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

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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^{-1}, 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

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1. 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 [1]. 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 [2,3], 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 [4]. Some even discuss it as the next functional food promoting health and as a valuable source of antioxidants [5–7]. The potential application of *L. sulphureus* extracts as a preservative in food has also been reported [8,9].

The name *sulphureus* refers to the characteristic sulphur-like color. Responsible for this color are orange-yellowish polyenes, the laetiporic acids [10,11] that were long presumed to be a carotenoid pigment named laetiporxanthin [12]. 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. [13].

Large-scale intensive cultivation of *L. sulphureus* fruiting bodies for commercial production was described [14], 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^{-1}) [10,11]. As different sources discuss the high variability in compounds that occur in different *L. sulphureus* species [11,15], 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.

2. 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.

2.1. 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 [16]. Mycelia were grown and maintained on standard nutrient liquid agar (SNL, cf. Supporting Information) according to Sprecher [17]. 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. Supporting Information). 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).

2.2. 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 [18]. 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^{-1}. A sample (10 mL) was taken every weekday. Samples were analyzed as described in Sections 2.2 and 2.4.

2.3. 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 2.2. 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 2.4).

2.4. 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^{-1} cm^{-1}) [10]. For verification, a methanolic extract of the dried biomass was measured via mass spectrometry, as described in Zschätzsch et al. [19]. The main pigment was laetiporic acid A with m/z 421 $[M + H]^+$ and 419 $[M - H]^+$ (Scheme S1).

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.

2.5. 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 [20]. In addition, the samples were tested for their color fastness during washing according to DIN EN ISO 105-C06:2010-08 [21]. 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 (t0). A ΔE value below 1 generally implies a color change unnoticeable to the human eye [22].

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^{-1} 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.

2.6. 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^{-1} and 479 mg L^{-1} 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.

3. Results

3.1. Production Strain

Four strains of *L. sulphureus* were compared concerning their potential for generation of laetiporic acids (Figure 1a). Strain B was found to produce both the highest amount of dry matter and pigment, with 10.1 g L^{-1} biomass (day 20) and 27.7 mg g^{-1} 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^{-1} (Figure 1b).

Strain A was approximately equal to strain B in biomass production with a maximum of 9.9 g L^{-1}, but produced significantly lower amounts of pigment. Strain C produced the second highest amount of pigment (55.1 mg L^{-1}), but yielded only about half the biomass. The lowest amount of pigment was produced by strain D with 1.0 mg L^{-1}. 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^{-1}, respectively) and pigment yield (30.8 vs. 1.0 mg L^{-1}, respectively). A picture of unprocessed *L. sulphureus* culture samples on day 15 illustrates the high variation in pigment yields (insert in Figure 1a).

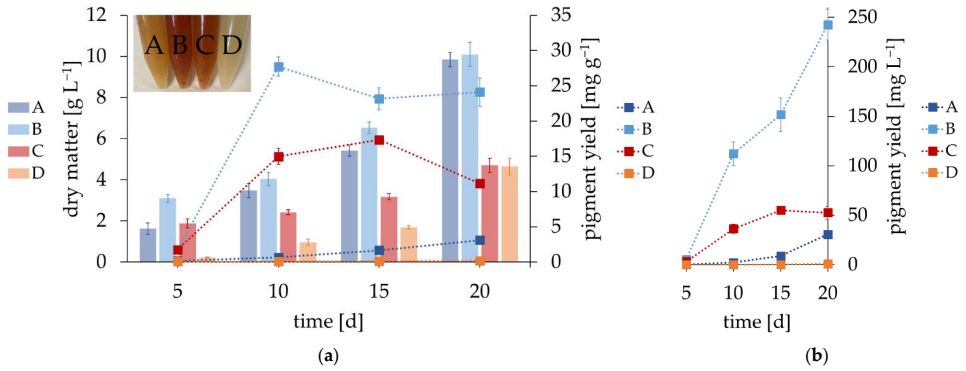

Figure 1. (**a**) Productivity of different *L. sulphureus* strains regarding biomass (bars, left ordinate) and pigment yield (squares, right ordinate) in shake flask culture. Insert on the top left side: A picture of the unprocessed cultures (biomass and supernatant) on cultivation day 15 with visible color differences. (**b**) Total pigment yield per culture volume over the course of cultivation.

3.2. 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^{-1}, respectively; Figure 2a,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^{-1} and the overall highest biomass of 7.7 g L^{-1} (PDB: 4 g L^{-1} on day 12; Figure S1). The addition of 5 g L^{-1} 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^{-1} laetiporic acid concentration on day 12 (Figure 2c). Controlling the pH with sodium hydroxide to prevent a pH drop below 2 (Figure 2d, addition of 400 mL on cultivation day 7) inhibited laetiporic acid synthesis and yielded a maximum concentration of 0.5 g L^{-1} 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 (Figure S1).

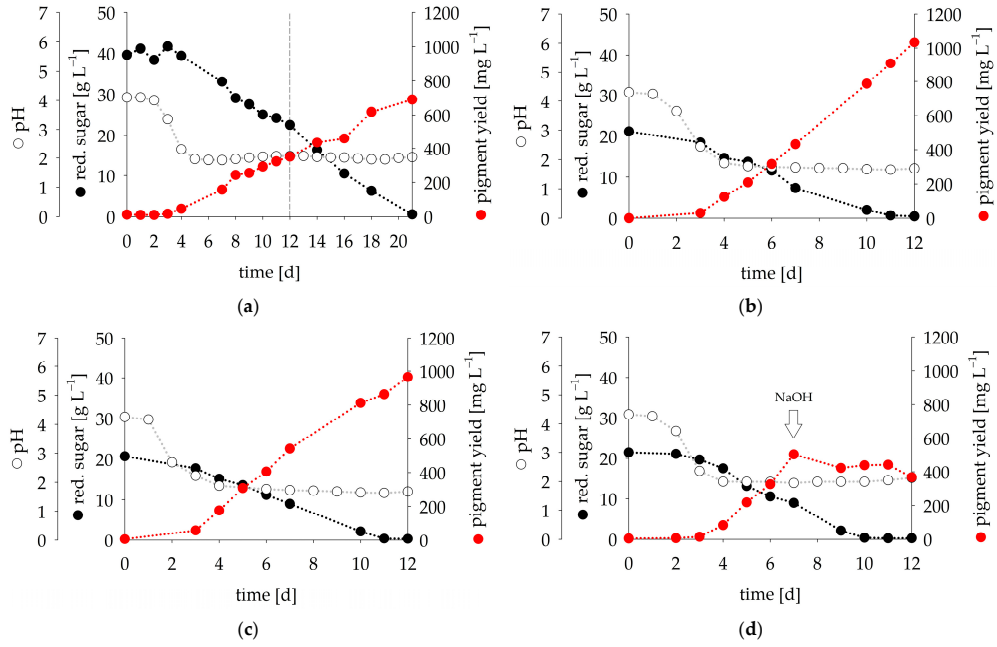

Figure 2. Influence of different media and pH regulation on laetiporic acid yield during cultivation in a 7 L STR. (**a**) Moser b, dashed line marks day 12; (**b**) PDB; (**c**) PDB + 5 g L^{-1} beech sawdust; (**d**) PDB + pH regulation.

3.3. 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 3), 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 (Figure S2).

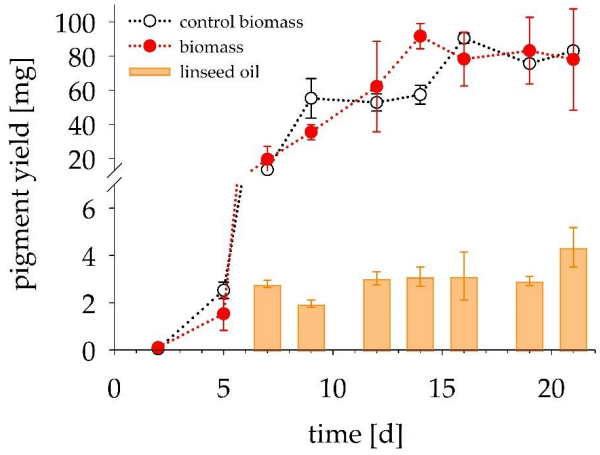

Figure 3. Laetiporic acid yields in biomass and lipophilic phase in 250 mL shake flask culture over the course of the cultivation during in situ extraction. White dots depict the measurements for the control without second phase. The yield for the 10 mL extractive phase on a certain day is the yield in the phase that was removed (and then renewed) on that day of sampling.

3.4. Pigment Stability

Pigment extraction from biomass can be successfully performed with ethanol, as reported previously [19]. 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 (Figure S3). Storing the freeze-dried biomass at room temperature but in the dark, resulted in a loss of 82% color within 6 months (Figure 4a). 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 4b). Additional experiments with ascorbic acid improved pigment stability as well, but were less effective compared to N_2 stabilization (data not shown).

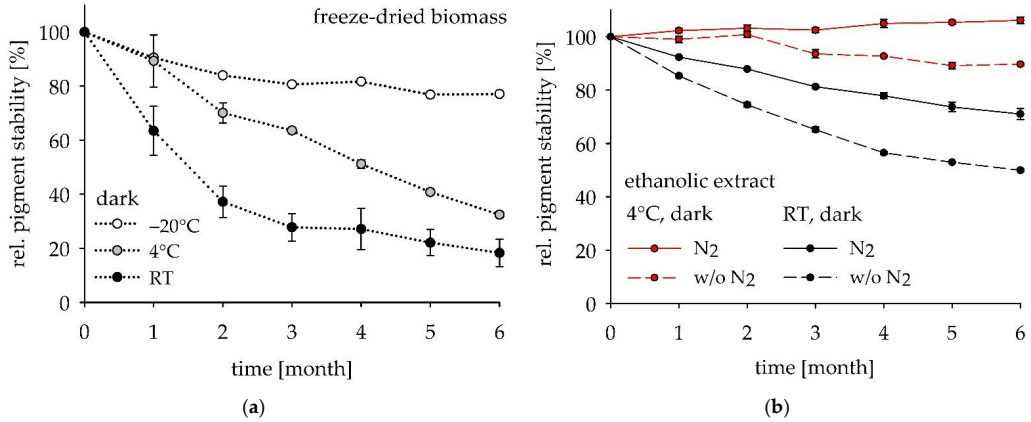

Figure 4. Stability of ethanolic *L. sulphureus* extracts and freeze-dried biomass over time, stored under different conditions. The difference in absorbance compared to t_0 is displayed as the percentage of remaining absorbance. Stabilities of (**a**) freeze-dried biomass stored at different temperature conditions in the dark; (**b**) ethanolic extracts stabilized with nitrogen.

3.5. Color Fastness of Dyed Silk

As shown in our previous paper [19], 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 1 and S1). 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 5a). 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.

Table 1. 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 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 ($\Delta E \approx 1$).

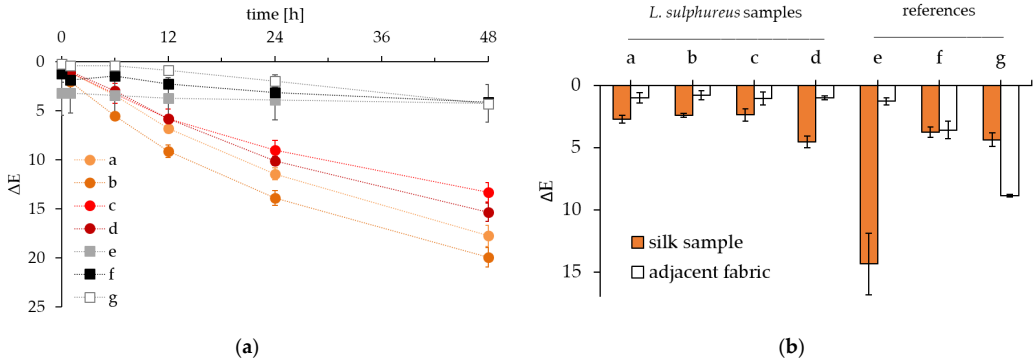

Figure 5. Comparison of the (**a**) light and (**b**) washing stability of silk samples dyed with *L. sulphureus* (a, b, c, d) and conventional textile dyes (e, f) against industrially dyed silk (g).

3.6. 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 6). 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 [3]) were added to the picture of samples (Figure 6, foreground).

Figure 6. 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. 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 [23–25]. The special case of two identical strains obtained from the same strain collection at different time points (A and D in Figure 1), 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 [26].

Davoli et al. reported a maximum laetiporic acid A content of 6.7 mg g^{-1} for *L. sulphureus* strain 79110, with a dry matter concentration of around 3 g L^{-1} after 17 days of cultivation in shake flasks using a peptone corn steep glucose medium [11]. Seibold et al. described a maximum polyene concentration of 7.5 mg g^{-1} [13]. 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^{-1} dry matter, 27.7 mg g^{-1} laetiporic acid; Figure 1a).

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^{-1} in the shake flask culture at day 20 (Figure 1b), and were able to increase the yield to 690 mg L^{-1} at day 21 with Moser b medium and up to 1000 mg L^{-1} 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 2). 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 [27]. 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 [28]. In the present work, regulation of the pH by addition of NaOH, when values below 2 were reached, drastically reduced pigment yield (Figure 2d). Likewise, excessive sugar concentrations negatively impacted color formation (Figure 2a, 40 g L^{-1} total concentration of reducing sugars in Moser b). PDB yielded the highest product concentration (Figure 2b) 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^{-1} 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 *Saccharomyes cerevisiae* by applying a biphasic in situ extraction [29]. 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 [30], 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") [31].

Davoli et al. (2005) described the laetiporic acids to be "surprisingly stable in the presence of oxygen and light" [11] (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 [13]. The herein presented extract storage (Figure 4) and light stability trials with dyed silk (Figure 5a) 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 4). 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 5 [19]). 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 6). 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 [5]. 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 [32]. Further studies on the safety profile of the dyed goods appear mandatory.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8120684/s1, Scheme S1: Chemical structure of laetiporic acid A; Figure S1: Influence of different media and pH regulation on biomass yield in a 7 L STR; Figure S2: Laetiporic acid yields during in situ extraction; Figure S3: Preliminary stability trials; Table S1: Pictures of the silk samples during light stability test. The graphical abstract was created with BioRender.

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