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A lignolytic Fungi with Laccase Activity Isolated from Malaysian local Environment for Phytochemical Transformation Purposes

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Abstract

The main aim of the current study is to isolate a fungi with laccase activities to be used for transformation of phytochemicals isolated from the same environment as an approach to produce novel pharmaceutical products. In our study, laccase producing fungi was isolated from samples taken from a tropical trees barks which grown in the International Islamic University Malaysia, Kuantan campus using different chromogenic substrates on the agar plates such as ABTS, Tannic Acid, Azure B and Guaiacol. It was found the isolated fungi has excellent ability to produce laccase. The conclusion of this study confirm here that using ABTS is very useful to isolate laccase producing fungus. The microscopic visualization showed the characteristics of white-rot fungi. Further molecular identification still ongoing to check the complete identity of our fungi.

Keywords: Laccase, biotransformation, ABTS, Guaiacol, fungi.

Introduction

Organisms that are capable to degrade wood in the ecosystem are not so many, and most of them are from the white and brown rot fungi, White-rot fungi are the most interested for biological pulping and bleaching, *Phlebia radiata, Pleurotus ostreatus* and *Trametes versicolor* are all from White-rot fungi that produces a lignolytic enzymes White-rot fungi known to be have a complex enzymatic systems that help them in lignin degrading, laccase enzyme (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) is an active and interesting member of these systems. Laccases are multi-copper enzymes belong to the blue oxidases group. They known as oxidoreductases, which oxidizes diphenol and allied substances[1-3]. Laccases catalyze a hydrogen atom removal from the hydroxyl group of ortho- and para- substituted mono and polyphenolic substrates and from aromatic amines by one-electron abstraction to form free radicals capable of undergoing further depolymerization, repolymerization, demethylation or quinone formation[4]. Laccase has potential for industrial and biotechonlogical applications, since it has the ability to degrade phenolic and nonphenolic lignin structures and the ability to make some modification on the plant phytochemical which might produces potential pharmaceutical products. At this moment laccases have of great interest in synthetic chemistry as well as green chemistry in the future[5,6], laccases also have been suggested to be used for oxidative and production of complex polymers and pharmaceutical products through processes like biological transformation and biotechnological modifications of interested photochemical. Transformation Asiaticoside to produce oxy-asiaticosides -a potential anti-tuberculosis compound- is an interesting example of using laccases to produce medical agents. Laccases are extracellular glyco-proteins, which makes its removal from fungal biomass easy and fast[2,7,8]. The use of Laccase catalysis in different applications such as textile dye bleaching, pulp bleaching, bioremediation and biotransformation might be an environmentally friendly way that can replace the used chemical processes[9,10]. The aim of present study was to isolate lignolytic fungi with high laccase activity from trees barks in Malaysia to use it in the microbial transformation of phytochemicals extracted from local plants in order to produce an interesting vital compounds.

Material and Methods

Sample collection: Different samples were collected in sterile tube from the trees barks in the neighborhood of Kulliyyah (faculty) of Science, International Islamic University Malaysia, Kuantan campus. Sample collection was done during the torrential rain season in Malaysia as it was easy to see the growing fungi on the tree bark.

Media conditions and screening: Potato dextrose agar (PDA) medium supplemented with 0.01% W/V peptone, 0.001% W/V yeast extract and 5 ml of 20% W/V aqueous glucose solution was added a septically to each 500 ml of growth media, and Sabouraud-4 % - dextrose agar (SDA) media were used to cultivate fungi, chloramphenicol (0.01 %) was added to the SDA after autoclaving in order to inhibit the growth of uninterested organisms. 0.1 % W/V ABTS (2,2-Azinobis-3-ethylbenzthiazoline-6-sulphonate) have been used as a lignolytic indicator compound. Serial dilutions were made and the plates were incubated at 30°C in a dark place, the positive result to us was the observing of the fungal growth with a carpet like shape single colony produced pale green color zone to dark purple color zone. The color producing fungal colonies were purified by serial sub-culturing from the carpet edges until we got the

most unadulterated and most rapid growing strain and then four more indicator compounds were used to authenticate the production of laccase enzyme. Media that has been used in this experiment desingd according to Pointing, 1999 methodology with some modifications[6,11-14].

Preparation of indicator compounds used to validate laccase production: Special indicators have been used on the solid media with the purpose of proving the laccase production with the isolated fungi, at this point Pointing 1999 method with some adaptation have been used. 0.1% W/V ABTS, 0.01 W/V Azure B, 0.25% W/V lignin and 0.01% W/V Guaiacol have been added to the medium before autoclaving, where, 5 ml of separately sterilized 1% W/V aqueous tannic acid solution has been added to each 500 ml media after autoclaving. Taking into consideration that all experiments have been done under aseptic conditions and every indicator compound has been used alone in each media[6,11-14].

Pointers in the direction of laccase discharge: Positive results have been experiential through the different variations which came into view on the diverse solid medium. ABTS colorless agar media should curved into a colored surface started with pale green color up to dark purple which indicated a positive result to us, while Azure B blue agar media should give negative result to confirm the presence of laccase activity, thus fungi should not remove the stain of Azure B on the media, additionally, the interested enzyme activity can also be proved by the changes on colorless tannic acid agar media which produces brown colored zone around the colony as a result of laccase activity, however, the using of guaiacol, tannic acid reagent is good to prove that there are alignolytic enzyme activities but as brown color can make some doubt about the results, there should be some care during the results interpreting[11] (table1) go over the main points the findings and the results of this study.

Table-1
The results of the fungi reaction with the indicators

Indicator name	Result	Zone color
ABTS	+	Ranged from bright green to dark purple
Tannic Acid	+	Brown
Guaiacol	+	Brown
Azure B	_	No change in the agar color

Laccase presense test using well test method: Potato dextrose agar (PDA) medium supplemented with 0.01% W/V peptone, 0.001% W/V yeast extract and 5 ml of 20% W/V aqueous glucose solution was added a septically to each 500 ml of growth media. then the isolated fungi was inoculated and incubated for 5 days at 30^{o}C, after that well test were carried out by cutting the wells in the agar growth medium with more or less 5 mm in diameter, followed by few drops of 0.1% W/V ABTS solution (in 95% ethanol). Few drops of 0.1 W/V ABTS solution (in 95% ethanol) in addition to a few drops of 0.5% W/V aqueous H_2O_2 to a well in order to test the peroxidase activity. along with, only 95% ethanol added to a test well as a control. Then laid for 30 minutes under investigating the stain intensity and the rapidity of it appearance[11].

Morphological recognition of the isolated fungi: The classical method of identifying fungi is by using light microscopy. the fungi with their telomorphic stages were identified base on the morphology of their spores (conidial ontogeny) and fruiting bodies. Petri dish which has the fungi was incubated for further time to follow up the growth of the fruit bodies and then the fungal culture visualized under the light microscope.

Results and Discussion

The current study was mainly aimed to isolate an active fungi with preferred laccase actions from the local environment of Malaysia, this fungi proposed to be used for the microbial transformation of an interested phytochemicals from the tropical plants in the country, such as asiaticoside which reported to have a promising bioactivities and pharmaceutical uses. In this study dyes and colored indicators have been used as it makes possible visual recognition of lignolytic enzymes actions, this made a straightforward way of enzymes screening as no measurement is necessary[15]. The use of ABTS as substrate for laccase provided for the rapid visual expression of the enzyme positives, however, peroxidase enzymes can also oxidize the ABTS in the presence of H_2O_2 which might be produced endogenously, as a result it was necessary to use other laccase action markers. The isolation method from tree bark sample on our media with ABTS produced one attracted active fungi. The growth of fungi could be seen clearly by three days and the development of the snowy white mate was very fast after fourth day. Pure culture of the isolated fungi was established and kept for the molecular identification and biotransformation experiments (table1) and (figure 1) shows the findings of our study.

In solid media of PDA supplemented with ABTS substrate, the organism formed pale green up to dark purple zone which due to the oxidation activity of the enzyme. Tannic acid agar also called (Bavendamm test) was modified to offer an indication of overall polyphenoloxidase and lignolytic activity. the outcome of the enzyme existence is the assembly of a brown oxidation zone around the colonies. However, tannic acid cannot be used alone to confirm that the produced enzyme is laccase, and should be used with some doubt since it is not specific to any one of the lignolytic enzymes (LE), besides, the production of a brown oxidation zone might be similar to many naturally produced fungal pigments. Azure-B agar was associated with the production of (LE) peroxidase, even though, it is not a substrate for laccase. Accordingly the negative result of decolonization proves that the positive enzyme on the ABTS media is laccase enzyme. Guaiacol is another indicator which reported to be used for laccase production. Reddish brown color due to the oxidative polymerization of guaiacol in the presence of extracellular fungal laccase started to appear straight away

after about five minutes of the loading the agar disk into the guaiacol agar plate (figure 1). The well test results showed a very good result as the green color start to become visible without delay after dipping the 0.1% W/V ABTS in the well and after 30 minutes the color became light green then went to the dark purple (figure 2).

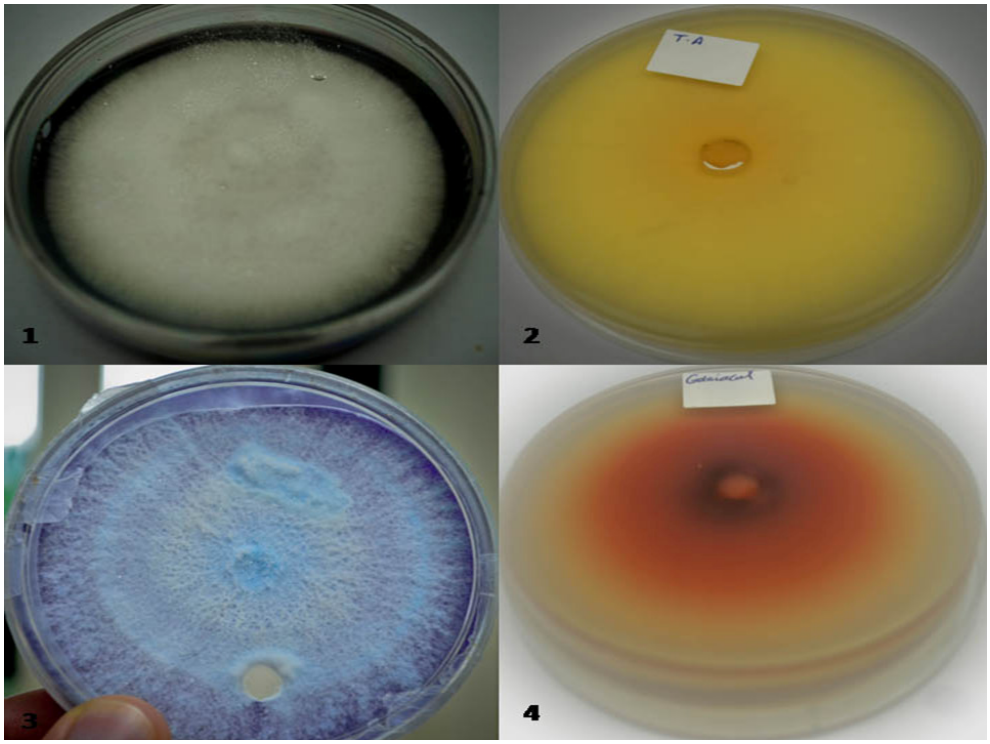

Figure-1
ABTS agar plate after the agar full color change [1], Tannic Acid agar plate [2], Azure B agar plate [3], Guaiacol agar plate [4]

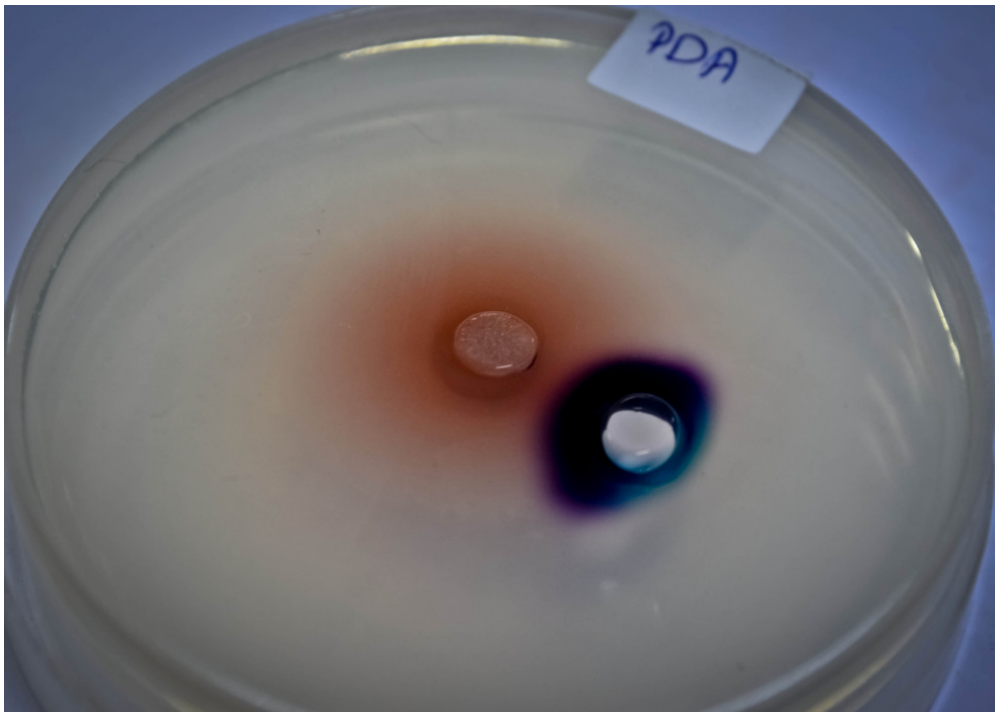

Figure-2
The well test result, it shows the gradual color change from green to dark purple

It is efficient to state that the results of this study were in step the results of[6,11-14] and there was no conflict with the others' results, nevertheless, we here report that the best test for laccase producing fungi isolation is ABTS test followed by Azure B test to confirm that the isolated fungi producing the laccase not peroxidase producing fungi, taking into consideration that only one of them might not be effective to make decision, more over we can say that guaiacol can be used to confirm that the isolated fungi is laccase producer but with some doubt in interpreting the results.

The selected strain was identified Under the microscope, the cross-section of the fruiting body slice showed clear, bright colored tubular channels. In the channels, there were spores evenly distributed. This is a characteristic feature of white rot fungi[12]. More molecular identification will be done in this ongoing research.

Conclusion

The present research work aimed at isolation of laccase producing fungi to be used in the biological transformation of the major bio-compounds isolated especially from *Centella asiatica* which is grown in the local environment of Malaysia and which traditionally used as an effective herbal remedy such as wound healing, and anti microbe, in addition to this plant was reported to has the ability to fight tuberculosis, tumor cells and Alzheimer. The study investigated the possibility to isolate a white rot fungi from natural sources too. During this study, different cultivation techniques have been also done in order to facilitate efficient screening. ABTS substrate showed a very strong ability to facilitate the growth and the isolation of the interested fungi with the laccase activity, and the microscopic identification also show the characteristics of the white rot fungi. The isolated fungi showed that it has a promised activity to be used for the main purpose of our ongoing research. Molecular identification also is still ongoing to determine the exact fungi identity. To sum up, laccase producing fungus have many biotechnological applications and the isolated fungi might be used for further research projects.

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