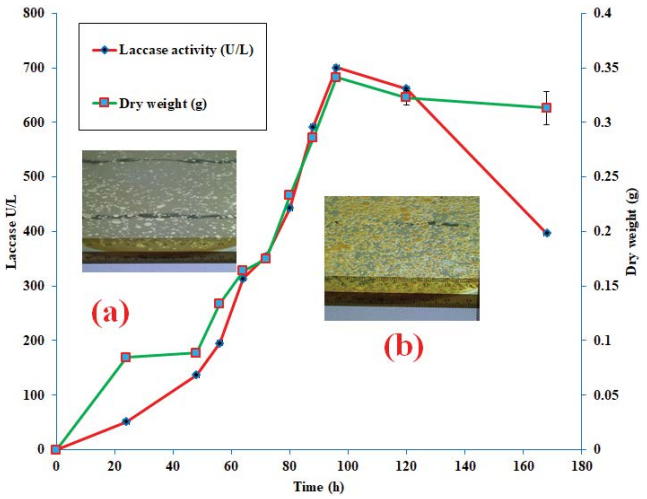

Fig. 1. (a) Growth kinetic (dry mass) and catalytic activity of the free *T. versicolor* calls and (b) growth image of mycelium of *Trametes versicolor* in the medium.

inducers, temperature and rotating speed [26]. Therefore, these important factors are optimized through one-factor-at-a-time experiments. However, in this article, the main emphasis on the type of medium, medium composition and pH of medium solution.

3.2.1. Effects of lignocellulosic substrates and concentration on laccase production

In order to determine the optimal conditions for laccase production of *T. versicolor*, the effects of different carbon sources were studied. In this research, biomass was used as a carbon source, mainly rice straw, coconut fibre, oil palm trunk (OPT), and oil palm empty fruit bunch (OPEFB). The observed results are shown in Fig. 2. After 120 h of incubation with different substrates, the highest laccase activity was determined in case of OPEFB and glucose medium 2051.189 U/L which was 3.23 folds higher than using the basal medium. The presence of lignocellulosic substrates had a positive effect on the culture broth towards the laccase yield. Laccase activity in OPEFB was observed maximum. It may be due to the lignin degradation by laccase to small molecular substrates such as ferulic acid and *p*-coumaric acid, which were considered as laccase inducers [28]. Furthermore, the composition of lignin in OPEFB is higher as compared to oil palm trunk (OPT) [29]. Laccase production was also facilitated by cellulose and lignocelluloses residues and results were summarized in earlier studies [30]. At the same time, part of these small molecules is completely degraded to H_2O and CO_2 to supply energy [31]. Thus, OPEFB will be an effective and future medium composition for *T. versicolor*.

To obtain the optimum concentration of OPEFB for laccase production, different OPEFB concentrations were added to the medium. The laccase activity was measured after 160 h. The results are shown in Fig. 3. The production of laccase activity was increased when the OPEFB concentration increased and reached the maximum, 2,172.349 U/L at 30 g/L of OPEFB, which was found to be 1.37 fold higher than at 10 g/L of OPEFB. It may be due to nutrient composition in the culture medium increased. The laccase production decreases when the OPEFB concentration increases. According to Brijwani et al. [32], excess carbon sources may reduce the laccase production by blocking its induction and only allowing constitutive production of the enzyme. Thus, the optimum concentration of OPEFB is 30 g/L. From this result, OPEFB is an excellent substrate to produce laccases, and it can be reused instead of becoming a wasteland.

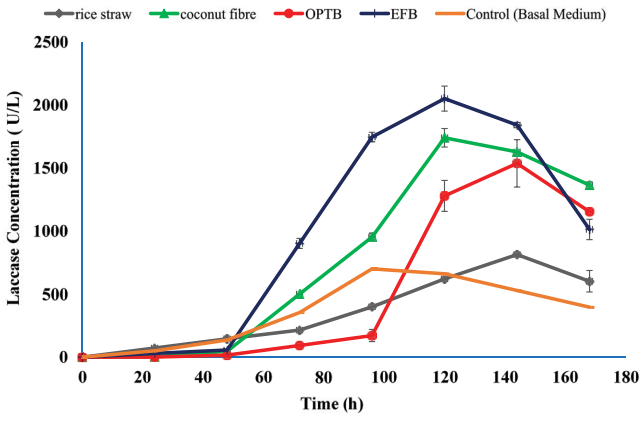

Fig. 2. Laccase production for different lignocellulosic substrates (rice straw, coconut fibre, oil palm trunk, oil palm empty fruit bunch and basal medium). Reaction: *T. versicolor* was incubated in different type of substrates in a 250 mL conical flask at pH 5.5 for 7 d.

3.2.2. Effects of pH on laccase production

The initial pH of solution play an important role and selected the range of pH from 4.5 to 6.5. The experiment was carried out to check the effect of pH on laccase production. Laccase activity was monitored from 0 to 160 h. The results are shown in Fig. 4. The maximum laccase production was found at pH 5.5, up to 2,172.349 U/L. Laccase activity was lower at pH 8.5 compared to laccase activity at pH 4.5. Most studies reported that the initial pH for laccase production was in between 4 to 6 [33]. Li et al. reported that optimum pH for laccase production of fungus *Trametes* sp. LS-10C was pH 4.0 [34]. In previous studies, the maximum laccase activity measured with ABTS was observed in pH 4–6, such as ascomycete *Trichoderma harzanium* [35]. Most laccase activities were optimum in acidic conditions when ABTS was used as substrates. ABTS was oxidized by the laccase more efficiently since it lay in the acidic region [36,37]. Besides, culture medium in acidic conditions for fungal growth has been related to the production of organic acids [38,39].

3.3. Biodegradation of antibiotic

3.3.1. OTC degradation

Amounts of OTC in the different batches and at different times were measured by UV/VIS spectrophotometer. The first batch of OTC was injected at T0. The control experiment of basal medium contained 100 ppm OTC without mycelium, while the test batch of basal medium contained

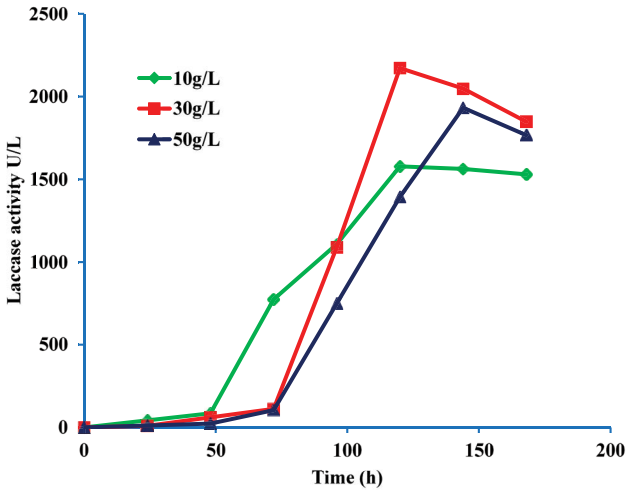

Fig. 3. Effect of different concentration of OPEFB on laccase activity. Reaction: Different concentration (10, 30 and 50 g/L) of OPEFB in medium culture of *T. versicolor* were incubated in 250 mL conical flask at pH 5.5 for 7 d.

100 ppm OTC with mycelium. Fig. 5a shows that the degradation of OTC takes longer time 96 h to reduce the concentration from 100 ppm to 21.29 ppm. It was similar to the removal of OTC by another white-rot fungus, *Pleurotus Ostreatus*, that required up to 4 d [24]. The results show the degradation was slow. At T0, while the culture medium removed OTC, the fungus also grew. Wen et al. [4] reported that the degradation of OTC is not entirely responsible by laccase from fungus since the degradation activity of a crude extract in which several enzymes and redox mediators were present. Besides, the characteristics of OTC were high-residual toxicity, and non-biodegradation influenced the slow rate of degradation process for OTC.

Meanwhile, test batch (basal medium and mycelium) where OTC was injected at T96, Fig. 5b shows a dramatic OTC reduction in the last 24 h and a further reduction in the previous 80 h, up to almost complete degradation of OTC was observed at T72 (residue 6.93 ppm). The concentration of OTC was mostly degraded during T72. A comparable trend of OTC degradation was also found in the control batch. In the culture media, a sharp reduction was observed in the first 24 h, and a further limited reduction occurred in the last 72 h. As a result, a very low OTC concentration was found at T72 (residue 6.93 ppm). However, in a flask contained OPEFB medium and mycelium, the reduction of OTC is significant and complete degradation almost occurred within 50 h at T48 (residue 1.058 ppm). The results indicated that mycelium growth before OTC treatment really improved the degradation process since extracellular enzyme and proved the presence of laccase in the culture medium (Fig. 5). Besides, the pellet produced during the cultivation help the degradation process itself. This is proved by Josep et al. [40] study, where fungal pellets were able to reduce both compounds such as OTC and CBF. Migliore et al. [24] reported the similar results as the *P. Ostreatus* mycelium absorbed by the mycelia and degraded the OTC. In the OPEFB medium with mycelium, the OTC degradation occurred faster due to the presence of the lignocellulosic substrate. Other research studies showed that lignocellulosic substrates could degrade pharmaceutical wastes, metals, and many more organic pollutants [41,42].

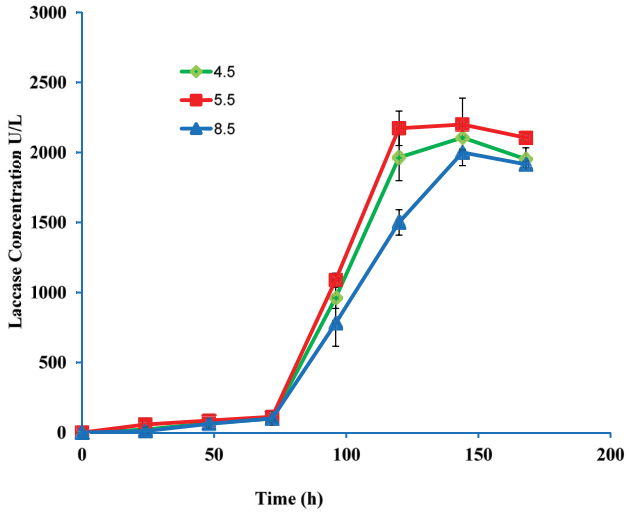

Fig. 4. Effect of different pH of the medium on laccase activity. Reaction: *T. versicolor* was incubated with different pH of OPEFB culture medium (4.5, 5.5, and 8.5) in a 250 mL conical flask for 7 d.

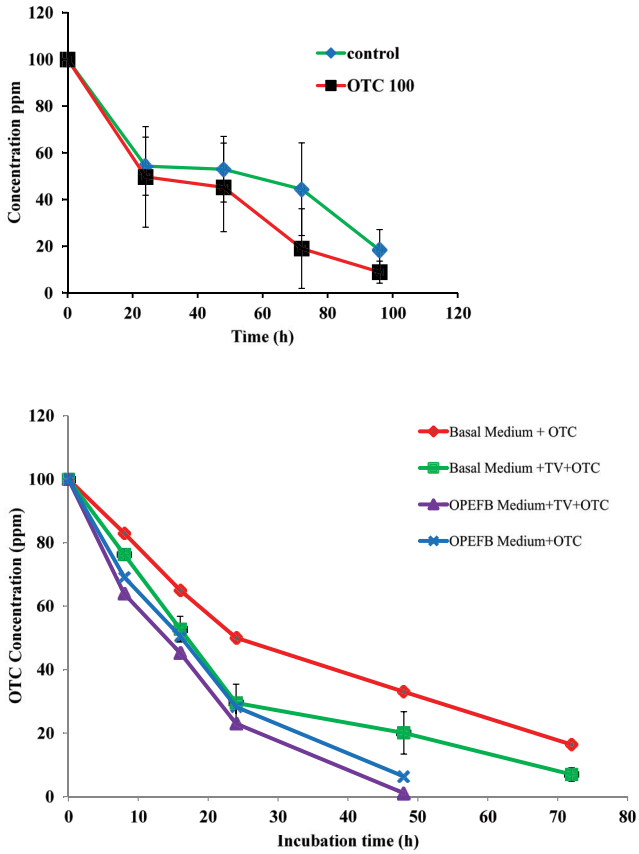

Fig. 5. (a) Degradation of 100 ppm OTC (injected OTC at T0). 50 mL of 100 ppm of OTC was injected at 0 d in a conical flask that contained *T. versicolor* and basal medium and (b) degradation of 100 ppm of OTC (injected OTC at T96) in OPEFB medium with *T. versicolor* and basal medium with *T. versicolor*. 50 mL of 100 ppm of OTC was injected after all *T. versicolor* were incubated for 96 h.

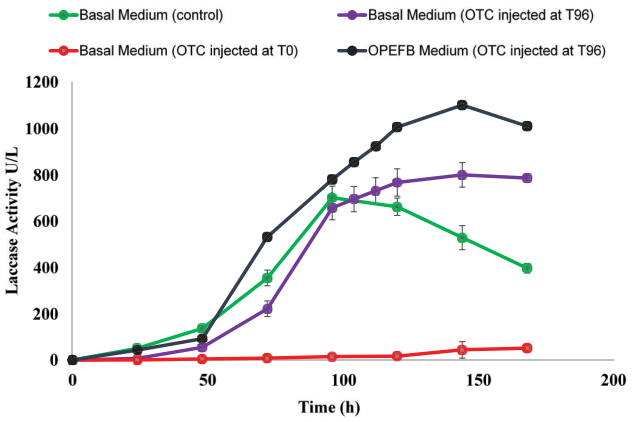

Fig. 6. Laccase concentration of *Trametes versicolor* mycelium in control batch, OTC injected at T0 and T96.

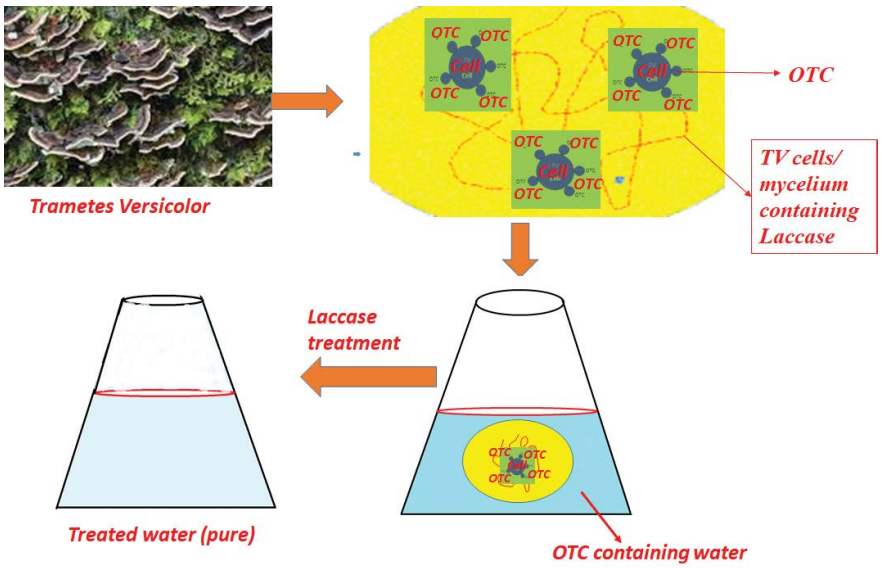

Fig. 7. Mechanism of oxytetracycline degradation after laccase activity.

3.3.2. Laccase activity in culture medium

The laccase activity was measured in the culture media of the control batch and test batch (basal medium and OPEFB medium). The amount of OTC was injected for the time interval at T0 and T96 (Fig. 6). The laccase activity was measured daily for all the flasks contain OTC. Flask (A) contained basal medium and mycelium, flask (B) contained basal medium, mycelium and OTC injected at T96, flask (C) contained basal medium, mycelium and OTC injected at T0, while flask (D) contained OPEFB medium, mycelium and OTC injected at T96. The laccase production for all the experiments showed a significance difference. In flask C, it was observed that the activity started to increase after 24 h and the production of laccase has slow progress. The OTC solution injected in flask C at 0 h of incubation inhibits the fungal growth. This may be due to the high concentration of OTC delaying the lag phase for *T. versicolor*. The system includes metabolism, protein synthesis, and enzyme activity. For the test batch, OTC injected at T96, the laccase activity was increased. OTC is also a carbon source since OTC has C–H–O chemical structure. Besides, it is also linked with amine chemical structure which can be a source of nitrogen during the cultivation process. The fed-batch process was applied at T96, which OTC as carbon source was feed on the 4th day of cultivation of *T. versicolor*, and the nutrient in the medium is increased again. Hence, laccase activity also increased [43]. The mechanism of oxytetracycline degradation after laccase activity is summarized in Fig. 7.

4. Conclusion

High laccase production of *T. versicolor* was obtained to remove OTC. This study illustrated the advantage of using different lignocellulosic agro wastes as substrates in laccase production. In this work, OPEFB showed good potential as a medium culture for laccase production. The optimum laccase activity was up to 2,051.189 U/L, which was 3.23 folds higher than other lignocellulosic substrates and basal medium. By using OPEFB as substrate, the environment could be conserved, and minimized the wasteland. For the removal of OTC experiment, the degradation efficiency of *T. versicolor* in OPEFB medium for OTC was found to be 99%. From the results, it was concluded that the OTC was absorbed by the mycelia of *T. versicolor* and then degraded. More studies can be attempted in order to investigate the degradation of OTC by using *T. versicolor* in real wastewater.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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