Application of Basidiomycota-derived biocatalysts in organic synthesis

Cumulative Dissertation

Presented by

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for the degree of

Doctor rerum naturalium (*Dr. rer. nat.*)

submitted to the

Faculty of Biology and Chemistry

prepared at the

Institute of Food Chemistry and Food Biotechnology

Justus Liebig University Giessen, Germany

2026

This thesis is accepted as a doctoral dissertation in fulfilment of the requirements for the degree of *Doctor rerum naturalium* by the Faculty of Biology and Chemistry, Justus Liebig University Giessen, Germany.

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Acknowledgements

I would like to express my sincere gratitude to everyone who supported me during my doctoral studies. Many people contributed to this work, either directly or indirectly, and I am grateful to all of them.

First and foremost, I would like to thank my supervisor, **Prof. Dr. Holger Zorn**, for giving me the opportunity to conduct my research at the institute and for creating a stimulating scientific environment. I am grateful to him for his support and trust, as well as for his valuable scientific input throughout this work.

I would like to thank **Prof. Dr. Richard Göttlich** for agreeing to evaluate the thesis as the second referee. I would also like to sincerely thank **Prof. Dr. Martin Rühl** and **Prof. Dr. Siegfried Schindler** for their kind willingness to be a part of the examination board and for their interest in my work.

I express my deepest gratitude to **Prof. Dr. Tetiana Zhuk** for her exceptional support throughout my doctoral studies, which extended far beyond academic matters. Her patience, belief in me, and assistance with scholarships, administrative matters, and numerous practical issues was invaluable for the successful completion of this doctoral dissertation.

Furthermore, I would like to extend my sincere thanks to **Prof. Dr. Andrey Fokin** for his assistance and support in fruitful cooperation with the Institute of Organic Chemistry. His expertise, insightful discussions, and guidance in the field of organic synthesis significantly contributed to this work.

I would also like to thank my colleagues **Dr. Marco Fraatz** and **Dr. Wendell Albuquerque** for their assistance, and the lively and constructive scientific exchanges.

Additionally, I would like to sincerely thank all my colleagues in the laboratory. I am grateful for the time we spent together, for the many scientific discussions, and also for the non-scientific conversations and moments that made everyday lab life enjoyable. These moments will remain pleasant memories for me. My thanks also go to the bachelor’s and master’s students who contributed to different parts of this work.

Finally, I would like to thank my family from the bottom of my heart. I am especially grateful to my parents, Valentin and Iuliia, and my sister Viktoriia for their constant encouragement and belief in me. I am also deeply grateful to my husband Vadym for pretending to understand my research over the past years and for forgiving my abrupt mood swings whenever experiments did not go as planned. The love, unconditional support and understanding of my family gave me the strength to continue, even in the most difficult times.

Abstract

The increasing demand for sustainable and selective methods of organic synthesis drives the replacement of traditional energy-intensive processes often based on toxic chemical reagents. Biocatalysis offers an attractive alternative that allows efficient transformations to be carried out under mild and environmentally friendly conditions. White-rot fungi represent a particularly promising source of such biocatalysts, as approaches based on these fungi exhibit high enzyme stability and unique enzymatic pathways compared to other biocatalytic methods based on bacteria or yeasts. Their non-toxic nature, along with straightforward application and accessibility, makes them attractive for various biotechnological processes. Nevertheless, the practical application of fungi as biocatalysts, including both growing cultures and storable lyophilized mycelium, remains insufficiently explored in preparative organic synthesis.

The present dissertation focuses on the disclosing of biocatalytic potential of the white-rot fungus *Bjerkandera adusta*, chosen for its non-toxic nature, rich enzymatic profile, and simple cultivation on inexpensive substrates. The work investigates the biocatalytic potential of both, the growing culture and the lyophilized mycelium with the aim of developing practical, selective, and green preparative approaches for the transformation of organic compounds. Particular attention is given to the ability of *B. adusta* to carry out oxidation and reduction reactions under mild and environmentally friendly conditions.

Lyophilized mycelium of *B. adusta* was shown to catalyze clean and selective oxidations of aromatic alcohols to the corresponding aldehydes under “on-water-on-air” conditions. The catalytic activity observed in the lyophilized mycelium was shown to be associated with the presence of aryl-alcohol oxidases. High conversion rates without overoxidation were achieved under mild conditions, and the lyophilisate retained its oxidative activity over extended storage periods, highlighting its practical value for further applications. The oxidative potential of the lyophilisate of *B. adusta* was further demonstrated through the selective conversion of α,β-unsaturated alcohols into valuable (*E*)-allylic aldehydes. The addition of small amounts of organic co-solvents enhanced substrate conversion while maintaining high chemoselectivity. Notably, *B. adusta* also mediated the isomerization of (*Z*)-2-nonenal to the corresponding (*E*)-diastereomer without further carbonyl group oxidation or CC-double bond reduction, which is rarely achieved under such conditions. Comparative experiments with lyophilisates of *Pleurotus* species confirmed the special catalytic efficiency of *B. adusta* in these reactions.

In contrast, growing cultures of *B. adusta* exhibited reductive activity, selectively converting anthranilic acid into 2-aminobenzaldehyde while completely suppressing overreduction to the corresponding alcohol. This transformation proceeds in aqueous aerobic conditions and displays strict regioselectivity, with only the *o*-isomer accepted as a substrate.

Zusammenfassung

Die zunehmende Nachfrage nach nachhaltigen und selektiven Methoden der organischen Synthese treibt den Ersatz traditioneller, energieintensiver Prozesse voran, die häufig auf toxischen chemischen Reagenzien basieren. Die Biokatalyse bietet eine attraktive Alternative, die effiziente Transformationen unter milden und umweltfreundlichen Bedingungen ermöglicht. Weißfäulepilze stellen eine besonders vielversprechende Quelle solcher Biokatalysatoren dar, da Ansätze auf Basis dieser Pilze im Vergleich zu anderen biokatalytischen Methoden, die auf Bakterien oder Hefen basieren, eine hohe Enzymstabilität und einzigartige enzymatische Reaktionswege aufweisen. Ihre ungiftige Natur sowie die einfache Anwendung und Verfügbarkeit machen sie attraktiv für verschiedene biotechnologische Prozesse. Dennoch bleibt die praktische Anwendung von Pilzen als Biokatalysatoren, einschließlich sowohl wachsender Kulturen als auch lagerfähiger lyophilisierter Myzelien, in der präparativen organischen Synthese bislang unzureichend untersucht.

Die vorliegende Dissertation konzentriert sich auf die Untersuchung des biokatalytischen Potenzials des Weißfäulepilzes *Bjerkandera adusta*, ausgewählt wegen seiner ungiftigen Natur, seines breiten enzymatischen Profils und der einfachen Kultivierung auf kostengünstigen Substraten. Die Arbeit untersucht das biokatalytische Potenzial sowohl der wachsenden Kultur als auch des lyophilisierten Myzels mit dem Ziel, praktische, selektive und umweltfreundliche präparative Ansätze für die Transformation organischer Verbindungen zu entwickeln. Besonderes Augenmerk liegt auf der Fähigkeit von *B. adusta*, Oxidations- und Reduktionsreaktionen unter milden und umweltfreundlichen Bedingungen durchzuführen.

Lyophilisiertes Myzel von *B. adusta* zeigte die Fähigkeit, saubere und selektive Oxidationen aromatischer Alkohole zu den entsprechenden Aldehyden unter in wässrigen Lösung zu katalysieren. Die beobachtete katalytische Aktivität im lyophilisierten Myzel wurde mit dem Vorhandensein von Arylalcohol-Oxidasen in Verbindung gebracht. Unter milden Bedingungen wurden hohe Umsetzungen ohne Überoxidation erreicht und das Lyophilisat behielt seine oxidative Aktivität über längere Lagerzeiten bei, was seinen praktischen Wert für weitere Anwendungen unterstreicht. Das oxidative Potenzial der Lyophilisate von *B. adusta* wurde weiterhin durch die selektive Umwandlung von α,β-ungesättigten Alkoholen in wertvolle (*E*)-Allylaldehyde demonstriert. Die Zugabe kleiner Mengen organischer Co-Lösungsmittel steigerte die Substratumsetzung bei gleichzeitig hoher Chemoselektivität. Bemerkenswerterweise vermittelte *B. adusta* auch die Isomerisierung von (*Z*)-2-Nonenal zum entsprechenden (*E*)-Diastereomer, ohne dass eine weitere Oxidation der Carbonylgruppe oder Reduktion der CC-Doppelbindung erfolgte, was unter solchen Bedingungen selten erreicht wird. Vergleichende Experimente mit Lyophilisaten von *Pleurotus*-Spezies bestätigten die besondere katalytische Effizienz von *B. adusta* in diesen Reaktionen.

Im Gegensatz dazu zeigten wachsende Kulturen von *B. adusta* eine reduktive Aktivität, indem sie Anthranilsäure selektiv in 2-Aminobenzaldehyd reduzierten, während eine Weiterreduktion zum entsprechenden Alkohol vollständig unterdrückt wurde. Diese Transformation erfolgt unter wässrigen, aeroben Bedingungen und zeigt strikte Regioselektivität, wobei nur das *o*-Isomer als Substrat akzeptiert wird.

List of publications

1. V. V. Babkina; W. Albuquerque; Y. M. Haiduk; W. Michalak; P. Ghezellou; H. Zorn; T. S. Zhuk, Fungal lyophilisates as catalysts for organic synthesis: Preparative oxidations with the white-rot fungus *Bjerkandera adusta*. *Mol. Catal.* **2023,** *549*, 113451.

2. V. V. Babkina; Y. M. Haiduk; Y. Kurtash; H. Zorn; T. S. Zhuk, Reduction of anthranilic acid to 2-aminobenzaldehyde by the white-rot fungus *Bjerkandera adusta* DSMZ 4708. *J. Biotech.* **2024,** *387*, 44-48.

3. L. Calza; V. V. Nikitenkova; W. Albuquerque; H. Zorn; T. S. Zhuk, Selective oxidation of α,β-unsaturated alcohols with lyophilisates of *Bjerkandera adusta*. *Chem. Biodivers.* **2025**, e01127.

Conference contributions

Poster

1. V. V. Babkina, Y. M. Haiduk, W. Albuquerque, H. Zorn, T. S. Zhuk. Preparative oxidation of primary alcohols by lyophilisates of the white-rot fungus *Bjerkandera adusta*. *13th Wartburg Symposium on Flavor Chemistry & Biology*, Eisenach (Germany), **2023**.

2. V. V. Babkina, Y. Kurtash, H. Zorn, T. S. Zhuk. Whole-cell production of 2-aminobenzaldehyde with the white-rot fungus *Bjerkandera adusta*. *17th International Weurman Flavour Research Symposium*, Wageningen (The Netherlands), **2024**.

3. V. V. Nikitenkova, L. Calza, W. Albuquerque, H. Zorn, T. S. Zhuk. Lyophilisate of *Bjerkandera adusta* as a biocatalyst for the oxidation of α,β-unsaturated alcohols. *Bioflavour*, Frankfurt am Main (Germany), **2025**.

4. V. V. Nikitenkova; L. Calza; W. Albuquerque; H. Zorn; T. S. Zhuk, Selektive Oxidation von Alkoholen zu wertvollen Aldehyden durch Lyophilisate des Weißfäulepilzes *Bjerkandera adusta*. *53. Deutsche Lebensmittelchemietage*, Halle (Saale) (Germany), **2025**.

5. T. S. Zhuk; V. V. Nikitenkova; Y. Kurtash; H. Zorn. Ganzzellreduktion von Carbonsäuren durch Weißfäulepilze. *53. Deutsche Lebensmittelchemietage*, Halle (Saale) (Germany), **2025**.

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List of abbreviations

α-DOX	*α*-dioxygenases
CAR	carboxylic acid reductase
ATP	adenosine triphosphate
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
AOR	aldehyde oxidoreductase
DAO	diamine oxidase
AOX	alcohol oxidase
AAO	aryl-alcohol oxidase
EC number	Enzyme Commission number
WRF	white-rot fungi
BRF	brown-rot fungi
UPO	unspecific peroxygenase
FAD	flavin adenine dinucleotide
GMC	glucose-methanol-choline
AADH	aryl-alcohol dehydrogenase/reductase
5-HMF	5-hydroxymethylfurfural
DFF	2,5-diformylfuran
FDCA	2,5-furandicarboxylic acid
PEF	polyethylene furanoate
PET	polyethylene terephthalate
FFCA	formylfurancarboxylic acid

1. Introduction

1.1 Background and Relevance

Organic chemistry underpins the synthesis of a wide range of essential compounds, including pharmaceuticals, polymers, cosmetics, and agrochemicals. Developing effective synthetic methods requires balancing key criteria such as efficiency, simplicity, chemo-, regio-, diastereo-, and enantioselectivity, as well as environmental safety. High selectivity of target transformations is among the main challenges in organic synthesis [1-3]. Competing reaction pathways most often produce mixtures of products, requiring additional separation and purification steps and reducing overall efficiency. Although classical methods are effective, they often do not provide the desired selectivity under mild and environmentally friendly conditions.

Modern syntheses must also follow the principles of green chemistry [4], which include, among others, reducing waste, minimizing the use of hazardous reagents, increasing energy efficiency, and using renewable resources wherever possible. Although these principles set clear goals for sustainable synthesis, their practical implementation is often difficult. Conventional organic synthetic methods are increasingly facing global challenges, such as limited natural resources, environmental concerns, and the need for sustainable production methods. Many classical methods rely on multi-step processes, generate large amounts of waste, and use hazardous reagents, which result in economic and environmental expenses.

1.1.1 Aldehydes as Key Compounds in Nature and Industry

The industrial production of aldehydes meets many of these challenges. In nature, they are widespread, particularly in plants [5], where they are responsible for characteristic aromas and serve as cosubstrates in enzymatic transformations [6]. Since they are quickly metabolized and do not accumulate in microorganisms due to their high toxicity [7], natural sources rich in aldehydes are relatively rare. Their sensory properties are remarkably diverse (Fig.1): (*E*)-2-hexenal imparts the fresh, green scent of cut grass, while (*E,E*)-2,4-decadienal provides a fatty, chicken-like aroma often associated with roasted meat. Piperonal, found in the essential oils of *Heliotropium* flowers [8], combines a sweet, floral, and slightly spicy smell with a benzodioxole moiety that also makes it a valuable precursor for pharmaceuticals, pesticides, and other industrial products [9-11]. Similarly, cuminaldehyde, the major aroma compound of *Cuminum cyminum* seeds [12-13], is widely used as a spice and is recognized for its beneficial health effects [12,14-15].

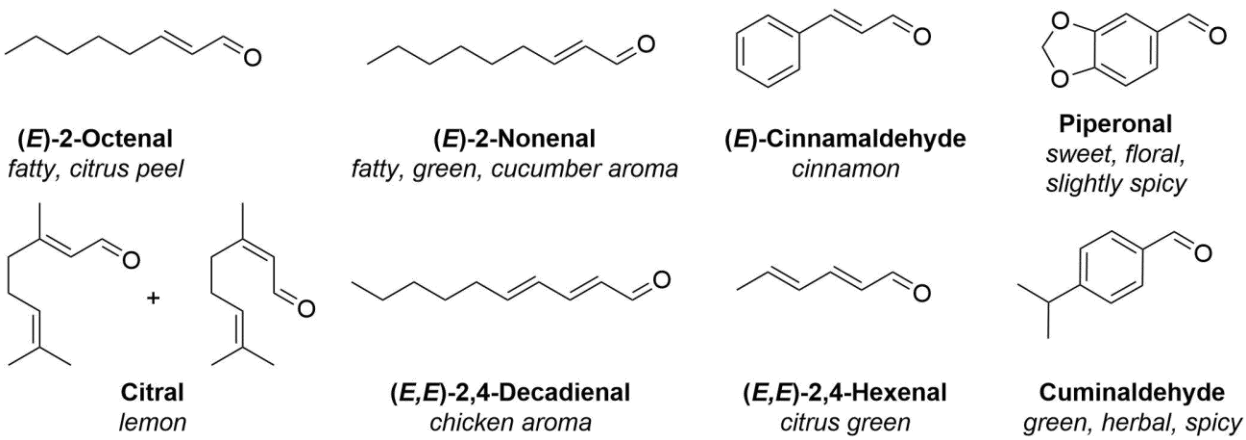

Figure 1. Representative aroma-active aldehydes and their characteristic odors.

Apart from their natural occurrence, aldehydes are also essential building blocks in organic synthesis because of the high reactivity of the carbonyl group [16-19]. Aldehydes are key flavor compounds in the food, cosmetics and beverage industry [20-22] due to their distinct organoleptic features [23]. The growing preference for healthy products labelled as “natural” further increases the importance of aldehydes in these sectors [24]. The biological and synthetic importance of aldehydes highlights the need for methods that allow their efficient and selective production.

The history of aldehyde syntheses dates back to the late 18th century. In 1774, Carl Wilhelm Scheele first synthesized acetaldehyde [25], although its structure remained unclear for decades. It was not until 1835 that Justus von Liebig determined the chemical constitution of the R–CHO group and introduced the term “aldehyde”, which means alcohol without hydrogen [26]. This work laid the foundation for the systematic study of aldehydes and their applications in various fields, including agriculture and chemical manufacturing [27]. It also gave a strong impulse to the development of modern perfumery [28]. Following early studies on aldehydes, several compounds quickly became central to the development of industrial chemistry. Benzaldehyde, a key aroma compound of *Prunus* species such as almonds, cherries, and apricots, was among the first to be identified and synthetically prepared [29]. Cinnamaldehyde was later recognized as the main contributor to the characteristic scent of cinnamon oil [30]. Its laboratory synthesis demonstrated the potential to recreate natural aromas chemically. Perhaps the most significant breakthrough was the production of vanillin from coniferin [31]. This marked the transition from extraction to large-scale industrial synthesis of aldehydes [32]. These advances not only expanded the availability of important compounds but also contributed the development of modern industrial chemistry, where aldehydes continue to play a key role in a wide range of applications.

The most common chemical routes to aldehydes involve the oxidation of the corresponding primary alcohols or extraction from natural sources. Every year, around $(110 \pm 23) \times 10^{12}$ g of

carbonyl compounds are produced worldwide, and most of them are obtained by oxidation of alcohols [33]. Traditional oxidations typically require toxic or harmful agents, such as chromium (VI) [34], manganese compounds [35], other strong inorganic oxidants [36-38] or transition metal catalysts [39-42]. Recent developments in photocatalysis also rely on scarce or complex materials, including graphitic C_3N_4-based polymers [43], Ti_3C_2/TiO_2 nanowires [44], or porous $Bi_{24}O_{31}Br_{10}(OH)_\delta$ catalysts [45]. Despite their effectiveness, these approaches often suffer from limitations such as overoxidation, high toxicity, low selectivity, long reaction times, and sensitivity to substrate structure [46], which restrict their suitability for producing aldehydes for use in food, beverage, and cosmetic applications. Therefore, promising alternative approaches such as biocatalysis are gaining attention due to their ability to achieve selective aldehyde transformations under mild and environmentally friendly conditions [47-48].

1.2 Biocatalysis

1.2.1 Fundamentals and Historical Development of Biocatalysis

Biocatalysis refers to the use of natural catalysts, such as enzymes or whole cells, to perform chemical transformations. Application of biocatalysis has a long history, dating back thousands of years. In ancient civilizations, such as Mesopotamia, China, and Japan, microorganisms were unknowingly used in processes like beer brewing, bread baking, wine fermentation, and cheese production [49]. The scientific understanding of enzymatic processes began much later. In 1814, Kirchhoff observed that a glutinous component of wheat could convert starch into sugar [49]. Shortly afterwards, Payen and Persoz systematically studied extracts from germinating barley that hydrolyzed starch into dextrin and sugar, establishing basic principles of enzyme action and paving the way for modern biocatalysis [50].

Nature has created a remarkable diversity of biocatalysts over millions of years. This long evolutionary history has given rise to an enormous biodiversity of microorganisms (bacteria, archaea, and fungi) that have developed a wide range of metabolic and physiological adaptations. As key drivers of biogeochemical cycles, they decompose organic matter and transform it into inorganic and low-molecular-mass organic compounds [51]. These microorganisms possess distinct enzymatic systems capable of converting substrates of various structural types, from simple aliphatic to complex polycyclic aromatic compounds. Enzymes, which are mostly proteins, are nature's highly efficient catalysts, evolved to accelerate biochemical reactions essential for life. They control and regulate metabolic reactions in microorganisms, plants, and animals [52]. Each enzyme possesses a unique active site that recognizes specific substrates and converts them into products with remarkable precision [53]. This molecular recognition results in exceptional chemo-, regio-, and stereoselectivity, which is difficult to achieve with traditional chemical catalysts [54].

In contrast to conventional chemical processes that often require extreme conditions such as high temperature, pressure, or the use of toxic reagents, enzymatic reactions typically proceed under mild and environmentally friendly conditions. Biocatalysts, including isolated enzymes and whole-cell systems such as bacteria, yeasts, or fungi used in viable or non-viable forms [52], can be derived from renewable resources. They are biodegradable and catalyze reactions with remarkable selectivity and efficiency [55]. This makes biocatalysis sustainable, energy-efficient, and compatible with the principles of green chemistry.

1.2.2 Applications and Industrial Significance of Enzymatic Catalysis

Modern biocatalysis combines these natural capabilities in both, industrial and laboratory applications. Its use ranges from large-scale production of fine chemicals [56] to the synthesis of pharmaceuticals [57], agrochemicals [58], and food [59], as well as to small-scale reactions in research laboratories, where new enzymes and reaction mechanisms are explored. One of the earliest examples of enzymatic synthesis was the enantioselective condensation of benzaldehyde with hydrogen cyanide to form (R)-mandelonitrile, catalyzed by hydroxynitrile lyase from almond (*Prunus amygdalus*) [60]. Over the next century, the field developed quickly, moving from crude extracts to purified and then recombinant enzymes [61]. Today, nitrile hydratase is used as an environmentally friendly alternative to metal-based catalysts in the large-scale production of acrylamide from acrylonitrile [62]. In food-related industries, β-galactosidases enable the production of lactose-free dairy products [63], while xylanases improve bread quality, and facilitate filtration and clarity during brewing [64]. In the pharmaceutical sector, transaminases, ketoreductases, and lipases catalyze stereoselective reactions leading to enantiomerically pure intermediates for drugs such as sitagliptin [65], atorvastatin [66], and ibuprofen [67-68]. Whole-cell systems are also utilized for multi-step cascade reactions, where several transformations proceed sequentially without the isolation of intermediates [55]. In research laboratories, such biocatalytic systems serve as versatile and sustainable tools to explore new reactivities and expand the synthetic utility of enzymes. Among the many types of compounds explored in these studies, aldehydes are of particular interest because of their unique combination of high reactivity and synthetic versatility.

1.2.3 Enzymatic Routes to Aldehyde Formation

Current enzymatic approaches are mainly based on a variety of enzyme systems that differ in their catalytic mechanisms, product selectivity, and the classes of precursor molecules that can be converted into aldehydes [69]. Aldehydes can occur in enzymatic systems either as intermediates or as the main reaction products. In many cases, they appear only as short-lived intermediates that are rapidly converted into alcohols or carboxylic acids. In several such biocatalytic systems, aldehydes are formed temporarily during reactions catalyzed by specific

enzymes. Among the best-known examples are alcohol dehydrogenases (ADHs), which catalyze the reversible oxidation of alcohols to aldehydes [69-71]. Although highly efficient and widely used in biocatalysis, they depend on costly and unstable cofactors and therefore require cofactor regeneration systems. The reaction equilibrium often favors the formation of alcohols, and a significant molar excess of the oxidizing agent is often required to shift the equilibrium toward aldehyde formation [72]. This tendency is further influenced by pH, as reduction predominates under neutral conditions, whereas higher pH values promote oxidation. Another important group are α-dioxygenases (*α*-DOX), which catalyze the oxidation of medium-chain fatty acids to 2-hydroperoxy fatty acids [73-74]. Spontaneous decarboxylation and dehydration lead to the formation of fatty aldehydes, commonly used as fragrance and aroma ingredients with fresh, citrus, or waxy notes [74]. In addition, decarboxylases represent another enzymatic route for aldehyde formation. Enzymes such as pyruvate decarboxylase [75-77], acetohydroxyacid synthase [78], and α-ketoisovalerate decarboxylase [7,79] catalyze the non-oxidative decarboxylation of α-keto acids, temporarily forming aldehydes, which are further converted through metabolic pathways. Another notable route involves the combined action of lipoxygenases and hydroperoxide lyases, which convert polyunsaturated fatty acids into volatile aldehydes responsible for characteristic green leaf aromas [80-81]. Despite the diversity of enzymatic routes leading to the formation of valuable aldehydes, practical application remains challenging due to narrow substrate scope, insufficient enzyme stability, and difficult reaction control.

In contrast, in another group of biocatalytic reactions, aldehydes are deliberately produced as the primary products. In this category, carboxylic acid reductases (CARs) are among the most extensively studied systems [82]. They catalyze the ATP (adenosine triphosphate)- and NADPH (nicotinamide adenine dinucleotide (phosphate)-dependent reduction of carboxylic acids to the corresponding aldehydes [83]. CARs show a broad substrate scope, ranging from aliphatic to aromatic, polycyclic, and heterocyclic acids [84], and they display high product selectivity, producing the aldehydes without further reduction. However, their strong dependence on cofactors and complex structure significantly limit their large-scale application. Aldehyde oxidoreductases (AORs) represent another enzyme class that catalyzes the reversible reduction of carboxylic acids to aldehydes [85]. AORs are oxygen-sensitive enzymes of bacterial and archaeal origin that operate under strictly anaerobic conditions using Fe-S clusters and redox mediators [86]. Because of this oxygen sensitivity, they have mainly been studied in native anaerobic hosts such as *Pyrococcus furiosu*s, where the produced aldehydes are often further reduced to alcohols. Despite their biotechnological potential, the functional expression of active AORs in heterologous systems like *Escherichia coli* or yeast remains an unsolved challenge [69]. Another class of enzymes is diamine oxidases (DAOs), formerly known as histaminases due to their role in histamine degradation to imidazole acetaldehyde.

They catalyze the oxidative deamination of polyamines, such as putrescine, cadaverine, spermidine, and histamine, forming the corresponding aldehydes along with ammonia and hydrogen peroxide [47,87-89]. These copper-containing homodimeric enzymes are highly selective for their substrates, making them useful for biocatalytic applications and for detecting biogenic amines in food and beverages. However, their applications are mainly limited by narrow substrate specificity and the challenges associated with recombinant expression in host cells. Among the enzymes directly yielding aldehydes are alcohol oxidases (AOXs) including aryl-alcohol oxidases (AAOs). They, in contrast, catalyze the oxidation of primary and secondary alcohols to aldehydes or ketones, using molecular oxygen as a cosubstrate. These enzymes are naturally found in methylotrophic yeasts and certain fungi, where they participate in methanol metabolism [90] or lignin degradation [91]. AOXs are particularly promising for biocatalysis because they require only oxygen and do not depend on costly cofactors. They have been successfully applied for the synthesis of vanillin [92], furan- derivatives [93-94], and oxidation of benzyl alcohol and its substituted derivatives [95]. AAOs exhibit high activity towards aliphatic unsaturated alcohols such as 2,4-hexadien-1-ol [96] and *trans*-2-hexen-1-ol [97], highlighting their potential for selective and sustainable aldehyde production. The key characteristics and limitations of these enzymes are summarized in Table 1.

Table 1. Main enzyme classes for aldehyde production.

Enzyme	EC number	Reaction type	Key features	Limitations	Ref.
ADHs	EC 1.1.1.1	reversible alcohol oxidation	High efficiency; widely used	Cofactor dependence; equilibrium favors alcohol; pH-sensitive	[69-72]
α-DOX	EC 1.13.11.92	Oxidation of medium-chain fatty acids	Aroma/fragrance production; medium-chain specificity	Narrow substrate scope	[73-74]
Pyruvate decarboxylase	EC 4.1.1.1				
Acetohydroxyacid synthase	EC 2.2.1.6	Non-oxidative α-keto acid decarboxylation	Forms aldehyde intermediates	Transient product; metabolically converted	[75-79]
α-Ketoisovalerate decarboxylase	EC 4.1.1.72				
Lipoxygenase + hydroperoxide lyase	EC 1.13.11.x EC 4.1.2.x	Sequential oxidation of polyunsaturated fatty acids	Generates green leaf aromas	Narrow substrate scope; enzyme instability	[80-81]
CARs	EC 1.2.1.30	Reduction of carboxylic acids	Broad substrate scope; high selectivity	Cofactor dependence; complex structure	[82-84]
AORs	EC 1.2.99.6	Reversible reduction of carboxylic acids	Anaerobic; use Fe-S clusters	Oxygen-sensitive; heterologous	[69,85-86]

Enzyme	EC number	Reaction type	Key features	Limitations	Ref.
				expression challenges	
DAOs	EC 1.4.3.22	Oxidative deamination of polyamines	Broad polyamine substrate	High substrate specificity; heterologous expression challenging	[87-89]
AOXs (AAOs)	EC 1.1.3.x (EC 1.1.3.7)	Oxidation of primary and secondary alcohols (O_2-dependent)	Cofactor-independent; high selectivity	Accumulation of hydrogen peroxide, heterologous expression challenging	[90-91,95-96]

Currently, most studies focus on isolated enzymes produced using recombinant expression systems. While the use of purified enzymes provides high selectivity, it often requires complex purification and cofactor regeneration steps, making the process expensive and less efficient for large-scale applications. In contrast, whole-cell biotransformations represent a more economical and operationally simpler alternative, as microbial cells naturally contain all the necessary cofactors and recycling systems in their natural environment. Production costs for whole-cell systems are significantly lower (35-100 €*kg^{-1}) compared to purified enzymes (250-1000 €*kg^{-1}), making them attractive for industrial applications. Moreover, the use of whole cells allows for the combination of sequential enzymatic steps, thereby avoiding costly intermediate isolation and enabling the conversion of complex natural substrates.

Whole-cell catalysts have been successfully used for the selective oxidation and reduction of various alcohols and aldehydes. For example, lyophilized *Janibacter terrae* cells were shown to catalyze the chemoselective oxidation of alcohols to aldehydes *via* a hydrogen transfer process using acetaldehyde as a hydride acceptor [98]. The cells exhibited strong substrate dependence: benzyl alcohols with small substituents (F, CH_3, OH) were efficiently transformed, while bulkier groups significantly reduced the activity. Similarly, acetic acid bacteria such as mutants of *Gluconobacter oxydans* demonstrated the ability to oxidize 2-methyl-1-butanol to isovaleraldehyde with good conversion yields [99], and the same species oxidized both aliphatic and aromatic alcohols in yields of 43–82% [100]. Two-phase systems (water/organic solvent) further improved product recovery by enabling *in situ* aldehyde removal, enabling the oxidation of 2-phenylethanol to phenylacetaldehyde and pseudocumene to 3,4-dimethylbenzaldehyde using acetic acid bacteria and recombinant *E. coli*, respectively [101-102].

Whole-cell systems based on yeasts have been applied for the transformation of terpene aldehydes. A broad range of yeast species were reported to convert (S)-perillaldehyde and α-methyl-cinnamaldehyde [103]. In these reactions, the double bond of (S)-perillaldehyde was

reduced to form dihydro-perillaldehyde; however, the resulting aldehyde was further undesirably reduced to dihydroperillic alcohol. Similarly, α-methyl-cinnamaldehyde was converted to α-methyl-dihydro-cinnamaldehyde and subsequently to α-methyldihydrocinnamyl alcohol [104]. Another notable example is *Komagataella phaffii*, whose alcohol oxidase exhibits broad substrate specificity and can oxidize benzyl alcohol to benzaldehyde in aqueous media. Due to low solubility and inhibition by both substrate and product, optimal performance was achieved in biphasic systems with 3–5% aqueous phase, where the enzyme retained high activity and could be immobilized on DEAE-Biogel to improve stability and reusability [105].

While bacteria and yeasts have been widely used for aldehyde synthesis, these systems often suffer from overreduction of the product to alcohols due to high dehydrogenase activities. Moreover, the coexistence of multiple enzyme classes within a single organism can lead to competing side reactions and lower selectivity. The main efforts in improving whole-cell biocatalysis have focused on the development of genetically modified host organisms. However, for many industrial applications, it is more desirable to avoid the use and handling of living genetically modified systems not only due to safety regulations, cost, and scalability reasons, but also because regulatory and labeling requirements may prevent the products from being marketed as ‘natural’.

To address these challenges, recent research has increasingly turned toward fungi, particularly basidiomycetes, as alternative biocatalysts. Although yeast and bacteria have been well-studied in various biocatalytic transformations, research on the potential of fungi to produce aldehydes remains limited. However, their enzymatic diversity makes them promising candidates for sustainable and selective biocatalysis.

1.3 Basidiomycota

1.3.1 Ecological and Taxonomic Overview

Fungi represent a diverse kingdom of eukaryotic organisms, estimated to comprise between 2.2 and 13 million species [106]. They are abundant worldwide and play a vital role in ecosystems, acting as decomposers, symbionts, and pathogens. By breaking down complex organic compounds into simpler molecules, fungi contribute significantly to carbon and nutrient cycling. Many form symbiotic associations with plants or algae, while others act as parasites of plants, animals, or other microorganisms [107].

Among the kingdom of fungi, the phyla Ascomycota and Basidiomycota together comprise the so-called higher fungi (Dikarya) [108], which are capable of forming complex fruiting bodies visible to the naked eye. The phylum Basidiomycota is one of the nine major phylum-level clades and currently includes more than 40,000 described species [109], although the real

number is likely much higher. Taxonomically, it is divided into three main subphyla: *Agaricomycotina*, *Ustilaginomycotina*, and *Pucciniomycotina* [110]. Most of the well-known edible and medicinal fungi belong to the Agaricomycotina, such as the oyster mushroom (*Pleurotus ostreatus*), white button mushroom (*Agaricus bisporus*), porcini (*Boletus edulis*), shiitake (*Lentinula edodes*), Ling Zhi (*Ganoderma lucidum*), and Fu Ling (*Wolfiporia cocos*) [111-112]. Morphologically, basidiomycetes are defined by basidia – specialized terminal cells of the hyphae where karyogamy and meiosis occur, giving rise to haploid basidiospores [109,113]. These structures are the distinctive feature of the phylum and the source of its name. Unlike Ascomycetes, which remain predominantly monokaryotic, basidiomycetes exhibit a prolonged dikaryotic stage, which contributes to their morphological plasticity and metabolic diversity [113].

Basidiomycota have enormous biotechnical and economic importance. Almost all edible fungi cultivated on a large scale belong to this phylum, and by 2013, the global production reached about 34 billion kilogram, with six main genera (*Lentinula*, *Pleurotus*, *Auricularia*, *Agaricus*, *Flammulina*, and *Volvariella*) accounting for nearly 90% of the total production [114]. In addition to their nutritional value, basidiomycetes produce a wide range of secondary metabolites with diverse structures and biological functions [115-116]. These include pigments [117], antimicrobial compounds [118], pharmacologically active substances [119], biosurfactants [120], and flavour compounds belonging to various chemical classes [121], such as terpenes, terpenoids, polyketides, and fatty acid or peptide derivatives [116]. This structural and functional diversity reflects their rich enzymatic profile.

1.3.2 Wood Decomposition Mechanisms of Basidiomycetes

Thanks to their extensive enzyme portfolios, Basidiomycota are capable of decomposing complex biopolymers such as cellulose and lignin [122], making them valuable decomposers in ecosystems. Wood-degrading fungi are broadly divided into white- and brown-rot types based on their lignocellulose degradation mechanisms. White-rot fungi (WRF) use oxidative enzymes to degrade all major wood components. These fungi depolymerize lignin, leaving a cellulose-rich residue [123-124]. Lignin is a highly complex, three-dimensional aromatic polymer composed mainly of methoxylated phenylpropanoid units derived from coniferyl, sinapyl, and *p*-coumaryl alcohol (Figure 2) [125]. The relative abundance of these monolignols varies among plant species, influencing both the structure and the resistance of lignin to degradation. Members of the white-rot group express a broad suite of oxidative enzymes involved in lignin metabolism, including heme-thiolate peroxygenases such as chloroperoxygenases and unspecific peroxygenases, as well as various copper-containing oxidoreductases, including laccases [126-127]. Some of these enzymes catalyze highly challenging oxidative transformations that are difficult to achieve even under harsh chemical

conditions with conventional reagents. These enzymes are capable of oxidizing both phenolic and/or non-phenolic aromatic compounds [128], initiating a cascade of reactions that break down the lignin polymer. Ultimately, lignin decomposition yields low-molecular-mass aromatic compounds, a process driven by extracellular enzymatic activity and complemented by subsequent intracellular metabolic conversions [129].

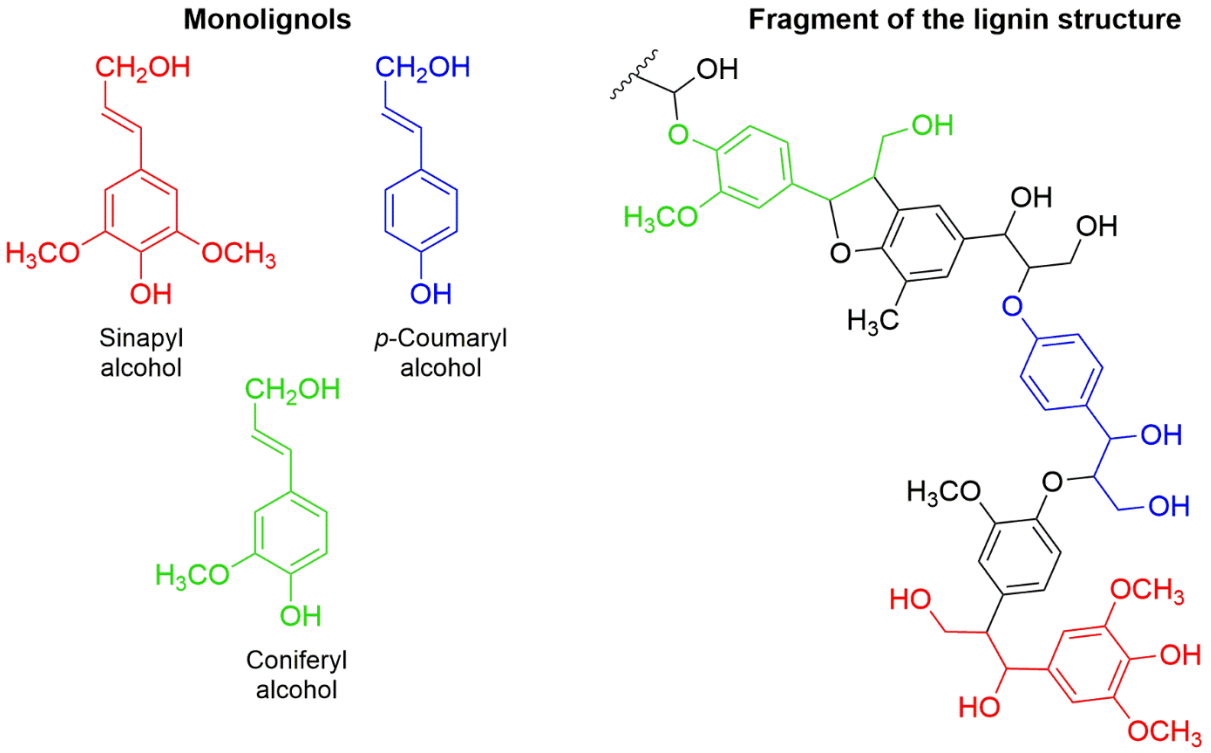

Figure 2. Representative fragment of lignin structure and its main monolignols.

In contrast, brown-rot fungi (BRF) primarily use non-enzymatic reactions to depolymerize cellulose and hemicellulose, while extensively modifying lignin [130]. In BRF, this process involves the Fenton reaction, in which Fe^{2+} ions are oxidized by hydrogen peroxide to generate hydroxyl radicals that modify the lignin structure and facilitate enzymatic degradation of (hemi)cellulose [131]. These distinct oxidative strategies underline the key ecological role of basidiomycetes as decomposers of plant biomass and recyclers of organic carbon.

1.3.3 Enzymatic and Biocatalytic Potential of Basidiomycetes

In addition to their ecological significance, fungi are also a rich source of ligninolytic and secondary metabolite biosynthetic enzymes, including terpene synthases, polyketide synthases, non-ribosomal peptide synthases, and cytochrome P450 monooxygenases [132]. Despite this enzymatic wealth, their potential has been explored only to a limited extent, particularly for synthetic and biocatalytic purposes. The unique oxidative and biosynthetic capabilities of basidiomycetous enzymes make them highly attractive tools for organic synthesis, particularly in reactions involving the selective oxidation and transformation of

aromatic substrates – key steps in the formation of aldehydes and other valuable intermediates.

Numerous studies have demonstrated the remarkable biocatalytic potential of basidiomycetes to carry out a variety of redox reactions under mild and environmentally favorable conditions. Whole-cell systems and isolated fungal enzymes have been successfully used for the reduction of carbonyl compounds and carboxylic acids, as well as for the oxidation of CH-bonds, epoxidation of olefins, and oxidative cleavage of alkenes, often with excellent chemo-, regio-, and stereoselectivity (Fig. 3) [133]. WRF have been extensively studied as effective tools for the degradation of toxic pollutants [134-135], and numerous examples demonstrate their ability to oxidize aromatic CH-bonds *en route*, with several studies also describing these types of oxidation for aliphatic compounds. In comparison to other biocatalytic systems such as bacteria or yeasts, WRF-based systems stand out due to their exceptional enzyme stability, non-toxic nature, and simplicity in use. Additionally, they are easily accessible even to synthetic laboratories with limited microbiological expertise.

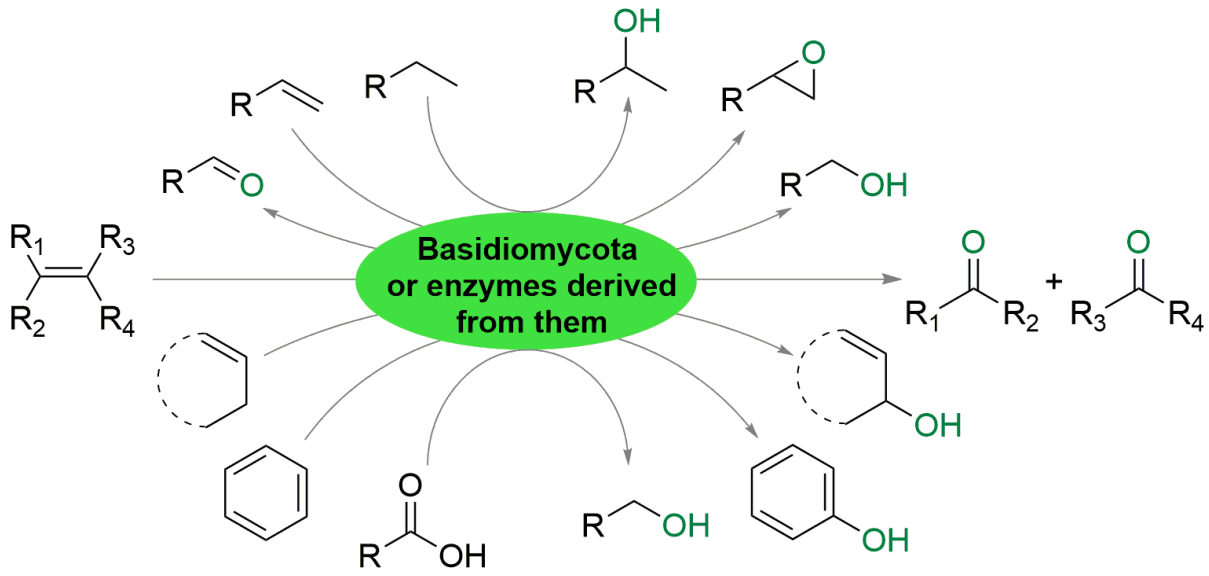

Figure 3. Representative reactions catalyzed by basidiomycetes or enzymes derived from them.

The first reduction catalyzed by a basidiomycetous fungus, *Trametes versicolor*, was reported as early as 1959 and described the transformation of aromatic carboxylic acids into the corresponding aldehydes and alcohols [136]. Since then, various fungi have been screened for their ability to reduce fluorinated, methoxy-, and amino-substituted benzoic acid derivatives, including isomeric anisic acids [137]. Basidiomycetous fungi are able to selectively reduce both, aromatic and aliphatic carbonyl compounds. For example, *Dichomitus albidofuscus* efficiently reduces both α,β-unsaturated and linear saturated aldehydes, such as 2-heptenal, dodecanal, and undecanal, to the corresponding alcohols in high yields. *D. albidofuscus* also exhibits high selectivity in the preparative, aqueous reduction of aromatic carbonyl compounds,

such as benzaldehyde, anisaldehyde, and vanillin, at room temperature, forming the corresponding alcohols in high yields. This fungus is capable of reducing benzoic acid and related derivatives to the corresponding alcohols [138]. This is remarkable because such a transformation typically requires the use of hazardous reagents or harsh reaction conditions in classical organic synthesis. Moreover, some functional groups remain intact under these mild biocatalytic conditions, which is favorable for the selective reduction of polyfunctional aromatic substrates. Recently, *Pycnoporus cinnabarinus*, *Pleurotus eryngii*, *Pleurotus sapidus*, *Laetiporus sulphureus*, and *Lepista nuda* were also found to convert substituted benzoic acids, such as *p*-anisic, vanillic, veratric, piperonylic, and eudesmic acids into the corresponding fragrant aldehydes, including *p*-anisaldehyde, vanillin, veratraldehyde, piperonal, and 3,4,5-trimethoxybenzaldehyde [139].

Basidiomycetes have also demonstrated remarkable potential in the enantioselective reduction of ketones, producing optically pure alcohols under mild and environmentally friendly conditions. Numerous studies have reported the ability of fungal whole cells and enzymes to catalyze stereoselective reductions of prochiral ketones. Whole cells of several Brazilian basidiomycetes, including *Irpex lacteus*, *Pycnoporus sanguineus*, *Trametes rigida*, *Trametes versicolor*, and *Trichaptum byssogenum*, were shown to efficiently catalyze the reduction of 2-(phenylseleno)cyclohexanone to its corresponding alcohol with high stereoselectivity [140]. In particular, *T. rigida* produces both, *cis*- and *trans*-isomers in excellent yields. Growing cells of the basidiomycete *Lentinus strigellus* also exhibited the ability to stereoselectively reduce a broad range of aromatic and aliphatic ketones, including 2-octanone, which was converted into enantiopure (S)-2-octanol with high conversion yields [141]. Furthermore, screening of 19 edible mushrooms revealed that *Pleurotus salmoneostramineus*, *Ganoderma lucidum*, and *Flammulina velutipes* act as particularly efficient biocatalysts in the reduction of α-keto esters to α-hydroxy esters, yielding products with high enantiomeric excess (>99%) [142]. In addition, fruiting bodies of *Clitocybe nebularis*, *Entoloma undatum*, *Lactarius deterrimus*, *Laccaria amethystina*, *Amanita muscaria*, *Boletus badius*, *Suillus bovinus*, and *Scleroderma citrinum* exhibited measurable carbonyl reductase activity toward substrates such as 3,4-hexanedione and acetophenone, yielding the corresponding chiral alcohols in moderate amounts [143].

Apart from whole-cell biotransformations, several basidiomycetous carboxylic acid reductases (CARs) have been identified and characterized. Heterologously expressed enzymes from *Serpula lacrymans* showed substrate specificity toward L-threonine and benzoic acid, while a reductase from *Heterobasidion annosum* efficiently converted phenylpyruvic acid [144]. Another enzyme, BvCAR from *Boreostereum vibrans*, involved in vibralactone biosynthesis, catalyzed the reduction of 3-prenyl-4-hydroxybenzoate to the corresponding aldehyde [145].

The selective oxidative cleavage of CC-double bonds is an important transformation that enables the conversion of olefinic substrates into aldehydes and ketones. Several basidiomycetes have demonstrated this ability. For instance, *Trametes hirsuta* was shown to oxidatively cleave alkenes adjacent to aromatic rings, forming the corresponding benzaldehyde derivatives [146]. Enzymes isolated from the same species, including a Mn(III)-dependent oxidase, were also capable of performing alkene cleavage using molecular oxygen [147]. Similarly, a dye-decolorizing peroxidase (DyP) from *Pleurotus sapidus* catalyzed the oxidative cleavage of aryl alkenes such as *trans*-anethole to yield aromatic aldehydes [148]. In addition, a lipoxygenase from *P. sapidus* was found to convert piperine into piperonal and 3,4-methylenedioxycinnamaldehyde *via* a co-oxidation mechanism [149].

Selective oxidation of CC-double bonds also represents a valuable transformation in basidiomycete-mediated biocatalysis involving cytochrome P450 enzymes. A P450 from *Punctularia strigosozonata* catalyzed the selective epoxidation of a *cis*-olefin in a model substrate [150], while another from *Panus rudis* participated in the biosynthesis of panepoxydone and exhibited broad substrate tolerance toward 2-substituted hydroquinones [151]. Fungi represent a rich source of cytochrome P450 monooxygenases for selective oxyfunctionalization reactions, but their industrial application is often limited by poor expression, low selectivity and stability. Unspecific peroxygenases (UPOs), first discovered in the basidiomycete *Agrocybe aegerita* [152], represent an attractive alternative since they catalyze similar reactions but use only hydrogen peroxide instead of expensive cofactors such as NAD(P)H [153]. Their remarkable catalytic potential has made UPOs highly attractive for biotechnological applications. They efficiently catalyze oxidation reactions, such as the conversion of cyclohexane to cyclohexanol and cyclohexanone, under mild conditions [154]. These enzymes also show high stability in organic solvents and excellent selectivity in alkane hydroxylation, allowing complete conversion of propane to 1-propanol [155]. Continuous discovery of new UPOs through genomic analysis and heterologous expression has further expanded their diversity and synthetic utility [156]. In addition to their use in selective oxyfunctionalization of inert CH-bonds, certain fungal UPOs also participate in natural product biosynthesis, such as the oxidative dimerization of torosachrysone leading to the formation of phlegmacins [157].

These examples highlight both the historical and contemporary importance of basidiomycetous fungi as versatile and robust biocatalysts. Their ability to perform complex redox transformations under mild conditions, combined with the natural diversity of their enzymatic systems, makes them highly promising for selective organic synthesis and biotechnological applications.

1.4 Aryl-alcohol oxidases of *Bjerkandera adusta*

1.4.1 The basidiomycetous fungus Bjerkandera adusta

Bjerkandera adusta, commonly known as the smoky polypore, is a widespread white-rot fungus found in North America, Europe, and Asia [158-159]. In traditional medicine, *B. adusta* is used for its antibacterial, antifungal, and anti-inflammatory properties [160-161] and is also considered the etiologic fungus responsible for chronic coughs [162]. The fungus forms annual sessile fruiting bodies with a gray-brown tubular layer contrasting with a lighter outer layer (Fig. 4). Although inedible, the mushroom emits a pleasant aroma when fresh. As a prominent member of the white-rot fungi, it plays an important ecological role by decomposing dead wood of deciduous trees and contributing to the global carbon cycle. Furthermore, this fungus is capable of degrading persistent organic pollutants, such as polycyclic aromatic hydrocarbons [163], pentachlorophenol [164], and 2,4,6-trinitrotoluene [165]. *B. adusta* is a rich source of extracellular oxidative enzymes [166], including, for example, dye-decolorizing peroxidases, which have been isolated and characterized from this fungus [167]. Accordingly, aryl-alcohol oxidases have recently been identified in its supernatants as a supplier of H_2O_2 for peroxidases [168-169]. These enzymatic properties make *B. adusta* a promising candidate for industrial applications, such as bioremediation, food, pharmaceutical, and cosmetics, as it is non-toxic and easily cultivated on inexpensive substrates.

Figure 4. Fruiting body of *Bjerkandera adusta* on a tree. (Image kindly provided by Dr. Alejandra Omarini (Asociación para el Desarrollo de Villa Elisa y Zona, CONICET, Argentina) and Dr. Gerardo L. Robledo (BioTecA3 – Centro de Biotecnología Aplicada al Agro y Alimentos, Ciudad Universitaria, Córdoba, Argentina).

1.4.2 Aryl-alcohol oxidases (AAOs): structure and mechanism

AAOs (also known as veratryl-, benzyl-, salicyl- and aromatic-alcohol oxidases) are FAD (flavin adenine dinucleotide)-dependent oxidoreductases that belong to the glucose-methanol-choline (GMC) oxidoreductase superfamily. Although many AAOs are secreted extracellularly, some enzymes from certain species are intracellular, highlighting the variability of their localization in fungi and other organisms. AAO was first detected in 1960 in cultures of the basidiomycete *Trametes versicolor* [170]. The enzyme was not purified, but its properties suggested the presence of a flavoprotein capable of transferring hydrogen from aromatic alcohols to molecular oxygen, producing hydrogen peroxide (H_2O_2) and the corresponding aldehydes. Since then, AAO activity has been reported in a wide range of basidiomycetes, including *Pleurotus* species [171-174], *Phanerochaete chrysosporium* [175], *Ustilago maydis* [176], *Coprinopsis cinerea* [177], and *Bjerkandera adusta* [169,178]. AAOs have also been described in ascomycetes, such as *Botrytis cinerea* [179] and *Aspergillus terreus* [180], and in non-fungal organisms, including insects (*Chrysomela populi*, *Phratora vitellinae*) [181] and gastropods (*Arion ater*, *Helix aspersa*, *Limax flavus*) [182].

In fungi, the physiological role of AAOs is primarily to generate H_2O_2, which activates ligninolytic peroxidases during white-rot decay or initiates Fenton chemistry in brown-rot fungi (Fig.5). The enzymes catalyze the oxidation of a broad spectrum of aromatic and primary alcohols, producing the corresponding aldehydes while reducing molecular oxygen to H_2O_2. In *B. adusta*, veratryl and *p*-anisyl alcohols serve as natural substrates, supporting continuous H_2O_2 production for ligninolytic activity. Aldehydes help maintain the redox cycle by being converted back to their corresponding alcohols through reduction by NAD(P)H-dependent aryl-alcohol dehydrogenases/reductases (AADHs). This process is crucial because excess H_2O_2 can inactivate peroxidases, whereas insufficient amounts slow down the rate of lignin degradation, which is the main energy source for WRF.

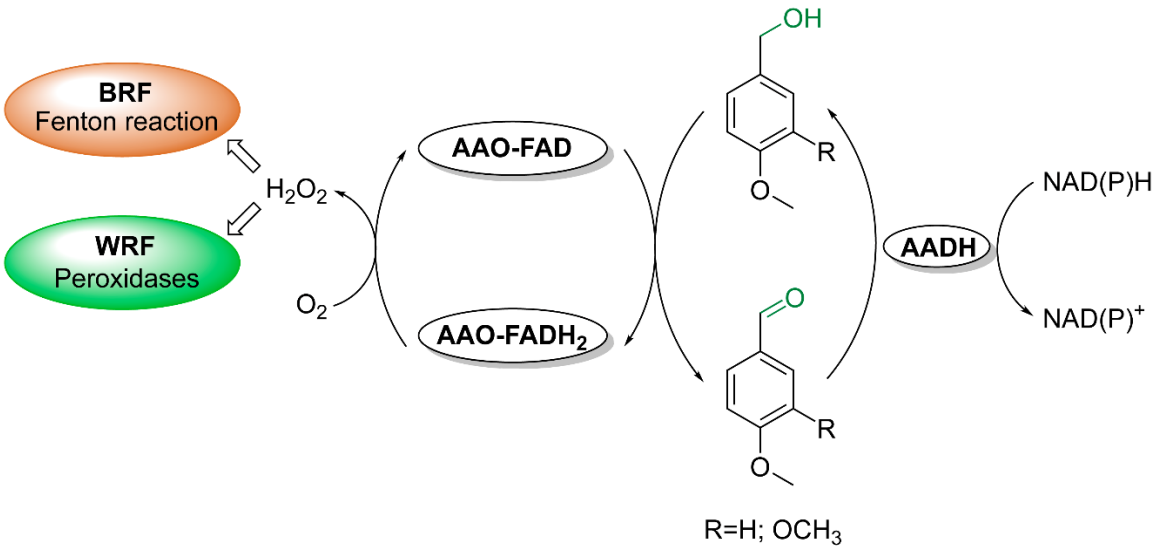

Figure 5. Scheme of the involvement of AAO in the key step of lignin degradation.

Structurally, AAOs are monomeric flavoproteins with a molecular mass typically ranging from 65 to 78 kDa [174,183]. Their catalytic cycle involves two half-reactions (Fig. 6B). In the reductive half-reaction, the enzyme oxidizes the alcohol substrate to the corresponding aldehyde, accompanied by the reduction of the flavin cofactor. In the oxidative half-reaction, molecular oxygen reoxidizes the flavin, yielding hydrogen peroxide as a by-product [95]. AAOs primarily oxidize aromatic alcohols to the corresponding aldehydes. In some cases, the enzyme is also capable of further oxidizing certain aldehydes to the corresponding carboxylic acids. In aqueous solution, aldehydes exist in equilibrium with their hydrated (*gem*-diol) forms, which can also serve as substrates for AAO (Fig. 6A, 6C). The hydration process is rapid and quickly reaches equilibrium, occurring faster in acid or base than at neutral pH. The rate of hydration also depends on the chemical nature of the substituents on the aromatic ring: electron-withdrawing groups stabilize the *gem*-diol intermediate, facilitating oxidation, whereas electron-donating groups destabilize it and reduce its reactivity [184].

Recent mechanistic studies support a concerted mechanism in which both, alcohol and *gem*-diol oxidation involve simultaneous transfer of a α-carbon “hydride” from the substrate to the isoalloxazine ring of FAD and a proton to a catalytic base in the enzyme’s active site (Fig.6B, 6C). This general base, typically a histidine residue, facilitates proton abstraction from the hydroxyl group of the substrate, thereby promoting hydride transfer and efficient flavin reduction [184]. Such a mechanism provides a unified explanation for the broad substrate specificity and high catalytic efficiency of fungal AAOs.

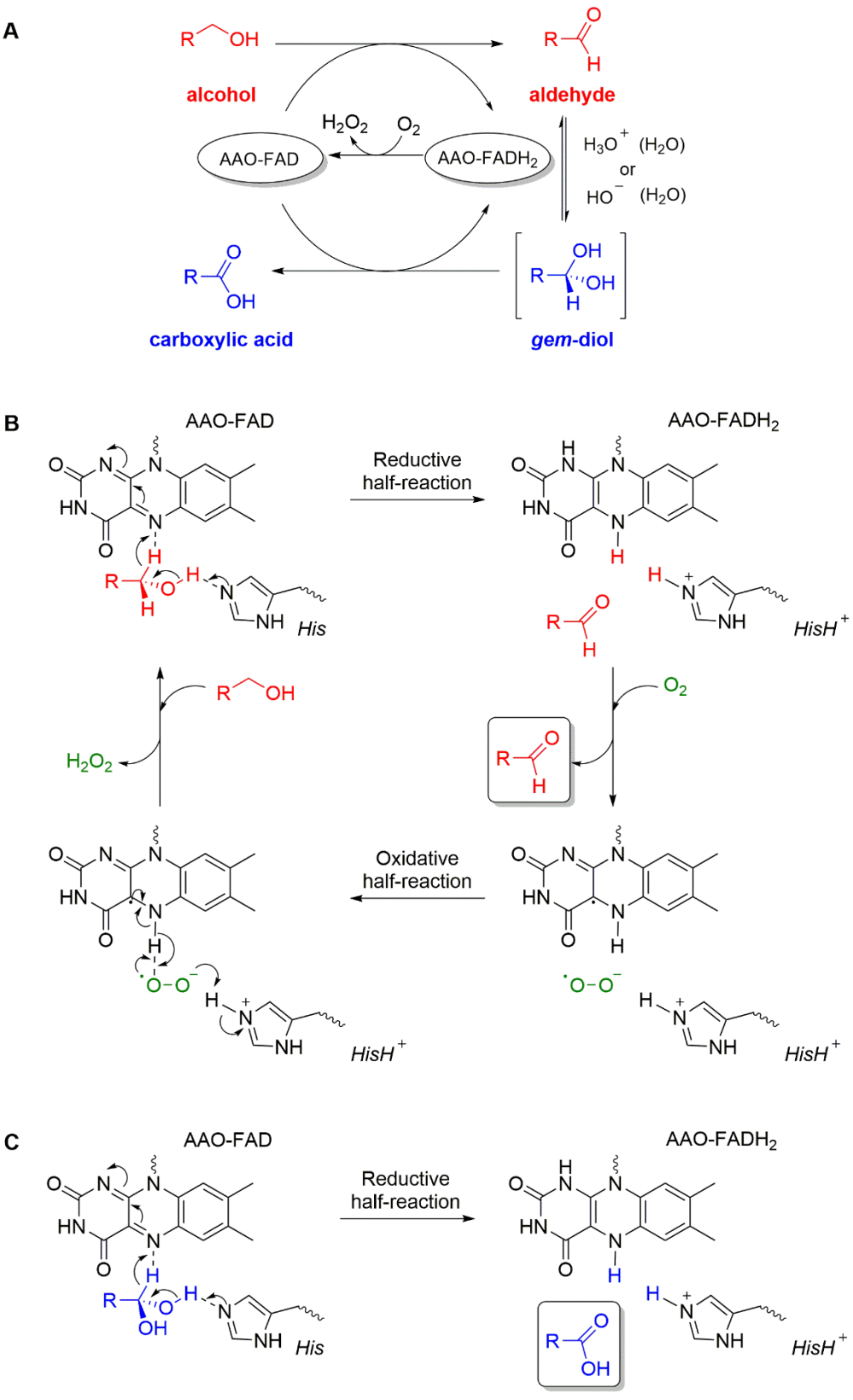

Figure 6. A. General scheme of the AAO-catalyzed oxidation of alcohol to aldehyde and its further transformation to a carboxylic acid *via* an unstable *gem*-diol. **B.** The catalytic cycle of AAO consists of two half-reactions. In the reductive half-reaction, an alcohol is converted to an aldehyde, while the oxidative half-reaction regenerates the flavin and produces hydrogen peroxide. **C.** The reductive half-reaction of the AAO catalytic cycle with a *gem*-diol substrate, which is converted to the corresponding carboxylic acid.

Since the oxidation of gem-diols proceeds analogously to alcohol oxidation, AAOs can also catalyze multi-step oxidative transformations. A well-known example is their activity toward 5-hydroxymethylfurfural (5-HMF). In aqueous solution, the 2,5-diformylfuran (DFF) generated from 5-HMF undergoes rapid hydration to form its corresponding *gem*-diol, which can then enter subsequent oxidation cycles. As a result, AAOs are capable of performing a full sequence of three consecutive oxidation reactions, converting HMF into 2,5-furandicarboxylic acid (FDCA). In recent years, the synthesis of FDCA has received increased attention due to its potential for producing polyethylene furanoate (PEF), a biobased alternative to mineral oil-derived plastics used in polymer manufacturing such as polyethylene terephthalate (PET). It has been demonstrated that AAOs readily catalyze the first two oxidation steps, namely from 5-HMF to DFF and further to formylfurancarboxylic acid (FFCA), while the final oxidation of FFCA to FDCA is significantly slower. This limitation can be overcome either by minimizing the inhibitory effect of H_2O_2 produced during the earlier steps [185] or by modifying the catalytic properties of the enzyme [186]. Additionally, engineered AAO variants with enhanced activity toward FFCA show markedly improved overall 5-HMF conversion, enabling efficient production of FDCA through the *gem*-diol. However, these reactions have only been demonstrated at low substrate concentrations up to 15 mM.

AAOs have shown significant potential in the synthesis of flavors, fragrances, and other high-value compounds [187]. Despite the identification of numerous AAO-encoding genes in the genomes of wood-degrading fungi, most catalytic studies have focused on a single representative, *Pe*AAO from *Pleurotus eryngii* [188], and its mutant *Pe*AAO2 [189]. Recently, the biocatalytic potential of these enzymes has been highlighted. PeAAO2 demonstrated high stability, retaining activity in the presence of up to 30% (v/v) organic solvents and elevated H_2O_2 levels. Under optimized conditions, it efficiently converted piperonyl alcohol to piperonal, reaching a concentration of ~245 mM within 3 h, while preparative-scale reactions provided 95% conversion and 85% yield of isolated product after crystallization. Additional aromatic, heterocyclic, and allylic primary alcohols at concentrations up to 300 mM were likewise oxidized to the corresponding aldehydes with excellent conversion yields [190]. These results underscore that AAOs are promising biocatalyst for the production of valuable aldehydes. However, the production of these enzymes in sufficient amounts remains challenging. Heterologous expression of *Pe*AAO in *Komagataella phaffii* often failed, whereas *Pe*AAO2, differing by only seven amino acids, could be expressed at 315 mg/L.

Interestingly, AAO sequences generally show low identity, suggesting that their catalytic properties may differ significantly. So far, only a few other AAOs were purified and characterized in respect to their biochemical properties and substrate spectra. Previously, AAO was detected in *B. adusta* cultures both extracellularly [168] and intracellularly [191]. The purified extracellular AAO showed relatively high activity only towards *p*-methoxybenzyl

alcohol. Another extracellular AAO was purified from an arthroconidial fungus identified as an anamorph of *B. adusta*, exhibiting relatively high activity towards methoxy- and chloro-substituted benzylic alcohols [178]. At the same time, activity of these two enzymes towards benzyl alcohol was low. In both cases, activities were measured spectrophotometrically, and no preparative experiments were performed. The purification of AAOs from fungal cultures remains challenging, and low yields during heterologous expression limit their large-scale application. Whole-cell fungal cultures have been explored as an alternative, as they can catalyze selective biotransformations despite the presence of numerous enzymes. Similarly, fungal lyophilisates, which can be obtained from mycelium grown in submerged cultures by freeze-drying in unlimited amount, retain enzymatic activity over long-term storage. Lyophilisates of the edible basidiomycete *Pleurotus sapidus* have been reported as efficient biocatalysts for oxyfunctionalization reactions, including the highly regioselective allylic oxidation of (+)-valencene to the grapefruit flavor (+)-nootkatone, with α- and β-nootkatol as the only relevant side products [192]. In addition, lyophilized mycelium of *Pleurotus sapidus* has been applied for efficient and regioselective oxidations of mono- and sesquiterpenes, functionalized terpenoids, cyclohexene derivatives, and benzylic substrates to the corresponding allylic alcohols, enones, epoxides, or ketones [193].

Structurally, AAOs are relatively underexplored. For decades, the only available crystal structure was that of *Pe*AAO, initially solved in its free form and later in complex with *p*-anisic acid, the final product of its natural substrate oxidation [188,194]. More recently, additional structures have been determined, including *Mt*AAO from *Thermothelomyces thermophilus* [195], *Ba*AAO from *Bjerkandera adusta* [196], and the first bacterial AAOs, *Sh*AAO from *Streptomyces hiroshimensis,* and *Sd*AAO from *Sphingobacterium daejeonense*, some co-crystallized with substrate analogues [197]. These structures provide essential insights into substrate binding and catalysis, although the overall number of resolved AAO structures remains limited.

2. Objectives of the study

The present dissertation aims to explore sustainable and selective biocatalytic strategies for the production of valuable aldehydes using basidiomycetous white-rot fungi, with a particular focus on *Bjerkandera adusta* as a non-toxic and efficient source of multiple enzymes. The study will provide environmentally friendly alternatives to conventional chemical oxidation and reduction reactions, avoiding the use of toxic and hazardous reagents while maintaining high selectivity and compatibility with various functional groups under mild reaction conditions.

The research will investigate both, whole-cell growing cultures and lyophilized mycelium of *B. adusta*, as these systems are expected to exhibit different catalytic behaviors. In growing cultures, the reduction of carbonyl compounds is expected to dominate, enabling the selective formation of products such as 2-aminobenzaldehyde from anthranilic acid while avoiding overreduction. Lyophilized mycelium, on the other hand, will be evaluated as a convenient and storable source of aryl-alcohol oxidase for the selective oxidation of primary and allylic alcohols to aldehydes under mild conditions without undesired overoxidation or reduction of CC-double bonds. The study will also examine how reaction conditions, including the use of co-solvents, substrate structures and concentrations influence the selectivity and efficiency of both biocatalytic systems.

Overall, the work aims to develop a comprehensive understanding of *B. adusta* as a versatile biocatalyst, identifying factors that determine substrate specificity, reaction efficiency and stability. The insights obtained are expected to facilitate to the development of sustainable biocatalytic approaches for aldehyde synthesis and demonstrate the potential application of white-rot fungi in organic synthesis, as well as in food, cosmetic, and pharmaceutical industries, in accordance with the principles of green chemistry.

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4. Fungal lyophilisates as biocatalysts for organic synthesis

Fungal lyophilisates as catalysts for organic synthesis: Preparative oxidations with the white-rot fungus *Bjerkandera adusta*

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Mol. Catal. **2023,** *549*, 113451.

https://doi.org/10.1016/j.mcat.2023.113451

Molecular Catalysis 549 (2023) 113451

Contents lists available at ScienceDirect

Molecular Catalysis

journal homepage: www.journals.elsevier.com/molecular-catalysis

Fungal lyophilisates as catalysts for organic synthesis: Preparative oxidations with the white-rot fungus *Bjerkandera adusta*

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ARTICLE INFO

Keywords:
Biocatalytic oxidation
Metal-free
Aldehyde
Alcohol
Aryl-alcohol oxidase

ABSTRACT

A clean and selective "on-water-on-air" oxidation of primary alcohols to the corresponding aldehydes with mycelium lyophilisates of the non-toxic white-rot fungus *Bjerkandera adusta* was developed. The biocatalytic protocol is simple and the catalyst is readily accessible as a powder, which could be stored at least for 7 months without changes of activity. The catalytic activity of the fungus is associated with the presence of aryl-alcohol oxidase, that was isolated from the lyophilisate and characterised. Remarkably, a dichotomy in the fungal reactivity was observed, as the corresponding growing cultures catalyse the reverse process, selectively reducing aldehydes to the corresponding alcohols. Enzymatic oxidations by *B. adusta* are an excellent non-toxic alternative to metal-mediated oxidations in the lab with potential in food, beverage, cosmetic, and pharmaceutical industries, as it tolerates many functional groups.

1. Introduction

Aldehydes are valuable building blocks in organic synthesis due to the broad reactivity of the carbonyl group [1–4]. Apart from that, aldehydes possess distinct organoleptic features and thus represent an important group of chemicals for the flavour and fragrance industries [5–7]. The most common way to aldehydes is based on the oxidation of the respective primary alcohols with often expensive, toxic or harmful agents like chromium (VI) [8] and manganese [9] reagents and other strong inorganic oxidants [10,11]. The industrial approaches, namely aerobic oxidations of alcohols, include various metals as catalysts, mainly belonging to the transition metals [12–15]. Photocatalytic alcohol oxidations, which are currently intensely studied, are either also based on transition metal catalysts or require scarce catalysts such as graphitic C_3N_4-based polymers [16], Ti_3C_2/TiO_2 nanowires [17] or porous $Bi_{24}O_{31}Br_{10}(OH)_\delta$ photocatalyst [18]. Consequently, the food, beverage, and cosmetic industries attempt to shift the field of aldehyde production to microbial and enzymatic strategies [19].

In vivo, aldehydes mostly serve as a co-substrate for enzymes and do not accumulate in microorganisms because of high toxicity [20]. A fascinating tactic has been developed by white-rot fungi (WRF) for lignin degradation [21–23], where aldehydes are involved in reduction/oxidation peroxidase cycles [24,25] (Scheme 1). In such a H_2O_2 production process veratraldehyde and anisaldehyde are the products of aryl-alcohol oxidase (AAO)-catalysed [26–29] oxidation of the corresponding alcohols by molecular oxygen. The aldehydes propagate the cycle by reducing back to alcohols by NAD(P)H (nicotinamide adenine dinucleotide (phosphate)-dependent aryl-alcohol dehydrogenases/reductases (AADH). This tool is fabulously tuned by fungi as higher amounts of H_2O_2 deactivate peroxidases [30] whereas lower amounts reduce the rate of lignin degradation which is the main source of energy for WRF. Lignin digestion by fungi leads to several substituted aromatics, including aryl alcohols and aldehydes that can also be synthesised *de novo* by some fungi [31,32]. In particular, *B. adusta* (BAD) produces veratryl and *p*-anisyl alcohols under such circumstances [33].

AAOs are FAD (flavin adenine dinucleotide)-dependent oxidoreductases and display a great potential in biocatalytic processes, as they only require molecular oxygen for substrate oxidation and generate hydrogen peroxide as a by-product without need for additional cofactors. Producing the corresponding aldehydes, AAOs have a diverse

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https://doi.org/10.1016/j.mcat.2023.113451
Received 7 May 2023; Received in revised form 4 August 2023; Accepted 6 August 2023

substrate scope including benzyl alcohol and its substituted derivatives [34], as well as aliphatic α-unsaturated alcohols, *e.g. trans*-hex-2-en-1-ol [35]. AAOs were isolated mainly from basidiomycetes, such as *Pleurotus eryngii* [36] and *Coprinopsis cinerea* [29]. AAO from the oyster mushroom *Pleurotus ostreatus* takes part in detoxifying 5-hydroxymethylfurfural (the inhibitor of cellulolytic enzymes) catalysing the oxidation of both alcohols and aldehydes directly to carboxylic acids [37]. It should be mentioned that another type of enzymes dependent only on molecular oxygen and discovered in WRF, namely laccases, are able to catalyse the oxidation of benzylic alcohols to the corresponding aldehydes [38,39]. However, laccases exhibit an extraordinary broad substrate spectrum and are non-specific with regard to aromatic or allylic alcohols, but also oxidise phenols to quinones, etc.

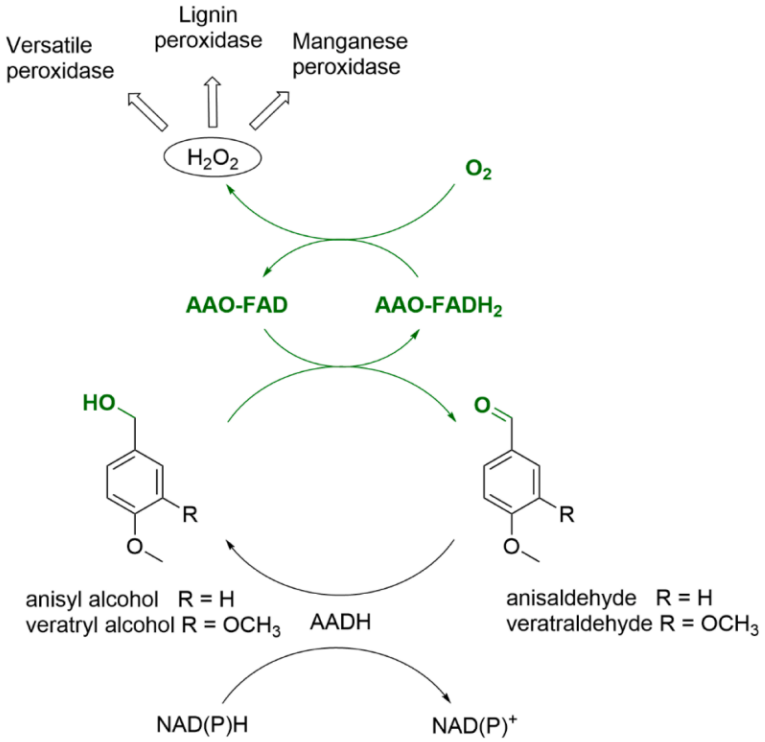
Considering their high stability, broad substrate spectrum, independency of costly cofactors and the use of molecular oxygen as a final electron acceptor, AAOs seem to be powerful enzymes for applications in synthetic chemistry. However, despite numerous studies, these valuable enzymes are still not applied in chemical laboratory routines. The main problem in the production of AAO in sufficient amounts is the purification from the fungal cultures; additionally, heterologous expression of AAOs often suffers from low yields [40]. Recently, an AAO from the fungus *Pleurotus eryngii* was heterologously expressed and secreted in *Pichia pastoris* (recently reclassified as *Komagataella phaffii*) with only 315 mg/L yield [41]. A possible solution may be found in the utilisation of whole-cell fungal cultures, that, despite of the presence of various enzymes, can catalyse a number of highly selective biotransformations [42–44], *e.g.* the oxidation of benzyl alcohol [41] as well as the reduction of carbonyls [45]. As mentioned above, AAOs are comparatively stable and need only molecular oxygen, while their partner in the cycle of redox reactions, AADHs, are dependent on the cofactor NAD(P)H, which is not stable enough and should be regenerated if the purpose is to keep AADHs activity on a high level. Therefore, we tested fungal lyophilisates as a source of AAOs for preparative organic transformations. Generally, fungal lyophilisates can be obtained in unlimited amounts by freeze-drying the fungal mycelium grown in submerged cultures, and they may be stored for a long time without loss of activity. The aim of this work was to find an effective sustainable approach for the production of valuable aldehydes using white-rot fungi as efficient suppliers of AAO whilst avoiding genetically modified species.

B. adusta (BAD) was chosen as it is known as a source of extracellular oxidative enzymes [46]. For example BAD is able to degrade very stable organic compounds, such as 2,4,6-trinitrotoluene [47], pentachlorophenol [48], and phthalocyanine dyes [49]. In addition, a dye-decolorizing peroxidase has been purified and characterised from BAD [50]. Accordingly, AAOs as effective supplier of H_2O_2 for peroxidases have been detected in BAD supernatants recently [51,52]. This fungus is non-toxic and its applications in the food, pharmaceutical and cosmetic industries are simple and safe. BAD cultures could be prepared through a rather simple microbiological technique, as they grow on a variety of inexpensive substrates.

2. Experimental

2.1. Organism

The filamentous fungus *Bjerkandera adusta* was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ 4708), Braunschweig, Germany. The stock culture was maintained on a solid medium containing 15 $g \cdot L^{-1}$ malt extract (Fluka, Neu-Ulm, Germany) and 15 $g \cdot L^{-1}$ agar-agar (Roth, Karlsruhe, Germany).



Scheme 1. Involvement of AAO in the key step of lignin degradation as supplier of H_2O_2 for peroxidases.

2.2. Chemicals

Benzyl alcohol, benzaldehyde, 4-methylbenzyl alcohol, 3-methylbenzyl alcohol, cumin alcohol, 4-chlorobenzyl alcohol, 3-chlorobenzyl alcohol, 2-chlorobenzyl alcohol, 4-trifluoromethyl benzyl alcohol, 3-trifluoromethyl benzyl alcohol, 2-trifluoromethyl benzyl alcohol, *p*-anisyl alcohol, *m*-anisyl alcohol, gastrodigenin, 3-hydroxybenzyl alcohol, 4-nitrobenzyl alcohol, piperonyl alcohol, veratryl alcohol, 2-naphthalenemethanol, 1-naphthalenemethanol, 4-methoxy-1-naphthalenemethanol were purchased from Merck KGaA (Darmstadt, Germany).

2.3. Submerged cultures

The culture medium was prepared by dissolving malt extract (20 g) in 1 L of deionized water. For the preparation of the precultures, a 1 cm^2 agar plug from the leading mycelial edge was transferred into 100 mL medium (in a 250 mL Erlenmeyer flask) and then homogenized with a T 25 digital Ultra-Turrax homogenizer (IKA, Staufen, Germany; 30 s, 10.000 $r{\cdot}min^{-1}$). The precultures were grown on an incubation shaker (Orbitron, Infors HAT, Bottmingen, Switzerland; 150 $r{\cdot}min^{-1}$, deflection 25 mm) under the exclusion of light at 24 °C for 7 days. Afterwards, the precultures were homogenized, and 10% (v/v) of the homogenate was inoculated for submerged cultivation on 100 mL (in 250 mL Erlenmeyer flasks) scale.

2.4. Lyophilized mycelium

After 6 days of growth in submerged culture, the mycelium was harvested by filtration and washed with distilled water. The obtained biomass was freeze-dried (Vaco2, Zirbus Technology, Bad Grund, Germany) and stored at −20 °C until usage.

2.5. Biotransformation with growing cultures

Substrate (1 mmol) was added to submerged cultures of BAD grown in malt extract (3%) medium on the X (X = 2–6) culture day. The reaction mixture was placed on an incubation shaker at 150 $r{\cdot}min^{-1}$ (deflection 25 mm) under the exclusion of light at 24 °C for 8 days. After supplementation of the growing culture with the substrate, samples (5 mL) were taken every 24 h, 0.5 g of NaCl was added to the media, and the mixture was stirred for 10 min at 800 rpm (magnetic stirrer). For extraction, 5 mL of Et_2O was added, and the resulting mixture was stirred for 20 min at 800 rpm (magnetic stirrer) and centrifuged for 5 min at 1690 × g to separate the organic layer. The extraction was repeated three times. The combined organic layers were washed with brine (1 × 30 mL) and water (1 × 30 mL), dried over Na_2SO_4, concentrated to 2 mL and analysed by GC–MS (Agilent Technologies 7890A GC, column Agilent VF-WAXms (30 m × 0.25 mm, 0.25 μm) and Agilent 5975C MSD Triple-Axis mass spectrometer). Every biotransformation was repeated three times to verify the reproducibility of the experiments.

2.6. General procedure for the biotransformation

BAD lyophilisate (400 mg) was suspended in 15 mL phosphate buffer (0.1 M, pH = 7). The dried cell mass was rehydrated by stirring at 800 rpm for 10 min, and 0.5 mmol (final concentration is 20 mM) of the substrate was added. The reaction mixture was stirred at 24 °C and 800 rpm for 24 h. Subsequently, additional lyophilisate (400 mg) and phosphate buffer (10 mL) were added, reaction mixture was stirred for further 24 h, 30 mL Et_2O was added and the resulting mixture was stirred for 30 min. The resulting suspension was centrifuged to separate the phases. Then 30 mL Et_2O was added to the aqueous phase, stirred for 15 min, centrifuged, and the resulting layers were separated. The combined organic phases were dried over Na_2SO_4 and evaporated to dryness. The resulting reaction mixtures were analysed by GC-MS and NMR spectroscopy. The isolation of products was performed as described below:

A. The aldehydes were isolated through their bisulfate derivatives. The reaction mixture was extracted with ether (5 × 10 mL) and combined extracts were evaporated. The residue was shacked with saturated solution of $NaHSO_3$ (2 mL) for 5 h, diluted with water to dissolve the precipitate, washed with ether (2 × 5 mL) and 20%-aqueous NaOH solution was added to adjust pH = 12. A free aldehyde was extracted by ether (3 × 5 mL), the combined extracts washed with brine (2 × 3 mL), dried over Na_2SO_4 and evaporated to give respective aldehyde.

B. The residue after the ether extraction from the reaction mixture was purified by column chromatography on silica gel (eluent (pentane/ether) changed gradually: 10/1, 7/3, 1/1) to isolate the major components. The respective fractions were combined, concentrated in vacuum, and the ^1H and ^{13}C NMR spectra of the products were compared with those of authentic reference samples. Representative biotransformations were repeated three times to verify the reproducibility of the experiments. The detailed information about experimental data and yields is collected in Electronic Supplementary Information.

2.7. Up-scaled biotransformation of piperonyl alcohol

BAD lyophilisate (2 g) was suspended in 30 mL phosphate buffer (0.1 M, pH = 7). The dried cell mass was rehydrated by stirring at 1300 rpm for 10 min, and 380 mg (2.5 mmol, final concentration of 83.3 mM) of piperonyl alcohol was added. The reaction mixture was stirred at 24 °C and 1300 rpm for 15 h, Et_2O (20 mL) was added to reaction mixture, the resulting mixture was stirred for 15 min, and layers have been separated. The extraction was repeated 5 times. The combined organic phases were dried over Na_2SO_4 and evaporated to dryness. The product was isolated by Method A through the reaction with a saturated aqueous solution of sodium bisulphite ($NaHSO_3$, 30 mL) to give piperonal (235 mg, 63%) as a white solid. The identity of the solid has been confirmed by comparing of its ^1H and ^{13}C NMR spectra with those of an authentic reference compound.

2.8. Enzyme purification

Purified enzyme fractions were obtained through two chromatographic steps based on ion-exchange (IEX) and size exclusion (SEC) performed by Fast Protein Liquid Chromatography (FPLC, NGC Chromatography Systems, Biorad, Germany), with protein peaks monitored at 280 nm. For IEX, proteins were separated through a DEAE Sepharose column (Merck, Darmstadt, Germany) equilibrated with 0.1 M Tris-HCl buffer pH 7. Different protein fractions were eluted by isocratic flows of 0.1 M Tris-HCl buffer pH 7 added by 20%, 50% and 100% of 1 M NaCl. After chromatography, each peak was concentrated by freeze-drying the purified fractions and by micro-centrifugal filtration (MWCO of 10 kDa) and desalted by dialysis (Float-A-Lyzer G2, SpectrumLabs with a MWCO of 10 kDa) in a buffer exchanging system against phosphate buffer at neutral pH. Each protein peak was tested for activity for AAO (GC-MS and ABTS assay) and the peak with enzymatic activity was further submitted to a SEC purification step. SEC was performed using a superdex 200 gel filtration column (Merck) eluted by 0.1 M Tris-HCl at a flow rate of 1 mL/min. Each eluted peak in the SEC was tested for activity and subjected to SDS-PAGE analysis and LC-MS-based proteomics identification.

2.8.1. Activity of the fractions

4-Methylbenzyl alcohol (**7**) (0.5 mg) was added to every fraction after IEX (100 μL). Mixtures were shaked for 24 h at room temperature and 500 rpm. The mixtures were extracted by Et_2O, and analysed by GC-MS. Activity of every fraction after SEC has been analysed in the same way.

2.8.2. Gel electrophoresis

SDS-PAGE was used to determine the purity of the proteins after the two purification steps. Protein separation in polyacrylamide gels (12%) was performed according to Laemmli [53]. For that, proteins were denatured [in a solution containing β-mercaptoethanol and sodium dodecyl sulfate (SDS)] to be separated according to their molecular mass by a current of 20 mA. Proteins bands were visualised by staining with Coomassie brilliant blue.

3. Results and discussion

3.1. Biotransformations of benzyl alcohol and benzaldehyde with whole-cell growing cultures of BAD

We first estimated the oxidative activity of growing BAD cultures towards aromatic alcohols, utilising benzyl alcohol (**1**) as the simplest model compound. Alcohol **1** is not a typical substrate for white-rot fungi due to the absence of methoxyl and hydroxyl functions in the aromatic rings present in lignin. Due to the fact that the quality and quantity of produced enzymes do vary substantially during the culture growth, alcohol **1** was added to the submerged cultures on various culture days (2–6 days). The reaction mixtures were analysed every 24 h by GC-MS (Fig. 1). We detected the presence of benzaldehyde (**2**) in all reaction mixtures. As a by-product, *p*-anisaldehyde (**3**) was identified if **1** was added to the growing culture on the 2nd to 4th day. If **1** was supplemented to the cultures on the 5th or 6th culture day, **3** was not detected, but benzoic acid (**5**), which had not been identified in younger cultures, was found. Additionally, the traces of a well-known metabolite of BAD, namely 3-chloro-4-methoxybenzaldehyde (**4**) [31], has been identified in every reaction mixture with alcohol **1** as a supplement. This is in agreement with the previous suggestion [54] that compounds **2** and **3** are intermediates in the biosynthetic pathway to **4** from phenylalanine produced by BAD. The reason for the relatively low amounts of **2** in the reaction mixtures may be associated with the ability of the growing cultures of BAD to reduce aromatic aldehydes [45].

To test this hypothesis, we evaluated the activity of BAD towards benzaldehyde (**2**) by its supplementation to the growing cultures on the 2nd, 3rd, 4th, 5th and 6th culture day (Fig. 2). The reduction of the carbonyl group proceeded efficiently, especially when **2** was added between the 3rd and 6th culture day, whereas the share of **4** as a by-product was less than 1%, **3** was not detected and the amounts of benzoic acid (**5**) were increasing daily. A new component detected in the reaction mixture was assigned to 1-hydroxy-1-phenylpropan-2-one (**6**) which could be a precursor of the corresponding diol formed by BAD as described previously [55]. Thus we concluded that in the growing whole-cell cultures of BAD the reduction of carbonyl group dominates.

3.2. Biotransformations with lyophilized cultures

As we disclosed the inefficiency of BAD growing cultures for aldehyde production, we performed studies on lyophilisates prepared from BAD mycelium. The production of an extracellular AAO by BAD and its activity towards several aromatic alcohols, especially veratryl alcohol, has been already described [51,52]. However, lyophilised mycelia of BAD have not been tested before as a catalyst for alcohol oxidation despite they could represent a promising alternative: lyophilisates may be obtained in any desired amounts by freeze-drying of the mycelium and stored at –20 °C for months. We analysed the AAO activity of

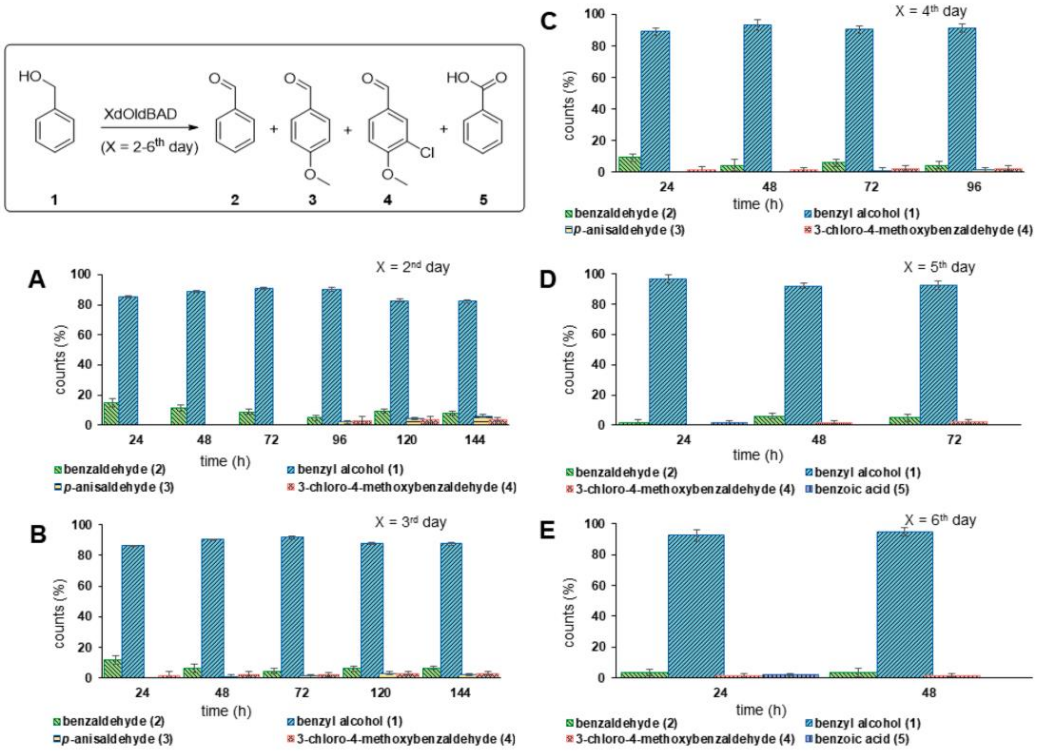


Fig. 1. Biotransformation of benzyl alcohol (**1**) with whole-cell cultures of *B. adusta* (according to GC-MS data). Substrate **1** was added to the growing culture on the 2nd (**A**), 3rd (**B**), 4th (**C**), 5th (**D**), 6th (**E**) day. Error bars represent standard deviations based on triplicate experiments.

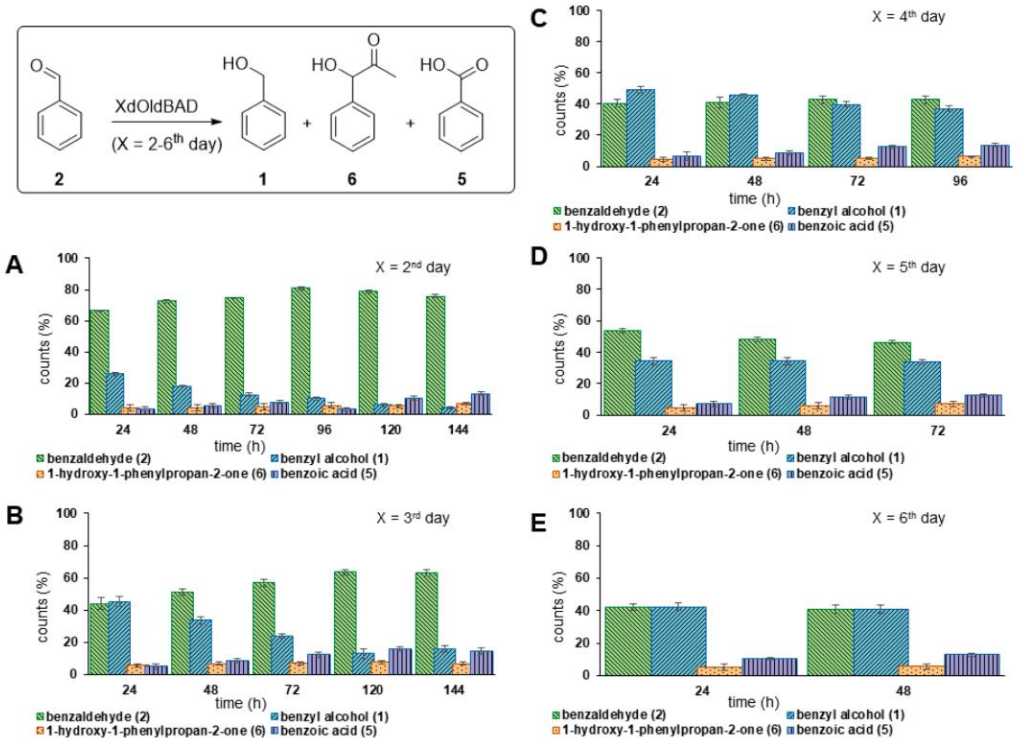


Fig. 2. Biotransformation of benzaldehyde (**2**) with whole-cell cultures of *B. adusta* (according to GC–MS data). Substrate **2** was added to the growing culture on the 2nd (**A**), 3rd (**B**), 4th (**C**), 5th (**D**), 6th (**E**) day. Error bars represent standard deviations based on triplicate experiments.

lyophilised mycelium collected on the different culture days (see Supplementary data), and found the lyophilisates from the 6th day as the most promising for aldehyde production. Several experiments were performed on a small scale with benzylic alcohol (**1**), and the reaction mixtures were analysed by GC-MS. After optimisation of the reaction conditions, the biotransformations were scaled up and the series of oxidations were performed at the preparative level. The aldehydes were isolated through the reaction with $NaHSO_3$ or by column chromatography in several cases. Unlike growing cultures, the lyophilised mycelia of BAD demonstrated high oxidative activities towards substituted benzyl alcohols (Table 1). Alcohol **1** and alkylbenzyl alcohols **7, 9, 11** were oxidised to corresponding aldehydes **2, 8, 10, 12** with isolated yields *ca.* 70–80%. Our bioconversions allow obtaining *p*- (**14**) and *m*-(**16**) chlorobenzaldehydes with yields of 86% and 72%, correspondingly, however only traces of the *o*-derivative **18** were detected under the same conditions. Traces of benzaldehyde (**2**) have been identified in reaction mixtures of the oxidation of chloro-benzyl alcohols. In the case of trifluoromethyl-substituted benzyl alcohols, higher yield of the *m*-substituted isomer **22** was observed. The *p*- (**25**) and *m*-(**26**) methoxy alcohols mimic the suitable substrates for WRF due to the presence of the CH_3O-group and were oxidised with yields of more than 90%. *p*-Nitrobenzaldehyde (**32**) has been obtained with 92% yield. The alcohols **28, 29, 33** with HO-groups in the aromatic rings were not oxidised effectively and *m*-hydroxybenzaldehyde (**30**) was formed with a yield of 52%. The presence of *p*-hydroxyl groups has been previously reported to negatively influence the oxidation by AAO [36]. Accordingly, no products were detected with *p*-hydroxybenzyl alcohol (**28**) as a substrate and only traces were found with vanillyl alcohol (**33**).

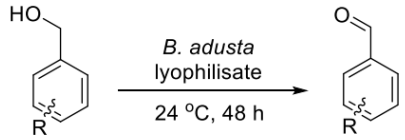
Piperonal (**36**), which is a sweet-flowery and spicy odour component of the essential oils of *Heliotropium* genus flowers, has been obtained from the corresponding alcohol **35** with 67% yield. Due to the presence of benzodioxole fragment, **36** serves as a precursor for several products of industrial importance like insecticides, pesticides, and pharmaceuticals [56–58]. The typical AAO substrate veratryl alcohol (**37**) gave the corresponding aldehyde **38** with a yield 84%. Expansion of the aromatic system to two condensed aromatic rings in *β*-naphtalenemethanol (**39**) led to one of the highest yields of the corresponding aldehyde **40**. Surprisingly, the substituted naphtalenes **41** and **43** are almost unreactive under the same conditions. Cinnamyl alcohol (**44**) that contains an aromatic system conjugated with a double bond, readily reacted with BAD lyophilisate to give cinnamic aldehyde (**45**) with high yield.

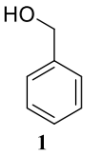
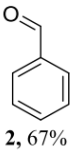
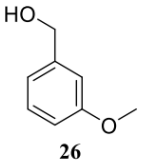
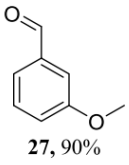
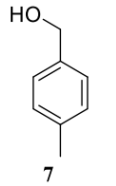
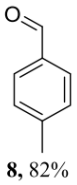
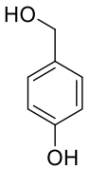
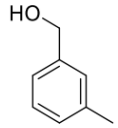
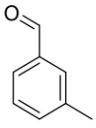
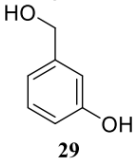
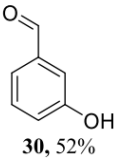
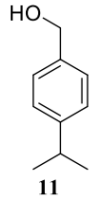
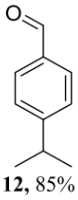
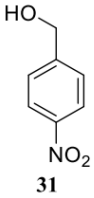
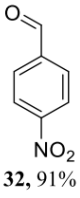
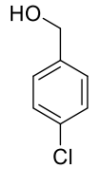
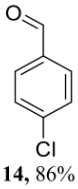
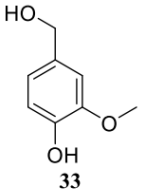
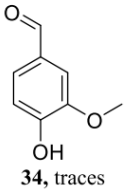
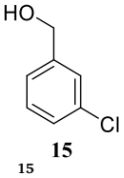
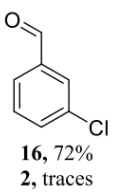
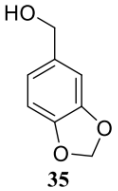
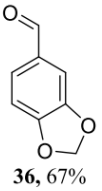
The substrate scope of BAD lyophilisate is quite similar to those of extracellular AAOs from *B. adusta* and *P. eryngii*, as they demonstrate high activity towards *p*-anysil (**25**), piperonyl (**35**) and β-naphthalenemethyl (**39**) alcohols and low activity towards vanillyl alcohol (**33**) [41,52]. It should be mentioned that in case of 4-hydroxybenzyl alcohol (**28**) the properties of BAD lyophilisate are more similar to *P. eryngii* AAO, as the extracellular AAO from BAD demonstrated moderate activity towards **28**. We found the highest yields of the products with electron-donating substituents, which is in good agreement with the preference of native AAOs toward substrates like *p*-methoxybenzyl (**25**) and veratryl (**37**) alcohols. Notably, the observed high yields with the substrates with electron-withdrawing groups may be associated with the fact that reactivity is influenced by the position of the substituent, rather then electronic effects. We also assume that alcohols **18** and **23** with *o*-substituents give only traces of products due to steric hindrance.

3.3. Purification of aryl-alcohol oxidase from BAD lyophilisate

We assumed that the catalytic activity of the BAD lyophilisate could

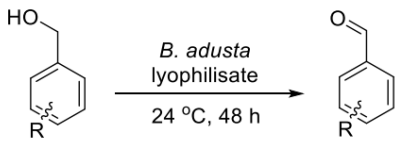
Table 1
Structures of alcohols and corresponding products (aldehydes) with isolated yields.

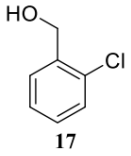
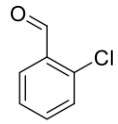
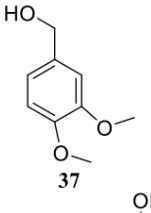
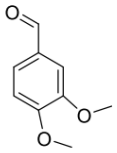
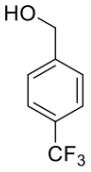
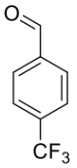
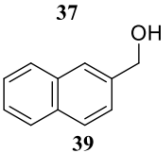
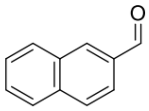
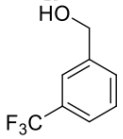
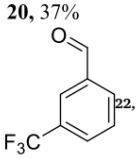
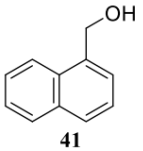
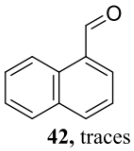
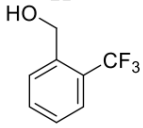
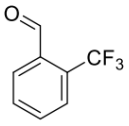
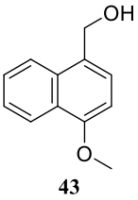
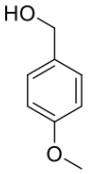
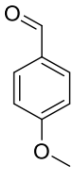
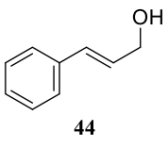
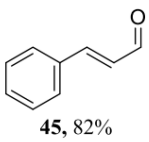


starting compound	product, yield	Absolute amount of isolated product, mg	starting compound	product, yield	Absolute amount of isolated product, mg
 **1**	 **2,** 67%	36	 **26**	 **27,** 90%	61
 **7**	 **8,** 82%	49	 **28**	no products	-
 **9**	 **10,** 77%	46	 **29**	 **30,** 52%	32
 **11**	 **12,** 85%	58	 **31**	 **32,** 91%	77
 **13**	 **14,** 86% **2**, traces	67	 **33**	 **34,** traces	-
 **15** 15	 **16,** 72% **2,** traces	50	 **35**	 **36,** 67%	50

(*continued on next page*)

Table 1 (*continued*)



starting compound	product, yield	Absolute amount of isolated product, mg	starting compound	product, yield	Absolute amount of isolated product, mg
 **17**	 **18,** traces **2,** traces	-	 **37**	 **38,** 83%	70
 **19**	 **20,** 37%	32	 **39**	 **40,** 90%	76
 **21**	 **22,** 82%	71	 **41**	 **42,** traces	-
 **23**	 **24,** traces	-	 **43**	no products	-
 **25**	 **3,** 99%	67	 **44**	 **45,** 82%	54

result from the presence of AAO. Previously, AAO was detected in BAD cultures both, extracellularly [44,53] and intracellularly [33]. The purified extracellular AAO demonstrated relatively high activity only towards *p*-methoxybenzyl alcohol (**25**) [44]. Another extracellular AAO has been purified from *Bjerkandera arthroconidial* anamorph [59], and its activity was relatively high towards methoxy- and chloro-substituted benzylic alcohols. In contrast, towards benzyl alcohol (**1**) the activities of these two enzymes were low. In both cases, the activities have been measured spectrophotometrically, and no preparative experiments have been performed. To disclose the nature of the catalytic properties of the BAD lyophilisate, we attempted to isolate the active enzymes. Standard protocols for enzyme isolation were applied, and the catalytic properties of the obtained proteins were characterised utilising *p*-methylbenzyl alcohol (**7**) as model substrate.

The ion-exchange (IEX) chromatographic step of crude extract provided four fractions (Fig. 3a; IEX 1–4) whose activity towards **7** was tested by GC-MS. The active fraction IEX3 was used for further purification. The protein concentration of fraction IEX3 was estimated at 400 μg/mL. An additional purification step by size exclusion chromatography (SEC, superdex 200) provided four main peaks (Fig. 3b) from which fraction SEC2 was active. The collected protein fractions (IEX3 and SEC2) were visualised as bands in a SDS polyacrylamide gel electrophoresis (SDS-PAGE) and compared to the crude extract of BAD (Fig. 3c).

SDS-PAGE of the purified enzyme showed a single band with a molecular mass of 60–65 kDa. The same molecular mass has been identified

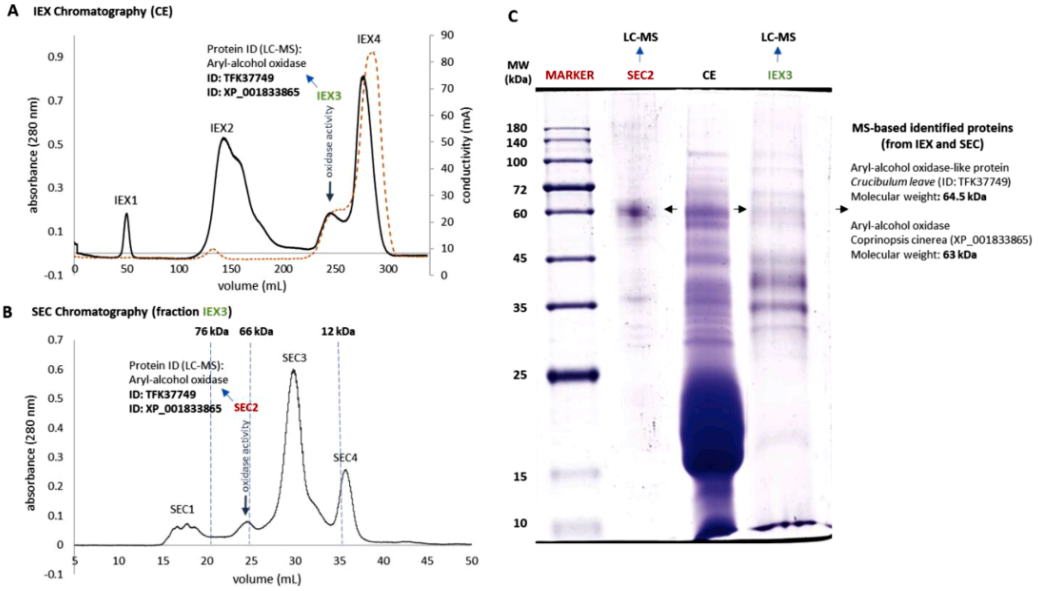


Fig. 3. AAO purification: (**A**) IEX chromatographic step; (**B**) SEC (superdex 200); (**C**) SDS-PAGE gels.

for the deglycosylated protein (after Superdex-75) assigned as AAO from *Bjerkandera arthroconidial* anamorph [59]. The identification of the alcohol oxidase in the purified fractions was performed by LC-MS/MS-based bottom-up proteomics (see Sections 1.5 and 5 of supplementary information). The MS data confidently detected identical unique peptides belonging to an AAO (NCBI ID: **TFK37749,** available at https://www.ncbi.nlm.nih.gov/protein/TFK37749) from *Crucibulum leave* and an AAO (NCBI ID: **XP_001833865,** available at https://www.ncbi.nlm.nih.gov/protein/XP_001833865) from *Coprinopsis cinerea* in both purification steps (IEX3 and SEC2). We thus demonstrated that the oxidative properties of BAD lyophilisate is associated with the presence of AAOs, that are effective enzymes for preparative synthesis of aldehydes allowing to avoid an overoxidations.

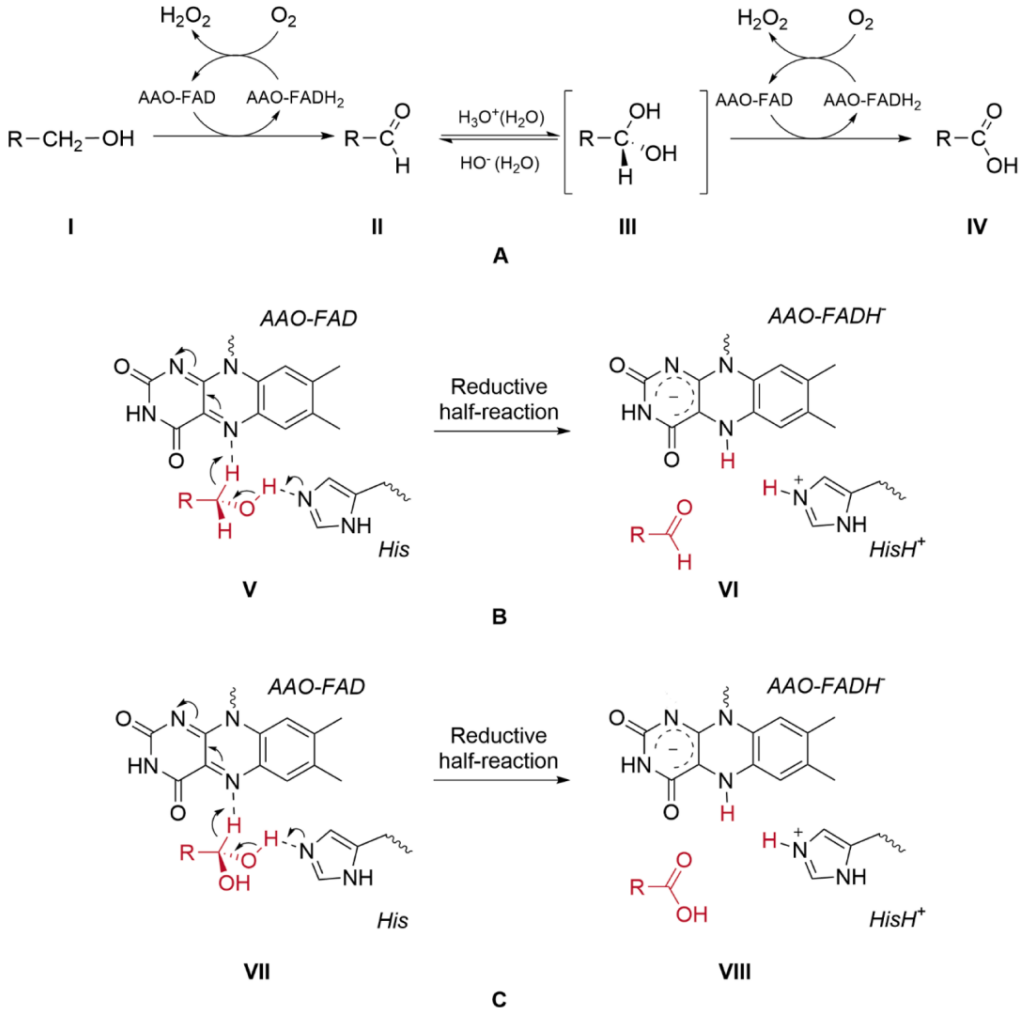
3.4. Influence of the limiting factors

Based on the results of the LC-MS/MS-based bottom-up proteomics of the active fraction, we proved the presence of AAO-type enzymes in BAD lyophilisates. The absence of the carboxylic acids amongst the products that sometimes accompany the alcohol oxidation by purified AAO [60] can be attributed to the neutral pH value of the reaction medium. An aldehyde might serve as a substrate for AAO in its hydrated form (*gem*-diols) when water is added to carbonyl group (Scheme 2A). Recently, Ferreiro at al [60] reported that the mechanism of the rate-limiting reductive half-reaction would probably be similar to the previously described [27] for AAO oxidation of alcohols. Both mechanisms include the concerted transfer of the α-carbon "hydride" to flavin and the proton of the hydroxyl group to a catalytic base (Scheme 2B for alcohol, 3C for *gem*-diols). Hydration of aldehydes is a rapid reaction, quickly reaching equilibrium, but faster in acid or base than in neutral solution . The hydration rates of aromatic aldehydes are generally low compared with those of aliphatic ones, and that for benzaldehyde is often considered as negligible; however, for aromatic substrates, the presence of electron-withdrawing or electron-donating ring substituting groups, respectively, stabilise or destabilise the *gem*-diol intermediate [60]. With the BAD lyophilisate, we did not detect any traces of acids even with chloro- and nitro- substituents that are common electron-withdrawing ring substituents.

In order to minimise the probability of the formation of *gem*-diols, a pH value of **7** was chosen as an optimum for the proposed method. Furthermore, the influence of the temperature and several organic solvents on the efficiency of the catalytic properties of BAD lyophilisate was evaluated, using benzyl alcohol (**1**) as a model compound (Fig. 4). The evaluation of the reaction mixture has been performed after 8 h, and it was shown that at 20−24 °C, the reaction mixture was composed of benzyl alcohol (**1**, around 60%) and benzaldehyde (**2**, 40%). With increasing temperature, the conversion of **1** to **2** decreased, and at 50 °C no traces of **2** were detected in the reaction mixture after 8 h. Concerning organic solvents, we decided to avoid hydroxyl-containing solvents and chose dimethylsulfoxide (DMSO) and dimethylformamide (DMF). It was found that after 8 h in the presence of 5% DMSO, the yield of **2** was increased by 9% compared to the reference biotransformation without organic solvent. The presence of DMF decreased the catalytic activity of lyophilisate. It should be mentioned that intensive stirring increases the reaction rate and yields.

3.5. Preparative biooxidation of piperonyl alcohol

For the upscaling and evaluation of the sustainability of the developed approach, piperonyl alcohol was chosen as a model alcohol. The amount of substrate was increased to 2.5 mmol (5 times compared to the general procedure) and the amount of lyophilisate was adjusted to 2 g (2.5 times compared to the general procedure). The biotransformation was performed with 83.3 mM substrate and stirred for 15 h at 1300 rpm. Under the optimised conditions, the preparative yield and conversion of the upscaled transformation were 63% and 68%, respectively. It should be noted that, compared to waste produced in other approaches, lyophilisate and potassium phosphates are not a toxic waste and may be even used as a compost. An additional benefit of the developed approach is that a non-GMO is used for the preparative scale oxidation reactions. This is highly important for industries that strive for marketing their products as 'EU-natural' compounds, specifically in the food, beverage,



Scheme 2. **A**. General scheme of transformation of alcohol (**I**) to aldehyde (**II**) catalysed by AAO including further oxidation of **II** to carboxylic acid (**IV**) *via* unstable *gem*-diol (**III**). **B.** The reductive half-reaction of the AAO catalytic cycle with an alcohol as a substrate (**V**) transformed to aldehyde (**VI**). **C.** The reductive half-reaction of the AAO catalytic cycle with a gem-diol as a substrate (**VII**) transformed to carboxylic acid (**VIII**).

and cosmetic industries.

4. Conclusions

We identified the ability of whole-cell growing cultures of the white-rot fungus *B. adusta* to produce benzaldehyde from benzylic alcohol. However, the presence of fungal reductases causes the reverse reaction during the fungal growth. In contrast, lyophilisate obtained from fungal mycelia displays exclusive oxidative activity towards substituted benzyl alcohols and gives the respective aldehydes in high preparative yields. The reactions proceeded without over-oxidation to the corresponding carboxylic acids and tolerated many functional groups (Alk, Hal, NO_2, CH_3O, CF_3, etc.). We isolated and characterised an aryl-alcohol oxidase (AAO) as active enzyme responsible for aldehyde production from lyophilisate. Notably, the preparative oxidations could be achieved without the isolation of pure enzymes, as lyophilisate provides them in significant quantities. The catalytically active lyophilisate may be obtained in any desired amounts by freeze-drying of the fungal mycelium grown in submerged culture and can be stored for long times without loss of activity. We thus have developed an on-air-on-water biocatalytic methodology readily available for the preparation of practically-valuable aldehydes in organic labs even without microbiological expertise. The high activity of BAD lyophilisates in the presence of DMSO along with high storage stability are clear advantages which make BAD lyophilisates a promising biocatalyst for biotechnological applications. The extension of our approach for preparative oxidations of other classes of organic substrates is currently underway in our laboratories.

CRediT authorship contribution statement

Valeriia V. Babkina: Visualization, Investigation, Methodology, Writing – review & editing. **Wendell Albuquerque:** Visualization, Methodology, Writing – review & editing. **Yana M. Haiduk:** Methodology. **Weronika Michalak:** Methodology. **Parviz Ghezellou:**

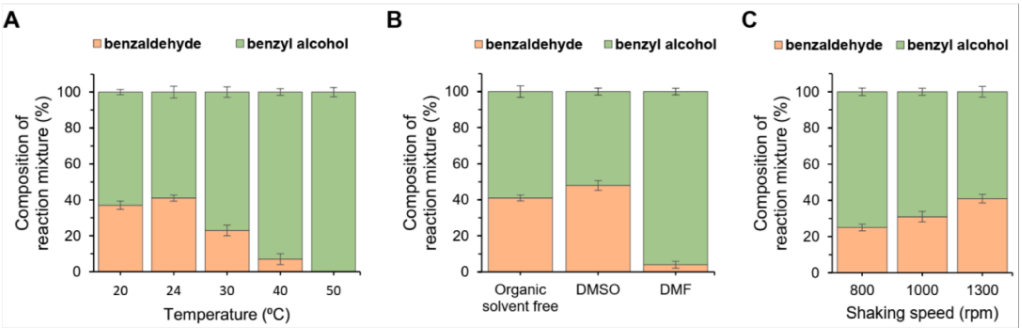


Fig. 4. Compositions of the reaction mixtures: (**A**) at different temperatures: 20°C, 24 °C, 30 °C, 40 °C, and 50 °C; (**B**) with different solvents (5% *v/v*): dimethylsulfoxide (DMSO), dimethylformamide (DMF); (**C**) with different stirring rates (800, 1000, 1300 rpm). Compositions of the reaction mixtures (%) were calculated from ^{1}H NMR spectra of the reaction mixtures utilising *m*-xylene as internal standard. Error bars represent standard deviations based on triplicate experiments.

Validation, Methodology. **Holger Zorn:** Resources, Conceptualization, Writing – review & editing. **Tatyana S. Zhuk:** Conceptualization, Methodology, Formal analysis, Supervision, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

TSZ gratefully acknowledges financial support by the Alexander von Humboldt Foundation (Georg Forster Research Fellowship). VVB and YMH were financed by Aid Fund by the Central Hessian Universities and the Research Campus of Central Hessen (FCMH) and JLU Aid Fund. We sincerely thank to Dr. Alejandra Omarini (Asociación para el Desarrollo de Villa Elisa y Zona, CONICET, Argentina) and Dr. Gerardo L. Robledo (BioTecA3 - Centro de Biotecnología Aplicada al Agro y Alimentos, Ciudad Universitaria, Córdoba, Argentina) for the providing the picture of *B. adusta*.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mcat.2023.113451.

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5. Biocatalytic reduction of carboxylic acids

Reduction of anthranilic acid to 2-aminobenzaldehyde by the white-rot fungus *Bjerkandera adusta* DSMZ 4708

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J. Biotech. **2024,** *387*, 44-48.

https://doi.org/10.1016/j.jbiotec.2024.03.015

Journal of Biotechnology 387 (2024) 44–48

Contents lists available at ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Reduction of anthranilic acid to 2-aminobenzaldehyde by the white-rot fungus *Bjerkandera adusta* DSMZ 4708

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ARTICLE INFO

Keywords:
Biocatalytic reduction
Aldehyde
Growing culture
Flavor compound

ABSTRACT

The biocatalytic aerobic "in-water" reduction of anthranilic acid to 2-aminobenzaldehyde by growing cultures of the basidiomycetous white-rot fungus *Bjerkandera adusta* has been studied. The high specific activity of *Bjerkandera adusta* towards the carboxylic group of anthranilic acid that allows avoiding the formation of the corresponding alcohol has been demonstrated using different substrate concentrations. The presence of ethanol as co-solvent allows increasing the yield of target product. In contrast to chemical reducing agents that usually yield 2-aminobenzyl alcohol, an overreduction of anthranilic acid is completely suppressed by the fungus and gives the target flavor compound in satisfactory preparative yields. It was shown that the activity of *Bjerkandera adusta* towards anthranilic acid does not apply to its *m*- and *p*-isomers.

1. Introduction

2-Aminobenzaldehyde (**1**) is a pleasant fragrance component imparting a floral odor. It has been identified as an essential component of many flowers including broom (*Spartium junceum*), false acacia (*Robinia pseudoacacia*), European bird cherry (*Padus avium*), lily (*Lilium candidum*), seringat (*Philadelphus coronarius*), *Pittosporum tobira*, and *Hypecoum imberbe* (Joulain, 1987). In *Robinia pseudoacacia*, the biosynthetic pathway towards **1** includes the transformation of anthranilic acid (**2**) to indole (**3**) followed by oxidative ring opening and hydrolysis of the resulting *N*-formyl-2-aminobenzaldehyde (**4**) (Spiteller and Steglich, 2001). The chemical synthesis of **1** is based on the reduction of 2-nitrobenzaldehyde (**5**) with aqueous ferrous sulfate and ammonia (Scheme 1) (Foy et al., 1993). An alternative approach based on the reduction of the much less expensive precursor anthranilic acid (**2**) is not successful as it proceeds directly to 2-aminobenzyl alcohol due to overreduction (Wiklund and Bergman, 2006). This is due to the well-known fact that aldehydes are much more reactive towards reductants than carboxylic acids.

The preparation and handling of 2-aminobenzaldehyde (**1**), which is more reactive than many other aldehydes, is difficult. Additionally, it is complicated by the presence of both, amino and aldehyde groups, causing intermolecular condensation reactions. Formed under acidic conditions, the dimer **6** and the tetramer of **1** have been known for around 100 years, (Seidel, 1926) giving rise to McGeachin-type bisaminals chemistry (Chen et al., 2020). Several spectrophotometric assays are based on the ability of **1** to react spontaneously with δ-1-pyrrolines and δ-1-piperidines generating the triple aromatic ring structure **7** (Schöpf et al., 1948). For example, **1** reacts with pyrrolines forming yellow compounds that are used in spectrophotometric methods for the determination of diamine oxidase activity (Bolmstedt and Tham, 1959; Boehm et al., 2020a). The same characteristic of **1** makes it possible to simply and quickly determine the amount of δ-1-piperidine-6-carboxylate in the urine of patients with antiquitin deficiency, which causes the most common vitamin B_6-dependent epileptic encephalopathy (Boehm et al., 2020b). Moreover, **1** is routinely used as a highly specific tool for dihydrodipicolinate synthase purification due to the formation of the purple adduct (**8**) with 2,3-dihydrodipicolinate (Mitsakos et al., 2011).

Previous data suggest that utilization of living organisms for the preparation of 2-aminobenzaldehyde (**1**) should be pretty challenging. However, it is already known that **1** is produced by several basidiomycetous fungi, namely *Hebeloma Sacchariolens* (Wood et al., 1992) and *Wolfiporia cocos* (Sommer et al., 2021). Recently, we demonstrated the ability of white-rot fungi to reduce efficiently aromatic carboxylic acids to the corresponding aldehydes and alcohols, (Zhuk et al., 2021, 2022) and the aim of current study was to develop an effective and inexpensive

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https://doi.org/10.1016/j.jbiotec.2024.03.015
Received 31 December 2023; Received in revised form 30 March 2024; Accepted 30 March 2024
Available online 4 April 2024

method for the production of 2-aminobenzaldehyde with potential for direct use by humans. Herein, we propose the first biocatalytic approach to 2-aminobenzaldehyde (**1**) by selective reduction of anthranilic acid (**2**) with growing cultures of the white-rot fungus *B. adusta.*

2. Materials and methods

2.1. Organism

The filamentous fungi *Bjerkandera adusta* (DSMZ, 4708), *Irpex consors* (DSMZ, 7382), *Trametes versicolor* (DSMZ, 11309), *Marasmius cohortalis* (DSMZ, 8257), *Stereum complicatum* (DSMZ, 5182), *Flammulina velutipes* (DSMZ, 1658), *Daedalea quercina* (DSMZ, 4953), *Pleurotus sapidus* (DSMZ, 8266), *Pleurotus erymgii* (DSMZ, 8264), *Pleurotus cornucopiae* (DSMZ, 5342), *Pleurotus ostreatus* (DSMZ, 1833), *Dichomitus squalens* (DSMZ, 9615), *Auricularia fuscosuccinea* (DSMZ, 9844), and *Auricularia polytricha* (DSMZ, 6918) were obtained from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. The stock cultures were maintained on a solid medium containing 15 $g{\cdot}L^{-1}$ malt extract (Fluka, Neu-Ulm, Germany) and 15 $g{\cdot}L^{-1}$ agar-agar (Roth, Karlsruhe, Germany).

2.2. Chemicals

Anthranilic acid (99,5%), 2-aminobenzaldehyde (98%), 3-aminobenzoic acid (98%), and 4-aminobenzoic acid (99%) were purchased from Merck (Darmstadt, Germany) and used without further purification.

2.3. Screening of fungal reductive activity towards anthranilic acid using surface cultures

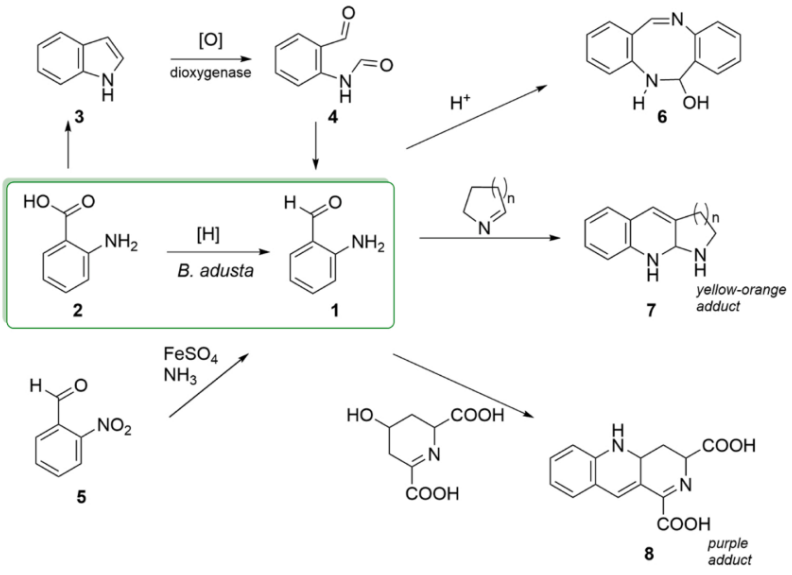
The fungi were maintained on malt extract agar (MEA; 20 $g{\cdot}L^{-1}$ malt extract, 15 $g{\cdot}L^{-1}$ agar-agar) or minimal medium (MMA; 15 $g{\cdot}L^{-1}$ agar-agar, 6.24 $g{\cdot}L^{-1}$ monosodium L-aspartate·H_2O, 2.4 $g{\cdot}L^{-1}$ NH_4NO_3, 1.5 $g{\cdot}L^{-1}$ KH_2PO_4, 0.5 $g{\cdot}L^{-1}$ $MgSO_4{\cdot}H_2O$, 400 $\mu g{\cdot}L^{-1}$ ethylenediaminetetraacetic acid (EDTA), 90 $\mu g{\cdot}L^{-1}$ $ZnSO_4{\cdot}7\ H_2O$, 80 $\mu g{\cdot}L^{-1}$ $FeCl_3{\cdot}6\ H_2O$, 30 $\mu g{\cdot}L^{-1}$ $MnSO_4{\cdot}H_2O$, 5 $\mu g{\cdot}L^{-1}$ $CuSO_4{\cdot}5\ H_2O$, pH=6) without and with glucose (15 $g{\cdot}L^{-1}$). For inoculation, an agar plug (a 0.4 cm diameter) was transferred from a freshly overgrown plate to agar plates with corresponding media containing anthranilic acid (137 $\mu g{\cdot}L^{-1}$, approximately 3.5 mg per agar plate). As anthranilic acid is a toxic compound and poses danger in dust form, we kept it in agar layer. It is not volatile as it is characterized by a low vapor pressure of 0.001 hPa (52.6 °C). Cultivation was performed at 24 °C in the dark for up to 14 days. The formation of 2-aminobenzaldehyde was determined organoleptically, as the compound is characterized by specific strong floral odor.

2.4. Submerged cultures

The culture medium was prepared by dissolving malt extract (20 g) in 1 L of deionized water. For the preparation of the precultures, a 1 cm^2 agar plug from the leading mycelial edge was transferred into 100 mL medium (in a 250 mL Erlenmeyer flask) and then homogenized with a T 25 digital Ultra-Turrax homogenizer (IKA, Staufen, Germany; 30 s, 10.000 $r{\cdot}min^{-1}$). The precultures were grown on an incubation shaker (Orbitron, Infors HAT, Bottmingen, Switzerland; 150 $r{\cdot}min^{-1}$, deflection 25 mm) under the exclusion of light at 24 °C for 7 days. Subsequently, the precultures were homogenized, and 10% (v/v) of the homogenate was inoculated for submerged cultivation on 100 mL (in 250 mL Erlenmeyer flasks) scale.

2.5. Biotransformation of anthranilic acid with growing cultures

Anthranilic acid (22 mg (1.5 mM), 30 mg (2 mM), 37.7 mg (2.5 mM), 45 mg (3 mM), 52.7 (3.5 mM), 60 mg (4 mM)) was added to submerged cultures of *B. adusta* (110 mL) grown in malt extract (2%) medium in 250 mL Erlenmeyer flasks on the 3rd culture day. The reaction mixture was placed on an incubation shaker at 150 $r{\cdot}min^{-1}$ (deflection 25 mm) under exclusion of light at 24 °C for 2 days. After supplementation of the growing culture with the substrate, samples (3 mL) were taken every 4 h. 0.5 g of NaCl was added to the sample, and the mixture was stirred for 5 min at 800 rpm (magnetic stirrer). For extraction, 3 mL Et_2O that contained 2.5 mM internal standard was added, and the resulting mixture was stirred for 10 min at 800 rpm



Scheme 1. Strategies for the production of 2-aminobenzaldehyde (**1**) and some chemical properties.

(magnetic stirrer) and centrifuged for 5 min at 3000 × g to separate the organic layer. The extraction was repeated three times. The combined organic layers were washed with brine (1 × 20 mL) and water (1 × 20 mL), dried over Na_2SO_4, concentrated to 2 mL, and analysed by GC-MS (Agilent Technologies 7890 A GC, column Agilent VF-WAXms (30 m × 0.25 mm, 0.25 μm) and Agilent 5975 C MSD Triple-Axis mass spectrometer). Every experiment was repeated three times to verify the reproducibility of the results.

2.5.1. Biotransformation of anthranilic acid with growing cultures: blank experiments

A. The experiment described in Section 2.5 has been performed with autoclaved media without fungus and repeated three times. No traces of product have been identified.

B. The experiment described in Section 2.5 has been performed with growing culture autoclaved prior to the addition of substrate and repeated three times. No traces of product have been identified.

2.6. Biotransformation of anthranilic acid with growing culture in the presence of ethanol

Anthranilic acid (30 mg (2 mM), 37.7 mg (2.5 mM)) was dissolved in ethanol (2 mL) and added to submerged culture of *B. adusta* (110 mL) on the 3rd culture day, and biotransformation proceeded for 2 days. The reaction mixture was processed and analysed as described in Section 2.5. Every experiment was repeated three times to verify the reproducibility of the experiments.

2.7. Up-scaled biotransformation of anthranilic acid

Anthranilic acid (120.7 mg, 0.88 mmol, final concentration of 2 mM) dissolved in 2 mL of ethanol was added to 440 mL submerged cultures of *B. adusta* grown in malt extract medium in 1 L Erlenmeyer flasks on the 3rd culture day. The reaction mixture was placed on an incubation shaker at 150 r·min^{-1} (deflection 25 mm) under exclusion of light at 24 °C for 2 days. 20 g of NaCl was added to the medium, and the mixture was stirred for 15 min at 800 rpm (magnetic stirrer). For extraction, 150 mL Et_2O was added, and the resulting mixture was stirred for 20 min at 800 rpm (magnetic stirrer), and layers have been separated. The extraction was repeated 5 times. The combined organic phases were dried over Na_2SO_4 and evaporated to dryness. The product was isolated by column chromatography on silica gel (hexane/ether = 1/1) to give 2-aminobenzaldehyde (41 mg, 39%) as a yellow solid. The identity of the solid has been confirmed by comparison of its ^1H and ^{13}C spectra with those of an authentic reference compound.

2.8. Biotransformations of isomers of anthranilic acid with growing culture

3-Aminobenzoic acid (30 mg, (2 mM)) was added to submerged culture of *B. adusta* (110 mL) on the 3rd culture day, and biotransformation proceeded for 2 days. The reaction mixture was processed and analysed as described in Section 2.5.

4-Aminobenzoic acid (30 mg, (2 mM)) was added to submerged culture of *B. adusta* (110 mL) as described above. The reaction mixture was processed and analysed as described in Section 2.5.

Anthranilic acid (15 mg, (1 mM)) and 4-aminobenzoic acid (15 mg, (1 mM)) were added to submerged culture of *B. adusta* (110 mL) as described above. After 24 h the reaction mixture was processed and analysed as described in Section 2.5.

Every experiment was repeated three times to verify the reproducibility of the experiments.

2.9. Biotransformation of anthranilic acid with resting cells

The mycelium was collected from the submerged cultures of *B. adusta* after three days of growing and washed three times with sterilized distilled water. Then the corresponding sterilized buffer was added (the volume was twice less then volume of supernatant). The next buffers have been tested: HEPES (pH = 7, 0.05 mM and 0.1 mM), phosphate (pH = 7, 0.05 mM and 0.1 mM), TRIS-HCl (pH = 7, 0.05 mM and 0.1 mM). Then anthranilic acid (2 mM) was added. The reaction mixtures were placed on an incubation shaker at 150 r·min^{-1} (deflection 25 mm) under exclusion of light at 24 °C for 20 h. After 24 h the reaction mixture was processed and analysed as described in Section 2.5. Every experiment was repeated three times to verify the reproducibility of the results.

3. Results and discussion

3.1. Screening of basidiomycetous fungal reductive activity towards anthranilic acid (2)

In a broad screening of various basidiomycetes, 14 different fungi from the orders Agaricales, Polyporales, Russulales and Auriculariales were tested for the reduction of **2**. Either malt extract (ME) or minimal medium with or without glucose were tested for fungal growth. The first screening round has been performed using surface cultures, and *B. adusta* has been identified as the most promising fungus for the reduction of anthranilic acid (**2**). The results of the screening have been presented in Table S1. Then the process has been transferred into submerged cultures in which 2-aminobenzaldehyde (**1**) has been identified without traces of the corresponding alcohol as a product of further reduction of the aldehyde group. The most efficient reduction of anthranilic acid (**2**) was obtained in malt extract (2%) medium and addition of **2** on the third culture day.

The reductive activity of *B. adusta* towards **2** may be associated with the presence of specific carboxylic acid reductases (CARs), that are common enzymes for white-rot fungi and other organisms. CARs are large multi-domain proteins that catalyze the ATP- and NADPH-dependent reduction of a wide range of acids to the corresponding aldehydes. Carbonyl reductase activity towards the corresponding aldehyde **1** was not observed, as no traces of the alcohol have been identified in the reaction mixtures.

3.2. Time course for anthranilic acid (2) biocatalytic reduction

In the next step, *B. adusta* was grown submerged in malt extract medium in 250 mL Erlenmeyer flasks (110 mL medium). The formation of 2-aminobenzaldehyde (**1**) was monitored quantitatively using o-xylene as an internal standard by GC-MS analysis. The range of initial concentration of anthranilic acid (C_0) was 1.5 mM - 4 mM (Fig. 1). Samples was taken every four hours during 2 days. The maximum concentrations of **1** in A (C_0 = 1.5 mM) and B (C_0 = 2 mM) were achieved after 8 h and 24 h, and amounted to 0.85 mM and 1.1 mM respectively, corresponding to roughly 55% yield. In C (C_0 = 2.5 mM) and D (C_0 = 3 mM), the highest concentrations of **1** were observed after 28 h (0.78 mM and 0.54 mM). In E (C_0 = 3.5 mM) the maximum concentration of **1** (1.3 mM) was found after 32 h of transformation. With C_0 = 4 mM (F) the concentration of **1** was very low (0.2 mM).

The above experiments clearly showed that the time to reach the maximum concentration increased with increasing substrate concentration. This could be a result of toxic effects of anthranilic acid on the culture that slow down the expression of the corresponding reductases, or the gradual dissolution of the anthranilic acid (**2**) at higher concentrations. Also, the yield of the aldehyde **1** decreased with increasing substrate concentrations. The optimal concentration was C_0 = 2 mM, when the yield of **1** was roughly 55%. It was clearly shown that after reaching the maximum, the concentration of **1** decreased till its complete disappearance in all experiments. This may be caused by further transformation of the aldehyde **1**, e.g., by oxidative degradation or condensation. The further reduction of **1** can be excluded, as no traces of

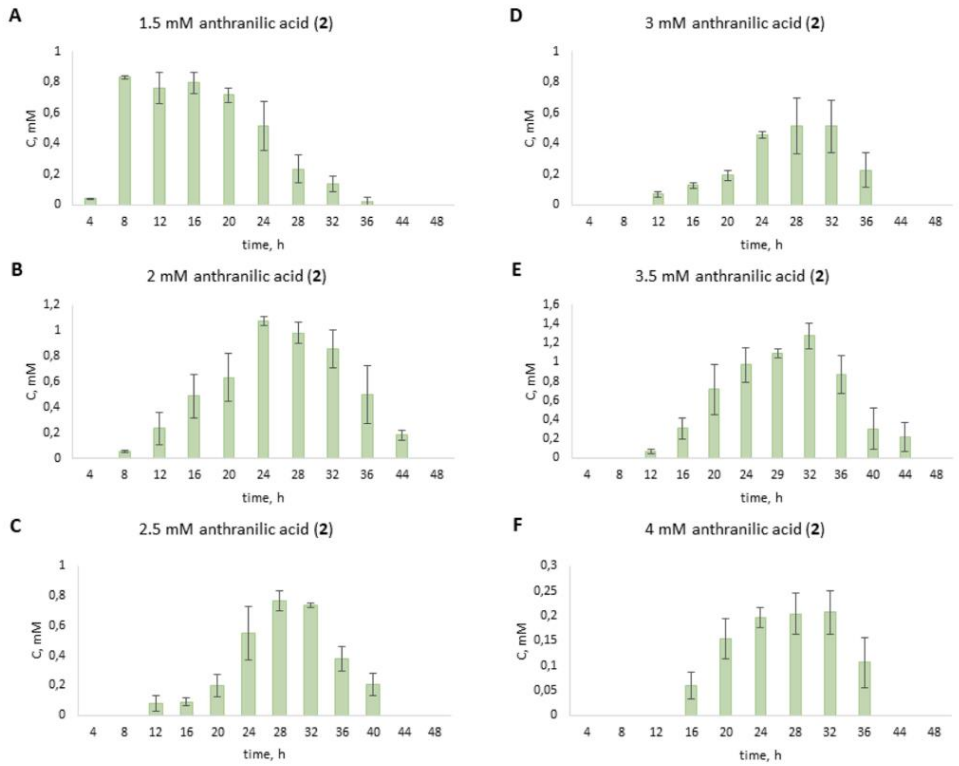


Fig. 1. Formation of 2-aminobenzaldehyde (**1**) in submerged cultures of *B. adusta* containing varying initial concentrations of anthranilic acid (**2**) as precursor: A) 1.5 mM, B) 2 mM, C) 2.5 mM, D) 3 mM, E) 3.5 mM, F) 4 mM. Error bars represent standard deviations based on triplicate experiments.

the corresponding alcohol have been detected in the reaction mixtures. The further transformation of 2-aminobenzaldehyde in cultures of BAD are subject of our future research.

3.3. Reduction of anthranilic acid (2) in the presence of ethanol as co-solvent

Previously, it was shown that white-rot fungi can survive and efficiently transform the compounds of interest in the presence of alcohols as co-solvents (Zhuk et al., 2015). Moreover, the presence of co-solvents may increase the yields of the biotransformation reactions. Therefore, the reduction of **2** was studied in the presence of ethanol. The yields of 2-aminobenzaldehyde (**1**) were significantly increased, and the presence of the co-solvent substantially decreased the reaction times. The observed effect of the presence of ethanol as co-solvent could be a result of better and faster dissolution of the substrate in the culture media (Fig. 2).

3.4. Preparative transformations

To evaluate the potential of the developed biotransformation for preparative applications, the reduction of anthranilic acid (**2**) by *B. adusta* in the presence of ethanol as a co-solvent was scaled-up (Scheme 2). Using 2 mM as the initial concentration, acid **2** was added

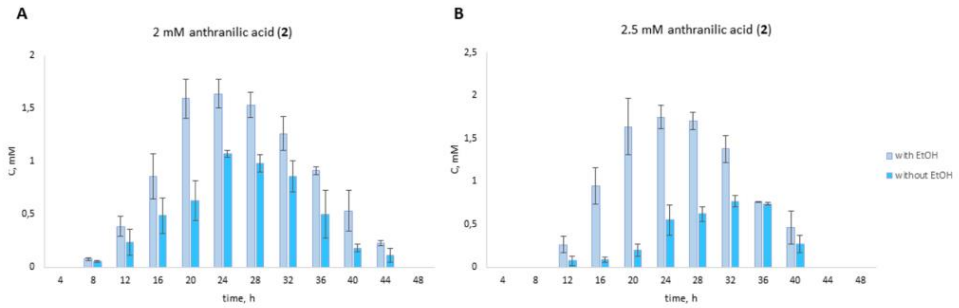
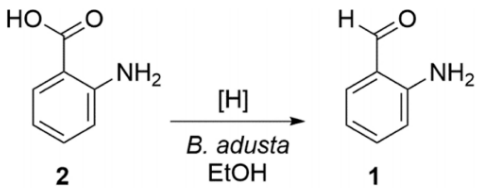


Fig. 2. Formation of 2-aminobenzaldehyde (**1**) in submerged cultures of *B. adusta* with ethanol as co-solvent. Initial concentrations of anthranilic acid (**2**): A) 2 mM, B) 2.5 mM. Error bars represent standard deviations based on triplicate experiments.



Scheme 2. The reaction of preparative biotransformation of anthranilic acid (**2**).

to 440 mL culture on the 3rd culture day. The concentration of aldehyde **1** increased during 2 days, and started to decrease after 48 h. The reaction was stopped by the addition of NaCl to the reaction medium. The extraction of the reaction mixture (including culture supernatant and mycelium) with diethyl ether resulted in 50 mg of crude extract. After combining all organic extracts and purification by column chromatography, 41 mg of pure 2-aminobenzaldehyde (**1**) with GC-MS and NMR spectra identical to those of the reference compound were obtained. To the best of our knowledge, this is the first example of the preparative biocatalytic reduction of anthranilic acid that allows the production of 2-aminobenzaldehyde (**1**) without formation of even traces of 2-aminobenzyl alcohol.

3.5. Biocatalytic reduction of isomers of anthranilic acid (2)

In order to clarify if the developed approach could be also applied for the synthesis of isomers of **1**, the following experiments were performed. Under the conditions developed for the reduction of **2**, isomers of anthranilic acid, namely 3-amino- and 4-aminobenzoic acid were subjected to the biotransformation process. After supplementation of 3-aminobenzoic acid, only trace amounts of the corresponding aldehyde were detected, and 4-aminobenzoic acid was not reduced to the corresponding aldehyde at all. 3- and 4-aminobenzyl alcohol were also not detected in the reaction mixtures. Experiments with simultaneous addition of anthranilic and 4-aminobenzoic acid to the fungal culture were also performed. **1** was identified as a single aldehyde in reaction mixture.

3.6. Biotransformation of anthranilic acid with resting cells

We decided to analyse the ability of resting cells to reduce the anthranilic acid (**2**). We tested three buffers keeping neutral pH in every case. Neutral pH should help to avoid the further transformations of the target product 2-aminobenzaldehyde (**2**). In the reaction mixtures with HEPES and phosphate buffers only the traces of **2** have been detected. With TRIS-HCl buffer the yield of **2** was 15–20% according to GC-MS. These results clearly show that responsible for the biotransformation enzymes are produced by *B. adusta* not only in presence of anthranilic acid (**1**), but they are its regular enzymes.

4. Conclusions

Growing cultures of the basidiomycetous fungus *B. adusta* demonstrate the ability to selectively reduce anthranilic acid to 2-aminobenzadehyde with satisfactory chemical yields and without overreduction to the corresponding benzyl alcohol. This biocatalytic approach is potentially useful for the production of this highly demanded aroma component with a floral scent by the aerobic “in-water” approach. In addition, our experimental results offer clear paths for the further development this green technology for the production of 2-aminobenzadehyde, *e.g.,* through the use of co-solvents and/or identification and further application of the enzyme responsible for the disclosed biotransformation.

CRediT authorship contribution statement

Yuliya Kurtash: Methodology. **Holger Zorn:** Writing – review & editing, Resources, Conceptualization. **Valeriia Babkina:** Writing – original draft, Visualization, Methodology. **Yana Hajduk:** Methodology. **Tatyana Zhuk:** Writing – review & editing, Validation, Supervision, Project administration, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgements

VB, YH and YK were financed by Erasmus+ EU funds. TZ gratefully acknowledges financial support by the Alexander von Humboldt Foundation (Georg Forster Research Fellowship) and Philipp Schwartz Initiative. The work was partially supported by MES of Ukraine (# 0123U104223).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiotec.2024.03.015.

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6. *Bjerkandera adusta* lyophilisate as catalysts for the oxidation of α,β-unsaturated alcohols

Selective Oxidation of α,β-Unsaturated Alcohols With Lyophilisates of Bjerkandera adusta.

L. Calza; V. V. Nikitenkova; W. Albuquerque; H. Zorn; T. S. Zhuk

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Chem. Biodivers. **2025**, *22* (12), e01127.

https://doi.org/10.1002/cbdv.202501127

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RESEARCH ARTICLE OPEN ACCESS

Selective Oxidation of α,β-Unsaturated Alcohols With Lyophilisates of *Bjerkandera adusta*

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Received: 31 March 2025 | **Revised:** 28 July 2025 | **Accepted:** 30 July 2025

Funding: Letizia Calza was financed by Erasmus+ EU funds, and Valeriia Nikitenkova was supported by DBU. Tatyana Zhuk gratefully acknowledges financial support from the Philipp Schwartz Initiative.

Keywords: alcohol oxidation | allylic aldehydes | flavor compounds | whole cells

ABSTRACT

The biocatalytic aerobic production of (*E*)-2-allylic aldehydes from their corresponding alcohols using lyophilisates of the basidiomycetous fungus *Bjerkandera adusta* is reported. The addition of small amounts of organic solvents to the reaction media increased the reaction and substrate conversion rates, allowing for to produce (*E*)-aldehydes under sustainable conditions. Citral (mixture of (*E*)- and (*Z*)-3,7-dimethylocta-2,6-dienal) was found as a result of the oxidation of geraniol ((*E*)-3,7-dimethyl-2,6-octadien-1-ol) as well as of nerol ((*Z*)-3,7-dimethyl-2,6-octadien-1-ol). In the case of (*Z*)-2-nonene-1-ol, the formation of (*E*)-2-nonenal was detected as the only product. The isomerization of (*Z*)-2-nonenal to the corresponding (*E*)-diastereomer with fungal lyophilisate under the applied reaction conditions has been shown in additional experiments. No products of further oxidation of the formed aldehydes or reduction of the activated CC-double bond were detected in the reaction mixtures. A comparative analysis of the catalytic activity of lyophilisates obtained from *Pleurotus sapidus* and *Pleurotus eryngii*, as well-known producers of oxidative enzymes, was performed in model experiments.

1 | Introduction

α,β-Unsaturated aldehydes are well-known building blocks in organic synthesis [1–3] as well as flavor compounds used in food and beverages [4]. They are responsible for a wide variety of odor impressions, from the fresh green notes of (*E*)-2-hexenal to the fatty, chicken-like flavor of (*E,E*)-2,4-decadienal. Despite their widespread occurrence in nature, especially in plants [5], they are not accumulated in organisms but rather serve as cosubstrates for *in vivo* enzymatic transformations [6]. For this reason, rich natural sources of aldehydes are rare.

A number of chemical methods for the synthesis of α,β-unsaturated aldehydes have been developed [7–9]. However, even mild approaches are based on the utilization of toxic metals, which makes them inappropriate for using the produced aldehydes in food, beverages, and cosmetics. Recently, biocatalytic transformations attracted much attention as promising sustainable alternatives to traditional chemical approaches [10]. Nature developed a portfolio of proteins to transform different types of precursors to target aldehydes, and many researchers attempt their application at laboratory and industrial scale [11–14]. The simplest and most common method for the preparation

Letizia Calza and Valeriia Nikitenkova should be considered the first authors equally.

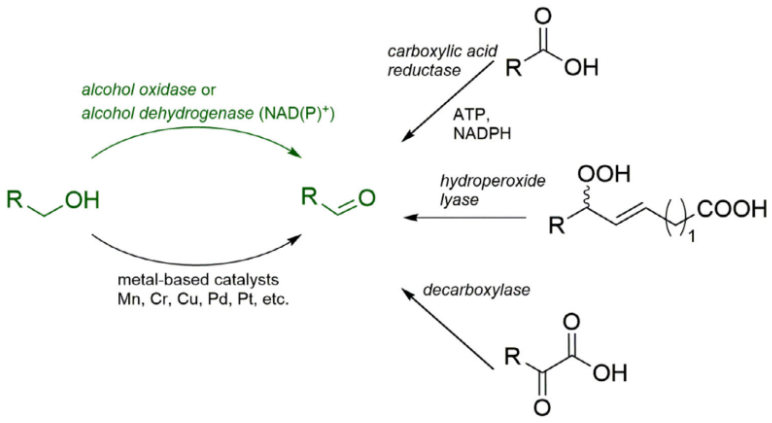


FIGURE 1 | Selected biocatalytic approaches for aldehyde production (ATP – adenosine triphosphate; NADPH – nicotinamide adenine dinucleotide phosphate). These cofactors are required in stoichiometric amounts.

of aldehydes is based on the oxidation of primary alcohols that, in many cases, suffer from overoxidation, sensitivity to the alkyl chain length, long reaction times, as well as low conversion and space–time yields [10]. Current biocatalytic approaches are mainly based on the utilization of alcohol oxidases [15, 16], alcohol dehydrogenases [17, 18], carboxylic acid reductases [19, 20], decarboxylases (for α-keto acid decarboxylation) [21, 22], and the combination of lipoxygenases with hydroperoxide lyases [23] (Figure 1). Alcohol dehydrogenases and carboxylic acid reductases are well-studied catalysts; however, they require the presence of stoichiometric amounts of expensive cofactors or the implementation of cofactor regeneration systems. Decarboxylases and hydroperoxide lyases are appropriate for specific substrates, namely ketoacids and hydroperoxides of polyunsaturated fatty acids, respectively. Aryl alcohol oxidases, which are FAD (flavin adenine dinucleotide)-dependent oxidoreductases, seem to be the most promising biocatalysts as they require only molecular oxygen as a cosubstrate for alcohol oxidation and do not depend on costly external cofactors [24, 25]. Alcohol oxidases have been successfully applied for the synthesis of furan-based derivatives [26–28], vanillin [29], and for the oxidation of methanol and glycerol [30].

Currently, the main research activities on biocatalytic approaches are associated with the construction of recombinant expression systems for target enzyme production, followed by enzyme purification, which is still economically challenging. Recently, the examination of the dependency of the manufacturing costs of whole-cells *vs.* enzymes showed that prices for industrial (non-purified) enzymes range between 250 and 1000 €*kg^{-1}, whereas whole-cells are considerably cheaper (35–100 €*kg^{-1}) [31]. The main efforts in whole-cell production are focused on the implementation of modified host organisms, but there are many potential industrial applications in which it would be more desirable to avoid the handling of living genetically modified systems.

An alternative protocol for the production of aromatic aldehydes from the corresponding alcohols using *Bjerkandera adusta* (BAD) lyophilisate has been described by us recently [32]. The approach allows overcoming the challenges of recombinant expression systems and enzyme purification, as well as uses the main advantages of whole-cells, namely low cost, enzyme stability, and independence from additional cofactors. The high catalytic activity of BAD lyophilisate is associated with the confirmed presence of aryl-alcohol oxidases that preserve their catalytic activity after the freeze-drying process and long-term storage. One of the main challenges of the application of this approach to the oxidation of allylic alcohols could be the presence of an activated CC-double bond in the substrates, which may be reduced easily, unlike the aromatic ring of benzyl alcohol. Previously, the ability to reduce CC-double bonds in α,β-unsaturated substrates was shown for various fungi from the Phylum Basidiomycota [33, 34].

The current study aims to explore the prospects of BAD lyophilisates to oxidize allylic alcohols to the corresponding carbonyl compounds. The scope and limitations of allylic alcohol oxidation depending on their molecular structures are disclosed as well.

2 | Results and Discussion

2.1 | Influence of the pH and Presence of Cosolvents

The oxidative activity of BAD lyophilisates towards (*E*)-2-octen-1-ol (**1a**) as a model compound was estimated using the procedure developed previously for aromatic alcohols [32]. The biotransformation of **1a** was studied at pH 6, 7, and 8, which are optimal for the enzymatic oxidation of alcohols, as previously described [32, 35]. The conversion of the alcohol to the aldehyde was analyzed after 15 min, 2, 4, 6, 10, and 24 h. (Figure 2) Product concentrations were determined utilizing gas chromatography-mass spectrometry (GC-MS) via an internal standard (1-adamantanol). Calibration curves were established for the target aldehydes using commercially available standards. Notably, after 15 min, the yield of aldehyde **2a** already reached 10% in all experiments. The

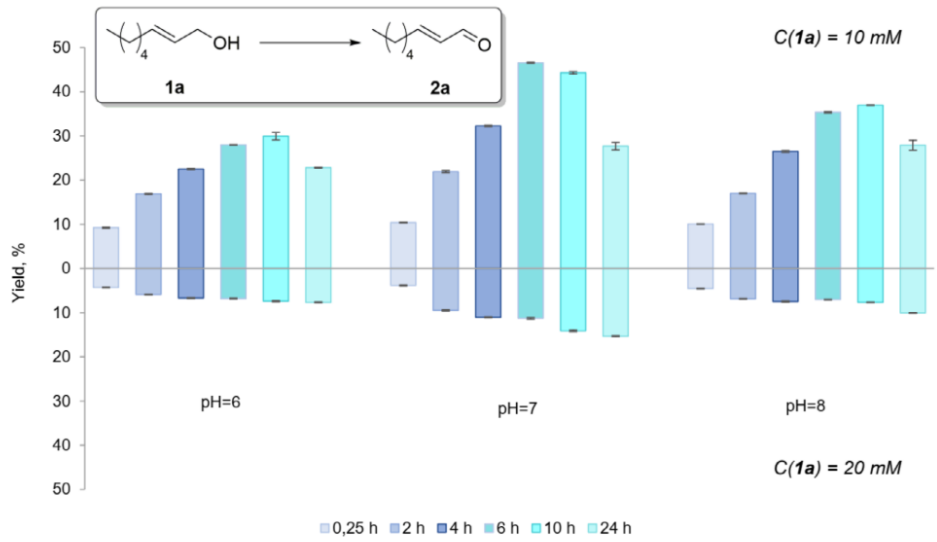


FIGURE 2 | Yields of (*E*)-2-octenal (**2a**) after 15 min, 2 h, 4 h, 6 h, 10 h, and 24 h at pH 6, 7, and 8. The initial concentrations of the substrate (*E*)-2-octen-1-ol (**1a**) were 10 mM (upper part) and 20 mM (bottom part).

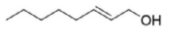
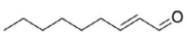
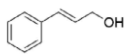
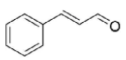
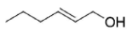
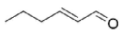
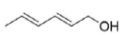
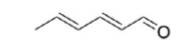
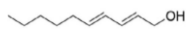
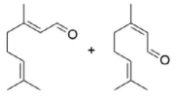
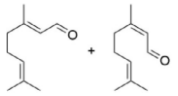
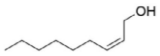
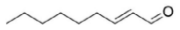
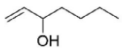
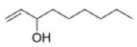
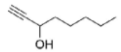
content of **2a** reached maximum values between 6 and 10 h of reaction time. Among the tested pH values, the highest yield (up to 45%) was observed at pH 7. Decreasing the concentration of the substrate increased the yield; thus, at pH 7, experiments with 10 mM of (*E*)-2-octen-1-ol (**1a**) gave a higher yield of aldehyde **2a**, namely up to 45 % compared to 15 % with 20 mM. Notably, the reaction is remarkably selective as **2a** was observed as the only product. The simplicity of the work-up of the reaction mixture by simple filtration/extraction opens certain perspectives for this methodology. However, high conversion rates of starting alcohol **1a** were not reached through the applied "on-water" methodology, and attention was thus turned to the use of organic cosolvents.

Methanol, ethanol, and isopropanol were tested as cosolvents in concentrations of 10 and 30 volume % (Figure 3). The presence of 30 % of every solvent decreased the conversion of substrate **1a** significantly, likely due to enzyme denaturation at such high cosolvent concentrations. In contrast, with 10 % of cosolvent, the reaction proceeded smoothly, suggesting that the enzyme remained stable under these milder conditions. Among the tested alcohols, isopropanol proved to be the most effective, likely due to its moderate polarity and relatively bulky structure, which together provided a favorable balance between enzyme stability and substrate solubility. Under these conditions, a ca. 90 % yield of **2a** was reached after 4 h. This yield was confirmed by performing preparative experiments, in which (*E*)-2-octenal (**2a**) was isolated and characterized through nuclear magnetic resonance (NMR) spectroscopy. These reaction conditions were further used for the study of the substrate scope. In control reactions with autoclaved lyophilisates, only the substrate **1a,** without any traces of products, was detected in the reaction mixtures.

2.2 | Biotransformation of α,β-unsaturated Primary Alcohols

After optimization of the reaction conditions using (*E*)-2-octen-1-ol (**1a**), the activity of BAD lyophilisates was tested toward a number of substrates of different structures (Table 1). Product formation was monitored utilizing GC-MS with an internal standard (1-adamantanol). For substrates **1a** and **1d–i**, the oxidation reactions were performed on a preparative scale, and the products were isolated using column chromatography. (*E*)-2-Nonen-1-ol (**1b**) and (*E*)-cinnamyl alcohol (**1c**), as well as model alcohol **1a**, were fully converted to the corresponding aldehydes **2b** and **2c**. (*E*)-2-Hexen-1-ol (**1d**), which contains a shorter carbon chain, demonstrated low conversion, giving only 20% of (*E*)-2-hexenal (**2d**) while the yield of (*E,E*)-2,4-hexadienal (**2e**) from the corresponding alcohol **1e** reached 65%. With *(E,E)*-2,4-decadien-1-ol (**1f**), traces of diastereomeric aldehyde **3f** were identified, together with the main product *(E,E)*-2,4-decadienal (**2f**) with 68% yield. Citral (3,7-dimethyl-2,6-octadienal, **2g**), which is a mixture of geranial and neral [36, 37], was found as a product of the oxidation of geraniol (**1g**) as well as of nerol (**1h**). However, the conversion of nerol (**1h**) reached only 57%, while geraniol (**1g**) was fully converted. Notably, the oxidation of (*Z*)-2-nonen-1-ol (**1i**) gave selectively (*E*)-2-nonenal (**2b**), the product of isomerization without any traces of the *Z*-configured aldehyde, with a conversion of the substrate of 86%. No oxidation products were found with 1-hepten-3-ol (**1j**), 1-nonen-3-ol (**1k**), 1-octyn-3-ol (**1l**), and cyclohexen-1-ol (**1m**). These findings clearly demonstrate the selectivity of the transformation of primary alcohols. Since possible by-products, such as the respective acids or starting/saturated alcohols, could either be adsorbed by the lyophilisate or be highly water-soluble, preparative experiments with selected alcohols ((*E*)-2-hexen-1-ol (**1d**), *(E,E)*-2,4-hexadien-1-ol (**1e**), *(E,E)*-2,4-

TABLE 1 | Biotransformation of α,β-unsaturated alcohols.*

#	Substance	Conversion, %	Product	Yield, %
1	(*E*)-2-octen-1-ol (**1a**) 	100	(*E*)-2-octenal (**2a**) 	87§
2	(*E*)-2-nonen-1-ol (**1b**) 	100	(*E*)-2-nonenal (**2b**) 	99
3	(*E*)-cinnamyl alcohol (**1c**) 	100	(*E*)-cinnamaldehyde (**2c**) 	99
4	(*E*)-2-hexen-1-ol (**1d**) 	23	(*E*)-2-hexenal (**2d**) 	20§
5	(*E,E*)-2,4-hexadien-1-ol (**1e**) 	73	(*E,E*)-2,4-hexadienal (**2e**) 	65§
6	(*E,E*)-2,4-decadien-1-ol (**1f**) 	84	(*E,E*)-2,4-decadienal (**2f**)  Diastereomeric 2,4-decadienal (**3f**)	68§ traces
7	Geraniol (**1g**) 	100	Citral (**2g**)[a]  62 : 38	95§
8	Nerol (**1h**) 	57	Citral (**2g**)[a]  48 : 52	52§
9	(*Z*)-2-nonen-1-ol (**1i**) 	86	(*E*)-2-nonenal (**2b**) 	83§
10	1-hepten-3-ol (**1j**) 	0	—	0
11	1-nonen-3-ol (**1k**) 	0	—	0
12	1-octyn-3-ol (**1l**) 	0	—	0
13	2-cyclohexen-1-ol (**1m**) 	0	—	0

[a]Determined by GC-MS from relative peak areas.

*All reactions were performed under identical conditions as described in the Experimental Section 3.6 (General procedure for biotransformation).

§Isolated yields.

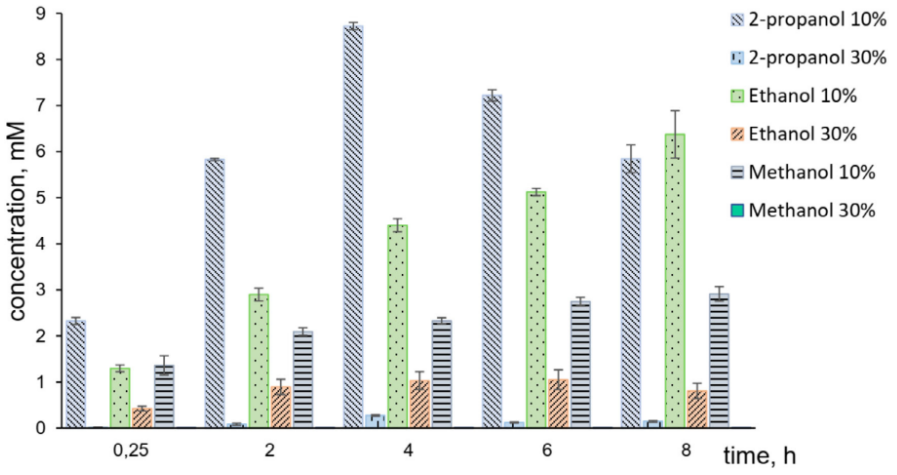


FIGURE 3 | Concentration of (*E*)-2-octenal (**2a**) as a product of biotransformation of alcohol **1a** (10 mM) performed in the presence of different cosolvents (methanol, ethanol, and 2-propanol) in concentrations of 10% and 30%.

decadien-1-ol (**1f**), nerol (**1h**), geraniol (**1g**), and (*Z*)-2-nonen-1-ol (**1i**)) were performed displaying high reaction yields.

2.3 | Biotransformation of (*Z*)-/(*E*)-2-nonen-1-ol

For geraniol (**1g**), nerol (**1h**), and (*Z*)-2-nonen-1-ol (**1i**), the partial or full conversion of one geometric isomer to another has been observed. It is likely that amino acids, acting as zwitterions, contribute to the stabilization of intermediates during the isomerization process. The catalytic ability of several amino acids, as well as of bovine serum albumin, in promoting the isomerization of geranial and neral has already been demonstrated [38]. Notably, neither the presence of vitamin C nor conducting the reaction under an inert atmosphere affected the isomerization rate. As the fungal lyophilisates contain numerous proteins, the reaction conditions were tested using *(Z)*-2-nonenal (**1n**), which was synthesized using Dess-Martin periodinane from *(Z)*-2-nonen-1-ol (**1i**). Under standard reaction conditions with BAD lyophilisate, *(Z)*-2-nonenal (**1n**) gave (*E*)-2-nonenal (**2b**) as the only product. (Figure 4) This result is in accordance with the above-mentioned isomerization of geranial and neral.

2.4 | Characterization of Further Fungal Lyophilisates

Pleurotus eryngii and *Pleurotus sapidus* are known as sources of oxidative enzymes. As only supernatants of the corresponding fungi have been well studied for the presence of oxidative enzymes, the ability of their lyophilisates to catalyze allylic alcohol oxidation reactions similar to BAD lyophilisates was investigated. The standard procedure for lyophilisate production and (*E*)-2-octen-1-ol (**1a**) as a model compound was used (Figure 5). The results showed that these lyophilisates are not as efficient as those from BAD, but have some potential as well. In particular, while the lyophilisate of *P. eryngii* gave low conversions of the starting compounds, the lyophilisates of *P. sapidus* displayed certain activity, still much lower than that observed for the BAD lyophilisate. The activities of *P. eryngii* and *P. sapidus* lyophilisates were studied under the conditions that are the most efficient for BAD lyophilisate, thus giving a rough estimation of their activity. By varying the cultivation time and conditions, as well as cosolvent and pH values, it could be possible to increase their catalytic activity. However, the developed simple conditions clearly show the advantage of the BAD lyophilisate.

3 | Experimental

3.1 | Organism

The filamentous fungi BAD (DSMZ 4708), *P. eryngii* (DSMZ 8264), and *P. sapidus* (DSMZ 8266) were obtained from the German Collection of Microorganisms and Cell Cultures, Brunswick, Germany. The stock cultures were maintained on a solid medium containing 15 g/L malt extract (Fluka, Neu-Ulm, Germany) and 15 g/L agar-agar (Roth, Karlsruhe, Germany).

3.2 | Chemicals

1-Adamantanol (99%), *(E)*-2-nonen-1-ol (96%), 2-cyclohexen-1-ol (95%), Dess-Martin periodinane (97%), and *(E)*-2-nonenal were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). 1-Hepten-3-ol (98%), 1-nonen-3-ol (98%), 1-octyn-3-ol (98%), 2,4-hexadien-1-ol (99%), *(Z)*-2-hexen-1-ol (94%), *(E)*-2-octen-1-ol (97%), citral (*E+Z*, 95%), and *(E,E)*-2,4-hexadienal (95%) were purchased from Alfa Aesar, Thermo Fisher (Kandel, DE). 2,4-Decadien-1-ol (95%) and *(Z)*-2-nonen-1-ol (95%) were obtained from abcr GmbH (Karlsruhe, DE). Cinnamyl aldehyde (98%) and *(E)*-2-hexen-1-ol (97%) were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, DE). Geraniol (99%), nerol (97%), *(E)*-2-

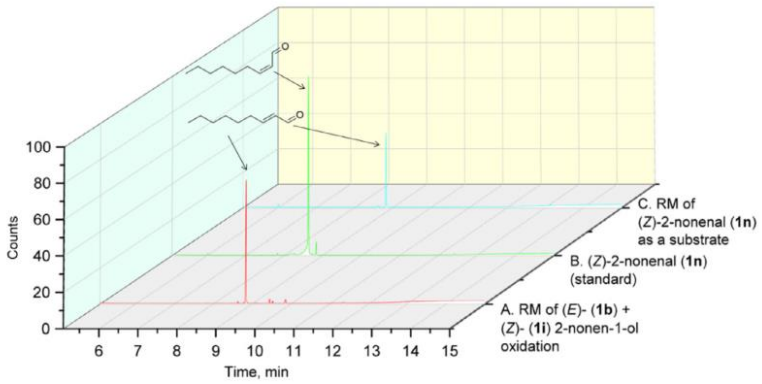


FIGURE 4 | Gas chromatography-mass spectrometry (GC-MS) chromatograms of: (A) The reaction mixtures (RM) of biotransformation of an equimolar mixture of (*Z*)- (**1i**) and (*E*)- (**1b**) 2-nonen-1-ols; (B) Standard of (*Z*)-2-nonenal (**1n**); (C) The reaction mixtures (RM) of the transformation of (*Z*)-2-nonenal (**1n**).

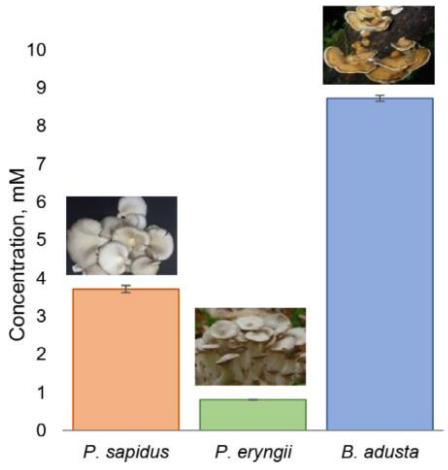


FIGURE 5 | Comparative analysis of oxidation of (*E*)-2-octen-1-ol (**1a**) with lyophilisates of *P. sapidus, P. eryngii*, and *B. adusta*.

hexenal (99%), and *(E)*-2-cinnamyl alcohol (98%) were bought from Acros Organics (New Jersey, USA). *(E)*-2-Octenal (98%) was purchased from BLD Pharmatech GmbH (Reinbek, DE). *(E,E)*-2,4-Decadienal (95%) was obtained from Fisher Scientific GmbH (Schwerte, DE).

3.3 | NMR and MS Instrumentation

The following instruments were used during this study: Rotavapor from Heidolph (Schwabach, Germany) for the evaporation procedure; T 25 digital Ultra-Turrax homogenizer (IKA, Staufen, Germany) for the homogenization of submerged cultures; shaker (Orbitron, Infors HAT, Bottmingen, Switzerland) for the incubation of cultures, freeze dryer Vaco2 (Zirbus Technology, Bad Grund, Germany) for lyophilization of mycelium.

NMR spectra were recorded at 298 K on a Bruker Avance II 400 MHz WB instrument (Billerica, Massachusetts, USA). The $CHCl_3$ signal (s, 7.26 ppm) was set as reference for the spectra in $CDCl_3$. NMR spectra were reported in Figures S1–S14.

GC-MS was performed using an Agilent 7890A gas chromatograph equipped with an Agilent VF-WAXms capillary column (30 m × 0.25 mm, 0.25 µm) and an Agilent 5975C MSD Triple-Axis mass spectrometer.

3.4 | Lyophilized Mycelium

The culture medium was prepared by dissolving malt extract (20 g) in 1 L of deionized water. For the preparation of the precultures, a 1 cm^2 agar plug from the leading mycelial edge was transferred into 100 mL medium (in a 250 mL Erlenmeyer flask) and homogenized with a T 25 digital Ultra-Turrax homogenizer (IKA, Staufen, Germany; 30 s, 10.000 $r{\cdot}min^{-1}$). The precultures were grown on an incubation shaker (Orbitron, Infors HAT, Bottmingen, Switzerland; 150 $r{\cdot}min^{-1}$, deflection 25 mm) under the exclusion of light at 24°C for 7 days. Afterwards, the precultures were homogenized, and 10% (v/v) of the homogenate was inoculated for submerged cultivation on a 400 mL (in 1 L Erlenmeyer flasks) scale. After 6 days of growth in submerged culture, the mycelium was harvested by filtration and washed with distilled water. The obtained biomass was freeze-dried (Vaco2, Zirbus Technology, Bad Grund, Germany) and stored at -20°C until usage.

3.5 | Influence of the pH Value on the Biotransformation

BAD lyophilisate (320 mg) was rehydrated in 20 mL potassium phosphate buffer (100 mM) at different pH values ranging from 6.0 to 8.0 (24°C) by stirring at 650 rpm for 10 min. (*E*)-2-Octen-1-ol (**1a**) was added in different concentrations (10 mM and 20 mM), and the reaction mixture was further stirred for 24 h.

Samples were taken after 15 min, 2 h, 4 h, 6 h, 10 h, and 24 h. For analysis, 1 mL of the reaction mixture was taken, the pH was adjusted to 7, and sodium chloride (100 mg) was added to the reaction mixture. 1 mL of pentane containing 1-adamantanol (8 mM) was added as an internal standard, and the resulting mixture was shaken and centrifuged (4000 × g, 2 min, 4°C) to separate the phases. The extraction process was repeated three times. The combined organic phases were dried over Na_2SO_4 and analyzed by GC-MS. Blank transformations were performed with autoclaved lyophilisates as controls. Every experiment was performed in triplicate.

3.6 | Influence of Organic Cosolvents on the Biotransformation

BAD lyophilisate (320 mg) was rehydrated in 18 mL (10% of cosolvent) or 14 mL (30% of cosolvent) potassium phosphate buffer (100 mM, pH = 7) by stirring at 650 rpm at 24°C for 10 min. (*E*)-2-Octen-1-ol (**1a**) was added to the reaction mixture as a solution in 2 mL (10% of cosolvent) or 6 mL (30% of cosolvent) of organic solvent to a final concentration of 10 mM. The reaction mixture was stirred at 24°C and 650 rpm for 8 h. Samples were taken after 15 min, 2 h, 4 h, 6 h, and 8h. The samples were processed as described in section 3.5.

3.7 | General Procedure for Biotransformation

BAD lyophilisate (320 mg) was rehydrated in 18 mL potassium phosphate buffer (100 mM, pH = 7) by stirring at 650 rpm at 24°C for 10 min. The substrate was added to the reaction mixture as a solution in 2 mL of 2-propanol to a final concentration of 10 mM. The reaction mixture was stirred at 24°C and 650 rpm for 4 h. For analysis, 1 mL of the reaction mixture was taken, and sodium chloride (100 mg) and 1 mL of pentane containing 1-adamantanol (8 mM) as an internal standard were added, and the resulting mixture was shaken and centrifuged (4000 × g, 2 min, 4°C) to separate the phases. The extraction was repeated three times. The combined organic phases were dried over Na_2SO_4 and analyzed by GC-MS.

3.8 | Up-Scaled Procedure for Biotransformation

BAD lyophilisate (1.6 g) was rehydrated in 90 mL potassium phosphate buffer (100 mM, pH = 7) by stirring at 650 rpm at 24°C for 20 min. The substrate was added to the reaction mixture to a final concentration of 10 mM as a solution in 10 mL of 2-propanol. The reaction mixture was stirred at 24°C and 650 rpm for 4 h. Sodium chloride (5 g) was added, and the reaction mixture was further stirred for 10 min. The mixture was extracted with pentane (3 × 40 mL), and the suspension was separated by centrifugation. The combined organic phases were dried over Na_2SO_4 and analyzed by GC-MS. After evaporation of the organic phase to dryness, the products were purified by column chromatography on silica gel (eluent – pentane/ether changed gradually: 10/1, 7/3, 1/1) to isolate the major components. The respective fractions were combined, concentrated in vacuum, and ^1H and ^{13}C NMR spectra of the products were compared with those of authentic reference samples.

3.9 | Dess-Martin Oxidation of (*Z*)-2-nonen-1-ol (1i)

To a solution of (*Z*)-2-nonen-1-ol (**1i**) (650 mg, 4.57 mmol) in dichloromethane (100 mL), Dess-Martin periodinane (3.88 g, 9.15 mmol) was added in one portion. The reaction mixture was stirred at RT for 5 h, quenched with 20% aq. Na_2SO_3 (10 mL) and with saturated aq. $NaHCO_3$ (80 mL). The mixture was stirred until the aq. layer was clear. The aq. layer was extracted with DCM (3 × 30 mL). The combined organic phases were dried (Na_2SO_4) and concentrated in vacuum [39].

3.10 | Catalytic Activity of Lyophilisates of *P. sapidus* and *P. eringii*

Lyophilisates of the fungi were prepared according to the procedure described in 2.3. The corresponding lyophilisate (320 mg) was rehydrated in 18 mL potassium phosphate buffer (100 mM, pH = 7) by stirring at 650 rpm at 24°C for 10 min. The substrate was added to the reaction mixture as a solution in 2 mL of 2-propanol to a final concentration of 10 mM. The reaction mixture was stirred at 24°C and 650 rpm for 4 h. For analysis, 1 mL of the reaction mixture was taken, sodium chloride (100 mg) was added, and the reaction mixture was shaken. 1 mL of pentane containing 1-adamantanol (8 mM) as internal standard was added, and the resulting mixture was shaken and centrifuged (4000 × g, 2 min, 4°C) to separate the phases. The extraction was repeated three times. The combined organic phases were dried over Na_2SO_4 and analyzed by GC-MS.

4 | Conclusions

In conclusion, an oxidation protocol for allylic primary alcohols that utilizes fungal lyophilisates as a source of stable oxidative enzymes, aqueous media with 10% isopropanol as a green cosolvent, and atmospheric oxygen as an oxidant was developed. The approach relies on mild conditions and allows obtaining target aldehydes, avoiding overoxidation as well as reduction of activated CC-double bonds. While the retention of configuration was observed for *(E)*-configured alcohols, the (*Z*)-forms were isomerized to the more stable *(E)*-diastereomers. No products of oxidation were observed for the tested non-primary allylic alcohols, namely 1-hepten-3-ol, 1-nonen-3-ol, 1-octyn-3-ol, and cyclohexen-1-ol. The reaction times are short, the workup is simple, and the concentration of the substrate reaches 5 mM of substrate for 1.6 g of lyophilisate. Continued efforts will aim to broaden the substrate scope, including more complex primary alcohols and secondary allylic alcohols with diverse structures. Additionally, exploring immobilization strategies or the reuse of the lyophilisate may enhance the economic and practical value of the method. Given its high selectivity, mild reaction conditions, and environmentally friendly nature, this approach holds promise as a sustainable alternative for industrial

production of flavor and fragrance aldehydes, especially where diastereoselectivity is important.

Author Contributions

Letizia Calza: visualization, investigation, and methodology. **Valeriia Nikitenkova**: visualization, investigation, methodology, and writing – review and editing. **Wendell Albuquerque**: methodology. **Holger Zorn**: resources and writing – review and editing. **Tatyana Zhuk**: conceptualization, methodology, formal analysis, supervision, writing – original draft, and review and editing.

Acknowledgments

Letizia Calza was financed by Erasmus+ EU funds, and Valeriia Nikitenkova was supported by DBU. Tatyana Zhuk gratefully acknowledges financial support from the Philipp Schwartz Initiative.

Open access funding enabled and organized by Projekt DEAL.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and its Supporting Information section.

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