Journal of Chemical Learning Innovation
Vol. 1, No. 2, December 2024, pp. 117~125
ISSN: 3063-0886, DOI:10.37251/jocli.v1i2.3053

Preliminary Study on Laccase-Catalyzed Oxidative Transformation of Anethole: Evidence from GC-MS Analysis

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Article Info

Article history:

Received Oct 17, 2024
Revised Nov 25, 2024
Accepted Dec 21, 2024
OnlineFirst Dec 31, 2024

Keywords:

Anethole
Biocatalyst
Laccase Enzyme
White Oyster Mushroom

ABSTRACT

Purpose of the study: This research was conducted with the aim of synthesizing anethole dimers using the laccase enzyme biocatalyst.

Methodology: In this study, anise oil containing 90% anethole, laccase enzyme as a biocatalyst, and hydroquinone as a mediator were used. The laccase enzyme used was isolated from white oyster mushroom (Pleurotus ostreatus) which has an activity of 712.758 U/L. The anethole dimer formation reaction was carried out in a biphasic medium (ethyl acetate: phosphate buffer = 4:1) which was carried out for 24 hours and 48 hours. The reaction results were then extracted with ethyl acetate and produced a thick brownish liquid with a more intense color intensity in the 48-hour reaction.

Main Findings: Comparison of the GC test results on anise oil, 24-hour reaction, and 48-hour reaction showed an increase in peaks and changes in peak height in the 48-hour reaction. Anethole and p-anisaldehyde compounds had a smaller % area than in the 24-hour reaction. It is suspected that the 48-hour reaction produced a new compound derived from the oxidation reaction, namely caryophyllene oxide, but the compound that is the anethole dimer has not been identified.

Novelty/Originality of this study: This study introduces a biocatalytic approach for anethole transformation using laccase from Pleurotus ostreatus, integrating mechanistic insights with product characterization. It reveals the limited reactivity of anethole under laccase catalysis while identifying alternative oxidation pathways, such as caryophyllene oxide formation. These findings advance understanding of substrate specificity and expand knowledge of laccase-mediated transformations in non-phenolic systems.

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1. INTRODUCTION

Biocatalysis has emerged as a powerful strategy in modern organic chemistry, offering selective and environmentally benign alternatives to conventional synthetic methods [1], [2]. Enzymes enable complex transformations under mild conditions, often with high regio- and stereoselectivity [3], [4]. Among these transformations, oxidative coupling reactions are particularly important for constructing C–C and C–O bonds [5], [6]. Such processes are essential in the synthesis of bioactive compounds and functional materials. Therefore, exploring enzymatic approaches for oxidative transformations continues to attract significant scientific attention.

Laccase (EC 1.10.3.2) is a multicopper oxidase widely studied in the field of biocatalysis due to its ability to oxidize a broad range of phenolic and non-phenolic substrates [7], [8]. This enzyme utilizes molecular oxygen as the terminal electron acceptor, producing water as the only by-product [9], [10]. The catalytic versatility of laccase makes it a promising tool for green oxidation reactions. It has been successfully applied in polymerization, degradation of pollutants, and organic synthesis. Consequently, laccase-based systems have become increasingly relevant in sustainable chemistry research [11], [12].

Anethole, a naturally occurring phenylpropanoid compound, is commonly found in essential oils such as those from anise and fennel [13], [14]. It has attracted interest in natural product chemistry due to its biological activities and structural features [15], [16]. The presence of an electron-rich aromatic ring makes anethole a suitable substrate for oxidative transformations. In particular, its potential to undergo dimerization opens pathways to novel compounds with enhanced or altered bioactivity [17], [18]. However, the controlled synthesis of anethole dimers remains a challenging task.

Oxidative dimerization represents a key transformation in synthetic chemistry, enabling the formation of structurally complex molecules from simple precursors [19], [20]. Traditional chemical methods for dimerization often require harsh conditions, toxic reagents, or metal catalysts. These limitations raise concerns regarding environmental impact and selectivity. In contrast, enzymatic oxidation offers a greener and more selective alternative [21], [22]. Thus, laccase-catalyzed dimerization presents a promising approach for sustainable synthesis.

Mechanistic understanding plays a crucial role in advancing enzymatic transformations within physical chemistry. Elucidating the reaction pathway of laccase-catalyzed oxidation can provide insights into intermediate species and product distribution [23], [24]. Such knowledge is essential for optimizing reaction conditions and improving yield and selectivity [25], [26]. Furthermore, mechanistic studies contribute to the rational design of biocatalytic systems. Despite its importance, detailed mechanistic investigation of anethole dimerization remains limited.

Structural characterization is equally important in confirming the identity and properties of synthesized compounds [27], [28]. Techniques such as nuclear magnetic resonance (NMR), Fourier-transform infrared spectroscopy (FTIR), and gas chromatography–mass spectrometry (GC–MS) are commonly used in analytical chemistry [29], [30]. These methods provide complementary information about molecular structure and functional groups. Accurate characterization is essential to validate the formation of dimeric products. It also enables correlation between structure and potential functionality.

Despite growing interest in laccase-mediated transformations, several research gaps remain. Previous studies have largely focused on phenolic substrates, with limited attention given to compounds like anethole. Moreover, comprehensive investigations integrating mechanistic insights and detailed structural characterization are still scarce. This study addresses these gaps by exploring the laccase-catalyzed oxidative dimerization of anethole in a systematic manner. The novelty of this research lies in combining mechanistic analysis with rigorous structural identification of the resulting dimers. The urgency of this work is underscored by the increasing demand for sustainable synthetic methods in modern chemistry. This research was conducted with the aim of synthesizing anethole dimers using the laccase enzyme biocatalyst.

2. RESEARCH METHOD

2.1. Research Materials and Tools

This research used white oyster mushrooms and anise oil as the primary substrates. White oyster mushrooms served as the source of the laccase enzyme, while anise oil served as the base material containing anethole [31]. These two materials were chosen due to their relatively easy availability and potential for biocatalytic reactions. Utilizing these natural materials also supports an environmentally friendly approach to the synthesis process [32], [33]. Therefore, selecting these primary materials is a crucial first step in the success of the research.

In addition to the primary materials, this research also involved various supporting chemicals. These chemicals included phosphate buffer as a pH regulator, as well as organic solvents such as acetone, n-hexane, ethyl acetate, and ethanol. Aquadem was used as a common solvent in various stages of the process. Hydroquinone and ABTS were used as reagents in enzyme activity testing. The use of these chemicals was adjusted according to the needs of each stage of the research to ensure optimal process operation.

The equipment used in this research included various laboratory instruments for preparation, separation, and analysis. The initial process used a blender and Erlenmeyer flask, while separation was carried out using a separating funnel and filter paper. Sample concentration was performed using a rotary vacuum evaporator, while a centrifuge was used to separate components based on density [34], [35]. Compound analysis was performed using GC-MS and UV-Vis instruments. In addition, chromatography techniques such as TLC and a chromatotron were used for further separation.

Other supporting equipment included micropipettes for accurate small-volume measurements, aluminum foil to protect samples from light, and a cooling system to maintain temperature stability. Environmental parameters such as pH and temperature were controlled using a pH meter and thermometer [36], [37]. Samples were stored in a refrigerator to maintain material stability. The entire research process was also supported by a variety of standard laboratory glassware. With this combination of tools and materials, the research could be carried out systematically and in a controlled manner.

2.2. Work Procedures

2.2.1. Isolation of Laccase Enzyme from White Oyster Mushrooms

Isolation of the laccase enzyme began by grinding 300 grams of white oyster mushrooms using a 0.2 M phosphate buffer solution at pH 6.0. The homogenization process was carried out using a homogenizer at a low temperature (0–5°C) for 15 minutes to maintain enzyme stability. The resulting homogenate was then filtered through a filter cloth to separate the solid residue. The filtrate was then centrifuged at 3500 rpm for 20 minutes to obtain the supernatant, which served as the crude enzyme extract. This crude extract was then stored at 0–2°C to maintain enzyme activity.

Enzyme purification was carried out using an organic solvent, namely cold acetone, to increase enzyme activity. The acetone was cooled to below −10°C before being mixed with the crude enzyme extract in a 1:3 ratio (enzyme:acetone). The mixture was then stirred for 7–12 minutes and allowed to stand until a precipitate formed at a temperature of −10 to 0°C. The mixture was then centrifuged to separate the precipitate from the supernatant. The resulting precipitate was then resuspended in pH 6.0 phosphate buffer. This suspension was then used as an enzyme extract for the next stage of analysis.

2.2.2. Determination of Laccase Enzyme Activity

Laccase enzyme activity was determined using the ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)) substrate oxidation method. The principle of this test is the oxidation of ABTS by laccase to a blue-green cation radical ($ABTS^+$). The intensity of the resulting color was measured using a UV-Vis spectrophotometer at a wavelength of 420 nm. The resulting absorbance is directly proportional to the laccase enzyme activity. Thus, changes in absorbance can be used as an indicator of enzyme activity.

The measurement was performed by mixing 160 µL of a 0.4 mM ABTS solution in pH 6 phosphate buffer with 40 µL of enzyme extract in a 1.5 mL cuvette. The mixture was shaken until homogeneous, then the absorbance was measured for 5 minutes at a wavelength of 420 nm. The change in absorbance was recorded every minute to determine the reaction rate. Enzyme activity values are expressed in International Units (IU), where 1 IU is defined as the amount of enzyme capable of oxidizing 1 µmol of ABTS per minute. Activity calculations are performed by considering changes in absorbance, total reaction volume, enzyme volume, and the ABTS extinction coefficient.

2.2.3. Anethole Dimerization Reaction with Laccase Enzyme Catalyst

The anethole dimerization reaction was carried out in a biphasic medium system to increase reaction efficiency and product stability. The biphasic medium was prepared by mixing ethyl acetate and phosphate buffer in a 4:1 ratio. The mixture was then placed into two test tubes, each containing 5 mL. Next, 1 mL of anethole solution was added to each tube as a substrate. The use of a biphasic system allows for easier product extraction into the organic phase. To the first tube, 1 mL of hydroquinone (0.5 mg/mL) was added as a mediator and 4 mL of laccase enzyme extract. Meanwhile, the second tube was used as a control without the addition of enzymes and mediators. Both mixtures were stirred until homogeneous and allowed to settle until two layers of phases formed. The reaction process was observed through color changes that occurred in the system. These changes are indicative of the ongoing oxidative reaction by the laccase enzyme.

2.2.4. Isolation of Reaction Products

To obtain a larger product, the dimerization reaction was carried out on a larger scale. The reaction medium was prepared by mixing phosphate buffer and ethyl acetate in a 1:4 ratio. 20 mL of enzyme extract, 5 mL of anethole, and 20 mL of hydroquinone were added to the mixture. The mixture was then stirred for 30 minutes to ensure homogeneity of the reaction system. After that, the solution was left for 24 to 48 hours to allow the reaction to proceed optimally. The resulting product was then separated through an extraction process using ethyl acetate. The organic phase containing the product was collected and then evaporated to remove the solvent. The evaporation process was carried out at a temperature of 50–60°C until a residue in the form of a crude product was obtained. This step aims to concentrate the reaction product. The crude product was then used for further analysis.

2.2.5. Analysis of Reaction Products

Initial product analysis was performed using thin-layer chromatography (TLC) to identify the compound components in the mixture. The mobile phase used was a mixture of n-hexane and ethyl acetate in various ratios, namely 1:1, 2:1, 3:2, 4:1, and 5:1. This variation aimed to determine the most optimal separation conditions. Observed parameters included the number of spots and the degree of separation between components. TLC results were used as a basis for selecting the best conditions for further separation. After the optimum conditions were obtained, the reaction products were analyzed using a GC-MS instrument to determine the composition and structure of the compounds. This technique allows for compound identification based on mass fragmentation patterns. The data obtained were then used to confirm the formation of anethole dimers. This analysis is a crucial step in ensuring the success of the reaction. Thus, comprehensive product characterization can be carried out.

3. RESULTS AND DISCUSSION

3.1. Isolation of Laccase Enzyme from White Oyster Mushroom

Laccase is an oxidase enzyme found abundantly in fungi and higher plants, particularly those from the Ascomycetes, Deuteromycetes, and Basidiomycetes groups. In this study, the source of the laccase enzyme was obtained from the white oyster mushroom (Pleurotus ostreatus), a member of the Basidiomycetes group. In this organism, laccase plays a crucial role in lignin degradation and various other biological functions such as pigmentation, fruiting body formation, sporulation, and defense mechanisms. This indicates that the white oyster mushroom is a potential source of laccase enzymes for biocatalytic applications. Therefore, isolating the enzyme from this source is a crucial initial step in the research.

The isolation process begins with the destruction of the fungal tissue in a 0.2 M phosphate buffer solution at pH 6.0. This buffer is used to maintain the optimum pH of the laccase enzyme so that it remains stable during the extraction process. Next, homogenization is carried out at a low temperature (0–5°C) to prevent protein denaturation due to increased temperature. Low temperature conditions also serve to maintain enzyme activity and prevent inactivation before use in the reaction. Therefore, controlling pH and temperature is crucial for successful enzyme isolation.

The next step is separating the enzyme extract from other cellular components. This process involves filtering through cotton cloth to remove coarse particles. The resulting filtrate is then centrifuged at 3500 rpm to produce a supernatant. This supernatant represents the crude laccase enzyme extract, which is generally brownish-yellow in color. To maintain its stability, the enzyme extract is stored at low temperatures (0–2°C) until use in the next step.

3.2. Precipitation of Crude Laccase Enzyme Extract

To increase enzyme activity and purity, a partial purification stage was performed using a protein precipitation method. The method used in this study was precipitation using an organic solvent, acetone. Generally, enzyme purification can be performed using various methods, such as salt precipitation (ammonium sulfate) and chromatography. However, acetone was chosen because it is relatively simple and effective in separating proteins based on their solubility. This method is also widely used in initial enzyme purification.

The principle of acetone precipitation is related to changes in the hydrophobic interactions of protein molecules. The addition of acetone displaces water molecules around the protein surface, disrupting these hydrophobic interactions. As a result, the protein aggregates and precipitates from the solution. This process is influenced by electrostatic interactions and dipole forces between protein molecules. Larger proteins tend to aggregate more easily due to their more complex charge distribution.

Temperature plays a critical role in this process, as precipitation must be carried out at temperatures below −10°C. At higher temperatures, organic solvents can interact directly with the hydrophobic regions of the protein and cause denaturation. Therefore, low temperature conditions were maintained to maintain the stability of the enzyme structure and activity. In this study, the enzyme extract was mixed with acetone in a 1:3 ratio until a precipitate formed. The resulting precipitate was then separated by centrifugation and resuspended in phosphate buffer at pH 6.0 as a purified enzyme extract.

3.3. Determination of Laccase Enzyme Activity

Laccase enzyme activity in this study was determined using the ABTS substrate oxidation method. This method is based on the ability of laccase to oxidize ABTS into a stable, blue-green cation radical ($ABTS^+$). The intensity of the resulting color was measured using a UV-Vis spectrophotometer at a wavelength of 420 nm. The resulting absorbance is proportional to the number of cation radicals formed. Therefore, the absorbance value can be used as an indicator of enzyme activity.

Observations were made by recording changes in absorbance every minute for five minutes. The obtained data were then used to determine the reaction rate based on the gradient of the absorbance-time curve. The results showed that the maximum gradient occurred in the first minute of observation, indicating high enzyme activity in the initial phase of the reaction. This is consistent with enzyme kinetics, which generally show a maximum reaction rate at the beginning of interaction with the substrate. This analysis provides a quantitative overview of the catalytic ability of the laccase enzyme.

Based on the calculations, the laccase enzyme activity obtained was 712.758 U/L. This value indicates that the isolated enzyme has sufficiently high activity and meets the requirements as a biocatalyst in organic reactions. Compared with the literature, the minimum activity required for the formation of dimers, trimers, or polymers is approximately 68 U/L, while the maximum reported activity reaches 2200 U/L. Thus, the enzyme activity in this study is within the effective range to support the anethole dimerization reaction. These results indicate that the laccase enzyme from white oyster mushrooms has good potential for application in biocatalysis-based synthesis.

3.4. Anethole Dimerization Reaction with Laccase Enzyme Catalyst

The laccase enzyme used in this study belongs to the oxidoreductase class of enzymes capable of catalyzing oxidation and reduction reactions. In its mechanism, laccase oxidizes aromatic compounds, particularly phenolics, into reactive free radicals. These radicals can then undergo coupling reactions to form dimers, oligomers, and even polymers. This process is the basis of the anethole dimerization reaction studied in this study. Thus, laccase plays a crucial role in initiating the formation of more complex derivative compounds.

In its catalytic system, laccase requires a mediator compound to increase reaction efficiency, especially with less reactive substrates. The mediator used in this study was hydroquinone, which functions as an electron shuttle. This compound is first oxidized by laccase, then transfers electrons, accelerating the oxidation of the anethole substrate. The use of a mediator is known to significantly increase oxidation efficiency compared to the system without a mediator. This is due to the smaller size of the mediator molecule, which allows it to interact more easily with the active site of the enzyme.

Structurally, laccase is a metalloenzyme containing copper (Cu) ions in its active center. These Cu ions play a role in the electron transfer process from the substrate to oxygen as the final acceptor. The laccase catalytic mechanism involves several steps: electron transfer from the substrate to the mononuclear copper center, then to the trinuclear center, and finally, the reduction of oxygen to water. The presence of this metal system allows the reaction to proceed without producing toxic intermediates such as peroxide. Therefore, laccase is an efficient and environmentally friendly catalyst.

The anethole dimerization reaction was carried out in a biphasic medium system consisting of a mixture of ethyl acetate and phosphate buffer in a 4:1 ratio. The use of a biphasic medium aims to increase product stability while facilitating the extraction of the reaction compounds into the organic phase. Compared with a monophasic system, this approach has proven more effective in increasing product yield. Ethyl acetate was chosen as the organic solvent due to its ability to dissolve organic compounds and its compatibility with the enzymatic system. Therefore, the reaction conditions were designed to optimize the interaction between the substrate, enzyme, and mediator.

Qualitative test results showed significant changes between the reaction system and the control. The system containing the laccase enzyme and mediator produced a more intense brownish color compared to the control system without the enzyme. This color change indicates an oxidation reaction that produces new compounds. Meanwhile, in the control system, the changes were relatively minimal. This indicates that the laccase enzyme plays a direct role in the chemical transformation of anethole.

To obtain a larger product, the reaction was carried out on a larger scale with incubation times of 24 and 48 hours. After the reaction, the mixture was extracted using ethyl acetate to separate the organic and aqueous phases based on differences in polarity. The organic phase was then evaporated to remove the solvent, resulting in a thick, brownish liquid. The color intensity of the product after the 48-hour reaction was significantly more intense than after the 24-hour reaction. This indicates that the reaction progressed further with increasing incubation time.

3.5. Analysis of Reaction Products

Initial product analysis was conducted qualitatively by observing changes in color and aroma. Furthermore, thin-layer chromatography (TLC) was used to identify the components in the product mixture. Separation in TLC is based on differences in compound distribution between the stationary and mobile phases. In this study, a mixture of n-hexane and ethyl acetate was used as the mobile phase in varying proportions. This variation was intended to obtain optimal separation conditions.

However, TLC results indicated that component separation did not provide significant indications of new product formation. This was likely due to the relatively small amount of product compared to the other components in the mixture. Furthermore, the complexity of the mixture can also affect the resolution of TLC separation. Therefore, a more sensitive analytical method is needed to identify reaction products. In this case, GC-MS analysis was used for further characterization.

3.6. GC-MS Analysis

Initial analysis of anise oil using GC-MS revealed several main components, with a dominant peak at a retention time of approximately 18.96 minutes. This peak was identified as anethole based on an m/z value of 148. In addition to anethole, other compounds such as p-anisaldehyde and aromatic ketone derivatives were also detected. This composition indicates that the anise oil used contains main components suitable as reaction substrates. This data serves as a reference for analyzing compositional changes after the reaction.

GC-MS analysis results from the 24-hour reaction showed that anethole remained the dominant component with a high area percentage. This indicates that the dimerization reaction had not yet progressed significantly during this time. However, oxidizing compounds such as p-anisaldehyde were detected, indicating the onset of an initial reaction. Therefore, the 24-hour reaction time was insufficient for optimal transformation.

In the 48-hour reaction, the chromatogram showed an increase in the number of peaks, indicating the formation of new compounds. The percentage of anethole decreased compared to the 24-hour reaction, indicating that some anethole had undergone transformation. In addition, several oxidation compounds were detected, such as aromatic derivatives and new compounds suspected to be coupling products. This indicates that the reaction progresses further with increasing incubation time.

One of the identified compounds is one with a spectral similarity to 1-(4-methoxy-phenyl)-2-phenyl-ethene-1,2-dione. The presence of two phenyl groups in the structure indicates the possibility of an oxidative coupling reaction between aromatic molecules [38], [39]. However, this identification is still tentative because it is based solely on mass spectrum data. To definitively confirm the structure, further analysis such as NMR or FTIR is required. Therefore, these results provide an initial indication of the formation of a dimer compound, although it cannot yet be definitively confirmed.

In general, anethole exhibits lower reactivity than other phenolic compounds such as eugenol. This is due to the absence of a hydroxyl group that can increase radical stability during the oxidation reaction. Consequently, the anethole dimerization process is slower and produces a limited amount of product. Nevertheless, the results of this study still demonstrate the potential use of laccase enzymes in the transformation of anethole. Further research is needed to optimize reaction conditions and improve dimerization yield.

The reactivity of anethole in the laccase catalytic system exhibits different characteristics compared to classic phenolic substrates. This is primarily due to the absence of a hydroxyl group on the aromatic ring, which generally plays a key role in stabilizing radicals through resonance. Without this stabilization, the resulting cation radical tends to be more reactive and less selective, thus more susceptible to side reactions such as fragmentation or further oxidation rather than directional coupling to form dimers [40]. This explains the relatively low efficiency of anethole dimerization in this system.

The role of the mediator in the laccase system is also a crucial factor in determining the reaction pathway. Hydroquinone, as a mediator, works by transferring electrons between the substrate and the enzyme's active site, thus enabling the direct oxidation of substrates that are less reactive to laccase. However, the use of a mediator can also increase the complexity of the reaction system by generating additional radical species that can interact with various components in the mixture. Consequently, selectivity toward the formation of specific products, including dimers, decreases and the product distribution becomes more widespread.

The two-phase system used in this reaction offers advantages in terms of product separation and organic compound stability, but it can also affect reaction kinetics [41]. The distribution of the substrate, mediator, and enzyme across two distinct phases can potentially limit effective interactions between them. Furthermore, mass transfer between the phases is a limiting factor that can slow the formation of reactive intermediates. Therefore, although a biphasic system offers practical advantages, further optimization is needed to improve overall reaction efficiency.

From a mechanistic perspective, the oxidation of anethole by laccase likely involves the formation of cation radicals in the aromatic system, which then undergo limited delocalization. These radicals can follow several reaction pathways, including further oxidation to carbonyl compounds or addition reactions that yield other derivatives [42]. However, without adequate stabilization, the likelihood of intermolecular radical coupling is less likely than degradation or partial transformation reactions. This suggests that substrate structure plays a key role in determining the direction of reactions in biocatalytic systems.

The implications of these findings suggest that the success of oxidative coupling reactions using laccase is highly dependent on the compatibility of the substrate structure with the enzyme's catalytic mechanism. To increase the likelihood of dimer formation, approaches such as modifying the reaction conditions, such as the use of more selective mediators, adjusting the pH and temperature, or even modifying the substrate structure through the introduction of electron-donating groups, can be considered. Thus, this study provides an initial basis for the development of more effective strategies in the biocatalysis-based synthesis of non-phenolic compounds.

This research provides an initial contribution to understanding the oxidation behavior of non-phenolic compounds by laccase enzymes, specifically anethole, which has a phenylpropanoid structure without a hydroxyl group. These findings broaden insights into substrate limitations in laccase-based biocatalytic systems, which have previously focused primarily on phenolic compounds. Conceptually, these results confirm that the success of oxidative coupling reactions is determined not only by the presence of a catalyst but also by the suitability of the substrate's electronic structure to the radical formation mechanism [43], [44]. Thus, this research has important implications for the development of enzyme-based green synthesis strategies, particularly in designing more selective reaction systems for compounds with low reactivity.

However, this study has several limitations that require consideration. Product identification relies on GC-MS analysis without the support of advanced spectroscopic techniques such as NMR or FTIR, so the structure of the resulting compound cannot be definitively confirmed [45], [46]. Furthermore, no in-depth kinetic analysis or mechanistic studies were performed, so the proposed reaction pathway remains hypothetical. Another limitation lies in the lack of systematic optimization of reaction conditions, including variations in pH, temperature, substrate concentration, and mediator type. The use of enzymes in crude extract form also has the potential to affect reaction selectivity due to the presence of other components in the system. Therefore, further research with a more comprehensive experimental approach is urgently needed to strengthen mechanistic interpretations and increase the reliability of the results.

4. CONCLUSION

Based on the results of the research that has been conducted, it was obtained that the laccase enzyme isolated from white oyster mushrooms showed an activity of 712.758 U/L, with one unit of activity (U) defined as the amount of enzyme capable of oxidizing 1 μmol ABTS per minute at room temperature and pH 6. This activity value indicates that the laccase enzyme produced has the potential as a biocatalyst in the oxidation reaction of organic compounds. However, in this study the laccase enzyme has not shown an effective ability to catalyze the formation of anethole dimer compounds. However, the enzyme was proven to be able to catalyze the oxidation reaction of other compounds, namely converting caryophyllene to caryophyllene oxide. This indicates that the isolated laccase still has good catalytic activity, although its specificity towards the anethole substrate is still limited. Therefore, further optimization of the reaction conditions is needed to increase the success of the synthesis of anethole dimers. Further research is recommended to characterize the product using advanced spectroscopic techniques such as NMR and FTIR to definitively determine the compound's structure. Furthermore, optimization of reaction conditions and more in-depth mechanistic studies are needed to improve the selectivity and efficiency of product formation.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the support of their supervisors, laboratory staff, and institution for providing guidance, facilities, and resources. Appreciation is also extended to colleagues and all parties who contributed to this research.

AUTHOR CONTRIBUTIONS

Conceptualization, S.Z.R. and W.P.; Methodology, S.Z.R.; Software, S.Z.R.; Validation, S.Z.R. and W.P.; Formal Analysis, S.Z.R.; Investigation, S.Z.R.; Resources, W.P.; Data Curation, S.Z.R.; Writing – Original Draft Preparation, S.Z.R.; Writing – Review & Editing, S.Z.R. and W.P.; Visualization, S.Z.R.; Supervision, W.P.; Project Administration, S.Z.R.; Funding Acquisition, W.P.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

Not applicable.

REFERENCES

[1] U. Hanefeld, F. Hollmann, and C. E. Paul, "Biocatalysis making waves in organic chemistry," *Chem. Soc. Rev.*, vol. 51, no. 2, pp. 594–627, 2022, doi: 10.1039/d1cs00100k.

[2] C. K. Winkler, J. H. Schrittwieser, and W. Kroutil, "Power of biocatalysis for organic synthesis," *ACS Cent. Sci.*, vol. 7, no. 1, pp. 55–71, Jan. 2021, doi: 10.1021/acscentsci.0c01496.

[3] Y. Ding, G. Perez-Ortiz, J. Peate, and S. M. Barry, "Redesigning enzymes for biocatalysis: Exploiting structural understanding for improved selectivity," *Front. Mol. Biosci.*, vol. 9, no. July, pp. 1–17, 2022, doi: 10.3389/fmolb.2022.908285.

[4] R. Mu *et al.*, "Application of enzymes in regioselective and stereoselective organic reactions," *Catalysts*, vol. 10, no. 8, pp. 1–25, 2020, doi: 10.3390/catal10080832.

[5] L. E. Zetzsche *et al.*, "Biocatalytic oxidative cross-coupling reactions for biaryl bond formation," *HHS Public Access*, vol. 603, no. 7899, pp. 79–85, 2022, doi: 10.1038/s41586-021-04365-7.Biocatalytic.

[6] L. Lu, H. Li, and A. Lei, "Oxidative cross-coupling reactions between two nucleophiles," *Chinese J. Chem.*, vol. 40, no. 2, pp. 256–266, Jan. 2022, doi: 10.1002/cjoc.202100396.

[7] P. Aza and S. Camarero, "Fungal laccases: Fundamentals, engineering and classification update," *Biomolecules*, vol. 13, no. 1716, pp. 1–21, 2023, doi: 10.3390/biom13121716.

[8] M. Mahuri, M. Paul, and H. Thatoi, "A review of microbial laccase production and activity toward different biotechnological applications," *Syst. Microbiol. Biomanufacturing*, vol. 3, no. 4, pp. 533–551, Oct. 2023, doi:

10.1007/s43393-023-00163-6.

[9] J. Jayakumar, D. Priyadarshini, A. Parthasarathy, and S. R. Reddy, “Recent advances in molecular oxygen assisted laccase catalyzed sustainable organic transformations,” *Asian J. Org. Chem.*, vol. 12, no. 1, pp. 1–27, Jan. 2023, doi: 10.1002/ajoc.202200564.

[10] C. Garrido and S. Leimkühler, “The inactivation of human aldehyde oxidase 1 by hydrogen peroxide and superoxide,” *Drug Metab. Dispos.*, vol. 49, no. 9, pp. 729–735, Sep. 2021, doi: 10.1124/dmd.121.000549.

[11] D. M. Scheibel, I. P. I. Gitsov, and I. Gitsov, “Enzymes in ‘green’ synthetic chemistry: Laccase and lipase,” *Molecules*, vol. 29, no. 5, pp. 1–33, 2024, doi: 10.3390/molecules29050989.

[12] T. Itoh and Y. Takagi, “Laccase-catalyzed reactions in ionic liquids for green sustainable chemistry,” *ACS Sustain. Chem. Eng.*, vol. 9, no. 4, pp. 1443–1458, 2021, doi: 10.1021/acssuschemeng.0c07097.

[13] A. Zayed, M. Sobeh, and M. A. Farag, “Dissecting dietary and semisynthetic volatile phenylpropenes: A compile of their distribution, food properties, health effects, metabolism and toxicities,” *Crit. Rev. Food Sci. Nutr.*, vol. 63, no. 32, pp. 11105–11124, Dec. 2023, doi: 10.1080/10408398.2022.2087175.

[14] M. Sharafan *et al.*, “Illicium verum (star anise) and trans-anethole as valuable raw materials for medicinal and cosmetic applications,” *Molecules*, vol. 27, no. 650, pp. 1–16, 2022, doi: 10.3390/molecules27030650.

[15] S. M. Umer, M. Solangi, K. M. Khan, and R. S. Z. Saleem, “Indole-containing natural products 2019–2022: Isolations, reappraisals, syntheses, and biological activities,” *Molecules*, vol. 27, no. 21, pp. 1–45, 2022, doi: 10.3390/molecules27217586.

[16] W. Hou and H. Xu, “Incorporating selenium into heterocycles and natural products─from chemical properties to pharmacological activities,” *J. Med. Chem.*, vol. 65, no. 6, pp. 4436–4456, Mar. 2022, doi: 10.1021/acs.jmedchem.1c01859.

[17] A. V. Atoki, P. M. Aja, E. N. Ondari, and T. S. Shinkafi, “Advances in alzheimer’s disease therapeutics: Biochemistry, exploring bioactive compounds and novel approaches,” *Int. J. Food Prop.*, vol. 26, no. 1, pp. 2091–2127, 2023, doi: 10.1080/10942912.2023.2243050.

[18] I. Lopes, C. Campos, R. Medeiros, and F. Cerqueira, “Antimicrobial activity of dimeric flavonoids,” *Compounds*, vol. 4, no. 2, pp. 214–229, 2024, doi: 10.3390/compounds4020011.

[19] S. K. Sinha *et al.*, “Transition-metal catalyzed C–H activation as a means of synthesizing complex natural products,” *Chem. Soc. Rev.*, vol. 52, no. 21, pp. 7461–7503, 2023, doi: 10.1039/D3CS00282A.

[20] S. E. Dibrell, Y. Tao, and S. E. Reisman, “Synthesis of complex diterpenes: Strategies guided by oxidation pattern analysis,” *Acc. Chem. Res.*, vol. 54, no. 6, pp. 1360–1373, Mar. 2021, doi: 10.1021/acs.accounts.0c00858.

[21] J. Teržan, A. Sedminek, Ž. Lavrič, M. Grilc, M. Huš, and B. Likozar, “Selective oxidation of biomass-derived carbohydrate monomers,” *Green Chem.*, vol. 25, no. 6, pp. 2220–2240, 2023, doi: 10.1039/d2gc04623g.

[22] G. D. Yadav, R. K. Mewada, D. P. Wagh, and H. G. Manyar, “Advances and future trends in selective oxidation catalysis: a critical review,” *Catal. Sci. Technol.*, vol. 12, no. 24, pp. 7245–7269, 2022, doi: 10.1039/D2CY01322C.

[23] D. den Boer, H. C. de Heer, F. Buda, and D. G. H. Hetterscheid, “Challenges in elucidating the free energy scheme of the laccase catalyzed reduction of oxygen,” *ChemCatChem*, vol. 15, no. 1, pp. 1–27, Jan. 2023, doi: 10.1002/cctc.202200878.

[24] D. Zhu *et al.*, “Insight into depolymerization mechanism of bacterial laccase for lignin,” *ACS Sustain. Chem. Eng.*, vol. 8, no. 34, pp. 12920–12933, 2020, doi: 10.1021/acssuschemeng.0c03457.

[25] S. G. Newman, “Optimizing chemical reactions,” *Chem. Rev.*, vol. 124, no. 7, pp. 3645–3647, Apr. 2024, doi: 10.1021/acs.chemrev.4c00231.

[26] L. Y. Chen and Y. P. Li, “Machine learning-guided strategies for reaction conditions design and optimization,” *Beilstein J. Org. Chem.*, vol. 20, pp. 2476–2492, 2024, doi: 10.3762/bjoc.20.212.

[27] N. F. Shamsudin *et al.*, “Flavonoids as antidiabetic and anti-inflammatory agents: A review on structural activity relationship-based studies and meta-analysis,” *Int. J. Mol. Sci.*, vol. 23, no. 20, pp. 1–35, 2022, doi: 10.3390/ijms232012605.

[28] Ç. Karabacak Atay, Ö. Dilek, T. Tilki, and B. Dede, “A novel imidazole-based azo molecule: synthesis, characterization, quantum chemical calculations, molecular docking, molecular dynamics simulations and ADMET properties,” *J. Mol. Model.*, vol. 29, no. 8, p. 226, Aug. 2023, doi: 10.1007/s00894-023-05625-1.

[29] K. P. Baviskar *et al.*, “A Review on hyphenated techniques in analytical chemistry,” *Curr. Anal. Chem.*, vol. 18, no. 9, pp. 956–976, Nov. 2022, doi: 10.2174/1573411018666220818103236.

[30] M. S. J. Maftuh, U. S. Lawal, M. Ade, A. V. Lama, and A. F. Adzim, “Understanding learning strategies: A comparison between contextual learning and problem-based learning,” *Educ. J. Educ. Learn.*, vol. 01, no. 01, pp. 54–65, 2023, doi: 10.61987/educazione.v1i1.496 Understanding.

[31] A. Syafawi and M. Rahmi, “Formation of anethole dimer compound using biocatalyst laccase from white oyster mushroom,” *Chem Trendz J.*, vol. 1, no. 1, pp. 1–16, 2024.

[32] S. Sfameni, G. Rando, and M. R. Plutino, “Sustainable secondary-raw materials, natural substances and eco-friendly nanomaterial-Based approaches for improved surface performances: An overview of What They Are and How They Work,” *Int. J. Mol. Sci.*, vol. 24, no. 6, pp. 1–33, 2023, doi: 10.3390/ijms24065472.

[33] A. Khosravi, A. Zarepour, S. Iravani, R. S. Varma, and A. Zarrabi, “Sustainable synthesis: natural processes shaping the nanocircular economy,” *Environ. Sci. Nano*, vol. 11, no. 3, pp. 688–707, 2024, doi: 10.1039/D3EN00973D.

[34] B. Cochachin-Carrera, J. Moreno-Cuevas, and N. Carvajal-Mena, “Effects of concentration by block freezing and vacuum evaporation on the physicochemical properties and digestibility of whey,” *CYTA - J. Food*, vol. 21, no. 1, pp. 313–320, 2023, doi: 10.1080/19476337.2023.2196321.

[35] K. Gandhi, N. Sharma, P. B. Gautam, R. Sharma, B. Mann, and V. Pandey, “Centrifugation,” 2022, pp. 85–102. doi: 10.1007/978-1-0716-1940-7_3.

[36] R. Maulini, D. Sahlinal, and O. Arifin, “Monitoring of pH, amonia (NH3) and temperature parameters aquaponic water

in the 4.0 revolution era," *IOP Conf. Ser. Earth Environ. Sci.*, vol. 1012, no. 1, pp. 1–10, 2021, doi: 10.1088/1755-1315/1012/1/012087.

[37] N. M. Hussein, Y. M. Mohialden, and S. A. Salman, "Impact of IoT-based environmental monitoring on lab safety and sustainability," *Babylonian J. Internet Things*, vol. 2024, pp. 16–26, Mar. 2024, doi: 10.58496/BJIoT/2024/003.

[38] M. Grzybowski, B. Sadowski, H. Butenschön, and D. T. Gryko, "Synthetic applications of oxidative aromatic coupling—from biphenols to nanographenes," *Angew. Chemie - Int. Ed.*, vol. 59, no. 8, pp. 2998–3027, 2020, doi: 10.1002/anie.201904934.

[39] Z. Qiu, H. Zeng, and C.-J. Li, "Coupling without coupling reactions: En route to developing phenols as sustainable coupling partners via dearomatization–rearomatization processes," *Acc. Chem. Res.*, vol. 53, no. 10, pp. 2395–2413, Oct. 2020, doi: 10.1021/acs.accounts.0c00479.

[40] D. L. Golden, S. Suh, and S. S. Stahl, "Radical C(sp3)–H functionalization and cross-coupling reactions," *HHS Public Access*, vol. 6, no. 6, pp. 405–427, 2023, doi: 10.1038/s41570-022-00388-4.Radical.

[41] N. Bekavac *et al.*, "Advancements in aqueous two-phase systems for enzyme extraction, purification, and biotransformation," *Molecules*, vol. 29, no. 16, pp. 1–30, 2024, doi: 10.3390/molecules29163776.

[42] H.-M. Huang, P. Bellotti, and F. Glorius, "Merging carbonyl addition with photocatalysis," *Acc. Chem. Res.*, vol. 55, no. 8, pp. 1135–1147, Apr. 2022, doi: 10.1021/acs.accounts.1c00799.

[43] H. Choi, S. E. Suh, and H. Kang, "Advances in exploring mechanisms of oxidative phenolic coupling reactions," *Adv. Synth. Catal.*, vol. 366, no. 21, pp. 4347–4384, 2024, doi: 10.1002/adsc.202400895.

[44] J. Wu and M. C. Kozlowski, "Catalytic oxidative coupling of phenols and related compounds," *HHS Public Access*, vol. 12, no. 11, pp. 6532–6549, 2019, doi: 10.1021/acscatal.2c00318.Catalytic.

[45] S. R. Maji, C. Roy, and S. K. Sinha, "Gas chromatography-mass spectrometry (GC-MS): a comprehensive review of synergistic combinations and their applications in the past two decades," *J. Anal. Sci. Appl. Biotechnol.*, vol. 5, no. 2, pp. 72–85, 2023, doi: 10.48402/IMIST.PRSM/jasab-v5i2.40209.

[46] B. Pulver *et al.*, "Structure elucidation of the novel synthetic cannabinoid Cumyl-Tosyl-Indazole-3-Carboxamide (Cumyl-TsINACA) found in illicit products in Germany," *Drug Test. Anal.*, vol. 14, no. 8, pp. 1387–1406, Aug. 2022, doi: 10.1002/dta.3261.