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Formation of Anethole Dimer Compound Using Biocatalyst Laccase from White Oyster Mushroom

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ABSTRACT

Due to their specificity and environmental friendliness, enzymes are frequently used as biocatalysts for organic compound synthesis. The goal of this research is to synthesize anethole dimer using the laccase enzyme as the biocatalyst. Laccase is a widely used enzyme that belongs to the oxidoreductase group. This study employed anise oil containing 90% anethole, laccase isolated from the white oyster mushroom (Pleurotus ostreatus) with an activity of 712,758 U/L, and hydroquinone as a mediator. The anethole dimerization was conducted in a biphasic medium (ethyl acetate and phosphate buffer in a 4:1 ratio) for 24 and 48 hours. The reaction mixture was extracted with ethyl acetate, yielding a brownish, viscous liquid that exhibited increased color intensity after 48 hours. Gas chromatography analysis of anise oil and the reactions after 24 and 48 hours revealed increased peak intensity and changes in peaks after 48 hours. The percentage area of anethole and p-anisaldehyde was smaller in the 48-hour reaction than in the 24-hour reaction. Identification of the compounds after 48 hours suggested the formation of new compounds resulting from oxidation reactions. Examples of these new compounds include caryophyllene oxide; however, the dimerization of the anethole compound was not conclusively identified.

Keywords: white oyster mushroom; biocatalyst; laccase enzymes; anethole

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1. Introduction

Indonesia is an archipelagic nation known for having the world's second largest tropical forest, rich in biodiversity and recognized as one of the seven megabiodiverse countries alongside Brazil. The distribution of higher plants in Indonesia's tropical forests accounts for more than 12% (30,000 species) of the global total (250,000 species) [–]. These facts underscore Indonesia's role in meeting global demand for palm oil, spices, and essential oils.

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In recent years, Indonesia has seen a significant increase in the export of essential oils since 2005 [4]. Essential oils are commonly used in pharmaceuticals, cosmetics, and perfumes, as well as in the food and beverage industries. Essential oils are secondary metabolites of plants that serve as defensive mechanisms and attractants for pollinators [17].

Among the many essential oil products produced, Indonesia is recognized for producing predominantly anise oil and star anise oil, mainly found in Yogyakarta, Boyolali, and Sumatra [18]. Anise oil is known to contain propenyl phenol compounds, specifically anethole, which comprises 80–90% [32]. Anethole is commonly used as a key ingredient in baby oils and medicines. Moreover, anethole also exhibits antioxidant, anti-inflammatory, and gastroprotective activities [13].

Secondary metabolites in essential oils include terpenoids, phenolics, and aryl propanoids [38]. In nature, aryl propanoid compounds are found in several groups based on their structures, such as lignans, allyl phenols, propenyl phenols, and cinnamates. Lignans, as more complex compounds, can be synthesized from simpler aryl propanoid groups like propenyl phenols [24].

Currently, much research is focused on transforming aryl propanoid compounds into other compounds with higher biological activity. One approach involves synthesizing propenyl phenol dimer compounds. This was undertaken by Arifin (2008), who synthesized dimer compounds from eugenol. The dimer compound of eugenol was subsequently tested for its antioxidant activity. The results indicated that eugenol itself exhibited a lower IC50 value compared to its dimer compound. Therefore, the formation of eugenol dimer compounds could enhance antioxidant activity.

The coupling reaction of propenyl phenol compounds to form dimer compounds can be achieved using acid catalysts such as HCl [26] or through radical formation using UV. However, the latter method has a drawback in that the resulting products are a mixture of more than 5 compounds [6]. The use of catalysts in coupling reactions is costly and environmentally unfriendly, prompting extensive research to discover more environmentally friendly catalysts.

The dimerization reaction of propenyl phenol derivatives can also be conducted using the biocatalyst enzyme oxidoreductase, such as the laccase enzyme [39]. Based on previous research, laccase enzymes have been isolated from wood-decaying fungi [27], mold from empty oil palm fruit bunches (Dewi, 2011), and white oyster mushrooms (Arifin, 2008). Laccase enzyme isolated from white oyster mushrooms exhibits a specific activity of 0.56 units/mg protein (Arifin, 2008). In its catalytic process, laccase enzyme utilizes oxygen and only produces water as a byproduct, making it an environmentally friendly catalyst [28].

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2. Materials and Method

Research materials

The materials used in this research were white oyster mushrooms and anis oil.

Chemical material

The chemicals used in this research are buffers phosphate, acetone, aquadem, hydroquinone, n-hexane, ethyl acetate, ethanol, and ABTS.

Research tools

The research tools used in this study include a blender, an Erlenmeyer flask, a separating funnel, a rotary vacuum evaporator, filter paper, a centrifuge, a gas chromatography-mass spectrometry (GC-MS) instrument, a UV-visible spectrophotometer, a thin-layer chromatography (TLC) plate, a Chromatotron, a micropipette, aluminum foil, coolers, pH meters, thermometers, refrigerators, and glassware commonly found in laboratories.

3. Result

Isolation of laccase enzyme from white oyster mushroom

Laccase enzymes are widely produced by fungi and higher plants. They have been isolated from fungal groups such as Ascomycetes, Deuteromycetes, and Basidiomycetes. In this study, laccase enzyme was isolated from white oyster mushroom (Pleurotus ostreatus), which belongs to the Basidiomycetes group. In white oyster mushrooms, laccase plays a role in lignin degradation and other functions, including pigmentation, fruiting body formation, spore formation, and defense mechanisms [15].

The isolation of laccase enzyme from white oyster mushrooms begins with grinding the mushrooms in a 0.2 M phosphate buffer at pH 6.0. The use of this pH 6.0 phosphate buffer helps maintain the enzyme's pH optimum range, as laccase from white oyster mushrooms functions optimally within this range [25]. The ground mushrooms are then homogenized while maintaining a temperature of 0–5°C. This temperature range is crucial to prevent protein denaturation and to keep the enzyme inactive until it is used in the reaction.

Next, the laccase enzyme extract is separated from cellular debris. Filtration is performed using cotton cloth and centrifugation at 3500 rpm to obtain the supernatant. This supernatant contains crude laccase enzyme, which appears yellowish-brown. To prevent denaturation, the enzyme is stored at temperatures between 0 and 2°C.

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Deposition of crude extract of laccase enzyme

To enhance the enzyme's activity, purification is necessary. There are several methods for enzyme purification, including protein precipitation with ammonium sulfate or organic solvents, as well as chromatography. Chromatography methods include ion exchange, affinity, filtration, and hydrophobic chromatography [16]. In this study, protein precipitation was carried out using the acetone precipitation method.

Partial purification using acetone involves reducing the hydrophobic molecules on the enzyme when acetone is added. Water molecules around the hydrophobic surface of the protein are replaced by acetone molecules, thereby disrupting hydrophobic interactions.

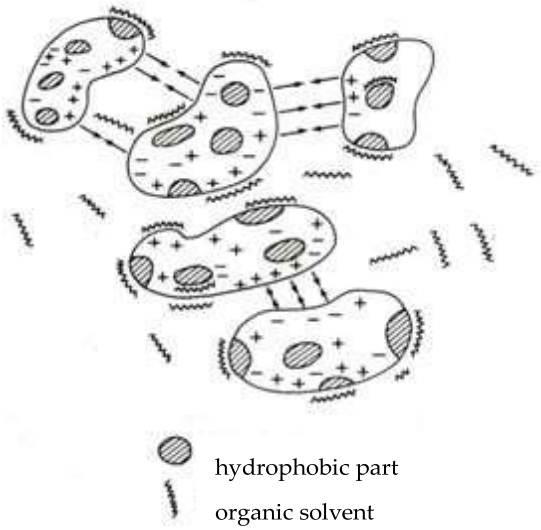

Figure 1. Protein aggregation due to the interaction of opposite charges one protein with another protein

The primary factors causing protein molecule aggregation by acetone are electrostatic bonds and dipolar forces. The presence of acetone in the protein solution leads to aggregation between oppositely charged surfaces of different protein molecules. The precipitation process with organic solvents occurs due to the electrostatic state of the protein. Larger protein molecules aggregate more easily because they have higher charge differences. Larger molecules aggregate quickly due to significant changes in surface charges between different protein molecules.

In the acetone purification process, the temperature must be below -10°C. At higher temperatures, molecules of the organic solvent enter the protein through its surface and interact with hydrophobic residues of the protein. These interactions lead to protein denaturation [16].

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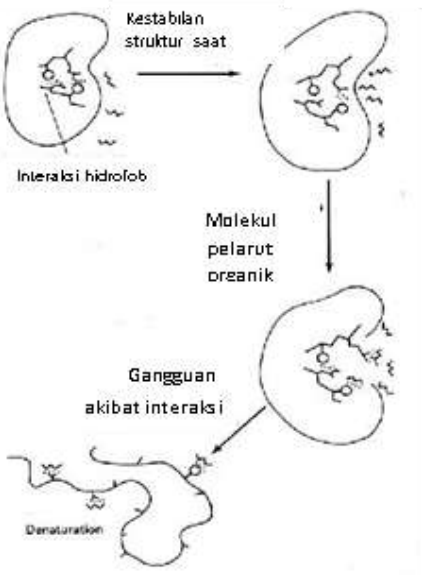

Figure 2. Protein denaturation due to organic solvents

Protein precipitation was carried out by adding acetone to the enzyme extract in a ratio of acetone-to-enzyme extract (3:1). This ratio was determined based on research conducted by Mustafa et al. [25] for the purification of laccase enzyme, where the acetone to enzyme extract ratio was maintained at 3:1. The mixture was then stirred until precipitation occurred. After the precipitation step, a whitish precipitate was obtained, which was subsequently centrifuged to separate the enzyme from the organic solvent and suspended in pH 6 phosphate buffer.

Determination of laccase enzyme activity

In this study, the activity of the laccase enzyme was determined using the method described by Bourbonnais and Paice [31]. The principle of this assay is as follows: A non-phenolic dye, 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS), is oxidized by laccase to form a more stable radical cation (ABTS+). The concentration of the blue-green colored radical cation (measured at a wavelength of 420 nm) correlates with laccase activity [–]. Below is the reaction showing the conversion of ABTS to ABTS radical cation.

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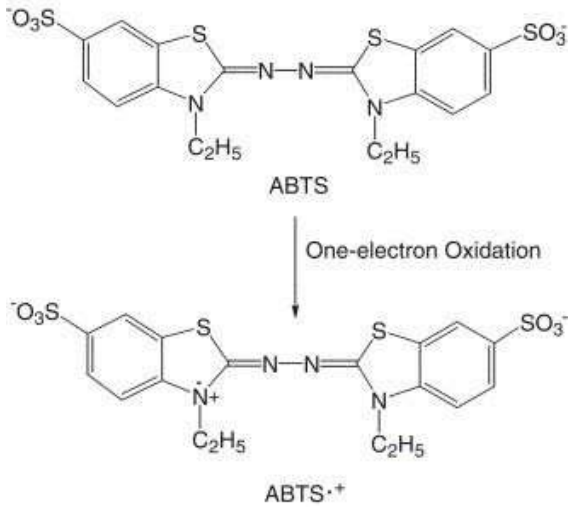

Figure 3. Acid oxidation reaction of 2,2'-Azinobis-di-(3-ethylbenzthiazoline- 6-sulfonate) (ABTS)

The change in absorbance of the radical cation was observed every minute for five minutes. Laccase activity was expressed as international units (IU) per liter, where one IU is defined as the amount of enzyme that can oxidize one micromole of ABTS per minute at room temperature and a pH of six. The graph below illustrates the relationship between ABTS radical cation absorbance and time.

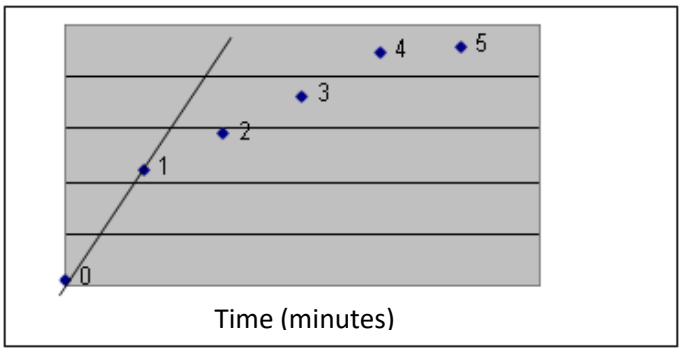

Figure 4. Graph of the relationship between ABTS cation radical absorbance and time

From the graph above, it can be observed that the maximum gradient is obtained from the point 0–1. Based on calculations, the activity of the laccase enzyme obtained is 712.758 U/L. This enzyme activity level falls within the category capable of acting as a biocatalyst in reactions forming organic compound dimers, trimers, or polymers. This assessment is based on comparing values from various enzyme isolations used as biocatalysts in organic reactions. To catalyze the formation of dimer, trimer, and polymer compounds, a minimum

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activity of 68 U/L is required, whereas the maximum activity reported reaches up to 2200 U/L [38].

Anethol dimerization reaction catalyzed by laccase enzyme

The laccase enzyme employed in this study is an oxidoreductase enzyme capable of catalyzing oxidation and reduction reactions. Laccase enzymes catalyze the oxidation of phenolic compounds into radicals, which can subsequently form dimers, oligomers, or polymers.

In the catalytic reaction of phenolic compounds, laccase enzymes require another compound to act as a mediator. This mediator compound is typically a simpler structured phenolic compound compared to the substrate [39]. In this study, the mediator used is hydroquinone. Hydroquinone acts as an electron shuttle, undergoing oxidation by the laccase enzyme. The oxidized mediator then accelerates the oxidation of the substrate.

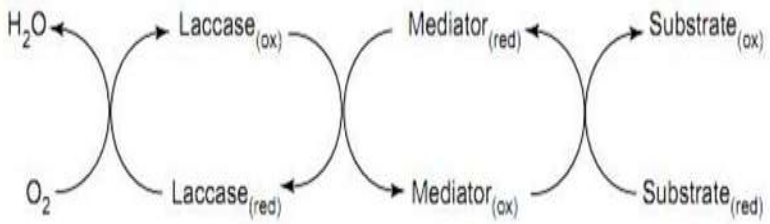

Figure 5. Schematic of the laccase mediator system

According to Johannes [40], hydroquinone can enhance oxidation by up to 13% compared to reactions conducted without a mediator. The small size of hydroquinone allows it to interact more readily with enzymes compared to substrates. This reactive hydroquinone radical can then oxidize phenolic substrates such as anethole into anethole radicals, while O_2 acts as a hydrogen acceptor and is reduced to H_2O.

Laccase enzyme, as a biocatalyst, is a metalloenzyme, meaning it is an enzymatic protein that forms strong bonds between its protein component and a metal embedded within the enzyme molecule. The metal in the enzyme facilitates electron transfer, charge transfer, substrate transfer, oxygenation, and detoxification processes. In holoenzymes, laccase can exist as monomers, dimers, or trimers, each monomer containing four Cu atoms distributed across three redox sites, with each site playing a distinct catalytic role [41].

The presence of Cu metal in laccase facilitates electron transfer from the reduced substrate to oxygen molecules without releasing toxic peroxide intermediates. The enzymatic mechanism involves three main steps: reduction at the mononuclear Cu atom to trinuclear Cu, followed by the reduction of O_2 by trinuclear Cu to form H_2O [42].

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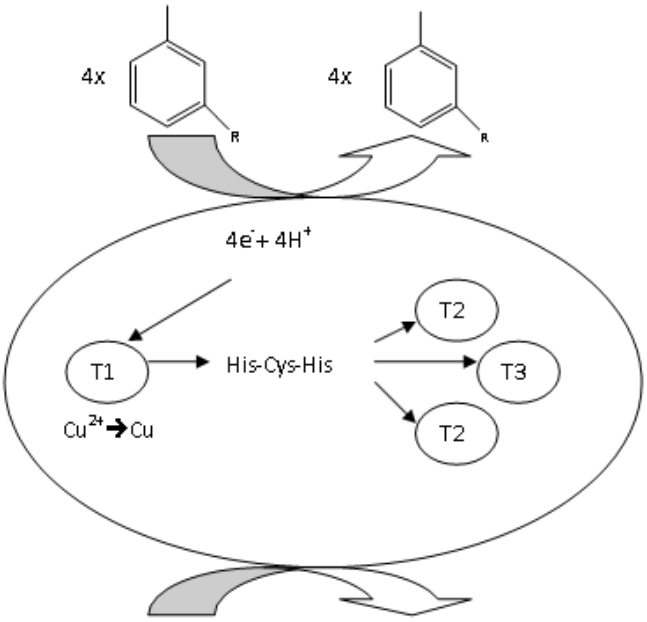

Figure 6. Mechanism of action of Cu on the laccase enzyme [5]

Dimerization of anethole is conducted in a biphasic medium. The use of a biphasic medium enhances product stability and allows for higher extraction efficiency in the organic phase compared to a monophasic system [25]. According to Adelakun et al. [43], a biphasic medium for organic compound synthesis using laccase biocatalysis involves mixing a buffer with an organic solvent. The organic solvents used include acetone, ethyl acetate, ethanol, dioxane, and methanol. Based on their findings, ethyl acetate provides the highest product yield. Therefore, in this study, the biphasic medium used is a mixture of ethyl acetate and phosphate buffer in a ratio of 4:1.

Qualitative testing by comparing reaction compounds in reaction tube II and the control tube I shows observable physical differences. In tube II, which contains a biphasic solution of laccase enzyme, hydroquinone, and anise oil, a darker brown color is observed in the lower layer compared to tube I, which has the same composition but lacks laccase enzyme. This indicates a change has occurred, likely due to the formation of a new compound in tube II.

To isolate the formed products, the reaction was scaled up using 5 mL of anise oil in a biphasic medium (ethyl acetate: phosphate buffer = 4:1). The mixture was stirred for 2 hours and allowed to stand for 24 and 48 hours. Subsequently, extraction was performed using ethyl acetate to separate the organic and aqueous phases. The separation process is based on the polarity differences between the components in the solution.

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The extracted organic phase was then evaporated to remove the solvent, yielding a thick, brownish liquid. The reaction at 48 hours showed a higher concentration intensity compared to the reaction at 24 hours.

Analysis of reaction products

Qualitative observation of the reaction product can be determined by changes in color and aroma. Additionally, identification can be done using thin-layer chromatography (TLC). This method can also indicate the number of compound components in the reaction product. The separation process is based on the differential distribution of each component in a mixture into the mobile and stationary phases.

In this study, the developing solution (mobile phase) used was a mixture of n-hexane and ethyl acetate in various ratios. Generally, the TLC results did not show significant changes in the reaction. This could be due to the small amount of reaction products and the complexity of the mixture, making it difficult to identify the reaction products.

GC-MS analysis

Analysis of anis essential oil using GC-MS produces chromatogram with 5 compound peaks as shown in figure 7 as follows:

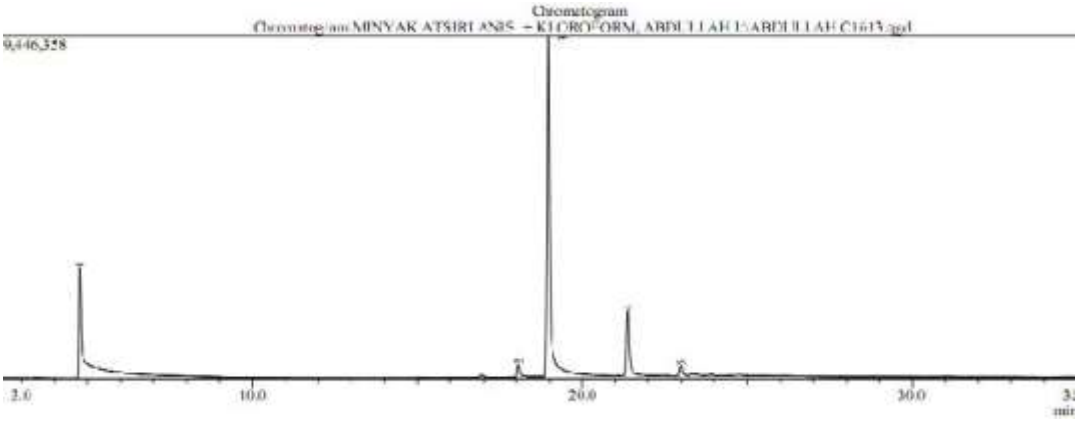

Figure 7. Anis essential oil chromatogram

Based on the chromatogram above, it is noted that the highest peak appears at a retention time of 18.96 minutes with an area percentage of 65.71%. From the MS analysis results, this peak at m/z 148 corresponds to anethole compound. Peak no. 1 corresponds to the solvent, chloroform. Peaks 2 to 4 correspond, respectively, to anethole, p-anisaldehyde, and p-methoxybenzyl methyl ketone. The MS spectrum of anethole is depicted in Figure 7.

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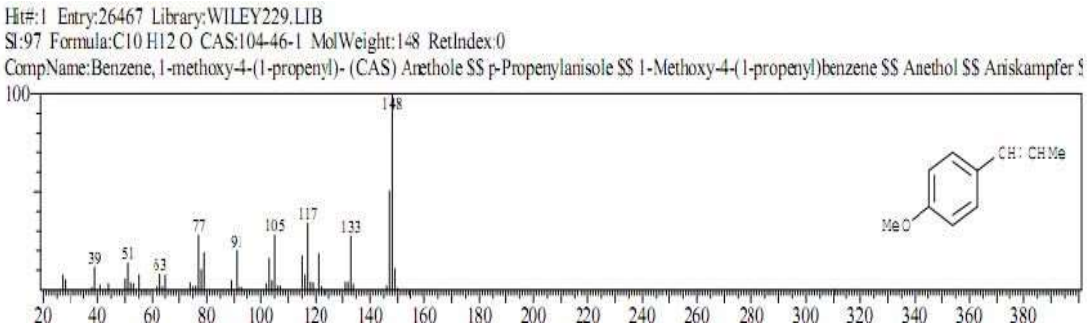

Figure 8. Mass spectrum of the anethol compound

In this study, anise oil was reacted in a biphasic medium using the biocatalyst laccase and hydroquinone as a mediator to form dimeric compounds of anethole. The reaction was conducted for 24 hours and 48 hours. Here are the results from the GC-MS analysis of the anise oil reaction after 24 hours.

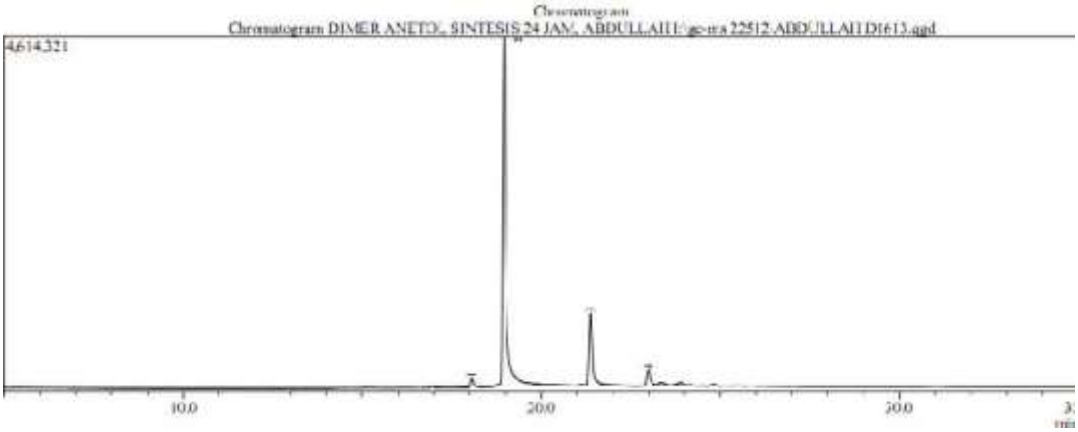

Figure 9. Chromatogram of anis oil reaction results for 24 hours

The chromatogram of the reaction product of anise oil after 24 hours shows the highest peak at a retention time of 18.958 minutes, covering an area of 77.68%, and was identified by MS as anethole. Here are the compound data generated from the 24-hour reaction.

Tabel 1. Data on compound content in 24 hours reactions

No Peak	Retention Time (minutes)	Compound Name	% Area
1	18,056	Anetol	1,59
2	18.960	Anetol	77,68
3	21,379	*p*-Anisaldehis	17,25
4	22,992	Anisil aseton	3,47

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GC-MS analysis for the reaction of anis essential oil for 48 hours can be seen in Figure 10 below

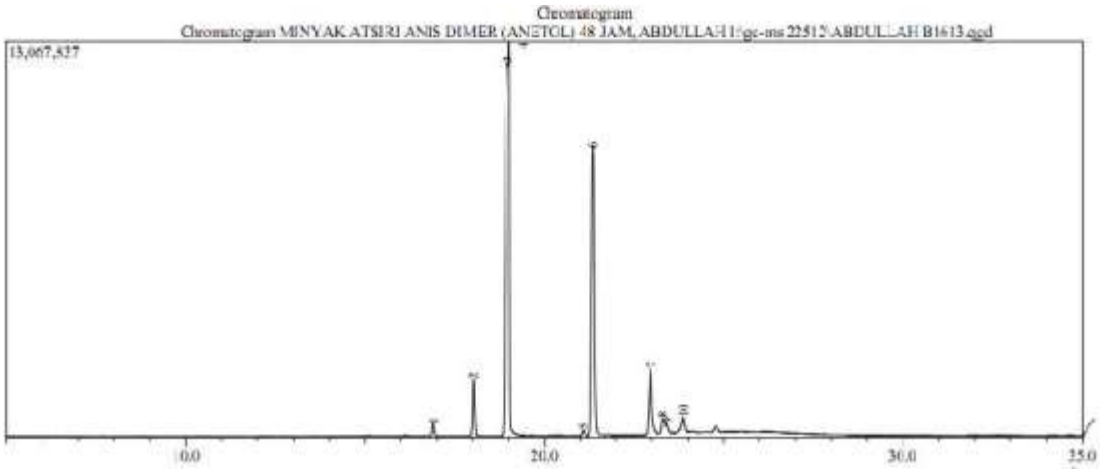

Figure 10. Chromatogram of anis oil reaction results for 48 hours

The GC analysis chromatograph for the 48-hour reaction had many more peaks than the 24-hour reaction, so it can be concluded that there are new compounds. The following differences are produced:

Tabel 2. Differences in compound yields

Retention Time (minutes)	Compound Name	% Area	
		24 hour reaction	48 hour reaction
18,958	Anetol	77,68	37,42
21,347	*p*-anisaldehid	17,25	27,27

The anethole and p-anisaldehyde compounds in the 24-hour reaction had a higher percentage area than those in the 48-hour reaction. This suggests that the amount of anethole decreased in the 48-hour reaction, possibly due to its conversion into other compounds. Below is the compound data generated from the 48-hour reaction:

Tabel 3. Data on compound content in the 48 hours reaction

No Peak	Retention Time (minutes)	Compound Name	% Area
1	16.897	Anetol	0.86
2	18.024	Estragol	3.82
3	18.958	Anetol and Estragol	37.42
4	19.009	Anetol and Estragol	19.30
5	21.080	Kariofilena	0.42

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6	21.347	*p*-Anisaldehis	27.27
7	22.955	Anisil Aseton	6.92
8	23.867	p-metoksipropiofenon dan 1-(4-Metoksi-fenil)-2-fenil-eten-1,2-dion	1.39

The above data indicates the presence of different compounds in the 24-hour reaction product. Three of these compounds are oxidation reaction products with a retention time of 21.083 and an area of 0.42%, representing caryophyllene oxide. Caryophyllene oxide can be synthesized from caryophyllene through oxidative reactions using H_2O_2 (Kadarohman et al., 1999). The formation of this compound in the 48-hour reaction suggests that oxidative reactions were catalyzed by laccase enzymes. Below is a diagram of the formation process.

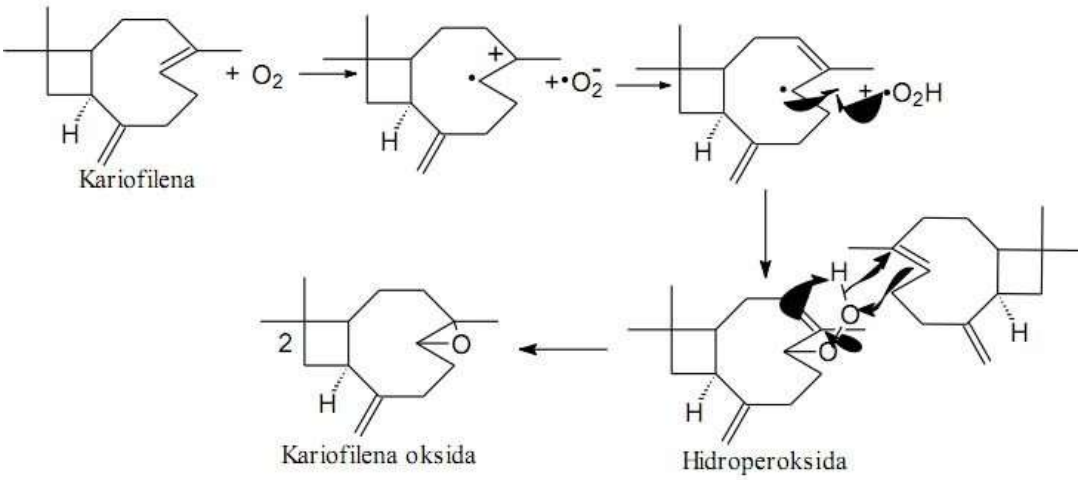

Figure 11. The reaction mechanism for the oxidation of caryophyllene with oxygen becomes caryophyllene oxide

The following is the spectrum of the compound caryophyllene oxide

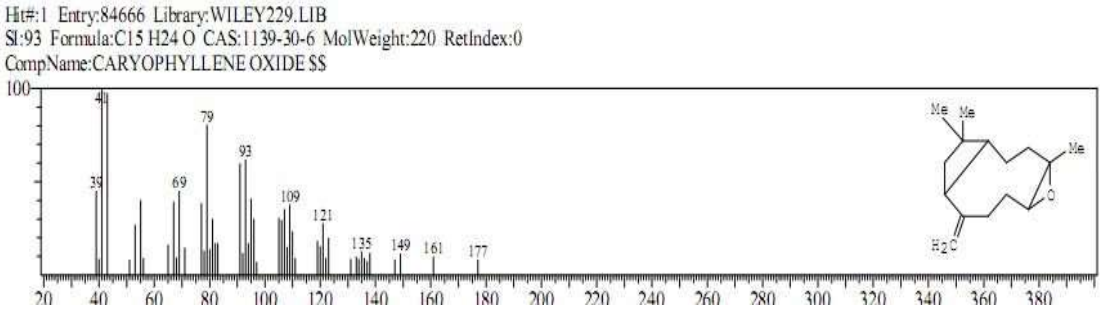

Figure 12. Mass spectrum of caryophyllene oxide compounds

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Then, at a retention time of 23.86 with an area percentage of 1.39, it is thought that it can be produced through oxidative coupling processes. The results of the MS analysis for these compounds are shown below.

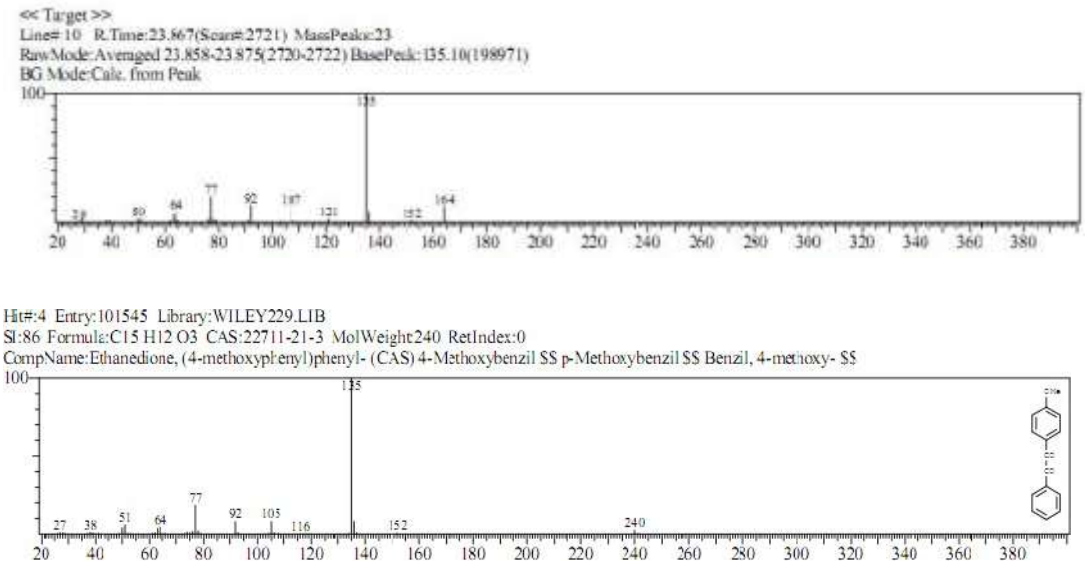

Figure 13. Mass spectrum of the compound 1-(4-Methoxy-phenyl)-2-phenyl-ethene-1,2-dione

This hypothesis is based on the MS spectrum data of a compound with an 86% similarity to 1-(4-Methoxyphenyl)-2-phenyl-ethen-1,2-dione found in the library. The structure of the reference compound is shown in the following figure:

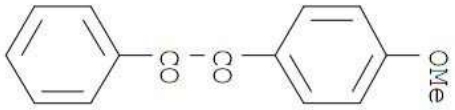

Figure 14. Structure of the compound 1-(4-Methoxy-phenyl)-2-phenyl-ethene-1,2-dione

The presence of two phenyl groups in the compound indicates a coupling reaction between two compounds, each of which has a phenyl group similar to anetol. Because the analysis only uses MS data, the structure of the synthesized compound cannot yet be determined. Although anethole constitutes a large percentage of anise oil, it is less reactive compared to phenolic compounds that contain hydroxyl groups. This makes anethole more difficult to react compared to eugenol, which has successfully synthesized its dimer using the same process with the biocatalyst enzyme laccase.

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4. Conclusions

The application of computational chemistry in understanding reaction mechanisms of biochemical processes has emerged as a powerful tool in modern biochemistry. By combining theoretical modeling with experimental validation, we can deepen our understanding of complex biological systems and pave the way for innovative applications in drug discovery, biotechnology, and personalized medicine.

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