PRELIMINARY STUDYONENZYMATIC DEGRADATION OF MODEL OF PHARMACEUTICAL WASTE BY LACCASE FROM TRAMETES SP

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Review Article

PRELIMINARY STUDY ON ENZYMATIC DEGRADATION OF MODEL OF PHARMACEUTICAL WASTE BY LACCASE FROM TRAMETES SP

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Abstract: Chemical synthesis processes of the pharmaceuticals industry produce wastewaters which are variable in character and highly strong. Disposal of waste materials such as unused drugs, pharmaceutical production facilities, hospital wastes are the origin of the pharmaceutical pollution. Laccase is a copper-containing phenol oxidase, which can oxidize electron-rich substrates of phenolic and non-phenolic origin with a concomitant reduction of oxygen to water through a radical-catalyzed reaction mechanism. Laccases are present in higher plants, fungi, bacteria and insects,and the most studied group of enzymes to date is from fungal origin, also the functions of laccases are diverse. The chemical model chosen for the study were diclofenac and ibuprofen, as it is one of the drugs that appears with the most frequency in the analyses of waste waters due to its high consumption as an anti-inflammatory and analgesic. A model sample for representative as common active materials was experimentally evaluated to assess the simple biological degradation, using crude laccase from *Trametes* species. Optimal results were achieved as the crude enzyme was precipitated with 60% acetone method ; the specific activity was found to be 0.67 (unit/mg), and showed can be exploited for handling the reduce of hazardous pharmaceutical waste materials.

Keywords: Enzymatic Degradation, Pharmaceutical Waste, Laccase, Trametes Sp.

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Introduction

Laccase enzyme (E.C. 1.0.10.3.2) is produced by various fungi and higher plants [1]. White decay fungus is known capable of degrading lignin by producing laccase enzyme [2]. This enzyme is first found in the sap of lacquer tree growing in Japan, i.e. *Rhus vernicira*, in 1883, and is then known distributed in plants and fungi. This enzyme has the structure of benzendiol, oxygen reductase and partly extracellular glycoprotein containing copper atom and its enzyme cluster is called blue copper oxidase. Laccase is a monomeric, dimeric, tetrameric enzyme with molecule weight of 60 – 80 kDa [3].

This enzyme is easily found on fungi in our surrounding and take no time to grow on dead/fallen tree trunk and are known as decay fungi of *trametes* species. In addition, laccase enzyme is also found on is consumable and non-toxic white oyster mushroom. This mushroom belongs to *pleurotus* species [4] [5].

Research Methods

This research is intended to be a preliminary study of the potential of white oyster mushroom locally growing in Jakarta to be used as an environmentally friendly catalyst in the reduction of waste content of drug materials containing ibuprofen and diclofenac with laccase enzyme simple extraction and analysis on the biodegradation of drug materials in waste by using UV-Visible spectrophotometer.

White oyster mushroom, ammonium sulfate, ethanol, ethyl acetate, BSA, Lowry reagent, acetate buffer. Diclofenac sodium, ibuprofen. Aquades. Tool: UV-Vis. FT-IR Spectrophotometer and GCMS.

Laccase Extraction.

Daily analysis on enzyme activity starts with enzyme extraction from 250 grams of white oyster mushroom, using 500 mL acetate buffer solution at pH 4.6 on *shaker* at speed of 100 rpm for 1 hour. The solution and substrate are decanted from the flask into centrifuge tube and chilled and centrifuged for 15 minutes at 2000 rpm.

Analysis on Laccase Activity.

Enzyme activity is measured by inserting 50 µL supernatant model into 150 µL, 0.5 mM guaiacol solution. The dimer absorbance is observed at wavelength of 500 nm using UV-Visible spectrophotometer. Any change in the dimer absorbance of guaiacol product is observed every minute for 10 minutes. One activity unit is defined as the amount of enzyme which may accommodate 1 µmol guaiacol every minute at 28°C.

Analysis on Protein Concentration

The protein is tested using Lowry solution. 100 µL supernatant (centrifuged liquid sample) is mixed with 2 mL Bradford solution in cuvette. This mixture is then homogenized using *vortexmixer* for 10 seconds and left for 10 minutes before measurement using spectrophotometry at wavelength of 595 nm.

Biodegradation reaction of pharmaceutical waste solution model

Water-ethanol based solution is prepared (1:1) respectively 100 mL, containing 10,000 ppm ibuprofen or diclofenac sodium compound. Isolate laccase enzyme 10% solution is then prepared in 20 mL water, mixed respectively with 50 mL ibuprofen or diclofenac sodium compound for 30 minutes, and added with 2500 ppm beta-naphthol as mediator. The mixture is inserted into separating funnel, added with 15 mL ethyl acetate, shaken, added with sodium sulfate dryer, and then filtered. The filtrate of each sample is evaporated, and then analyzed using KLT with hexane raising solvent: ethylacetate (2:1).

Results and Discussion

Laccase Rough Enzyme Extraction

Laccase enzyme extraction from trametes fungus starts with cutting of relatively hard mushroom body to small pieces, smashed using blender, added with aquades, and then filtered using rough cloth to withhold big sized flakes [6] [7].

Figure 1.*Trametes sp.* sample

The filtrate produced is added with cool acetone and then left for deposition and the deposition result is separated from the filtrate. The filtrate is then added with ammonium sulfate 70% to obtain second phase deposition and measured for protein content using spectrophotometer and for enzyme rough activity [8].

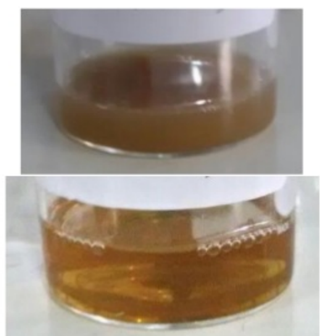

Figure 2. Acetone extract of laccase rough enzyme

Laccase activity is examined by measuring guaiacol change pursuant to the method. The absorbance change in rated lambda-maximum is 460 nm, in which guaiacol product dimer as the result of specific activity is observed once a minute for 10 minutes. One unit of activity is defined as the amount of enzyme which may oxidize 1 μmol guaiacol every minute at 28°C. Partial purification is conducted in two stages, comprising deposition with cold acetone and followed with addition of ammonium sulfate 70%. According to the calculation using its protein content, the specific activity of laccase enzyme is found 0.67 (unit/mg) [9].

Diclofenac and Ibuprofen Modification

This chain reaction event is known with a coupling reaction. To return the enzyme's functions, this coupling reaction is equipped with additional reaction system, in which some compound is available, ready reduce the laccase enzyme [10].

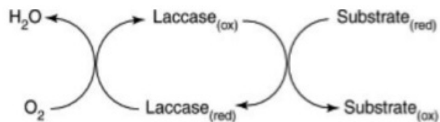

Figure 3. Laccase enzyme reaction mechanism

The research results show that the compound group called substrate in the figure above is compound with phenolic based structure, and it is known that phenolic has good reducing character since it has hydroxyl group bound to aromatic. An example of this substrate is benzendiol [11].

Na-diclofenac Ibuprofen

Figure 4. Na-diclofenac and ibuprofen

A substrate oxidation is, in this case diclofenac or ibuptofen, expected to occur with existing reaction which involves oxygen, substrate and enzyme. This oxidation result is known with biomodification [12].

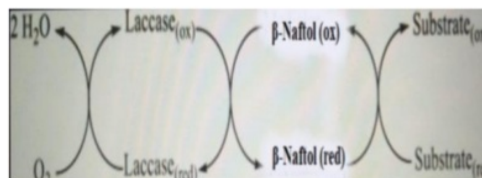

Figure 5. Utilization of intermediate compound beta-naphthol in biomodification reaction

Beta-naphthol may directly to reduce the laccase enzyme, the enzyme may be active continuously, and the expected final result is that the substrate (diclofenac or ibuprofen) will be more effectively oxidized.The biomodification product is shown in the liquid top phase dissolved in the added ethyl acetate solvent [13].

Figure 6. Extraction of diclofenac biomodification product

Analysis with thin layer chromatography as given, it is known that the 5x2 hours of reaction process is concluded to remove diclofenac in real, as marked with no existence of dot, which is expected to have transformed to other compound.

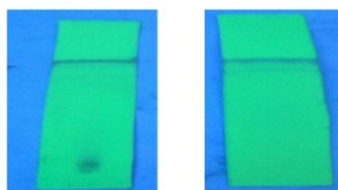

Figure 7. Analysis on KLT diclofenac and biomodification product

Sample model, ibuprofen, as given, there is a change to the dot after experiencing reaction condition for 8x 2 hours. According to the chromatographic data, we may observe that other product has very different polarity with ibuprofen, and some residual ibuprofen is still seen [14] [12].

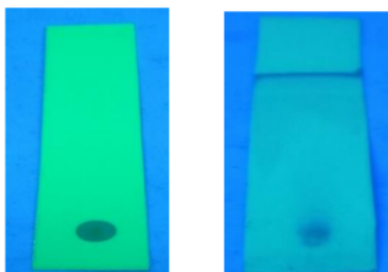

Figure 8. Analysis on KLT ibuprofen and biomodification product

Diclofenac substrate is known to experience biomodification more than ibuprofen. The laccase enzyme from *Trametes sp,* extraction potentially modifies organic compounds, which in this case is modeled as pharmaceutical waste.

Analysis on product modified from Diclofenac and Ibuprofen

An advanced analysis is using the instrument gas chromatography mass spectrometry (GC-MC) with condition as given in the figure product ibuprofen and diclofenac.

Ibuprofen Biomodification

Model II, in figure 9 which is diclofenac, with help of gas chromatography generates the following result:

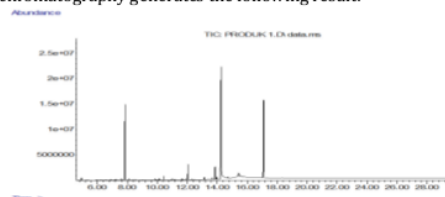

Figure 9. Chromatographic gas of diclofenac reaction product

According to the data of this research which employs the rough enzyme of fungus trametes sp., it shows organic structural modification which may be designed as that in Figure 10. From the compound model of diclofenac, the biomodificaiton pathway is known as follows:

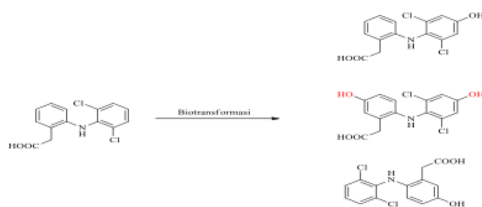

Figure 10. Hydroxylation reaction model of diclofenac biomodification product.

The hydroxylation may also be detected both in dihydroxy and monohydroxy. This enzymatic oxidative reaction is examined with, among others, the main role of cytochrome P450 enzyme. In this research, the GC-MS data shows newly identified product with selection of main reaction product with retention time of 17 minutes, with fragmentation data as given in the figure below, with detection of value m/z 279 and with fragmentation result as follows, shown in figure.11.

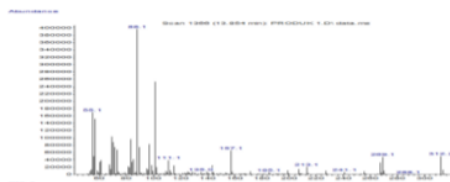

Figure 11. Spectrometric mass of diclofenac reaction product.

This result is in accordance with the possibility that trametes laccase enzymatic reaction which may modify the aromatic structure of diclofenac compound experiences hydroxylation, as shown in the figure below.

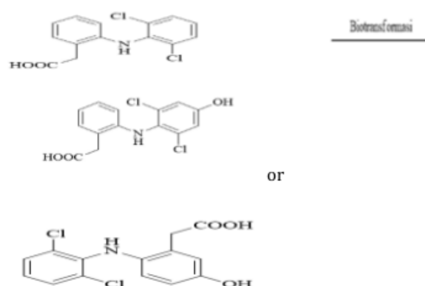

Figure 12. Structure of diclofenac compound experiences hydroxylation.

Thin Layer Chromatography Conditions :Stationary Phase : Silica-gel Plate 20 x 20 cm F234 , Mobile Phase ; Hexane : Ethyl Acetate 2 : 1, Chamber : Beaker glass volume size 250, equipped with a cover, Saturation condition ; 15 minute left before use.Condition of Instrumentation Analysis GCMS Figure 13. Hydroxylation product of diclofenac compound by the laccase enzyme of trametes sp.

Kromatogram GC-MS : Ibuprofen product

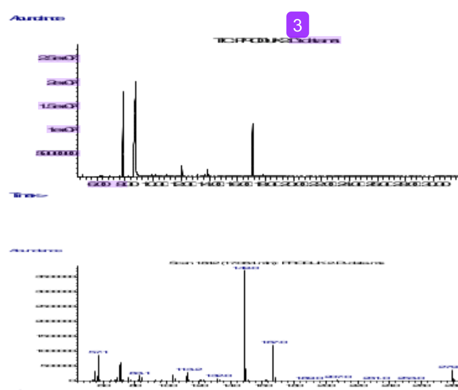

Figure 13. Kromatogram GC-MS : Ibuprofen product.

Diclofenac Product

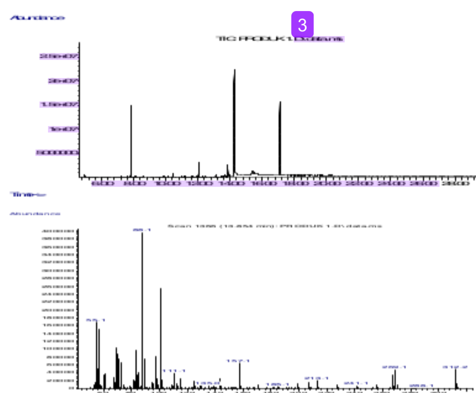

Figure 14. Diclofenac Product.

4. Conclusion

According to the research employing two organic compound models ibuprofen and diclofenac, we may conclude that wood decay fungus (trametes sp.) contains laccase enzyme. Laccase rough extract with 5 hours of reaction contact with the model compounds above is known to be able to serve as biomodification catalyst with hydroxylation reaction ability.Laccase enzyme belongs to the oxidoreductase group, thus with detection of hydroxyl group (-OH) in intermediate product, we may conclude that the model compounds designed in this research experience biotransformation. Based on the foregoing, we may conclude that the laccase rough enzyme of fungus trametes sp. species has the ability to modify organic compound, such as from diclofenac to hidroxy-diclofenac.

Suggestion

It is highly important that next researchers conduct further researches with regard to toxicity characteristics.

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