Supplementary Information

Mixture Design of Experiments to Improve Fungal Degradation of Cosmetic Pigments

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EXPERIMETAL SECTION

Materials and Reagents. Toluene-2,5-diamine sulfate (PTD), *p*-aminophenol (PAP), 2-methylresorcinol (2MR), and 4-amino-2-hydroxytoluene (PAOC), all > 99,9% purity, were kindly provided by Huwell Chemicals S.p.A. (Milan, Italy). Lactic acid (≥ 88% purity) and *i*-propyl alcohol (≥ 99.9% purity) were purchased from Carlo Erba Reagents (Milan, Italy). $MgSO_4$ (98.4% purity), NaCl (99.9% purity), and sodium citrate (≥ 99.0% purity) were obtained from VWR International (Milan, Italy). Glacial acetic acid (≥ 99.8% purity), sodium acetate and acetonitrile (both ≥ 99.0% purity), and ammonium acetate (≥ 98% purity) were from Sigma-Aldrich (Milan, Italy). Methyl alcohol (> 99.8% purity) and citric acid (≥ 99.5% purity) were from Honeywell International Inc. (Morristown, USA). Milli-Q water was produced by means of a Millipore Milli-Q Element A10 water purification system (Merk-Millipore, Milan, Italy). Mycelium of species M9911 *Trametes versicolor* (mix with ± 1% spawn), M2191 *Pleurotus ostreatus* (mix with ± 2% spawn), and M9720 *Ganoderma lucidum* (mix with ± 1% spawn) were acquired from Mycelia (Deinze, Belgium). Cardboard and the ingredients for the formulation of hair dyes were all kindly provided by Davines S.p.A. (Parma, Italy). QuEChERS PSA/C18 + $MgSO_4$ and PSA/C18/GCB + $MgSO_4$ dSPE devices were obtained from Restek (Milan, Italy), whereas the Supel™ QuE Z-Sep/C18 dSPE devices were from Supelco (Bellefonte, USA).

Optimization of fungal growth conditions. For each trial, a total of 9.50 g of culture was prepared in porcelain capsules and 10 mL of an aqueous lactic acid solution was added. The acid concentration was adjusted proportionally to the amount of dye present within the culture. After homogenization, each culture was covered with perforated Parafilm® (Bemis Company Inc., Wisconsin, USA). The cultures were incubated under aerobic conditions at 28 °C, at 85% relative humidity, and in the dark, using a PID system incubator (Instruments s.r.l., Milan, Italy). Prior to culture preparation, all the tools were sterilized in a POLEAX EC 10 L autoclave (Quirumed, Valencia, Spain) at 121 °C for 18 min. Between the preparation of each culture, the work surface and tools were disinfected with a 70% *v/v* aqueous solution of isopropyl alcohol.

Model regression. A first-order model was postulated for t_G and S_f:

$$y = b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3 + e$$

Equation 1

where the terms denoted as b_i are the regression coefficients and e represents the residuals.

A coefficient was deemed statistically significant if its absolute value exceeded the semi-amplitude of its 95% confidence interval. Model performance was evaluated in terms of the fraction of explained variance (R^2) and predictive capability using leave-one-out cross-validation (Q^2). Model validity was assessed by carrying out a lack-of-fit F-test ($\alpha = 0.01$).

Single desirability functions (d_i) were calculated for t_G as shown in Equation 2, and for S_f and m_d, as shown in Equation 3.

$$d_i = \begin{cases} 0, & y_i > U_i \\ \dfrac{U_i - y_i}{U_i - L_i}, & \text{otherwise} \end{cases}$$

Equation 2

$$d_i = \begin{cases} 0, & y_i < L_i \\ \dfrac{y_i - L_i}{U_i - L_i}, & \text{otherwise} \end{cases}$$

Equation 3

The optimization boundaries were defined as follows:

- t_G: L_i was defined as the upper limit of the 95% confidence interval of the minimum predicted response within the experimental domain, whereas U_i was set at 240 h.
- S_f: L_i was set at 2, and Ui at 6.
- m_d: L_i corresponded to the minimum amount of dye used in the culture formulation (*i.e.*, 0.95 g), whereas U_i corresponded to the maximum amount (*i.e.*, 4.75 g).

The optimal conditions were those corresponding to the maximum global desirability, calculated as the weighted geometric mean of the single desirability functions.[1] The weights assigned to t_G, S_f, and m_d were 3, 1, and 2, respectively.

QuEChERS extraction. The performance of QuEChERS extraction was evaluated by varying the extraction salts mixtures and the sorbent for dSPE. The binary mixture PTD-2MR was selected as model combination. Three different extraction salt mixtures were tested: i) unbuffered (UB): 4 g of $MgSO_4$ and 1 g of NaCl;[2] ii) AOAC: 6 g of $MgSO_4$ and 1.5 g of sodium acetate;[3] iii) EN: 4 g of $MgSO_4$, 1 g of NaCl, 1 g of sodium citrate and 0.32 g of citric acid[4]. Three different sorbents were tested: i) $MgSO_4$ + PSA/C18; ii) $MgSO_4$ + PSA/C18/GCB; iii) $MgSO_4$, Z-Sep/C18.

The extracts were diluted 1:20 with Milli-Q water and submitted to UV-vis analysis using an Evolution 260 Bio spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) in the 210–800 nm range, operating in transmittance mode. The sample was placed into a quartz semi-micro cuvette (Hellma Analytics, Milan, Italy) with a light path of 10 mm. Both bandwidth and resolution were set to 1 nm, while the integration time was set to 250 ms. For each treatment, a blank culture was also submitted to extraction for background correction. The wavelength values for each dye, used as response variable, are reported in Table S3.

A two-way ANOVA was carried out to highlight differences in the average responses for each factor under investigation. Normality and homoscedasticity of data were tested using Shapiro-Wilk test and Hartley's test, respectively. For statistically significant results, effect size was estimated using Cohen's η^2 [5] and post-hoc multiple pairwise comparisons were carried out by applying Student's t-tests with Bonferroni correction ($\alpha = 0.05$).

Degradation experiments. Ten grams of each sample (8.05 g if mycelium was absent) were weighed, followed by the addition of 2.50 mL of cold Milli-Q water (4.62 mL if mycelium was absent). Subsequently, 10 ml of cold acetonitrile were added, and the sample was vigorously shaken for 1 min. Four g of $MgSO_4$ and 1 g of NaCl were added, and the tube was shaken again for 1 min. The tubes were stored at -20 °C for 1 h to facilitate the separation of fats and proteins. Finally, it was centrifuged at 5000 rpm for 5 min at 4 °C. One milliliter of supernatant was transferred to the PSA/C18 + $MgSO_4$ dSPE clean-up tube, agitated for 30 s and centrifuged at 5000 rpm for 5 min at 4 °C. The extract was diluted 1:20 with Milli-Q water and submitted to UHPLC-DAD analysis using a

Thermo Scientific UHPLC 3000 RS (Thermo Fisher Scientific, Monza, Italy), equipped with a quaternary pump (GPL-3400RS), a WPS-3000 TRS autosampler, a TCC-3000 RS temperature controller column compartment, and a DAD 3000 RS detector. Instrument control, data acquisition and processing were performed using Chromeleon software.

Reversed phase chromatographic separation was carried out using a pentafluorophenyl Nucleudur PFP (150 mm x 2.0 mm, 3.0 μm) column equipped with the same pre-column (both by Marcherey-Nagel, Dueren, Germany) and maintained at 30 °C. The operating conditions were as follows: solvent (A), ammonium acetate (0.1 M, pH = 5.4); and solvent (B), methanol. The flow rate was 0.3 mL min^{-1}, and the injection volume was 5 μL. Chromatographic separation was carried out under gradient elution as follows: 5–70% B at 5–22 min, 70–5% B at 22–25 min (total runtime: 35 min). The detection wavelength was set at 230 nm.

Method validation. Detection (yD) and quantitation limits (yQ) expressed as signals were calculated by performing 10 replicate measurements of the blank matrix, as follows: yD = xb + 3 s0' and yQ = xb + 10 s0', where $\bar{x}b$ and s0' are the mean and corrected standard deviation (s0'= s0/√n) calculated on the basis of the blank responses (s0) and the number of replicate observations (n) averaged when reporting results. Finally, LODs and LOQs were obtained by projection of the corresponding yD and yQ through a calibration plot y = f(x) onto the concentration axis.

Linearity was evaluated for each analyte on k = 5 concentration levels in the LOQ-2500 mg kg^{-1} range (LOQ, 500, 1000, 2000, 2500 mg/kg-1, n = 3 independent replicated measurements per level). Homoscedasticity was assessed using Bartlett's test. The goodness of fit and linearity were further verified through lack-of-fit and Mandel's tests. The significance of the intercept was evaluated by running a t-test (α = 0.05). Precision was assessed in terms of repeatability and intermediate precision. Relative standard deviations ($RSD_{\%}$) were evaluated at three concentration levels (LOQ, 160 mg/kg^{-1} and 2500 mg/kg^{-1} for each analyte) by performing n = 6 replicated measurements *per* level. Intermediate precision was estimated over three days verifying homoscedasticity of data and performing the analysis of variance (ANOVA) at a 95% confidence level. Trueness was evaluated in

terms of recovery rate ($RR_{\%}$) by spiking the blank matrix at the same concentration levels used for precision testing. RR% were calculated as follows: RR%=c1/c2·100 where c1 is the observed concentration and c2 is the concentration obtained by spiking the blank sample. Ten replicate measurements per level were performed. Selectivity was assessed by analyzing blank samples spiked with potentially interfering dyes to verify the absence of interfering compounds.

Matrix effect was evaluated as follows:

i) by comparing the response of the analytes in a standard solution was compared with that of a blank matrix extract spiked with the analytes at the same concentration

$ME = (A-B)/A \times 100$

where A is the response (peak area) of the analyte in the standard solution, and B is the response in the blank matrix extract spiked with the analyte at the same concentration.

ii) by comparing the slopes of calibration curves obtained from standard solutions and matrix-matched standard solutions were compared. A Student's *t*-test ($\alpha = 0.05$) was performed to assess the presence of significant differences between the slopes.

Life cycle assessment. The Ecoinvent version 3.10 database contains up to 18000 reliable processes covering a wide range of industries, including agriculture and animal husbandry, building and construction, chemicals and plastics, energy, forestry and wood, metals, textiles, transportation, tourist accommodation, waste treatment and recycling, and water supply.[6] In particular, processes from the “Ecoinvent, cut-off by classification, system” database were used for the SimaPro modeling.

Goal and Scope Definition. Consumption in terms of materials, both constituting the substrate and for laboratory equipment, energy consumption for machinery operation, and transport steps for supply of primary materials constituting the substrate were included in the analysis. According to the reference ISO, the defined Functional Unit (F.U.) is the reference unit against which inventory flows are normalized. In the present case study, the selected F.U. consists of 100 g of substrate (substrate composition; 29% hair dye, 40% mycelium and 31% cardboard) incubated for 4 days, i.e., a material

composition and period considered optimal for degradation. The reference year chose for analysis is 2024.

Life Cycle Inventory. Once the scope, functional unit and system boundaries of the study are established, the inventory analysis is carried out according to the ISO references for LCA methodology. This step involves quantifying the input and output streams bounded by the system boundaries and normalized to the functional unit. The data related to laboratory operations are primary data from direct on-site measurements. All material consumption related to the equipment used were quantified: ceramic capsule and pestle, steel spatula and prop, plastic tray, parafilm cover, lactic acid in the inoculation phase, and distilled water in the sterilization phase. Regarding the electricity consumption, these were quantified based on the data sheets of the machines and their operating times. The operations considered are as follows: scales for weighing the materials, autoclave for sterilization of the equipment, inoculation of the substrate for 4 days, and finally oven drying of the final substrate. The information on the final substrate constituent materials, such as the cardboard layer and hair dye, were modeled using secondary data from the Ecoinvent 3.10 library. In addition, also the mycelium production process is based on secondary data and was modeled based on the study CIT from the literature.[7,8] Finally, concerning the transportation of the materials, the distance for transporting the substrate material (mycelium, cardboard, hair dye and lactic acid) from the production site to the laboratory were measured.[9] The transport medium selected from the Ecoinvent library is "Transport, freight, lorry 16-32 metric ton, EURO6 {RER}| market for transport, freight, lorry 16-32 metric ton, EURO6 | Cut-off, S". On the other hand, auxiliary materials used during these transport phases were not considered, due to the lack of information and to the low level of contribution of these elements. Production of equipment, buildings, and other capital goods, staff business travel, and staff commuting to work were excluded from the analysis, in line with the reference ISO.[10] The detailed description of the input data used in the inventory phase is provided in Table S7.

Life Cycle Impact Assessment. The impact assessment method used in this study is Environmental Footprint (E.F.) 3.1.[11] This is the environmental impact assessment method recommended by the European Commission, consisting of 16 impact categories with a midpoint approach, each of which refers to a given independent characterization model.

Software. Multilinear regression and multicriteria optimization were carried out by using custom scripts in MATLAB environment (v. R2022a, Mathworks, Massachusetts, USA). The evaluation of effects and iso-response curves was performed using the CAT software,[12] in the R development environment (v.3.1.2, The R Foundation).

Development and characterization of bio-composite materials: The fungal substrate powder was mixed with PBAT granules in an analytical mill and heat-treated overnight at 40 °C, before being extruded using a twin-screw extruder (Zamak MercatorRES-2 P/12A Explorer twin-screw Extruder)). Filaments obtained during extrusion were cooled, pelletized by a Scheer SGS 25-E4 pelletizer before being introduced in the Babyplast 10/12 injection molding machine (Rambaldi+Co it srl, Italy) for the injection molding process to produce 5A type dumbbell specimens. According to the UNI EN ISO 527-2:2012 [13], a tensile test was performed on ten replicates of each PBAT/fungal substrate mixture, including pure PBAT. The uniaxial tensile tests were performed with a TesT dynamometer (Model 112, TesT GMBH Universal Testing Machine, Germany). The specimens are clamped at both ends using hydraulic jaws approximately at the broad parallel-sided portions. Pre-tensioning of 5 N at 5 mm/min was carried out to align the specimens. The test began with an elongation rate of 10 mm/min until breakage. The engineering stress-strain curve for the determination of mechanical properties is obtained reporting the stress σ [MPa], defined as the ratio between the applied force (F) and the initial cross section area (A_0), as a function of the strain ε [mm/mm], derived from the displacement Δl (mm) of the specimen over its starting length (l_0).

TABLES

Table S1. Composition of the pseudo-components expressed as weight fraction (see Fig. S1 top-left)

Pseudo-component	Cardboard	Dye	Mycelium
X_1	0.10	0.10	0.80
X_2	0.10	0.50	0.40
X_3	0.50	0.10	0.40

Table S2. Experimental matrix. The composition of each experiment is reported as weight fraction in the pseudo-component domain as depicted in Fig. S1 (top-right)

Experiment	X_1	X_2	X_3
1	1.00	0.00	0.00
2	0.00	1.00	0.00
3	0.00	0.00	1.00
4	0.67	0.33	0.00
5	0.67	0.00	0.33
6	0.33	0.67	0.00
7	0.00	0.67	0.33
8	0.33	0.00	0.67
9	0.00	0.33	0.67
10	0.67	0.17	0.17
11	0.17	0.67	0.17
12	0.17	0.17	0.67
13	0.33	0.33	0.33
14	0.33	0.33	0.33
15	0.33	0.33	0.33

Table S3. Wavelengths used for the detection of the investigated pigment

Dye	Compound	Wavelength (nm)
Primary dyes	PTD	256
	PAP	230
Couplers	2MR	279
	PAOC	230

Table S4. Single desirability values and global desirability

Response	Species	d_i
t_G	*TV*	1.00
	PO	0.96
	GL	1.00
S_f	*TV*	0.55
	PO	0.87
	GL	0.42
m_d		0.47
D		0.79

Table S5. LOD, LOQ, linearity range and calibration curves of precursors and couplers

Pigment	LOD (mg kg^{-1})	LOQ (mg kg^{-1})	Linearity range	b_1 (± s_e)
PAP	2.3	7.5	LOQ-2500	y = 0.0159 (± 0.0003) x
PTD	4.8	16		y = 0.0099 (± 0.0002) x
2MR	5.6	19		y = 0.0112 (± 0.0002) x
PAOC	2.2	7.5		y = 0.0256 (± 0.0003) x

Table S6. Impact of each component in the substrate production process

Impact categories	Hair dye	Mycelium	Cardboard	Lactic acid	Plastic tray for mycelium	Steel spatula	Ceramic capsules	Ceramic pestle
AC [mol H+ eq]	1,17E-03	2,40E-04	1,07E-04	2,22E-03	5,61E-07	1,08E-05	2,14E-05	7,24E-06
GWP [kg CO_2 eq]	2,53E-01	2,35E-02	2,32E-02	5,98E-01	1,48E-04	2,57E-03	6,77E-03	2,29E-03
ECF [CTUe]	7,70E+00	4,24E-01	2,22E-01	4,47E+00	6,04E-05	8,58E-02	1,90E-02	6,42E-03
PM [disease inc.]	1,16E-08	1,78E-09	1,18E-09	2,01E-08	6,27E-12	2,33E-10	7,37E-09	2,49E-09
EUm [kg N eq]	1,46E-03	5,34E-04	4,91E-05	5,73E-04	9,23E-08	2,43E-06	4,78E-06	1,62E-06
EUf [kg P eq]	5,01E-05	7,39E-06	1,35E-05	1,27E-04	1,63E-09	1,05E-06	1,37E-06	4,64E-07
EUt [mol N eq]	1,76E-03	9,08E-04	3,85E-04	4,46E-03	9,95E-07	2,50E-05	5,21E-05	1,76E-05
HTc [CTUh]	2,48E-09	1,09E-10	9,44E-11	1,66E-09	3,40E-14	2,61E-10	1,68E-11	5,66E-12
HTnc [CTUh]	1,88E-09	2,07E-09	3,72E-10	4,62E-09	1,58E-13	4,85E-11	4,65E-11	1,57E-11
IR [kBq U-235 eq]	1,06E-02	9,63E-04	2,23E-03	4,19E-02	7,20E-08	7,54E-05	3,70E-04	1,25E-04
LU [Pt]	5,12E-01	2,42E+00	1,56E+00	1,22E+00	3,75E-05	8,50E-03	2,81E-02	9,48E-03
OD [kg CFC11 eq]	2,23E-08	6,22E-10	5,62E-10	1,74E-08	8,62E-14	1,23E-11	1,17E-10	3,95E-11
POF [kg NMVOC eq]	8,78E-04	9,85E-05	1,08E-04	2,83E-03	4,10E-07	8,37E-06	1,95E-05	6,60E-06
RUf [MJ]	4,06E+00	2,37E-01	3,16E-01	1,07E+01	3,33E-03	2,62E-02	9,59E-02	3,24E-02
RUm [kg Sb eq]	1,28E-06	1,93E-07	8,55E-08	4,58E-06	2,67E-11	1,73E-08	3,32E-08	1,12E-08
WU [m^3 depriv.]	6,45E-02	2,92E-01	1,03E-02	2,12E-01	8,91E-05	4,85E-04	1,13E-03	3,83E-04

Table S6. Impact of each component in the substrate production process (continue)

Impact categories	Deionised water	Parafilm	Glass jar	Tin cap	Steel prop	Plastic tray for incubation	Sterilizer	Hair dye transport	Mycelium transport
AC [mol H+ eq]	9,46E-07	1,21E-05	2,57E-06	1,97E-06	7,71E-06	4,02E-06	5,39E-07	1,43E-07	1,76E-05
GWP [kg CO_2 eq]	8,91E-05	2,66E-03	3,18E-04	1,61E-04	1,84E-03	1,06E-03	5,08E-05	6,88E-05	8,46E-03
ECF [CTUe]	1,12E-02	1,63E-02	1,74E-03	4,67E-03	6,15E-02	4,32E-04	6,39E-03	2,63E-04	3,24E-02
PM [disease inc.]	7,48E-12	1,33E-10	3,64E-11	2,26E-11	1,67E-10	4,49E-11	4,26E-12	5,07E-12	6,23E-10
EUm [kg N eq]	7,38E-08	2,37E-06	4,17E-07	4,56E-07	1,74E-06	6,61E-07	4,21E-08	3,44E-08	4,23E-06
EUf [kg P eq]	3,42E-08	5,86E-07	4,69E-08	3,76E-07	7,52E-07	1,17E-08	1,95E-08	4,66E-09	5,73E-07
EUt [mol N eq]	7,37E-07	2,49E-05	4,75E-06	6,13E-06	1,79E-05	7,12E-06	4,20E-07	3,71E-07	4,57E-05
HTc [CTUh]	6,52E-13	7,95E-12	7,95E-13	4,11E-13	1,87E-10	2,44E-13	3,72E-13	4,88E-13	6,00E-11
HTnc [CTUh]	1,30E-12	2,09E-11	3,28E-12	6,22E-12	3,47E-11	1,13E-12	7,41E-13	6,08E-13	7,47E-11
IR [kBq U-235 eq]	1,09E-05	1,21E-04	1,29E-05	1,43E-05	5,40E-05	5,16E-07	6,23E-06	1,26E-06	1,54E-04
LU [Pt]	3,84E-04	7,93E-03	2,95E-03	1,37E-03	6,09E-03	2,69E-04	2,19E-04	5,85E-04	7,19E-02
OD [kg CFC11 eq]	3,35E-11	6,38E-11	5,11E-12	1,43E-12	8,82E-12	6,18E-13	1,91E-11	1,37E-12	1,68E-10
POF [kg NMVOC eq]	2,90E-07	1,41E-05	1,52E-06	1,41E-06	5,99E-06	2,94E-06	1,65E-07	2,38E-07	2,93E-05
RUf [MJ]	1,16E-03	8,94E-02	4,07E-03	2,04E-03	1,88E-02	2,38E-02	6,59E-04	9,68E-04	1,19E-01
RUm [kg Sb eq]	1,19E-09	2,00E-08	1,60E-09	4,12E-07	1,24E-08	1,91E-10	6,80E-10	2,24E-10	2,75E-08
WU [m^3 depriv.]	9,73E-03	1,14E-03	5,37E-05	9,05E-05	3,47E-04	6,38E-04	5,55E-03	4,02E-06	4,94E-04

Table S6. Impact of each component in the substrate production process (continue)

Impact categories	Incubation energy consumption	Heater energy consumption	Autoclave energy consumption	Lactic acid transport	Cardboard transport	Scale energy consumption	Total
AC [mol H+ eq]	1,10E-02	1,60E-03	3,74E-06	7,50E-07	1,55E-07	5,88E-05	1,65E-02
GWP [kg CO_2 eq]	2,62E+00	3,82E-01	8,91E-04	3,60E-04	7,46E-05	1,40E-02	3,94E+00
ECF [CTUe]	8,13E+00	1,19E+00	2,77E-03	1,38E-03	2,86E-04	4,35E-02	2,24E+01
PM [disease inc.]	5,41E-08	7,91E-09	1,85E-11	2,65E-11	5,49E-12	2,90E-10	1,08E-07
EUm [kg N eq]	1,64E-03	2,39E-04	5,58E-07	1,80E-07	3,73E-08	8,78E-06	4,52E-03
EUf [kg P eq]	5,81E-04	8,50E-05	1,98E-07	2,44E-08	5,05E-09	3,11E-06	8,73E-04
EUt [mol N eq]	1,79E-02	2,61E-03	6,09E-06	1,95E-06	4,03E-07	9,57E-05	2,83E-02
HTc [CTUh]	6,73E-09	9,83E-10	2,29E-12	2,56E-12	5,29E-13	3,60E-11	1,26E-08
HTnc [CTUh]	3,25E-08	4,74E-09	1,11E-11	3,18E-12	6,59E-13	1,74E-10	4,66E-08
IR [kBq U-235 eq]	3,11E-01	4,54E-02	1,06E-04	6,57E-06	1,36E-06	1,66E-03	4,15E-01
LU [Pt]	1,53E+01	2,23E+00	5,20E-03	3,06E-03	6,34E-04	8,18E-02	2,34E+01
OD [kg CFC11 eq]	6,01E-08	8,78E-09	2,05E-11	7,17E-12	1,48E-12	3,22E-10	1,11E-07
POF [kg NMVOC eq]	7,65E-03	1,12E-03	2,61E-06	1,25E-06	2,58E-07	4,10E-05	1,28E-02
RUf [MJ]	4,18E+01	6,11E+00	1,43E-02	5,07E-03	1,05E-03	2,24E-01	6,39E+01
RUm [kg Sb eq]	3,26E-05	4,77E-06	1,11E-08	1,17E-09	2,43E-10	1,75E-07	4,42E-05
WU [m^3 depriv.]	1,89E+00	2,76E-01	6,44E-04	2,10E-05	4,36E-06	1,01E-02	2,78E+00

Table S7. LCI INPUT inventory data referred to 1 kg of substrate

	Inventory	Unit	Value	Data source process	Data source
Materials	Hair dye	kg	0.2873	Ecoinvent 3.10	On site measurement
	Mycelium	kg	0.401	Ecoinvent 3.10	On site measurement
	Cardboard	kg	0.3115	Ecoinvent 3.10	On site measurement
	Lactic acid	kg	1.272	Ecoinvent 3.10	On site measurement
	Plastic tray for mycelius	kg	0.00039	Ecoinvent 3.10	On site measurement
	Steel spatula	kg	0.0121	Ecoinvent 3.10	On site measurement
	Ceramic capsules	kg	0.0343	Ecoinvent 3.10	On site measurement
	Ceramic pestle	kg	0.0115	Ecoinvent 3.10	On site measurement
	Deionised water	kg	2.187	Ecoinvent 3.10	On site measurement
	Parafilm	kg	0.0132	Ecoinvent 3.10	On site measurement
	Glass jar	kg	0.0029	Ecoinvent 3.10	On site measurement
	Tin cap	kg	0.00015	Ecoinvent 3.10	On site measurement
	Steel prop	kg	0.0086	Ecoinvent 3.10	On site measurement
	Plastic tray for incubation	kg	0.0028	Ecoinvent 3.10	On site measurement
	Sterilizer	kg	1.246	Ecoinvent 3.10	On site measurement
Processes	Hair dye transport	km	12.3	Ecoinvent 3.10	Calculated
	Mycelium transport	km	1110	Ecoinvent 3.10	Calculated
	Cardboard transport	km	12.3	Ecoinvent 3.10	Calculated
	Lactic acid transport	km	149	Ecoinvent 3.10	Calculated
	Incubation energy consumption	kWh	73.68	Ecoinvent 3.10	Calculated
	Heater energy consumption	kWh	10.77	Ecoinvent 3.10	Calculated
	Autoclave energy consumption	kWh	0.0251	Ecoinvent 3.10	Calculated
	Scale energy consumption	kWh	0.000375	Ecoinvent 3.10	Calculated

Table S8. Sensitivity analysis on energy sources, Scenario 1 and Scenario 2 comparison

Impact categories	S1 Laboratory energy consumption	S2 Laboratory energy consumption	Reduction/Increase
AC [mol H+ eq]	1,26E-02	4,92E-03	61%
GWP [kg CO2 eq]	3,01E+00	6,39E-01	79%
ECF [CTUe]	9,36E+00	7,66E+00	18%
PM [disease inc.]	6,24E-08	4,47E-08	28%
EUm [kg N eq]	1,89E-03	7,31E-04	61%
EUf [kg P eq]	6,70E-04	3,92E-04	41%
EUt [mol N eq]	2,06E-02	7,71E-03	63%
HTc [CTUh]	7,75E-09	5,60E-09	28%
HTnc [CTUh]	3,74E-08	3,09E-08	17%
IR [kBq U-235 eq]	3,58E-01	5,22E-02	85%
LU [Pt]	1,76E+01	2,77E+00	84%
OD [kg CFC11 eq]	6,92E-08	4,33E-08	37%
POF [kg NMVOC eq]	8,82E-03	2,82E-03	68%
RUf [MJ]	4,82E+01	7,92E+00	84%
RUm [kg Sb eq]	3,76E-05	4,41E-05	-15%
WU [m3 depriv.]	2,18E+00	6,17E-01	72%

FIGURES

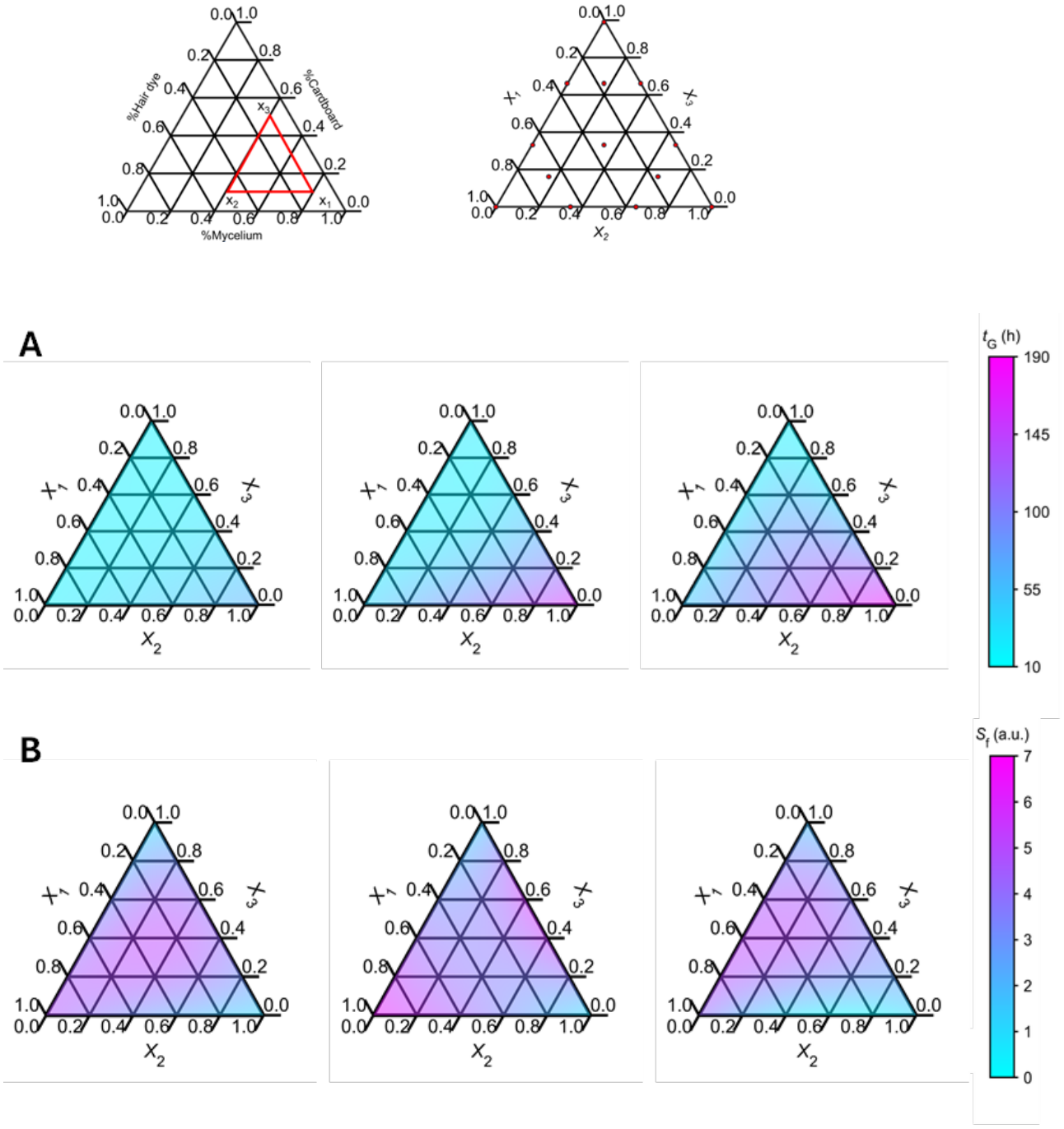

Fig. S1. The red triangle illustrates the pseudo-component domain used in this study (top-left) and the utilized experimental points are depicted in red (top-right). Ternary contour plots for: A) germination time and B) shape factor. TV is reported on the left, PO in the middle, and GL on the right. For each response variable, all the plots were rescaled to use the same color bar for comparison purposes

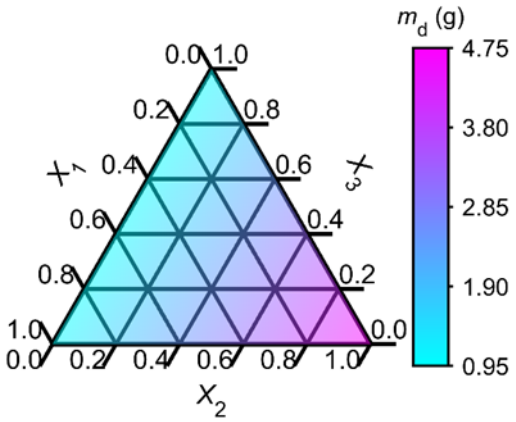

Fig. S2. Ternary contour plots for the amount of hair dye in the pseudo-component domain.

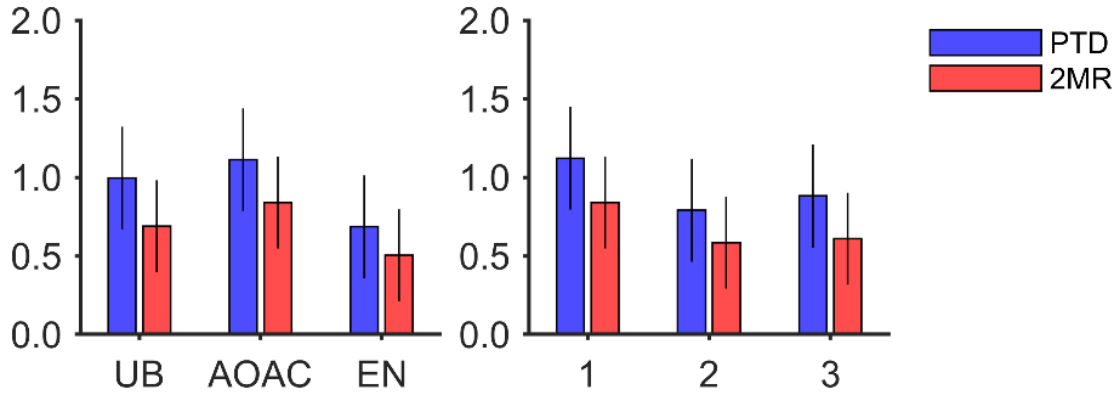

Fig. S3. Two-ways ANOVA after background correction. Left: effect of the buffer (UB: unbuffered; AOAC: acetic buffer; EN: citrate buffer). Right: effect of the dSPE clean-up (1: PSA/C18 + MgSO4; 2: Z-Sep/C18; 3: PSA/C18/GCB + MgSO4). The results are reported as means with respect to each treatment for the investigated combinations of dyes: PTD + 2MR. Absorbance values were recorded at the wavelengths reported in Table S3. The error bars show the amplitude of the 95% confidence interval estimated with respect to the residual variance**.**

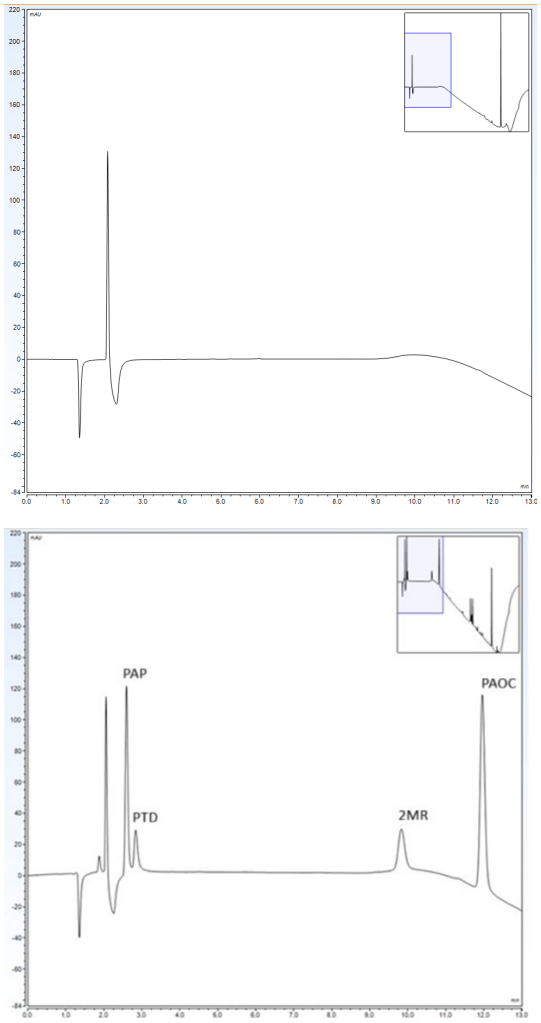

Fig. S4. QuEChERS-UHPLC-DAD chromatograms of: blank matrix (top) and blank matrix spiked with the binary mixtures PTD-2MR and PAP-PAOC at 2500 mg kg^{-1} (bottom)

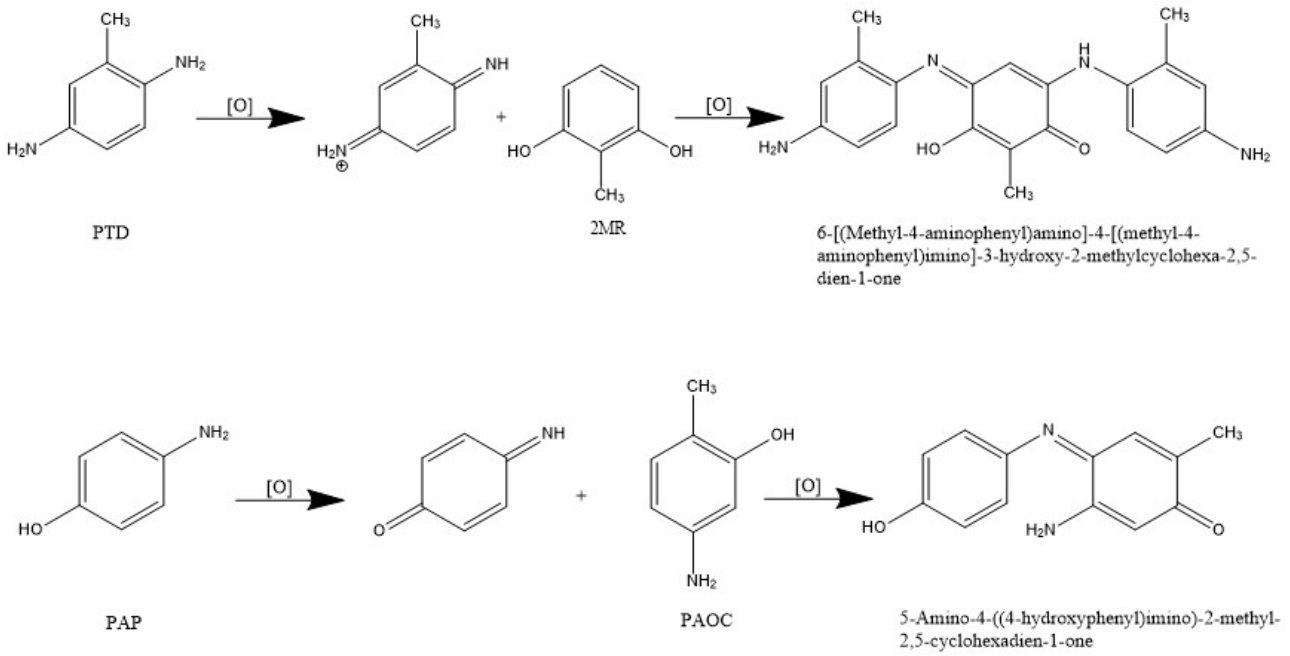

Fig. S5. Reaction scheme related to the formation of the coupling products of the binary mixtures PTD-2MR (top) and PAP-PAOC (bottom) [14]

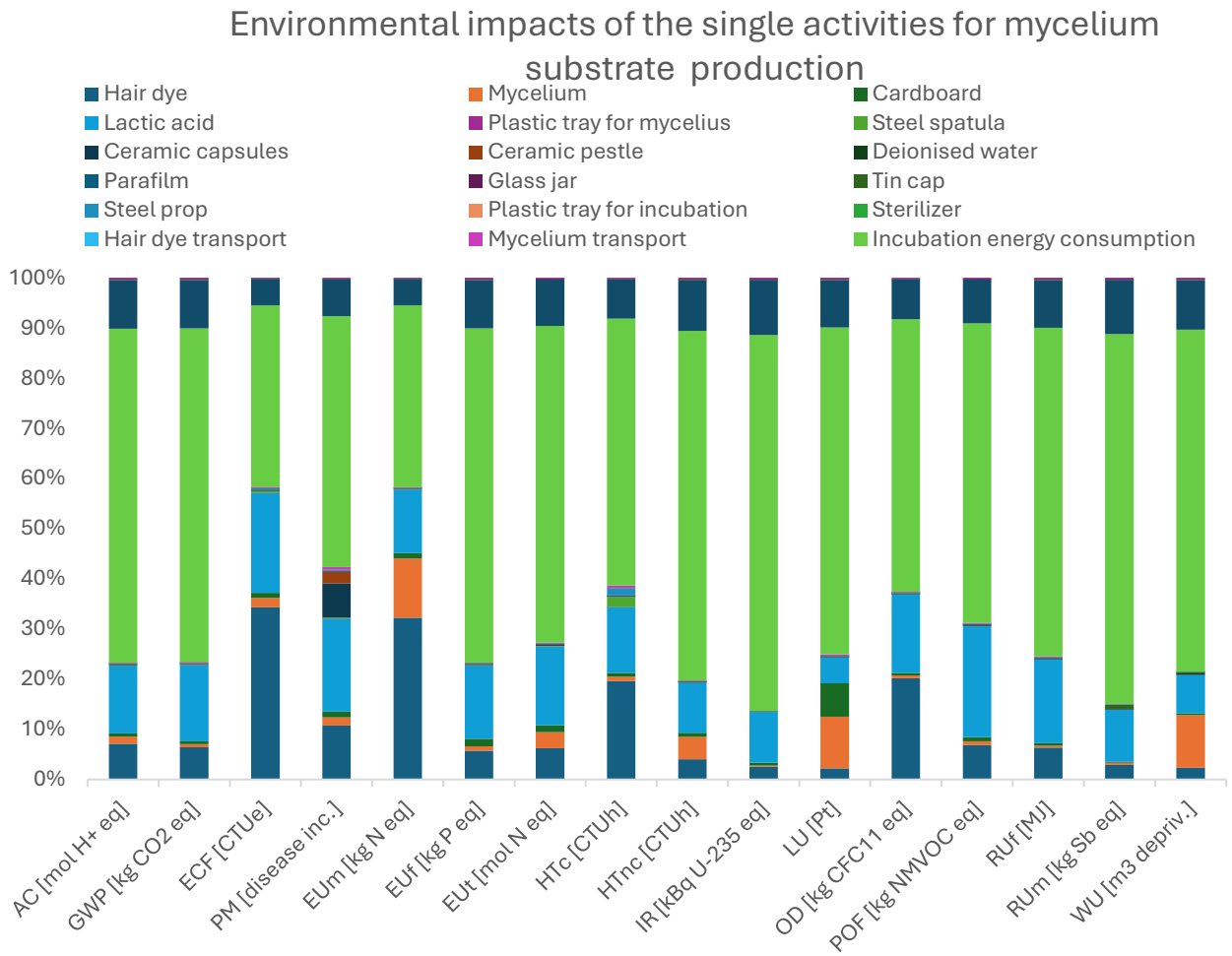

Fig. S6. Environmental impact of each activity for mycelium substrate production.

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