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The degradation of carbazole and the production of ligninolytic enzyme by isolated marine fungi

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ABSTRACT

Biodegradation of carbazole heterocyclic hydrocarbon by isolated marine fungi were tested. Out of the 64 fungal isolates tested, 5 fungi were able to decolorize more than 50% of 0.01% Remazol Brilliant Blue R. Isolate B3 were able to decolorize 99% of RBBR in all concentrations tested. Isolate B3 and B4 showed the highest removal of carbazole at 88% and 53%, respectively as observed with GCMS in the degradation trial. Laccase enzyme was produced in high concentration of 528.00±11.33 U/L and 642.67±11.43 U/L for isolate B3 and B4. It is observed that the presence of carbazole triggered the production of laccase as it was produced only at 106.67±3.33 U/L and 14.00 U/L for isolate B3 and B4 without carbazole. Results suggested that isolate B3 belonged to the Basidiomycota. The prospects of carbazole biodegradation by these isolates are suspected to be contributed through the production of laccase (Lac).

Keywords: heterocyclic hydrocarbon, fungi, carbazole.

INTRODUCTION

The oil industry deals with the global processes of exploration, production, transportation, refining and marketing of natural hydrocarbons (crude oil and natural gas). Hydrocarbons, besides being the basic raw materials for the chemical industry represents the largest source of energy on the planet. Release of hydrocarbons into the environment whether accidentally or due to human activities is a main cause of water and soil pollution. Polycyclic aromatic hydrocarbons (PAHs) and heterocyclic aromatic compounds (HACs) are two major group of chemical pollutant present in the environment. Activation of electrophilic metabolites are required for PAH and HAC to exert their mutagenic or carcinogenic effects. Polycyclic aromatic compound and heterocyclic aromatic compound can be easily present in the petroleum compounds. Polycyclic aromatic hydrocarbons (PAHs) are composed of two or more condensed aromatic rings of carbon and hydrogen atoms; the rings are linked together in linear and angular arrangements. While, heterocyclic have a cyclic structure of five- or six-membered rings containing at least one or more heteroatom of sulfur (S), nitrogen (N), and oxygen (O). These heterocyclic compounds co-occur in PAHs mixtures and can constitute 1 to 10% of the total PAH concentration in contaminated sediments, and tend to make up higher percentages in mixtures derived from creosote or tar. However, the focus of research has been more into the degradation of global pollution by polycyclic aromatic hydrocarbon (PAH) such as naphthalene and phenanthrene. Although many heterocyclic hydrocarbon compounds especially sulfur, nitrogen and oxygen heterocycles have been found widely in seawater at sites contaminated with petroleum, little awareness has been taken to degrade heterocyclic hydrocarbon compounds.

Hydrocarbons in the environment are biodegraded primarily by bacteria, yeast, and fungi. The reported efficiency of biodegradation ranged from 6% to 82% for soil fungi, 0.13% to 50% for soil bacteria, and 0.003% to 100% for marine bacteria. Over the last decade, marine-derived fungi have become a great interest in mycoremediation. Fungi cometabolize PAHs to a wide variety of oxidized products and in some cases to CO_2. There are two identified and widely reported mechanisms of fungal PAHs metabolism, one involving cytochrome p450 system and another one is using extracellular enzymes. The ligninolytic fungus produces soluble extracellular enzymes that directly attack the CAR and other PAHs whereas bacterial CAR degrading enzymes are intracellular in nature. These extracellular ligninolytic enzymes, includes lignin peroxidases (LiP), manganese peroxidases (MnP), and laccases (Lac). Moreover, ligninolytic fungi can also incorporate hydroxyl group in the aromatic ring of heterocycles. These enzymes may oxidize PAHs to form transient PAH diphenols. Hence lead to the detoxification. However, as much as the pathway of bacterial CAR degradation has been well studied, data regarding similar activities in fungi is limited till date. No data has ever recorded in the pathways of fungi in the degradation on carbazole heterocycles. In fact, these organisms can grow in stressful habitats, characterized by high salinity and pH, low water activity, high concentration of sodium ions and high pressure. In response to these stimuli they produce a wide range of different and structurally complex products. Hence, the objective of the present study is to isolate potential fungal isolates that may help in the degradation of carbazole heterocyles. Two isolates with the ability to produce high level of laccase enzyme has been isolated and studied. Degradation trial was done to quantify the loss of carbazole heterocycles and also the production of laccase enzyme by these fungi.

MATERIALS AND METHODOLOGY

Isolation and Screening: Seawater samples were collected at around 0.5-1 meters deep from three different locations in coast of Sarawak. The samples were coded as DS, PP and B which shows samples from Damai Sentral area, Pasir Pandak area and Buntal area, respectively. The seawater were kept into empty screw-capped gallons bottle and brought to the laboratory to be stored in the cold room. Seawater samples were filtered by using 0.22 µm pore size filter membrane (MILIPORE EXPRESS™ PLUS, Milipore, USA) to obtain the fungi. The filter paper was then cut before transferring into a sterile petri dish. Later it was washed with 1 mL filtered seawater in a petri dish.

Isolation was done in 4 different typed of media. Artificial sea water (ONR7a medium). Glucose peptone yeast agar (1g of yeast, 1 g of peptone, 10 g of glucose and 15 g agar in 1 L of filtered marine water), 3% Malt agar (with 30g of malt extract , 5 g of peptone ,15g of agar in 1L filtered marine water) and Glucose Minimal Media Agar (1g of K_2HPO_4, 3 g of $NaNO_3$, 0.01g of $ZnSO_4.7H_2O$, 0.005g of $CuSO_4.5H_20$, 0.0005g of $MgSO_4.7H_20$, 0.01g of $FeSO_4.7H_2O$, 0.5g KCl, and 10g glucose). About 0.1 mL of sample from the petri dish was spread on each agar. The plate with most sufficient and prolific growth is selected. Fungi that are apparently different are chosen based on growth rate and morphology from the plate; and purified on a new agar plate.

RBBR Decolorization in Liquid Medium

Decolorization activity of RBBR was carried out in 50 ml falcon tube with 10ml of glucose minimal media in filtered seawater with an addition of 0.01 % RBBR. Each flask was inoculated with 3 plugs of fungi in 0.3 cm^3 in size. After 14 days of incubation in static condition with room temperature, aliquots of 1.5 ml were taken from each flask were centrifuged at 10,000 rpm, 4ºC for 10 minutes and absorbance of supernatants was measured in UV-spectrophotometer at the corresponding maximum wavelength 595 nm for RBBR. The uninoculated dye free medium was used as blank. The uninoculated dye containing controls were used as reference to correct abiotic color disappearance. This step is repeated to all the isolated fungi samples at 14 days. Different concentrations of RBBR are also used in this study which is 0.03% and 0.05%. The best fungi that show the highest degradation of RBBR is selected as the inoculum to be implemented in subsequent experiments.

Degradation Performance of the Isolated Marine Fungi

The 1 % (w/v) solution of carbazole compounds were prepared each and dissolved in dimethlyformamide (DMF). 1 g of substrate were weighed and kept into 15ml screw-capped falcon tube. 1 ml of dimethlyformamide (DMF) will be pipetted into the falcon tube and the substrate will be vortex to dissolve. For the degradation performance of the cultivation of heterocyclic hydrocarbon utilizing fungi, the selected fungi were first cultivated in a glucose minimal media supplemented with carbazole that was prepared earlier at a final concentration of 2500 ppm which previously diluted with dimethlyformamide (DMF). The reaction was conducted in static condition at room temperature for 14 days.

The heterocyclic hydrocarbon compounds degradation by the isolated marine fungi was analyzed quantitatively by GCMS. Carbazole was extracted with ethyl acetate after acidification to pH 2.0 with 1M HCl. A 5 ml aliquot were sampled from each flasks every third day from day 0 to day 14 of the experiment. The control was the flasks supplemented with 0.1 % (w/v) of heterocyclic hydrocarbon compounds with no fungal culture. GC-MS QP2010 equipped with a capillary column INTERCAP-5 (0.25 mm x 15 m, 0.25 µm film thickness, GL Science, Tokyo, Japan) was used and conducted as described previously.

Ligninolytic Enzyme Assay

Ligninase peroxidase assay was measured by oxidation of veratryl alcohol (VA) to veratryl aldehyde with absorbance increase at 310 nm with extinction coefficient value of 9300 $M^{-1}cm^{-1}$. Manganese peroxidase was measured by oxidation of 2,6-dimethoxyphenol (2,6-DMP) by MnP system to form a quinone dimer with absorbance increase at 469 nm with extinction coefficient value of 6500 $M^{-1}cm^{-1}$. Laccase activity was measured based on the oxidation of 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) at 420 nm with extinction coefficient value of 36 000 $M^{-1}cm^{-1}$.

Fungal Morphology Identification: Morphological observations were made from selected isolate grown on glucose minimal media with filtered sea water at about 24°C under laboratory conditions of diffuse daylight. The macroscopic and microscopic characteristics was observed 5 days after inoculation. Morphology identification of the isolate was done according to the method described by Dubey and Maheswari and confirmed by comparison with cultures of fungi in the molecular genetic laboratory UNIMAS.

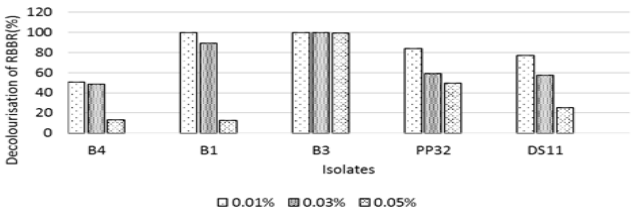

Fig.1.The graph shows the decolorization of 0.01%, 0.03% and 0.05% of RBBR by isolated fungi

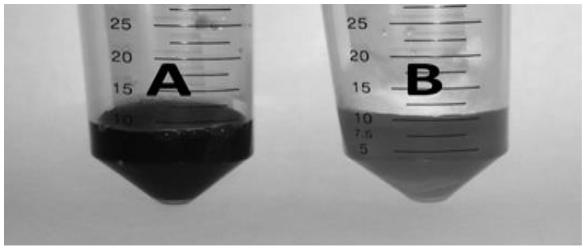

Fig.2.Decolorization of 0.05% rbbr by isolate b3. falcon tube a is the control that were not inoculated with fungi.while b is the decolorization of rbbr after 14 days of incubation.

RESULTS AND DISCUSSION

Isolation and Screening: In the present study totally 64 fungal species were isolated based on its growth rate and morphology from 3 different areas of Damai Sentral area, Pasir Pandak area and Buntal area, respectively. These isolated fungi were then subjected to screening of ligninolytic producer using RBBR decolorization technique.

RBBR DecolorizationThe decolorization of Remazol Brilliant Blue R (RBBR), by isolated marine fungi and the relationship of their ability to produce ligninolytic activity were analyzed. Out of the 64 fungal isolates tested, only 5 fungi were able to decolorize up to more than 50% of 0.01% concentration of RBBR. They were identified as fungal isolates of B1, B3, B4, PP32 and DS11. Isolate B3 were able to degrade 99% of RBBR in all 3 RBBR concentration (0.01, 0.03 and 0.05%).

RBBR has been widely used as a model compound in degradation studies. It is the raw material in the production of polymeric dyes and, as a derivative of anthracene, represents an important member of toxic and recalcitrant organo-pollutants. The identity of enzymes involved in the degradation of RBBR is still not completely established, with evidence indicating the involvement of lignin peroxidase and laccases. Decolorizing ability was strongly correlated to ligninase production according to Sundman test. These 5 isolates were chosen for the degradation trial of carbazole and ligninolytic enzymatic assay.

Fig. 1 shows the decolorization of RBBR by all the selected isolate of fungi. The decolorization has been done in 3 concentrations namely 0.01%, 0.03% and 0.05%. Isolate B3 shows the highest degradation of RBBR in all 3 concentration, which is 100% in 0.01% RBBR, 99.824% in 0.03% RBBR and 99.155% in 0.05% RBBR. Isolate B1 shows the second highest decolorization.

Fig 2. shows the decolorization that happens after 2 weeks of incubation of isolate B3 in 10 mL glucose minimal media amended together with 0.05% RBBR concentration. The blue colour of dye are decolorize to the original colour of the medium.

Degradation of Carbazole: Heterocyclic aromatic hydrocarbon compounds are relatively recalcitrant to microbial attack, though they are known to be oxidized by bacteria and a considerable body of experimental data has accumulated during the past 20 years. In these study, out of 5 isolates that has showed the ability to decolorize RBBR, only 2 fungi has shown the ability to oxidize carbazole as quantified using GCMS. Fig. 3 shows the chromatography graph obtained after 2 weeks of incubation. Isolate B3 (B) showed 88% oxidation of carbazole, and isolate B4 (C) has showed 53% oxidation of carbazole. The carbazole was efficiently oxidized after 2 weeks of incubation, but oxidized products remained undetectable.

Bressler has reported fungal oxidation of carbazole and its alkylated derivative (N-ethylcarbazole) by purified extracellular ligninolytic enzyme, laccase, produced by white-rot fungus Coriolopsis gallica. Carbazole and N-ethylcarbazole was observed to be completely oxidized in 1 hour when 5 laccase units were used. Unfortunately, no oxidized products were detected after the completion of the experiment Apart from that, complete and efficient oxidation will only happen in the presents of accelerators. No oxidation of carbazole was detected even after a week of incubation in the absence of accelerators.

However in this study no activators were needed in the removal and oxidation of carbazole thus indicating that the isolate were able to oxidized carbazole without the presents of any accelerators but still the product remains undetectable. On no occasion were precipitates observed in our reaction mixtures. Since laccase oxidation employs a radical oxidation mechanism, the product of carbazole oxidation may become sufficiently condensed and polymerized so as to be undetectable by the methods we used.

Diverse fungi including filamentous fungi, white-rot fungi, basidiomycetes and deuteromycetes have been reported to degrade or detoxify various PAHs. Two known and extensively reported mechanisms of fungal PAHs metabolism, one involving cytochrome p450 system and other utilizing some soluble extracellular enzymes, like lignin peroxidase (LiP), manganese peroxidases (MnP) and laccases (Lac). Owing to nonspecific characteristics, these extracellular enzymes can oxidize a wide range of organic pollutants thus, enzyme assay were conducted to the selected isolates.

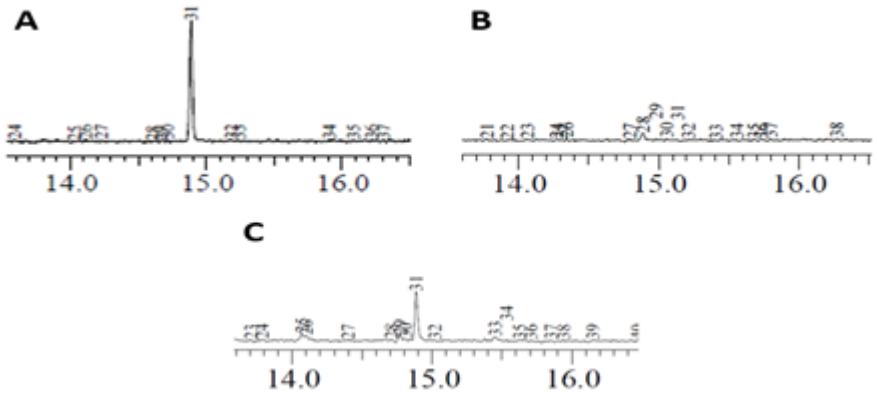

Fig.3.A) Control. Carbazole was detected at peak 31 with a total of 17.81%. B) Isolate B3 were able to degrade carbazole up to 88% as shown at peak number 28 C). Isolate B4 were able to remove up to 53% of carbazole with peak of carbazole at peak number 31.

Ligninolytic Enzyme Assay: White rot fungi have been studied for their ability to initiate degradation of recalcitrant organo-pollutants such as polycyclic aromatic hydrocarbons (PAHs). These fungi degrade lignin by secreting extracellular redox enzymes, which are laccase, manganese peroxidase and lignin peroxidase which play a key role in lignin biodegradation. In this study, isolate B3 shows the highest secretion of laccase enzyme and is gradually increase with time.

Manganese peroxidase and laccase were the major enzyme secreted detected during the incubation time. Laccase enzyme were produced in a very high concentration. It is gradually increased over time. Manganese peroxidase (MnP) enzyme activities dropped at day 15 for isolate B3 and day 12 for isolate B4. The first possibilities are may be due to enzyme inhibiting substance in the EFB extracts was supposed to prevent or decreases assays of MnP activity. The second possibilities in difficulties in the detection of MnP can also be due to the colored aromatic compounds that derived from solid substrates. Lignin peroxidase were not detected during the entire degradation trial. Manganese peroxidase and laccase are known to be able to oxidize and catalyze ring cleavage of aromatic compound. The study of the laccase-mediator system in the bioremediation of polycyclic aromatic hydrocarbons (PAHs) has been extensively reported. Laccase is an enzyme that catalyzes the oxidation of a wide number of phenolic compounds and aromatic amines but its substrate range have been extended to non-phenolic compounds in the presence of low molecular mass compounds acting as mediators. Laccases from white rot fungi have been also used to oxidize alkenes, carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene in the presence of HBT and ABTS as mediators.

Fig.4 shows the enzyme activity of laccase for isolate B3, B4, control B3 and control B4. Control B3 and control B4 were the inoculated tubes without the addition of carbazole as substrate. Laccase was the dominant enzyme with the production of 528.00±11.33 U/L and 642.67±11.43 U/L enzyme produced for isolate B3 and isolate B4 at day 15. The enzymes were gradually increased and starting to get static at day 12 to 15 for isolate B3. Enzyme assay showed that the control for B3 and control B4 produced laccase at lower amount without the addition of carbazole.

Fig. 5 shows manganese peroxidase enzyme activity for isolate B3, B4, control B3 and control B4. Manganese peroxidase was produced lower than laccase enzyme. The highest recorded enzyme activity was at day 12 with 14.516±2.053 U/L by isolate B3. It is still observed that manganese peroxidase enzyme activity were lower in control without the addition of carbazole thus indicating the substrate triggered the production of this enzyme as well.

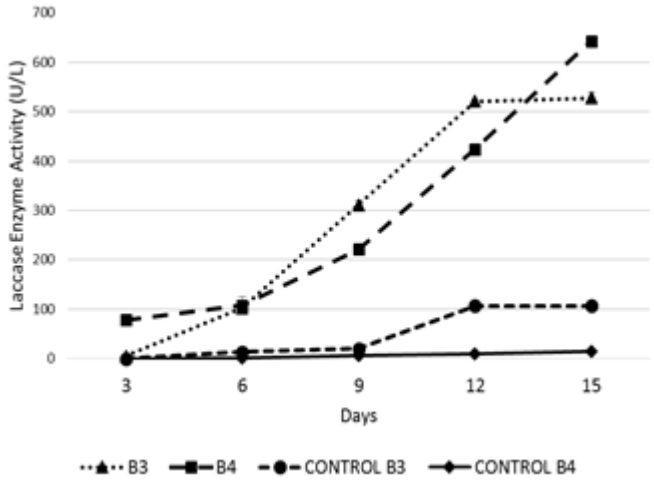

Fig.4.Data (mean ±S.D) showing the laccase enzyme activity of the isolates.

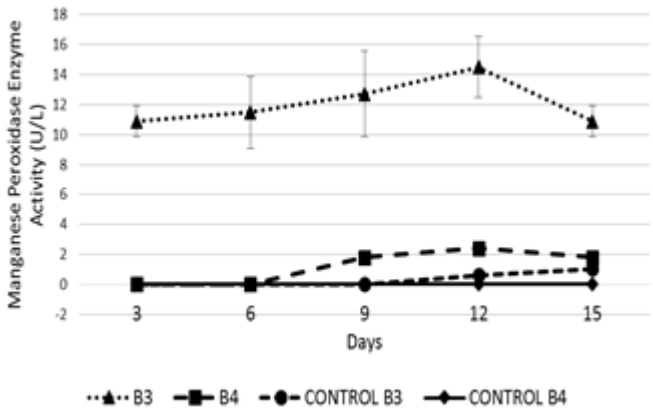

Fig.5.Data (mean ±S.D) showing the manganase peroxidase enzyme activity of the isolates.

Isolate B4 shows highest laccase activity at day 15 compared to isolate B3 with 642.67±11.43 U/L Isolate B3 shows its highest amount of laccase produce at day 15 with 528.00±11.33 U/L. The enzyme increased gradually with time and starting to get static at day 12 to 15 for isolate B3. The highest recorded enzyme activity were at day 12 with 14.516±2.053 U/L by isolate B3 but dropped at day 15. Manganese peroxidase were also detected in isolate B4 with 2.42 U/L at day 12.

From the graph, laccase enzyme produced by isolate B4 were higher compared to isolate B3. However isolate B3 shows the highest amount of carbazole removal. Manganese peroxidase enzyme were produced a bit higher in isolate B3 compared to B4. Thus indicating that isolate B3 were able to efficiently remove and oxidize carbazole with lower laccase enzyme activity.

Fungal Morphology Identification

Isolate B3 was selected for identification based on its ability to decolorize 99% of all concentration of RBBR It also produces the second highest activity of laccase and oxidize 88% of carbazole. Isolate B3 also has the ability to grow in 100% salinity of seawater. Isolate B3 is a fast growing fungi and are able to cover whole plate of PDA, MEA and glucose minimal media with 100% filtered seawater agar in 7 days. The colonies were white and the mycelium developed only at the surface of the media used. The mycelium texture appeared smooth and did not exude pigments or droplets. Clamp connections were presents and is short, large and slightly curved. The presence of clamp connections suggested the isolate to be Basidiomycota fungi. No spore like formation were observed and thus further morphology identification is restricted.

CONCLUSION

The results from this study showed the ability of fungal crude enzyme which are high in activity of laccase to oxidize carbazole. These preliminary study is crucial in determining which fungi are able to oxidize carbazole which are a heterocycle PAH. The heterocycle PAH carbazole biodegradation potential of the isolate B3 positively correlated with their potential to express laccase (Lac) enzyme thus suggesting its involvement in the process. The isolate is subjected for molecular identification. The data regarding fungi in the degradation of heterocycles PAH are scarce. This is the first report of the oxidation of carbazole by a crude fungal laccase and also first without any accelerators added.

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