Biotechnological Production of Fungal Colorants

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Für meine Mutter.

Dedicated to my mother.

Zusammenfassung

Da eine globale Transformation in Richtung zirkulärer Bioökonomie auf nachhaltige Prozesse und umweltfreundliche Produkte angewiesen ist, werden in dieser Arbeit Alternativen für aktuell eingesetzte Farbstoffe untersucht. Die biotechnologische Produktion von Farbstoffen aus Pilzen wird als Lösungsstrategie vorgeschlagen.

Zwei verschiedene Spezies wurden auf ihr Potential als Farbstoff-Produzenten untersucht: der Gemeine Schwefelporling (*Laetiporus sulphureus*) und der Zottige Schillerporling (*Inonotus hispidus*). Beide Arten sind bekannt dafür, sowohl im Fruchtkörper als auch im Myzel in flüssiger Nährkultur farbige Moleküle zu synthetisieren. *L. sulphureus* produziert die orangen Laetiporsäuren und *I. hispidus* bildet das gelbe Hispidin, ein bekanntes Antioxidant mit vielen weiteren Bioaktivitäten.

Die Kultivierung beider Spezies wurde in dieser Arbeit vom Schüttelkolben bis zum 4 bzw. 7 L Maßstab im Bioreaktor vergrößert und die Nährmedien optimiert. Durch den Vergleich vier verschiedener *L. sulphureus* Stämme wurde der potenteste Produktionsstamm ausgewählt und erzielte Ausbeuten von rund 1 g/L Laetiporsäure. Bei der Kultivierung von *I. hispidus* wurden verschiedene physikalische und chemische Einflussfaktoren zur Steigerung der Hispidin-Ausbeute getestet. Der Einsatz von Belichtung und oxidativem Stress stimulierte die Farbstoffsynthese, genau wie dem Nährmedium zugesetzte Präkursoren. Alle Effektoren wurden in parallelen Kultivierungen in Bioreaktoren zweier verschiedener Bauarten getestet und eine Ausbeute von 5,5 g/L Hispidin erzielt.

Verschiedene Produktaufarbeitungs-Strategien wurden etabliert, von der klassischen Extraktion bis zum Zwei-Phasen-System. Die zuvor geringe Stabilität der Laetiporsäure-Extrakte konnte durch Stickstoff und Lagerung bei niedrigen Temperaturen stabilisiert werden und das Potential für die Anwendung in Textilien, Kosmetika und Lebensmitteln wurde demonstriert.

Zusammenfassend wurde in dieser Arbeit anhand von zwei Beispielen gezeigt, dass die biotechnologische Produktion von Pilzfarbstoffen möglich ist. Die Maßstabsvergrößerung vom Labor bis zur Pilotanlage wurde berichtet und die Kombination von chemischen und physikalischen Einflussfaktoren sorgten für Ausbeuten im g/L Bereich, die einen entsprechenden Bioprozess wettbewerbsfähig mit konventionellen Prozessen für natürliche Farbstoffe machen.

Schlagworte: Basidiomycota; Bioprozess; natürliche Farbstoffe; *Laetiporus sulphureus*; Laetiporsäure; *Inonotus hispidus*; Hispidin; Bioökonomie

Abstract

Since a global transformation towards circular bioeconomy relies on sustainable processes and environmentally friendly products, alternatives to currently used colorants and dyes are explored in this thesis. The proposed solutions are biotechnologically produced pigments from fungi.

Two different species and their potential to produce colorants were investigated: the sulphur shelf (*Laetiporus sulphureus*) and shaggy bracket (*Inonotus hispidus*). Both species are known to synthesize colorful molecules in their fruiting bodies and in the mycelium grown in liquid culture. *L. sulphureus* synthesizes several orange laetiporic acids and *I. hispidus* produces the yellow hispidin, which is a known antioxidant with several other bioactivities.

In this thesis, the cultivation of both species was scaled up from shake flask to a 4 and 7 L bioreactor and nutrition medium was optimized. By comparing four different *L. sulphureus* strains, the most potent pigment producer was identified and achieved yields of around 1 g/L laetiporic acid. Different physical and chemical influencing factors for improved hispidin yield were tested for the cultivation of *I. hispidus*. Irradiation and oxidative stress stimulated pigment synthesis, as well as supplemented precursors. The effects were combined in a comparison of two different types of bioreactors and a yield of 5.5 g/L hispidin was achieved.

Different product recovery strategies were investigated, from conventional extraction to a biphasic system. The prior poor stability of laetiporic acid extracts was improved by addition of nitrogen and storage at low temperatures. The potential for application in textiles, cosmetics and food was demonstrated.

In conclusion, the biotechnological production of fungal colorants was demonstrated using two examples in this thesis. Upscaling from laboratory to pilot scale was reported and optimization of chemical and physical parameters resulted in g/L-scale product titers, which render bioprocesses competitive to conventional production processes for natural colorants.

Keywords: Basidiomycota; Bioprocess; Natural Colorants; *Laetiporus sulphureus*; Laetiporic acid; *Inonotus hispidus*; Hispidin; Bioeconomy

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List of Abbreviations

Abbreviation	Meaning
APCI	atmospheric pressure chemical ionization
abs.	absorbance
approx.	approximately
BM	biomass
BMBF	German Federal Ministry of Education and Research (dt. Bundesministerium für Bildung und Forschung)
BTM	dry matter (dt. Biotrockenmasse)
^{14}C	radioactive isotope of carbon, used for radiocarbon dating
°C	degree Celsius
CaA	caffeic acid
$CaCl_2$	calcium chloride
CiA	cinnamic acid
CIE Lab	color space according to the International Commission on Illumination
C/N ratio	carbon-to-nitrogen ratio
$CO_{2,L}$	oxygen concentration [%]
$CO_{2,L}^*$	oxygen saturation concentration [%]
CuA	*p*-coumaric acid
$CuSO_4$	copper(II) sulfate
cf.	compare (lat. *confer*)
D	diameter [m]
DAD	diode array detector
DCM	dichloromethane
demin. water	demineralized water
dest. H_2O	distilled water
DO	dissolved oxygen
DSMO	dimethyl sulfoxide
DSMZ	German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig/DE (dt. Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)
ΔE	color difference between two colors [-]

EDTA	ethylenediaminetetraacetic acid
e.g.	for example (lat. *exempli gratia*)
ESI-MS	electrospray ionization - mass spectrometry
EtOH	ethanol
$FeCl_3$	iron(III) chloride
× g	times gravity, relative centrifugal force
Glu	glucose
GMO	genetically modified organism
GRAS	generally recognized as safe
H/D ratio	height to diameter ratio
HPLC	high performance liquid chromatography
H_2O_2	hydrogen peroxide
ITS	internal transcribed spacer (sequencing)
kDa	kilo Dalton
KH_2PO_4	potassium dihydrogen phosphate
K_2HPO_4	potassium hydrogen phosphate
k_La	oxygen transfer coefficient [h^{-1}]
LC-MS	liquid chromatography - mass spectrometry
LED	light-emitting diode
LSU	*Laetiporus sulphureus*
M	[mol/L]
Mb	mega bases
MEK	methyl ethyl ketone
MeOH	methanol
$MgSO_4$	magnesium sulfate
mM	[mmol/L]
$MnSO_4$	manganese(II) sulfate
m/z	mass-to-charge ratio
N	stirrer speed [rpm] [1/min]
n	number of stirring blades [-]; number of experiments [-]
N_2	nitrogen
$NaNO_3$	sodium nitrate
NaOH	sodium hydroxide

NH_4NO_3	ammonium nitrate
NL/min	normal liters per minute; normalized air flow
Np	stirrer factor 6 for Rushton turbine [-]
OD	optical density
O/N	over night
PAHs	polycyclic aromatic hydrocarbons
PAL	phenylalanine ammonia-lyase
PCBs	polychlorinated biphenyls
PDB	potato dextrose broth
P	energy input [W]
pH	negative decimal logarithm of the proton concentration
Phe	L-phenylalanine
pO_2	partial pressure of oxygen
P_V	volume-related energy input [W/m^3]
R^2	coefficient of determination
Ref.	reference
rel.	relative
Ret.	retention time [min]
ROS	reactive oxygen species
RP	reversed phase
rpm	revolution per minute
RT	room temperature
SD	standard deviation
SmF	submerged fermentation
SNL	standard nutrient liquid
sp.	species
STR	stirred tank bioreactor
temp.	temperature
TN	total nitrogen
TOC	total organic carbon
TU	Technical University
Tyr	L-tyrosine
UV	ultraviolet light

V_{reactor}	working volume of the reactor [m^3]
v_{Tip}	tip speed [m/s]
v/v	volume concentration (volume per volume) [%]
vvm	unit of the aeration rate; volume of air per volume of medium per time
wave	rocking motion bioreactor
w/o	without
WOF	weight of fiber [%]
w/v	mass concentration (weight per volume) [%]
w/w	mass fraction (weight per weight) [%]
ΔX	biomass yield [g/L]
$ZnSO_4$	zinc sulfate
ε	molar extinction coefficient [L/mol·cm]
λ	wavelength of light [nm]
λ_{max}	wavelength with the maximal absorbance of a substance [nm]
μ	growth rate [d^{-1}]
ρ_{Fluid}	density of the medium [kg/m^3]

1 Introduction

In this chapter, the theoretical background relevant for this thesis is summarized. An introduction into Basidiomycota and their characteristics is given, as well as their biotechnological challenges. Fungal colorants and their induction as secondary metabolites are presented briefly and put into context with the state of the art.

1.1 Status Quo

Colors are essential to our daily lives as they influence our emotions, moods, and behavior. They create psychological and physiological expectations and are therefore one of the most important factors in the decision-making process [1]. Facing the progressing climate change and an increasing world population, we need to reflect on the sustainability of colorants. After the industrialization, chemically synthesized colorants were produced in large quantities in Germany (*e.g.* Agfa and Farbwerke Hoechst). Even BASF, today's largest chemical company, started its business with the production of colorants. The global colorants market is predicted to reach 79 billion $ in 2027 [2]. Among them are textile dyes, with a forecast market size of 10 billion $ by 2026 [3].

Most of our textiles are dyed with petrochemically synthesized colorants, mainly azo dyes that pollute the environment during and after their application. One of many examples is the report of Umbuzeiro *et al*., who found the mutagenic activity of a river in Brazil to be a direct consequence of the local textile processing plant that released azo dye containing wastewater [4]. Its treatment with chlorine in the drinking water plant led to colorless but even more harmful compounds, and a population of 60,000 was exposed to the contaminated water [5,6]. However, the uncontested stability, wide range of hues, availability and price of these synthetic dyes make them hard to replace in the textile industry. Nowadays, there is growing interest addressing the degradation of synthetic dyes with *e.g.* microbial enzymes, to reduce the environmental impact of the textile industry [7].

In the European food sector, the concerns regarding artificial colorants have almost eliminated them from the market. The food industry responded to people's awareness after the "Southampton study" that was published in 2007 [8], accused artificial dyes to affect children's attention. Azo dyes were widely replaced with plant-based colorants, although their field of application is often limited due to stability issues regarding light, heat and/or acidity. Those colorants, perceived as natural and sustainable alternatives, are heavily

dependent on the climate, can only be harvested seasonally, and their production competes directly with feed and food supply. Trying to overcome this dilemma, microbial pigment production has gained interest in recent years, resulting in several publications [9–12].

While all kinds of microorganisms from algae to bacteria can be considered for fermentation purposes, fungi are a widely underestimated kingdom for the exploration of new colorants. Most of the literature on fungal pigments deals with filamentous fungi belonging to the division of Ascomycota [13–15], including *Chlorociboria aeruginascens* (*cf.* chapter 3). Some ascomycetous pigments have already made their way to the market with *e.g.* large-scale fermentation of *Blakeslea trispora* for *ß*-carotene production. The production process was approved by the European Union and the product permitted as food colorant E 160 a (iii) or CI Food Orange 5 [16]. Another, more recent example for the commercialization efforts is *Talaromyces atroroseus*, which produces monascus-like pigments without the accompanying monascus-toxins [17]. The process is currently being scaled up by the Danish company Chromologics [18].

However, what about the mushroom-forming (and sometimes edible) fungi? Their colorful compounds have been subject to research for a long time and many reviews describe chemical structures and biosynthesis in fruiting bodies [19–22]. The palette includes various pigments that are not produced or used industrially yet. As neither the extensive harvest of naturally growing, nor the cultivation of fruiting bodies is conductive for a sustainable large-scale production of new colorants, this thesis focusses on the biotechnological perspective.

1.2 Basidiomycota

The division of Basidiomycota is the second largest within the kingdom of fungi, next to the division of Ascomycota. Together with the Entorrhizomycetes, they represent the subkingdom of "higher fungi", the Dikarya. The division of Basidiomycota can further be split into the subphyla Agaricomycotina, Pucciniomycotina, Ustilaginomycotina, Wallemiomycotina and Basidiomycota *incertae sedis*, with the latter not assigned yet [23–25]. They all have in common that attributed families are ecologically and taxonomically diverse and colonize all terrestrial ecosystems. Their unique ability to decompose wood, degrading the xylem cell wall components including cellulose, hemicellulose and lignin, makes Basidiomycetes key players in the global carbon cycle [26]. Different estimations reckon that approx. 950 of the more than 30,000 representatives are edible mushrooms.

Basidiomycota exhibit an average genome size of 47 Mb (Ascomycota 37 Mb) that comes with a vast metabolic diversity [27,28]. They are known to have a complex secretome and highly active secondary metabolic pathways that enable production of a wealth of bioactive compounds [21,29–31]. Exceptional (and distinguished from other taxonomic divisions) is their ability to form large subterrestrial networks of mycelium, which, under certain circumstances, break through the soil, or bark of a tree to form the sexually reproductive fruiting bodies (**Figure 1**). The life cycle of a generic basidiomycetous fungus begins with the germination of a spore, which gives rise to monokaryotic mycelium. The mycelium grows and eventually fuses with another compatible mycelium, forming a dikaryotic mycelium. The dikaryotic mycelium grows and differentiates into primordia, which are small, mushroom-like structures that develop into the mature fruiting body. The mature fruiting body produces and releases spores from the basidia, which are dispersed by wind or other means, and can then germinate and give rise to new monokaryotic mycelia.

For the biotechnological production of target compounds, the outlasting form of the fungus, the mycelium, is of most interest. Under sterile laboratory conditions, the mycelium of many basidiomycetous species can grow on solid or liquid substrates; meaning emerged on agar plates or in a solid-state fermentation [32] or submerged in nutrition medium in shake flasks or bioreactor cultivations (**Figure 2**).

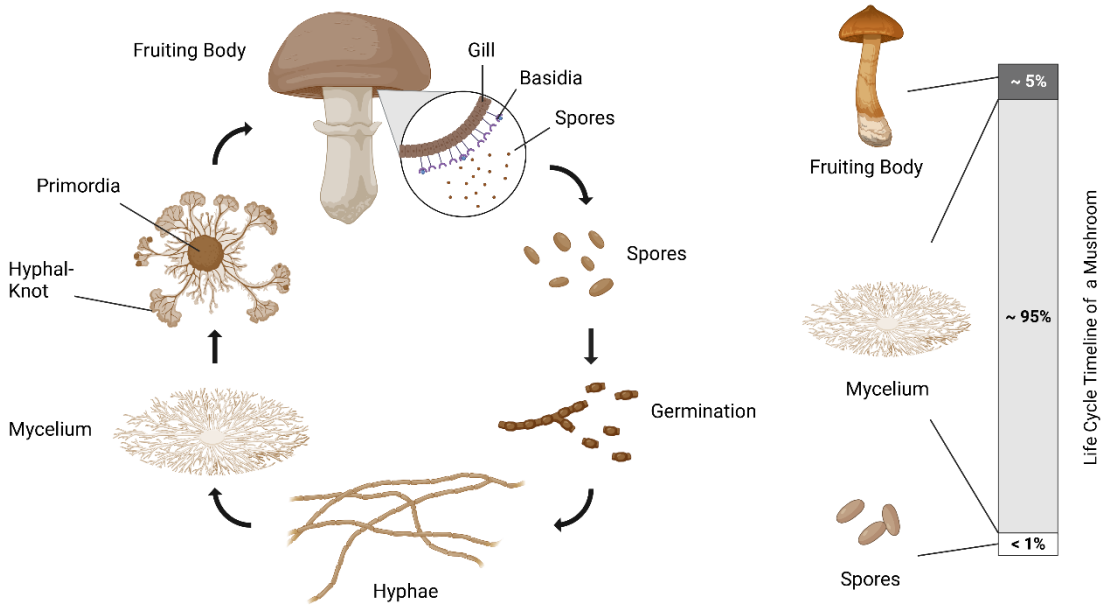


Figure 1. Schematic life cycle of a generic basidiomycetous fungus and timeframe of different development stages.[1]

(**a**) (**b**) (**c**) (**d**) (**e**)

Figure 2. Pictures of *Laetiporus sulphureus* (**a**) fruiting body, (**b**) mycelium on agar plate, (**c**) mycelium in a 250 mL shake flask culture, (**d**) mycelium in a 4 L stirred-tank bioreactor and (**e**) mycelium in a 4 L cell-culture bag cultivation.

[1] Modified after Soe, Z. (2023). Life Cycle of Mushroom, Biorender.com

1.2.1 Cultivation of Basidiomycota

Serving as a freely available food source or being used as traditional medicine, the fruiting bodies of Basidiomycota have always been harvested from nature. By creating the ideal growing conditions in a solid-state fermentation, the fruiting bodies of many mushrooms can be harvested under artificial conditions as well [33]. As demonstrated in **Figure 1**, the lifespan of the fruiting body is relatively short, compared to the outlasting mycelium-form, making the production of target compounds or fungal-based materials on an industrial large-scale suboptimal.

The interest in fermentation of mycelium has grown in recent years, along with the awareness of the urgent and necessary transition towards a circular bioeconomy. As Meyer *et al*. (2020) pointed out, there are many potential fields of application for fungal biotechnology [34] and thus a new market for fungal products emerged. Start-ups like Mushlabs or Kynda are following the trend of investigating alternatives for animal-based products by producing basidiomycetous biomass as meat substitutes [35,36]; MycoWorks, Bolt Threads and Zvnder are producing leather-like material from mycelium [37–39]; NEFFA is producing a mycelium-based material for fashion, and Ecovative produces amongst others compostable packaging from hemp hurd and mycelium [40,41].

For other aims than biomass production, the submerged cultivation of Basidiomycota as a whole-cell production organism is published in scientific literature for many target compounds (*e.g.* aroma molecules [42–45] and active pharmaceutical ingredients [46–48]) but is still considered as a niche in the industrial context. There are only a few companies specialized in the large-scale production, with one example being ASA Spezialenzyme, who are producing the enzyme Laccase from *Agaricus bisporus* and *Trametes* sp. [49] as a result of previous collaboration with the Institute of Food Chemistry, Leibniz University Hannover.

1.2.2 Biotechnological Challenges in the Cultivation of Basidiomycota

There are many reasons why the vast metabolic potential of Basidiomycota remained or remains biotechnologically untouched. To name a few, their submerged cultivation can be difficult, as hyphae cling to built-in components of bioreactors. The growth of basidiomycetous strains is relatively slow, compared to bacterial growth. Furthermore, the desired secondary metabolites (like pigments) that are synthesized in the fruiting body are not necessarily synthesized in the non-differentiated mycelium in submerged culture. It is not astonishing that fungi cultivated in liquid nutrition media and samples from nature vary in their compound constitution. As Berger *et al*. pointed out, cell growth under different physical and chemical conditions results in differences in gene expression and hence morphological differences [50]. Transferring the strains into liquid media often results in different profiles of secondary metabolites or no growth at all.

To obtain the desired compounds, the inducing factor for secondary metabolite synthesis needs to be identified (*cf.* chapter 1.3.2). Microorganisms can further be altered by traditional chemical or physical mutagenesis (*e.g.* radiation) or nowadays, genetic engineering. Kirchgaessner *et al.* recently reported a genetic tool to tap new basidiomycetous secondary metabolites, whose encoding genes are often too long for conventional heterologous expression [51]. In this thesis, only wild type strains were investigated, as GMOs (genetically modified organisms) are still a controversial topic in our society; however, strain engineering and heterologous expression might be necessary to achieve yields that are economically viable and therefore, competitive with petrochemical synthesis. Other strategies to improve growth and product yields include comparing the productivity of different strains as presented in chapter 4 or strain improvement by the creation of monokaryons, as reported by Krahe *et al*. [52].

For the implementation of a bioprocess as indicated in **Figure 3** (see also **Figure 2d** and **Figure 2e)**, the strain specific preferences for optimal growth and product formation need to be elucidated. Factors to be investigated include the pH and temperature optimum, agitation and aeration levels, and C/N sources and ratio. As mentioned, the construction of bioreactors needs to be considered, as built-in components can be overgrown by filamentous organisms. If the biosynthesis is light-dependent, the reactor needs to be transparent or lights need to be installed, which adds further challenges for the up-scaling of processes. If the cultivated microorganisms are agitation sensitive, they can be rocked instead of stirred. As Jonczyk *et al*. showed, different bioreactor types can deliver different results. They found the shear stress

sensitive basidiomycete *Flammulina velutipes* to grow better and exhibit a higher enzymatic activity in the rocking motion bioreactor [53]. For hispidin production with *Inonotus hispidus*, no difference was found, when cultivating the strain in a stirred tank or the rocking motion bioreactor [54].

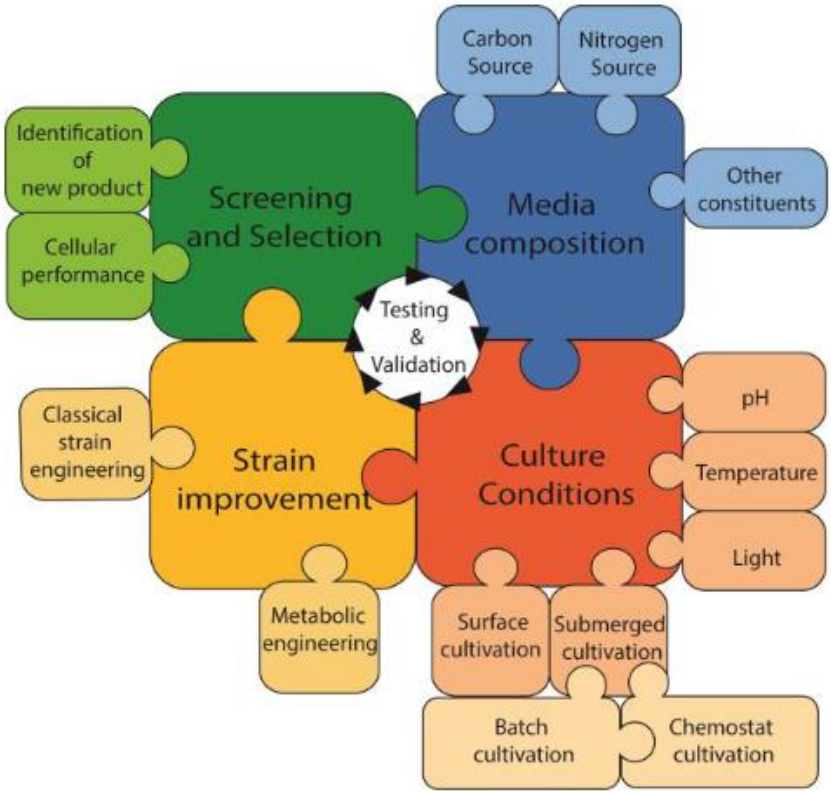


Figure 3. Schematic overview of factors that need to be considered, when implementing a bioprocess. Replicated from [55].

1.2.3 Sustainable Production Processes with Basidiomycota

Regarding the sustainability of bioprocesses, the nutrition media used for cultivation is problematic. Sugars (C-source) and other components are mainly derived from agricultural products and thus compete with food production. Agro-industrial by-products or side-streams could be established as a green alternative [56]. Side-streams like peels, straws, press cakes, bagasse, whey and cooking oil occur in large volumes often ending up as waste and are partly already discussed for colorant production [50,56,57]. In contrast to chemically defined nutrition medium, the chemical composition of these substrates varies naturally. In addition, they normally need to be pretreated with alkali, acid and/or heat before fermentation, as most microorganisms cannot degrade them directly due to missing enzymes. In this regard, Basidiomycota are exceptionally suited, as they can grow on lignocellulose-rich waste without pretreatment by producing degradative enzymes for lignin and/or hemicellulose. The mild reaction conditions for a biocatalytic degradation come along with lower costs, which is advantageous for basidiomycetous bioprocesses [58,59].

The term "downstream process" refers to the recovery and purification steps of the multicomponent fermentation broth, essential for achieving high product concentration and purity. It plays a crucial role in determining the overall production costs of the final product and can account for a significant portion of the total production costs, mainly due to low product concentration and high energy demands for recovery. Therefore, selecting an appropriate downstream process is of great economic interest [60]. Different strategies can be applied after the cultivation, to efficiently recover and purify the desired products, *e.g.* filtration, centrifugation or chromatography, depending on the specific application.

In situ product recovery is a downstream process strategy that can be applied to integrate product recovery into the bioreactor itself by two or more phase partitions. This strategy aims to facilitate recovery by liquid-liquid extraction, and can, in some cases, overcome end-product inhibition. Different techniques for *in situ* product recovery have been explored, including classical and hybrid techniques, which offer advantages such as improved process integration, reduced downstream operations, and cost savings [61].

1.3 Colorants

Colorants are substances that are used to add color to various materials such as food, cosmetics, textiles, and plastics. They can be naturally derived from plants, animals or microorganisms, or synthesized chemically.

In food, colorants are used to enhance the appearance of the product and make it more appealing to consumers. They are also used to compensate for color loss that occurs during processing and storage. As mentioned in chapter 1.1, the use of synthetic colorants in food has been a topic of debate due to their potential health risks. In cosmetics, colorants have a decorative focus and are used to add color to products such as eye shadows, lipsticks, and nail polishes. They are also used in hair dyes to change the color of hair. However, some synthetic colorants used in cosmetics have been found to cause skin irritation and allergic reactions. Textile colorants are used to dye fabrics and create a wide range of colors and patterns. Natural colorants such as indigo and madder have been used for centuries to dye fabrics. Synthetic colorants are also widely used in the textile industry due to their ease of use and availability. So, colorants are an important part of our daily lives, adding color and vibrancy to the products we use.

The terms “pigment” and “colorant” are technically distinguished by their solubility, with pigments being insoluble and colorants or dyes being soluble. In the biological context, no such terminological difference is made and the words are used synonymous.

Pigments appear colorful, as they absorb light from the electromagnetic spectrum between 400 and 800 nm (**Figure 4**). The light that is not absorbed is reflected and visible for the human eye, resulting in the observing of the complementary color. Molecules that are able to absorb visible light exhibit a system of conjugated double bonds that result in delocalized π-electrons.

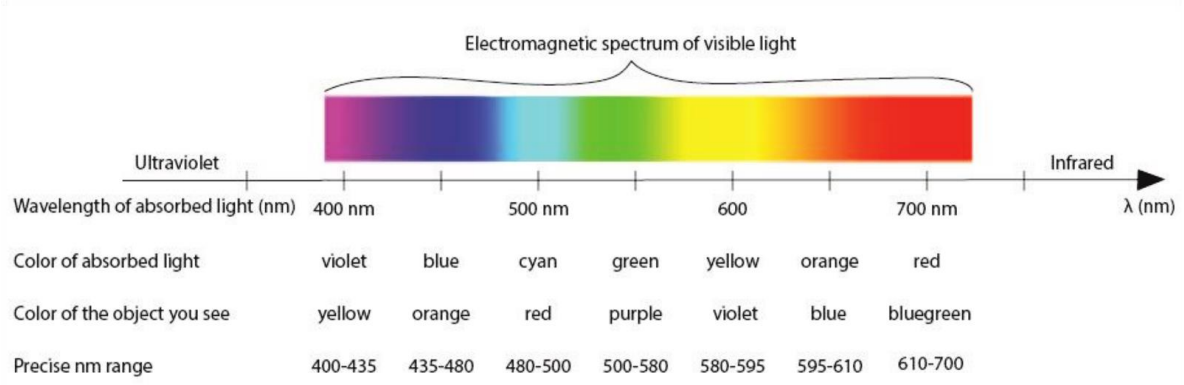


Figure 4. The electromagnetic spectrum of visible light, including the range of wavelengths absorbed by an object and the color of this object observed. Replicated from [55].

1.3.1 Fungal Pigments

Many fungi are able to synthesize compounds with large conjugated systems, chromophores that make them appear colorful (**Figure 5**). Their coloration is an essential characteristic used for identification of the species. Fungal pigments are mainly classified after their chemical structure and biosynthetic pathway. The working group around Wolfgang Steglich is to be mentioned here, who pioneered the structure determination for many basidiomycetous colorants [19,22].

Pigments derived from the shikimate pathway (from aromatic amino acids like phenylalanine and tyrosine) include terphenylquinones like polyporic acid, atromentin and, as reported in this thesis, hispidin from *Inonotus hispidus* (**Figure 6b**). Pigments from the malonate pathway comprise the well-known anthraquinones from *Cortinarius* sp., polyketides like the laetiporic acids from *Laetiporus sulphureus* (**Figure 6a**) or the unique octaketide xylindein from *Chlorociboria aeruginascens* (*cf.* chapter 3). Other classes are pigments derived *via* the mevalonate/isoprenoid pathway (sesquiterpenoids and carotenoids) and pigments containing nitrogen like indole derivates (*e.g.* melanin).

The fruiting bodies of many basidiomycetes have been used by mushroom-enthusiasts to dye fabrics on a small artisanal scale [62–64], but there have been no approaches to transfer this knowledge to an (impactful) industrial scale yet.

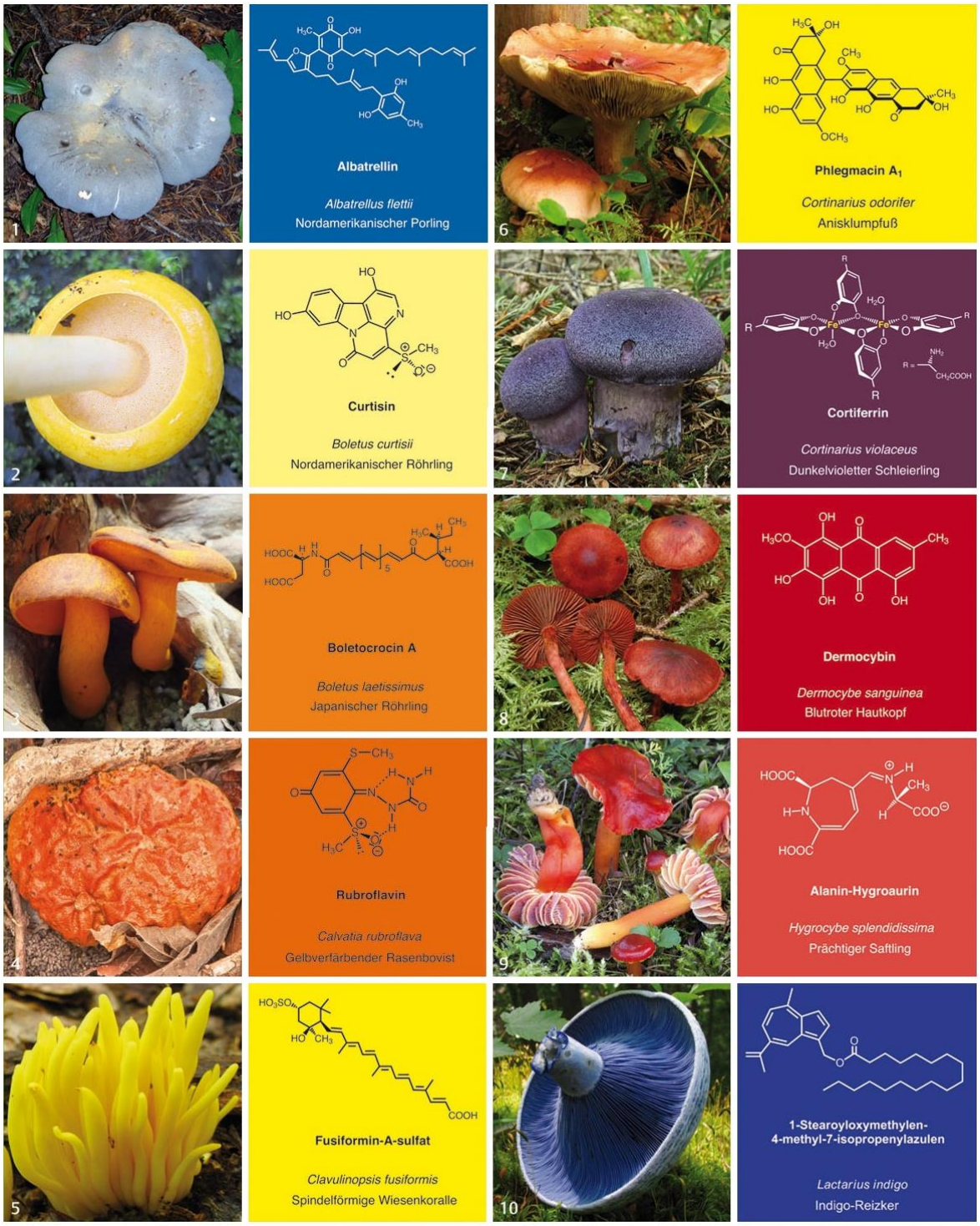


Figure 5. The variety of fungal pigments in fruiting bodies.1) *Albatrellus flettii* with the chemical structure of Albatrellin, 2) *Boletus curtisii* with Curtisin, 3) *Boletus laetissimus* with Boletocrocin A, 4) *Calvatia rubroflava* with Rubroflavin, 5) *Clavulinopsis fusiformis* with Fusiformin-A-sulfat, 6) *Cortinarius odorifer* with Phlegmacin A_1, 7) *Cortinarius violaceus* with Cortiferrin, 8) *Dermocybe sanguinea* with Dermocybin, 9) *Hygrocybe splendidissima* with Alanin-Hygroaurin, 10) *Lactarius indigo* with 1-Stearoyloxymethylen-4-methyl-7-isopropenylazulen. Replicated from [22].

Figure 5 (continued). The variety of fungal pigments in fruiting bodies. 11) *Lactarius lilacinus* with the chemical structure of Lilacinon, 12) *Lactarius necator* with Necatoron, 13) *Leccinum crocipodium* with Crocipodin, 14) *Leucocoprinus birnbaumii* with Birnbaumin B, 15) *Mycena haematopus* with Haematopodin, 16) *Russula flavida* with Russulaflavidin, 17) *Retiboletus ornatipes* with Retipolid A, 18) *Stephanospora caroticolor* with Stephanosporin, 19) *Xerocomus badius* with Badion A Kaliumkomplex, 20) *Suillus tridentinus* with Tridentochinon. Replicated from [22].

1.3.2 Induction of the Secondary Metabolite Synthesis

Immobile organisms like fungi need another strategy than flight to counter enemies and competitors. Different compounds, among them also colorful ones, are synthesized as a chemical defense mechanism in the fruiting body [65,66]. These secondary metabolites are not necessarily produced by the mycelium in nature or in a completely different environment, under aquatic conditions. Nevertheless, the submerged production of pigments can be induced by different stressors.

A well-known physical induction mechanism is light, as many pigments serve as photon scavengers and thus as UV protection [67,68]. A contribution to photo-activated defense has recently been discussed as well [69,70]. The influence of light of different wavelengths on mycelium and coloration has been investigated in different species, among them *Inonotus* sp. [71–73] and *Terana caerulea* [74]. Other physical elicitors comprise thermal and mechanical stress (wound-related pigment formation) [75–77]. Brandt *et al*., for instance, reported a polyketide synthase that was upregulated upon injury of the mycelium [78].

Chemical stressors have been discussed for ascomycetous pigment production (*e.g. Monascus ruber* and sodium octanoate [79]), however there are only a few reports on basidiomycetous pigment production. One of those examples is the addition of hydrogen peroxide, which has been investigated in submerged *Inonotus* cultures [54,80]. Moreover, color synthesis can be induced biologically as shown for *Serpula lacrymans* upon co-cultivation. Certain metabolites of *Bacillus subtilis* were identified as inducers of pulvinic acid pigment formation in this strain [81–83]. Understanding the metabolic pathways and elicitors for gene expression is key for a biotechnological production of fungal colorants.

1.3.3 Biosynthesis of Laetiporic acid and Hispidin

This thesis deals with the pigments produced in the fruiting bodies of *Laetiporus sulphureus* and *Inonotus hispidus* in particular (**Figure 6**). In the following chapters, the synthesis of the target compounds laetiporic acid and hispidin in submerged cultivated mycelium is reported and discussed.

Hispidin is synthesized as secondary metabolite by certain fungi, including species of the genus *Inonotus*. The biosynthesis involves the phenylpropanoid pathway, which is a metabolic sequence starting from L-phenylalanine and leading to phenylpropanoid secondary products. The first step in this pathway is the enzyme phenylalanine ammonia-lyase (PAL), which catalyzes the deamination of phenylalanine to cinnamic acid [84].

The biosynthesis of laetiporic acid specifically involves a highly reducing polyketide synthase enzyme, identified and named LpaA by the working group around Dirk Hoffmeister. This enzyme consists of eight domains and produces different laetiporic acids (A, B, C, D) with a methylbranched C_{26}-C_{32} main chain. The function of LpaA as a multi-chain length polyene synthase has been verified through heterologous pathway reconstitution in *Aspergillus nidulans* and *Aspergillus niger*. The reconstitution experiments confirmed that LpaA is the sole enzyme necessary for laetiporic acid biosynthesis [85].

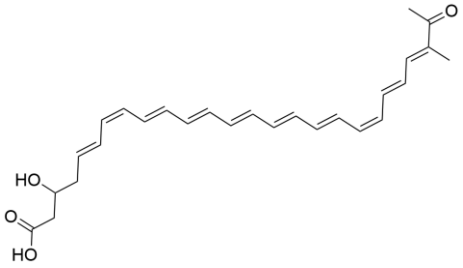
Hispidin, is a known precursor of luciferin in fungal bioluminescence. Different possible building mechanisms are described and illustrated in **Figure 7**. The biosynthesis of hispidin involves the conversion of caffeic acid through the styrylpyrone pathway. The hydrolysis of oxyluciferin in bioluminescence produces caffeic acid, which can be recycled in the biosynthesis of hispidin [86]. The discovery of the biosynthetic pathway for hispidin and luciferin in fungi is described to have significant implications for synthetic biology and the development of autonomously glowing organisms [87].

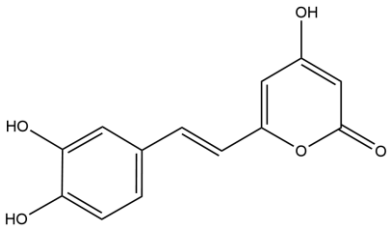
Light is an essential factor for hispidin biosynthesis in *I. hispidus.* It was shown to initiate a sequential increase in the activity of enzymes involved in production of styrylpyrones; including cinnamate and *p*-coumarate hydroxylase activity as well as tyrosine ammonia-lyase and aminotransferase activity for phenylalanine [73,88].

L. sulphureus *I. hispidus*

[2]

[3]





(**a**) (**b**)

Figure 6. The fungal species dicussed in this thesis and their pigments. (**a**) *Laetiporus sulphureus* with its main pigment laetiporic acid A and (**b**) *Inonotus hispidus* with the yellow hispidin.

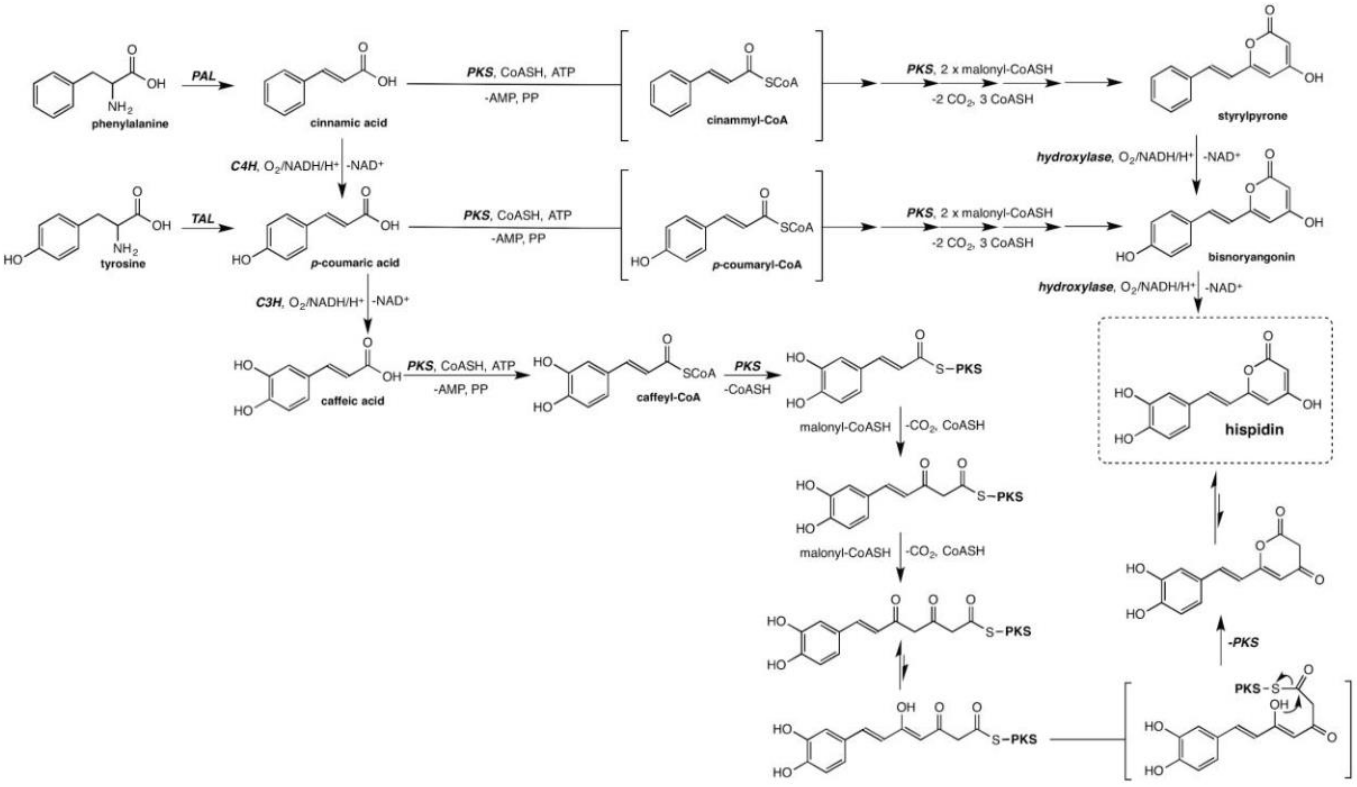


Figure 7. Proposed biosynthesis of hispidin. Scheme replicated from [87], adapted from [89].

[2] Lee Collins (2006), https://commons.wikimedia.org/wiki/File:Laetiporus_sulphureus_1.jpg (last accessed 22nd August 2023)

[3] Rob Hille (2008), https://commons.wikimedia.org/wiki/File:Afbeelding_187.jpg (last accessed 27th April 2023)

2 Aim of This Thesis

The aim of this thesis was to gain a better understanding of the cultivation and secondary product formation of submerged cultivated Basidiomycota. As target compounds, the biosynthesis of colorants was investigated in two different species, *Inonotus hispidus* and *Laetiporus sulphureus*. *Chlorociboria aeruginascens*, an Ascomycete producing the blueish pigment xylindein is also included in chapter 3, however all the credit for this research belongs to my project partners at the Technical University Dresden.

Different strategies were tested to improve productivity of the cultures to economically attractive levels: the induction of secondary metabolite synthesis was investigated by applying different elicitors and stressors like light and oxidative stress. Precursors of the desired substances were supplemented to exclude bottlenecks in the biosynthetic pathway and improve the yield. The species were cultivated in different bioreactors to investigate the influence of shear stress on growth and product formation. Different extraction procedures and product recovery strategies were tested to design a feasible bioprocess. Moreover, practical dyeing trials were conducted to assess color intensity, stability and applicability. Ultimately, the aim was to explore and exploit the merely untouched metabolic potential of Basidiomycota as microbial cell factories to extend the scope of industrial biotechnology.

3 Production of Natural Colorants by Liquid Fermentation with *Chlorociboria aeruginascens* and *Laetiporus sulphureus* and Prospective Applications

3.1 Preamble to the Publication

In this article, we shed light on *Chlorociboria aeruginascens*, a wood-decaying Ascomycete that is commonly known as the green-stain fungus. It is found in temperate regions worldwide and is commonly associated with hardwood trees, such as oak, beech and maple [90]. The fungus has been studied for its potential use in bioremediation and for its production of the blue-green pigment xylindein, which can be applied in veneer or in organic semiconductors [91]. The pigment has been found to have a superior stability compared to other pigments like indigo [92]. The growth and pigmentation of *C. aeruginascens* were influenced by various environmental factors, including temperature, pH value, oxygen level, light intensity and the availability of organic nitrogen sources [93]. This publication reports the upscaling of the bioprocess for xylindein production.

Laetiporus sulphureus is a basidiomycetous fungus that is edible at a young stage and occurs worldwide [94]. It is a saprophyte and grows on deciduous trees [95]. The chemical composition of *L. sulphureus* fruiting bodies has been studied and compounds exhibiting antimicrobial, antifungal and anti-inflammatory properties have been found [85,95–97]. *L. sulphureus* forms linear polyene pigments that have been identified in both fruiting bodies and liquid cultures [85,98]. In this article, we report the first ever biotechnological upscaling approaches.

This article is a joint work of the Institute of Natural Materials Technology, Technical University Dresden (Marlen Zschätzsch, Susanne Steudler, Olena Reinhardt, Stephanie Stange, André Wagenführ, Thomas Walther and Anett Werner) and the Institute of Food Chemistry, Leibniz University Hannover (Pia Bergmann, Franziska Ersoy and Ralf G. Berger). The reported data dealing with *Chlorociboria aeruginascens* (*cf.* chapters 3.5.1, 3.5.2 and 3.5.3) originates solely from the colleagues at TU Dresden and is therefore not considered in further chapters of this thesis.

I contributed to the article through identification of the strain of *L. sulphureus* with the highest pigment production (DSMZ 11211, *cf.* chapter 4). I investigated the pigment to biomass ratio in standard nutrient liquid and Moser b medium (*cf.* chapters 3.5.4 and 3.7.1) and confirmed

the extraction of different laetiporic acids via LC-MS (*cf.* chapters 3.5.5, 3.7.4 and **Supporting Figure 4**).

The article was published in the peer-reviewed journal *Engineering in Life Sciences* on 26th of January 2021 as part of the special issue dedicated to Prof. Thomas Bley on the occasion of his 70th birthday and can be accessed online via https://doi.org/10.1002/elsc.202000079.

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RESEARCH ARTICLE
Engineering in Life Sciences

Production of natural colorants by liquid fermentation with *Chlorociboria aeruginascens* and *Laetiporus sulphureus* and prospective applications

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3.2 Abstract

The replacement of potentially hazardous synthetic dyes with natural dyes and pigments are of great interest for a sustainable economy. To achieve cost-effective, environmentally friendly, and competitive products, improvements in cultivation, extraction and dyeing processes for pigment producing organisms are necessary. In our study, we were able to scale up the production of xylindein by *Chlorociboria aeruginascens* from 3 L to 70 L bioreactor cultivations. We have identified important bioprocess parameters like low shear stress (150 rpm, tip speed < 0.5 m/s) for optimal pigment yield (4.8 mg/L/d). Additionally, we have demonstrated the potential of laetiporic acid production by *Laetiporus sulphureus* in various cultivation systems and media, achieving dried biomass concentrations of almost 10 g/L with a 7 L bioreactor cultivation after 17 days. Extractions performed at 70 °C and 15 min incubation time showed optimal results. To the best of our knowledge, we have described for the first time the use of this pigment in silk dyeing, which results in a brilliant hue that cannot easily be produced by natural pigments.

Keywords: *Chlorociboria aeruginascens*, xylindein, *Laetiporus sulphureus*, laetiporic acid, natural dye

3.3 Introduction

Colors play a special role in our everyday life. Already in the middle of the 19th century the synthetic dye industry emerged [99]. Further advances in chemical synthesis replaced previously used colors made from natural raw materials. Synthetic dyes have a petrochemical origin and certain azo dyes have been shown to pose harmful health effects. Even more

alarming is that their production and use often cause environmental hazards around the textile industry through the release of toxic substances, especially in developing countries. In order to improve environmental conditions and provide the prerequisites for a circular bio-economy, research is needed to advance the industrial production of natural dyes [100].

Various natural sources have been used for dyeing before the synthetic era. For example, inorganic mineral pigments like ultramarine and malachite exist next to dyes from biological origin. Main sources are plants, algae, microbes, and animals like cochineal. Well known examples are alizarin from madder plant, phycocyanin from cyanobacteria, and red pigments from *Monascus* species (Ascomycota) [14,101]. Although natural dyes have considerable ecological advantages over synthetic dyes, their industrial production has remained difficult until today, mainly due to low yields [102]. There are also disadvantages in the production of colors from plants, such as seasonal fluctuations or the conflict with the food industry, especially with regard to the growing area.

The group of fungi holds great potential to exploit their natural dyes or pigments, which represent an interesting alternative to other natural sources [103]. These pigments exhibit several key properties, for example colorfastness [100] and ecological production using agricultural by-products, which contributes to a circular bioeconomy. Additionally, fungal dyes and pigments as secondary metabolites have an immense chemical variation. Besides the color appearance, they can exhibit antibacterial, anticarcinogenic, insecticidal, fungicidal (mycotoxins) or antioxidant properties [85,104]. However, the fungal potential as high value metabolite producer has hardly been used so far.

Fungal species capable of producing pigments are divers and comprise Ascomycota and Basidiomycota. Two examples are xylindein producing *Chlorociboria aeruginascens* and the pigment family of laetiporic acids produced by *Laetiporus sulphureus*. Xylindein can be found in historical intarsia work. Since the 15th century wood colored by *Chlorociboria* sp. has been used for these artworks [105,106]. Recently, especially the group around Sara C. Robinson [107,108] investigated the production of xylindein by cultivating *Chlorociboria* sp. to produce biomass and thereby achieved high xylindein yields [109]. Next to solid state fermentation, addition of wood substrates to agar plates or liquid cultures using malt extract medium have been used for cultivation [110–112]. Our previous studies identified orange juice in liquid cultures as a suitable substrate for xylindein production [113]. Furthermore, we could show that xylindein formation is triggered by nitrogen limitation [113]. Organic solvents such as dichloromethane (DCM) or chloroform can be used for pigment extraction

[93,114]. The optimization of xylindein extraction might allow to further advance the natural dyeing of textiles, which has already been shown for several materials, for example, cotton, wool, and polyamide [108,115]. Application of xylindein as paint color was accomplished using DCM extracts and native oils, especially raw linen oil, have been identified as suitable carriers for xylindein [116]. However, a successful transfer to paints was not yet possible [117]. Xylindein is not only promising for mycological wood discoloration and textile dyeing, but also as a fluorescent marker or an organic semiconductor [91,109,118]. Of particular interest are also the approaches of Maeda *et al*. [119] to use xylindein for medical and pharmacological applications. So far, xylindein can only be purchased in small quantities through the working group around Sara C. Robinson [120]. The production of xylindein by large-scale liquid cultivation is a prerequisite for the industrial use of the pigment.

Laetiporic acid has been described in literature as a polyene for the first time in 2004 [121], with the identification of several variants one year later [98]. These laetiporic acids therefore form a whole family of non-carotenoid orange-yellow polyene pigments. Stirred bioreactor cultivations have been described, but the mentioned studies concentrated on pigment identification and the insulinogenic properties of the produced extracellular polysaccharides. Coloration of the fungal mycelium was not mentioned, but an optimal mycelium growth at a very acidic pH of around two was identified [122]. A recent study showed antifungal effects by *Laetiporus* polyenes [85]. To the best of our knowledge, these pigments have not been produced for subsequent use as natural colorants.

In the present study, we investigated the cultivation of *C. aeruginascens* and *L. sulphureus* in bioreactors up to 70 L with respect to enhanced pigment production. Furthermore, we analyzed parameters for pigment extractions and the possible application of the pigments in wood coloring or fabric dyeing.

3.4 Materials and Methods

3.4.1 Microorganism and Cultivation

C. aeruginascens (A 39 provided by IHI Zittau, Germany) was maintained on 50% (*v/v*) orange juice agar plates (50% orange juice from Sonniger®, 100% fruit content, manufactured for ALDI Nord, Germany; 30 g/L Agar-Agar, Roth, Germany). For submers cultivation, two 1 cm^2 of a 14-day-old fungal agar culture were transferred to 150 mL 5% (*v/v*) orange juice medium (orange juice from Sonniger®, ALDI Nord, Germany; 22 °C, 120 rpm, 14 d) according to Stange *et al*. [93]. For the fermentation experiments, 200 mL of

submers culture (120 rpm, 14 days) was used to inoculate 2 L 5% (*v/v*) orange juice medium containing 1 mL/L silicone antifoam emulsion (Roth, Germany) in a 3 L bioreactor (Z611000310, Applikon, Netherland). These were cultivated at 22 °C and 150 rpm for 14 days. Every 2 days a sample (5 mL) was taken. For the scale up experiments, 500 mL or 5 L of submers culture was used to inoculate 5 or 55 L 5% (*v/v*) orange juice medium containing 1 mL/L antifoam in a 7 L (Z611000720, Applikon, Netherland) or 70 L bioreactor (Z620003070, Applikon, Netherland), respectively. The cultivations were carried out at 22 °C with 150 rpm and an aeration rate of 0.5 vvm (7 L bioreactor) or 100 rpm and 0.27 vvm (70 L bioreactor). Samples were taken every 2 days (5 mL, 7 L bioreactor) or every day (50 mL, 70 L bioreactor). The equipment of the reactors is summarized in Table S1.

L. sulphureus (DSMZ 11211, Germany) was maintained on 1.5% (*w/v*) agar plates with standard nutrient liquid (SNL) medium at 24 °C. Information on media composition see Section 3.4.2 and Supporting Information 3.7.1. For submerse cultivation, 1 cm^2 of a 7-day-old agar plate was transferred to 200 mL medium. Cultures were harvested after 14 days. For the fermentation experiments, 5% (*v/v*) submers culture was used to inoculate 4 L Moser b medium in a 7 L bioreactor (Z611000720, Applikon, Netherland). These were cultivated at 26 °C with an agitation rate of n = 300 rpm and an aeration rate of pO_2 = 1 or 2 L/min. A sample (10 mL) was taken every weekday.

3.4.2 Media Optimization for *L. sulphureus* Cultivation

Standard media like SNL and Moser b, both containing 30 g/L glucose and trace elements (content see supporting information 3.7.1) have been tested for mycelium cultivation in shaking flasks (170 rpm, 26 °C) and compared to simple media like PDB (potato dextrose broth, Roth, Germany) containing 13.25 g/L broth and PDB supplemented with 5 g/L ground beech (< 0.5 mm).

3.4.3 Characterization of 3 L Bioreactor System Cultivating *C. aeruginascens*

For the characterization in the 3 L bioreactor (Z611000310, Applikon, Netherland), the working volume (0.5 to 2 L), aeration rate (0.5 to 2 vvm) and stirrer speed (0 to 800 rpm) were varied. The experiments were performed for water, culture medium, and culture medium with antifoam (1 mL/L, Silicone antifoam emulsion, Roth, Germany).

The k_La value was determined using the dynamic method by alternating air and nitrogen in double determination. The calculation of the k_La (oxygen transfer coefficient [h^{-1}]) was performed by integrating Equation (1) with $CO_{2,L}$ (oxygen concentration [%]) and $CO_{2,L}^*$ (saturation concentration [%]) in the liquid.

$$\frac{dCO_{2,L}}{dx} = k_L a \cdot (CO_{2,L}{}^* - CO_{2,L}) \quad (1)$$

The mixing time was determined using the decolorization method in triple determination. Water was stained blue using 2.5 mL/L starch and 0.5 mL/L iodine potassium iodide solution. Then, 1.25 mL/L sodium thiosulfate was added and the time measured until the solution was "completely" decolorized.

The energy input was calculated according to Equation (2), neglecting the Newton number, with P_V (volume-related energy input [W/m^3], V_{reactor} (working volume of the reactor [m^3]), $P_{ez\text{-}control}$ (energy input [W]), ρ_{Fluid} (density of the medium [kg/m^3]), N (stirrer speed [rpm]), D (stirrer diameter [m]), Np (stirrer factor 6 for Rushton turbine [-]) and n (number of stirring blades [-]).

$$P_V = \frac{P_{ez-control}}{V_{reactor}} = \frac{\rho_{fluid} \cdot (\frac{N}{60})^3 \cdot D^5 \cdot N_p \cdot n}{V_{reactor}} \quad (2)$$

The calculation of the tip speed was based on stirrer diameter and speed according to Equation (3) with v_{Tip} (tip speed [m/s]), N (stirrer speed [1/min]) and D (diameter of the stirring blade [m]).

$$v_{Tip} = \frac{N}{60} \cdot \pi \cdot D \quad (3)$$

3.4.4 Process Analytics

The *C. aeruginascens* biomass concentration was determined gravimetrically. For this purpose, biomass suspensions (2 mL using 3 or 7 L bioreactor, 40 mL using 70 L bioreactor) were separated via a 0.45 µm filter and dried at 103 °C. To determine the biomass dry weight of *L. sulphureus*, 5 mL sample was centrifuged (10 min, 5000 g, 25 °C), the pellet was washed once and then dried O/N. The reducing sugars were determined by the method of Miller [123]. In addition, the sugar composition was determined by HPLC (Rezex™ RPM Monosaccharide Pb^{+2} column, Phenomenex, USA) at 85 °C and a flow rate of 0.6 mL/min. The total nitrogen (TN) was determined by disintegration with the Laton total nitrogen cuvette test (LCK338, Hach-Lange, Germany). The determination of the total organic carbon

(TOC) was determined by the difference method of the TOC cuvette test (LCK338, Hach-Lange, Germany). Freeze-drying (Christ, Germany) was performed after centrifuged biomass was frozen at -80 °C O/N.

3.4.5 Extraction Process for Xylindein

Disruption of the fresh biomass was carried out using a high-pressure homogenizer at 2.6 kbar in the French Press (Constant Cell Disruption System TS 6, Constant Systems LTD, UK). Alternatively, the harvested biomass was gently dried at 60 °C and then ground in an ultracentrifugal mill (ZM 200, Retsch GmbH, Germany). For solvent screening, 1 ml of pretreated fresh biomass was incubated with 3 mL solvent (30 min, overhead shaker; 1 h, still state). After centrifugation for 5 min at 2600 g (Heraeus Biofuge stratos, Thermo Fisher Scientific GmbH, Germany), the absorption spectrum (300 – 800 nm) of the supernatant was recorded with a spectrophotometer (Beckmann DU 640, Beckman, USA). The following solvents were examined: acetone, acetylacetone, benzyl alcohol, 2-butanone (methyl ethyl ketone, MEK), dichloromethane (DCM), 1,2-dichloroethane, dimethyl sulfoxide (DSMO), ethanol, ethyl acetate, glacial acetic acid, isopropanol, methoxypropanol, n-hexane, phenol, 1,2-propanediol, toluene, and water. The following parameters for fresh biomass were examined: ratio of solvent (DCM) to extraction material of 1:1, 1:2, 1:3, 1:4; and extraction time of 5, 10, 30, and 60 min (overhead shaker, solvent ratio 1:1), both treated after extraction as described above. In comparison, 10 mg dried biomass was incubated with 3 mL of the solvents MEK and DCM. The contact time of solvent and extraction material was varied (0.5, 1, 1.5, 2, 2.5, 3, 4, and 5 h). The extraction material was centrifuged (5 min at 2600 g) and the absorption spectrum of the supernatant determined. Analysis of extraction cycles is described in the supporting information.

To determine the temperature stability, both the purified pigment and fresh biomass were dried at 40, 50, 60, 70, 80, and 103 °C. A photo documentation of the samples was made after 30, 60, 120, 180 min, and 24 h. To assess the storage stability, both the xylindein powder and two extracts (xylindein solved in DCM or MEK) were examined. All materials were stored in a cold room (8 °C) and checked regularly over a period of 9 months and documented.

3.4.6 Extraction Process for Laetiporic Acids

For bioreactor experiments, 1 mL sample was centrifuged (10 min, 5000 g, 25 °C), washed with dest. H_2O and centrifugation was repeated. Biomass was ground and resolved in 10 mL absolute ethanol (99.8%, Roth, Germany). After centrifugation (5000 g, 10 min) the absorption spectrum of the supernatant was recorded with a spectrophotometer (Beckmann DU 640, Beckman, USA). For media optimization experiment, the culture was homogenized using an ultra-turrax (IKA, Germany). Then, 2.5 mL sample was incubated with 2.5 mL absolute ethanol and absorbance of extract was determined as described before.

For extraction optimization several parameters were analysed. Pretreatment always involved homogenization of the sample. Extractions with 100% solvent were carried out with 7.2 g/L fresh biomass or 0.36 g/L freeze-dried biomass. Biomass was centrifuged (5000 g, 10 min), the pellet resuspended in solvent (ethanol, methanol, acetonitrile) and incubated for 30 min. After centrifugation (5000 g, 10 min) the absorption of the supernatant was determined. For extractions with 50% (*v/v*) ethanol biomass was diluted 1:2 with solvent to achieve a final concentration of 7.2 g/L (fresh) or 0.36 g/L (freeze-dried). Extraction temperatures of 25, 50, 70, and 90 °C and extraction times of 15, 30, 45, and 60 min were tested with freeze-dried biomass (0.36 g/L). For stability experiments, extracted solutions (freeze-dried, 50% ethanol, 30 min at 70 °C) were kept at room temperature and absorbance was determined after 1 h, 2 h, 1 d, and 7 d. Extract stability was also assessed for 50% acetone and 50% acetonitrile after 3 days at room temperature. Color stability of laetiporic acid extracts at different pH values was determined according to Stange *et al*. [93]. The extract was diluted 1:10 in different pH buffers. Due to this dilution, an extract of 3.6 g/L freeze-dried biomass (10x higher concentrated) was used. After an incubation of 10 min the absorbance was measured. All extractions have been performed in duplicate and data points represent the mean ± SD.

3.4.7 Application of Xylindein (Veneer Dyeing) and Laetiporic Acids (Silk Dyeing)

Three samples each of birch veneer (*Betula* sp.*)* and beech veneer (*Fagus sylvatica*) with a diameter of 88 mm (veneer thickness 0.9 mm, tangential section) were cut and then soaked in tap water for 10 min and autoclaved at 103 °C for 30 min. Additionally, six cellulose filter papers were autoclaved. Under sterile conditions, 50 mL mycelium suspension (liquid culture as described before) was transferred to a filter paper and covered with a veneer (area-related dry biomass concentration 1.6 mg_{BM}/cm^2). These samples were then transferred to a petri

dish (Ø 92 mm) and incubated at 23 °C ± 2 °C for 12 weeks in the dark. Regular microscopic examination using a binocular (40 × magnification) were performed.

For the application of laetiporic acids, 50 or 200 mg/mL biomass (fresh, centrifuged) diluted in 50% ethanol were treated with an ultra-turrax. Silk samples (2 cm^2) were dyed individually in conical flasks (50 mL) at 70 or 90 °C for 60 min in a thermomixer (Eppendorf, Germany).

3.5 Results and Discussion

3.5.1 Fermentation of *Chlorociboria aeruginascens* in Bioreactors up to 70 L

In our previous studies, we have identified optimal growth parameters using 12-well plates and shaking flask cultures. The following parameters were retained: liquid fermentation (bubble column or stirred reactor), batch process, 5% orange juice as medium, pH 3.5-5.0 and temperature 20 ± 2 °C.

For the scale-up, various bioprocess engineering parameters have been analyzed using a 3 L bioreactor screening. This included the consumption of nutrients (carbon source, nitrogen source, etc), mixing time, k_La value as a measure for oxygen input, as well as energy input and stirrer tip speed (measure for shear stress) calculated from stirrer speed, reactor and stirrer geometry.

A fast mass transfer and a good oxygen supply are of elementary importance to avoid unwanted limitations and require rapid mixing and sufficient k_La values. However, the challenge is to find the right balance between mixing, aeration and fungal growth as well as product formation. The aim was therefore to identify an optimum between mycelium growth and the necessary bioprocess parameters. For this purpose, the bioreactor was first characterized in different modes. For shear-sensitive organisms, the use of bubble columns has proven successful. To simulate a bubble column, the 3 L reactor was operated without stirring. The k_La values were in the range of 7-20 h^{-1} depending on the aeration rate (0.5-2.0 vvm) and the working volume (0.5-2 L). The mixing times were in the range between 5 and 15 s. For the characterization of the 3 L bioreactor with installations and agitator, a moderate aeration rate of 1 NL/min was chosen. In **Table 1** the different parameters are depicted. As expected, by increasing the stirrer speed (150-800 rpm), the k_La values increased and reached a maximum of more than 110 h^{-1}. The mixing time played a rather minor role in the stirred version and was in the range between 1-6 s, which indicates short mixing times

and good mixing. Subsequently, the tip speed and the power input were determined on the basis of the selected parameters and the reactor geometry.

In all experiments, we could exclude oxygen limitation based on the online measurement of the pO_2 content. An increase of biomass and a decrease of the carbon and nitrogen source over time was detected. The most important parameter to determine optimal fermentation parameters was the production of pigment. Visual observations during cultivations in the bioreactor showed that the onset of pigment production coincides with a lowering of the pH-value, which starts to increase again after only few hours (**Supporting Figure 1**, see also **Figure 8**). This temporal pH lowering could function as a primary indicator for successful pigment induction in industrial fermentations. Additionally, this online measurable parameter is independent of visual observations or pigment extraction.

Table 1. Overview of the detected scale-up criteria as a function of the stirrer speed in the 3 L bioreactor for *C. aeruginascens* cultivation (performed with working volume 2 L, ring gasifier, 2 disc stirrer and 3 baffle).

Scale-up criteria	unit	SmF1	SmF2	SmF3	SmF4
Stirrer speed	[rpm]	800	450	150	0
Aeration rate	[vvm]	0.5	0.5	0.5	0.5
K_La value	[h^{-1}]	114	25	4.5	3.6
Mixing time	[s]	1.4	2.4	6.1	8.9
Tip-Speed	[m/s]	2.01	1.13	0.38	0
Energy input	[kW/m^3]	3.44	0.61	0.02	-
Biomass yield	[g/L]	1.04	1.2	1.66	1.3
pH drop (= start of pigment formation)	[d]	6	6	4	5

Next to the already mentioned parameters pH and pO_2, the time course of dry weight (biomass), nitrogen and carbon content were determined. The chronological progression of these parameters is presented in **Figure 8** for the largest fermentation (70 L bioreactor), but similar results were obtained in the 3 L screening (compare also with **Table 1**). As described

in our previous work [113], pigment production is triggered by nitrogen limitation. Indeed, in these fermentations the total nitrogen content drops to a constant level at the time of the color change. Since the method detects total nitrogen, even if it is bound in certain molecules, the nitrogen content is not reduced to zero. Therefore, it is assumed that the nitrogen is no longer bioavailable for the organism. At this time between day 4 and 6, the carbon source is still sufficiently available. Of great importance is a suitable uptake of carbon and nitrogen by the fungal biomass and sufficient oxygen supply usually characterized by high k_La values. An insufficient oxygen supply inhibited the formation of the desired pigment [93]. In the case of *C. aeruginascens*, a high k_La value is not the crucial parameter for successful cultivation under the given conditions but rather the tip speed as a measure of shear stress, which needs to be moderate to prevent damage to the biomass.

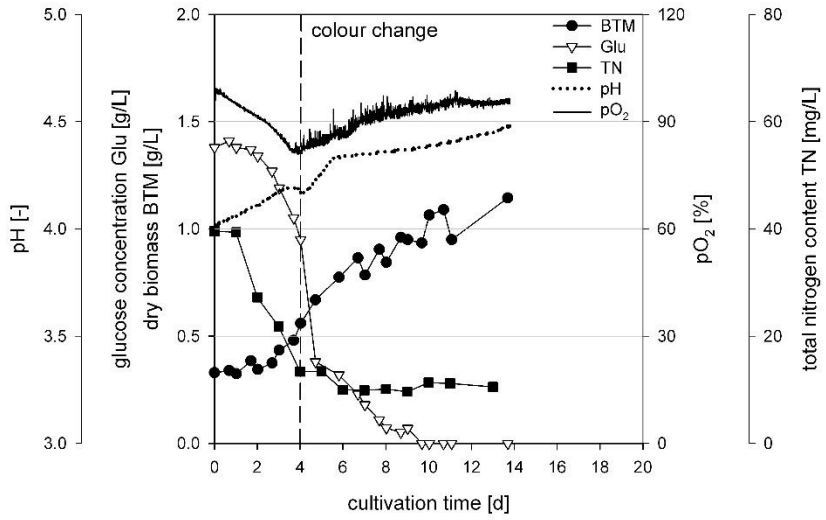


Figure 8. Comparison of the chronological progression of different cultivation parameters and their relationship with regard to the formation of xylindein in a 70 L bioreactor cultivation of *C. aeruginascens*.

The results showed that at a low stirrer speed (150 rpm, tip speed < 0.5 m/s) *C. aeruginascens* had both the fastest pigment formation and the highest biomass concentration. Compared to the three other fermentations with 1.0-1.3 g/L biomass and pigment induction at day 5-6, we achieved the highest biomass concentration of about 1.7 g/L and the earliest start of pigment production at day 4 at 150 rpm (**Table 1**). Thus, with this screening we could show that too much energy input resulted in reduced biomass yield due to shear stress. This reduction also resulted in a lighter color of the biomass obtained.

Considering the similarity theory, the results of the experiments in the 3 L bioreactor were first transferred to a 7 L bioreactor (working volume 5 L) and then to a 70 L bioreactor (working volume 55 L) (**Table 2**, **Supporting Table 1**). K_La values between 4.5 and 7.9 $h^{-1}$and tip speeds of 0.38 to 0.52 m/s for the different bioreactor volumes were calculated.

As shown in **Supporting Figure 2** all cultivations show a similar pH-value curve and biomass concentration. A similar growth rate was also detected for all scales, which was also reflected in the biomass formed per liter of medium used (3 L bioreactor: μ = 0.206 d^{-1}, ΔX = 1.14 g/L; 7 L bioreactor: μ = 0.151 d^{-1}, ΔX = 0.9 g/L; 70 L bioreactor: μ = 0.233 d^{-1}, ΔX = 1.34 g/L). Only minimal delays in pigment formation with increasing working volumes were observed. Therefore, we could show the successfully implemented scale-up and that the "tip speed", controlled by the stirrer speed and stirrer geometry, is one of the most important scale-up criteria in the cultivation of *C. aeruginascens*. Conditions like working volume, aeration rate, and stirrer speed influence the shear stress and have to be optimized to achieve as little damage to the biomass as possible for ideal pigment yield when transferred to industrial scale.

Table 2. Overview of the set scale-up criteria of the different scales cultivating *C. aeruginascens*.

Description	unit	3 L bioreactor	7 L bioreactor	70 L bioreactor
Working volume	[L]	2	5.5	55
Stirrer speed	[rpm]	150	150	100
Aeration rate	[vvm]	0.5	0.5	0.27
K_La value	[h^{-1}]	4.5	7.9	4.7
Tip-Speed	[m/s]	0.38	0.46	0.52
Energy input	[kW/m^3]	0.02	0.04	0.02
Biomass yield	[g/L]	1.66	1.44	1.7
pH drop (= start of pigment formation)	[d]	4	4.5	5

3.5.2 Analysis of Xylindein Extraction

The pigment xylindein is primarily located in the hyphae and is only released into the environment in small quantities. Cell disruption is therefore of fundamental importance for

successful extraction. For this purpose, mechanical treatment, in particular disruption using a high-pressure homogenizer for fresh biomass and grinding when using dried biomass, has proven to be effective. If fermentation is not directly followed by pigment extraction, dry biomass is easier to handle, for example, for storage and transport. The currently most widely used solvent for xylindein is dichlormethan (DCM) [93,124]. The quantities required for industrial application, however, can only be used under very complex and expensive conditions due to the toxicity of the solvent. This also applies to other solvents suitable for xylindein extraction, for example, chloroform, acetonitrile, or glacial acetic acid [125]. To find an alternative solvent for DCM, an extensive screening was carried out. Solvents of different groups such as hydrocarbons, halogenated hydrocarbons, carboxylic acids, carboxylic acid esters, ketones and diketones, alcohols and alkanols, aromatic organic compounds, carbon-sulphur compounds, and water were examined. The extracts have a bluish or greenish color depending on the product concentration. Extracts exhibited a characteristic absorption spectrum with two peaks at approx. 610 and 650 nm (**Figure 9a**), which is identical to values reported in literature [105,119]. Solvents from the ketone/diketone range, especially acetone, acetylacetone, 2-butanone (MEK, methyl ethyl catone), proved to be suitable for extraction. Benzyl alcohol also showed a very good extraction capacity, which was even better than DCM. Due to the later separation of the solvents using rotary evaporation, acetone, ethyl acetate, and MEK, as well as DCM as reference were further investigated. We identified the following parameters as optimal: 30 min extraction time per cycle, a 1:1 ratio of solvent to extraction material, and 3-6 extraction cycles. MEK was found to be the preferred solvent besides DCM. Rotary evaporation produced a dark green powder ready for various applications. For future industrial use, we identified that further processing of the culture supernatant by ultrafiltration (10 kDa or less) allows the recovery of pigment released during cultivation. The complete extraction process is summarized in **Figure 9b**.

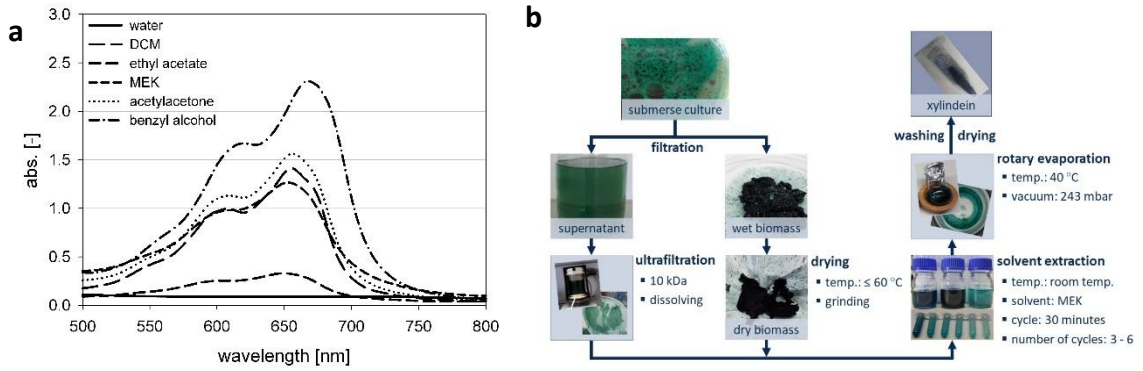


Figure 9. Extraction of xylindein. (**a**) Absorption spectra of extracts with different solvents and (**b**) extraction scheme.

Analysis of the extracts showed that it was free of fat, sugar, and protein. Furthermore, a temperature stability of up to approx. 100 °C has been proven. Harrison *et al*. [118] were even able to report a stability up to 190 °C. If the pigment is still bound to the biomass, irreversible damage to the dye (brown coloring) was observed above 60 °C. Therefore, if biomass needs to be dried before pigment extraction, it is advised to use low temperature conditions. The extracted pigment (green powder) was stored stably for months, while liquid extract stability depends on the solvent. The DCM extracts were stable for 9 months showing no loss of color, while the MEK extracts increasingly lost intensity, and showed a change in color (blue to pink) after more than five months. In general, solvent and product concentration affected color shade and intensity (solvatochromic, hyper-chromatic, and batho-chromatic effects).

During the cultivation in the 70 L bioreactor of *C. aeruginascens* in 5% orange juice, approximately 1.3 g/L dry biomass was obtained within 2 weeks. The contained xylindein was extracted by the method developed and described above using MEK (**Figure 9b**). One gram of dry biomass contained approx. 50 mg xylindein. This in turn corresponds to a product yield of 5%. In comparison, natural dye precursors for indigo production, which can be obtained from dried woad leaves, only resulted in yields of 0.3% [126]. Comparing yields of xylindein, Boonloed *et al*. [109] achieved 62 mg xylindein from a liquid culture (working volume 250 mL). The biomass concentration was not specified, but the cultivation time was much longer with 10 weeks. Therefore, a productivity of 3.5 mg/L/d of xylindein was achieved. In our study, a productivity of 4.8 mg/L/d was obtained by optimizing the cultivation (medium, parameters) and through the transfer to a much larger scale (working

volume 55 L), as well as significant reduction of the cultivation time. The identification of xylindein production resulted in publishing of our patent [127].

3.5.3 Coloration of Veneer by Fungal Surface Growth

The extracts can be applied to manufactured artwork as done previously by the group around Sara C. Robinson [114]. In our study, we analyzed the coloring of inconspicuous and light types of wood, such as birch or beech, by using of *C. aeruginascens* mycelium cultivation on veneer. We could show that solid wood and veneers were successfully stained by xylindein produced during a combination of solid and liquid cultivation (**Figure 10**). The most effective staining was achieved by placing wood samples in liquid cultures [128]. Within 12 weeks, surface-colored solid wood and veneers could thus be produced.

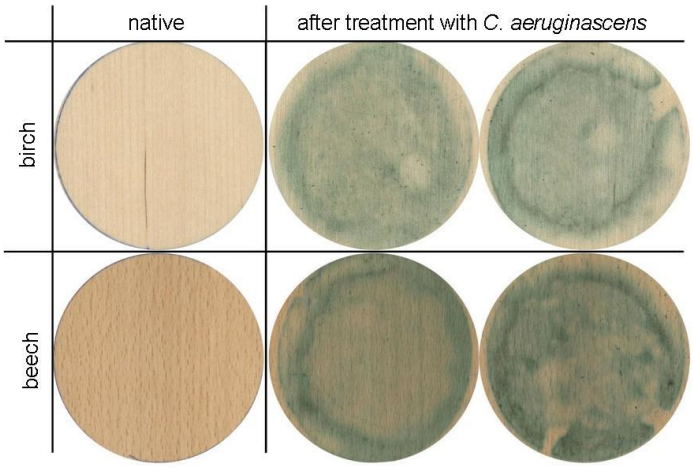


Figure 10. Coloration of veneers with xylindein. Two types of wood have been used for liquid-solid cultivation of *C. aeruginascens* for a period of 12 weeks.

3.5.4 Optimization Process for the Fermentation of *Laetiporus sulphureus*

In a screening of 25 different Basidiomycota using submerged cultivation in shaking flasks, we identified *Laetiporus sulphureus* as a potent pigment producer. The pigments have been previously identified as a whole family of different laetiporic acids and have been characterized exhaustively [98]. Compared to other Basidiomycota, biomass growth and pigment production was achieved on a satisfactory level without specific elicitors or tedious media optimization. To optimize pigment generation, we compared the final biomass concentration of *L. sulphureus* cultures from two different media in shaking flasks and analyzed the extracted pigment, which can be identified by the absorbance peak at 445 nm

[98]. Compared to standard nutrient liquid (SNL) medium, we achieved a higher pigment to biomass ratio with Moser b medium containing solely glucose as sugar source (data not shown). In both media, most of the pigment is located in the biomass and therefore pigment yield depends on biomass growth. To obtain first insights into the scale up of the cultivation, we utilized Moser b and analyzed biomass growth in a 7 L bioreactor. The bioreactor samples were analyzed for dried biomass and pigment (extract from 1 mL sample), which is shown in **Figure 11**.

After 7 days, a decrease of the determined biomass was observed, which was caused by an inhomogeneous distribution of the biomass in the bioreactor due to floating. At the end of the cultivation, a final probe was taken after harvesting the bioreactor culture. For this purpose, the biomass was mechanically removed from built-in components and walls and mixed into the liquid phase. Thereby, we achieved a final biomass of 9.54 g/L and an OD_{445nm} = 55.8. Without pH regulation, strong acidification up to pH 2 occurs by the fungus itself, as described previously [122].

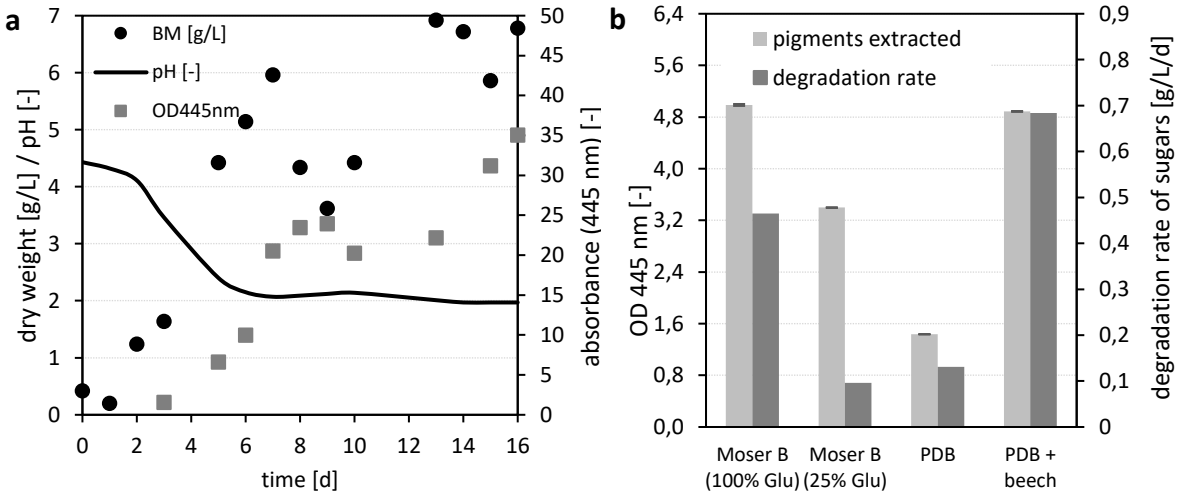


Figure 11. Cultivation of *L. sulphureus* (**a**) in a bioreactor showing dry weight, absorption of extract and pH and (**b**) media optimization in shaking flasks after 14 days of cultivation (Glu: glucose, PDB: potato dextrose broth).

Bioeconomy depends on the identification of cost-effective processes to provide products from sustainable and renewable sources. Compared to complex media like SNL or Moser b, it is therefore advantageous to use cheap and sustainable substrates like agricultural residues or by-products. We have compared the previously used Moser b and simple media like PDB

(potato dextrose broth) with or without additives like ground beech wood. In addition, previous research work ([129], under revision) showed that metabolite pathways may be inhibited by an abundant amount of sugars. Therefore, we tested Moser b with a sugar content of 30 g/L (100%) and 7.5 g/L (25%) glucose and PDB with 10 g/L glucose.

To obtain high nutrient exchange shaking was increased to 170 rpm, which resulted in higher aggregation of the biomass except for cultivations with solid material (**Supporting Figure 3**). The latter induced higher shear forces, resulting in broadly dispersed mycelium and increased nutrient uptake (higher fungal surface). Accordingly, the sugar degradation rate increased with ground beech by 70-80% compared to PDB without beech (**Figure 11b**), with an almost complete consumption of sugar after 14 days of cultivation. Due to biomass aggregation, the complete culture was homogenized with an ultra-turrax before sample taking and 2.5 mL sample was extracted with 2.5 mL ethanol (f.c. 50% ethanol). Highest pigment yields were achieved with both Moser b (100% glucose) and PDB with beech. Further investigations, especially regarding optimal growth conditions in bioreactor systems could further improve time- and cost-optimized pigment production. This will focus in particular on the use of sustainable media, known to enhance certain metabolic pathways in Basidiomycota ([129], under revision).

3.5.5 Analysis of Laetiporic Acids Extraction

Various solvents were analyzed for their suitability to extract pigment from biomass of *L. sulphureus*. We identified an optimal biomass/solvent ratio of 7.2 g/L freshly harvested biomass in 5 mL solvent (data not shown). This corresponds to a concentration of 0.36 g/L freeze-dried biomass. Due to problems with dissolving freeze-dried biomass in the different solutions, we rehydrated the biomass before further treatment. The results for extractions with aqueous ethanol solution (50% (*v/v*)) and pure ethanol, methanol, and acetonitrile are shown in **Figure 12a**. Absorption peaked around 445 nm for all solvents (**Supporting Figure 4a**), which is identical to laetiporic acids reported in literature [98]. The extracts contained different laetiporic acid pigments with absorption peaks around 460 nm, as the spectra shows a shoulder at this wavelength (most pronounced with acetonitrile). This was also confirmed by LC-MS (**Supporting Figure 4b**). Since we achieved very good extraction rates with aqueous ethanol (especially for freeze-dried samples), extraction temperature and time for this system was further optimized. Further advantages were the reduced solvent consumption and omission of the centrifugation step. We identified an optimal extraction

temperature of 70 °C (**Figure 12b**), an optimal extraction time of 15 min (**Figure 12c**) and determined the extracts' stability at room temperature to be in the range of a few hours (**Figure 12d**). The stability increased when aqueous solutions (50% (*v/v*)) of acetone and acetonitrile were used (**Supporting Figure 5**). For further applications, it is important to determine the color stability at different pH values. According to Stange *et al*. [93] we performed experiments for the solvents ethanol, acetone, and acetonitrile in buffers at different pH (1:10 ratio). In the acidic and neutral range, no change of absorption was detected. In the alkaline buffer solutions (pH 9-13), an increase in absorption was observed, but this has no effect on the color stability itself (**Supporting Figure 6**). The absorbance peak did not shift, therefore no change in color was detected.

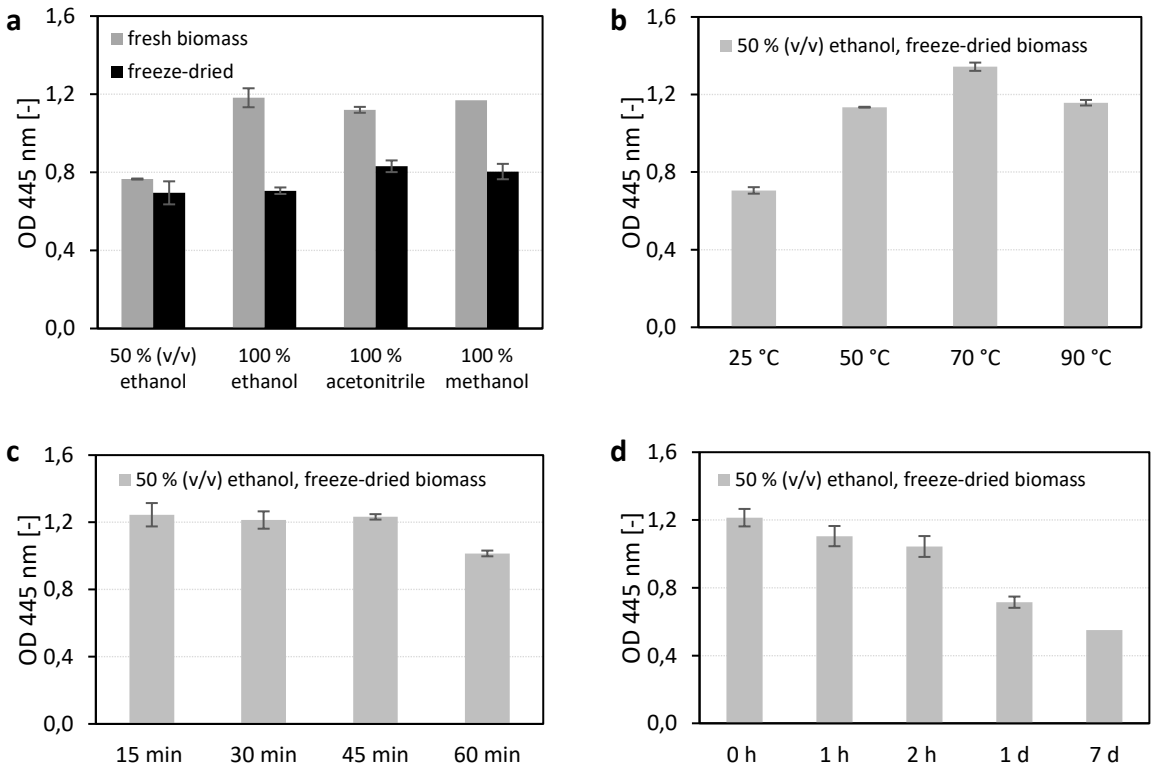


Figure 12. Extraction parameters of laetiporic acid showing the absorption maximum of extracts at OD_{445nm}. (**a**) Extracts with different solvents comparing fresh and freeze-dried biomass. (**b**) Comparison of extraction temperature. (**c**) Comparison of extraction time. (**d**) Time dependent stability of extract at room temperature.

3.5.6 *In Situ* Pigment Extraction for Textile Application

Materials like wool and silk can be dyed sustainably using heat to fixate the colorant. Since fruiting bodies of mushrooms have been historically used for dyeing textiles [62], we combined *in situ* extraction with dyeing. In short, freshly harvested biomass was diluted with ethanol (f.c. 50% (*v/v*)) to achieve biomass concentrations of 50 or 200 g/L. This mixture was immediately used as a dyeing solution during the 60 min long dyeing process. The color intensity increased with increasing biomass (**Figure 13a**). Increased temperature (90 °C instead of 70 °C) resulted in an increase of the red value (a) for high biomass concentration. The color of the silk is very brilliant, which is not often seen with natural pigments (**Figure 13b**).

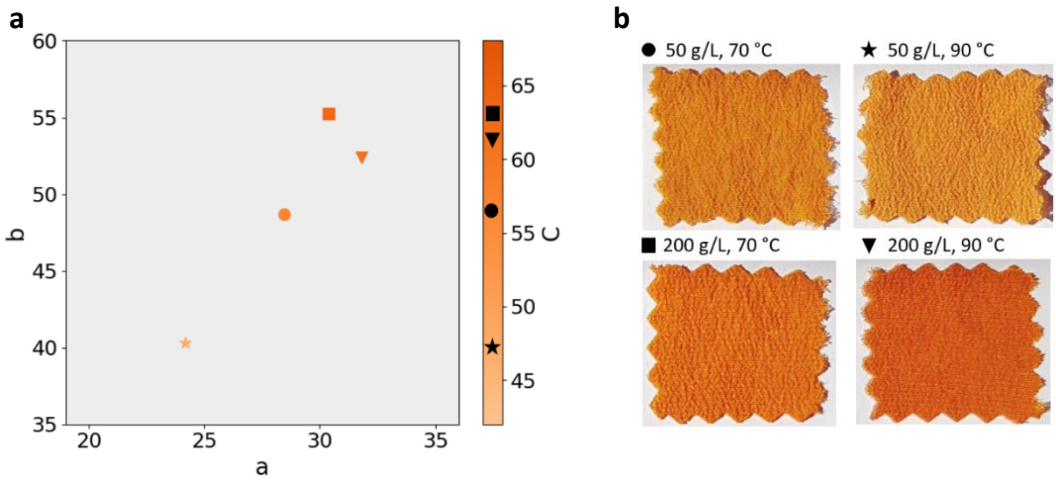


Figure 13. Application of laetiporic acid pigment. (**a**) CIE lab measurements with the values a, b and C of silk samples dyed with L. sulphureus biomass shown in (**b**). (CIE: International Commission on Illumination; a: color values from green (−) to red (+); b: color values from blue (−) to yellow (+); C: chroma, relative saturation).

3.6 Concluding Remarks

The blue-green xylindein is a very versatile pigment, which cannot be produced synthetically. So far, it can only be purchased in small quantities through the working group around Sara C. Robinson [120]. Effective biotechnological production is therefore of fundamental importance for industrial use. In our study we were able to successfully scale up our cultivation in the 70 L bioreactor and identify key process parameters, such as the “tip speed” (<0.5 m/s). We were able to reduce the cultivation time significantly to 2 weeks and increase productivity (4.8 mg/L/d). Until now, xylindein has been extracted using DCM. However, DCM can only be used under very complex and expensive conditions due to the toxicity of

the solvent. Our extraction optimization has enabled us to identify MEK as a suitable extraction agent for industrial use. Besides the extraction of the pure pigment, the mycological modification of wood by cultivation with *C. aeruginascens* is of great interest, but wood coloring is a long process. Cultivations on solid wood with deeper wood coloring took up to 2.5 years [90]. Additionally, the long-term cultivations were susceptible to contamination, ineffective coloring, or wood degradation. With our approach using a combination of liquid and solid *C. aeruginascens* cultivation, first results of surface colored wood and veneers were achieved within 3 months. Next to the blue-green color of xylindein by *C. aeruginascens*, we investigated the pigment family of laetiporic acids produced by *L. sulphureus*. In our study, upscaling of the pigment production through bioreactor cultivation was achieved. The successful extraction of laetiporic acids with simple and nontoxic solvents and the feasibility to dye silk samples provide a new possibility for natural textile dyeing. In contrast to other fungal pigments mainly produced by mold fungi [100] the toxicity is reduced because *L. sulphureus* is an edible mushroom, known as "chicken of the woods". Further work will focus on the toxicity of the pigments, as well as evaluation of color fastness, UV resistance, and washing stability. It is also intended to study different materials to extend the application variety. This study provides more chances for eco-friendly natural dyes from sustainable resources and offers a solution to counter the hazardous effects of synthetic colorants on the environment and human health.

3.7 Supporting Information

3.7.1 Media Composition

SNL: 30 g/L glucose monohydrate, 4.5 g/L L-asparagine monohydrate, 3 g/L yeast extract, 1.5 g/L KH_2PO_4; 0.5 g/L $MgSO_4$; 5 µg/L $CuSO_4 \cdot 5\ H_2O$; 80 µg/L $FeCl_3 \cdot 6\ H_2O$; 30 µg/mL $MnSO_4 \cdot H_2O$; 90 µg/mL $ZnSO_4 \cdot 7\ H_2O$; 400 µg/mL EDTA

Moser b: 30 g/L D-glucose$\cdot\ H_2O$; 10 g/L malt extract; 2 g/L peptone; 0.15 g/L K_2HPO_4; 0.35 g/L KH_2PO_4; 1 g/L NH_4NO_3; 0.3 g/L $NaNO_3$; 0.5 g/L $MgSO_4 \cdot 7\ H_2O$; 0.1 g/L $CaCl_2$; 0.001 g/L biotin; 0.05 g/L inositol; 0.0018 g/L $ZnSO_4 \cdot 7\ H_2O$; 0.017 g/L $FeCl_3 \cdot 6\ H_2O$; 0.0056 $MnSO_4 \cdot\ H_2O$; 0.05 g/L thiamine.

3.7.2 Purification of Culture Supernatant

The culture supernatant was filtered using different filter sizes to obtain the xylindein released in the medium. Syringe filter units (0.45 µm) and centrifuge tube filters (10 kDa, 5 kDa) were tested. The tube filters were centrifuged for 10 min at 2600 g. The absorption spectra of the filtrates and starting solutions were recorded.

3.7.3 Determination of Extraction Cycles

To determine the number of cycles, pretreated fresh biomass was incubated in a ratio of 1:1 with solvent (DCM) for 30 min an overhead shaker, centrifuged (5 min at 2600 g) and the supernatant separated. The pellet was again mixed with 3 mL solvent and incubated for 30 minutes. This cycle was repeated up to 6 times. Afterwards, the absorption spectrum of the supernatants was recorded. For comparison, 10 mg ground dried biomass was mixed with 3 mL each of MEK or DCM and incubated for 30 min in an overhead shaker. The biomass was then centrifuged and separated from the supernatant. For the second extraction cycle, 3 mL of the respective solvent was added to the pellet. This protocol was repeated for up to 7 extraction cycles. Afterwards, the absorption spectra of the supernatants were recorded.

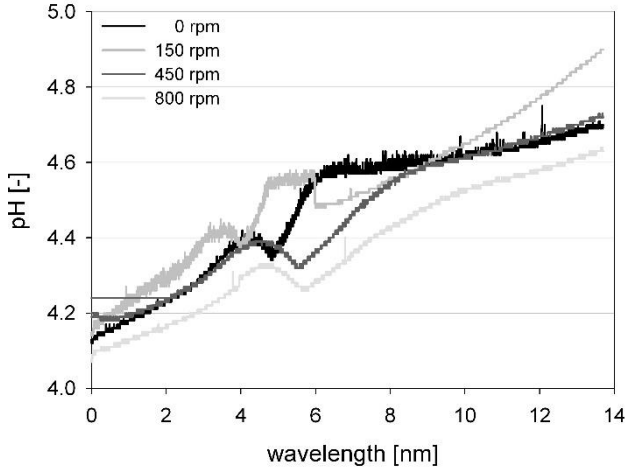
3.7.4 Chromatographic Measurement

For LC-MS analysis, approx. 20 mg dried biomass of *L. sulphureus* was extracted with 3·1,5 mL methanol and the extract shaken with 5 mL hexane for 5 min. The methanol phase was dried under nitrogen and the residue taken up using 2 mL acetonitrile. Measurements were performed using a Varian LC-MS/MS system (Pump 212, autosampler 460 and MS 320 with APCI housing; Varian) equipped with a Nucleodur Pyramid RP column (150/2 mm, 3 µm, Macherey-Nagel, Düren, Germany) at 30 °C. Elution was performed using (A) aqueous 0.1% formic acid (LC-MS grade, Carl Roth, Karlsruhe, Germany) and (B) acetonitrile with 0.1% formic acid (LC-MS grade, Carl Roth, Karlsruhe, Germany) with the following gradient: 0 - 5 min 50% B, 5 - 20 min 50 - 100% B, 20 - 48 min 100% B, 48 - 50 min 100 - 50% B, 50 - 55 min 50% B at a flow rate of 500 µL /min. Detection occurred using APCI housing 50 °C, drying gas 200 °C, capillary 100 V, collision gas pressure 200 mPa, and detector 1500 V at m/z: SIM: 403, 421, 447, and 473 and scan 300 - 500 in addition to detection at 445 and 460 nm.

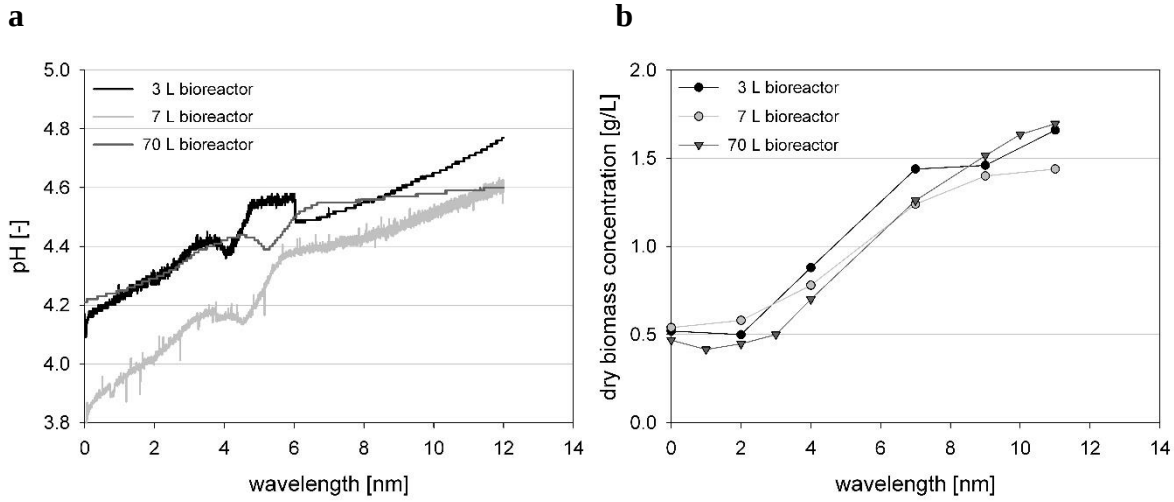
3.7.5 Supporting Data

Supporting Table 1. Detailed parameters for scale-up from 3 L to 70 L bioreactor.

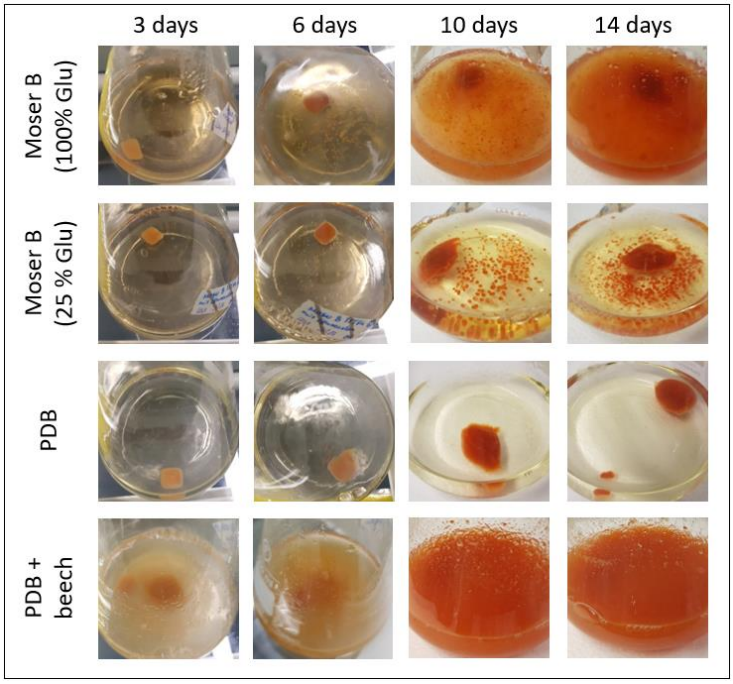
Description		3 L bioreactor	7 L bioreactor	70 L bioreactor
				
Volume	[L]	3	7	70
Work volume	[L]	2	5.5	55
H/D ratio		1.5	1.8	2.2
Stirrer type		Disc stirrer	Disc stirrer	Disc stirrer
Number of stirrers		2	3	3
Stirrer diameter	[cm]	4.8	4.9	10
Stirrer speed	[rpm]	150	150	100
Installations		3 baffle	3 baffle	4 baffle
Aeration trype		Ring gasifier	Ring gasifier	Ring gasifier
Aeration rate	[vvm]	0.5	0.5	0.27
Temperature control		Heater / heating jacket	Double Jacket	Double Jacket
K_La value	[h^{-1}]	4.5	7.9	4.7
Tip-Speed	[m/s]	0.38	0.46	0.52
Energy input	[kW/m^3]	0.02	0.04	0.02



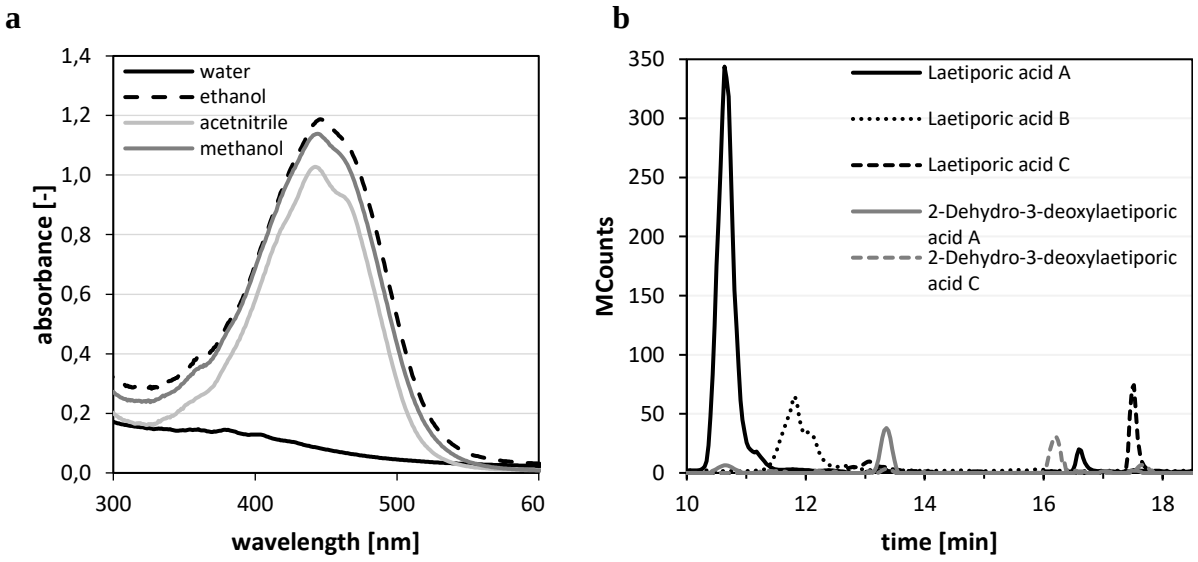
Supporting Figure 1. Time course of pH values as a function of the stirrer speed of the *C. aeruginascens* cultivations in the 3 L bioreactor.



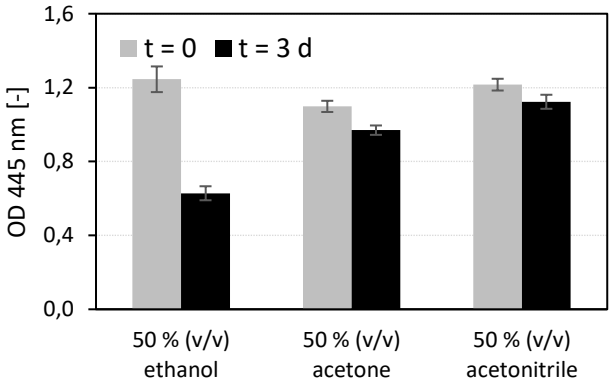
Supporting Figure 2. Up scaling bioreactor experiments for *C. aeruginascens*. (**a**) Time course of pH-values and (**b**) correlating biomass development as a function of cultivation scale.



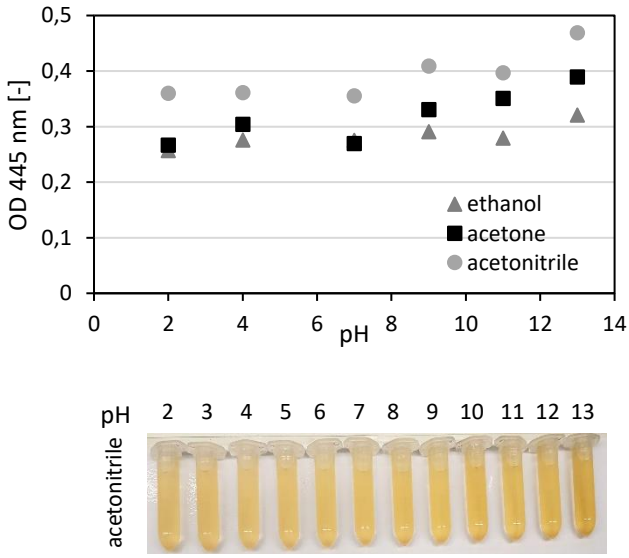
Supporting Figure 3. Cultivation of *L. sulphureus* with different media in shaking flasks. Pictures of the cultures at day 3, 6, 10, and 14.



Supporting Figure 4. Spectrophotometric and chromatographic analysis of pigment extracts. (**a**) Absorption spectra of extracts with various solvents from freeze-dried LSU biomass. (**b**) LC-MS analysis of the methanol extract of the dried biomass. Shown are the selected ion masses of the different laetiporic acids as described by Davoli *et al*. [20].



Supporting Figure 5. Time dependent stability of extracts using various aqueous solvent solutions. Extract remained for three days at room temperature.



Supporting Figure 6. Color stability of laetiporic acid at different pH values (1:10 dilution of extract in buffer with certain pH).

3.8 Disclosures

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Conflicts of Interest: The authors declare no conflict of interest.

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Data Availability Statement: Data available on request from the authors. The data that support the findings of this study are available from the corresponding author upon reasonable request.

4 Pilot-Scale Production of the Natural Colorant Laetiporic Acid, Its Stability and Potential Applications

4.1 Preamble to the Publication

In the second publication of this thesis, our work on *Laetiporus sulphureus* was continued. As described in chapter 3, *L. sulphureus* forms orange polyene pigments both in fruiting bodies and submerged cultivated mycelium. In this article, we improved the 7 L bioprocess after identifying the most potent production strain. Nutrition media, product recovery and storage were optimized and application trials expanded.

This article is a joint work of the Institute of Natural Materials Technology, Technical University Dresden (Olena Reinhardt, Anett Werner and Marlen Zschätzsch) and the Institute of Food Chemistry, Leibniz University Hannover (Pia Bergmann, Christina Frank, Meike Takenberg, Franziska Ersoy, and Ralf G. Berger).

I conceptualized the study and all published data was generated and interpreted by me. Olena Reinhardt, Anett Werner and Marlen Zschätzsch performed the fermentation experiments and tested the stability of color extracts. They prepared silk samples for experiments on light and washing stability. Meike Takenberg and Christina Frank were involved in investigation and formal analysis of shake flask cultures. Franziska Ersoy and Ralf G. Berger were involved in writing the original draft of the manuscript that was reviewed and edited by Marlen Zschätzsch and Anett Werner. The resources and funding for the project were acquired by Ralf G. Berger and Anett Werner.

The manuscript was published as part of the special issue “Pigment Production in Submerged Fermentation” in the journal *Fermentation* on the 28th of November 2022 and can be accessed online via https://doi.org/10.3390/fermentation8120684. After publication, the article was chosen as cover story for the December edition (*Fermentation* volume 8, issue 12), with the graphical abstract on the front page. The issue can be accessed via https://www.mdpi.com/2311-5637/8/12.

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Pilot-Scale Production of the Natural Colorant Laetiporic Acid, Its Stability and Potential Applications

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4.2 Abstract

Laetiporus sulphureus, a wood-decaying basidiomycete, produces yellow-orange pigments in fruiting bodies and, as was recently shown, in submerged cultivated mycelia. Out of four strains, the most potent laetiporic acid producer was identified and its yield compared in different media. The complex Moser b medium was replaced by potato dextrose broth, achieving higher yields at a lower cost. Cultivation was then scaled up from shake flask to a 7 L stirred tank bioreactor. Optimization of parameters led to increased product concentrations up to 1 g/L, the highest yield reported so far. An *in situ* product recovery strategy with a biphasic system was established, increasing the yield by 19% on the shake flask scale. A crude ethanolic extract of the biomass was examined for color stability and application trials. In contrast to what has been suggested in the past, the pigment showed limited long-term stability to oxygen and light, but was stable under storage in the dark at 4 °C under nitrogen. The orange extract was successfully incorporated into different matrices like foods, cosmetics and textiles. Laetiporic acid can potentially replace petrochemical based synthetic dyes, and can thus support the development of a circular bioeconomy.

Keywords: laetiporic acid; *Laetiporus sulphureus*; natural colorants; natural dye; basidiomycete; bioprocess; submerged fermentation; biotechnology

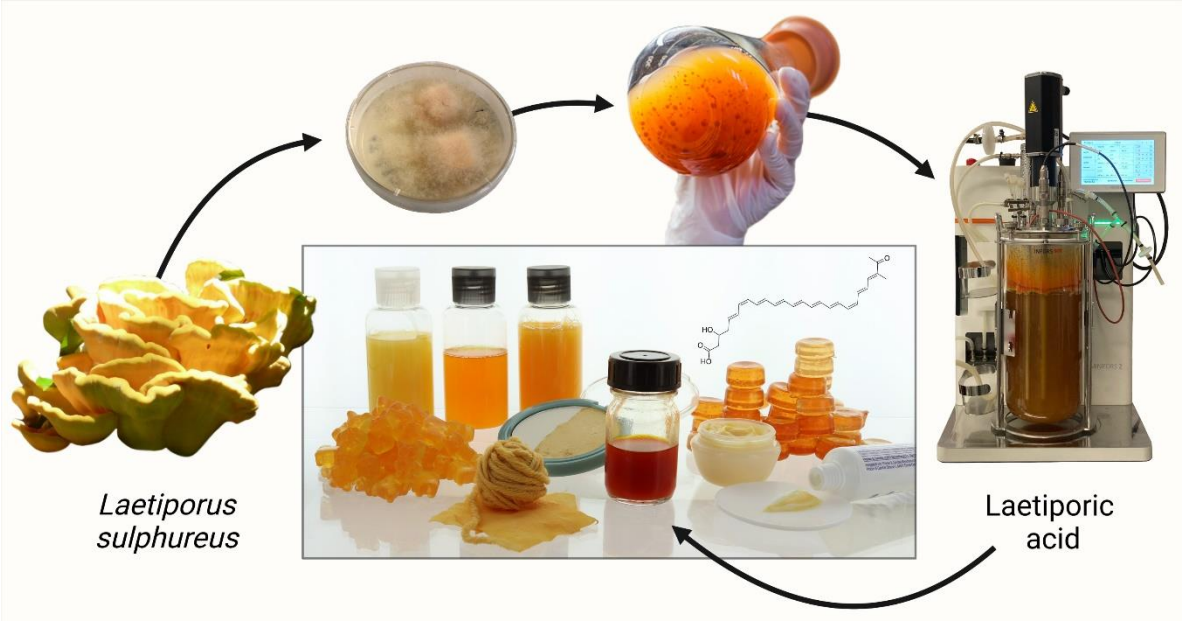


Figure 14. Graphical abstract of the publication [130].

4.3 Introduction

Consumers' demand for natural ingredients and "clean label" products remains high, fueling the search for alternative colorants. Regarding the range of possible hues and price, synthetic dyes are still hard to beat and are widely used in textiles, despite the well-known negative environmental and health effects [131]. Natural, plant-based colorants suffer from challenges like seasonal availability and dependency on climate. In addition, their production competes with food production due to the need for farmland. An alternative are microbial colorants, for example from fungi. Fruiting bodies have long been used for textile dyeing [62,63], but, like dye bearing plants, can be harvested only once. The biotechnological generation in submerged cultivated mycelium is a promising alternative for sustainable and natural colorants.

The basidiomycete *Laetiporus sulphureus* is edible when young and is supposed to taste like chicken, as the common name "chicken of the woods" suggests. It is known for its use in Asian and European folk medicine and biologically active compounds, like antimicrobial triterpenes [132]. Some even discuss it as the next functional food promoting health and as a valuable source of antioxidants [133–135]. The potential application of *L. sulphureus* extracts as a preservative in food has also been reported [136,137].

The name sulphureus refers to the characteristic sulphur-like color. Responsible for this color are orange-yellowish polyenes, the laetiporic acids [98,121] that were long presumed to be a

carotenoid pigment named laetiporxanthin [138]. Interestingly, *L. sulphureus* synthesizes a group of colorants: the predominant laetiporic acid A, laetiporic acid B, C, and D, which differ in chain length, and their respective 2-dehydro-3-deoxy derivates. Their biosynthesis has been investigated and the responsible polyketide synthase was described by Seibold *et al*. [85].

Large-scale intensive cultivation of *L. sulphureus* fruiting bodies for commercial production was described [139], but submerged fermentation is more efficient for color production in general. Davoli and Weber *et al*. discovered that a *L. sulphureus* strain produced 15 times more laetiporic acid A in submerged culture than in its fruiting body (3.71 vs. 0.25 mg/g) [98,121]. As different sources discuss the high variability in compounds that occur in different *L. sulphureus* species [98,140], we investigated several strains to identify the most promising pigment producer. The media and process control in a 7 L stirred tank bioreactor (STR) were optimized to produce laetiporic acids for application and stability trials. A pigment extraction process using a second phase (biphasic system) during the cultivation was established. This *in situ* product recovery facilitates the downstream process by avoiding the post-harvest extraction step. In addition, the color fastness regarding light and washing, the stability of the laetiporic acid extract, and different matrices to explore possible applications were evaluated.

4.4 Materials and Methods

All chemicals were purchased in pro analysis grade (Sigma Aldrich, Seelze, Germany; Merck, Darmstadt, Germany; Carl Roth, Karlsruhe, Germany), if not stated otherwise.

4.4.1 Species and Media Preparation

Four strains of the basidiomycete *Laetiporus sulphureus* were investigated: (A) DSMZ 2785, purchased 2006 from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Braunschweig, Germany); (B) DSMZ 11211, purchased 2007; (C) a proprietary isolate obtained from a fruiting body growing on *Populus tremula* in the region of Hannover, Germany in 2010, and (D) DSMZ 2785, purchased in 2012. All of the strains were verified by internal transcribed spacer (ITS) sequencing [141]. Mycelia were grown and maintained on standard nutrient liquid agar (SNL, *cf.* 4.7.1 Media Composition) according to Sprecher [142]. The plates were incubated for 7 days at 24 °C, before transferring 1 cm^3 of the

overgrown agar into 150 mL Moser b medium in a 300 mL Erlenmeyer shake flask as preparatory culture (*cf.* 4.7.1 Media Composition). The piece of agar was homogenized for 5 s at 11,000 min^{-1} (MiniBatch D-9, Miccra GmbH, Heiterheim, Germany) and the culture incubated on an orbital shaker (Multitron, Infors AG, Bottmingen, Switzerland) for 7 days at 24 °C and 150 rpm. It was homogenized again as described above and used to inoculate the main cultures (10% *v/v*).

4.4.2 Cultivation Parameters

For comparison of potential production strains, four *L. sulphureus* strains were cultivated for 21 days at 24 °C and 150 rpm on an orbital shaker in 250 mL Moser b medium in 500 mL shake flasks. 10 mL sample were collected on cultivation day 5, 10, 15 and 20 and analyzed for dry matter, pH, reducing sugar and pigment content. The samples were centrifuged at 4 °C and 5000× *g* for 15 min to separate biomass and supernatant. The biomass concentration was determined gravimetrically as dry matter, after washing the biomass with 5 mL demin. water and freeze drying (Alpha 1–4, Christ, Osterode am Harz, Germany). The pH was measured in the supernatant (pH 211, Hanna Instruments, Vöhringen, Germany), and the total reducing sugars determined after filtration with a 0.45 µm filter according to the method developed by Miller [123]. The shake flask cultivations were conducted in triplicates and data expressed as mean ± standard deviation.

For bioreactor experiments, 5% (*v/v*) preparatory culture was used to inoculate 3.6 L medium in a 7 L bioreactor (Z611000720, Applikon, Netherlands). These were cultivated at 26 °C with an agitation rate of 300 rpm and an aeration rate of pO_2 = 2 L/min. A sample (10 mL) was taken every weekday. Samples were analyzed as described in Section 4.4.2 and Section 4.4.4.

4.4.3 *In Situ* Product Recovery

To test the *in situ* extraction of laetiporic acids during submerged cultivation, *L. sulphureus* strain B was cultivated in a biphasic system. Safflower oil, refined linseed oil, tung oil and a binder agent containing safflower oil, dehydrogenized castor oil, soy oil and tung oil were provided by Biopin Vertriebs GmbH, Germany. Lauryl alcohol, as well as cultures without addition of a second phase were used as references. The cultures containing lauryl alcohol were incubated at 26 °C due to its melting point, while the other cultures were incubated at

24 °C, as described in Section 4.4.2. Samples were taken every second or third day, and from day 5 on 10 mL oil was added to the cultures. During every sampling, the extractive phase was removed and freshly added. For the exchange, a 50 mL sample from the surface of the culture was centrifuged (15 min, 4 °C, 5000× *g*) to separate extractive phase and aqueous culture supernatant. The supernatant was returned to the cultures and the concentration of laetiporic acids in the lipophilic phase was determined photometrically after extraction with methanol (*cf.* Section 4.4.4).

4.4.4 Analysis of Pigment Production

For all shake flask experiments, the freeze-dried biomass was extracted exhaustively with methanol and the extract dried under nitrogen. The samples were stored at −20 °C until further use. The residue was taken up in a defined volume of 2–10 mL HPLC-grade methanol and filtered with a 0.45 µm syringe filter. Absorbance was measured in a photometer and pigment content calculated as laetiporic acid A, with the extinction coefficient reported by Weber *et al*. (λ_{max}(MeOH) = 442 nm; ε = 26,669 L/mol·cm) [10]. For verification, a methanolic extract of the dried biomass was measured via mass spectrometry, as described in Zschätzsch *et al*. [143]. The main pigment was laetiporic acid A with *m/z* 421 $[M + H]^+$ and 419 $[M - H]^+$ (**Supporting Figure 7**).

For samples from the bioreactor experiments, 1 mL was centrifuged (10 min, 5000× *g*) and washed with demin. water. After repetition of the centrifugation and washing step, the biomass was ground and resolved in 10 mL ethanol (99.8%). It was centrifuged once more (5000× *g*, 10 min) and the absorption spectrum of the supernatant was recorded. Extraction and measurement of the pigment yield gave identical results for both methanol and ethanol.

4.4.5 Stability Trials

Accelerated light and washing stability tests were performed with dyed silk in triplicates. Silk samples (5 cm^2, untreated, purchased at Schmusewolle, Celle, Germany) were dyed with *L. sulphureus* dry matter (samples a, b, c) or wet biomass (d). As references, silk dyed with madder (e) (WEJA Färbeset, Livos Pflanzenchemie, Germany) and commercially available textile dye (f) (Simplicol Textil expert-India-Orange, Brauns-Heitmann GmbH & Co. KG, Warburg, Germany) were prepared and tested against industrially dyed silk (g) (Pietro Baldini). For all self-prepared samples, silk was dyed at 90 °C for 1 h, with 1% (a, b, d) or

30% (c) ethanol in demin. water. Sample b was treated with vinegar essence (5%, Surig, Speyer & Grund GmbH & Co. KG, Mainz, Germany) for 2 h at room temperature after the dyeing process.

Accelerated light stability tests were performed, as described previously [54]. In addition, the samples were tested for their color fastness during washing according to DIN EN ISO 105-C06:2010-08 [144]. For washing stability trials, color was measured with LUCI 100 (Dr. Lange; light source D65, 10° observer) and analyzed with the software Spectral QC. The ΔE value is defined as the color difference of, in this case, a sample (t_x) compared to its reference (t_0). A ΔE value below 1 generally implies a color change unnoticeable to the human eye [145].

Pigment stability in freeze-dried biomass at different storage conditions was investigated. Aliquots (protected from light) of 500 mg freeze-dried biomass were stored at room temperature (RT, around 20 °C, n = 3), 4 °C (n = 3) and −20 °C (n = 1). At sampling points, 10 mg biomass was extracted with 10 mL 100% ethanol for 15 min at 70 °C and the absorbance (λ = 445 nm) of the extract was measured.

Finally, ethanolic extracts of submerged cultivated *L. sulphureus* mycelium (2 g wet biomass, twice extracted with 30 mL 100% ethanol, 15 min at 70 °C) were stored under different conditions to investigate extract stability. Extracts were adjusted to 0.15 g/L laetiporic acid and stored in 10 mL aliquots in glass vials. Samples from the aliquot were measured repeatedly for up to 1 year (λ = 445 nm, photometer DU 640, Beckman). Extracts were stored at RT in light, in the dark, at 4 °C and −20 °C and flushed with nitrogen for 1 min before storage and after taking samples.

4.4.6 Application of Laetiporic Acids

A crude ethanolic extract of the harvested biomass (hot extraction with 98% ethanol, 25 cycles; Extraction System B-811, BÜCHI Labortechnik AG, Flawil, Switzerland) with 746 mg/L and 479 mg/L laetiporic acid A was used for application tests in foods and cosmetics, respectively. Hard candy was prepared by boiling 300 g isomalt (Isomalt STM, BENEO-Palatinit GmbH, Mannheim, Germany) and 6 g 50% citric acid solution (S.A. Citrique Belge N.V., Tienen, Belgium) in 100 g tap water at 165 °C. The laetiporic acid extract was added at a concentration of 0.3 or 1.7% (*w/w*) to the cooling sugar mass at 130 °C and poured into metal molds. Jelly gum was prepared by boiling 1000 g sucrose (Nordzucker

AG, Braunschweig, Germany) and 1335 g glucose syrup (40 DE, Scandic Food A/S, Vejle, Denmark) in 500 g tap water at 117 °C. 200 g of porcine gelatin (225 bloom, PB Gelatins GmbH, Nienburg, Germany) was dissolved in 470 g tap water and added to the sugar mass at 90 °C. 3% (*w/w*) color extract was added to 70 g 50% citric acid solution, incorporated into the jelly gum mass, molded using stamped starch beds (C*Clean Set 03703, Cargill BV, Haven, The Netherlands) and dried for 3 days at room temperature. After removing the starch, the jelly gums were polished (Capol 4468 D, Capol GmbH, Elmshorn, Germany).

As model matrices for cosmetics, product samples from the German market were obtained at a Rossmann drug store and colored with the laetiporic acid extract by incorporating it into the product. Soap (lavera Naturkosmetik Milde Pflegeseife) was dyed with 2% (*w/w*) color extract, transparent and opaque shampoo (Alterra Naturkosmetik everyday shampoo; Isana med Shampoo jeden Tag) with 7 and 4% (*w/w*), toothpaste (Blend a med classic) with 16.7% (*w/w*), body lotion (Nivea soft) with 6.7% (*w/w*). Powder (Alterra Naturkosmetik finish powder) was scraped out of the package and mixed 1:1 with the ethanolic color extract. It was dried at 50 °C and pressed back into the compact.

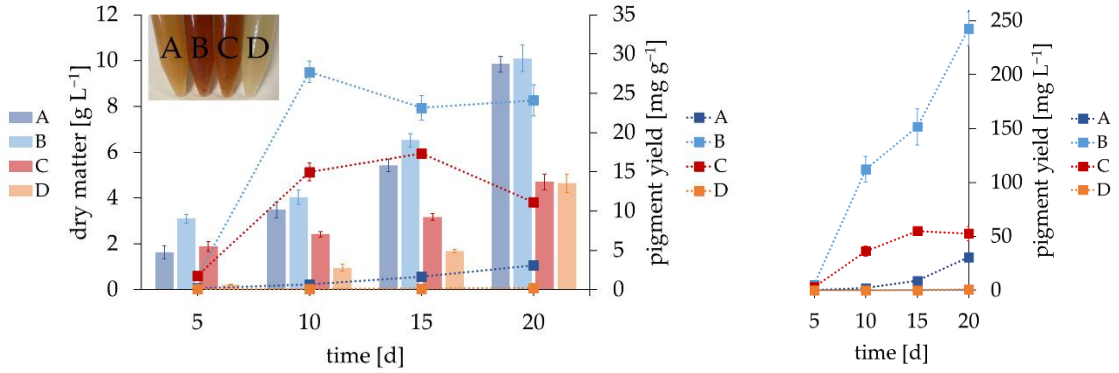
4.5 Results

4.5.1 Production Strain

Four strains of *L. sulphureus* were compared concerning their potential for generation of laetiporic acids (**Figure 15a**). Strain B was found to produce both the highest amount of dry matter and pigment, with 10.1 g/L biomass (day 20) and 27.7 mg/g laetiporic acid (day 10), respectively. The combination of high productivity in both biomass and pigment synthesis generated the overall highest laetiporic acid yield with 242.7 mg/L (**Figure 15b**).

Strain A was approximately equal to strain B in biomass production with a maximum of 9.9 g/L, but produced significantly lower amounts of pigment. Strain C produced the second highest amount of pigment (55.1 mg/L), but yielded only about half the biomass. The lowest amount of pigment was produced by strain D with 1.0 mg/L. Thus, the difference between the best and the worst performing strain regarding laetiporic acid production amounted to a factor of about 240. The two strains with an identical strain number, that were obtained from the same strain collection at different time points (A, purchased in year 2006 and D, in 2012) also differed significantly in both biomass production (9.9 vs. 4.7 g/L, respectively) and pigment yield (30.8 vs. 1.0 mg/L, respectively). A picture of unprocessed *L. sulphureus*

culture samples on day 15 illustrates the high variation in pigment yields (insert in **Figure 15a**).



(**a**) (**b**)

Figure 15. (**a**) Productivity of different *L. sulphureus* strains regarding biomass (bars, left ordinate) and pigment yield (squares, right ordinate) in shake flask culture. Insert on the top left side: A picture of the unprocessed cultures (biomass and supernatant) on cultivation day 15 with visible color differences. (**b**) Total pigment yield per culture volume over the course of cultivation.

4.5.2 Fermentation Experiments

L. sulphureus strain B, which was the most potent pigment producer, was cultivated in a 7 L STR with different media compositions. The use of potato dextrose broth (PDB) increased the pigment yield on cultivation day 12 by a factor of 3 compared to Moser b medium (1.03 and 0.34 g/L, respectively; **Figure 16a,b**). In the latter, it took 21 days until the sugar was depleted completely, resulting in a final laetiporic acid concentration of 0.69 g/L and the overall highest biomass of 7.7 g/L (PDB: 4 g/L on day 12; **Supporting Figure 8**). The addition of 5 g/L beech sawdust to the PDB resulted in a slightly quicker pH drop on day 2, but otherwise led to no significant changes of growth parameters and product yields, with both reaching 1 g/L laetiporic acid concentration on day 12 (**Figure 16c**). Controlling the pH with sodium hydroxide to prevent a pH drop below 2 (**Figure 16d**, addition of 400 mL on cultivation day 7) inhibited laetiporic acid synthesis and yielded a maximum concentration of 0.5 g/L on day 7, which was only half of the yield of the other PDB fermentations. The biomass production was slightly elevated in comparison to the fermentation without pH regulation (**Supporting Figure 8**).

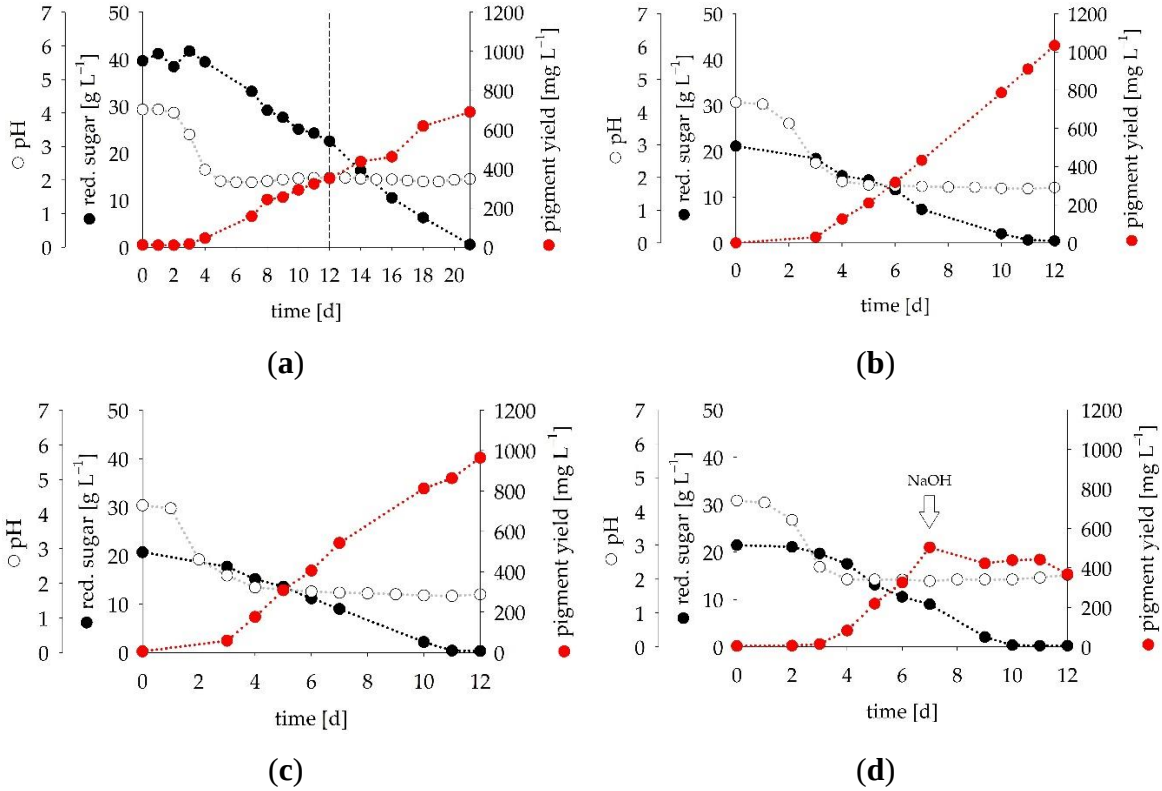


Figure 16. Influence of different media and pH regulation on laetiporic acid yield during cultivation in a 7 L STR.(**a**) Moser b, dashed line marks day 12; (**b**) PDB; (**c**) PDB + 5 g L^{-1} beech sawdust; (**d**) PDB + pH regulation.

4.5.3 *In Situ* Product Recovery

To test an *in situ* extraction of pigments for product recovery during the cultivation, a lipophilic phase of 10 mL was added to the shake flask cultures from day 5 on, and was renewed during each sampling. Oils traditionally used for glazing wood like safflower, refined linseed and tung oil were tested as a second phase. Lauryl alcohol and a cultivation without a second phase were used as controls. All yields, including those for the (smaller volume) extractive phase are given for one 250 mL culture. The addition of different oils resulted in a visible migration of laetiporic acid from the biomass to culture supernatant and extractive phase. Refined linseed oil had no significant effect on the pigment yield in the biomass compared to the control (78 and 83 mg on day 21, respectively). Nonetheless, it generated an additional 21 mg of pigment in the oil extraction phases over the duration of the cultivation (**Figure 17**), which increased the overall pigment yield by 19% in comparison to the control cultivation. Both tung and safflower oil generated similar amounts of pigment in the extractive phase but reduced the yield in the biomass, thus reducing the overall final yield (43 and 61 mg, respectively). Lauryl alcohol resulted in a significantly reduced pigment yield in the extractive phase due to an inhibitory effect on the biomass (**Supporting Figure 9**).

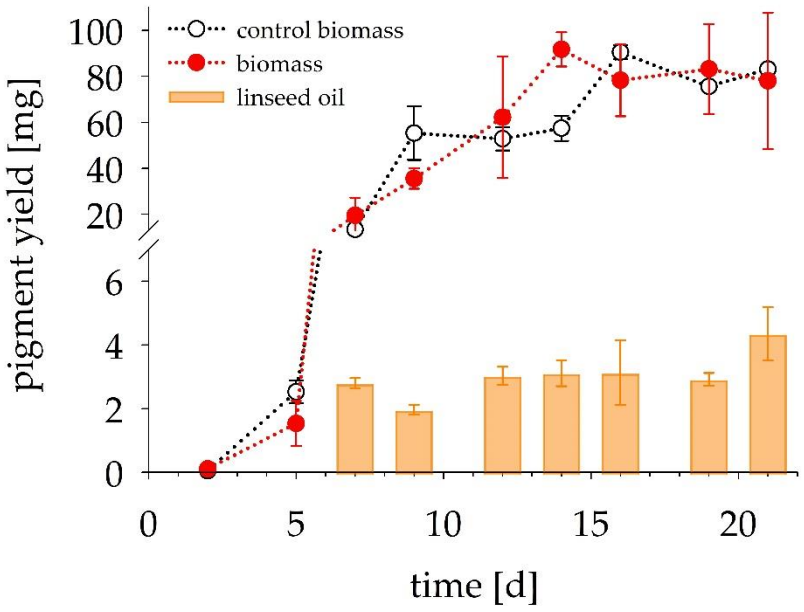


Figure 17. Laetiporic acid yields in biomass and lipophilic phase in 250 mL shake flask culture over the course of the cultivation during *in situ* extraction. White dots depict the measurements for the control without second phase. The yield for the 10 mL extractive phase on a certain day is the yield in the phase that was removed (and then renewed) on that day of sampling.

4.5.4 Pigment Stability

Pigment extraction from biomass can be successfully performed with ethanol, as reported previously [143]. To identify the best storage conditions for laetiporic acids, freeze-dried *L. sulphureus* biomass (containing pigments) and pigment extracts were tested in long-term experiments. Various parameters, such as light and temperature were tested. Preliminary stability trials of freeze-dried biomass, as well as extracts stored in light and at room temperature, showed a severe loss within a few weeks (**Supporting Figure 10**). Storing the freeze-dried biomass at room temperature but in the dark, resulted in a loss of 82% color within 6 months (**Figure 18a**). However, chilling the biomass stabilized the color, with 4 °C and −20 °C maintaining 32 and 77% of the original absorbance over the same time, respectively.

Compared to low stability of the pigment in the biomass over time, storage of ethanolic pigment extracts at 4 °C in the dark and flushing the extract with nitrogen resulted in 100% pigment stability over 6 months. Nitrogen also showed a stabilizing effect on extracts stored at room temperature (**Figure 18b**). Additional experiments with ascorbic acid improved

pigment stability as well, but were less effective compared to N_2 stabilization (data not shown).

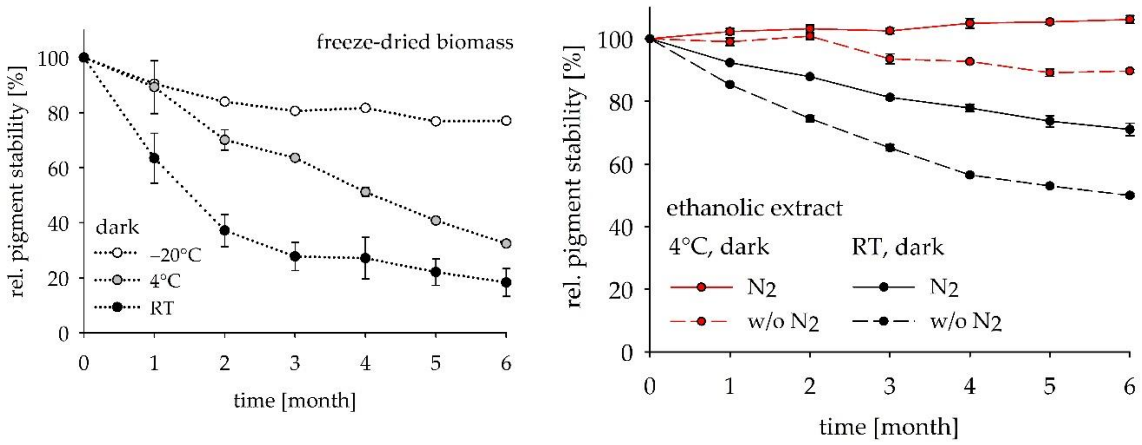


Figure 18. Stability of ethanolic *L. sulphureus* extracts and freeze-dried biomass over time, stored under different conditions. The difference in absorbance compared to t_0 is displayed as the percentage of remaining absorbance. Stabilities of (**a**) freeze-dried biomass stored at different temperature conditions in the dark; (**b**) ethanolic extracts stabilized with nitrogen.

4.5.5 Color Fastness of Dyed Silk

As shown in our previous paper [143], silk can be dyed directly with *Laetiporus sulphureus* biomass. To assess the stability of the pigment after heat dependent fabric dyeing, an accelerated light stability test was performed with differently dyed samples (**Table 3** and **Supporting Table 2**). Results showed that, independent of whether freeze-dried or wet biomass was utilized and of the dyeing conditions used, silk samples dyed with *L. sulphureus* biomass were not as light fast as the reference samples (**Figure 19a**). Changes in color appearance noticeable to the human eye occurred in all samples ($\Delta E \geq 1$), even the uncolored reference (data not shown). Control samples stayed below a ΔE of 5, while *L. sulphureus* samples faded faster. This resulted in higher ΔE values between 13 and 20, depending on the dyeing process used.

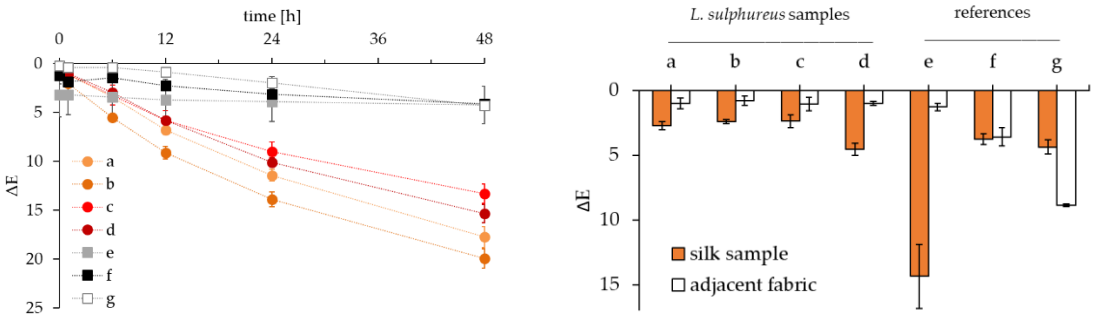
A washing stability test was undertaken with identically prepared silk samples. Silk dyed with madder (e) lost a lot of color during washing, resulting in ΔE values of about 14, while all other samples stayed below 5. Samples dyed with *L. sulphureus* freeze-dried biomass gave slightly better results than the sample dyed with fresh biomass ($\Delta E \approx 2.5$ for samples a, b, c and $\Delta E = 4.5$ for sample d). The staining of adjacent fabrics during washing was also

investigated. Industrial dyed silk (g) and Simplicol textile dye (f) perceptibly stained the adjacent samples, resulting in ΔE values of 9 and 4, respectively. The adjacent fabrics for all other silk samples remained undyed (ΔE ≈ 1).

Table 3. Silk samples were dyed with either biomass from *L. sulphureus* or commercially available dyes for 1 h at 90 °C, with varying amounts of ethanol added and differing after treatments.The industrially dyed silk (g) was not dyed in-house and thus also not according to these specifications.

Label	Dye	WOF [1]	Ethanol	Curing
a	*L. sulphureus*, freeze-dried biomass	20%	1%	-
b	*L. sulphureus*, freeze-dried biomass	20%	1%	vinegar
c	*L. sulphureus*, freeze-dried biomass	20%	30%	-
d	*L. sulphureus*, wet biomass	20%	1%	-
e	Dyer's madder	50%	-	-
f	Simplicol textile dye	40%	-	-
g	Industrially dyed silk	-	-	-

[1] Weight of Fiber (WOF): Amount of dyestuff used, given as the ratio of fiber.



(**a**) (**b**)

Figure 19. Comparison of the (**a**) light and (**b**) washing stability of silk samples dyed with *L. sulphureus* (a, b, c, d) and conventional textile dyes (e, f) against industrially dyed silk (g).

4.5.6 Application in Various Matrices

A crude ethanolic extract of *L. sulphureus* biomass was used to dye various products from the food and cosmetics category (**Figure 20**). Jelly gums and hard candies were chosen as examples for foods. Their successful coloration showed that laetiporic acids withstood production temperatures of up to 130 °C and retained their coloring abilities in low water activity matrices. A light and dark orange hard candy variant was obtained by incorporating 0.3 or 1.7% laetiporic acid extract. For cosmetics, soap, shampoo, powder, body lotion and toothpaste were dyed. The obvious dependency of the final coloring on the amount of extract

used for the dyeing process becomes evident when comparing the three small bottles containing soap, opaque shampoo and transparent shampoo that were stained with 2, 4 and 7% extract, respectively. To display the whole application spectrum tested so far, wool and silk dyed with unprocessed culture broth (biomass and supernatant; 90 °C for 1 h according to [62]) were added to the picture of samples (**Figure 20**, foreground).

Figure 20. Ethanolic extract of submerged cultivated *L. sulphureus* (bottle in the middle) used for application trials. From left to right: Jelly gum, soap, transparent and opaque shampoo, wool and silk, powder, hard candy, body lotion, toothpaste.

4.6 Discussion

Out of four *Laetiporus sulphureus* strains, the most potent laetiporic acid producer (strain B, DSMZ 11211) was selected. The results underline the significant differences between strains of the same species, and stress the importance of choosing the most suitable one for the metabolite/activity of interest as has been reported before, for example, for antimicrobial and laccase activity [146–148]. The special case of two identical strains obtained from the same strain collection at different time points (A and D in **Figure 15**), points out the effect of the conditions and methods used for and during strain maintenance that are normally not addressed in the method sections of publications. High deviations in enzyme activity of daughter generation monokaryons was reported for the basidiomycete *Pleurotus sapidus* (DSMZ 2866), stressing the intraspecific variability of monokaryotic strains [52].

Davoli *et al*. reported a maximum laetiporic acid A content of 6.7 mg/g for *L. sulphureus* strain 79110, with a dry matter concentration of around 3 g/L after 17 days of cultivation in shake flasks using a peptone corn steep glucose medium [98]. Seibold *et al*. described a maximum polyene concentration of 7.5 mg/g [85]. The herein presented *L. sulphureus* strain B exceeded the reported yields in biomass and pigment production by a factor of around 3 and 4, respectively (10.1 g/L dry matter, 27.7 mg/g laetiporic acid; **Figure 15a**).

Up-scaling from shake flask to bioreactor often leads to an overall growth and product yield improvement. With *L. sulphureus* strain B and Moser b medium, we achieved a final laetiporic acid yield of 250 mg/L in the shake flask culture at day 20 (**Figure 15b**), and were able to increase the yield to 690 mg/L at day 21 with Moser b medium and up to 1000 mg/L at day 12 with potato dextrose broth in a 7 L stirred tank bioreactor. Without regulation, *L. sulphureus* acidifies the culture to a pH of 2 or lower (**Figure 16**). Optimal pH values for the growth have been discussed in literature. Luangharn *et al*. investigated optimal growth conditions on agar plates by comparing colony diameters, and found pH 6 to 8 optimal for emersed mycelial growth [149]. Hwang *et al*. found the highest dry matter and exopolysaccharide concentration for an initial pH of 2 for *Laetiporus sulphureus* var. *miniatus* in shake flask cultures and STR [122]. In the present work, regulation of the pH by addition of NaOH, when values below 2 were reached, drastically reduced pigment yield (**Figure 16d**). Likewise, excessive sugar concentrations negatively impacted color formation (**Figure 16a**, 40 g/L total concentration of reducing sugars in Moser b). PDB yielded the highest product concentration (**Figure 16b**) and is slightly cheaper than Moser b (0.1 € per liter, roughly 3%). More severe is the personnel cost needed to prepare Moser b medium with its 15 different ingredients, in contrast to the ready-to-use PDB. Additionally, PDB could potentially be produced from the side stream potato peels and glucose. Therefore, the most cost-effective process for laetiporic acids production concerning yield and cultivation time is a bioreactor cultivation with PDB (12 days, 1 g/L pigment).

Downstream processing is often the most cost-intensive part in fermentative processes. Facilitating the product removal therefore leads to a more economic process that can compete better with petrochemical syntheses. During *in situ* extraction, the product (here laetiporic acid) was removed from the cells, hence shifting the balance towards product production, as described by the Law of Mass Action. A possible feedback inhibition by the product is avoided, resulting in higher product titers. The biotechnological production of vitamin A, for example, was improved by a factor of 2 in an engineered *Saccharomyces cerevisiae* by applying a biphasic *in situ* extraction [150]. The lipophilic product yield was improved by providing a lipophilic storage phase, as the intercellular storage capacity of the cells was limited. Inspired by the work of Große *et al*., who achieved a tenfold increase of the desired product *α*-ylangene by cultivation of *Tyromyces floriformis* in a biphasic system [151], different oils used for wood glazing were examined as a second, lipophilic phase during the submerged cultivation. Refined linseed oil use increased the final pigment yield by 19% in

comparison to the control culture, which could be used without cell disruption or solvent extraction of the biomass. The extractive phase could therefore be applied directly for dyeing purposes without additional downstream steps. Up-scaling of a biphasic system is difficult, but DAB, a Dutch biotechnology spin-off from TU Delft, is currently developing a suitable bioreactor concept ("Fermentation Accelerated by Separation Technology") [152].

Davoli *et al*. (2005) described the laetiporic acids to be "surprisingly stable in the presence of oxygen and light" [98] (p. 821) and concluded that they might be good food dyes. Seibold *et al*. (2020) supported these results. They exposed the isolated laetiporic acids A and B to light for 24 h and found no photoisomerization [85]. The herein presented extract storage (**Figure 18**) and light stability trials with dyed silk (**Figure 19a**) do not support these assessments. The reference set for the evaluation of stability led to a different interpretation. While Davoli *et al*. compared the laetiporic acids to very sensitive carotenoids, we chose robust textile dyes as control samples. Nevertheless, the ethanolic color extracts can be stabilized by exclusion of oxygen when stored in the dark and in cold temperatures (**Figure 18**). From visual observations, laetiporic acids seemed to be more stable when incorporated into a matrix, rather than applied on a surface. Thus, further studies were performed on the dyeing of different matrices. Keeping the ease of application, storage and transport for a potential industrial use in mind, all application trials were performed with the ethanolic extract instead of methanolic extracts or instead of the biomass, which had been used previously (**Figure 19** [143]). All tested matrices were dyed successfully. To the best of our knowledge, this is the first application of laetiporic acid as a colorant in food and cosmetic matrices (**Figure 20**). Klaus *et al*. investigated different extracts of *L. sulphureus* fruiting body and concluded that it could be applied as a food colorant, as no cytotoxic effects were provable [133]. Even though *L. sulphureus* fruiting bodies are edible at a young stage, and different sources state that mycelial extracts should therefore be generally recognized as safe (GRAS), the natural origin of fungal dyes does not automatically imply food safety [153]. Further studies on the safety profile of the dyed goods appear mandatory.

4.7 Supplementary Materials

4.7.1 Media Composition

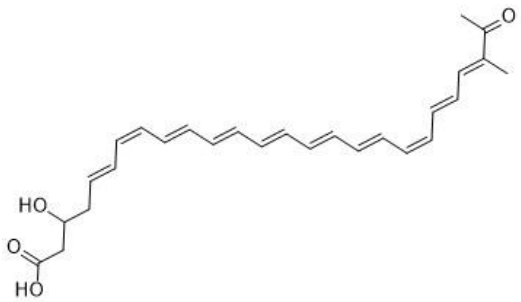
SNL Agar: 1 L SNL contains 30 g D-glucose monohydrate, 4.5 g L-asparagine monohydrate, 3 g yeast extract, 1.5 g KH_2PO_4, 0.5 g $MgSO_4$, 5 µg $CuSO_4 \cdot 5\ H_2O$, 80 µg $FeCl_3 \cdot 6\ H_2O$, 30 µg $MnSO_4 \cdot H_2O$, 90 µg $ZnSO_4 \cdot 7\ H_2O$, 400 µg EDTA and 20 g agar-agar.

pH is adjusted to 6.0

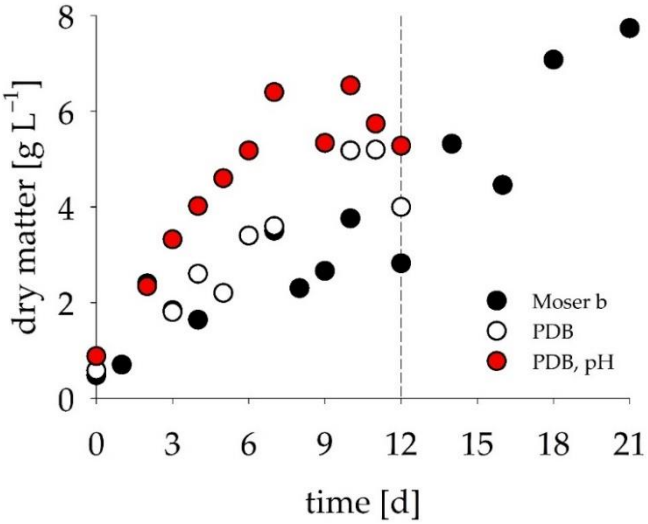
Moser b Medium: 1 L Moser b contains 30 g D-glucose monohydrate, 10 g malt extract, 2 g peptone, 0.15 g K_2HPO_4, 0.35 g KH_2PO_4, 1 g NH_4NO_3, 0.3 g $NaNO_3$, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 0.1 g $CaCl_2$, 1 mg biotin, 50 mg inositol, 18 mg $ZnSO_4 \cdot 7\ H_2O$, 10 mg $FeCl_3$, 5.6 mg $MnSO_4 \cdot H_2O$ and 50 mg thiamine hydrochloride.

pH is not adjusted

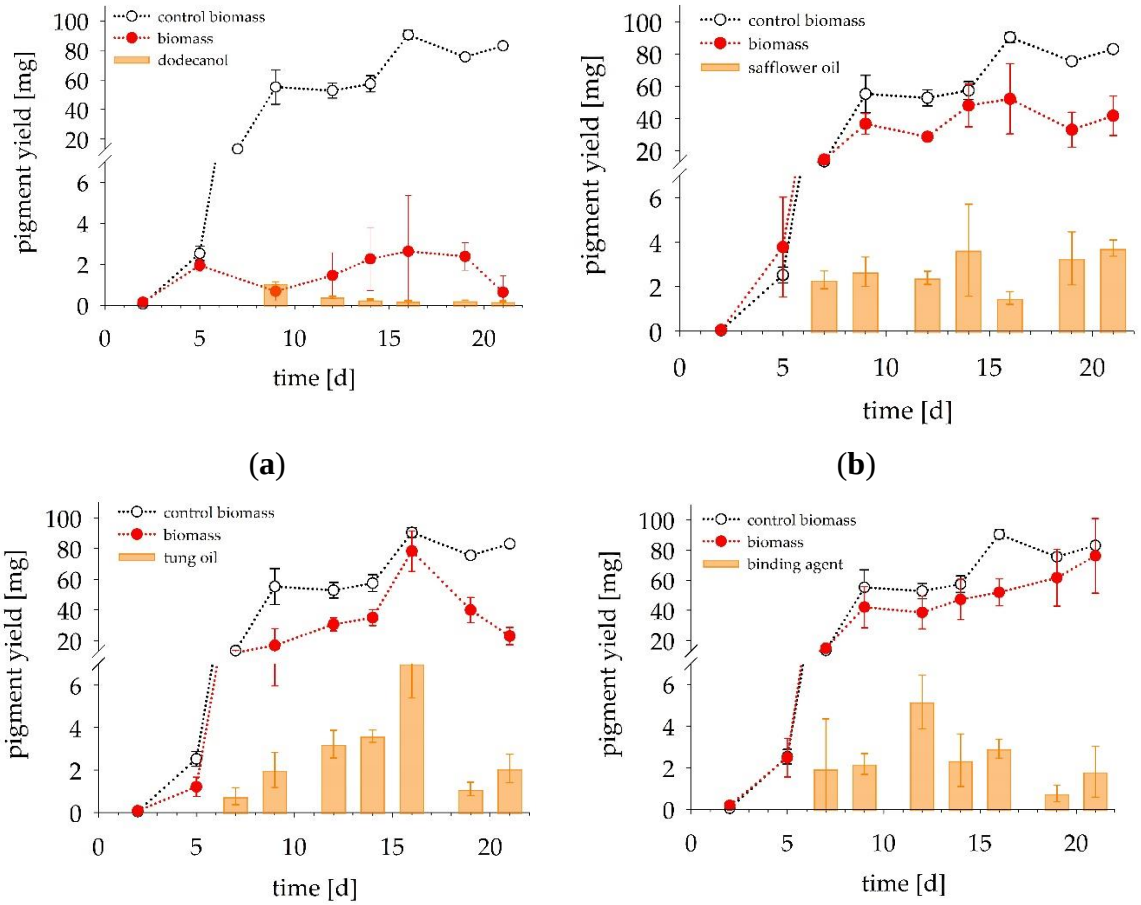
4.7.2 Supporting Data



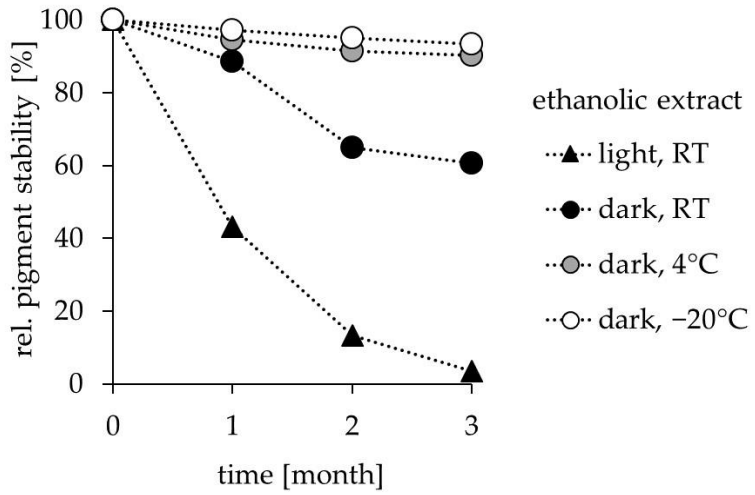
Supporting Figure 7. Laetiporic acid A, the dominant pigment found in submerged cultures of *Laetiporus sulphureus*.



Supporting Figure 8. Influence of different media (Moser b and PDB) and pH regulation (PDB, pH) on biomass yield in a 7 L STR.The harvest of PDB cultures is marked with a dashed line.

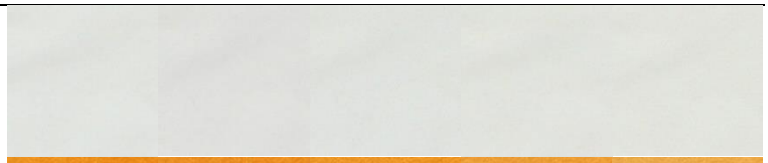
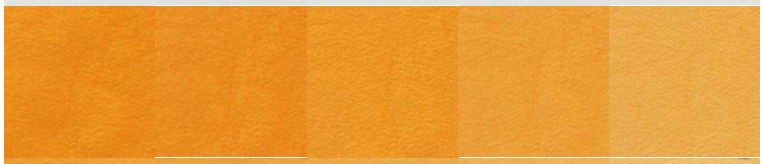


Supporting Figure 9. Laetiporic acid yields in biomass and lipophilic phase over the course of the cultivation during *in situ* extraction for (**a**) lauryl alcohol; (**b**) safflower oil; (**c**) tung oil and (**d**) binding agent. White dots depict the measurements for the control without second phase. The yield for the extractive phase on a certain day is the yield in the phase that was removed (and renewed) on that day of sampling.



Supporting Figure 10. Preliminary stability trials with ethanolic *L. sulphureus* extracts stored under different conditions (n = 1).

Supporting Table 2. Pictures of the silk samples over time.Samples were dyed with a) *L. sulphureus* dry matter and 1% EtOH; b) *L. sulphureus* dry matter, vinegar and 1% EtOH; c) *L. sulphureus* dry matter and 30% EtOH; d) wet biomass and 1% EtOH; e) dyer's madder; f) commercially available textile dye; g) industrially dyed silk.

time [h]	1	6	12	24	48
Reference					
a					
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4.8 Disclosures

Author Contributions: Conceptualization, P.B., A.W. and R.G.B.; methodology, P.B. and M.Z.; validation, P.B. and M.Z.; formal analysis, P.B., M.T., C.F. and O.R.; investigation, P.B., M.T., C.F. and O.R.; resources, A.W. and R.G.B.; data curation, M.Z. and P.B.; writing—original draft preparation, P.B., F.E. and R.G.B.; writing—review and editing, M.Z. and A.W.; visualization, M.Z. and P.B.; supervision, F.E., M.Z., A.W. and R.G.B.; project administration, F.E. and R.G.B.; funding acquisition, A.W. and R.G.B. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

5 Cultivation of *Inonotus hispidus* in Stirred Tank and Wave Bag Bioreactors to Produce the Natural Colorant Hispidin

5.1 Preamble to the Publication

Inonotus hispidus is a fungus belonging to the division of Basidiomycota that is used in traditional medicine and has been studied for its antimicrobial, anti-cancer and immuno-modulatory properties [154,155]. *I. hispidus* is known to cause soft-rot decay in wood, and its mechanisms of growth have been studied on beech wood [156]. The fungus mainly grows on five different tree species: *Morus alba*, *Ulmus macrocarpa*, *Fraxinus mandshurica*, *Ziziphus jujuba*, and *Malus pumila* [157]. It is rich in phenolic compounds, which contribute to its antioxidant activity [158]. Most of the bioactivities are attributed to hispidin, a polyphenol synthesized in fruiting bodies and liquid cultures of *I. hispidus*. Hispidin has been found in various other fungi like *Phellinus linteus* and *Phaeolus schweinitzii*, also known as the dyer's polypore [159,160]. In this article, we report the stimulation of hispidin synthesis in *I. hispidus*, upscaling of the bioprocess and application trials.

This article is a joint work of the Institute of Natural Materials Technology, Technical University Dresden (Marlen Zschätzsch and Anett Werner) and the Institute of Food Chemistry, Leibniz University Hannover (Pia Bergmann, Meike Takenberg, Christina Frank, Franziska Ersoy, and Ralf G. Berger).

I conceptualized the study and all published data was generated and interpreted by me. As part of her Bachelor thesis, Christina Frank investigated the influence of light and oxidative stress on hispidin yields of *I. hispidus* cultures under my supervision. Meike Takenberg was involved in the analysis of sugar consumption and experiments on the influence of precursors on pigment yields. Franziska Ersoy and Ralf G. Berger supervised the work scientifically and contributed to writing the original draft of this article. Marlen Zschätzsch and Anett Werner then reviewed and edited the manuscript. The resources and funding for the project were acquired by Ralf G. Berger and Anett Werner.

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Article

Cultivation of *Inonotus hispidus* in Stirred Tank and Wave Bag Bioreactors to Produce the Natural Colorant Hispidin

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5.2 Abstract

Hispidin (6-(3,4-dihydroxystyrl)-4-hydroxy-2-pyrone) production in submerged cultured mycelia of the basidiomycete *Inonotus hispidus* was doubled in shake flasks through irradiation with white light. The daily addition of 1 mM hydrogen peroxide as a chemical stressor and a repeated supplementation of the shake flask cultures with 2 mM caffeic acid, a biogenetic precursor, further increased the hispidin synthesis. These cultivation conditions were combined and applied to parallel fermentation trials on the 4 L scale using a classical stirred tank bioreactor and a wave bag bioreactor. No significant differences in biomass yield and colorant production were observed. The hispidin concentration in both bioreactors reached 5.5 g/L, the highest ever published. Textile dyeing with hispidin was successful, but impeded by its limited light stability in comparison to industrial dyes. However, following the idea of sustainability and the flawless toxicity profile, applications in natural cosmetics, other daily implements, or even therapeutics appear promising.

Keywords: *Inonotus hispidus*; hispidin; bioprocess; induction; elicitation; precursor; stirred tank reactor; wave bag reactor; natural dye

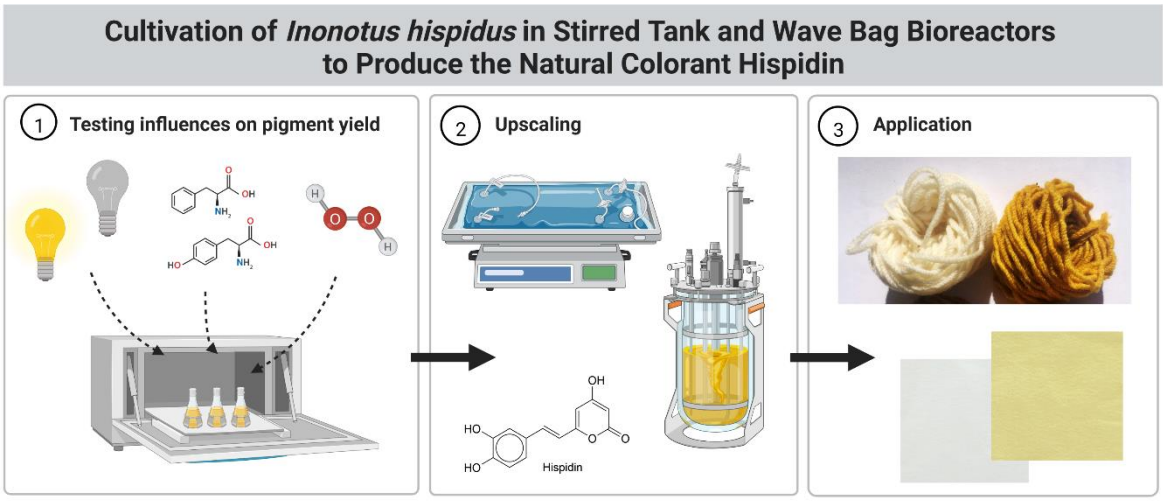


Figure 21. Graphical abstract of the publication [54].

5.3 Introduction

Inonotus hispidus, a basidiomycete from the class of Agaricomycetes, is popular in Asian folk medicine and supposed to cure many kinds of illnesses. Modern pharmacological studies have confirmed various bioactivities, which were frequently attributed to the occurrence of the yellow pigment hispidin [89,154,161–163]. While it is dominant in the metabolite spectrum, a number of other compounds seem to contribute to the observed antimicrobial, antiviral, antioxidant, anti-inflammatory, immunomodulatory, antiproliferative, and cytotoxic activities of extracts from this species [164–166]. Keto–enol tautomerism may result in an equilibrium between the lactone and the 4-pyrone form (isohispidin), a reaction giving rise to the formation of further, possibly bioactive oligomer compounds [164]. Much less work has been devoted to the use of hispidin as a natural colorant, with few sources reporting a potential application for dyeing [167]. The fruiting bodies of *I. hispidus* were recently used for textile dyeing, thus confirming earlier findings [64].

Natural dyes have been used since ancient times for the coloring of art and everyday objects of all kinds. The commencing industrialization in the 19th century changed the focus to the new synthetic dyes with an enormous potential for different hues combined with affordable prices. Currently, toxicity and environmental issues caused by synthetic dye production and the dyeing process, as well as a newfound appreciation for natural and sustainable products by consumers, have revived the search for natural dyes. Colorants extracted from plants or fruiting bodies of mushrooms provide alternatives, but suffer from numerous imponderabilities and can, with few exceptions, only be gained once per season [62,63]. Fruiting bodies from higher fungi (Basidiomycota) display a full color spectrum, but most

fungal cells stop to produce the colorants when transferred to submerged cultivation, where they form cell clusters.

As hispidin is synthesized in both the fruit body and the mycelium, cultures of *I. hispidus* were chosen as a prototype to study its production more closely [165,168]. Hispidin is partly derived from acetate via the polyketide pathway [20,169] and, as *I. hispidus* decays wood, from lignin degradation products [89]. Perrin *et al*. suggested already 50 years ago that hispidin was synthesized from cinnamic, *p*-coumaric, or caffeic acid [170]. In the closely related *Inonotus obliquus*, the addition of hydrogen peroxide led to a higher yield of hispidin derivates [171]. It was further found that hispidin synthesis was light-dependent, with blue light and a 30 min light period per day stimulating biosynthesis best [73,88,170]. Various cell culture models convincingly demonstrated the protective effects of hispidin, acting as a scavenger of reactive oxygen species (ROS) [172–174].

The purpose of this study was to fuse the findings detailed above to set up a bioprocess producing high yields of hispidin using *I. hispidus*. A comparative, parallel fermentation in a stirred tank and wave bag reactor was explored, as our previous work on the shear stress sensitive basidiomycetes showed that cultivation in a wave bag reactor resulted in highly dispersed mycelia, followed by higher biomass production and a doubled formation of peptidases [53]. The established bioprocess would present a sustainable cell factory to be run anywhere, anytime, and, in contrast to solid-state fermentation, on any scale without using arable land or jeopardizing endangered wild specimens.

5.4 Materials and Methods

5.4.1 Cultivation Parameters and Media

Inonotus hispidus (DSMZ 8658, verified by ITS sequencing after [175]) was incubated for 10 days at 24 °C on standard nutrient liquid agar plates (SNL; 30 g of D-glucose monohydrate, 4.5 g of L-asparagine monohydrate, 3 g of yeast extract, 1.5 g of KH_2PO_4, 0.5 g of $MgSO_4$, 5 µg of $CuSO_4 \cdot 5\ H_2O$, 80 µg of $FeCl_3 \cdot 6\ H_2O$, 30 µg of $MnSO_4 \cdot H_2O$, 90 µg of $ZnSO_4 \cdot 7\ H_2O$, 400 µg of EDTA, and 20 g of agar per liter, pH 6.0). All chemicals were purchased in pro analysis grade (Sigma Aldrich, Seelze, Germany; Merck, Darmstadt, Germany; Carl Roth, Karlsruhe, Germany), if not stated otherwise. Then, 1 cm^3 of the overgrown agar plate was transferred into 150 mL of Moser b medium (30 g of D-glucose monohydrate, 10 g of malt extract, 2 g of peptone, 0.15 g of K_2HPO_4, 0.35 g of KH_2PO_4, 1 g of NH_4NO_3, 0.3 g of $NaNO_3$, 0.5 g of $MgSO_4 \cdot 7\ H_2O$, 0.1 g of $CaCl_2 \cdot 2\ H_2O$, 1 mg of biotin,

50 mg of myo-inositol, 1.8 mg of $ZnSO_4 \cdot 7\ H_2O$, 10 mg of $FeCl_3$, 5.6 mg of $MnSO_4 \cdot H_2O$, and 50 mg of thiamine hydrochloride per liter) and homogenized (MiniBatch D-9 with DS-20/PF-SMIR, Miccra GmbH, Heiterheim, Germany) at 11,000 min^{-1} for 5 s. The preparatory culture was incubated on an orbital shaker (Multitron, Infors AG, Bottmingen, Switzerland) for 10 days at 24 °C and 150 rpm and used subsequently to inoculate the main cultures (10% *v/v*, 250 mL culture volume in total) after homogenization. All fermentations were carried out in triplicates. Bioreactors were sampled in triplicate.

5.4.2 Investigation of Different Influences on Pigment Yield

To test the influence of different light conditions, the window of an orbital shaker was obscured and equipped with LED stripes (1 m long, 60 LEDs, 4 W/m, PUR-LED GmbH & Co. KG), emitting light of different wavelengths. Wavelengths given by the manufacturer (400 nm, 470 nm, white light/full visible spectrum) were verified with a spectrometer (OceanOptics HR4000, wavelength range 200–1100 nm, resolution ~240 pm). The LED stripe labeled as "400 nm" exhibited a sharp intensity maximum measured at 394 nm, that labeled as "470 nm" exhibited a maximum at 464 nm, and "white light" exhibited a broad range from 420 to 740 nm with a maximum at around 590 nm. The agar plate, pre-culture, and main culture were kept in the dark. The main cultures were irradiated every day for 30 min, as described by Nambudiri *et al*. [73,168,175,175], for 6 days a week. The control culture was cultivated in an orbital shaker with windows, as were the cultures for the determination of the influence of hydrogen peroxide and the precursors.

Hydrogen peroxide was added from a 30% stock bottle to the cultures as described by Kavitha and Chandra [176]. Briefly, 10 or 25 mM final concentration was added once on day 4 of the cultivation. Alternatively, 1 mM hydrogen peroxide was added on days 4, 5, 7, 8, 9, 10, 11, 13, and 14 of the cultivation (a total of 252 µL 30% H_2O_2). For supplementation of precursors, L-tyrosine, L-phenylalanine, cinnamic acid, *p*-coumaric acid, and caffeic acid were dissolved in 1 mL of 70% ethanol and added to the cultures on days 4, 7, 9, and 11 to give a final concentration of 2 mM. For all shake flask experiments, 10 mL samples were taken on days 1, 5, 10, and 15.

5.4.3 Fermentation Experiments

For parallel fermentation experiments, *I. hispidus* was cultivated in a 6 L stirred tank bioreactor (STR) (Minifors 2, Infors AG, CH) and a 10 L single-use cultivation bag (ReadyToProcess Wave 25, Cytiva, Marlborough, MA, USA; utilized without lid) simultaneously, both filled with 4 L of Moser b medium and inoculated with 5% (*v/v*) starter culture (see Supporting Figure 5-1). For both systems, cultivation temperature was 24 °C with a constant air flow of 0.5 L/min and no specific light regulation. The agitation rate was set to 20–40 rpm at 9° platform angle for the bag cultivation and 150–500 rpm for the STR, controlled via the minimum dissolved oxygen value, which was set to 20% DO. For the bag cultivation, the DO was measured via optical sensors outside the bag; for STR cultivation, the reactor was equipped with an internal DO sensor (VisiFerm DO Sensor, Hamilton Bonaduz AG, Bonaduz, Switzerland). The pH value was measured externally for the bag cultivation; for STR, an Easyferm 325 pH electrode (Hamilton Bonaduz AG) was used. Moreover, the STR was equipped with a Rushton turbine. Then, 10 mL of rapeseed oil was added to both systems on day 2 of the cultivation as an antifoam agent. To dampen excessive foam formation, 30 mL of rapeseed oil was added to the STR on day 7. Caffeic acid and hydrogen peroxide (0.25 and 1 mM) were added on a daily basis after cultivation day 4, as the preceding shake flask experiments suggested a yield improvement. A sample was taken every day and measured in triplicate (3 × 5 mL).

5.4.4 Analytics

Samples taken in triplicate during the cultivation were centrifuged (15 min, 4 °C, 5000× *g*; Rotina 380 R, Hettich GmbH & Co KG. Tuttlingen, Germany) to separate biomass and supernatant. The supernatant was used for pH measurement (pH 211, Hanna Instruments, Vöhringen, Germany), and the total reducing sugars were determined after filtration with a 0.45 μm filter according to the method developed by Miller [123]. The filtered supernatant was analyzed by HPLC, but only negligible hispidin concentrations, presumably from cell lysis, were found. The biomass was washed with 5 mL of demineralized water once and freeze-dried (Alpha 1–4, Christ, Osterode am Harz, Germany). The dried biomass was weighed, and the concentration was determined. The dry matter was then extracted exhaustively with methanol (Carl Roth, Karlsruhe, Germany), and the extract was concentrated. After filtration, it was measured via HPLC, equipped with a system control unit (Shimadzu CBM-20A, Kyoto, Japan), degasser (Shimadzu DGU-20A5R), autosampler

(Shimadzu SIL-20ACHT), column (Chromolith® Performance, RP-18 endcapped, 100–4.6 mm), and DAD (Shimadzu Nexera X2 SPD-M30A) using the following conditions: flow 1.5 mL/min, injection volume 10 µL, detector 220–450 nm, oven 35 °C. The eluent gradient consisted of A (acetonitrile) and B (water + 0.1% formic acid) according to the following scheme: 0 min 98% B, 8 min 80% B, 13 min 0% B, 15 min 0% B, 16 min 98% B, and 17 min 98% B. Hispidin was measured at 9.4 min at λ_{max} = 369 nm. Hispidin concentration was calculated via external calibration with a hispidin standard (≥98%, Sigma Aldrich Chemie GmbH, Germany) using eight concentrations between 4 and 240 mg/L (regression: $y = 18564x - 83881$, $R^2 = 0.9896$). Cinnamic acid (Ret. 11.0 min, λ_{max} = 277 nm), *p*-coumaric acid (Ret. 7.2 min, λ_{max} = 309 nm), and caffeic acid (Ret. 5.7 min, λ_{max} = 323 nm) were measured with the same method, using external standards for quantification. L-Tyrosine and L-phenylalanine concentrations were determined as described by Rottmann *et al*. [177], and the identity of hispidin was confirmed by mass spectrometry using conditions as previously detailed.

5.4.5 Application of Cultivation Extracts

An accelerated light stability test was performed in triplicate with dyed silk. Samples were dyed with *I. hispidus* culture broth and tested against silk samples dyed with Dyer's madder (WEJA Färbeset Resedgelb, Livos Pflanzenchemie, Germany) and commercially available textile dyes (Simplicol Textilfarbe expert–Mais Gelb, Brauns-Heitmann GmbH & Co. KG; Marabu Easy Color gelb, Marabu GmbH & Co. KG; Dylon Sunflower Yellow, Henkel AG & Co. KGaA, Germany). Samples were prepared by heating 100 g of silk or wool at 90 °C for 1 h in the respective liquor. Samples were not treated with mordants or fixing agents other than those present in the commercial products. The samples were radiated for 48 h with 400 W/m^2 at 15 °C in an Atlas Suntest XLS+, equipped with a Xenon lamp and window glass filter (Atlas Material Testing Technology GmbH, Linsengericht-Altenhaßlau, Germany). The color was measured after 1, 6, 12, 24, and 48 h with a Konica Minolta CM-600d (Geometry 8°, measurement area 8 mm, reflection mode, specular component excluded) and analyzed with the program Colibri, version 3.8.12. The given ΔE values, as defined by the International Commission on Illumination, reflect the color difference of a sample (t_x) compared to a reference (t_0). For documentation, pictures were taken with a Canon EOS 800D with an 18–55 mm EF-S zoom lens, in a PackshotStart Mark II (Sysnext, Levallois-Perret, France) (see **Supporting Table 3**).

5.5 Results

In an early stage of the research, it was observed that *I. hispidus* cultures grew morphologically different when cultivated in Moser b medium as compared to standard nutrient liquid (SNL). While both cultures yielded around 100 g of wet biomass per liter after 11 days (SNL 110 ± 19 g/L, Moser b 91 ± 6 g/L), absorbance of the supernatant at λ = 365 nm differed significantly, indicating a much stronger color formation in Moser b medium. Thus, all subsequent experiments were performed using Moser b medium.

5.5.1 Influence of Light

I. hispidus was cultivated under different light conditions. Changes in irradiation slightly delayed biomass production compared to the control setup (incubator with windows, but without extra illumination), but had no effect on the final dry matter concentration of about 9 g/L after 15 days of cultivation (**Figure 22**). Hispidin generation started around day 10, when biomass production ceased. Pigment yields were dramatically reduced in the dark. Illumination with λ = 400 nm restored them to roughly the same amount as under control conditions (49.1 and 60.2 mg/g, respectively). An increase of about 50% on day 15 was obtained by illumination with 470 nm (89.8 mg/g), and white light more than doubled the yield to a final 131.6 mg/g hispidin.

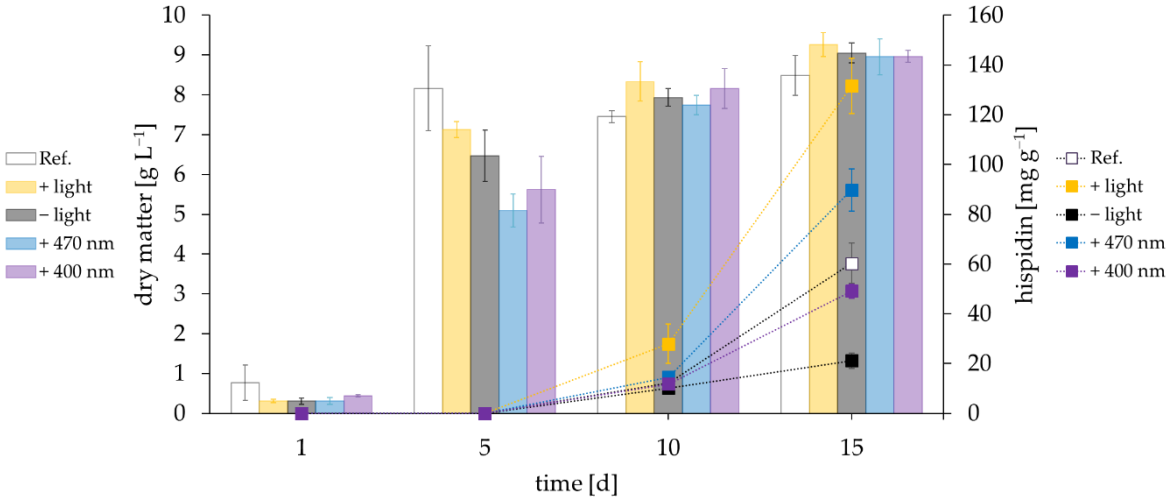


Figure 22. Biomass (bars, left ordinate) and hispidin yields (squares, right ordinate) of differently irradiated cultures of *I. hispidus*. Cultures were either kept in the dark (−light, depicted in black/gray) or irradiated with LEDs, specified as 400 nm (purple), 470 nm (blue), and white light (+light, depicted in yellow). The reference culture (Ref., depicted in white) was cultivated in an incubator with a glass window, but without specific light regulation.

5.5.2 Influence of Oxidative Stress

Different concentrations of hydrogen peroxide were added either daily (1 mM) or once on day 4 to determine the influence of oxidative stress. Both 10 and 25 mM slightly reduced biomass generation (**Figure 23**). As seen before (**Figure 22**), pigment generation started again on day 10. The single dosages of H_2O_2 decreased the hispidin yield on day 15, but daily addition of 1 mM H_2O_2 increased the yield by 45% up to 87.8 mg/g when compared to the control.

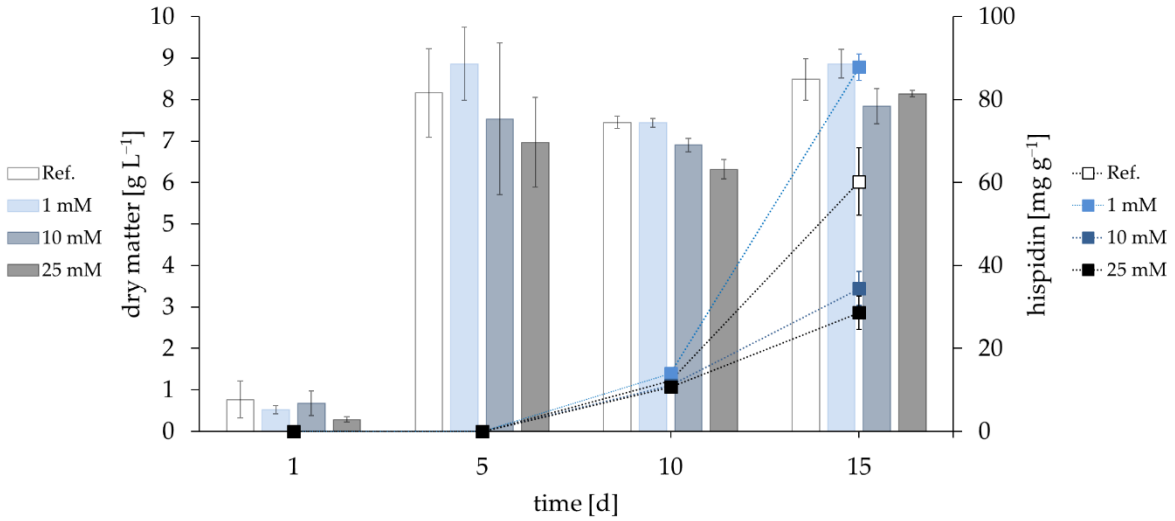


Figure 23. Biomass (bars, left ordinate) and pigment yields (squares, right ordinate) of cultures of *I. hispidus* exposed to oxidative stress through addition of hydrogen peroxide. The indicated concentration of H_2O_2 was added either daily (1 mM) or once during the cultivation on day 4 (10 mM and 25 mM). As the cultivations evaluating the influences of light and oxidative stress were performed in parallel, the control in **Figure 22** and **Figure 23** was identical.

5.5.3 Influence of Precursors

Following earlier biogenetic work [89,168] (**Figure 24**), 2 mM L-tyrosine, L-phenylalanine, cinnamic (CiA), *p*-coumaric (CuA), or caffeic acid (CaA) was supplemented to *I. hispidus* cultures on cultivation day 4, 7, 9 and 11. All cultures developed equally until the precursors were added on day 4 (**Figure 25**). Both cinnamic and *p*-coumaric acid completely inhibited pigment formation, but also negatively affected both biomass generation and sugar consumption, with cinnamic acid having a more severe effect. This was also reflected by the pH of the cultures, which was slightly (2.7, *p*-coumaric acid) and significantly (3.6, cinnamic acid) increased compared to all other cultivations (pH~2.5).

Cultures supplemented with L-tyrosine, L-phenylalanine, and caffeic acid showed no significant differences to the reference (Ref.) regarding biomass generation, pH, or

consumption of reducing sugars. All of them reached final dry matter concentrations of around 10 g/L. L-Phenylalanine slightly raised pigment yield to 25.2 mg/g on day 14 compared to the control (19.4 mg/g). Caffeic acid enhanced pigment synthesis significantly, yielding 53.4 mg hispidin per gram dry matter on day 14.

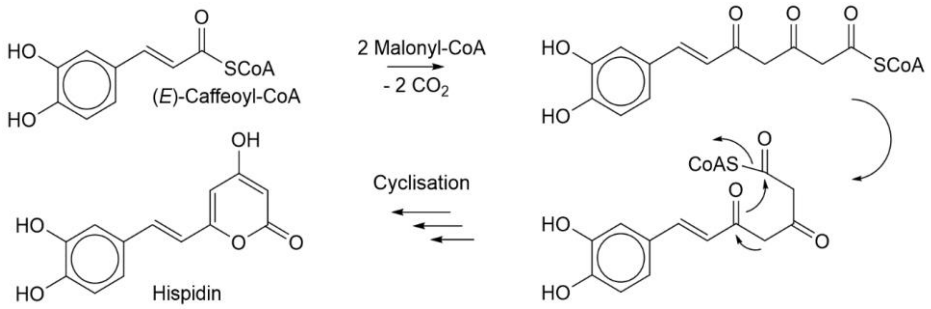


Figure 24. Proposed biogenesis of hispidin in *I. hispidus* after [89]. Caffeic acid is elongated by two molecules (malonyl-CoA) with the release of carbon dioxide. After cyclization, the target molecule hispidin is formed. A detailed portrayal of the proposed pathways can be found in Lee and Yun (2011).

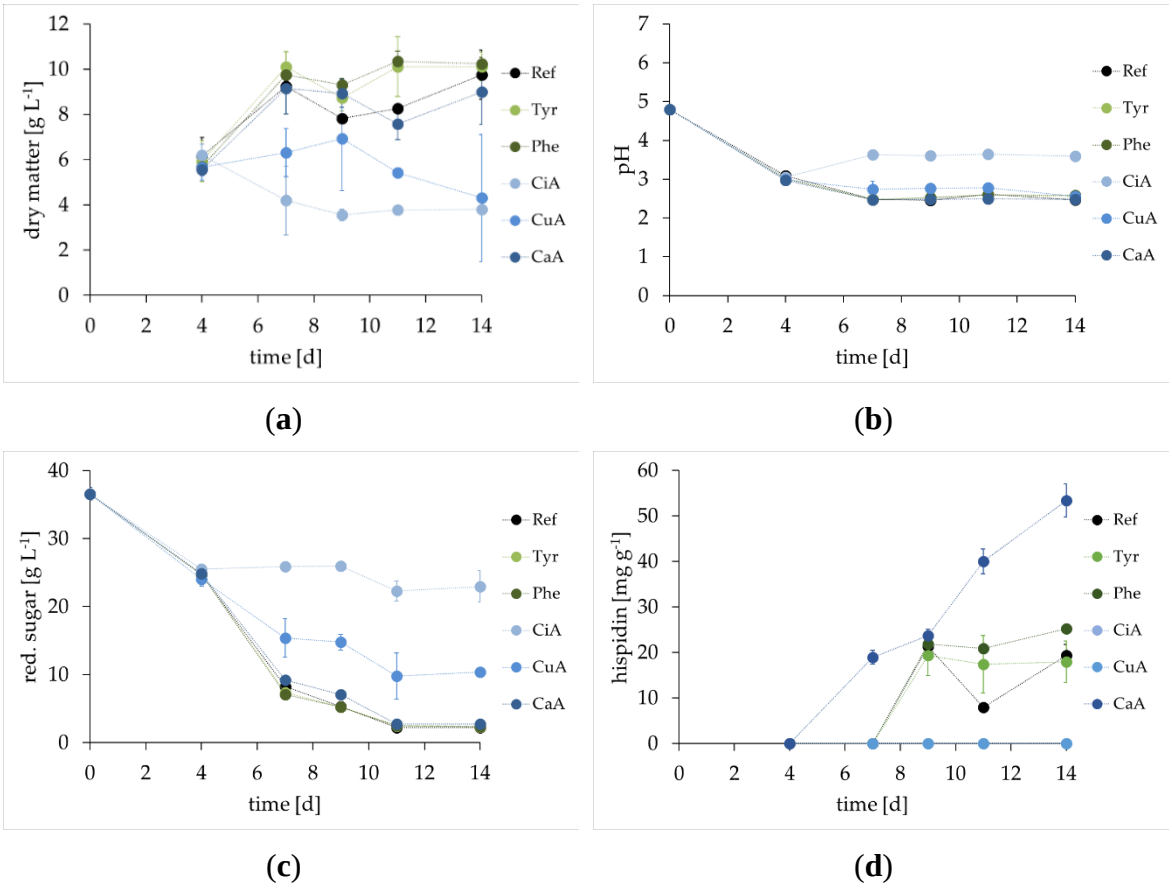


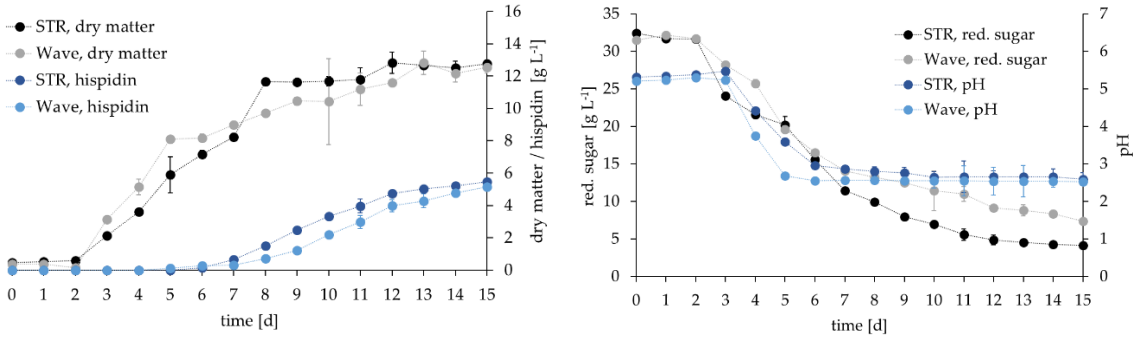
Figure 25. Cultivation of *I. hispidus* with precursors (2 mM supplemented on days 4, 7, 9, and 11). The control (Ref.) is depicted in black, L-tyrosine (Tyr) in light green, L-phenylalanine (Phe) in dark green, cinnamic acid (CiA) in light blue, *p*-coumaric acid (CuA) in blue, and caffeic acid (CaA) in dark blue. Comparison of (**a**) biomass yield, (**b**) pH value, (**c**) total content of reducing sugars, and (**d**) hispidin concentration.

5.5.4 Comparative Cultivation in Two Types of Bioreactors

Full-spectrum white light and the addition of 1 mM H_2O_2 (daily) and 2 mM caffeic acid on days 4, 7, 9, and 11 increased hispidin biosynthesis when applied separately (see above). To verify if the effects could be combined to increase the overall pigment yield and to compare two different bioreactor types, parallel cultivations were set up in a stirred tank (STR) and a bag bioreactor (Wave). The same preculture was used, and every effort was made to operate the two bioreactors under identical biological and chemical conditions to filter out merely the effects of the different bioreactor constructions (**Supporting Figure 11**).

Initially, the mycelium grew slightly better in the wave as indicated by the higher biomass and lower pH around day 4, but this was not reflected by the consumption of reducing sugars (**Figure 26**). From day 7 on and, thus, during the active synthesis of hispidin, the STR showed

slightly quicker biomass and hispidin generation. Both reactor types reached similar final concentrations of 12.8 and 12.6 g/L dry matter, and 5.5 and 5.2 g/L hispidin for the STR and wave, respectively (**Figure 26a**). The slightly better growth in the STR matched the more pronounced decrease in the content of reducing sugars (**Figure 26b**). In comparison to shake flask cultivations (9 to 10 g/L, **Figure 22** and **Figure 23**), the cultivation in both bioreactors led to a slight increase of the final biomass (>12 g/L). The hispidin yields were significantly improved: The g/L data indicated in **Figure 26a** correspond to 428.5 (STR) and 410.9 mg hispidin per gram dry matter (wave), respectively, an increase by a factor of seven compared to the control (**Figure 22** and **Figure 23**) and by a factor of three compared to the best single-parameter change in shake flasks, with full illumination (**Figure 22**). Both biomass and hispidin generation kinetics were comparable to those observed in the shake flasks.



(**a**) (**b**)

Figure 26. Parallel cultivation of *I. hispidus* in a stirred tank (STR, depicted in black and dark blue) and cell-bag bioreactor (wave, depicted in gray and light blue). (**a**) Pigment and biomass yield, (**b**) consumption of reducing sugars, and pH over the course of cultivation.

5.5.5 Application of Hispidin as a Colorant

For a long time, extracted fruiting bodies of *I. hispidus* have been used for textile dyeing. Any large-scale application, however, would require a permanent and economical hispidin source. Submerged cultures serve this purpose. Wool and silk were dyed with *I. hispidus* culture broth (for silk samples see **Supporting Table 3**), and a preliminary stability test against commercial textile dyes was conducted with the silk samples. The initially obtained coloring on silk appeared easy on the eye, but accelerated light stability tests demonstrated that hispidin was not as lightfast as the plant-based or synthetic dyes. Changes in color appearance noticeable for the human eye occurred in all samples (even in the noncolored reference). Control samples stayed below a ΔE of 5 during the 48 h of illumination, while

I. hispidus samples faded more quickly. They reached ΔE values over 5 within 6 h and of about 17 after 48 h (**Figure 27**).

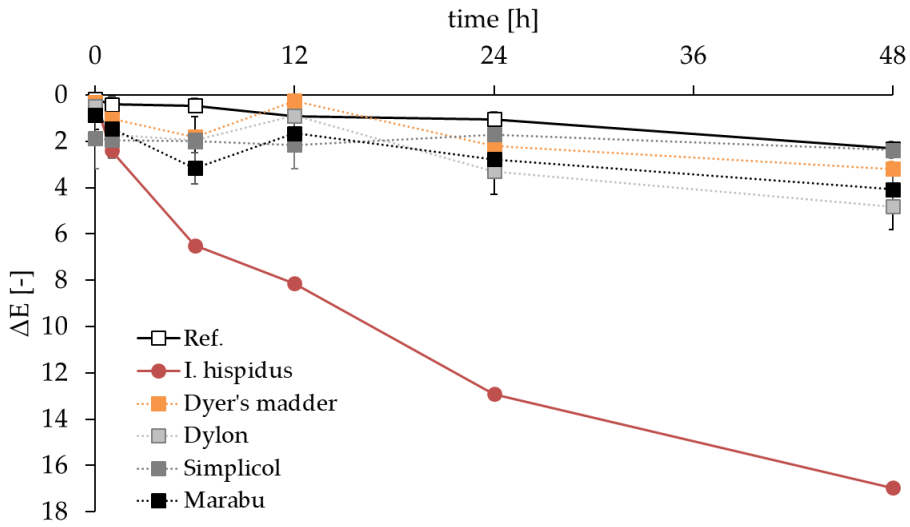


Figure 27. Comparison of the light stability of silk samples dyed with *I. hispidus* (red) and commercially available plant-based (Dyer's madder, depicted in orange) and synthetic textile dyes (Dylon, Simplicol, and Marabu depicted in gray, dark gray, and black, respectively). The noncolored reference (Ref.) is shown in white squares.

5.6 Discussion

In plants, the key step from L-phenylalanine to phenylpropenoid acids is catalyzed by the blue-light dependent phenylalanine/tyrosine ammonia-lyase (PAL). A maximum promotor activity in carrot was observed using UV-B light [178], which agreed with earlier findings for hispidin formation in *I. rheades*. This species accumulated hispidin in mycelium under blue light, yielding 8.1 mg styrylpyrones per gram dry matter [72]. In this work, a final concentration of 131.6 mg hispidin per gram dry matter was obtained under white light (**Figure 22**). Comparison of chromatographic and mass spectrometry data (ESI-MS: $M + H^+$ = m/z 247, $M - H^+$ = 245; a pair of signals indicating (E/Z)-isomers in similar concentration) with the reference compound confirmed the identity [72].

As the laboratory glass of the Erlenmeyer flasks and the stirred tank used for the submerged cultures in this study showed a blue-light cutoff at around 400 nm, only wavelengths >400 nm were examined. White light with an emission maximum at around 590 nm gave the best results (**Figure 22**). At the same time, the best biomass yields were recorded, indicating that the observed improvement was less attributable to PAL induction, but rather to a nonspecific

stimulation of the cell metabolism, perhaps to protect against ROS [172–174]. Illumination of the cell cultures with UV light would be possible by mounting waterproof UV lamps inside the bioreactor, but at the risk of promoting the mutation rate of the cultures. Nevertheless, such an experiment, perhaps with an intermittent illumination regime, seems appealing, especially to prepare for an upscaling of the bioprocess.

Hispidin generation started around day 10, when biomass production was nearly finished, suggesting a shift to secondary metabolism in the stationary phase of the culture. This poses the question for the ecological role of hispidin. The discovery of luminous fungi could provide an answer. Fruiting bodies of *Mycena chlorophos*, *Omphalotus japonicus*, and *Neonothopanus gardneri* shared a common mechanism of bioluminescence using hispidin as a luciferin precursor [179]. Hydroxylation of hispidin to 3-hydroxyhispidin, the "fungal luciferin", may also occur in *I. hispidus* and related hispidin-producing fungi [180].

Inspired by the work of Zheng *et al*. on *I. obliquus*, hydrogen peroxide was applied as a chemical stressor (**Figure 23**) [171]. The use of this chemical was preferred over others not only because basidiomycetes, as is commonly known, generate this compound through secreted hexose and aryl oxidases and dispose a surplus of the oxidant via catalase activity, but also because heavy-metal ions or fungicidal compounds would not have fitted the idea of a sustainable and natural process. Furthermore, the phenolic structure of hispidin confers the known antioxidative capacity and, in turn, a defense mechanism against exogenous hydrogen peroxide [172–174,181]. Despite the small size of the dataset, it can be concluded that higher concentrations of the stressor (10 and 25 mM) exceeded the tolerance of the cells resulting in growth retardation. The subliminal stress exerted by the daily application of 1 mM hydrogen peroxide, however, gave a significantly higher hispidin yield, confirming the earlier results [171].

The hispidin molecule is built from a shikimate derived phenylpropenoid unit by a polyketide-type chain elongation [20,165,168,169], as proven by ^{14}C-tracer studies [170] and the detection of hispolon, the intermediate after elongation by one malonate unit, in some extracts [163]. It was hypothesized that the acetate pool of well-growing cells should be filled up, while mycelia growing in vitro should lack the typical phenylpropenoid lignin degradation products liberated by a parasitizing fruiting body growing on wood. The parallel cultures thrived well until 2 mM L-tyrosine, L-phenylalanine, cinnamic (CiA), *p*-coumaric (CuA), or caffeic acid (CaA) was supplemented on day 4 (**Figure 25**). Both CiA and CuA immediately inhibited growth and hispidin formation. Although known as fungicides, it was

hypothesized that *I. hispidus* would possess enough hydroxylase activities to convert them to nontoxic caffeic acid, but this could not be confirmed as the mycelia did not recover until the end of cultivation. Both aromatic amino acids showed negligible effects compared to the control culture, another piece of evidence for the missing involvement of a PAL activity. Only caffeic acid, the phenylpropenoic acid structure closest to the product structure, had a pronounced effect, lifting the hispidin yield from around 19 mg/g (control) to 53 mg/g after 2 weeks (**Figure 25**). With the principle of circular bioeconomy in mind, the residual caffeic acid present in coffee grounds could serve as a source of caffeic acid in the future. Our data showed that around 1.8 of the 7 g/kg of caffeic acid remained in spent grounds after a common hot-water extraction of coffee powder. The decrease in hispidin yield of the reference experiment in comparison to the reference in **Figure 22** and **Figure 23** (19.4 vs. 60.2 mg/g, respectively) was most likely due to the addition of the precursors solved in 70% ethanol (final 0.28% ethanol in the culture on the day of addition). Further experiments with final 1.4% ethanol in the cultures strongly inhibited pigment production (data not shown). For future applications, care has to be taken to remove or minimize this effect by choosing another solvent.

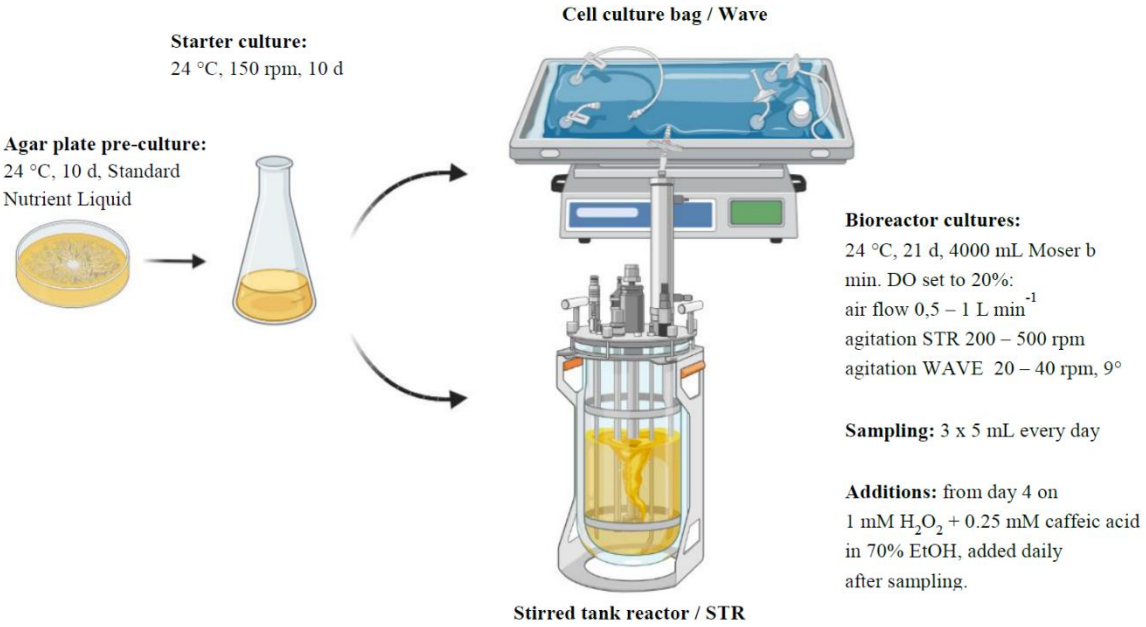
The combined effects of illumination and addition of 1 mM H_2O_2 and 2 mM caffeic acid were compared in parallel cultivations using a STR and a wave bioreactor (**Figure 26**). The wave was originally developed for the cultivation of shear sensitive animal cells. The thin cells of fungal mycelia could equally benefit from this cultivation mode as confirmed previously for the production of peptidases [53]. In contrast to expectations, this was not observed. Both bioreactors produced similar concentrations of biomass (STR vs. wave: 12.8 vs. 12.6 g/L), and hispidin (STR vs. Wave: 5.5 vs. 5.2 g/L) (**Figure 26a**). For comparison, the overall highest yield was reported by Park *et al*. (2004), who achieved a maximum of 2.5 g/L hispidin yield using *Phellinus linteus* cultivated in shake flasks [166]. The more controlled cultivation in the bioreactors resulted in a better biomass yield compared to the shake flask cultivations (12.8 vs. 9 g/L, **Figure 22**). The hispidin yields increased even more significantly when compared to shake flask cultures, from 131.6 to 428.5 mg/g dry matter. Earlier experiments with the wave bioreactor without addition of caffeic acid and H_2O_2 reached 3.9 g/L hispidin on cultivation day 13 (data not shown). These values are not fully comparable to the data presented in this manuscript, as different precultures were used. Nonetheless, the addition of both inducers resulted in 4.3 g/L hispidin on day 13 (**Figure 26a**), which amounts to an increase of 10%.

The successful cultivation of basidiomycetes in an STR goes along with numerous problems; inactivation of probes due to adherence of cells, excessive foaming, and subsequent plugging of waste air filters are among the worst. As a result, it took several attempts to operate the STR properly. On the other hand, the wave is a single-use device, and scale-up is currently limited to 300 L. In solid-state fermentation trials using *Phellinus linteus*, 1.107 mg hispidin per gram was achieved after 6 weeks [182]. This corresponds to a roughly 390-fold increased yield compared to the highest yield obtained in the STR after 2 weeks in this study (428.5 mg/g). In addition, obstacles of the solid-state approach are the lack of fermentation control and the problematic scale-up. A 20 ton submerged fermentation was reported, but the yield of 3 mg/g hispidin was low [183].

All colorants bleach when exposed to light (and oxygen) over time, because they absorb electromagnetic waves from the visible spectrum; otherwise, they would not appear colorful. The absorbed energy is usually converted into heat, but in part also responsible for the excitation of bonds to react with oxygen and, in the UV range, for the direct cleavage of covalent bonds, which results in colorless products. Natural colorants are well known to bleach faster, which was also the case for hispidin (**Figure 27**). However, it was stable for months in the refrigerator (data not shown). Neither the Ames test, in vitro chromosome aberration test, acute oral toxicity test, nor bone marrow micronucleus test detected toxicological properties of hispidin [183]. It may, thus, be favorably used to dye enclosed or opaque products, such as soap, shampoo, body lotion, or toothpaste with additional health benefits (see Section 5.3). Textile or wood dyeing [64,167] would require UV filters, perhaps mordants, or other fixing agents.

In conclusion, a combination of physical and chemical parameters combined in an optimized bioprocess may yield hispidin in concentrations that would allow to substitute petrochemical-based, toxicologically problematic synthetic dyes for a number of applications. In addition to its utilization for dyeing purposes, the reported bioprocess might also be used to generate hispidin to study or implement its plethora of previously reported bioactivities.

5.7 Supplementary Materials



Supporting Figure 11. Overview of the experimental steps to inoculate the two bioreactor types with mycelia from the same pre-culture, and operating conditions for the comparison of the two bioreactors for hispidin production.

Supporting Table 3. Pictures of the silk samples over time.

time [h]	0	1	6	12	24	48
Reference						
I. hispidus						
Dyer's madder						
Dylon						
Marabu						
Simplicol						

Supporting Figure 12. Left: undyed wool, right: wool dyed with *I. hispidus* submerged culture.

5.8 Disclosures

Author Contributions: Conceptualization, P.B., A.W. and R.G.B.; methodology, P.B.; validation, P.B.; formal analysis, M.T., C.F. and P.B.; investigation, M.T., C.F. and P.B.; resources, R.G.B.; data curation, P.B. and F.E.; writing—original draft preparation, P.B., F.E. and R.G.B.; writing—review and editing, M.Z. and A.W.; visualization, C.F., M.T., and P.B.; supervision, F.E., M.Z., A.W. and R.G.B.; project administration, F.E. and R.G.B.; funding acquisition, A.W. and R.G.B. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

6 Outlook

In this thesis, the biotechnological production of fungal colorants was presented in two examples. The biotechnology of Basidiomycota contributes another piece to the circular bioeconomy puzzle, with advantageous attributes like independency from fossil oils, cheap and sustainable substrates, synthesis at mild conditions and no toxic solvents needed. The extracted biomass could finally be deployed in biogas plants or processed further. Biotechnologically produced colorants will not be dependent of seasonal harvest and be operable at any scale at any time.

As Basidiomycota thrive well on non-food side-streams of the agro-industry, such as straw, sawdust, flax, peels, press cakes, pomace, molasses and fibres; those substrates could be used in future. They occur in large volumes and typically end up as fertilizer, feed additives or in biogas plants. As the nutrition medium and the downstream process (product recovery) are the most cost-intensive parts of a bioprocess, the use of cheap substrates can make the production more cost-efficient.

The fact that many other research institutions and companies are working on the replacement of petrochemically synthesized dyes demonstrates the urgency of this topic. Other approaches than presented in this thesis are being investigated: for example the heterologous production of plant colorants for food applications by Phytolon [184], aiming to spare the large scale extraction of high value edible resources. The fermentative production of textile dyes from (engineered) microorganisms is pursued by several start-ups [185–187]. Last but not least, the existing dyeing processes are being reviewed for improvement with new technologies like super critical CO_2 [188] or incorporating the pigment into the fibre [189] for waterless textile dyeing.

But before any of the reported colorants can be used in products for the European market, the safety needs to be examined thoroughly. The same restrictions apply for hispidin and laetiporic acid, as no toxicity tests were conducted in this thesis.

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8 Curriculum Vitae

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Education

2019 – 2023	Doctoral Research Study Leibniz University Hannover, Institute of Food Chemistry Dissertation: Biotechnological Production of Fungal Colorants
2017 – 2019	Master of Science Ostwestfalen-Lippe University of Applied Sciences Life Science Technologies / Bioprocessing
2013 – 2017	Bachelor of Engineering Bremerhaven University of Applied Sciences Food Technology / Food Economics
2005 – 2013	General Qualification for University Entrance Marion-Dönhoff Secondary School Nienburg

Work Experience

2023 –	Associate Lecturer Hochschule Hannover - University of Applied Sciences and Arts, Department of Bioprocess Engineering
2019 – 2023	Research Associate Leibniz University Hannover, Institute of Food Chemistry (Prof. Dr. Ralf G. Berger)
2019	Research Assistant Technical University Braunschweig, Institute of Food Chemistry (Prof. Dr. Peter Winterhalter)
2018 – 2019	Research Assistant Ostwestfalen-Lippe University of Applied Sciences, Institute of Food Chemistry (Prof. Dr. Jürgen Zapp)
2016 – 2017	Student Assistant Chr. Hansen GmbH, Nienburg/DE

2015	Internship Semester Chr. Hansen A/S, Hørsholm/DK
2014 – 2016	Student Assistant Bremerhaven University of Applied Sciences, Institute of Applied Microbiology and Biotechnology (Prof. Dr. Matthias Nagel)

9 List of Publications

Bergmann, P.; Frank, C.; Reinhardt, O.; Takenberg, M.; Werner, A.; Berger, R.G.; Ersoy, F.; Zschätzsch, M. Pilot-Scale Production of the Natural Colorant Laetiporic Acid, Its Stability and Potential Applications. *Fermentation* **2022**, *8*, 684, doi:10.3390/fermentation8120684.

Bergmann, P.; Takenberg, M.; Frank, C.; Zschätzsch, M.; Werner, A.; Berger, R.G.; Ersoy, F. Cultivation of *Inonotus hispidus* in Stirred Tank and Wave Bag Bioreactors to Produce the Natural Colorant Hispidin. *Fermentation* **2022**, *8*, 541, doi:10.3390/fermentation8100541.

Januschewski, E.; Bischof, G.; Thanh, B.N.; Bergmann, P.; Jerz, G.; Winterhalter, P.; Heinz, V.; Juadjur, A. Rapid UV/Vis Spectroscopic Dye Authentication Assay for the Determination and Classification of Reactive Dyes, Monascus Pigments, and Natural Dyes in Coloring Foodstuff. *J. Agric. Food Chem.* **2020**, *68*, 11839–11845, doi:10.1021/acs.jafc.0c03676.

Zschätzsch, M.; Steudler, S.; Reinhardt, O.; Bergmann, P.; Ersoy, F.; Stange, S.; Wagenführ, A.; Walther, T.; Berger, R.G.; Werner, A. Production of natural colorants by liquid fermentation with *Chlorociboria aeruginascens* and *Laetiporus sulphureus* and prospective applications. *Eng. Life Sci.* **2021**, *21*, 270–282, doi:10.1002/elsc.202000079.

Parts of this dissertation were presented at the following events:

Regional Association Conference 2023 - North and Northeast, Hannover/DE, 20th – 21st March 2023
Organized by GDCh e.V., Lebensmittelchemische Gesellschaft (German Chemical Society, Society of Food Chemistry)
Contribution: P. Bergmann: „Biotechnologische Produktion von Farbstoffen aus Basidiomyceten“ (Lecture)

Leibniz AnsprechBAR, Hannover/DE, 5th November 2022
Organized by Leibniz University Hannover
Contribution: P. Bergmann: „Farbstoffe – Das Auge isst mit! Färben wir zukünftig mit Naturfarben aus Speisepilzen?“ (Poster)

(Bio)Process Engineering - a Key to Sustainable Development, Aachen/DE, 12th – 15th September 2022
Organized by DECHEMA e.V. (Society for Chemical Engineering and Biotechnology)
Contribution: P. Bergmann, M. Takenberg, C. Frank, F. Ersoy and Ralf G. Berger: “Biotechnological Production of Basidiomycetous Pigments for Textile Dyeing” (Poster)

Leibniz University Chemistry Symposium, Hannover/DE, 20th July 2022
Organized by GDCh e.V. (German Chemical Society), Local Section Hannover
Contribution: P. Bergmann, M. Takenberg, C. Frank, F. Ersoy and Ralf G. Berger.: „Biotechnological Production of Natural Colorants from Basidiomycota for Industrial Application” (Poster)

Himmelfahrtstagung on Bioprocess Engineering 2022 - Future Bioprocesses for a Sustainable Industry, Mainz/DE, 23rd – 25th May 2022
Organized by DECHEMA e.V. (Society for Chemical Engineering and Biotechnology)
Contribution: P. Bergmann, M. Takenberg, C. Frank, F. Ersoy and Ralf G. Berger.: „Biotechnological Production of Natural Colorants from Basidiomycota for Industrial Application” (Poster)

Bioeconomy Camp 2021, Berlin/DE and Online, 30th September – 1st October 2021
Organized by the German Federal Ministry of Education and Research (BMBF) and University of Hohenheim
Contribution: “FungiColor - Natural Colors from Basidiomycota” (Video)

Global Bioeconomy Summit 2020, Berlin/DE and Online, 16th – 20th November 2020
Organized by the Bioeconomy Council of the German Government
Contribution: “FungiColor - Natural Colors from Basidiomycota” (Video)