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Article

Biosynthesis of Benzylic Derivatives in the Fermentation Broth of the Edible Mushroom, *Ischnoderma resinosum*

Purni C. K. Wickramasinghe and John P. Munafo, Jr.*

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ABSTRACT: Employing isotope incubation studies, the biosynthetic pathway leading to a series of benzylic derivatives was elucidated in the fermentation broth of the edible mushroom *Ischnoderma resinosum* (P. Karst). Twenty-six hydroxy- and methoxy-benzylic derivatives were screened by gas chromatography−mass spectrometry (GC−MS) of which 13 were detected in the culture media. Results from the isotope incubation studies showed the transformation of both benzyl alcohol and benzoic acid into benzaldehyde. Benzaldehyde was then converted into 4-methoxybenzaldehyde via hydroxylation and subsequent methylation of the 4-C position. The resulting 4-methoxybenzaldehyde was then hydroxylated in the 3-C position followed by methylation into 3,4-dimethoxybenzaldehyde. Based on these findings, a novel metabolic scheme for the biosynthesis of benzylic derivatives in *I. resinosum* was proposed. The knowledge of the biosynthetic pathway was utilized to produce 4-hydroxy-3-methoxybenzaldehyde (vanillin) from 4-hydroxy-3-methoxybenzoic acid (vanillic acid). This is the first report to elucidate the biosynthetic pathway of benzyl derivatives and production of vanillin from *I. resinosum*.

KEYWORDS: *I. resinosum, biosynthesis, benzaldehyde, 4-methoxybenzaldehyde, 3,4-dimethoxybenzaldehyde, vanillin*

INTRODUCTION

The demand for natural flavor compounds has grown in recent years due to increased consumer preference for products of natural origin. Although plants were considered the major source of flavor compounds historically, the difficult and expensive nature of isolation, as well as low yields, make natural flavor production from plants a less sustainable option than microbial fermentation. These circumstances have led to the utilization of fungi, such as white-rot basidiomycetes, as a viable alternative for biotechnological production of natural flavor compounds.[1,2] Natural flavor compounds are defined as compounds obtained from living cells, including food-grade microorganisms and their enzymes according to United States and European legislature.[3,4] For example, synthetic 4-hydroxy-3-methoxybenzaldehyde (vanillin) is primarily used in the food and flavor industry due to the high cost of natural vanillin production.[5] However, in recent years, the consumer demand for naturally derived ingredients has stimulated the search for alternative means of natural vanillin production.[6−10] An alternative source to produce natural vanillin is white-rot fungi. For example, *Phanerochaete chrysosporium* can convert ferulic acid to vanillin that can be marketed as "natural vanillin" due to its definition in the United States and European legislature.[3,4,11,12]

Fungi are a source of compounds with food, flavor, and pharmaceutical applications. Examples of compounds with commercial applications include benzyl alcohol and benzaldehyde from *Bjerkundera adusta*, *Ischnoderma benzoinum*, *Dichomitus squalens*, *Polyporus tuberaster* as well as 4-methoxybenzyl compounds from *Bjerkundera* and *Pleurotus* spp.[2,6,11] *Ischnoderma resinosum* (P. Karst), a ligninolytic fungus, belongs to the order Polyporales and family Ischnodermataceae (Jülich).[13] *Ischnoderma resinosum* is capable of fermenting natural substrates, such as L-phenylalanine or tyrosine, to produce a wide variety of flavor compounds.[14,15] Benzylic derivatives including benzaldehyde, 4-methoxybenzaldehyde, and 3,4-dimethoxybenzaldehyde were previously reported in the *I. resinosum* fermentation broth with a distinctively pleasant "candy-like" odor in our laboratory.[16]

Fungal benzylic derivatives are enzyme-dependent and species-specific.[6,17−19] For example, peroxidase enzymes, including lignin peroxidase (LiP) and manganese peroxidase (MnP), have been identified in a wide variety of fungi, including *I. benzoinum*.[20] These enzymes are involved in the biosynthesis of hydroxylated benzylic derivatives.[19] Furthermore, enzymes that oxidize aryl alcohols, including benzyl, 4-methoxybenzyl, and 3,4-dimethoxybenzyl alcohols, to their corresponding aldehydes such as aryl-alcohol oxidase (AAO) and laccase have been characterized in fungi.[21,22] Other intracellular dehydrogenase enzymes such as aryl-aldehyde dehydrogenase (AADD) and aryl-alcohol dehydrogenase (AAD), which reduce aromatic acids to aldehydes and aromatic aldehydes to alcohols, have also been identified in fungi such as *Pleurotus eryngii*.[23,24]

While biosynthetic pathways for the production of benzylic derivatives in other basidiomycetes have been investigated in

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the past, there have been no studies determining the biosynthetic pathway for the benzylic derivative production in *I. resinosum*.[25,26] Therefore, the aim of this study was to elucidate the biosynthetic pathway to produce benzaldehyde, 4-methoxybenzaldehyde, and 3,4-dimethoxybenzaldehyde in a fermentation broth. In addition, this study also aimed to utilize the knowledge of the biosynthetic pathway in production of natural benzylic derivatives, using production of commercially valuable natural vanillin as one example. Accordingly, the objectives were to; (1) cultivate the fungus in liquid broth and incubate the fermentations with isotopically labeled precursors, (2) elucidate the biosynthetic pathway using labeled isotope studies coupled with gas chromatography−mass spectrometry (GC−MS), and (3) utilize the knowledge of the biosynthetic pathway to produce natural vanillin via fermentation.

MATERIALS AND METHODS

Microorganism. The strain, UT-PW019, used in a previous study, was isolated from a mature basidiocarp. Internal transcribed spacer (ITS) sequencing results and phenotypical characteristics led to the identification of UT-PW019 as *I. resinosum*.[16] The fungal isolate was decontaminated using 10% bleach solution for 10 min and sterile deionized water for 15 min. Upon decontamination, the specimen was cultured on Petri dishes containing potato dextrose agar (PDA) (Himedia, India).[16] Fermentations were maintained at 25 °C in a MIR-254 cooled incubator (Panasonic Healthcare Co., Ltd., Japan). Species confirmation for UT-PW019 was conducted via internal transcribed spacer (ITS) sequencing with primers, ITS 4 (5′-TCCTCCGCTTATTGATATGC) and ITS 5 (5′-GGAAG-TAAAAGTCGTAACAAGG) as described previously.[16,19] Fungal isolates were cryopreserved at −80 °C in potato dextrose broth (PDB) (Himedia, India), supplemented with glycerol (10%, v/v) as frozen agar plugs using a Mr. Frosty freezing container (Thermo Fisher Scientific, Fair Lawn, NJ) for future use. The ITS sequence was deposited in the national center for biotechnology information (NCBI) database under the GenBank accession number, MN633306.

Medium and Culture Conditions. Erlenmeyer flasks containing PDB (85 mL) were inoculated with 7-day-old mycelia grown on PDA homogenized in 100 mL of sterile deionized water (1 mL). Fermentations were maintained aerobically at 25 °C and 120 rpm on an advanced digital shaker (VWR, Radnor, PA).

Reference Compounds. Reference standards **1**−**26** (Table 1) purchased from Sigma-Aldrich (St. Louis, MO) were used to identify putative precursor and intermediate compounds involved in the biosynthesis of benzylic derivatives in fungal fermentations. Each compound was identified using mass spectra of authentic reference standards as well as retention indices (RI) on both FFAP and DB-5 columns. A mixture of *n*-alkanes (C9−C40) was analyzed using GC−MS to obtain retention time for each hydrocarbon to calculate linear retention indices.

Isotopically Labeled Compounds. ($^2H_5$)-**2**, ($^2H_5$)-**3**, ($^2H_3$)-**5**, ($^2H_5$)-**8**, and ($^2H_3$)-**25** were purchased from C/D/N isotopes (Quebec, Canada), and ($^2H_6$)-**7** was purchased from aromaLAB (Planegg, Germany). Positions of isotopic labels are as shown in Figures 1, 3−6. Isotopically labeled compounds were dissolved in 5 mL volumetric flasks containing freshly distilled diethyl ether. Compounds **2**, **3**, **8**, and **25** were dissolved at known concentrations, whereas compounds **5** and **7** were quantitated using isotopically unmodified compounds as reference standards. The ions used for each compound were as follows (unlabeled/labeled standard): **2**, *m*/*z* 105/110; **3**, *m*/*z* 79/84; **5**, *m*/*z* 135/138; **7**, *m*/*z* 166/172; **8**, *m*/*z* 105/110; **25**, *m*/*z* 151/154.

Solvents and Other Chemicals. Chromatographic-grade diethyl ether and pentane were obtained from Honeywell Burdick & Jackson (Muskegon, MI) and Millipore Sigma (St. Louis, MO), respectively. Solvents were distilled in-house using a 250 mL CG-1233 series distillation head from Chemglass Life Sciences (Vineland, NJ) prior to use. Anhydrous sodium sulfate was purchased from Fisher Scientific (Fair Lawn, NJ). A mixture of *n*-alkanes C9−C18 was obtained from Phenomenex (Torrance, CA), and individual *n*-alkanes C19−C26 as well as a mixture of *n*-alkanes C21−C40 were purchased from Millipore Sigma (St. Louis, MO).

Table 1. List of Putative Precursor and Intermediate Compounds Produced in Fungal Fermentations and Their Linear Retention Indices

no.[a]	compound[b]	RI[c] FFAP	RI[c] DB-5
1	anisole*	1275	921
2	benzaldehyde*	1569	960
3	benzyl alcohol*	1829	1078
4	phenol*	1973	988
5	4-methoxybenzaldehyde*	1982	1254
6	4-methoxybenzyl alcohol*	2256	1282
7	3,4-dimethoxybenzaldehyde*	2397	1479
8	benzoic acid*	2405	1168
9	4-hydroxy-3-methoxybenzaldehyde	2552	1400
10	3,4-dimethoxybenzyl alcohol*	2613	1500
11	3-hydroxybenzaldehyde	2679	1371
12	4-hydroxy-3-methoxybenzyl alcohol	2694	1450
13	4-methoxybenzoic acid*	2751	1225
14	3,4-dihydroxybenzaldehyde	2755	2487
15	3-hydroxy-4-methoxybenzaldehyde*	2758	1455
16	3-hydroxy-4-methoxybenzyl alcohol	2768	1463
17	4-hydroxybenzaldehyde*	2839	1362
18	4-hydroxybenzyl alcohol	2849	1345
19	3-hydroxybenzyl alcohol	2868	1349
20	3-hydroxy-4-methoxybenzoic acid	2894	1590
21	3,4-dihydroxybenzyl alcohol	2984	2363
22	3,4-dimethoxybenzoic acid*	3085	1591
23	3-hydroxybenzoic acid	3158	2340
24	3,4-dihydroxybenzoic acid	3442	2523
25	4-hydroxy-3-methoxybenzoic acid	3497	2179
26	4-hydroxybenzoic acid	3657	2374

[a]Compounds numbered per retention time on FFAP column. [b]Identified by comparing retention indices on FFAP and DB-5 columns. [c]Linear retention index (RI). *Indicates compound was detected in fungal fermentations.

Isotope Incubation Studies. Two fungal fermentation broth cultures (75 g) were incubated with 1 mL of isotopically labeled and unlabeled precursor compounds (1000 ppm) prepared in diethyl ether at 25 °C and 120 rpm for a designated number of additional days on an advanced digital shaker (VWR, Radnor, PA). The isotope used, culture age at addition/isotope incubation time is as follows: ($^2H_5$)-**2**, 1/5 days; ($^2H_5$)-**2**, 1/12 days; ($^2H_5$)-**3**, 1/5 days; ($^2H_5$)-**3**, 1/12 days; ($^2H_3$)-**5**, 7/17 days; ($^2H_6$)-**7**, 7/14 days; ($^2H_5$)-**8**, 1/5 days; ($^2H_5$)-**8**, 1/12 days; and ($^2H_3$)-**25**, 7/14 days. The fermentation broth without the biomass from each incubation was then sequentially extracted with freshly distilled diethyl ether (150 mL) on an autoshaker (VWR) at ambient temperature for 10 min. Upon extraction, the fermentations were centrifuged using a Sorvall RC5B plus refrigerated centrifuge (Marshall Scientific, Hampton, NH) operated at 2489 × *g* for 5 min and supernatants were collected. Organic layers were isolated using a separatory funnel. The final extracts were subjected to high vacuum distillation using solvent assisted flavor evaporation (SAFE) maintained at 10^{-3} Pa, as described previously.[16] Briefly, each sample was gradually released into the temperature-controlled evaporation flask (41 °C) until completion. Upon completion, the distillate was thawed at room temperature and dried over anhydrous sodium sulfate. Final samples were then concentrated to ∼2 mL using a Vigreux column (50 × 1 cm^2) and to 200 μL under a gentle stream of nitrogen as described previously.[16] High vacuum distillates were analyzed using GC−MS.

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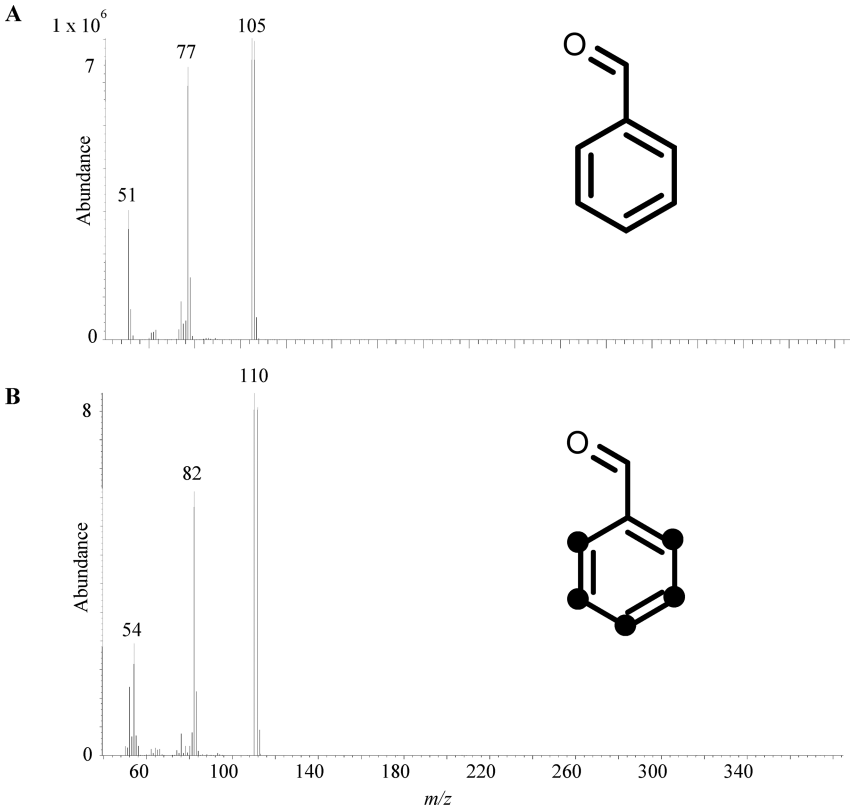

Figure 1. EI-MS spectra of (a) unlabeled and (b) labeled benzaldehyde produced when fermentations were incubated with ($^2H_5$)-benzyl alcohol.

Table 2. List of Isotopically Labeled Precursor Compounds, Corresponding Isotopically Labeled Products and Major Ions Present in Their Electron Ionization-Mass Spectra (EI-MS) Identified after Incubation Studies

no.[a]	labeled precursor[b]	labeled product[c]	unlabeled ion[d]	labeled ion[e]
2	$^2H_5$ -**2**	$^2H_5$-benzyl alcohol	108	113
		$^2H_4$-4-hydroxybenzaldehyde	121	125
		$^2H_4$-4-methoxybenzaldehyde	135	139
		$^2H_3$-3,4-dimethoxybenzaldehyde	166	169
3	$^2H_5$ -**3**	$^2H_5$-benzaldehyde	105	110
		$^2H_5$-benzoic acid	105	110
		$^2H_4$-4-methoxybenzaldehyde	135	139
		$^2H_3$-3,4-dimethoxybenzaldehyde	166	169
5	$^2H_3$ -**5**	$^2H_3$-4-methoxybenzyl alcohol	138	141
		$^2H_3$-3-hydroxy-4-methoxybenzaldehyde	151	154
		$^2H_3$-3,4-dimethoxybenzaldehyde	166	169
7	$^2H_6$ -**7**	$^2H_6$-3,4-dimethoxybenzyl alcohol	168	174
8	$^2H_5$ -**8**	$^2H_5$-benzaldehyde	105	110
		$^2H_4$-4-methoxybenzaldehyde	135	139
		$^2H_3$-3,4-dimethoxybenzaldehyde	166	169
25	$^2H_3$ -**25**	$^2H_3$-4-hydroxy-3-methoxybenzaldehyde	151	154
		$^2H_3$-4-hydroxy-3-methoxybenzyl alcohol	154	157

[a]Compounds numbered per retention indices on FFAP column. [b]Isotopically labeled precursor compounds used. [c]Isotopically labeled products detected upon incubation. [d]Major ion detected in the EI-MS spectra of the unlabeled product. [e]Major ion detected in the EI-MS spectra of the labeled product after incubation studies.

Each incubation experiment was performed in at least triplicate. Incubation studies with each isotopically labeled compound were designed based on a 30-day time course study evaluating the production of benzaldehyde, 4-methoxybenzaldehyde, and 3,4-dimethoxybenzaldeyde and conducted as described previously.[16]

Gas Chromatography–Mass Spectrometry (GC–MS). An Agilent gas chromatograph (7820A series) coupled with an Agilent mass spectrometer detector (5977B) was used for GC–MS analysis. A Zebron ZB-FFAP GC capillary column from Phenomenex and a HP-5MS capillary column from Agilent Technologies (both, 30 m × 0.25 mm o.d. × 0.25 μm film thickness) were used for separations. On-column injections of concentrated high-vacuum distillates (1 μL) were made using an autosampler equipped with a 5 μL syringe at an initial oven temperature of 35 °C. Helium gas at a constant flow rate of 1 mL/min was used as the carrier gas. After the sample was injected, oven temperature was increased to 60 °C at a 60 °C/min

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rate, then ramped-up to 250 °C at a 6 °C/min rate, and held for 5 min. The MS was operated in electron impact (EI) ionization mode at 70 eV with a scan range of m/z 50−350. The MS source and MS quadrupole were maintained at 230 and 150 °C, respectively.

RESULTS AND DISCUSSION

Identification of Benzylic Precursors and Intermediates. Prior to investigating the biosynthetic pathway, 26 potential precursors and intermediate compounds prepared in diethyl ether (10 ppm) were subjected to GC−MS. These compounds were used as analytical standards to screen for metabolites involved in the biosynthetic pathway.[16] Out of the 26 reference standards, 13 compounds including **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, **10**, **13**, **15**, **17**, and **22** were identified in the fermentations.

Isotope Incubation Studies. Isotope incubation studies conducted using putative benzylic derivative precursors ($^2H_5$)-**2**, ($^2H_5$)-**3**, ($^2H_3$)-**5**, ($^2H_6$)-**7**, ($^2H_5$)-**8**, and ($^2H_3$)-**25** led to the identification of isotopically labeled intermediate compounds involved in the biosynthetic pathway (Table 2).

($^2H_5$)-Benzyl Alcohol. Isotopically labeled benzaldehyde was detected in the 5-day old incubations with ($^2H_5$)-benzyl alcohol. Findings suggested that benzyl alcohol is a precursor to benzaldehyde (Figures 1−3). Aryl-alcohol oxidase (AAO)

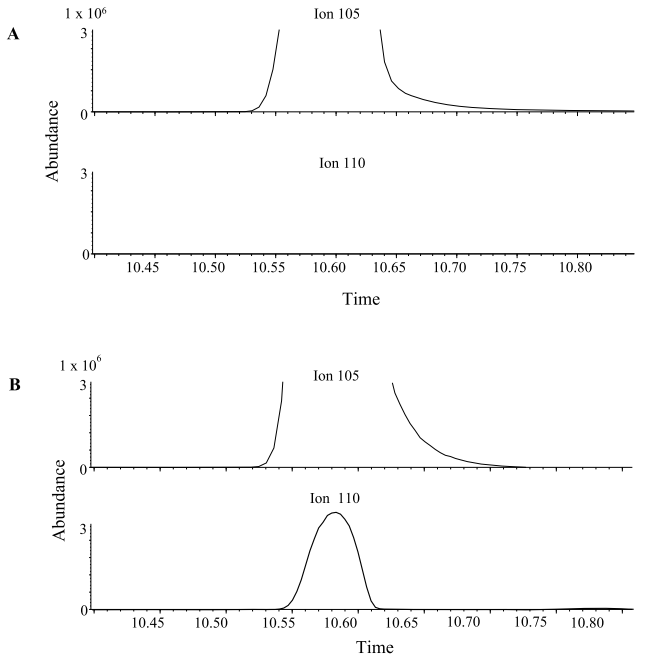

Figure 2. Extracted ion chromatograms of (a) unlabeled and (b) labeled benzaldehyde produced when incubated with ($^2H_3$)-benzyl alcohol.

has been reported to convert benzyl alcohol into benzaldehyde via oxidation.[21] The high-vacuum distillates prepared from the incubations with unlabeled benzyl alcohol were used as a control to ensure that the fungal growth was not inhibited due to the carrier or the elevated compound concentrations. Furthermore, the incubation with the unlabeled benzyl alcohol also served as a control to evaluate the production of isotopically labeled intermediates in the experimental incubations with labeled benzyl alcohol. However, it is worth noting that incubations with unlabeled benzyl alcohol still produced unlabeled benzaldehyde, as *I. resinosum* has

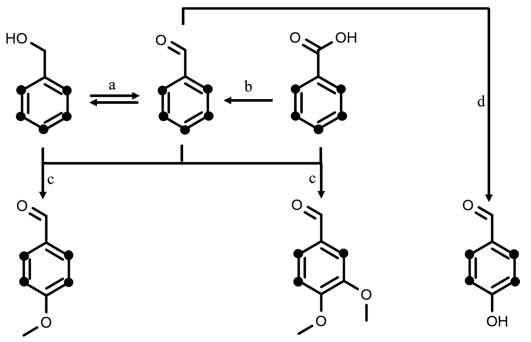

Figure 3. Conversions including, isotopically labeled (a) benzyl alcohol to benzaldehyde, and vice versa, (b) benzoic acid to benzaldehyde and benzyl alcohol, (c) benzyl alcohol, benzoic acid, and benzaldehyde to 4-methoxybenzaldehyde and 3,4-dimethoxybenzaldehyde, and (d) benzaldehyde to the transient intermediate 4-hydroxybenzaldehyde were observed.

previously reported to be able to produce benzaldehyde de novo[6,16] (Figures 1 and 2).

Additionally, results from the 12-day old incubations with ($^2H_5$)-benzyl alcohol revealed the presence of isotopically labeled 4-methoxybenzaldehyde and 3,4-dimethoxybenzaldehyde, suggesting that benzyl alcohol is a precursor of these downstream benzylic derivatives (Figure 3).

($^2H_5$)-Benzoic Acid. Isotopically labeled benzaldehyde and benzyl alcohol were identified when the incubations with ($^2H_5$)-benzoic acid were analyzed after 5 days. These findings suggested that benzoic acid is a precursor for both benzaldehyde and benzyl alcohol (Figure 3). A previous study showed that intracellular dehydrogenases, such as aryl-aldehyde dehydrogenase (AADD), reduced aromatic acids to their corresponding aldehydes in another fungus, *P. eryngii*.[22,24] Furthermore, the presence of isotopically labeled benzyl alcohol also suggested that benzaldehyde is then reduced to its corresponding alcohol as observed in *P. eryngii*, potentially using aryl-alcohol dehydrogenase (AAD).[21] Isotopically labeled 4-methoxybenzaldehyde and 3,4-dimethoxybenzaldehyde were also detected when incubations were extended to 12 days. These results support benzoic acid is a precursor of downstream benzylic derivatives (Figure 3).

($^2H_5$)-Benzaldehyde. The analysis of the incubations with ($^2H_5$)-benzaldehyde after 5 days led to the detection of isotopically labeled benzyl alcohol and 4-hydroxybenzaldehyde (Figure 3). The presence of labeled benzyl alcohol when incubated with the respective aldehyde suggested the possible presence of a mechanism involving the joint activity of AAO and AAD, converting alcohol into aldehyde and vice versa as previously reported.[25] In addition, isotopically labeled 4-hydroxybenzaldehyde was identified in the high vacuum distillate. A previous study reported that fungi produced an enzymatic complex involved in lignin biodegradation including lignin peroxidase (LiP).[25] This enzyme has been identified to be responsible for the biosynthesis of hydroxylated aldehydes including 4-hydroxybenzaldehyde from benzaldehyde as an intermediate in the biosynthetic pathway, in a wide variety of white-rot fungi, potentially including *I. resinosum*.[20]

Interestingly, 4-hydroxybenzaldehyde was not observed in incubations analyzed after 12 days, indicating that 4-hydroxybenzaldehyde is more likely an intermediate in production of 4-methoxybenzaldehyde and downstream benzylic derivatives. To the best of our knowledge, this is

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the first study to report the presence of 4-hydroxybenzaldehyde in *I. resinosum* fermentations, though 4-hydroxybenzaldehyde has been previously identified as an intermediate in the L-phenylalanine degradation pathway of *Bjerkandera adusta*.[25] While both 4-hydroxybenzyl alcohol and 4-hydroxybenzoic acid were also identified in *B. adusta* fermentations, they were not detected during the current study.[25] These results suggested that 4-hydroxybenzaldehyde gets methylated to 4-methoxybenzaldehyde due to the potential activity of 4-*O*-methyltransferase as reported previously.[27] Studies have shown that 4-C methylation of hydroxyl groups is important, as free-hydroxyl phenolic compounds are known to inhibit LiP activity.[28] Therefore, *O*-methyltransferases, such as 4-*O*-methyltransferase, are known to accelerate lignin degradation in white-rot fungi by converting the inhibitory groups into less toxic methoxylated phenols.[28] Both isotopically labeled 4-methoxybenzaldehyde and 3,4-dimethoxybenzaldehyde were identified in extended incubations after 12 days, indicating that benzaldehyde is a precursor to downstream benzylic derivatives.

($^2H_3$)-4-Methoxybenzaldehyde. When the incubations with ($^2H_3$)-4-methoxybenzaldehyde were analyzed, isotopically labeled 4-methoxybenzyl alcohol, 4-methoxy-3-hydroxybenzaldehyde, and 3,4-dimethoxybenzaldehyde were identified (Figure 4). The presence of labeled 4-methoxybenzyl alcohol

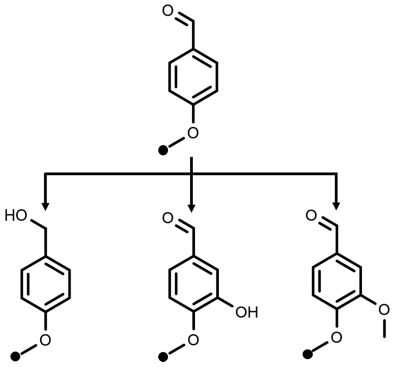

Figure 4. Isotopically labeled 4-methoxybenzaldehyde was converted to 4-methoxybenzyl alcohol, 3-hydroxy-4-methoxybenzaldehyde, and 3,4-dimethoxybnzaldehyde.

suggested that AAD may convert 4-methoxybenzaldehyde into 4-methoxybenzyl alcohol. While no incubations with isotopically labeled 4-methoxybenzoic acid were conducted during this study, unlabeled 4-methoxybenzoic acid was detected during the initial screening of potential precursor and intermediate compounds. The presence of unlabeled 4-methoxybenzoic acid suggested that AADD may convert 4-methoxybenzoic acid into 4-methoxybenzaldehyde similarly to benzoic acid conversion to benzaldehyde.[25]

Isotopically labeled 3-hydroxy-4-methoxybenzaldehyde was also identified. While no studies have previously identified 3-hydroxy-4-methoxybenzaldehyde in *I. resinosum*, current findings suggested that 3-hydroxy-4-methoxybenzaldehyde is an intermediate in the conversion of 4-methoxybenzaldehyde to 3,4-dimethoxybenzaldehyde. Harper et al. identified a 3-*O*-methyltransferase, responsible for 3-*O*-methylation of 3-hydroxy-4-methoxybenzoic acid by *S*-adenosylmethionine (SAM) in *P. chrysosporium*.[29] 3-*O*-methyltransferase is regiospecific, only methylating the 3-hydroxyl group of several benzoic acids and aldehydes substituted with a hydroxy or a methoxy substituent at the 2-C or 4-C position.[29] 3-*O*-methyltransferase was also shown to be induced later than 4-*O*-methyltransferase in the fungal growth cycle.[27]

Isotopically labeled 3,4-dimethoxybenzaldehyde was also detected in the incubations. While enzymatic conversion of 4-methoxybenzaldehyde into 3,4-dimethoxybenzaldehyde has been reported previously, intermediaries involved in the pathway have not been reported.[25] This is the first study to observe the conversion of 4-methoxybenzaldehyde into 3,4-dimethoxybenzaldehyde via methylation of the intermediate 3-hydroxy-4-methoxybenzaldehyde in *I. resinosum*.

($^2H_6$)-3,4-Dimethoxybenzaldehyde. Isotopically labeled 3,4-dimethoxybenzyl alcohol was identified when incubations with ($^2H_6$)-3,4-dimethoxybenzaldehyde were analyzed (Figure 5.). Intracellular AAD, known to reduce 3,4-dimethoxy-

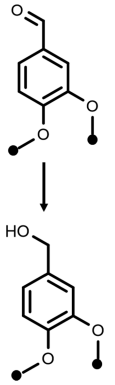

Figure 5. Isotopically labeled 3,4-dimethoxybenzaldehyde was converted into 3,4-dimethoxybenzyl alcohol.

benzaldehyde to 3,4-dimethoxybenzyl alcohol using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor, was previously purified.[20,21,24,30] Another report demonstrated that AAD is able to reduce aryl aldehydes (benzyl-, 4-methoxybenzyl-, and 3,4-dimethoxybenzyl- alcohols) into their corresponding alcohols, similarly to current findings.[31,32] Unlabeled 3,4-dimethoxybenzoic acid was identified in the fermentations during precursor identification studies. While no incubation studies with isotopically labeled 3,4-dimethoxybenzoic acid were performed, findings from previous studies support that the AADD may convert 3,4-dimethoxybenzoic acid into 3,4-dimethoxybenzaldehyde in the fermentations.[25]

($^2H_3$)-4-Hydroxy-3-methoxybenzoic Acid (Vanillic Acid). Fermentations did not appear to produce vanillin de novo. However, 3-hydroxy-4-methoxybenzaldehyde (isovanillin) was detected in the fermentations leading to the conclusion that the fungus is able to add a hydroxyl group at the 3-C position when the 4-C position is methoxylated and not vice versa. One potential mechanism of vanillin production in fungal fermentations includes methylation of the 3-C hydroxyl group in 3,4-dihydroxybenzaldehyde, alcohol, or acid via the activity of the metaspecific 3-*O*-methyltransferase.[29] Another potential mechanism of vanillin production includes the conversion of 3-hydroxybenzaldehyde, alcohol, or acid into their corresponding dihydroxybenzyl derivative via hydroxylation at the 4-C position followed by methylation at the 3-C position.[30] However, none of the potential precursors, including 3,4-dihydroxybenzaldehyde, 3,4-dihydroxybenzyl alcohol, 3,4-dihydroxybenzoic acid, 3-hydroxybenzaldehyde, 3-hydroxybenzyl alcohol, and 3-hydroxybenzoic acid, were

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detected during the precursor identification studies. Therefore, we concluded that the fermentations are limited in vanillin production due to the lack of precursor compounds in the early stages of the biosynthetic pathway. No studies linking the site-specific activity of LiP preferring the 4-C position over the 3-C position could be found in the literature.

The fungus's ability to convert aromatic acids into their corresponding aldehydes in combination with the successful methylation of the 3-C hydroxyl group led to the evaluation of vanillic acid to vanillin conversions in the fermentations. When the incubation with ($^2H_3$)-4-hydroxy-3-methoxybenzoic acid was analyzed, both isotopically labeled vanillin and vanillyl alcohol were detected (Figure 6). A previous study reported

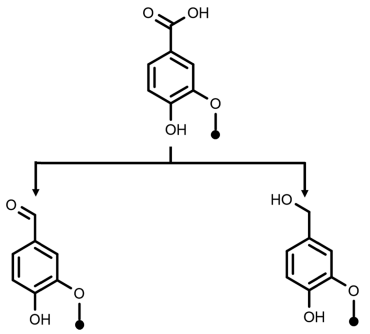

Figure 6. Isotopically labeled vanillic acid was converted to both vanillin and vanillyl alcohol.

that *Pycnoporus cinnabarinus* was able to successfully produce natural vanillin from vanillic acid. The study utilized natural vanillic acid produced by *Aspergillus niger* via the conversion of ferulic acid, thereby creating a two-step bioreactor system to produce natural vanillin.[33] This current study demonstrates natural vanillin production from *I. resinosum* via vanillic acid fermentation. This suggests that vanillin may be produced through the co-cultivation of vanillic acid producing fungi with *I. resinousm*. Studies are currently underway on co-cultivation, growth dynamics, and production of benzylic derivatives among different strains of *I. resinosum* and will be published separately.

In conclusion, this is the first study to describe the biosynthetic pathway for benzylic derivatives in *I. resinosum* fermentation. Results from the study demonstrated conversion of both benzyl alcohol and benzoic acid into benzaldehyde, as well as benzaldehyde into 4-methoxybenzaldehyde via hydroxylation and subsequent methylation at the 4-C position. The resulting 4-methoxybenzaldehyde then transformed to 3,4-dimethoxybenzaldehyde via subsequent hydroxylation and methylation at the 3-C position. Based on the available literature, it is postulated that the benzylic derivatives may be hydroxylated and/or methoxylated by intracellular enzymatic activities of LiP, 4-*O*-methyltransferase, 3-*O*-methyltransferase, AAD, AADD, and extracellular activities of AAO. However, additional studies are required to support these hypotheses. Based on isotope incubation studies, a novel biosynthetic pathway for production of benzylic derivatives in *I. resinosum* was proposed (Figure 7). The knowledge of the biosynthetic pathway was utilized to produce vanillin from vanillic acid. This study provides a foundation for future research investigating the biosynthesis and potential applicability for the commercial production of compounds with flavor and pharmaceutical applications.

AUTHOR INFORMATION

Corresponding Author

John P. Munafo, Jr. − *Department of Food Science, The University of Tennessee, Knoxville, Tennessee 37996, United States;* orcid.org/0000-0003-4702-2955; Phone: 865-974-7247; Email: jmunafo@utk.edu; Fax: 865-974-7332

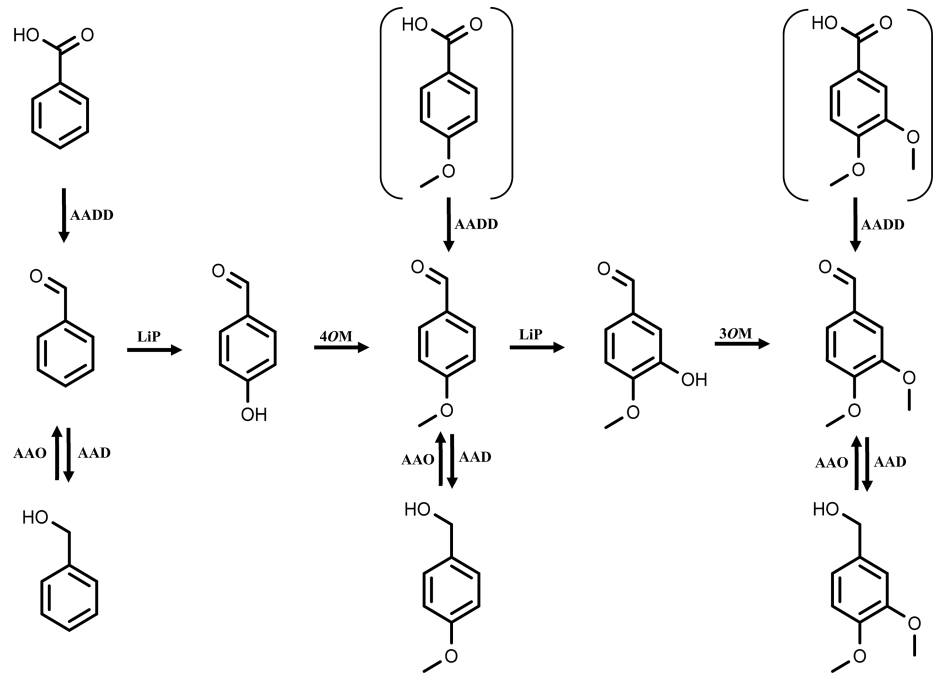

Figure 7. Proposed biosynthetic pathway of major benzylic derivatives produced by *I. resinosum*. Compounds within brackets are putative intermediates. Aryl-alcohol oxidases (AAO), aryl-alcohol dehydrogenase (AAD), aryl-aldehyde dehydrogenase (AADD), lignin peroxidase (LiP), 3-*O*-methyltransferase (3*O*M), and 4-*O*-methyltransferase (4*O*M) are all potentially involved in the biosynthetic pathway.

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Author

Purni C. K. Wickramasinghe − *Department of Food Science, The University of Tennessee, Knoxville, Tennessee 37996, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.9b07218

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

FFAP, free fatty acid phase; GC−MS, gas chromatography−mass spectrometry; ITS, internal transcribed spacer; NCBI, national center for biotechnology information; PCR, polymerase chain reaction

■ NOMENCLATURE

L-phenylalanine, (2*S*)-2-amino-3-phenylpropanoic acid; tyrosine, (2)-2-amino-3-(4-hydroxyphenyl)propanoic acid; vanillic acid, 3-methoxy-4-hydroxybenzoic acid; vanillyl alcohol, 3-methoxy-4-hydroxybenzyl alcohol; vanillin, 3-methoxy-4-hydroxybenzaldehyde

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