Fermentation Dynamics and Benzylic Derivative Production in *Ischnoderma resinosum* Isolates

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ABSTRACT: Fermentation dynamics and benzylic derivative production were evaluated in the fermentation broth of six different *Ischnoderma resinosum* (P. Karst) isolates over a period of 30 days to understand their potential applications in bioreactor optimization for natural flavor compound production. D-Glucose and D-fructose levels decreased from 20.4 ± 0.4 to 7.1 ± 1.4 g/L and 1.0 ± 0.1 to <0.1 g/L, respectively, in all fermentations. Isolate I₂ produced the highest concentration of ethanol (546. 4 ± 0.4 mg/L). l-Lactic acid production varied between 4.3 ± 0.6 and 3.7 ± 0.2 mg/L, whereas acetic acid concentrations decreased from 81.0 ± 3.3 to <40.0 mg/L. pH decreased from 4.9 ± 0.0 to 3.6 ± 0.4 at the end of 30 days in all fermentations. Isolate I₁ was the highest producer of benzaldehyde (BA) (12.0 ± 0.2 mg/kg) and 4-methoxybenzaldehyde (4-MBA) (239.6 ± 3.9 mg/kg), while isolate I₄ was the highest producer of 3,4-dimethoxybenzaldehyde (3,4-DMBA) (27.8 ± 0.2 mg/18 kg). Identification of isolate I₁ as a high BA and 4-MBA producer and isolate I₄ as a high 3,4-DMBA producer suggested differential benzylic derivative production among *I. resinosum* isolates. This study lays the foundation for future investigations evaluating additional *I. resinosum* isolates for benzylic derivative production as well as studies aimed at bioreactor optimization with potential commercial application.

INTRODUCTION

Biotechnological production of natural flavor ingredients has been an area of continual research interest since its introduction over 20 years ago.¹⁻³ There are over 100 estimated natural flavor ingredients produced via enzymatic or microbial processes available for consumption.¹⁻⁷ The expanding interest in natural flavors is driven primarily by increasing consumer demand for all-natural products. Flavor compounds produced by employing raw materials via microbial or enzymatic methods are labeled “natural” under both European and US legislation.²,⁶,⁷ Additionally, natural flavor production through biotechnological routes offers a more sustainable alternative to traditional cultivation, wild harvesting, and chemical synthesis.

A rising consumer preference for naturally derived flavor ingredients has increased the use of microbial sources such as white-rot fungi, which have become popular and more sustainable alternatives to the use of botanical materials.⁹⁻¹¹ Two methods of biotechnological flavor production include de novo synthesis and bioconversion. De novo synthesis refers to production via microbial fermentation in cultivation media not supplemented with precursor compounds. In contrast, bioconversion implies the biosynthesis of flavor compounds through the introduction of precursors to the cultivation media.⁴ A variety of metabolites with and without commercial applications are produced during de novo synthesis owing to the total enzymatic profile of the microorganism. However, targeted metabolites with a commercial value can be produced from specific precursors introduced to the microbial fermentations through one or many biochemical steps during bioconversion.⁴ Many microorganisms have been identified with metabolic potential for de novo biosynthesis or bioconversion of commercially viable flavor compounds in culture media.⁴,¹²⁻¹⁴ However, the concentrations in these natural microbial sources are usually too low for cost-effective applications.¹⁵ Thus, understanding the biosynthetic pathway, fermentation dynamics, and microbial potential for de novo synthesis and/or bioconversion of metabolites is essential to optimizing and increasing the yields of natural flavor ingredients with commercial interest.¹⁵,¹⁶

White-rot fungi contain lignin-degrading enzyme systems involving a variety of extracellular oxidative enzymes such as
laccase, lignin peroxidase, and manganese peroxidase.\textsuperscript{17−20} These enzymes produce a wide variation of commercially relevant aromatic compounds as byproducts during lignin degradation.\textsuperscript{21} For example, white-rot fungi such as \textit{Bjerkundera adusta}, \textit{Ischnoderma benzoinum}, \textit{Dichomitus squalens}, and \textit{Polyporus tuberaster} have been reported to produce benzy alcohol and benzaldehyde, whereas \textit{I. resinosum} has been reported to produce benzaldehyde (BA), 4-methoxybenzaldehyde (4-MBA), and 3,4-dimethoxybenzaldehyde (3,4-DMBA).\textsuperscript{22−24}

\textit{Ischnoderma resinosum} belongs to the order Polyporales and family Ischnodermataceae.\textsuperscript{25} The shelf-like fruit bodies can be identified in early to late fall and are widely distributed in North American hardwood forests. The fungal basidiocarp can be found singly, in groups, or overlapping clusters on fallen hardwood tree trunks and branches.\textsuperscript{25} The mature basidiocarp is velvety, with a tan-to-reddish brown resin encrusting the body, and a white interior.\textsuperscript{25} Younger fruitbodies are pale brown in color, fleshy, and soft to the touch. \textit{Ischnoderma resinosum} is edible when young because of its high water content. However, it becomes inedible and cork-like with age.\textsuperscript{25}

It has been reported that flavor compound production is dependent on both the fungal growth rate and growth conditions, thus necessitating a better understanding of the fermentation dynamics.\textsuperscript{26,27} \textit{Ischnoderma resinosum} has been reported previously to produce benzylic derivatives with applications in the food and flavor industry such as

Figure 1. \textit{Ischnoderma resinosum} isolates collected from six different geographical locations surrounding the Great Smoky Mountain range.

Figure 2. Phylogenetic analysis using a maximum likelihood method and general time reversible model comparing the sequences of the internal transcribed spacer ribosomal DNA (rDNA) region from six \textit{I. resinosum} isolates with other \textit{Ischnoderma} spp. retrieved from GenBank database.\textsuperscript{49} This analysis involved 17 nucleotide sequences. The numbers above the nodes are supporting percentages obtained from 1000 bootstrap replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X.\textsuperscript{50}
benzaldehyde and 4-methoxybenzaldehyde. However, evaluation of a variety of isolates is important for understanding the applicability of I. resinus fermentations in benzylic derivative production. However, to the best of our knowledge, no studies have yet investigated the fermentation dynamics or variations in benzylic derivative production among several I. resinus isolates. Therefore, the aim of this study was to compare the fermentation dynamics as well as the de novo production of benzylic derivatives among several I. resinus isolates collected from the southeastern United States over a 30 day period. Accordingly, the objectives were to (1) collect, identify, and cultivate the fungal isolates in liquid media, (2) evaluate fermentation dynamics of D-glucose, D-fructose, ethanol, l-lactic acid, acetic acid, and pH for each isolate over 30 days, and (3) quantitate the de novo production of benzaldehyde, 4-methoxybenzaldehyde, and 3,4-dimethoxybenzaldehyde using stable isotope dilution assays (SIDAs).

RESULTS AND DISCUSSION

Site Description and Sample Collection. Six I. resinus isolates growing on dead or decaying hard wood from the surrounding areas of the Great Smoky Mountain Range in south eastern United States were collected. Three isolates, namely, I1 (Sep 2017), I3 (Oct 2018), and I6 (Nov 2018), were collected from Cumberland, Knox, and Cheatham counties in Tennessee, whereas the remaining three isolates, namely, I2 (Aug 2018), I4 (Oct 2018), and I5 (Nov 2018), were collected from Durham, Wake, and Buncombe counties in North Carolina, respectively (Figure 1). Each isolate was collected, wrapped in wax paper, sealed, and stored on ice during transportation to the laboratory at the University of Tennessee, Knoxville. All six isolates were typical in appearance for I. resinus as described previously. Fungal basidiospores were dark brown in color, thick, and fleshy, with light brown rounded edges.

Fungal Culture Identification. Morphological characteristics combined with ITS sequencing allowed for the identification of isolates I1−I6 as I. resinus. Phylogenetic analysis showed that all six isolates clustered together with I. resinus isolate MH 979275.1 collected from the United States (Figure 2). ITS sequences for I. benzoinum, a European relative to the North American species, also were obtained from the NCBI database and incorporated into the phylogenetic analysis. All I. benzoinum isolates clustered separately from the I. resinus clade, further confirming the identity of the isolates from the current study. All ITS sequences were deposited in the NCBI database (Table 1).

Fermentation Dynamics. Fermentation dynamics for all six fungal isolates were assessed under aerobic conditions at ambient temperature over a 30 day time period. Utilization of D-glucose and D-fructose as well as changes in ethanol, l-lactic acid, acetic acid, and pH levels were investigated. Understanding the basal levels of each fermentation parameter under normal aerobic conditions is essential in future bioreactor optimization for production of benzylic derivatives from I. resinus.

D-Glucose. Although the D-glucose utilization varied among isolates, all six isolates consumed D-glucose present in potato dextrose broth (PDB), as indicated by the gradual reduction from 20.4 ± 0.4 g/L to an average of 5.1 ± 1.3 g/L for all isolates, except for I4 where it remained at 13.0 ± 0.2 g/L on day 30 (Figure 3).

Figure 3. Changes in (A) d-glucose and (B) d-fructose for isolates I1 through I6 (denoted as 1 through 6) reported as mean ± standard deviation.

D-Fructose. Similarly, all six isolates consumed D-fructose in a growth-dependent manner over the 30 day time course. Unlike D-glucose, the concentration of D-fructose in PDB prior to fungal growth averaged at 1.0 ± 0.1 g/L and was quickly reduced to <0.1 g/L between days 21 and 23 in all fermentations but I4. The D-fructose levels in I4 fermentations remained at 0.1 g/L on day 30, suggesting that the isolate is a slower consumer of both D-glucose and D-fructose (Figure 3).

Supplementation of fermentations with nutrients, especially carbon sources, has been reported to enhance the fungal growth cycle and subsequent production of compounds of interest. A previous study evaluating the production of BA by Rhizopus oligosporus in a solid-state fermentation system reported the supplementation of carbon sources led to enhanced growth and increased BA production. Among the carbon sources evaluated during the study including D-glucose, fructose, sucrose, lactose, and maltose, the highest BA productions at 14.84 ± 0.01 and 14.65 ± 0.00 mg/g were observed when fermentations were supplemented with D-glucose and fructose, respectively. Findings from the current study suggest that both I3 and I4, the highest producers of BA,

Table 1. Isolate, Origin, and NCBI Gen Bank Accession Numbers

| code  | isolate | location            | Gen Bank accession number |
|-------|---------|---------------------|---------------------------|
| I1    | UT-PW019| Cumberland County, TN| MN 633306                 |
| I2    | UT-PW081| Durham County, NC    | MN 999537                 |
| I3    | UT-PW093| Knox County, TN      | MN 999534                 |
| I4    | UT-PW094| Wake County, NC      | MN 999535                 |
| I5    | UT-PW096| Buncombe County, NC  | MN 999533                 |
| I6    | UT-PW097| Cheatham County, TN  | MN 999536                 |
4-MBA, and 3,4-DMBA, were able to efficiently utilize D-glucose and D-fructose in their fermentations. Consequently, supplementation of optimized bioreactor systems with high levels of D-glucose and D-fructose may be advantageous in enhancing benzylic derivative production from both I₃ and I₄ isolates; however, further studies are needed to support this hypothesis.

**Ethanol.** The fermentation dynamics of all six isolates were also evaluated according to the production of ethanol over 30 days. A maximum ethanol concentration of 546.4 ± 0.4 mg/L was reported in the I₂ fermentations on day 18 of the time course. Furthermore, concentrations of 517.3 ± 5.9, 388.8 ± 1.8, 371.7 ± 7.0, and 297.0 ± 5.6 mg/L were reported for I₀, I₄, I₃, and I₁ respectively. The lowest producer of ethanol was I₅ at a peak concentration of 14.4 ± 0.4 mg/L on day 6 (Figure 4).

![Figure 4. Changes in ethanol production for isolates I₁ through I₆ (denoted as 1 through 6) reported as mean ± standard deviation.](https://dx.doi.org/10.1021/acsomega.0c02550)

All six isolates from the current study utilized simple sugars such as D-glucose and D-fructose from PDB to produce ethanol as a byproduct. Other white-rot fungi have also been reported to produce ethanol directly from various sugars as well as lignocellulosic materials such as lignin, cellulose, and hemi-cellulose. For example, the white-rot fungi *Lentzites betulinus* have previously been evaluated for potential applications in bioethanol production from monosaccharides, disaccharides, and lignocellulosic biomass. Glucose was reported as the most efficient hexose sugar assimilated by *L. betulinus* to produce ethanol in comparison to mannose, galactose, and cellobiose. *L. betulinus* produced 7.68 g/L ethanol from 20 g/L glucose at a 75.1% theoretical yield. In addition to *L. betulinus*, other filamentous fungi such as *Aspergillus sojae*, *Rhizopus oryzae*, *Aspergillus oryzae*, and *Roseovarius indicus* are capable of assimilating glucose to produce ethanol. *I. resinosum* isolates from the current study produced significantly less ethanol concentrations to be considered in applications in bioethanol production.

**L-Lactic Acid.** L-Lactic acid concentrations gradually began to increase on day 3 prior to peak concentrations followed by a gradual decrease in all fermentations. Both I₄ and I₃ were responsible for the highest concentrations of L-lactic acid produced on day 21 at 4.3 ± 0.4 and 4.3 ± 0.6 mg/L, respectively. Fermentations I₅ and I₆ both peaked at 4.0 ± 0.3 and 4.0 ± 0.1 mg/L on days 21 and 6, respectively, whereas I₁ and I₂ produced the least concentrations of the organic acid at 3.7 ± 0.2 and 3.7 ± 0.6 mg/L on days 21 and 18, respectively (Figure 5).

![Figure 5. Changes in (A) l-lactic acid and (B) acetic acid concentrations for isolates I₁ through I₆ (denoted as 1 through 6) reported as mean ± standard deviation.](https://dx.doi.org/10.1021/acsomega.0c02550)

**Acetic Acid.** An average concentration of 81.0 ± 3.3 mg/L acetic acid was detected in PDB on day 0. The levels of acetic acid gradually decreased to <40.0 mg/L between days 12–21 in all fermentations (Figure 5).

**pH.** pH measurements of the fermentations were monitored also throughout the 30 days. Potato dextrose broth had an initial pH of 4.9 ± 0.0 and decreased to an average pH of 3.6 ± 0.4 by the end of the 30 day time course (Figure 6). Fungal production of organic acids has been reported to play key roles including pH acidification and direct environmental interactions depending on the type of fungus producing them. Previous studies have reported that fermentation of water-soluble carbohydrates such as D-glucose to L-lactic acid and/or acetic acid provided a conducive low pH environment for colonization and growth in *Pleurotus* spp. In the current study, L-lactic acid production was inversely related to the levels of D-glucose and D-fructose in fermentations in agreement with literature. However, acetic acid concentrations decreased throughout the time course in all fermentations and did not indicate inverse correlations with use of simple sugars. These observations suggested that the decreasing pH in fermentations may be partially driven by L-lactic acid, thereby allowing for favorable conditions for fungal growth and subsequent benzylic derivative production.

Interestingly, both acetic acid and L-lactic acid concentrations reached levels below detection by the end of the 30 day time course. Therefore, the decrease in pH throughout the 30 days could only be partially explained by the production of L-lactic acid. Saprobes such as white-rot fungi have previously been reported to secrete oxalic acid important in acid-catalyzed hydrolysis of holocellulose into their fermentation media. In addition, white-rot fungi including *Pycnoporus coccineus* have also been reported to produce formic acid, resulting in oxalate decarboxylation as well as pH acidification. Therefore, the pH acidification in the *I. resinosum* fermentations observed
During the current study could potentially be due to the secretion of other organic acids such as oxalic and/or formic acid to the fermentation media; however, additional fermentation studies are needed to explore these possibilities.

Bioreactors are important in many industries, including those involving fermentation, food, pharmaceuticals, and wastewater treatment. The main functions of a bioreactor include controlling the environmental conditions such as pH, temperature, and pressure as well as the nutrient/product concentrations during the bioprocess. An efficient bioreactor can maintain the desired biological activity by controlling the temperature, pH, fluid velocity, shear stress, O2, CO2, nutrient supply, reaction rate, and cell growth rate. Therefore, a well-optimized bioreactor system is important in manufacturing end products that meet government regulations and criteria regarding efficacy, safety, and quality, in addition to being cost-effective. The current study is the first investigation to evaluate the basal fermentation dynamics of several I. resinosum isolates in liquid cultures. Assessment of changes in D-glucose, D-fructose, ethanol, L-lactic acid, acetic acid, and pH at ambient temperature under aerobic conditions establishes a foundation for strain selection and future bioreactor optimization studies for production of benzylic derivatives from I. resinosum.

Production of Benzaldehyde. Benzaldehyde concentrations among the six isolates were quantitated using SIDAs over a 30 day time course. Fermentations from isolate I3 produced the highest concentration of BA at 12.0 ± 0.2 mg/kg on day 18, whereas I6 produced the lowest concentration of 3.7 ± 0.04 mg/kg on day 15 of the time course. Concentrations increased over the first 14 days, peaked on day 15, and decreased throughout the rest of the 30 days in all I1 (5.6 ± 0.4 mg/kg), I2 (4.5 ± 0.2 mg/kg), and I4 (4.2 ± 0.6 mg/kg) fermentations. Benzaldehyde concentration in I5 fermentations peaked at 5.9 ± 0.5 mg/kg on day 18 and decreased over the next 12 days (Figure 7).

Studies showed that white-rot fungi, including Agaricus bisporus, A. subrufescens, and D. squalens, produced <1 mg/L BA de novo.36−39 It was also reported that the addition of precursors such as L-phenylalanine or tyrosine to Poria xantha, I. benzoinum, D. squalens, and B. adusta fermentations significantly increased the BA production from <1 mg/L to 71–587 mg/L via bioconversion.22,40 Also, both benzyl alcohol and benzoic acid have been reported as precursors that can enhance the production of BA (Figure 8).41 Interestingly, de...
novel BA production in I₄ fermentations was higher at 12.0 ± 0.2 mg/kg than those reported earlier in A. bisporus, A. subrufescens, and D. squalens at <1 mg/L. 36,37 The above findings suggest that the addition of precursors such as benzyl alcohol, benzoic acid, or L-phenylalanine to I₄ fermentations may further increase BA production, which would support future bioreactor optimization studies for benzylic derivative production from I. resinosum.

**Production of 4-Methoxybenzaldehyde.** Isolate I₃ was also identified as the highest producer of 4-MBA. Concentrations in I₃ fermentations increased over the first 23 days, peaked on day 24 at 239.6 ± 3.9 mg/kg, and decreased over the remaining 6 days. Production continued to increase and then peaked on day 30 at 180 ± 1.6 mg/kg in I₃ fermentations, whereas concentrations peaked on day 27 for both I₄ and I₆ at 77.9 ± 5.8 and 197.2 ± 7.3 mg/kg, respectively. Isolate I₄ produced the lowest concentration of 4-MBA. Concentration in I₆ fermentations peaked on day 24 at 111.9 ± 5.1 mg/kg, whereas that in I₃ peaked on day 21 at 152.6 ± 10.8 mg/kg followed by subsequent decrease over the time course (Figure 9).

Concentration of 4-MBA in I₃ fermentations peaked on day 24, with a spike in production on day 21. A similar pattern was also evident in I₆ fermentations, the second highest producer of 4-MBA, with a spike in production on day 18. Benzaldehyde concentrations in both I₃ and I₆ fermentations declined from days 21 and 18, respectively. Previous investigations reported BA as a precursor of 4-MBA in I. resinosum fermentations, suggesting that BA produced in both I₃ and I₆ fermentations was subsequently converted to the corresponding high 4-MBA concentrations (Figure 8). 41 In addition, when grown in liquid media, Pleurotus spp. produced approximately 10–30 mg/L 4-MBA via bioconversion, whereas isolates I₃ and I₆ from the current study produced 239.6 ± 3.9 and 197.2 ± 7.3 mg/kg, respectively, with no additional precursors. 36 As a result, these findings successfully lay the groundwork for future bioreactor optimizations in the production of natural 4-MBA using I₃ and I₆ fermentations.

**Production of 3,4-Dimethoxybenzaldehyde.** Isolate I₄ was identified as the highest producer of 3,4-DMBA at 27.8 ± 0.2 mg/kg on day 30. Concentrations in I₃, I₄, and I₆ fermentations peaked on day 27 at 10.1 ± 0.1, 5.1 ± 0.1, and 5.8 ± 0.1 mg/kg, respectively, whereas the concentration in I₃ peaked on day 18 at 9.9 ± 2.1 mg/kg. Isolate I₄ was reported as the lowest producer of 3,4-DMBA at 3.6 ± 0.2 mg/kg on day 21 (Figure 10).

Isolate I₆ responsible for the lowest concentrations of BA and 4-MBA, was identified as the highest producer of 3,4-DMBA. Concentrations of BA and 4-MBA in I₄ fermentations decreased on day 18, whereas 3,4-DMBA concentrations continued to increase from day 18, suggesting the conversion of BA and 4-MBA to 3,4-DMBA in I₃ fermentations. These findings are consistent with the literature reporting BA and 4-MBA as precursors for 3,4-DMBA production (Figure 8). 41 Other white-rot fungi that included B. adusta, Bjerkandera sp., and D. squalens also produced 3,4-DMBA via bioconversion of metabolites such as 4-MBA from lignin degradation processes. 42 Outcomes from the current study allow for the applicability of I₄ isolate to optimization of natural 3,4-DMBA production from I. resinosum.

**Comparison of Benzylic Derivative Production.** Mean comparisons of BA, 4-MBA, and 3,4-DMBA concentrations in all six isolates on days 18, 24, and 30 were evaluated using one-way ANOVA and Tukey–Kramer HSD at a significance level of p < 0.05. Days 18, 24, and 30 were chosen based on the highest concentrations recorded for BA, 4-MBA, and 3,4-DMBA, respectively, during this study. All six isolates were significantly distinct from each other in their production of all three compounds (Figure 11). Isolate I₃ was the highest producer of BA and 4-MBA on days 18 and 24, respectively, whereas I₆ was the highest producer of 3,4-DMBA on day 30. This comparison among the six isolates highlights and evaluates their possible use in future optimization studies for increased benzylic derivative production from I. resinosum fermentations.

All fermentations were prepared and maintained under the same conditions. As a result, the observed differences in BA, 4-MBA, and 3,4-DMBA production could potentially be the result of variations in gene expression leading to secretome modifications in the biosynthetic pathway for benzylic derivatives (Figure 8). A previous study reported that both aryl-alcohol oxidase (AAO-1, AAO-4, and AAO-6) and aryl-alcohol dehydrogenase (AAD-1) gene expression in P. ostreatus were induced in the presence of secondary metabolites such as benzyl alcohol, 4-methoxybenzyl alcohol, and 3,4-dimethoxy-benzyl alcohol. 41,43 Furthermore, 9 out of 16 transcripts of Mtrase#1 and Mtrase#2 genes responsible for 4-O-methyltransferase and 3-O-methyltransferase involved in acceleration of lignin degradation have also been reported in B. adusta,
Ceriporiopsis subvermispora B, and Trametes versicolor. However, no transcriptome or secretome analyses in I. resinosum have been reported in the literature. Identification of specific isolates such as I3 and I4 responsible for the high production of BA, 4-MBA, and 3,4-DMBA, respectively, in combination with transcriptomics and functional analysis of enzymes involved in the biosynthetic pathway may prove useful in future bioreactor optimization studies.

In summary, all six isolates could assimilate D-glucose and D-fructose and produce ethanol as a byproduct. L-Lactic acid production was also observed to be inversely related to the use of D-glucose and D-fructose in fermentations. Interestingly, also contrary to the available literature, acetic acid concentrations decreased throughout the time course in all fermentations and did not indicate any inverse correlation with the use of simple sugars. In addition, pH also decreased in all fermentations, creating a conducive environment for fungal growth. Additionally, all six I. resinosum isolates produced BA, 4-MBA, and 3,4-DMBA in their fermentations at varying concentrations. Isolate I3 was identified as the highest BA and 4-MBA producer, whereas I4 was the highest 3,4-DMBA producer.

In conclusion, the fermentation dynamics suggested that supplementation with D-glucose and/or D-sucrose as well as the reduction of pH to levels conducive for optimum fungal growth can be utilized as bioreactor optimization processes to enhance the production of BA, 4-MBA, and 3,4-DMBA from the selected high producers of benzylic derivatives. This is the first report to evaluate the fermentation dynamics and production of benzylic derivatives among several I. resinosum isolates. While additional investigation into the fungal transcriptome and secretome are underway in our laboratory, the current study provides a basis for establishment and optimization of bioreactor systems to produce benzylic derivatives from I. resinosum fermentations.

**MATERIALS AND METHODS**

**Microorganism.** Six isolates including UT-PW019 (I1), UT-PW093 (I3), and UT-PW097 (I4) from Tennessee and UT-PW081 (I2), UT-PW094 (I4), and UT-PW096 (I6) from...
North Carolina were collected from mature basidiocarps. All specimens were decontaminated with 10% bleach solution prepared in water for 10 min, washed with sterile deionized water for 15 min, and then cultured on petri dishes containing potato dextrose agar (PDA) (Himedia, India). Cultures were incubated at 25 °C in a Panasonic MIR-254 Cooled Incubator (Panasonic Healthcare Co., Ltd., Japan). Internal transcribed spacer (ITS) sequencing with primers, ITS 4 (5'-TCTTCCGCTTATGATGC), and ITS 5 (5'-GGAAGTAAAAGCTGAAACAAGG) was used in species confirmation, as described previously. All six isolates were cryopreserved at −80 °C in PDB (Himedia, India) supplemented with glycerol (10%, v/v) as agar plugs by means of a Mr. Frosty Freezing Container (Thermo Fisher Scientific, Fair Lawn, NJ).

Medium and Culture Conditions. Three 250 mL Erlenmeyer flasks containing PDB (85 mL) were inoculated with homogenized 7 day-old mycelia grown on PDA plates (1 mL) for all isolates. Fermentations were maintained aerobically with homogenized 7 day-old mycelia grown on PDA plates (1 mL) for all isolates. Fermentations were maintained aerobically at 25 °C in a Panasonic MIR-254 Cooled Incubator (Thermo Fisher Scientific, Fair Lawn, NJ).

Reference Standards. BA, 4-MBA, and 3,4-DMBA from Sigma-Aldrich (St. Louis, MO) were used as authentic reference standards.

Isotopically Labeled Internal Standards. Both (2H5)-BA and (2H5)-4-MBA were purchased from C/D/N Isotopes (Quebec, Canada), whereas (2H5)-3,4-DMBA was purchased from aromaLAB (Planegg, Germany). All isotopically labeled compounds were dissolved in 5 mL volumetric flasks containing freshly distilled pentane. (2H5)-BA was dissolved at a known concentration, whereas both (2H5)-4-MBA and (2H5)-3,4-DMBA were quantitated using isotopically unmodified reference standards.

Solvents. Unstabilized diethyl ether from Honeywell, Burdick & Jackson (Muskegon, MI) and pentane from Millipore Sigma (St. Louis, MO) were freshly distilled in-house using a 250 mL CG-1233 series distillation head from Chemglass Life Sciences (Vineland, NJ) prior to general use. Anhydrous Na2SO4 was purchased from Thermo Fisher Scientific.

Fermentation Culture Dynamics. A Thermo Scientific Gallery discrete photometric analyzer was used to evaluate fermentation dynamics. Reagent systems including d-glucose (ref 984304), d-fructose (ref 984302), ethanol (ref 984300), L-lactic acid (ref 984308), and acetic acid (ref 984318) were purchased from Thermo Fisher Scientific. Undiluted fermentation broth samples, excluding the biomass (2 mL), were evaluated every 3 days for 30 days for each isolate. All tests were performed at 37 °C using a 340 nm filter. Findings were reported by means of triplicate measurements and standard deviations using Microsoft Excel for Office 365 MSO version 1811.

d-Glucose. Photometric determination of d-glucose concentrations was conducted with an enzymatic test system with hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6P-DH).

d-Fructose. Enzymatic test consisting of HK, phosphoglucose isomerase (PGI), and G6P-DH was employed to evaluate samples photometrically.

Ethanol. Photometric evaluation of ethanol concentrations was performed by an enzymatic test with alcohol dehydrogenase (ADH).

L-Lactic Acid. An enzymatic test with l-lactate dehydrogenase (L-LDH) was used to measure the l-lactic acid concentrations.

Acetic Acid. Acetic acid concentrations were measured using an enzymatic test with acetate kinase (AK), coenzyme A (CoA), phosphotransacetylase (PTA), ADP-dependent hexokinase (ADP-HK), and G6P-DH.

pH. Undiluted fermentation samples were evaluated for changes in pH with a pH meter from Denver Instruments (UltraBasic, Arvada, CO) operated at ambient temperature.

Stable Isotope Dilution Assay (SIDA). Fungal fermentation broth, excluding the biomass (2 g), was combined with freshly distilled diethyl ether (3 mL). Next, a mixture of (2H5)-BA, (2H5)-4-MBA, and (2H5)-3,4-DMBA at 1:1:1 (v/v) ratio was incorporated as an internal standard. The resulting mixtures were extracted for 10 min at ambient temperature. The organic fractions were then dried over 1.5 g of anhydrous Na2SO4 prior to gas chromatography–mass spectrometry (GC–MS) analysis. Concentrations of BA, 4-MBA, and 3,4-DMBA in each fermentation were calculated in mg/kg using the integrated area of the analyte peak, isotope labeled standard peak, mass of initial culture sample, volume of isotopically labeled standard added, and response factors (RF). Response factor (RF) and m/z (analyte/standard) for each analyte were as follows: benzaldehyde, m/z 105/110, RF 0.90; 4-methoxybenzaldehyde, m/z 135/138, RF 1.90; and 3,4-dimethoxybenzaldehyde, m/z 166/172, RF 0.90. Concentrations of BA, 4-MBA, and 3,4-DMBA in each isolate were evaluated every 3 days for a 30 day period. Values were reported as means of triplicate measurements and standard deviations using Microsoft Excel for Office 365 MSO version 1811. Statistical analysis was performed with JMP Pro 14.2 software (SAS Institute, Cary, NC).

Gas Chromatography–Mass Spectrometry (GC–MS). Volatile isolates were analyzed using a 78204 GC system coupled with a 5977B mass spectrometry detector (Agilent Technologies, Santa Clara, CA). Using an autosampler, samples (1 μL) were on-column injected cold into a Zebron ZB-FFAP GC capillary column (30 m × 0.32 mm o.d. × 0.25 μm), purchased from Phenomenex (Torrance, CA). Helium was the carrier gas at 1 mL/min flow rate. An initial oven temperature of 35 °C was held for 1 min. Temperature was then ramped to 60 °C at 60 °C/min followed by a second ramp at 6 °C/min to reach 250 °C and held for 5 min. The MS source and quadrupole were maintained at 230 and 150 °C, respectively, whereas the MS was operated in electron impact (EI) ionization mode at 70 eV with a scan range of m/z 50–350.

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Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS
We gratefully acknowledge the assistance of Steve Roberts, president of the Cumberland Mycological Society (CMS), Jay Englebach, Gwendolyn Casebeer, Laurie Jaegers, and Frank Bartucca from Asheville Mushroom Club (AMC), and Victoria Vito in providing the I. resinosum isolates used in this study. We would also like to acknowledge Gwendolyn Casebeer from AMC for providing the photograph of the fungal basidiospore used in the table of content graphic. This work was supported by the USDA National Institute of Food and Agriculture Hatch project #1016031.

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
D-fructose, (3S,4R,5R)-2-(hydroxymethyl)oxane-2,3,4,5-tetrol; D-glucose, (3R,4S,5S,6R)-6-(hydroxymethyl)oxane-2,3,4,5-tetrol; L-lactic acid, (2S)-2-hydroxypropanoic acid; L-phenylalanine, (2S)-2-amino-3-phenylpropanoic acid; tyrosine, (2R)-2-amino-3-(4-hydroxyphenyl)propanoic acid

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