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BIO-OIL CONVERSION FROM SUGARCANE BAGASSE LIGNIN UTILIZING CYTOCHROME MONOXYGENASE P450 ENZYMES

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ABSTRACT

The valorization of lignin is crucial for the economic and environmental sustainability of biorefineries. The main goal was to investigate a new P450 monooxygenase from an LNBR lignolytic consortium for transforming lignin monomers. Key steps included producing sugarcane bagasse lignin bio-oil, testing the enzyme on methoxylated monomers, and computationally analyzing enzyme-substrate interactions. This bio-oil was obtained from the hydrothermal depolymerization of sugarcane bagasse lignin (350 °C, 165 bar, 30 min, lignin/water ratio of 1:50). Both the lignin bio-oil and commercial guaiacol were tested as substrates for the enzymatic reactions. Some reaction parameters were assessed, such as the type of P450 monooxygenase (LNBR or a reference), oxygen presence, temperature, and time. The main methoxylated monomers quantified in the bio-oil were syringol, guaiacol, and 3-methoxy-catechol. The highest catechol production was 267 µM using 1 g/L of bio-oil (≈ 260 µM of guaiacol) as substrate, 50 µM of LNBR P450 monooxygenase, and 10 mM of ascorbic acid as reducing agent at 37 °C (66 h). Consumption of syringol and formation of 3-methoxy-catechol were also observed. The 3D structure of the LNBR monooxygenase enzyme was predicted with high reliability (>90%) by the AlphaFold program, except for the N-terminal region of the protein.

Keywords: Lignin. Bio-oil. P450 Monooxygenase. Demethylation. Computational Analysis.

1 INTRODUCTION

The valorization of lignin is essential for the sustainability of biorefineries. Mallinson[1] demonstrated the use of cytochrome P450 enzymes, like GcoA monooxygenase, in demethylating aromatic compounds from lignin, producing catechol from guaiacol and other derivatives based on the substrate. These compounds, including catechol, can be used in synthesizing valuable compounds like muconic acid and adipic acid, enhancing the overall value of lignin-derived products[2].

The importance of aromatic O-demethylation has spurred efforts towards discovering and characterizing enzymes capable of demethylating methoxyl groups from lignin-derived aromatics. A monooxygenase showing high identity (>70%) to the P450 system described by Mallison[1] was identified through genome mining of metagenomes from an LNBR lignolytic consortium[3]. This work aims to explore the potential of this new P450 O-demethylase enzyme system for the bioconversion of lignin aromatics.

2 MATERIAL & METHODS

The main steps of this study included (i) obtaining a bio-oil rich in aromatics from sugarcane bagasse lignin; (ii) evaluating LNBR P450 enzymes in the bioconversion of guaiacol and bio-oil; and (iii) computationally assessing the interactions between the LNBR monooxygenase and the major methoxylated aromatics from lignin bio-oil. The schematic flowchart of the experimental steps of this work is shown in **Figure 1**.

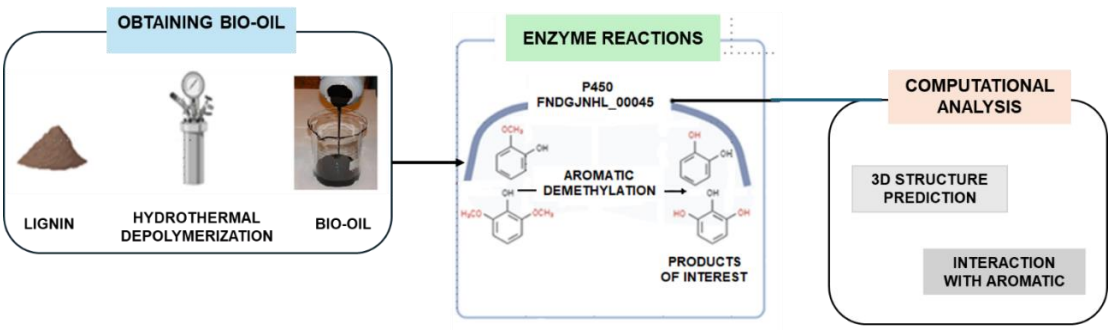


Figure 1 Representation of the main steps of this work.

Enzymatic assays for demethylation of aromatics used FNDGJNHL 00045 (LNBR consortium) and GCOA_AMYS7 (reference) monooxygenases. These enzymes were expressed in Escherichia coli in Terrific Broth medium containing 20 g/L tryptone, 24 g/L yeast extract, 5 g/L glycerol, and 90 mM potassium phosphate buffer at pH 7.5, supplemented with 500 µM thiamine, 50 µM FeCl3, and 50 mg/mL kanamycin. Cultures were grown at 37 °C until reaching an optical density of 600. After, Induction of enzyme production was achieved using δ-aminolevulinic acid (50 µM) and hemin (1.6 mM) for 18 hours at 20 °C and 250 RPM. Cell lysis was carried out with lysozyme and sonication, followed by purification on a nickel column. Some monooxygenase samples underwent additional purification on an ion exchange column to remove imidazole. The enzymatic activity of the purified enzymes was evaluated using a Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific) at 417 nm.

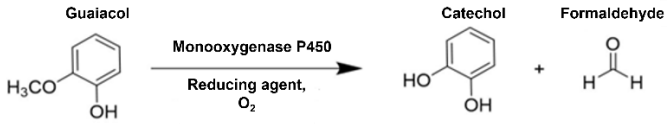
All reactions were conducted in duplicate in HEPES buffer (25 mM) and NaCl (50 mM) at pH 7.5, with agitation at 700 rpm at either 30 °C or 37 °C in a thermomixer, using 2 mL or 15 mL tubes, with or without bubbling oxygen. The substrates used were either 250 µM of commercial guaiacol or 1 g/L of sugarcane bagasse lignin bio-oil, which contained approximately 260 µM of guaiacol. Ascorbic acid and FNDGJNHL_00044 (redutase P450 LNBR) served as the electron donor for the monooxygenases. Catechol was quantified by GC-FID using the same method as for bio-oil characterization.

To ensure the comparability of computational results, both new enzymes from the lignolytic consortium of LNBR and those previously described underwent similar processing, editing, and analysis steps. DNA sequences were translated into protein sequences using web.expasy.org/translate/ to correct any translation errors. 3D structure prediction was conducted using the AlphaFold program in a Google Colab environment. The resulting structure files were downloaded for further analysis and discussion. In PyMOL, cofactors such as heme prosthetic group in monooxygenases and Fe-S, FAD, NADH, and BR in reductases were added. Docking, carried out in Dockthor, paired monooxygenases, either grouped with their respective reductases or not, with main aromatic monomers present in the bio-oil to assess interaction.

3 RESULTS & DISCUSSION

The main aromatic monomers identified in the bio-oil through CG-MS technique were quantified as syringol (1451 µM), guaiacol (1314 µM), phenol (1268 µM), 3-methoxycatechol (1235 µM), catechol (1048 µM), 4-ethylphenol (464 µM), pyrogallol (361 µM), 4-ethylguaiacol (332 µM), piceol (242 µM), and 4-methylguaiacol (213 µM). The main methoxylated monomers quantified in the bio-oil were syringol, guaiacol, and 3-methoxy-catechol. These monomers are susceptible to demethylation reactions catalyzed by P450 monooxygenases. The **Table 1** shows the main enzymatic reactions that produced catechol from guaiacol and bio-oil.

Table 1 Main enzymatic reactions that produced catechol from guaiacol or lignin bio-oil as substrate.



	P450	Substrate	Reducing agent	[Reducing agent] (mM)	T (°C)	t (h)	[c]O2	Catechol (µM)	Catechol Yield (%)
1	AMYS7	[a]Guaiacol	00044	0.05	30	18	-	6 ± 0.2	2
2	00045	Guaiacol	00044	0.05	30	18	-	5 ± 0.1	2
3	AMYS7	Guaiacol	AA	1.00	30	18	-	5 ± 0.1	2
4	00045	Guaiacol	AA	1.00	30	18	-	6 ± 0.7	2
5	AMYS7	Guaiacol	AA	10.00	30	18	-	5 ± 0.1	2
6	00045	Guaiacol	AA	10.00	30	18	-	20 ± 0.1	8
7	AMYS7	Guaiacol	AA	1.00	37	18	-	9 ± 0.1	4
8	00045	Guaiacol	AA	1.00	37	18	-	8 ± 0.4	3
9	00045	Guaiacol	AA	10.00	37	18	-	14 ± 1.0	6
10	00045	Guaiacol	AA	50.00	37	18	-	4 ± 0.1	2
11	00045	Guaiacol	AA	10.00	37	1	-	2 ± 0.1	1
12	00045	Guaiacol	AA	10.00	37	3	-	4 ± 0.8	2
13	00045	Guaiacol	AA	10.00	37	18	-	19 ± 9.0	8
14	00045	[b]Bio-oil	AA	10.00	37	18	-	59 ± 5.0	24
15	00045	Guaiacol	AA	10.00	37	64	+	52 ± 19.0	21
16	00045	Bio-oil	AA	10.00	37	64	+	267 ± 17.0	≈100

AMYS7: GCOA_AMYS7 (P450 monooxygenase from literature); 00045: FNDGJNHL_00045 (P450 monooxygenase from LNBR); 00044: FNDGJNHL_00044 (redutase P450 LNBR); AA: ascorbic acid. [a]Guaiacol concentration used was 250 µM; [b]Bio-oil concentration was 1 g/L containing approximately 260 µM of guaiacol; [c]Pure oxygen injection with atmospheric exchange for 15 minutes, immediately before the start of the reaction.

Analyzing the results of Table 1 we observed that varying the monooxygenase in reactions 5 and 6 showed that the LNBR monoxygenase outperformed the reference enzyme, producing 20 µM compared to 5 µM. The ascorbic acid performance with the LNBR monooxygenase was first assessed in reactions 2 and 4, where ascorbic acid slightly outperformed the LNBR reductase,

producing 6 µM of catechol compared to 5 µM. However, increasing the ascorbic acid concentration from 1 in reaction 4 to 10 in reaction 86 significantly favored catechol production, reaching 20 µM. Further analysis between reactions 6 and 10 showed that higher concentrations (50 mM) of ascorbic acid as a reducing agent reduced catechol production, decreasing from 20 µM to 4 µM. Based on these results, the use of the 00045 monooxygenase with 10 mM ascorbic acid as the reducing agent and a temperature of 37 °C was established. By varying the time (1, 3, 18, and 64 h), an increase in catechol production from 2 to 52 µM was observed in reactions 11, 12, 13, and 15. When using bio-oil as a substrate (reactions 12 and 14), we observed higher yields compared to reactions using guaiacol as the substrate (reactions 11 and 13). Reaction 13, using the LNBR consortium monooxygenase and a solution of 1 g/L of bio-oil as the substrate, achieved a yield of catechol from guaiacol present in the bio-oil that exceeded 100%. This suggests that other reactions might be occurring, potentially catabolizing other monomers into catechol, as well as transforming syringol into 3-methoxycatechol.

The translation of nucleotide sequences into protein sequences was performed accurately, and no deviation or error was detected when comparing the resulting sequences. The study successfully predicted the 3D structures of enzymes from the lignolytic consortium of LNBR using AlphaFold2, achieving high reliability scores above 90 on the pLDDT scale (**Figure 2**). The predicted structures of new proteins from LNBR were assigned scores of 94.8 (monooxygenase) and 92.2 (reductase), considered very good compared to previously reported enzymes. Molecular docking analyses were conducted to investigate the interaction of a new protein (00045) and compared with reference enzymes. Enzyme FNDGJHNL_00045 showed more favorable affinity values and electrostatic interactions compared to GcoA_AMYS7. Catechol and 3-methoxycatechol, products of lignin demethylation, exhibited the best interaction profiles with FNDGJNHL_00045, while guaiacol, a demethylation substrate, showed favorable energy profiles with GcoA_AMYS7.

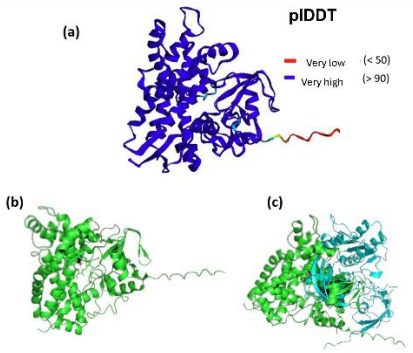


Figure 2 Graphical representation of the predicted structure in AlphaFold colored according to the model confidence score per residue (plDDT); (b) 3D structure of the LNBR monooxygenase FNDGJNHL_00045 visualized in the PyMOL program; (c) 3D complex formed by the LNBR monooxygenase FNDGJNHL_00045 and LNBR reductase FNDGJNHL_00044 visualized in PyMOL.

4 CONCLUSION

The bio-oil produced contained significant quantities of methoxylated monomers relevant for bioconversion, including syringol, guaiacol, and 3-methoxy-catechol. The highest catechol yield reached 267 µM using 1 g/L of bio-oil (approximately 260 µM of guaiacol), 50 µM of LNBR P450 monooxygenase, and 10 mM of ascorbic acid as a reducing agent at 37 °C over 64 hours. LNBR monooxygenase FNDGJNHL_00045 has shown promise for lignin bioconversion, with accurate prediction demonstrating a strong affinity with bio-oil monomers.

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