

Stacjonarne Studia Doktoranckie
Mikrobiologii, Biotechnologii i Biologii Eksperymentalnej

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Wykorzystanie grzyba *Nectriella pironii* do produkcji lakazopodobnej oksydazy wielomiedziowej i eliminacji toksycznych zanieczyszczeń przemysłu tekstylnego

The use of the fungus *Nectriella pironii* for the
production of a laccase-like multicopper oxidase
and elimination of toxic contaminants
in the textile industry

Praca doktorska

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Lista używanych skrótów

2,6-DMP	2,6-dimetoksyfenol;
ABTS	sól diamonowa kwasu 2,2'-azyno-bis(3-ethylobenzotiazolino-6-sulfonowego);
AO 7	Acid Orange 7;
AR 27	Acid Red 27;
B[a]A	(ang. <i>Benz[a]anthracene</i>) benz[a]antracen;
B[a]P	(ang. <i>Benzo[a]pyrene</i>) benz[a]piren;
BZT₅	biochemiczne zapotrzebowanie na tlen, mierzone po 5-ciu dniach;
FDA	(ang. <i>Food and Drug Administration</i>) Agencja Żywności i Leków;
Fen	(ang. <i>Phenanthrene</i>) fenantren;
HPLC-MS/MS	(ang. <i>High Performance Liquid Chromatography Coupled With Tandem Mass Spectrometry</i>) wysokosprawna chromatografia cieczowa sprzężona z tandemową spektrometrią mas;
IC	Indigo Carmine;
L1, L2	(ang. <i>Leachate</i>) próbki odcieków składowiskowych;
LiP	peroksydaza ligninowa;
LMCOs	(ang. <i>Laccase-like Multicopper Oxidases</i>) enzymy lakazopodobne;
MCOs	(ang. <i>Multicopper Oxidases</i>) wielomiedziowe oksydazy;
MnP	peroksydaza manganozależna;
PIs	(ang. <i>Phospholipids</i>) fosfolipidy;
PLFAs	(ang. <i>Phospholipid Fatty Acids</i>) fosfolipidy kwasów tłuszczowych;
RR 120	Reactive Red 120;
RBBR	Remazol Brilliant Blue R;
TBARS	(ang. <i>Thiobarbituric Acid Reactive Substances</i>) substancje reagujące z kwasem tiobarbiturowym;
WIOŚ	Wojewódzki Inspektorat Ochrony Środowiska;
WRF	(ang. <i>White-Rot Fungi</i>) grzyby białej zgnilizny;
WWA	(ang. <i>Polycyclic Aromatic Hydrocarbons</i> , PAHs) wielopierścieniowe węglowodory aromatyczne;
Z. P. B. „Boruta”	Zakładów Przemysłu Barwników „Boruta”.

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Wykaz publikacji wchodzących w skład rozprawy doktorskiej

P1 - Góralczyk-Bińkowska A., Jasińska A., Długoński J. 2019. Characteristics and use of multicopper oxidases enzymes. *Advancements of Microbiology*, 58, 1, 7–18. <https://doi.org/10.21307/PM-2019.58.1.007>.

IF₂₀₂₁ = 1,106; IF_{5-letni} = 1,955; punkty MEiN = 20

P2 - Góralczyk-Bińkowska A., Jasińska A., Długoński A., Płociński P., Długoński J. 2020. Laccase activity of the ascomycete fungus *Nectriella pironii* and innovative strategies for its production on leaf litter of an urban park. *PLoS ONE*, 15, 4, e0231453; errata w *PLoS ONE* 2020, 15, e0233553. <https://doi.org/10.1371/journal.pone.0231453>.

IF₂₀₂₁ = 3,752; IF_{5-letni} = 4,069; punkty MEiN = 100

P3 - Góralczyk-Bińkowska A., Długoński A., Bernat P., Długoński J., Jasińska A. 2021. Environmental and molecular approach to dye industry waste degradation by the ascomycete fungus *Nectriella pironii*. *Scientific Reports*, 11, 23829. <https://doi.org/10.1038/s41598-021-03446-x>.

IF₂₀₂₁ = 4,997; IF_{5-letni} = 5,516; punkty MEiN = 140

P4 - Góralczyk-Bińkowska A., Długoński A., Bernat P., Długoński J., Jasińska A. 2022. Accelerated PAHs transformation in the presence of dye industry landfill leachate combined with fungal membrane lipids changes. *International Journal of Environmental Research and Public Health*, 19, 13997. <https://doi.org/10.3390/ijerph192113997>.

IF₂₀₂₁ = 4,614; IF_{5-letni} = 4,799; punkty MEiN = 140

Sumaryczny IF₂₀₂₁ = 16,339

Sumaryczny IF_{5-letni} = 14,469

Łączna liczba punktów MEiN = 400

Wartości IF oraz punktację MEiN podano zgodnie listą obowiązującą w 2022 roku*

*(*Dane z bazy Journal Citation Reports™, stan na dzień 07.11.2022 r.)*

Źródła finansowania badań prowadzonych w ramach pracy doktorskiej

1. Dofinansowanie projektu pt. „*Charakterystyka indukcji lakazy grzyba *Myrothecium sp. IM 6443* oraz analiza jej potencjału biodegradacyjnego*” przyznane przez Narodowe Centrum Nauki w ramach konkursu Preludium 14. Lata 2017-2022 (UMO-2017/27/N/NZ9/02160), kierownik projektu
2. Dotacja celowa na działalność związaną z prowadzeniem badań naukowych lub prac rozwojowych oraz zadań z nimi związanych, służących rozwojowi młodych naukowców oraz uczestników studiów doktoranckich.
Rok 2018 (5811/E-345/M/2018), kierownik projektu

I Wprowadzenie

Powszechne wykorzystanie substancji barwiących w niemal wszystkich gałęziach przemysłu, w tym przede wszystkim w przemyśle farbiarskim, włókienniczym, kosmetycznym i papierniczym, przyczyniło się do ich znacznego rozprzestrzenienia w środowisku. Aktualnie znanych jest ponad 10 000 różnych barwników, a ich roczna produkcja wynosi 7×10^7 ton (Al-Tohamy i in. 2022; Ledakowicz i Paździor, 2021). W 2021 roku wartość światowego rynku barwników i pigmentów została wyceniona na 36,4 mld USD i zgodnie z prognozami oczekuje się, że w latach 2022–2030 osiągnie 57,2 mld USD (Dyes & Pigments Market Size, Share & Trends Analysis Report 2022-2030).

W trakcie procesów przemysłowych część wykorzystywanych barwników nie jest wiązana do barwionych materiałów, w efekcie czego może następnie przedostawać się wraz ze ściekami do środowiska wodnego (Ngo i Tischler, 2022). Oprócz barwników przemysł tekstylny wykorzystuje różnorodne związki chemiczne, które uwolnione do środowiska naturalnego stwarzają poważne zagrożenie dla organizmów w nim żyjących. Są to m. in. substancje pomocnicze, zmiękczacze i rozjaśniające tkaniny oraz metale przyspieszające proces barwienia (Al-Tohamy i in. 2022). W wyniku produkcji w zakładach przemysłu tekstylnego powstaje wiele różnych odpadów, które wymagają odpowiedniego zagospodarowania. Mogą być one poddane spalaniu, składowaniu lub przetworzeniu (Mihułka i in. 2003). Ze względu na olbrzymią skalę produkcji oraz nieefektywność procesów technologicznych do środowiska może przedostawać nawet do 50% stosowanego barwnika (Lellis i in. 2019).

W ostatnim czasie coraz więcej uwagi poświęca się również składowiskom odpadów niebezpiecznych, w których przez lata deponowane były różnorodne chemikalia bez właściwego nadzoru oraz ochrony przed uwolnieniem toksycznych substancji do środowiska naturalnego (Janicki i in. 2022). Na terenie województwa łódzkiego są to nieczynne już składowiska dawnych Zakładów Przemysłu Barwników „Boruta” (Z. P. B. „Boruta”) w Zgierzu oraz Zakładów Włókien Chemicznych „Wistom” w Tomaszowie Mazowieckim (Raport NIK, 2020). W założonej w Zgierzu w 1894 roku przez Jana Śniechowskiego i Ignacego Hordliczkę fabryce barwników zajmowano się produkcją (poza barwnikami) kwasu siarkowego, pikrytu, benzenu, fenolu i toluenu, co sprawiło, że zakłady te były ówczesnym największym w Polsce oraz znaczącym w Europie producentem barwników. Przy fabryce rozpoczęto również budowę pełnej, biologicznej oczyszczalni ścieków komunalnych i przemysłowych, której uruchomienie zaplanowano pierwotnie na grudzień 1996 roku. Jednak w I połowie lat 90-tych XX wieku opublikowano tzw. „Listę 80”, czyli listę największych trucicieli środowiska, wśród których znalazły się zakłady „Boruta”. Pomimo oddania w 1998 roku oczyszczalni do użytku, wraz z początkiem 1999 zakłady „Boruta” w Zgierzu zostały postawione w stan likwidacji (Przygucki 1994). Odpady zgromadzone w trakcie produkcji barwników już przed I wojną światową przechowywano w żelaznych pojemnikach, beczkach lub

koszach, a następnie składowano przykryte warstwą piasku, popiołu, azbestu i odpadów komunalnych (Raport NIK, 2020). Ze względu na niewłaściwe zabezpieczenie skarp, opady atmosferyczne przesiąkające przez warstwy ziemi wraz ze składowanymi odpadami, wypłukują z nich różne substancje. Powstają wtedy tzw. odcieki o złożonym składzie chemicznym. W związku z tym niezmiernie ważnym zadaniem jest opracowanie metody umożliwiającej efektywną eliminację toksycznych zanieczyszczeń. Duża różnorodność niebezpiecznych substancji sprawia, że nie ma uniwersalnej metody usuwania ich ze ścieków i zanieczyszczonych akwenów wodnych. Na wybór optymalnych technologii wpływają przede wszystkim rodzaj oraz stężenie zanieczyszczeń oraz planowane koszty procesu. Chociaż wyróżnia się trzy podstawowe metody eliminacji zanieczyszczeń: fizyczne, chemiczne i biologiczne, optymalnym rozwiązaniem jest połączenie wymienionych metod (Góralczyk i in. 2016).

Metodami najbardziej obiecującymi a zarazem przyjaznymi dla środowiska, które dodatkowo nie wymagają wysokich nakładów finansowych są metody biologiczne, zwłaszcza bioremediacja wykorzystująca do oczyszczania środowiska głównie potencjał metaboliczny drobnoustrojów. Wiele badań koncentruje się na wykorzystaniu w tym celu bakterii m. in. z rodzaju *Mycobacterium*, *Nocardia* i *Sphingomonas* (Lu i in. 2019). Również grzyby stanowią potężne narzędzie biotechnologiczne do usuwania zanieczyszczeń (de Lima i in. 2018). Wśród licznych zalet ich stosowania wskazać można przede wszystkim na biosyntezę enzymów m. in. lakaz, peroksydaz ligninowej (LiP) i manganozależnej (MnP) oraz monooksygenaz cytochromu P450 zdolnych do rozkładu złożonych organicznych związków o toksycznym charakterze (Ghosal i in. 2016; Lawal 2017). Grzyby charakteryzują się ponadto wysoką tolerancją na ekstremalne i zmienne warunki środowiska (Worrich i in. 2017; González-Abradelo i in. 2019; Peidro-Guzmán i in. 2020). Większość badań skupia się na zdolnościach grzybów białej zgnilizny (ang. *white-rot fungi*, WRF) do niespecyficznego rozkładu zanieczyszczeń za pośrednictwem zewnątrzkomórkowych enzymów ligninolitycznych. Wśród przykładowych gatunków WRF wskazać można *Phanerochaete chrysosporium*, *Pleurotus ostreatus* i *Trametes versicolor* (Cortés-Espinosa i in. 2013; Ghosal i in. 2016; Medaura i in. 2021). Szczególnie dużo uwagi poświęca się lakazom, należącym do grupy wielomiedziowych oksydaz (ang. *multicopper oxidases*, MCOs), które katalizują reakcje utleniania związków organicznych i nieorganicznych z jednoczesną redukcją tlenu cząsteczkowego do wody. Ze względu na szeroką specyficzność substratową oraz różne typy katalizowanych reakcji, lakazy stały się obiektem zainteresowania wielu badaczy. Zdolność utleniania mono- i difenoli, pochodnych aromatycznych, a także związków nieorganicznych, umożliwia szerokie zastosowanie tych enzymów w wielu gałęziach przemysłu, ochronie środowiska i medycynie (Szewczyk i Kowalski 2016). Lakazy grzybowe znalazły zastosowanie m. in. w przemyśle spożywczym, do usuwania z soków owocowych, piwa i wina związków fenolowych, występujących w zbyt dużych stężeniach i nadających tym napojom niepożądane cechy organoleptyczne (Upadhyay i in. 2016). Można je również wykorzystywać w procesach biodegradacji zanieczyszczeń

przemysłu farmaceutycznego (Lonappan i in. 2019), wielopierścieniowych węglowodorów aromatycznych (WWA) (Agrawal i in. 2018) i barwników tekstylnych (Jasińska i in. 2019).

Według Medaury i in. (2021) wiele grzybów należących do Ascomycota oraz Zygomycota, szeroko rozpowszechnionych w środowisku naturalnym, a także w zanieczyszczonych glebach, uległo przystosowaniu do niekorzystnych warunków poprzez wykształcenie zdolności do metabolizowania toksycznych związków organicznych m. in. poprzez produkcję enzymów. Jednym ze sposobów przystosowania się do niesprzyjających warunków są zmiany jakościowe i ilościowe kwasów tłuszczowych budujących lipidy błonowe tychże drobnoustrojów (Bernat i in. 2018). Kwasy tłuszczowe stanowią najbardziej dynamiczny składnik komórek. Na skutek oddziaływania różnych czynników zewnętrznych, lipidy tworzące struktury komórkowe ulegają modyfikacjom. Z tego też względu drobnoustroje łatwo przystosowują się do zmiennych warunków środowiska (Bernat 2020; de Kroon i in. 2013).

Głównym celem niniejszej rozprawy doktorskiej było ustalenie możliwości wykorzystania mikroskopowego grzyba strzępkowego *Nectriella pironii* IM 6443 do produkcji lakazopodobnej oksydazy wielomiedziowej oraz eliminacji zanieczyszczeń pochodzących z przemysłu tekstylnego takich jak barwniki przemysłowe, aminy aromatyczne oraz wielopierścieniowe węglowodory aromatyczne. Badany drobnoustrój został wyizolowany z gleby pobranej z terenów dawnych Zakładów Przemysłu Barwników „Boruta” w Zgierzu, gdzie przez wiele lat składowane były, w niewłaściwy sposób, odpady przemysłu tekstylnego. Również odcieki wykorzystane w pracy zostały pobrane ze składowiska odpadów niebezpiecznych zlokalizowanego na tym terenie.

II Cele pracy

Za główne cele badań przeprowadzanych podczas realizacji pracy doktorskiej przyjęto:

1. Skrining grzybów wyizolowanych z terenu dawnych Zakładów Przemysłu Barwników „Boruta” w Zgierzu wykazujących zdolność do produkcji enzymu o aktywności lakazy i eliminacji barwników przemysłu tekstylnego.
2. Wykorzystanie grzyba *Nectriella pironii* do eliminacji amin aromatycznych i barwników azowych oraz utylizacji odcieków ze składowiska odpadów niebezpiecznych.
3. Eliminacja wielopierścieniowych węglowodorów aromatycznych (WWA) przez grzyb *N. pironii* w obecności odcieków składowiskowych oraz ustalenie mechanizmów adaptacji grzyba do toksycznych zanieczyszczeń generowanych przez przemysł tekstylny.

III Realizacja poszczególnych celów pracy

III.1 Skrining grzybów wyizolowanych z terenu dawnych Zakładów Przemysłu Barwników „Boruta” w Zgierzu wykazujących zdolność do produkcji enzymu o aktywności lakazy i eliminacji barwników przemysłu tekstylnego

Charakterystykę wielomiedziowych oksydaz, w tym zaliczanych do nich lakaz opisano w artykule przeglądowym (P1) „Characteristics and use of multicopper oxidases enzymes” (Advancements of Microbiology, 58, 1: 7–18. <https://doi.org/10.21307/PM-2019.58.1.007>).

Wyniki badań obejmujące skrining drobnoustrojów wykazujących zdolność do produkcji enzymu o aktywności lakazy, wpływ warunków hodowli na aktywność enzymu, identyfikację lakazy, a następnie możliwości jej wykorzystania w procesach odbarwiania barwników przemysłowych przedstawiono w pracy eksperymentalnej (P2) „Laccase activity of the ascomycete fungus *Nectriella pironii* and innovative strategies for its production on leaf litter of an urban park” (PLOS ONE, 15, 4, e0231453; errata w PLOS ONE 2020, 15, e0233553. <https://doi.org/10.1371/journal.pone.0231453>).

Realizację pierwszego celu badawczego niniejszej pracy doktorskiej rozpoczęto od skringu grzybów, należących do Kolekcji Szczepów Katedry Mikrobiologii Przemysłowej i Biotechnologii UŁ, wyizolowanych ze skażonej gleby pobranej z terenu dawnych Z. P. B. „Boruta” w Zgierzu. Izolaty testowano pod kątem zdolności do produkcji enzymu o aktywności lakazy wykorzystując podłoże stałe Kirka-Farrella z dodatkiem substratów utlenianych przez lakazy do barwnych pochodnych, takich jak sól diamonowa kwasu 2,2'-azyno-bis(3-etylobenzotiazolino-6-sulfonowego) (ABTS) lub 2,6-dimetoksyfenol (2,6-DMP). W wybranych odstępach czasu dokonywano pomiarów średnicy grzybni oraz strefy zabarwienia wokół niej. W hodowlach szczepów IM 6443, IM 6467 oraz IM 6482 już po 24 godz. inkubacji zaobserwowano zabarwienie wokół grzybni wskazujące na syntezę enzymów przekształcających wymienione związki. Z tego względu wymienione izolaty użyto w kolejnym etapie badań, który polegał na ilościowej ocenie produkcji enzymu podczas hodowli w podłożu płynnym (Eggert i in. 1996). Spośród trzech badanych szczepów tylko szczep IM 6443 charakteryzował się najwyższą aktywnością enzymu, co przełożyło się na jego wybór jako modelu badawczego w prezentowanej pracy.

W celu ustalenia przynależności gatunkowej mikroorganizmu przeprowadzono analizę genetyczną w oparciu o sekwencje ITS. Na podstawie wyników stwierdzono, że wyznaczona sekwencja pozwala na zaklasyfikowanie grzyba oznaczonego symbolem IM 6443 jako *Nectriella pironii*. Dostępne dane literaturowe wskazują, iż *N. pironii* jest patogenem krzewów ozdobnych takich jak afelandra stercząca (*Aphelandra squarrosa*),

szczęśliw bungego (*Clerodendrum bungei*) i trójskrzyn pstry (*Codiaeum variegatum*) (Alfieri i Samuels, 1979).

Enzymy grzybowe o aktywności lakazy są wytwarzane stale, na pewnym poziomie, jednak możliwe jest uzyskanie ich nadprodukcji poprzez modyfikacje składu podłoża wzrostowego oraz warunków hodowli. Produkcja tych enzymów zależy od szeregu warunków takich jak stężenie i rodzaj źródła węgla jak i azotu oraz dodatek induktorów, czyli związków chemicznych powodujących zwiększenie produkcji enzymów (Piscitelli i in. 2011). Wśród powszechnie stosowanych induktorów wyróżnia się wybrane jony metali ciężkich, aminokwasy i związki aromatyczne. W badaniach dotyczących grzybów powszechną metodą zwiększania aktywności lakaz jest wykorzystanie jonów miedzi. Dowiedziono, że miedź odgrywa ważną rolę zarówno w zwiększeniu ekspresji genów kodujących lakazę, jak i ich regulacji na poziomie transkrypcji i translacji (Mahmoud i in. 2013). Dodatek tego metalu nie tylko wzmacnia syntezę lakaz, ale również pozytywnie wpływa na ich aktywność i stabilność. W niniejszej pracy wzbogacenie podłoża wzrostowego o siarczan miedzi (II) lub kwas ferulowy, skutkowało uzyskaniem aktywności lakazy na poziomie wynoszącym 1500 U L^{-1} , podczas gdy w układzie kontrolnym odnotowano 500 U L^{-1} . Natomiast skojarzone działanie tych dwóch induktorów spowodowało wzrost aktywności lakazy do 3500 U L^{-1} . Dane literaturowe wskazują, iż zwiększona produkcja lakazy w obecności związków fenolowych może być reakcją obronną drobnoustroju na wywołany ich obecnością stres oksydacyjny (Vrsanska i in. 2016). Związki fenolowe są naturalnymi produktami rozkładu ligniny, która zawarta jest np. w materiałach ligninocelulozowych. Interesującym aspektem wydaje się zatem wykorzystanie takich materiałów do przygotowania podłoży mikrobiologicznych, co stanowi tańszą alternatywę niż wykorzystanie podłoży komercyjnych. Dodatkowo takie podejście pomaga w utylizacji wybranych odpadów pochodzących z przemysłu rolno-spożywczego, wpisując się w koncepcję zrównoważonego rozwoju. Przykładem takiego materiału jest makuch rzepakowy - produkt uboczny otrzymywany po ekstrakcji oleju z nasion roślin oleistych, będący źródłem białek i tłuszczu (m. in. kwasu oleinowego, linolowego, linolenowego, erukowego). Również opadłe liście charakteryzujące się wyższą zawartością substancji fenolowych niż liście zielone, mogą być wykorzystywane do produkcji podłoży hodowlanych dla drobnoustrojów (Długoński i Góralczyk-Bińkowska, 2020). Z tego względu kolejny etap badań obejmował wykorzystanie bioodpadów takich jak makuch rzepakowy, siano oraz opadłe liście zebrane na Placu Komuny Paryskiej w Łodzi do hodowli grzyba strzępkowego *N. pironii*. Najwyższą aktywność enzymu (3330 U L^{-1}) uzyskano w 96 godz. hodowli w podłożu zawierającym ekstrakt z liści i była ona zbliżona do wartości otrzymanych podczas hodowli grzyba prowadzonych w gotowych podłożach mikrobiologicznych wzbogaconych jonami miedzi i kwasem ferulowym. Uzyskane wyniki stanowią pierwsze doniesienie dotyczące możliwości wykorzystania ekstraktu z liści do hodowli grzybów i wydajnej produkcji lakazy.

W kolejnym etapie pracy zrealizowanym dzięki uprzejmości Pana prof. dr. hab. Jarosława Dziadka i pomocy Pana dr. Przemysława Płocińskiego w Instytucie Biologii Medycznej Polskiej Akademii Nauk w Łodzi, opracowano metodę oczyszczania mieszaniny zewnątrzkomórkowych białek *N. pironii*. Procedura obejmowała wytrącanie białek siarczanem amonu, ultrafiltrację, a następnie chromatografię jonowymienną i filtrację żelową. Czystość i aktywność enzymatyczną otrzymanego białka potwierdzono obecnością pojedynczego prążka na zymogramie wybarwionym ABTS. Masa cząsteczkowa wynosiła około 50 kDa, co odpowiada danym literaturowym, opisującym właściwości innych lakaz. Identyfikację badanego białka potwierdzono również za pomocą spektrometrii mas porównując uzyskane widma fragmentacji z tymi dostępnymi w bazie danych, opisanymi jako „wielomiedziowe oksydazy” i/lub „lakazy”. Wyniki analiz sugerują, że sekwencja oczyszczonego białka jest najbardziej dopasowana do sekwencji białka KFA51000.1 z *Stachybotrys chartarum*. Wspomniane wielomiedziowe oksydazy stanowią bardzo zróżnicowaną grupę enzymów produkowanych zarówno przez organizmy pro-, jak i eukariotyczne, i charakteryzują się różnymi, nie do końca jeszcze poznanymi funkcjami biologicznymi. Ponieważ nadal nie ma jasnych kryteriów klasyfikacji, podział MCOs nie jest stały i usystematyzowany. Niemniej najbardziej odpowiedni wydaje się być podział zaproponowany przez Hoegger i in. (2006) wyróżniający 10 grup wielomiedziowych oksydaz: lakazy (Basidio- i Ascomycota oraz owadów), pigmenty grzybowe MCOs, ferroksozydazy grzybowe, roślinne i grzybowe oksydazy askorbinianowe, enzymy roślinne podobne do lakaz, białka odporne na miedź (CopA), oksydazy bilirubiny i białka wydzielania miedzi (CueO). Według Reiss i in. (2013) pojęcie „lakazy” należy stosować wyłącznie dla enzymu wydzielonego z soku drzewa *Rhus vernicifera*, z kolei inni autorzy (Brander i in. 2014) podkreślają, iż termin „lakazy” pierwotnie był stosowany w odniesieniu do roślinnych wielomiedziowych oksydaz, które posiadają trzy domeny. W pracy Ihssen i in. (2015) wskazane jest natomiast klasyfikowanie jako lakaz jedynie tych MCOs, które zostały wyizolowane w obecności nienasyconego alkilokatecholu - urushiolu. Z uwagi na odmienne, w pewnym stopniu, właściwości biochemiczne oraz nie do końca sprecyzowane funkcje biologiczne, należytem wydaje się być zatem wyróżnienie wśród MCOs także grupy enzymów lakazopodobnych (LMCOs) (Reiss i in. 2013). Aspekty te zostały wyczerpująco przedyskutowane w pracy przeglądowej (P1) wchodzącej w skład niniejszej rozprawy.

W kolejnym etapie badań przeprowadzono wstępną charakterystykę oczyszczonego białka obejmującą ocenę jego aktywności i stabilności w zakresie pH od 2,2 do 10,6 oraz analizę wpływu związków chemicznych opisywanych jako inhibitory enzymów. Enzym zachowywał aktywność w szerokim zakresie pH wynoszącym 3,0-10,6; natomiast jego najwyższą aktywność odnotowano w środowisku o pH równym 3,0. Z kolei lakaza najbardziej stabilna była przy pH równym 7,0 (wykorzystując do pomiarów 10 mM roztwór ABTS jako substrat). Enzym ulegał silnej inhibicji β -merkaptotanołem, azydkiem sodu, ditiotreitolem oraz kwasem kojowym (opisywanym jako typowy inhibitor lakaz).

Przeprowadzono również wstępne badania nad przydatnością oczyszczonej lakazy do dekoloryzacji barwników przemysłu tekstylnego. Analizie poddano barwniki zaliczane do różnorodnych grup chemicznych jak na przykład barwniki azowe (Reactive Orange 16 i Acid Red 27), indygidowe (Indigo Carmine) i antrachinonowe (Remazol Blue Brilliant R). Liczne doniesienia naukowe wskazują na niekorzystny wpływ tych związków na zdrowie zwierząt i ludzi. Na przykład, od 1976 r., barwnik Acid Orange 27 (AR 27) jest zakazany w Stanach Zjednoczonych przez Agencję Żywności i Leków (ang. *Food and Drug Administration*, FDA) jako potencjalny czynnik rakotwórczy (U.S. Government Accountability Office, 2022). Przeprowadzone badania pozwoliły na stwierdzenie, że enzym o aktywności lakazy (1 U mL^{-1}) zdolny jest do dekoloryzacji wymienionych barwników (w stężeniu 25 mg L^{-1}) już w ciągu 15 min. w zakresie 69-92%. Wskazuje to na ogromny potencjał lakazy *N. pironii* do dekoloryzacji różnorodnych barwników tekstylnych.

Badania wykonane w ramach pierwszego etapu pracy doktorskiej umożliwiły określenie warunków sprzyjających produkcji lakazy przez mikroskopowego grzyba strzępkowego *N. pironii*, a następnie wskazanie możliwości wykorzystania tego enzymu w procesach eliminacji barwników przemysłu tekstylnego. Nowatorskim podejściem było wykorzystanie w hodowli *N. pironii* i do produkcji enzymu, podłoża przygotowanego na bazie opadłych liści, co nie zostało do tej pory opisane w literaturze naukowej w odniesieniu do produkcji lakaz. Stanowi to niezmiernie korzystne rozwiązanie z uwagi na aspekt finansowy (w porównaniu z kosztami podłoży komercyjnych), a także gospodarkę odpadami.

Wnioski i stwierdzenia częściowe dotyczące skringu grzybów wyizolowanych z terenu dawnych Zakładów Przemysłu Barwników „Boruta” w Zgierzu wykazujących zdolność do produkcji enzymu o aktywności lakazy i eliminacji barwników przemysłu tekstylnego

1. Spośród badanych grzybów strzępkowych, trzy szczepy oznaczone symbolami IM 6443, IM 6467, IM 6482 wykazują zdolność utleniania ABTS i 2,6-DMP.
2. Hodowle prowadzone w podłożu płynnym wykazały, iż szczep IM 6443 charakteryzuje się najwyższą produkcją enzymu o aktywności lakazy.
3. Jony miedzi i kwas ferulowy indukują biosyntezę lakazy przez szczep IM 6443, a jednoczesny dodatek tych związków siedmiokrotnie zwiększa produkcję lakazy względem układu kontrolnego. Ponadto wykorzystanie niestandardowych podłoży (zwłaszcza ekstraktu z opadłych liści) skutkuje wytwarzaniem enzymu na podobnym poziomie jak dodatek chemicznych induktorów.
4. Badany drobnoustrój na podstawie analizy genetycznej został zidentyfikowany jako *N. pironii*.

5. Oczyszczona lakaza charakteryzuje się masą cząsteczkową wynoszącą 50 kDa oraz stabilnością w szerokim zakresie pH od 3,0 do 10,6.
6. Lakaza *N. pironii* charakteryzuje się zdolnością do dekoloryzacji barwników przemysłu tekstylnego zaliczanych do różnych grup chemicznych.

III.2 Wykorzystanie grzyba *Nectriella pironii* do eliminacji amin aromatycznych i barwników azowych oraz utylizacji odcieków ze składowiska odpadów niebezpiecznych

Rezultaty badań dotyczących możliwości wykorzystania grzyba *Nectriella pironii* do eliminacji amin aromatycznych, barwników azowych oraz utylizacji odcieków ze składowiska odpadów niebezpiecznych zostały opublikowane w pracy eksperymentalnej (P3) „Environmental and molecular approach to dye industry waste degradation by the ascomycete fungus *Nectriella pironii*” (Scientific Reports, 11, 23829. <https://doi.org/10.1038/s41598-021-03446-x>).

Przesłanką do realizacji badań w obrębie drugiego celu badawczego była publikacja raportu Najwyższej Izby Kontroli (NIK) pt. „Zapobieganie zagrożeniom ze strony składowisk pozakładowych z terenu województwa łódzkiego” opisującego stan środowiska na terenie składowiska po Z. P. B. „Boruta” w Zgierzu. Składowisko to opisywane jest jako jedno z czterech największych tzw. „bomb ekologicznych” w Polsce. Pozostałe trzy mieszczą się na terenie Bydgoszczy, Jaworzna i Tarnowskich Gór. Wyniki kontroli opisane we wspomnianym wcześniej raporcie ujawniły wiele nieprawidłowości m. in. obecność niewłaściwie zabezpieczonych odpadów, składowanych nielegalnie, z których wydostawały się odcieki przenikające do środowiska.

Ze względu na narastające ryzyko skażenia wód i gleby odciekami składowiskowymi w niniejszej pracy ocenie poddano zdolność grzyba *N. pironii* do eliminacji toksycznych zanieczyszczeń w nich zawartych. Próbkę odcieków (L1 oraz L2) pobranych ze składowiska odpadów niebezpiecznych dawnych Z. P. B. „Boruta” w Zgierzu zostały udostępnione dzięki uprzejmości Wojewódzkiego Inspektoratu Ochrony Środowiska (WIOŚ) w Łodzi. Pracownicy WIOŚ-u prowadzą stały monitoring odcieków ze składowiska, wód gruntowych (przy 9 zastosowaniach piezometrów), oczyszczonych ścieków oraz wód rzeki Bzury. Odcieki pobrane ze składowiska cechowały się podwyższoną zawartością chlorków i żelaza (dwukrotnie), lotnych fenoli (3 - 4-krotnie wyżej niż dopuszczalny limit) oraz przekroczeniem dopuszczalnej wartości dla wskaźnika BZT₅, odnoszącego się do biochemicznego zapotrzebowania na tlen, mierzonego po 5-ciu dniach. Dodatkowo przeprowadzone w ramach realizacji niniejszej pracy analizy HPLC-MS/MS badanych próbek wykazały obecność w odciekach *o*-tolidyny w stężeniach 0,3 i 0,43 mg L⁻¹. Związek ten jest aminą aromatyczną, powstałą na skutek degradacji barwników azowych, które jak wskazuje Raport NIK były produkowane przez wiele lat przez Z. P. B. „Boruta” z wykorzystaniem substancji o silnym działaniu kancerogennym, w tym benzydyny. Drugą aminą aromatyczną, której obecność stwierdzono w badanych próbkach, w stężeniach wynoszących odpowiednio 0,23 i 0,03 mg L⁻¹, była 4,4-oksydianilina. Substancja ta jest pochodną aniliny, wykorzystywaną w sieciowaniu polimerów oraz uznawana za potencjalny czynnik rakotwórczy dla ludzi [Wiley Online Library, 2022]. Według informacji uzyskanych z Głównego Inspektoratu Ochrony

Środowiska oraz Centralnego Laboratorium Badawczego (Oddział w Łodzi), amin aromatycznych nie wykryto w ściekach odprowadzanych z oczyszczalni do rzeki Bzury.

Następnie sprawdzono jak dodatek odcieków (10, 20 lub 40% zawartości) do podłoża hodowlanego wpływa na wzrost grzybni *N. pironii*. Wykazano, iż w 120 godz. hodowli w obecności 10% odcieków przyrost biomasy był dwukrotnie wyższy w porównaniu z układem kontrolnym. Natomiast wyższa zawartość odcieków (20 lub 40%) spowodowała spowolnienie procesów biosyntezy grzybni. Ocenie poddano również zdolność grzyba *N. pironii* do wzrostu oraz eliminacji *o*-tolidyny wprowadzonej do hodowli w stężeniach 0,5-50 mg L⁻¹. Przez trzy pierwsze doby hodowli produkcja biomasy w badanych układach była na podobnym poziomie i wynosiła 6-8 g L⁻¹. Po 72 godz. w hodowlach prowadzonych z dodatkiem *o*-tolidyny w stężeniu 50 mg L⁻¹ zaobserwowano nieznaczne ograniczenie produkcji biomasy. Analizy chromatograficzne umożliwiły określenie pozostałości *o*-tolidyny w hodowlach. Już po 24 godz. inkubacji w układach zawierających ksenobiotyk w stężeniu 0,5 mg L⁻¹ odnotowano 65% eliminację *o*-tolidyny. Istotny wzrost stopnia degradacji tego związku zaobserwowano po 96 godz. hodowli. Zawartość ksenobiotyku w hodowlach początkowo wynosząca 5, 10 i 50 mg L⁻¹ spadła odpowiednio do 0,48; 1,34 i 4,11 mg L⁻¹. Dane literaturowe dotyczące biodegradacji amin aromatycznych są ograniczone. Usuwanie tych związków opisano głównie dla amin aromatycznych wchodzących w skład pestycydów, leków i barwników, a w procesy te zaangażowane były różne gatunki bakterii, np. *Bacillus* sp., *Pseudomonas* sp., *Proteus* sp., *Serratia* sp., *Enterobacter* sp. (Barsing in. 2011; Fatima i in. 2019).

Uzyskane wyniki pozwalają stwierdzić, że w hodowli *N. pironii* *o*-tolidyna zostaje przekształcona głównie do 3,3'-dihydroksybenzydyny (*m/z* 217). Według Brüscheiler i Merlot (2017) 3,3'-dihydroksybenzydyna charakteryzuje się niższą toksycznością od *o*-tolidyny. Widma masowe otrzymanych metabolitów porównano z danymi literaturowymi i stwierdzono, że *o*-tolidyna może również ulegać hydroksylacji w hodowli *N. pironii* (np. przy udziale lakazy) i/lub być przekształcana w 3,3'-dihydroksybenzydynę i jej pochodne. Ponadto wykazano, iż w procesie biorą udział lakaza i cytochrom P450. Uzyskane rezultaty stanowią pierwsze doniesienie o biotransformacji *o*-tolidyny przez mikroskopowe grzyby.

Biorąc pod uwagę fakt, że ścieki przemysłu tekstylnego są mieszaniną zanieczyszczeń zawierających pozostałości barwników, soli, metali ciężkich i wielu innych chemikaliów stosowanych podczas barwienia, przeprowadzono dalsze badania w celu ustalenia wpływu wybranych metali na zdolność odbarwiania barwników azowych Acid Orange 7 (AO 7) i Reactive Red 120 (RR 120) w stężeniu 50 mg L⁻¹. Wybrano metale ciężkie (w stężeniu 0,75 mM Cd²⁺; 0,1 mM Cr⁶⁺; 1,75 mM Zn²⁺) powszechnie obecne w ściekach przemysłu barwiarskiego. Metale dodawano do hodowli *N. pironii* pojedynczo lub w postaci mieszaniny. Już w 48 godz. hodowli z dodatkiem barwnika RR 120 i mieszaniny jonów metali odnotowano 82% dekoloryzację. Odbarwianie AO 7 w 24 godz. hodowli *N. pironii* zachodziło nieznacznie wolniej w obecności metali dodawanych pojedynczo. Po 72 godz. hodowli w podłożu z dodatkiem AO 7 i mieszaniny jonów

metali uzyskano 83,5% dekoloryzację barwnika. W rzeczywistych ściekach z przemysłu włókienniczego barwnikom towarzyszą inne szkodliwe związki, np. metale ciężkie lub aminy stosowane jako prekursory barwników. Dlatego mikroorganizmy wykorzystywane w procesach biodegradacji barwników powinny wykazywać oporność wobec szkodliwego działania tych substancji i, co istotne, zdolność do przeprowadzania biodegradacji we wspomnianych warunkach.

Wyniki uzyskane w tym etapie pracy wskazują na zdolność mikroskopowego grzyba strzępkowego do biotransformacji *o*-tolidyny, w której uczestniczą lakaza i cytochrom P450. Stanowi to cenny wkład w rozwój badań nad eliminacją amin aromatycznych, która do tej pory była szeroko opisywana u bakterii. Co więcej, niezwykle ważnym aspektem okazało się wprowadzenie odcieków składowiskowych do hodowli *N. pironii*. Badania te wykazały, iż grzyb charakteryzuje się zdolnością do wzrostu w obecności toksycznych zanieczyszczeń pochodzących z przemysłu tekstylnego. Właściwość ta w przyszłości może być przydatna w opracowaniu systemu do bioremediacji środowisk skażonych różnorodnymi zanieczyszczeniami.

Wnioski i stwierdzenia cząstkowe dotyczące eliminacji amin aromatycznych i barwników azowych oraz utylizacji odcieków ze składowiska odpadów niebezpiecznych przez *N. pironii*

1. Ocieki pobrane ze składowiska odpadów niebezpiecznych charakteryzują się podwyższoną zawartością chlorków, żelaza, lotnych fenoli oraz przekroczeniem dopuszczalnej wartości dla wskaźnika BZT₅. Analiza chromatograficzna wykazuje obecność w odciekach amin aromatycznych – *o*-tolidyny oraz 4,4-oksydianiliny.
2. Mikroskopowy grzyb strzępkowy *N. pironii* IM 6443 wykazuje wzmożoną zdolność do wzrostu w obecności odcieków pobranych ze składowiska odpadów niebezpiecznych. Dodatek *o*-tolidyny do podłoża wzrostowego nie wpływał znacząco na produkcję biomasy.
3. *o*-tolidyna podczas hodowli *N. pironii* ulega hydroksylacji i/lub przekształceniu do mniej toksycznej pochodnej – 3,3'-dihydroksybenzydyny. Grzyb *N. pironii* odznacza się zdolnością do dekoloryzacji barwników Acid Orange 7 i Reactive Red 120 również w obecności jonów metali.

III.3 Eliminacja wielopierścieniowych węglowodorów aromatycznych (WWA) przez grzyb *N. pironii* w obecności odcieków ze składowiska odpadów niebezpiecznych po dawnych Zakładach Przemysłu Barwników „Boruta” w Zgierzu oraz ustalenie mechanizmów adaptacji grzyba do toksycznych zanieczyszczeń generowanych przez przemysł tekstylny

Wyniki badań dotyczące oddziaływania odcieków i związków zaliczanych do WWA na szczep mikroskopowego grzyba strzępkowego *N. pironii*, obejmujące kinetykę wzrostu grzybni, procesy biodegradacji WWA oraz zmiany adaptacyjne błon grzyba w odpowiedzi na obecność tych związków zostały opisane w pracy eksperymentalnej (P4) „Accelerated PAHs transformation in the presence of dye industry landfill leachate combined with fungal membrane lipids changes” International Journal of Environmental Research and Public Health 19, 13997. <https://doi.org/10.3390/ijerph192113997>.).

Realizację trzeciego celu pracy doktorskiej kontynuowano poddając analizie wpływ dwóch odcieków składowiskowych oraz trzech związków z grupy WWA – fenantrenu (Fen), benz[a]antracenu (B[a]A) oraz benz[a]pirenu (B[a]P) na wzrost grzybni *N. pironii* oraz efektywność biodegradacji badanych WWA. W pierwszej kolejności wykazano, iż w hodowlach prowadzonych w obecności B[a]A oraz B[a]P wzrost grzybni był na podobnym poziomie ($10,1$ i $10,4 \text{ g L}^{-1}$) jak w hodowli prowadzonej bez dodatku ksenobiotyku ($10,6 \text{ g L}^{-1}$). Natomiast wprowadzenie Fen do hodowli ograniczyło wzrost grzybni od 48 godz. hodowli. Dane literaturowe wskazują, iż różnice w tempie wzrostu grzybni w obecności Fen, B[a]A i B[a]P mogą wynikać ze struktury chemicznej tych związków. Odnosi się to do oporności na biodegradację, która wzrasta wraz z liczbą pierścieni aromatycznych budujących dany związek chemiczny (Fayeulle i in. 2019). Jednakże w prezentowanej pracy najbardziej szkodliwy wpływ na wzrost grzybni wywarł trójpierścieniowy fenantren, w przeciwieństwie do cztero- (B[a]A) i pięciopierścieniowych (B[a]P) ksenobiotyków. Według danych literaturowych czynniki fizyczne i chemiczne, w tym prężność pary, rozpuszczalność i adsorpcja wpływają na tolerancję WWA przez grzyby (Patel i in. 2020). Zatem wyższa wartość rozpuszczalności w wodzie Fen w porównaniu z B[a]A i B[a]P może wzmacniać adsorpcję tego związku z podłoża hodowlanego przez grzybnie. Wyniki analizy HPLC wykazały, iż pozostałości Fen, B[a]A i B[a]P wprowadzonych do hodowli w początkowym stężeniu 20 mg L^{-1} , po 72 godz. inkubacji stanowiły odpowiednio 12,3; 14,9 oraz 16,2 mg L^{-1} . Fen pomimo najbardziej negatywnego wpływu na produkcję biomasy *N. pironii*, był w największym stopniu eliminowany, co może wynikać z jego wysokiej rozpuszczalności w podłożu, a w konsekwencji większej przyswajalności przez grzybnie.

Następnie oceniono wpływ odcieków składowiskowych na wzrost grzybni *N. pironii* oraz eliminację WWA. Zaobserwowano, iż w obecności odcieków zawartość

biomasy w hodowlach *N. pironii* po 72 h inkubacji była zbliżona w układach zawierających odcieki i Fen (7,06 g L⁻¹ dla L1; 7,34 g L⁻¹ dla L2) do tej odnotowanej w hodowli zawierającej jedynie Fen (7,25 g L⁻¹). Uzyskane wyniki wskazują, iż badany drobnoustrój jest zdolny do adaptacji do niekorzystnych warunków środowiska takich jak obecność toksyn. Zależność ta skłoniła do przeprowadzenia badań nad wpływem w eliminacji WWA. Analiza HPLC-MS/MS wykazała, iż obecność odcieków znacznie zwiększa tempo degradacji WWA. Po 72 godz. hodowli pozostałość Fen (przy stężeniu wyjściowym 20 mg L⁻¹) wyniosła 2,69 i 2,99 mg L⁻¹ dla układów zawierających odpowiednio L1 i L2. Dodatek odcieków sprzyjał również degradacji B[a]A i B[a]P przez *N. pironii*. Po upływie 72 godz. hodowli z dodatkiem L1 i L2 pozostałość B[a]A wyniosła odpowiednio 0,98 i 0,65 mg L⁻¹ podczas gdy stężenie B[a]P w hodowli z dodatkiem L1 i L2 osiągnęło odpowiednio 3,09 oraz 1,01 mg L⁻¹. Wyższa skuteczność usuwania WWA w obecności odcieków może sugerować, że sprzyjają one degradacji Fen, B[a]A i B[a]P. Jednym z wyjaśnień tego zjawiska może być kometabolizm. Dodatek odcieków może stymulować sekrecję enzymów katabolicznych przez *N. pironii*, i tym samym wspomagać degradację WWA (Acevedo i in. 2011). Analizy widm masowych umożliwiły identyfikację czterech metabolitów Fen, B[a]A i B[a]P w hodowlach *N. pironii* prowadzonych z dodatkiem odcieków. Jednym ze zidentyfikowanych metabolitów we wszystkich badanych układach był kwas protokatechowy, będący związkiem pośrednim identyfikowanym w różnych szlakach metabolicznych fenantrenu podczas procesów rozszczepiania pierścieni (Liao i in., 2013). Torres-Farradà i in. (2019) wykazali, iż wymienione metabolity są związkami nietoksycznymi, o prostej budowie co umożliwia ich przekształcanie do CO₂ i H₂O na drodze reakcji biochemicznych przeprowadzanych przez mikroorganizmy. Kwas protokatechowy zidentyfikowano również w hodowlach zawierających odcieki wraz z B[a]An jak i B[a]P, co wskazuje na zdolność grzyba *N. pironii* do przekształcania B[a]P w mniej toksyczne pochodne. Na uwagę zasługuje fakt, że B[a]P składa się z pięciu pierścieni aromatycznych oraz charakteryzuje się wysoką stabilnością i niską biodostępnością, przez co nie jest łatwo degradowanym związkiem (Nzila i Musa, 2020). Jednak w przeprowadzonych eksperymentach pod wpływem aktywności grzyba *N. pironii*, B[a]P uległ przekształceniu w nietoksyczny kwas protokatechowy.

W pracach naukowych z ostatnich lat opisano wiele przykładów drobnoustrojów (w tym grzybów strzępkowych), które wykształciły szereg mechanizmów umożliwiających przetrwanie i przystosowanie się do niekorzystnych warunków w środowisku ich bytowania. Jednym ze sposobów adaptacji może być zmiana składu lipidów tworzących błony komórkowe (Bernat 2020). Ściana komórkowa i błona komórkowa mikroorganizmów mają bezpośredni kontakt z niebezpiecznymi substancjami otaczającymi komórkę. Drobnoustroje zmieniając strukturę błony mogą ułatwiać lub ograniczać przenikanie różnych substancji do jak i z wnętrza komórki (de Kroon i in. 2013). W celu wyjaśnienia zmian zachodzących w błonach komórkowych na skutek oddziaływania ksenobiotyków, prowadzi się badania obrazujące zmiany

ilościowe i jakościowe lipidów tworzących struktury komórkowe. Spośród lipidów najważniejszą klasę stanowią fosfolipidy (ang. *phospholipids*, PLs), które są podstawowym składnikiem budulcowym błon komórkowych i mogą odgrywać istotną rolę w adaptacji do niekorzystnych warunków (Bernat 2020). W związku z tym w pracy doktorskiej analizie poddano wpływ odcieków (w stężeniu 20%) i Fen, B[a]A i B[a]P (w stężeniu 20 mg L⁻¹) na skład PLs błonowych. U grzybów najważniejsze klasy PLs to: fosfatydylocholiny (PC), fosfatydyloetanolaminy (PE), fosfatydyloseryny (PS) fosfatydyloglicerol (PG), fosfatydyloinozytol (PI) oraz kwas fosfatydowy (PA). Analiza wykazała, iż głównymi klasami fosfolipidów błony *N. pironii* są PC i PE, których udział różnił się w zależności od warunków hodowli. W świetle danych literaturowych, zwiększenie zawartości PC w dwuwarstwie lipidowej stabilizuje jej strukturę, podczas gdy PE zmniejsza płynność błony i zwiększa przepuszczalność, co w konsekwencji może prowadzić do utraty integralności komórki (Li i in. 2006). Po 24 godz. hodowli badanego drobnoustroju w układzie kontrolnym (nie zawierającym ani WWA, ani żadnego z odcieków) zawartość PC wynosiła 36%. W hodowli z dodatkiem odcieku zawartość PC osiągnęła 40% całkowitej puli PLs podczas gdy w hodowlach prowadzonych w obecności wyłącznie Fen oraz Fen w połączeniu z L1 wynosiła odpowiednio 59,98 i 68,43%. Na podstawie uzyskanych wyników wyznaczono również stosunek PC/PE, który jest wskaźnikiem stopnia integralności i przepuszczalności błony komórkowej, a na jego wartość wpływają warunki środowiskowe (de Kroon i in. 2013). W hodowlach *N. pironii* zawierających wyłącznie Fen lub Fen w połączeniu z L1 z dodatkiem wyłącznie fenantrenu lub fenantrenu i odcieku, stosunek PC/PE był odpowiednio ponad 2- i 4-krotnie wyższy niż w układzie kontrolnym lub w hodowlach zawierających tylko odciek. Na podkreślenie zasługuje fakt, iż różnice te zaobserwowano jedynie w hodowlach inkubowanych z dodatkiem Fen. Natomiast w hodowlach *N. pironii* zawierających B[a]P lub B[a]A, stosunek PC/PE tylko nieznacznie różnił się od wartości ustalonej w układzie kontrolnym.

Równocześnie wykonywane analizy zawartości kwasów tłuszczowych tworzących cząsteczki fosfolipidów (ang. *phospholipid fatty acids*, PLFAs) ujawniły zmiany w ich nasyceniu w obecności Fen. Zmniejszeniu uległa zawartość nienasyconych kwasów tłuszczowych PC 18:2/18:2; PE 18:2/18:2 i PE 18:2/18:1 przy jednoczesnym wzroście zawartości kwasów nasyconych PC 16:0/18:2 i PC 16:0/18:1, co wskazuje na wzrost integralności błony. W oparciu o uzyskane wyniki wykazano, iż kwas palmitynowy (C16:0) był najbardziej dominującym nasyconym kwasem tłuszczowym, podczas gdy wśród nienasyconych były to kwas oleinowy (C18:1) i kwas linolowy (C18:2).

Z uwagi na wykazane w tym etapie pracy modyfikacje w składzie lipidów błonowych sprawdzono również, czy wprowadzenie fenantrenu i odcieków do hodowli *N. pironii* spowoduje oksydacyjne uszkodzenia lipidów badanego szczepu. Poprzez pomiar zawartości substancji reagujących z kwasem tiobarbiturowym (ang. *thiobarbituric acid reactive substances*, TBARS) oceniono stopień peroksydacji lipidów. W grzybnii *N. pironii* poddanej działaniu Fen (20 mg L⁻¹) stwierdzono podwyższony

poziom TBARS. Zawartość TBARS wzrosła z 2,34 (kontrola biotyczna) do 12,15 $\mu\text{M g}^{-1}$ mokrej biomasy (próbka z Fen) w pierwszych 24 godz. hodowli. Nie zaobserwowano natomiast zwiększonej ilości TBARS przy jednoczesnej obecności Fen i odcieków w hodowlach *N. pironii*. Po upływie 48 godz. inkubacji zawartość TBARS w grzybni poddanej działaniu Fen zmniejszyła się i we wszystkich badanych układach była na poziomie wynoszącym 1,8-2 $\mu\text{M g}^{-1}$ mokrej biomasy. Zaobserwowane w niniejszych badaniach zjawisko początkowego wzrostu poziomu peroksydacji lipidów pod wpływem Fen, a następnie jego spadku do poziomu odnotowanego w układzie kontrolnym może sugerować adaptację *N. pironii* do czynnika stresowego i/lub detoksykację zanieczyszczenia na skutek jego biotransformacji do mniej toksycznych produktów.

Szczególnie warte podkreślenia w tym etapie pracy wydają się być rezultaty odnoszące się do eliminacji WWA przez *N. pironii* w obecności odcieków składowiskowych. W wyniku wprowadzenia odcieków do hodowli, 5-krotnemu zwiększeniu uległa eliminacja Fen i B[a]P, natomiast w przypadku usuwania B[a]A wzrost był 15-krotny, w porównaniu do hodowli grzyba zawierających jedynie badane WWA. Uzyskane wyniki wskazują na potencjał *N. pironii* w przekształcaniu różnorodnych związków, nawet w obecności toksycznych zanieczyszczeń. Badania przeprowadzone w ramach niniejszej pracy doktorskiej mają charakter podstawowy, jednakże wskazują potencjalne możliwości wykorzystania grzyba *N. pironii* w procesach biodegradacji różnorodnych zanieczyszczeń generowanych przez przemysł tekstylny.

Wnioski i stwierdzenia cząstkowe dotyczące eliminacji WWA przez *N. pironii* w obecności odcieków ze składowiska odpadów niebezpiecznych po dawnych Zakładach Przemysłu Barwników „Boruta” w Zgierzu oraz mechanizmów adaptacji grzyba

1. Badany szczep jest w stanie eliminować trzy-, cztero- i pięciopierścieniowe WWA, a dodatek odcieków do hodowli przyspiesza tempo biodegradacji Fen, B[a]A i B[a]P.
2. Wykorzystanie przez *N. pironii* składników odżywczych zawartych w podłożu i odciekach składowiskowych świadczy o kometabolicznym charakterze biodegradacji badanych WWA.
3. Ekspozycja błon grzybowych na Fen w połączeniu z L1 silnie wpływa na profil fosfolipidowy. Pod wpływem Fen dodawanego oddzielnie lub w połączeniu z L1 zwiększeniu w błonach *N. pironii* ulega zawartość PC, podczas gdy zawartość PE zmniejsza się prowadząc do wzrostu integralności błony.
4. Analiza kwasów tłuszczowych wskazuje, iż w obecności Fen zmniejszeniu ulega zawartość nienasyconych kwasów tłuszczowych PC 18:2/18:2; PE 18:2/18:2 i PE 18:2/18:1 przy jednoczesnym wzroście zawartości kwasów nasyconych PC

16:0/18:2 i PC 16:0/18:1, co wskazuje na wzrost integralności błony komórkowej *N. pironii*.

5. W grzybni *N. pironii* poddanej działaniu Fen wzrósł poziom TBARS, co świadczy o peroksydacji lipidów. Natomiast po 48 godz. hodowli zawartość TBARS w biomacie wszystkich badanych układach utrzymuje się podobnym poziomie, co może wskazywać na adaptację mikroorganizmu do czynnika stresowego i/lub detoksykację zanieczyszczenia po jego biotransformacji do mniej toksycznych produktów.

IV Wnioski i stwierdzenia końcowe

1. Badany szczep mikroskopowego grzyba strzępkowego *N. pironii* IM 6443 wyizolowany z terenu dawnych Zakładów Przemysłu Barwników „Boruta” w Zgierzu wykazuje zdolność do produkcji enzymu o aktywności lakazy i eliminacji barwników przemysłu tekstylnego.
2. Mikroskopowy grzyb strzępkowy *N. pironii* IM 6443 wykazuje zdolność do przekształcenia *o*-tolidyny w obecności odcieków pobranych ze składowiska odpadów niebezpiecznych dawnych Z. P. B. „Boruta” w Zgierzu. Mikroorganizm odznacza się zdolnością do dekoloryzacji barwników Acid Orange 7 i Reactive Red 120 również w obecności jonów metali.
3. Grzyb wykazuje zdolność do eliminacji wielopierścieniowych węglowodorów aromatycznych (WWA), która wzmagana jest obecnością odcieków pobranych ze składowiska odpadów niebezpiecznych. Opisane zmiany dotyczące peroksydacji lipidów oraz zachodzące w profilu fosfolipidowym wskazują na adaptację grzyba *N. pironii* do czynnika stresowego i/lub detoksykację zanieczyszczenia po jego biotransformacji do mniej toksycznych półproduktów.

V Streszczenie

Substancje barwiące ze względu na powszechne wykorzystanie w wielu gałęziach przemysłu, przede wszystkim farbiarskim, włókienniczym, kosmetycznym i papierniczym, są szeroko rozpowszechnione w środowisku. Z powodu nieskuteczności procesów technologicznych do środowiska może przedostawać nawet do 50% substancji stosowanych w barwieniu. Procesy opierające się, przede wszystkim, na biologicznej eliminacji tych zanieczyszczeń stanowią najbardziej obiecujące, atrakcyjne ze względu na aspekt finansowy, a także przyjazne dla środowiska rozwiązanie problemu eliminacji zanieczyszczeń. Uwaga naukowców skupia się głównie na bioremediacji wykorzystującej do oczyszczania środowiska potencjał metaboliczny drobnoustrojów. Szczególnym zainteresowaniem cieszą się w głównej mierze drobnoustroje wyizolowane z terenów skażonych, z uwagi na wykształcone przez nie mechanizmy adaptacji, które umożliwiły im przetrwanie w zanieczyszczonym środowisku.

Niniejsza praca doktorska miała na celu wytypowanie mikroorganizmu, wykazującego zdolność do produkcji enzymu o aktywności lakazy i eliminacji barwników przemysłu tekstylnego, amin aromatycznych oraz wielopierścieniowych węglowodorów aromatycznych (WWA) z jednoczesnym wskazaniem mechanizmów adaptacji drobnoustroju do toksycznych zanieczyszczeń generowanych przez przemysł tekstylny. Charakterystyka lakaz oraz innych enzymów zaliczanych do wielomiedziowych oksydaz (MCOs), a także ich zastosowanie zostały opisane w pracy przeglądowej P1. W pierwszym etapie pracy, którego wyniki zostały przedstawione w pracy eksperymentalnej P2, przeprowadzono skryning grzybów wyizolowanych z terenu dawnych Zakładów Przemysłu Barwników (Z. P. B.) „Boruta” w Zgierzu, charakteryzujących się zdolnością do wytwarzania enzymu o aktywności lakazy. Izolat oznaczony jako IM 6443 odznaczał się najwyższą produkcją enzymu, zarówno podczas hodowli w podłożu stałym, jak i w hodowli płynnej. Wskazany drobnoustrój na podstawie analizy genetycznej został zidentyfikowany jako *Nectriella pironii*. W kolejnym etapie pracy, uprzednio wyizolowana mieszanina zewnątrzkomórkowych białek z hodowli *N. pironii* została poddana oczyszczaniu z wykorzystaniem chromatografii jonowymiennej oraz filtracji żelowej. Oczyszczone białko zidentyfikowano za pomocą spektrometrii mas jako lakaza. Enzym zachowywał aktywność w szerokim zakresie pH wynoszącym 3,0-10,6 oraz wykazywał zdolność do efektywnej dekoloryzacji (na poziomie wynoszącym 69-92%) barwników przemysłu tekstylnego zaliczanych do różnych grup chemicznych.

Zakres badań, opisanych w pracy eksperymentalnej P3, obejmował ocenę możliwości grzyba *N. pironii* do eliminacji amin aromatycznych i barwników azowych. Ponadto w eksperymentach wykorzystano próbki odcieków (oznaczonych symbolami L1 i L2) pobranych ze składowiska odpadów niebezpiecznych dawnych (Z. P. B.) „Boruta” w

Zgierzu. Odcieki te charakteryzowały się podwyższoną zawartością chlorków, żelaza, lotnych fenoli oraz przekroczeniem dopuszczalnej wartości dla wskaźnika BZT₅. Analiza chromatograficzna wykazała obecność w odciekach amin aromatycznych – *o*-tolidyny oraz 4,4-oksydianiliny. W wyniku przeprowadzonych analiz wykazano, że grzyb *N. pironii* charakteryzuje się zwiększoną zdolnością do wzrostu w obecności prób pochodzących ze składowiska odpadów niebezpiecznych. Ponadto dowiedziono, iż *o*-tolidyna podczas hodowli ulega hydroksylacji i/lub przekształceniu do mniej toksycznej pochodnej – 3,3'-dihydroksybenzydyny. Grzyb *N. pironii* odznaczył się zdolnością do efektywnej dekoloryzacji barwników AO 7 i RR 120 również w obecności jonów metali.

W ostatnim etapie pracy, którego rezultaty zostały zawarte w pracy eksperymentalnej P4, oceniono możliwość eliminacji WWA przez *N. pironii* w obecności odcieków składowiskowych oraz zidentyfikowano mechanizmy zachodzące w grzybni na skutek adaptacji mikroorganizmu do zanieczyszczeń. Uzyskane wyniki wykazały, iż badany szczep jest w stanie eliminować trzy-, cztero- i pięciopierścieniowe WWA. Ponadto, wprowadzenie odcieków do hodowli przyspiesza tempo biodegradacji fenantrenu (Fen), benz[a]antracenu (B[a]A) i benz[a]pirenu (B[a]P. W wyniku ekspozycji na Fen w połączeniu z L1 zidentyfikowano zmiany w profilu fosfolipidowym. Pod wpływem Fen, dodawanego oddzielnie lub w połączeniu z L1, zwiększeniu w błonach *N. pironii* ulega zawartość fosfatydylocholiny (PC), podczas gdy zawartość fosfatydyloetanolaminy (PE) zmniejsza się. Różnice odnotowano również w zmianie zawartości kwasów tłuszczowych. W obecności Fen zmniejszyła się zawartość nienasyconych kwasów tłuszczowych (PC 18:2/18:2; PE 18:2/18:2 i PE 18:2/18:1), podczas gdy zwiększeniu uległa zawartość kwasów nasyconych (PC 16:0/18:2 i PC 16:0/18:1), co również potwierdza wzrost integralności błony komórkowej *N. pironii*. Co więcej, w grzybni *N. pironii* poddanej działaniu Fen początkowo odnotowano wzrost poziomu TBARS, wskazującego stopień peroksydacji lipidów, natomiast po 48 godz. hodowli współczynnik ten utrzymywał się podobnym poziomie we wszystkich badanych układach. Uzyskane rezultaty mogą wskazywać na adaptację grzyba *N. pironii* do czynnika stresowego i/lub zdolność do detoksykacji zanieczyszczenia po jego uprzedniej biotransformacji do mniej toksycznych półproduktów.

Badania przeprowadzone w ramach pracy doktorskiej wskazują na potencjalne możliwości wykorzystania mikroskopowego grzyba strzępkowego *N. pironii* w procesach eliminacji różnorodnych zanieczyszczeń takich jak barwniki azowe, aminy aromatyczne czy WWA. Na podkreślenie zasługuje fakt, iż drobnoustrój ten zdolny jest do przekształceń wyżej wymienionych związków nawet w obecności odcieków pobranych ze składowiska odpadów niebezpiecznych, charakteryzujących się zawartością wielu toksycznych substancji.

VI Summary

Because of their widespread use in many industries (dyeing, textile, cosmetics and paper), dyes are widely found in the environment. Due to the ineffectiveness of treatment processes, up to 50% of the substances used in dyeing can enter the environment. Methods based on the biological elimination of these pollutants are the most promising, attractive in terms of financial aspects, and also environmentally friendly solution to the problem. Scientists focus primarily on bioremediation, which uses the metabolic potential of microorganisms to clean the environment. Microorganisms isolated from contaminated areas are particularly popular, due to the adaptation mechanisms that allow them to survive in a polluted environment.

The presented doctoral thesis has been based on four earlier publications marked as P1-P4.

Its aim was the selection of a microorganism showing the ability to produce enzyme with laccase activity and eliminate textile industry dyes, aromatic amines and polycyclic aromatic hydrocarbons (PAHs), as well as the indication of the mechanisms of this microorganism's adaptation to toxic pollutants generated by the textile industry. The characteristics of laccases and other enzymes classified as multicopper oxidases (MCOs) as well as their application had been described in review P1. The first stage of the work involved screening of fungi isolated from the area of the former "Boruta" Dye Industry Plant in Zgierz, and characterized by the ability to produce enzymes with lacase activity. The isolate designated as IM 6443 showed the highest enzyme production, both in solid and liquid cultures. Based on the genetic analysis, the indicated microorganism was identified as *Nectriella pironii*. The mixture of extracellular proteins isolated from the culture of *N. pironii* was purified using ion exchange chromatography and gel filtration. The purified protein was identified by mass spectrometry as laccase. The enzyme remained active in a wide pH range of 3.0-10.6 and showed the ability to effectively decolorize (at the level of 69-92%) textile industry dyes belonging to various chemical groups. These results were presented in publication P2.

The scope of the research described in publication P3 included the assessment of the ability of the tested fungus to eliminate aromatic amines and azo dyes. In addition, in these experiments samples of leachates (marked with symbols L1 and L2) collected from the hazardous waste landfill of the former "Boruta" Dye Industry Plant in Zgierz were used. They were characterized by an increased content of chlorides, iron and volatile phenols, and exceeded the allowed value for the BOD₅ index. Chromatographic analysis of leachates showed the presence of aromatic amines, *o*-tolidine, and 4,4-oxidianiline. It was shown that the filamentous fungus *N. pironii* is characterized by an increased ability to grow in the presence of samples from a hazardous waste landfill. Furthermore, it was proven that during cultivation *o*-tolidine underwent hydroxylation

and/or conversion to a less toxic derivative, 3,3'-dihydroxybenzidine. *N. pironii* showed the ability to effectively decolorize the dyes Acid Orange 7 and Reactive Red 120 also in the presence of metal ions.

In the last stage of the work, the possibility of PAH elimination by *N. pironii* in the presence of landfill leachates was evaluated. Additionally, the mechanisms occurring in the mycelium as a result of the adaptation to contamination were identified. The obtained results showed that the tested strain was capable of eliminating three-, four-, and five-ring PAHs. In addition, the introduction of the leachate into the culture accelerated the biodegradation rate of phenanthrene (Fen), benz[a]anthracene (B[a]A), and benz[a]pyrene (B[a]P). Under the influence of Fen added alone or in combination with L1, the phosphatidylcholine (PC) content increased in the *N. pironii* membranes, while the phosphatidylethanolamine (PE) content decreased. In the presence of Fen, the content of unsaturated fatty acids (PC 18: 2/18: 2; PE 18: 2/18: 2 and PE 18: 2/18: 1) decreased, while the content of saturated acids increased (PC 16: 0/18: 2 and PC 16: 0/18: 1), which also confirmed the increase in the integrity of the *N. pironii* cell membrane. Furthermore, the mycelium of *N. pironii* treated with Fen initially showed an increase in TBARS, indicating the degree of lipid peroxidation, while after 48 h of cultivation this ratio remained at a similar level in all tested systems. The results obtained might indicate the adaptation of *N. pironii* to stress factors and/or its ability to detoxify the contaminants after biotransformation to less toxic intermediates.

The research carried out as part of the doctoral work indicates the possibilities of using the microscopic filamentous fungus *N. pironii* in the elimination of various pollutants such as azo dyes, aromatic amines and PAHs. It should be emphasized that this microorganism is capable of transforming the above-mentioned compounds even in the presence of leachates collected from a hazardous waste landfill, which contain many toxic substances.

VII Literatura

- Acevedo, F., Pizzul, L., Castillo, M del. P., Cuevas R., Diez, M.C. 2011. Degradation of polycyclic aromatic hydrocarbons by the Chilean white-rot fungus *Anthracophyllum discolor*. J. Hazard. Mater. 185 (1), 212-219.
- Al-Tohamy, R., Ali, S.S., Li, F., Okasha, K.M., Mahmoud, Y.A.G., Elsamahy, T., Jiao, H., Fu, Y., Sun, J. 2022. A critical review on the treatment of dye-containing wastewater: Ecotoxicological and health concerns of textile dyes and possible remediation approaches for environmental safety. Ecotoxicol. Environ. Saf. 231, 113160.
- Alfieri, S.A., Samuels, G.J., 1979. *Nectriella pironii* and its *Kutilakesa*-Like Anamorph, A Parasite of Ornamental Shrubs. Mycologia 71, 6, 1178-1185,
- Agrawal, N., Verma, P., Shahi, S.K. 2018. Degradation of polycyclic aromatic hydrocarbons (phenanthrene and pyrene) by the ligninolytic fungi *Ganoderma lucidum* isolated from the hardwood stump. Bioresour. Bioprocess. 5, 11.
- Barsing, P., Tiwari, A., Joshi, T., Garg, S. 2011. Bioresource technology application of a novel bacterial consortium for mineralization of sulphonated aromatic amines. Bioresour. Technol. 2011102 (2), 765-771.
- Bernat, P. 2020. Zastosowanie lipidomiki w badaniu procesów detoksykacji u drobnoustrojów [w:] Biotechnologia drobnoustrojów w laboratorium i w praktyce. Teoria, ćwiczenia i pracownie specjalistyczne. Długoński, J. (red.). Wydawnictwo Uniwersytetu Łódzkiego, Łódź, Polska, ss. 478-485.
- Bernat, P., Nykiel-Szymańska, J., Stolarek, P., Słaba, M., Szewczyk, R., Różalska, S. 2018. 2,4-dichlorophenoxyacetic acid-induced oxidative stress: Metabolome and membrane modifications in *Umbelopsis isabellina*, a herbicide degrader. PLoS ONE 13(6), e0199677.
- Brander, S., Mikkelsen, J. D., Kepp, K.P. 2014. Characterization of an alkali- and halide-resistant laccase expressed in *E. coli*: CotA from *Bacillus clausii*. PloS ONE 9(6), e99402.
- Brüschweiler, B.J., Merlot, C. 2017. Azo dyes in clothing textiles can be cleaved into a series of mutagenic aromatic amines which are not regulated yet. Regul. Toxicol. Pharmacol. 88, 214-226.
- Cortés-Espinosa, D.V., Absalón, A.E. 2013. Phenanthrene removal from soil by a strain of *Aspergillus niger* producing manganese peroxidase of *Phanerochaete chrysosporium* [w:] Hydrocarbon. Kutcherov, V., Kolesnikov, A. (red.). IntechOpen, UK, London. <https://doi.org/10.5772/51944>.
- de Kroon, Al., Rijken, P.J., De Smet, C.H., 2013. Checks and balances in membrane phospholipid class and acyl chain homeostasis, the yeast perspective. Prog. Lipid. Res. 52(4), 374-394.

- de Lima, D., dos Santos, E.A., Marques, M.R., Giannesi, G.C., Beatriz, A., Yonekawa, M.K.A., Montanholi dos S.A. 2018. Fungal Bioremediation of pollutant aromatic amines. *Curr. Opin. Green Sustain. Chem.* 11, 34-44.
- Długoński, A., Góralczyk-Bińkowska, A. 2020. Wykorzystanie odpadów zieleni miejskiej do produkcji energii w lokalnych biogazowniach i spalarniach oraz syntezy lakaz grzybowych, [w:] Długoński, J., (Ed.), *Biotechnologia drobnoustrojów w laboratorium i w praktyce. teoria, ćwiczenia i pracownie specjalistyczne*. Wydawnictwo Uniwersytetu Łódzkiego: Łódź, Polska, pp. 350-355.
- Dyes & Pigments Market Size, Share & Trends Analysis Report By Product (Pigments, Dyes), By Application (Paints & Coatings, Printing Inks, Textiles), By Region (APAC, Europe), And Segment Forecasts, 2021-2028 <https://www.grandviewresearch.com/industry-analysis/dyes-and-pigments-market> [dostęp 22 maja 2022 r.]
- Eggert, C., Temp, U., Eriksson, K.E. 1996. The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. *Appl. Environ. Microbiol.* 62(4), 1151-1158.
- Fatima, M., Saeed, M., Aslam, M., Lindström, W.R., Farooq, R. 2019. Application of novel bacterial consortium for biodegradation of aromatic amine 2-ABS using response surface methodology. *J. Microbiol Methods* 174, 105941.
- Fayeulle, A., Veignie, E., Schroll, R., Munch, J.Ch., Rafin, C. 2019. PAH biodegradation by telluric saprotrophic fungi isolated from aged PAH-contaminated soils in mineral medium and historically contaminated soil microcosms. *J. Soil Sediments.* 19, 3056-3067.
- Ghosal, D., Ghosh, S., Dutta, T.K., Ahn, Y. 2016. current state of knowledge in microbial degradation of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *Front. Microbiol.* 7, 1369.
- González-Abradelo, D., Pérez-Llano, Y., Peidro-Guzmán, H., Sánchez-Carbente, M., Folch-Mallol, J.L., Aranda, E., Vaidyanathan, V.K., Cabana, H., Gunde-Cimerman, N., Batista-García, R.A. 2019. First demonstration that ascomycetous halophilic fungi (*Aspergillus sydowii* and *Aspergillus destruens*) are useful in xenobiotic mycoremediation under high salinity conditions. *Bioresour. Technol.* 279, 287-296.
- Góralczyk, A., Jasińska, A., Długoński, J. 2016. Mikroorganizmy w usuwaniu toksycznych barwników przemysłowych. *Post. Mikrobiol.* 55, 4, 424-432.
- Gunatilake, S.K. 2015. Methods of removing heavy metals from industrial wastewater. *J. Multidiscip. Eng. Sci. Stud.* 1, 12-18.
- Hoegger, P.J., Kilaru, S., James, T.Y., Thacker, J.R., Kües U. 2006. Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. *FEBS J.* 273, 2308-2326.

- Ihssen, J., Reiss, R., Luchsinger, R., Thöny-Meyer L., Richter M. 2015. Biochemical properties and yields of diverse bacterial laccase-like multicopper oxidases expressed in *Escherichia coli*. *Sci. Rep.* 12, 10465.
- Janicki, T., Długoński, A., Felczak, A., Długoński, J., Krupiński, M. 2022. Ecotoxicological estimation of 4-cumylphenol, 4-*t*-octylphenol, nonylphenol, and volatile leachate phenol degradation by the microscopic fungus *Umbelopsis isabellina* using a battery of biotests. *Int. J. Environ. Res. Public Health* 19, 4093.
- Jasińska, A., Góralczyk-Bińkowska, A., Soboń, A., Długoński, J. 2019. Lignocellulose resources for the *Myrothecium roridum* laccase production and their integrated application for dyes removal. *Int. J. Environ. Sci. Technol.* 16, 4811-4822.
- Lawal, A.T. 2017. Polycyclic Aromatic Hydrocarbons. A review, *Cogent Environ. Sci.* 3, 1, 1339841,
- Ledakowicz, S., Paździor, K. 2021. Recent achievements in dyes removal focused on advanced oxidation processes integrated with biological methods. *Molecules* 26(4), 870.
- Lellis B., Fávaro-Polonio C.Z., Pamphile J.A., Polonio J.C. 2019. Effects of textile dyes on health and the environment and bioremediation potential of living organisms. *Biotechnol. Res. Innov.* 3, 2, 275-290.
- Li, Z., Agellon, L.B., Allen, T.M., Umeda, M., Jewell, L., Mason, A., Vance, D.E. 2006. The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis. *Cell Metab.* 3(5), 321-231.
- Liao, S.G., Zhang, L.J., Sun, F., Wang, Z., He, X., Wang, A.M., Li, Y.J., Huang, Y., Lan, Y.Y., Zhang, B.L., Wang, Y.L. 2013. Identification and characterisation of phenolics in *Polygonum capitatum* by ultrahigh-performance liquid chromatography with photodiode array detection and tandem mass spectrometry. *Phytochem. Anal.* 24(6), 556-568.
- Lonappan, L., Rouissi, T., Laadila, M.A., Brar, S.K., Galan, L.H., Verma, M., Surampalli, R.Y. 2017. Agro-industrial-Produced Laccase for Degradation of Diclofenac and Identification of Transformation Products. *ACS Sustainable Chem. Eng.* 2017, 5, 5772-5781.
- Lu C, Hong Y, Liu J, Gao, Y., Ma, Z., Yang, B., Ling, W., Gatheru-Waigi, M. 2019. A PAH-degrading bacterial community enriched with contaminated agricultural soil and its utility for microbial bioremediation. *Environ. Pollut.* 251, 773-782.
- Mahmoud, M.G., Rifaat, H.M., El Sayed, O.H., El Beih, F.M., Selim, M.S. 2013. Effect of inducers and process parameters on laccase production by locally isolated marine *Streptomyces lydicus* from Red Sea, Egypt *International Journal of ChemTech Research* 5, 15-23.
- Medaura, M.C., Guivernau, M., Moreno-Ventas, X., Prenafeta-Boldú, F.X., Viñas, M. 2021. Bioaugmentation of Native Fungi, an Efficient Strategy for the

- Bioremediation of an Aged Industrially Polluted Soil With Heavy Hydrocarbons. *Front. Microbiol.* 12, 626436.
- Mihułka M. 2003. Charakterystyka technologiczna przemysłu włókienniczego w Unii Europejskiej. Ministerstwo Środowiska. Warszawa. https://www.ekoportal.gov.pl/fileadmin/Ekoportal/Pozwolenia_zintegrowane/poradniki_branzowe/opracowania/2._Charakterystyka_tehnologiczna_przemyslu_wlokiemmiczego_w_Unii_Europejskiej.pdf [dostęp 25 października 2022 r.]
- Ngo, A.C.R., Tischler, D. 2022. Microbial degradation of azo dyes: approaches and prospects for a hazard-free conversion by microorganisms. *Int. J. Environ. Res. Public Health* 19, 4740.
- Nzila, A., Musa, M.M., 2020. Current status of and future perspectives in bacterial degradation of benzo[a]pyrene. *Int. J. Environ. Res. Public Health* 18, 262.
- Patel, A.B., Shaikh, S., Jain, K.R., Desai, C., Madamwar, D. 2020. Polycyclic Aromatic Hydrocarbons: sources, toxicity, and remediation approaches. *Front. Microbiol.* 11, 562813.
- Peidro-Guzmán, H., Pérez-Llano, Y., González-Abradelo, D., Fernández-López, M.G., Dávila-Ramos, S., Aranda, E., Hernández, D., García, A.O., Lira-Ruan, V., Pliego, O.R. i in. 2020. Transcriptomic analysis of polyaromatic hydrocarbon degradation by the halophilic fungus *Aspergillus sydowii* at hypersaline conditions. *Environ. Microbiol.* 23(7), 3435-3459.
- Piscitelli, A., Giardina, P., Lettera, V., Pezzella, C., Sannia, G., Faraco, V. 2011. Induction and transcriptional regulation of laccases in fungi. *Curr. Genomics* 12, 104- 112.
- Pubchem. https://pubchem.ncbi.nlm.nih.gov/compound/4_4_-Oxydianiline [dostęp 25 października 2022 r.]
- Przygucki, T. 1994. Sto lat koloru: Zakłady Przemysłu Barwników "Boruta". Wydawnictwo Pryzmat, Łódź.
- Raport NIK. Zapobieganie zagrożeniom ze strony składowisk pozakładowych z terenu województwa łódzkiego" opisującego stan środowiska na terenie składowiska pozakładowego po Zakładach Przemysłu Barwników „Boruta <https://www.nik.gov.pl/plik/id,23253,vp,25961.pdf>. [dostęp 22 maja 2022 r.]
- Reiss, R., Ihssen, J., Richter, M., Eichhorn, E., Schilling, B., Thöny-Meyer, L. 2013. Laccase versus laccase-like multi-copper oxidase: a comparative study of similar enzymes with diverse substrate spectra. *PLoS One* 8, e65633.
- Szewczyk, R., Kowalski, K., 2016. Metabolomics and Crucial Enzymes in Microbial Degradation of Contaminants [w:] *Microbial Biodegradation: From Omics to Function and Application*. Długoński, J. (red.). Caier Academic Press, Norflok, UK, ss. 43-65.
- Torres-Farradá, G., Manzano-León, A.M., Rineau, F., Ramos Leal, M., Thijs, S., Jambon, I., Put, J., Czech, J., Guerra Rivera, G., Carleer, R., Vangronsveld, J. 2019. Biodegradation of polycyclic aromatic hydrocarbons by native *Ganoderma* sp.

- strains: identification of metabolites and proposed degradation pathways. *Appl. Microbiol. Biotechnol.* 103, 17, 7203-7215.
- Upadhyay, P., Shrivastava, R., Agrawal, P.K. 2016. Bioprospecting and biotechnological applications of fungal laccase. *3 Biotech* 6, 15.
- U.S. Government Accountability Office. <https://www.gao.gov/products/100521> [dostęp 22 maja 2022 r.]
- Vrsanska, M., Voberkova, S., Langer, V., Palovcikova, D., Moulick, A., Adam, V., Kopel, P., McPhee, D.J. 2016. Induction of laccase, lignin peroxidase and manganese peroxidase activities in white-rot fungi using copper complexes. *Molecules* 21, 1553–1564.
- Wiley Online Library - 4,4'-Oxydianiline [MAK Value Documentation] <https://onlinelibrary.wiley.com/doi/10.1002/3527600418.mb10180e0006> [dostęp 4 listopada 2022 r.]
- Worrich, A., Stryhanyuk, H., Musat, N., König, S., Banitz, T., Centler, F., Frank, K., Thullner, M., Harms, H., Richnow, H.H. 2017. Mycelium-mediated transfer of water and nutrients stimulates bacterial activity in dry and oligotrophic environments. *Nat. Commun.* 8, 15472.

VIII Całkowity dorobek naukowy

VIII.1 Publikacje

1. **Góralczyk-Bińkowska A.**, Długoński A., Bernat P., Długoński J., Jasińska A. 2022. Accelerated PAHs transformation in the presence of dye industry landfill leachate combined with fungal membrane lipids changes. *International Journal of Environmental Research and Public Health*, 19, 13997. <https://doi.org/10.3390/ijerph192113997>.
IF₂₀₂₁ = 4,614; IF_{5-letni} = 4,799; punkty MEiN = 140; brak cytowań
2. **Góralczyk-Bińkowska A.**, Długoński A., Bernat P., Długoński J., Jasińska A. 2021. Environmental and molecular approach to dye industry waste degradation by the ascomycete fungus *Nectriella pironii*. *Scientific Reports*, 11, 23829. <https://doi.org/10.1038/s41598-021-03446-x>.
IF₂₀₂₁ = 4,997; IF_{5-letni} = 5,516; punkty MEiN = 140; 3 cytowania
3. Skłodowska M., Mielczarz K., Chojak-Koźniewska J., Naliwajski M., Żyźniewska M., **Góralczyk-Bińkowska A.** 2021. Antioxidant response of cucumber leaf tissues treated with prohexadione-Ca to infection with *Pseudomonas syringae* pv. *lachrymans*. *Scientia Horticulturae* 289, 110452. <https://doi.org/10.1016/j.scienta.2021.110452>.
IF₂₀₂₁ = 4,342; IF_{5-letni} = 4,342; punkty MEiN = 140; brak cytowań
4. Nowak M., Zawadzka K., Szemraj J., **Góralczyk-Bińkowska A.**, Lisowska K. 2021. Biodegradation of chloroxylenol by *Cunninghamella elegans* IM 1785/21GP and *Trametes versicolor* IM 373: Insight into ecotoxicity and metabolic pathways. *International Journal of Molecular Sciences*. 22(9), 4360. <https://doi.org/10.3390/ijms22094360>.
IF₂₀₂₁ = 6,208; ; IF_{5-letni} = 6.628; punkty MEiN = 140; 7 cytowania
5. **Góralczyk-Bińkowska A.**, Jasińska A., Długoński A., Płociński P., Długoński J. 2020. Laccase activity of the ascomycete fungus *Nectriella pironii* and innovative strategies for its production on leaf litter of an urban park. *PLoS ONE*, 15, 4, e0231453; errata w *PLoS ONE* 2020, 15, e0233553 <https://doi.org/10.1371/journal.pone.0231453>.
IF₂₀₂₁ = 3,752; IF_{5-letni} = 4,069; punkty MEiN = 100; 18 cytowań
6. Jasińska A., Soboń A., **Góralczyk-Bińkowska A.**, Długoński J. 2019. Analysis of decolorization potential of *Myrothecium roridum* in the light of its secretome and toxicological studies. *Environmental Science and Pollution Research*. 26(25), 26313–26323. <https://doi.org/10.1007/s11356-019-05324-6>.
IF₂₀₂₁ = 5,190; IF_{5-letni} = 5,053; punkty MEiN = 70; 11 cytowań

7. **Góralczyk-Bińkowska A.,** Jasińska A., Długoński J. 2019. Characteristics and use of multicopper oxidases enzymes. *Advancements of Microbiology*. 58, 1, 7–18. <https://doi.org/10.21307/PM-2019.58.1.007>.
IF₂₀₂₁ = 1,106; IF_{5-letni} = 1,955; punkty MEiN = 20; 5 cytowań
8. Jasińska A., **Góralczyk-Bińkowska A.,** Soboń A., Długoński J. 2019. Lignocellulose resources for the *Myrothecium roridum* laccase production and their integrated application for dyes removal. *International Journal of Environmental Science and Technology*. 16(8), 4811–4822. <https://doi.org/10.1007/s13762-019-02290-x>.
IF₂₀₂₁ = 3,519; IF_{5-letni} = 3,463; punkty MEiN = 70; 7 cytowań
9. **Góralczyk-Bińkowska A.,** Jasińska A., Długoński J. 2019. Charakterystyka i kierunki wykorzystania enzymów z grupy wielomiedziowych oksydaz. *Postępy Mikrobiologii* 58, 1, 7–18.
IF₂₀₂₁ = 0,294; IF_{5-letni} = 0,443; punkty MEiN = 20;
10. Jasińska A., **Góralczyk A.,** Soboń A., Długoński J. 2018. Novel laccase-like multicopper oxidases from the *Myrothecium roridum* fungus - production enhancement, identification and application in the dye removal process. *Acta Biochimica Polonica*. 65(2), 287–295. https://doi.org/10.18388/abp.2017_2546
IF₂₀₂₁ = 2,349; IF_{5-letni} = 2,279; punkty MEiN = 40; 9 cytowań
11. **Góralczyk A.,** Jasińska A., Długoński J. 2016. Mikroorganizmy w usuwaniu toksycznych barwników przemysłowych. *Postępy Mikrobiologii*, 2016, 55(4), 424–432.
IF₂₀₂₁ = 0,294; IF_{5-letni} = 0,443; punkty MEiN = 20; 2 cytowania

Sumaryczny IF₂₀₂₁ = 36,665

Sumaryczny IF_{5-letni} = 38,99

Łączna liczba punktów MEiN = 900

Liczba cytowań = 62

Index H = 6

Wartości IF oraz punktację MEiN podano zgodnie listą obowiązującą w 2022 roku*

*(*Dane z bazy Journal Citation Reports™, stan na dzień 07.11.2022 r.)*

VIII.2 Rozdziały w podręcznikach akademickich

1. Paraszkievicz K., Jasińska A., **Góralczyk-Bińkowska A.** 2021. Use of microorganisms in the baking industry and for the production of fermented meat and vegetable products. W: Długoński J. (Red.): Microbial biotechnology in the laboratory and in practice. Theory, exercises and specialist laboratories. Wydawnictwo Uniwersytetu Łódzkiego - Wydawnictwo Uniwersytetu Jagiellońskiego, Łódź – Kraków 2021, ISBN 978-83-8220-150-5, str. 257-270.
2. Jasińska A., **Góralczyk-Bińkowska A.**, Paraszkievicz K. 2021. Asian food obtained by the use of microorganisms. W: Długoński J. (Red.): Microbial biotechnology in the laboratory and in practice. Theory, exercises and specialist laboratories. Wydawnictwo Uniwersytetu Łódzkiego - Wydawnictwo Uniwersytetu Jagiellońskiego, Łódź – Kraków 2021, ISBN 978-83-8220-150-5, str. 270-276.
3. Długoński A., **Góralczyk-Bińkowska A.** 2021. Use of municipal green waste for energy production in local biogas and incineration plants and the synthesis of fungal laccases. W: Długoński J. (Red.): Microbial biotechnology in the laboratory and in practice. Theory, exercises and specialist laboratories. Wydawnictwo Uniwersytetu Łódzkiego - Wydawnictwo Uniwersytetu Jagiellońskiego, Łódź – Kraków 2021, ISBN 978-83-8220-150-5, str. 330-335.
4. Jasińska A., **Góralczyk-Bińkowska A.** 2021. Dyes. W: Długoński J. (Red.): Microbial biotechnology in the laboratory and in practice. Theory, exercises and specialist laboratories. Wydawnictwo Uniwersytetu Łódzkiego - Wydawnictwo Uniwersytetu Jagiellońskiego, Łódź – Kraków 2021, ISBN 978-83-8220-150-5, str. 357-361.
5. Paraszkievicz K., **Góralczyk-Bińkowska A.**, Jasińska A. 2021. Use of industrial waste in microbiological biotechnology. W: Długoński J. (Red.): Microbial biotechnology in the laboratory and in practice. Theory, exercises and specialist laboratories. Wydawnictwo Uniwersytetu Łódzkiego - Wydawnictwo Uniwersytetu Jagiellońskiego, Łódź – Kraków 2021, ISBN 978-83-8220-150-5, str. 377-383.
6. Jasińska A., **Góralczyk-Bińkowska A.**, Długoński A. 2021. Characteristics and use of ligninolytic enzymes produced by fungi in environmental protection, industry and medicine. W: Długoński J. (Red.): Microbial biotechnology in the laboratory and in practice. Theory, exercises and specialist laboratories. Wydawnictwo Uniwersytetu Łódzkiego - Wydawnictwo Uniwersytetu Jagiellońskiego, Łódź – Kraków 2021, ISBN 978-83-8220-150-5, str. 391-397.
7. Paraszkievicz K., Jasińska A., **Góralczyk-Bińkowska A.** 2020. Wykorzystanie drobnoustrojów w przemyśle piekarniczym oraz do produkcji fermentowanych produktów mięsnych i warzywnych. W: Długoński J. (Red.): Biotechnologia

- drobnoustrojów w laboratorium i w praktyce. Teoria, ćwiczenia i pracownie specjalistyczne. Wydawnictwo Uniwersytetu Łódzkiego, Łódź 2020, ISBN 978-83-8142-954-2, str. 271-285.
8. Jasińska A., **Góralczyk-Bińkowska A.**, Paraszewicz K. 2020. Żywność azjatycka otrzymywana przy udziale drobnoustrojów. W: Długoński J. (Red.): Biotechnologia drobnoustrojów w laboratorium i w praktyce. Teoria, ćwiczenia i pracownie specjalistyczne. Wydawnictwo Uniwersytetu Łódzkiego, Łódź 2020, ISBN 978-83-8142-954-2, str. 285-292.
 9. Długoński A., **Góralczyk-Bińkowska A.** 2020. Wykorzystanie odpadów zieleni miejskiej do produkcji energii w lokalnych biogazowniach i spalarniach oraz syntezy lakaz grzybowych. W: Długoński J. (Red.): Biotechnologia drobnoustrojów w laboratorium i w praktyce. Teoria, ćwiczenia i pracownie specjalistyczne. Wydawnictwo Uniwersytetu Łódzkiego, Łódź 2020, ISBN 978-83-8142-954-2, str. 350-355.
 10. Jasińska A., **Góralczyk-Bińkowska A.** 2020. Barwniki. W: Długoński J. (Red.): Biotechnologia drobnoustrojów w laboratorium i w praktyce. Teoria, ćwiczenia i pracownie specjalistyczne. Wydawnictwo Uniwersytetu Łódzkiego, Łódź 2020, ISBN 978-83-8142-954-2, str. 379-384.
 11. Paraszewicz K., **Góralczyk-Bińkowska A.**, Jasińska A. 2020. Wykorzystanie odpadów przemysłowych w biotechnologii mikrobiologicznej. W: Długoński J. (Red.): Biotechnologia drobnoustrojów w laboratorium i w praktyce. Teoria, ćwiczenia i pracownie specjalistyczne. Wydawnictwo Uniwersytetu Łódzkiego, Łódź 2020, , ISBN 978-83-8142-954-2, str. 401-408.
 12. Jasińska A., **Góralczyk-Bińkowska A.**, Długoński A. 2020. Charakterystyka i wykorzystanie enzymów ligninolitycznych produkowanych przez grzyby w ochronie środowiska, przemyśle i medycynie. W: Długoński J. (Red.): Biotechnologia drobnoustrojów w laboratorium i w praktyce. Teoria, ćwiczenia i pracownie specjalistyczne. Wydawnictwo Uniwersytetu Łódzkiego, Łódź 2020. , ISBN 978-83-8142-954-2, str. 417-423.
 13. Jasińska A., **Góralczyk A.**, Długoński J. 2016. Dyes decolourisation and degradation by microorganisms. W: Długoński J. (Ed.): Microbial Biodegradation: From Omics to Function and Application. Caiser Academic Press, Norfolk UK, ISBN 978-1-910190-45-6, str. 119-141.

VIII.3 Przyznane nagrody i wyróżnienia

1. **Nagroda zespołowa Ministra Edukacji i Nauki** za znaczące osiągnięcia w zakresie działalności dydaktycznej przyznana za podręcznik akademicki **Biotechnologia drobnoustrojów w laboratorium i w praktyce. Teoria, ćwiczenia i pracownie specjalistyczne**. Wydawnictwo Uniwersytetu Łódzkiego, Łódź 2020 przyznana 19 lutego 2022 r.
2. **Nagroda II stopnia J. M. Rektora Uniwersytetu Łódzkiego** za monografię **Microbial Biodegradation: From Omics to Function and Application**. Caizer Academic Press, Norfolk UK 2016 przyznana 14 października 2017 r.
3. **Nagroda za najlepszy poster** w sesji Agricultural and urban soils – towards sustainable use and management podczas **Second Edition of Virtual International Conference „Plant productivity and food safety: Soil science, Microbiology, Agricultural Genetics and Food quality”** przyznana 16 września 2022 r.

VIII.4 Doniesienia konferencyjne

a) o zasięgu międzynarodowym

1. **Góralczyk-Bińkowska A.**, Długoński A., Bernat P., Długoński J., Jasińska A. Soil of dye industry landfill as a source of microorganisms capable of hazardous xenobiotics degradation, Second Edition of Virtual International Conference „Plant productivity and food safety: Soil science, Microbiology, Agricultural Genetics and Food quality” 15-16 września 2022 r., Toruń, str. 109. Poster
2. **Góralczyk-Bińkowska A.**, Długoński A., Długoński J. Application of leaf litter from a pocket park for fungal laccase production, Eurobiotech 2019, 7th Central European Congress of Life Sciences, 23-25 września 2019 r., Kraków, str. 33. Poster
3. **Góralczyk-Bińkowska A.**, Jasińska A., Długoński J. Application of *Myrothecium* sp. laccase for the elimination of industrial dyes, Eurobiotech 2019, 7th Central European Congress of Life Sciences, 23-25 września 2019 r., Kraków, str. 33. Poster
4. **Góralczyk A.**, Płociński P., Jasińska A., Długoński J. *Myrothecium* sp. IM 6443 laccase – isolation, purification and preliminary characterization. 4th Congress of Baltic Microbiologists, 10-12 września 2018 r., Gdańsk, str. 53, Poster
5. Jasińska A., Kędziora M., Jeżewicz M., Radzioch M., **Góralczyk A.**, Długoński J. Degradation of bisphenol A by whole culture of *Myrothecium roridum* and its crude laccase-like multicopper oxidases. The 4th Workshop on Microbiology in Health Care and Environmental Protection MIKROBIOT 2017, 19-21 września 2017 r., Łódź, str. 47. Poster
6. **Góralczyk A.**, Jasińska A., Soboń A., Długoński J. Production, characterisation and mass spectrometry identification of novel multicopper oxidases from *Myrothecium roridum*. The 6th Central European Congress of Life Sciences EUROBIOTECH 2017, 11-14 września 2017 r., Kraków, str. 141. Poster
7. Jasińska A., **Góralczyk A.**, Soboń A., Długoński J. Extracellular proteome of *Myrothecium roridum* – a valuable tool for dyes degradation. The 6th Central European Congress of Life Sciences EUROBIOTECH 2017, 11-14 września 2017 r., Kraków, str.139. Prezentacja ustna
8. **Góralczyk A.**, Jasińska A., Długoński J. Regulation of laccase biosynthesis using inducers in *Myrothecium* sp. IM 6443 culture. XI Copernican International Young Scientists Conference, 28-30 czerwca 2017 r., Toruń, str. 77. Prezentacja ustna
9. **Góralczyk A.**, Jasińska A., Długoński J. Comparison of extracellular proteins separation methods from the culture of *Myrothecium* sp. IM 6443. XI Copernican International Young Scientists Conference, 28-30 czerwca 2017 r., Toruń, str. 92. Poster

10. **Góralczyk A.**, Jasińska A., Długoński J. Spectroscopy as a versatile tool for exploring fungal Multicopper oxidases, VI International Conference of Biophysics Students, 19-21 maja 2017 r., Kraków. str. 43. Poster
11. **Góralczyk A.**, Jasińska A., Długoński J. Laccase production by the filamentous fungus IM 6443 isolated from contaminated soil. I BioChemMed Session, 25-27 listopada 2016 r., Gdańsk, str. 52. Poster
12. **Góralczyk A.**, Jasińska A., Soboń A., Długoński J. Multicopper oxidases from *Myrothecium roridum* with both laccase and bilirubin oxidase activity. 8th OxiZymes Meeting. 3-6 lipca 2016 r., Wageningen, str. 114. Poster
13. Jasińska A., Radzioch M., Nowak M., Nejman A., **Góralczyk A.**, Długoński J. Novel fungal laccases - screening, isolation and identification using new two-step method. 17th European Congress on Biotechnology, 3-6 lipca 2016 r., Kraków. Poster

b) o zasięgu krajowym

1. **Góralczyk-Bińkowska A.**, Długoński A., Bernat P., Długoński J., Jasińska A. Wpływ WWA i odcieków składowiskowych na błonę komórkową grzyba strzępkowego *Nectriella pironii* IM 6443. Ogólnopolska Konferencja Naukowa z okazji 10-lecia Polskiego Towarzystwa Mykologicznego, 24-28 września 2022 r., str. 74-75, Poster
2. **Góralczyk-Bińkowska A.**, Długoński A., Bernat P., Jasińska A., Długoński J. Eliminacja amin aromatycznych z toksycznych odcieków składowiskowych przez mikroskopowego grzyba strzępkowego *Nectriella pironii*. 54. Konferencja Mikrobiologiczna „MIKROORGANIZMY RÓŻNYCH ŚRODOWISK”, 20-21 września 2021 r., str. 36. Prezentacja ustna online
3. **Góralczyk-Bińkowska A.**, Długoński A., Bernat P., Jasińska A., Długoński J. Ocena oporności grzyba *Nectriella pironii* IM 6443 wobec toksycznych odcieków składowiskowych. 54. Konferencja Mikrobiologiczna „MIKROORGANIZMY RÓŻNYCH ŚRODOWISK”, 20-21 września 2021 r., str. 37. Prezentacja ustna online
4. **Góralczyk-Bińkowska A.**, Jasińska A., Bernat P., Długoński J. Wpływ barwników azowych i metali ciężkich na profil fosfolipidowy błony komórkowej *Nectriella pironii* IM 6443. MycoRise Up! Młodzi w Mykologii II Edycja, 23-25 kwietnia 2021 r., str. 111-112. Prezentacja ustna online
5. **Góralczyk-Bińkowska A.**, Jasińska A., Długoński J. Analiza mechanizmów enzymatycznych zaangażowanych w dekoloryzację barwników przez mikroskopowego grzyba strzępkowego *Nectriella pironii*. Kopernikańskie e-seminarium doktoranckie, 7 września 2020 r., str. 93. Prezentacja ustna online

6. **Góralczyk-Bińkowska A.**, Jasińska A., Długoński J. Dekoloryzacja to nie zawsze detoksykacja. Kopernikańskie e-seminarium doktoranckie, 7 września 2020 r., str. 94. Prezentacja ustna online
7. **Góralczyk-Bińkowska A.**, Jasińska A., Długoński J. Application of microbial species for the bioremediation of industrial wastes. National Scientific Conference „Science and Young Researchers” IV edition – on-line, 6 czerwca 2020 r., str. 62. Prezentacja ustna online
8. **Góralczyk-Bińkowska A.**, Jasińska A., Długoński J. Characterization and toxicity of azo dyes. National Scientific Conference „Knowledge – Key to Success” IV edition, 18 stycznia 2020 r., Toruń, str. 77. Prezentacja ustna
9. **Góralczyk-Bińkowska A.**, Jasińska A., Długoński J. Ocena zdolności dekoloryzacji barwników przemysłowych przez grzyb strzępkowy *Myrothecium* sp. IM 6443. III Konferencja Doktorantów Nauk Przyrodniczych. 25-28 czerwca 2019 r., Gdańsk, str. 41. Prezentacja ustna
10. **Góralczyk-Bińkowska A.**, Walaszczyk A., Sas Paszt L., Paraszkievicz K. Wpływ syntetycznych pestycydów na aktywność przeciwgrzybową ryzosferowych szczepów *Bacillus subtilis*. MycoRise Up! Młodzi w mykologii, 12-13 kwietnia 2019 r., Spała, str. 89-91. Poster
11. **Góralczyk-Bińkowska A.**, Jasińska A., Długoński J. Microbial multicopper oxidases – versatile enzymes with a broad application in biotechnology. National Scientific Conference Knowledge – Key to Success 2019. 19 stycznia 2019 r., Toruń, str. 116. Prezentacja ustna
12. **Góralczyk-Bińkowska A.**, Jasińska A., Długoński J. Zastosowanie podłoży przygotowanych na bazie odpadów ligninocelulozowych do produkcji lakazy przez grzyb mikroskopowy *Myrothecium* sp. IM 6443. Wkraczając w świat nauki 2018. 20-21 września 2018, Wrocław, str. 28, Prezentacja ustna
13. **Góralczyk-Bińkowska A.**, Jasińska A., Długoński J. Opracowanie wydajnej metody izolacji zewnątrzkomórkowych białek *Myrothecium* sp. IM 6443. Wkraczając w świat nauki 2018. 20-21 września 2018, Wrocław, str. 85, Poster
14. Markowska K., Grodzicka M., **Góralczyk A.**, Długoński J. Ekstrakt z makucha rzepakowego jako substrat w biosyntezie lakazy przez grzyba strzępkowego *Myrothecium* sp. IM 6443. II Sesja Młodych Mikrobiologów Środowiska Łódzkiego 8 czerwca 2018 r., Łódź, str. 12, Prezentacja ustna
15. **Góralczyk A.**, Jasińska A., Długoński J. Dekoloryzacja barwników przemysłowych z wykorzystaniem grzybowych wielomiedziowych oksydaz. III Konferencja Naukowa

ENZYMOS Enzymy w nauce i przemyśle. 18 listopada 2017 r., Lublin, str. 19-20,
Prezentacja ustna

16. **Góralczyk A.**, Jeżewicz M., Kędziora M., Jasińska A., Długoński J. Grzyby z rodzaju *Myrothecium* jako źródło enzymów utleniających toksyczne barwniki. I Sesja Młodych Mikrobiologów Środowiska Łódzkiego, 23 czerwca 2017 r., Łódź, str. 15,
Prezentacja ustna

VIII.5 Praktyki, kursy i szkolenia

1. Szkolenie „Zarządzanie wielokulturowością” zrealizowane w ramach projektu „Budowanie kompetencji kadry akademickiej i administracyjnej oraz podnoszenie zdolności instytucjonalnej w zakresie umiędzynarodowienia Uniwersytetu Łódzkiego”, Łódź 10 lutego 2021 r.
2. Pobyt w ramach umowy partnerskiej między Uniwersytetem Łódzkim a Uniwersytetem Justusa Liebiga w Giessen (Niemcy) w Instytucie Chemii Spożywczej i Biotechnologii Żywności w dniach od 30 stycznia do 5 lutego 2019 r.
3. Szkolenie medyczne z zakresu BLS - Basic Life Support, Łódź 26 czerwca 2018 r.
4. Szkolenie „Elektroforeza dwukierunkowa 2-DE metoda i zastosowanie”, Centrum Medyczne Kształcenia Podyplomowego, Warszawa 13–15 listopada 2017 r.
5. Praktyki w ramach programu Erasmus+ w Laboratorium Biochemii Uniwersytetu w Wageningen (Królestwo Niderlandów) w dniach od 13 lutego do 13 kwietnia 2017 r.
6. Warsztaty „Spektrometria mas w badaniach mikrobiologicznych, mikologicznych i biotechnologicznych”, Wydział Biologii i Ochrony Środowiska UŁ, Łódź 7 października 2016 r.

VIII. 6 Pozostała aktywność

a) członkostwo w stowarzyszeniach i organizacjach naukowych

1. Polskie Towarzystwo Mykologiczne od 2016 r. Od 5 grudnia 2020 r. funkcja Sekretarza Sekcji „Micromycetes w ochronie zdrowia, środowiska oraz w przemyśle”
2. Członkostwo w Uczelnianym Sądzie Koleżeńskim Uniwersytetu Łódzkiego, kadencja 2016-2018
3. Akademickie Zrzeszenie Studentów Biotechnologii od 2015 r.

b) działalność organizacyjna

1. V Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, 30-31.05.2019 r., Łódź.
2. IV Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, 24-05.05.2018 r., Łódź.
3. The 4th Workshop on Microbiology in Health Care and Environmental Protection MIKROBIOT 2017, 19-21 września 2017 r., Łódź.
4. Promocja studiów doktoranckich podczas I Dnia Doktoranta Uniwersytetu Łódzkiego 22 maja 2017 r.
5. III Ogólnopolska Konferencja Doktorantów Nauk o Życiu BioOpen, 11-12.05.2017 r., Łódź.
6. Opiekun grup badawczych w Studenckim Kole Naukowym Biotechnologiczno – Mikrobiologicznym „SKN Bio-Mik” od 2016 r.

Publikacje stanowiące podstawę rozprawy doktorskiej

P1 - Góralczyk-Bińkowska A., Jasińska A., Długoński J. 2019. Characteristics and use of multicopper oxidases enzymes. *Advancements of Microbiology*, 58, 1, 7–18. <https://doi.org/10.21307/PM-2019.58.1.007>.

P2 - Góralczyk-Bińkowska A., Jasińska A., Długoński A., Płociński P., Długoński J. 2020. Laccase activity of the ascomycete fungus *Nectriella pironii* and innovative strategies for its production on leaf litter of an urban park. *PLoS ONE*, 15:4 (e0231453). <https://doi.org/10.1371/journal.pone.0231453>.

P3 - Góralczyk-Bińkowska A., Długoński A., Bernat P., Długoński J., Jasińska A. 2021. Environmental and molecular approach to dye industry waste degradation by the ascomycete fungus *Nectriella pironii*. *Scientific Reports*, 11, 23829. <https://doi.org/10.1038/s41598-021-03446-x>.

P4 - Góralczyk-Bińkowska A., Długoński A., Bernat P., Długoński J., Jasińska A. 2022. Accelerated PAHs transformation in the presence of dye industry landfill leachate combined with fungal membrane lipids changes. *International Journal of Environmental Research and Public Health*, 19, 13997. <https://doi.org/10.3390/ijerph192113997>.

CHARACTERISTICS AND USE OF MULTICOPPER OXIDASES ENZYMES

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Abstract: Multicopper oxidases (MCOs) are a large family of blue copper proteins which contain from one to six copper atoms per molecule. Their catalytic centre consists of three domains which involve type I Cu, type II Cu and a pair of type III Cu's. They include laccases, ferroxidases, ascorbate oxidase, bilirubin oxidase, laccase-like multicopper oxidases. MCOs are capable of one-electron oxidizing of aromatic as well as non-aromatic compounds with a concomitant four-electron reduction of molecular oxygen to water. These properties make them a valuable tool in various industries (e.g. food, textile, pharmaceutical) medicine or environment protection.

1. Introduction. 2. Multicopper oxidases – classification, structure and properties. 3. Identification methods of MCOs. 4. Laccases vs. others MCOs. 5. Application of multicopper oxidases. 6. Summary

CHARAKTERYSTYKA I KIERUNKI WYKORZYSTANIA ENZYMÓW Z GRUPY WIELOMIEDZIOWYCH OKSYDAZ

Streszczenie: Wielomiedziowe oksydazy (MCO) stanowią dużą rodzinę niebieskich białek, a ich centrum katalityczne składa się z trzech domen: typu I Cu, typu II Cu oraz pary typu III Cu. Wśród MCO wyróżnia się lakazy, ferroksydazy, oksydazy askorbinianowe, oksydazy bilirubinowe oraz lakazopodobne wielomiedziowe oksydazy. Wielomiedziowe oksydazy katalizują reakcje utleniania aromatycznych jak i niearomatycznych związków z jednoczesną redukcją tlenu cząsteczkowego do wody. Właściwości te czynią je cennym narzędziem w wielu gałęziach przemysłu i ochronie środowiska.

1. Wprowadzenie. 2. Wielomiedziowe oksydazy – klasyfikacja, struktura i właściwości. 3. Metody identyfikacji enzymów z rodziny MCO. 4. Lakazy vs. inne MCO. 5. Zastosowanie wielomiedziowych oksydaz. 6. Podsumowanie

Key words: microorganisms, laccases, multicopper oxidases

Słowa kluczowe: drobnoustroje, lakazy, wielomiedziowe oksydazy

1. Introduction

Multicopper Oxidases, MCO are a family of enzymes that catalyse oxidation reactions of a substrate with simultaneous reduction of molecular oxygen to water. The universal catalytic centre is responsible for these reactions for all MCOs, composed of 4 or more copper atoms which form T1, T2 types and diatomic T3 types. Although the structure of the catalytic centre of most enzymes classified as MCO is similar, their biological functions and catalytic properties may be completely different.

So far (according to the UniProt database) circa 50 thousand various enzymes characterized by the presence of a catalytic centre typical of MCO and the ability to oxidize organic and inorganic compounds have been recognised. Many researchers have sought to analyse the characteristics on the basis of which it is possible to properly classify a newly identified enzyme

which displays MCO properties. However, this problem still seems to be unresolved for most MCOs due to the highly similar properties of most substrates and considerable homology of amino acid sequences (especially within the catalytic centre). The most commonly described MCOs include laccases (especially from fungi belonging to the *Basidiomycota* division), and so-called Laccase-like Multicopper Oxidases (LMCO), ascorbate oxidase (mainly described in higher plants), bilirubin oxidase (e.g. *Myrothecium verrucaria*), some fungal pigments and ferroxidases (e.g. Fet3p of *Saccharomyces cerevisiae* yeasts) [12, 26, 44].

The very high oxidation-reducing potential of MCOs and the diversity of reactions they catalyse have caused these enzymes to become highly popular among researchers worldwide and offer many possibilities of application. MCOs are relatively stable enzymes, easy to separate from a culture and purify, which, combined with their low substrate specificity, makes them

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a valuable tool in drug production processes [5], elimination of phenolic compounds from alcohol products [13], dye synthesis [19], degradation and decolorization of wood pulp [93] or detoxification of xenobiotics [40]. It is mainly laccases and laccase-like enzymes that have attracted the attention of both the scientific community and industry [40, 93, 94]. Little attention has been devoted so far to other groups of MCO enzymes, which, although relatively less widespread in the natural environment, are an equally interesting object of research and offer a possibility of practical application.

2. Multicopper oxidases – classification, structure and properties

A common feature of all enzymes included in the MCO family is the presence of a catalytic centre composed of at least four copper atoms divided – on account of their spectroscopic and magnetic properties – into three types: T1 and T2 types – containing one copper atom and the diatomic T3 type [67]. Type T1 gives the enzyme molecule a blue colouring and exhibits intense light absorption at a wavelength of 610 nm, resulting from a covalent copper-cysteine bond. In turn the T2 type is colourless and, similarly to the T1 type, detectable by means of electroparamagnetic resonance spectroscopy (EPR). The T3 type does not exhibit activity in EPR spectroscopy as a result of antiferromagnetic coupling of copper atoms. However, it is distinguished by a light absorption band at a wavelength of 330 nm [9] (Fig. 1).

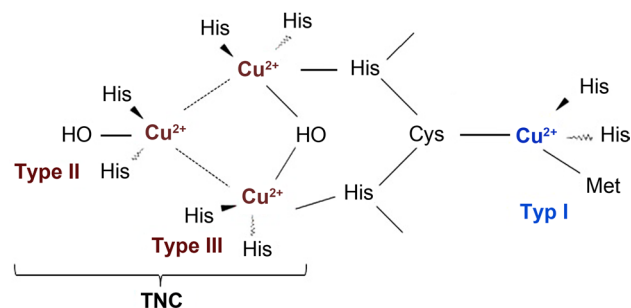


Fig. 1. Model of the catalytic cluster of MCO.

In the T1 centre, where the substrate undergoes oxidation, the copper atom is bound to two histidine residues and one cysteine forming a distorted trigonal pyramid structure. The His-Cys-His sequence mentioned, which is characteristic of MCO, links T1 with T3. Sometimes the fourth amino acid residue, with weaker binding (most commonly methionine, leucine or phenylalanine), may occur in an axial position, which affects the oxidoreduction potential of the enzyme, stabilizes it and regulates its activity. The copper atom of T2 type and two atoms of T3 type, located in close proximity, are coordinated by the so-called interdomain copper binding sites, composed of 2 and 6 histidine residues respectively, and forming a triatomic copper cluster (known as Trinuclear Cu Cluster, TNC). It is a structure unique for the MCO family and is the place where binding and the four-electron reduction of molecular oxygen into water occurs [76].

The majority of MCOs contains about 500 amino acid residues and adopts the β -sheet layout in its secondary structure, shaped into the characteristic motif of the Greek Key [32, 43]. Typically, an MCO molecule consists of three domains formed in this manner. The T1 copper centre is located in domain 3 (blue copper-binding domain), and the T2/T3 triatomic copper cluster is located at the interface between domains 1 and 3, which is farther away from the protein surface compared to domain 3. However, apart from the three-domain MCOs, proteins possessing two or six domains have also been characterized [61].

The MCO catalytic mechanism includes (1) the reduction of the T1 Cu site by capturing an electron from the oxidized substrate, (2) transferring the electron from the T1 site to the TNC and (3) reduction of O_2 with formation of two water molecules (Fig. 2).

MCOs oxidize a wide spectrum of substrates, such as phenol, methoxyphenol, aromatic amines, multi-aromatic compounds, metal ions [39, 51]. MCO-catalysed reactions may occur directly (reactions of simple phenolic compound oxidation) or in the presence of a compound called a mediator, which mediates the transfer of electrons from the substrate to the active enzyme centre (Fig. 3). If direct oxidation of the phenolic substrate leads to the formation of its reactive

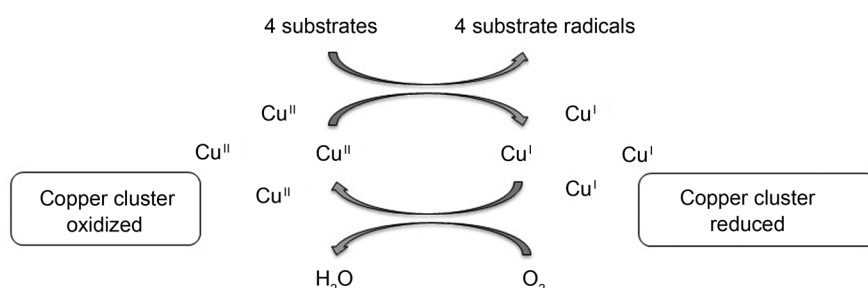


Fig. 2. Schematic of the catalytic mechanism of laccase.

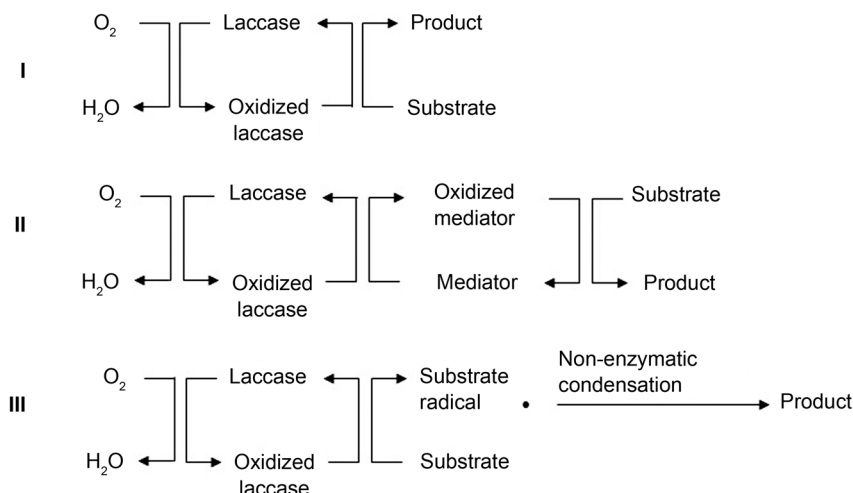


Fig 3. Mechanisms of reactions catalyzed by MCO.

I – reactions occurring directly, II – reactions occurring in the presence of a mediator, III – coupling reactions [Polak i Jarosz-Wilkolaka [66], modified].

and unstable radicals, these may, in the process of non-enzymatic, spontaneous coupling reactions combine to form dimers, oligomers or polymers [66].

MCOs are a very diverse group of enzymes produced by both prokaryotic organisms and *Eukaryota*, and are characterized by various, not yet fully understood, biological functions. Although all MCOs exhibit the capability for oxidizing aromatic compounds, two functional classes can be distinguished among them [86]. The first one is the enzymes that oxidize organic substrates more readily than metal ions. The group consists mainly of laccases and laccase-like enzymes. The latter, in turn, oxidize metal ions, such as Fe (II), Cu (I) and/or Mn (II), with higher efficiency, compared to organic substrates. The latter enzymes are referred to as metal oxidases, and the most common ones among them are human ceruloplasmin (Cp) and yeast ferroxidase (Fet3p) [53]. The MCO division is not permanent

and systematic, as there are no clear criteria for classification. For example, according to Hoegger *et al.* [26] multicopper oxidases form 10 enzyme groups: *Basidiomycota* laccases, *Ascomycota* laccases, insect laccases, MCO fungal pigments, fungal ferroxidases, plant and fungal ascorbate oxidases, plant-like enzymes like laccase, copper resistance proteins (CopA), bilirubin oxidases and copper efflux proteins (CueO) (Table I). In turn, Sirim *et al.* [83] distinguished within the MCO family: laccases, ferroxidases, ascorbate oxidases (AO) and bilirubin oxidases (BOD). After integration of the sequence data and MCO structures, the Laccase Engineering Database (LccED) was constructed (<https://lcced.biocatnet.de/>). Currently, the database contains 16 MCO superfamilies containing over 14,000 amino acid sequences of 10,415 various proteins (Tab. I).

The identification of laccases from among multicopper oxidases has remained ambiguous so far. Reiss

Table I
Classification of multicopper oxidases

Group of MCOs	Microorganism	Enzyme characteristic	References
<i>Basidiomycota</i> laccases	<i>Trametes pubescens</i>	pH 3–4.5; DMP, syringaldazine	[21]
<i>Ascomycota</i> MCOs	<i>Aspergillus niger</i>	pH 5–6; DMPPDA	[90]
Insect laccases	<i>Anopheles gambiae</i>	pH 6; ABTS	[46]
Fungal pigments MCOs	<i>Aspergillus niger</i>	pH 5; DMPPDA	[90]
Fungal ferroxidases (Fet3p)	<i>Saccharomyces cerevisiae</i>	pH 5; p-phenylenediamine	[86]
Ascorbate oxidases	<i>Brassica oleracea var. italica</i>	pH 7 ascorbic acid solution	[80]
Plants laccases	<i>Rhus vernicifera</i>	pH 9; syringaldazine	[96]
Bilirubin oxidases	<i>Myrothecium verrucaria</i>	pH 8; syringaldazine	[96]
Copper efflux proteins (CueO)	<i>Escherichia coli</i>	pH 6.5; DMP	[72]
Bacterial laccases (CotA)	<i>Bacillus subtilis</i>	T ½ in 80°C after 2–4 h	[61]
Copper-resistance proteins (CopA)	<i>Pseudomonas syringae</i>	pH 5; DMP	[84]

MCO classification according to Hoegger *et al.* [26] with characteristic of enzymes and examples of organisms producing them

et al. [71] proposed using the term “laccase” only for the enzyme isolated from the sap of *Rhus vernicifera* tree and introducing the term “laccase-like multi-copper oxidases” (LMCO) to account for the potential differences in their biological functions and biochemical features. In addition, Brander *et al.* [6] state that the term “laccases” was originally used in relation to plant-origin multicopper oxidases possessing three domains. Ihssen *et al.* [30] recommends classifying as laccases only those MCOs that have been isolated with urushiol – unsaturated alkyl catechol. The classification of enzymes in the MCO family is complex due to their broad substrate specificity, however, detailed biochemical characterization is necessary in order to organize the divisions proposed by the researchers. The division of MCOs is not permanent and systematic, as there are no clear criteria for classification. The division accepted by Hoegger *et al.* seems to be the most appropriate [26], however, due to slightly different biochemical properties and not fully specified biological functions, it seems reasonable to distinguish among the MCO also the group of laccase-like LMCO enzymes.

3. Identification methods of MCOs

Until recently, the identification of enzymes included in the MCO was based mainly on the characteristics of their biochemical features and catalytic abilities. MCO interactions with substrates can be broadly divided into two categories and one can distinguish enzy-

mes with low substrate specificity and enzymes with high specificity. The plant and fungal laccases belong to the first category and they can oxidize diphenols, aryl amines and aminophenols, and their K_m values are generally within the range of 1–10 mM. The remaining MCOs have a significant degree of substrate specificity ($K_m < 1$ mM) [85].

Some substances such as guaiacol, diammonium salt of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,6-dimethoxyphenol (DMP) and catechol have long been termed model laccase substrates [71]. However, it turned out that many of them are also oxidized by other enzymes from the MCO group, e.g. LMCO or bilirubin oxidases. Syringaldazine used to be considered to be a specific laccase substrate [64]. Syringaldazine and ABTS can be converted by MnP or LiP. However, the oxidation which depends on H_2O_2 allows for distinguishing these enzymes from MCO. MCO can also be distinguished from MnP using the leucoberbelin blue dye test. This compound reacts specifically with manganese ions released by MnP, resulting in the emergence of blue colour exhibiting the maximum absorption of light at the wavelength of 620 nm [18]. Figure 4 presents a scheme for the identification and differentiation of laccases from other ligninolytic enzymes proposed by Fernandes *et al.* [18].

While the ability to oxidize specific substrates allows one to quickly determine the activity of a given enzyme from the MCO group, in the era of the development of highly advanced molecular techniques, it should not be the only method of identification and characterization

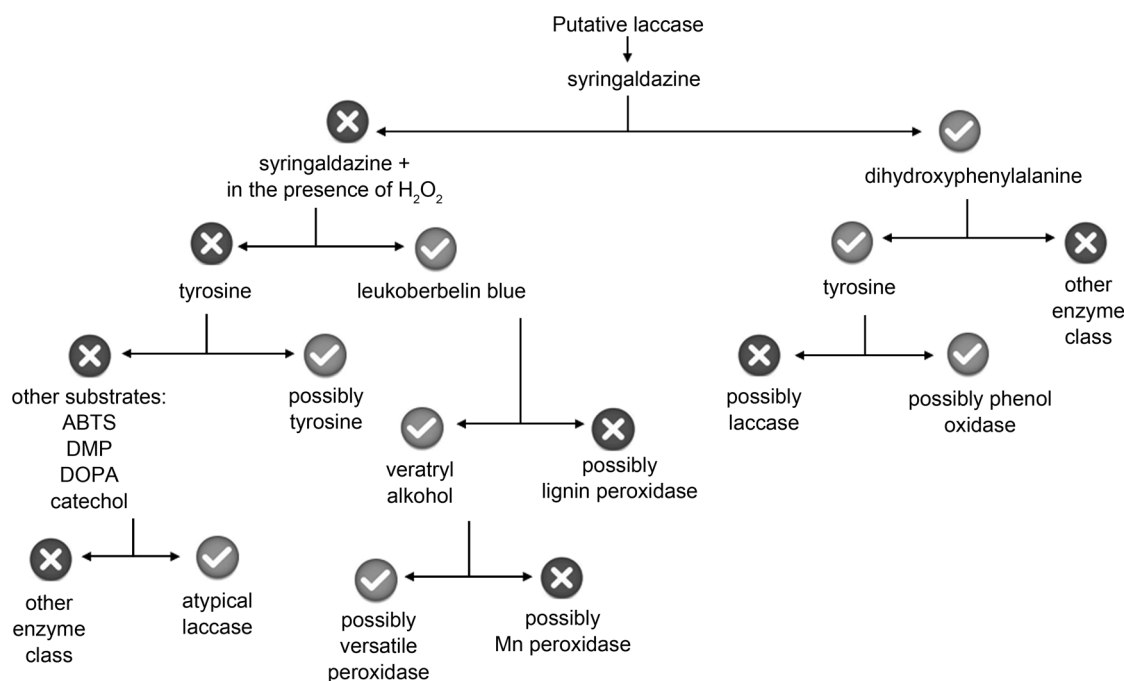


Fig. 4. The proposed scheme for the differentiation of laccases from other ligninolytic enzymes.

According to Fernandes *et al.* [18], modified.

of the newly recognized protein. The development of omics techniques, such as genomics, transcriptomics and proteomics has contributed to determining the genes responsible for encoding enzymes, studying their expression at the level of the transcriptome and quantitative and qualitative analysis of the MCO against the background of other proteins in the body. Perry *et al.* [65] was the first to identify two genes encoding laccases *Agaricus bisporus* and found that these enzymes may exist as isoforms and be encoded by multigene gene families. Hence, hundreds of genes encoding MCO have been identified in both fungi and bacteria. The presence of many genes encoding enzyme isoforms in one strain may indicate the involvement of these enzymes in various physiological processes. The confirmation of this hypothesis may be the fact that individual enzyme isoforms often differ in substrate specificity and their activity may be different in different pH or temperature variants. Some of them have a constitutive character, and some may be induced, for example, by aromatic compounds or copper ions. Genomic and proteomic techniques have made it possible to identify consensus sequences for MCOs which distinguish them from other enzymes. These sequences contain four (L1, L2, L3, L4) contiguous fragments of copper-binding amino acid residues, whose degree of similarity, depending on the organism from which the enzyme is derived, can range from 75 to 85%. The L2 and L4 regions allow the enzyme to be classified as MCO, whereas the L1 and L3 sequences indicate the subgroup of the enzyme [23].

4. Laccases vs. others MCOs

Among the microbial MCOs, laccases constitute the most studied and described group, being also the most numerous one. These are three-domain MCOs which were isolated for the first time by H. Yoshida in 1883 from resin from the *Rhus vernicifera* tree. Plant laccases, owing to the dehydrogenation mechanisms, play an important role in the polymerization of lignin-forming phenolic compounds, regeneration of damaged tissues and iron oxidation by converting Fe (II) to Fe (III) [15]. Although according to some researchers, the term laccase should be reserved exclusively for enzymes obtained from plants, also other three-domain MCOs, e.g. of microbial origin, are called laccase if only they exhibit the ability to oxidize aromatic compounds [61].

Laccases of fungal origin most often occur in the form of several monomers which oligomerize and then form multimeric complexes. The average molecular weight of the monomer ranges from about 50 to 110 kDa. An important feature of fungal laccases is a carbohydrate group with covalent binding, usually constituting 10 to 45% of the total enzyme molecule and

consisting mainly of mannose, N-acetylglucosamine and galactose. All these features protect fungal laccases against proteolysis, high temperatures, extremely high or low pH values and other unfavourable factors [15, 74].

Among the fungi capable of the biosynthesis of laccases, the most numerous groups are those from the *Basidiomycota* division, e.g. *Lentinus tigrinus*, *Agaricus bisporus*, *Trametes versicolor* [82]. Amongst them, there occur quite often the so-called fungi of white wood rot, incl. strains of the species *Phlebia radiata*, *Pleurotus ostreatus*, *Phanerochaete chrysosporium*, *Cerrena unicolor* [87]. The synthesis of these enzymes has also been described in the fungi belonging to the *Ascomycota* division, e.g. *Aspergillus niger*, *A. oryzae*, *Neurospora* sp., *Trichoderma atroviride* and *T. harzianum* [87]. In fungi, laccases are involved in the processes of morphogenesis, lignin degradation and defence reactions to stress. These enzymes produced by saprophytic and mycorrhizal fungi are involved in the circulation of organic matter in the soil by degrading plant litter polymers or the formation of humic compounds [26]. Bacterial laccases have been identified in the cultures of strains including *Azospirillum lipoferum*, *Escherichia coli*, *Bacillus subtilis* and several species of *Streptomyces* [7]. They were also described in *Anabaena azollae* cyanobacteria. Bacterial laccases are characterized by greater activity and stability than fungal enzymes at high temperatures, at alkaline pH and in the presence of high concentrations of chlorine and copper ions [15].

Both phenol and non-phenol substrates can undergo laccase-mediated catalytic reactions. In the case of molecules with high redox potential and with large size particles, which are not able to independently penetrate into the active enzyme centre, the action of the so called mediators is indispensable. They are organic compounds of low molecular weight which, when oxidized by laccase, form highly active cationic radicals capable of oxidising non-phenolic compounds. The most commonly used synthetic mediators are ABTS, hydroxyanthranilic acid (HAA), hydroxybenzotriazole (HBT) and hydroxyphthalimide (NPI). Natural mediators such as vanillin and syringaldehyde also have a similar effect [66].

Laccase-like multicopper oxidases (LMCO), similar to laccases, catalyse the oxidation of various substrates combined with the reduction of the O₂ molecule to two molecules of H₂O. Their biological functions are similar to the role of laccases, but not all of them have yet been recognized. LMCO have been described in many bacteria and fungi. The average molecular weight of LMCO is in the range of 51–66 kDa, while the number of amino acid sequences of enzymes is 470–600 aa. LMCO of *Streptomyces* bacterium have also been described, whose molecular mass is 32.6 kDa, and the amino acid sequence length is 297 aa. LMCOs of gram-negative bacteria differ from all other LMCOs through

the presence of proline between the two histidines and the additional histidine in the second position after the HXH motif. LMCOs of bacterial origin are considered to be more effective in the decomposition of organic compounds than fungal LMCOs. On the other hand, fungal LMCOs have a wider substrate range than LMCOs from gram-negative bacteria [49].

The optimum pH value for LMCO activity is not the same because it depends on the substrate used for its measurement [49]. For example, three types of LMCO originating from *Aspergillus niger*: McoA, McoB and McoG were purified and characterized for their biocatalytic potential. All three enzymes were monomers with molecular weights in the range of 80 to 110 kDa. The highest McoA activity was observed in the pH 5.0 environment, while pH 6.0 was optimal for McoB and McoG. Additionally, McoA and McoB oxidized DMP-PDA (N, N-dimethyl-p-phenylenediamine) in a wider pH range than McoG [89]. The LMCO isolated from *Myrothecium roridum* showed activity of both MCO and bilirubin oxidase [35].

Bilirubin oxidase is a thermostable enzyme containing a disulphide bond. BOD catalyses the oxidation of tetrapyrroles, e.g. bilirubin to biliverdin as well as diphenols or aryl diamines with simultaneous reduction of four oxygen atoms to water [10]. This enzyme was discovered in the non-ligninolytic fungus *Myrothecium verrucaria* MT-1 in 1981 by Noriaki Tanaka and Sawao Murao. Unlike laccases, BODs are characterized by higher activity and stability at neutral pH and high temperature, however not higher than 60°C. They are also characterised by high tolerance to chloride anions and other chelators [52]. For example, bilirubin oxidase isolated from *Myrothecium verrucaria* (MvBOD) exhibited the highest catalytic activity in the temperature range of 30–60°C and pH from 7 to 8.5. However, in solutions with pH above 9, a decrease in BOD catalytic activity by as much as 50% has been observed [91].

Ascorbate oxidase catalyses the oxidation of ascorbate to dehydroascorbic acid with formation of H₂O in the presence of oxygen. It has been isolated from higher plants, in which it occurs in the largest amount in the cell wall and is involved in their growth [80, 85]. Ascorbate oxidase also participates in defence reactions by modifying the apoplastic space [26]. Its activity and expression are induced by auxin and light, which suggests that it is involved in signal transduction [95]. AO has also been described in microorganisms, including *Myrothecium verrucaria*, *Aerobacter aerogenes*, *Acremonium* sp. HI-25 [60, 80]. In contrast to laccases which act as monomers, it is necessary to create a homodimer structure for proper functioning of the AO. Such a protein structure also performs stabilizing functions [61].

The copper efflux oxidase (CueO), which is present in periplasm in *E. coli*, may oxidize p-phenylene-

diamine and 2,6-dimethoxyphenol. Like some other three-domain MCOs, it possesses ferroxidase activity. As a result, the enzyme not only protects the cell against the adverse effects of copper ions (through the oxidation of Cu⁺ to less harmful Cu²⁺), but also participates in iron homeostasis. CueO has been shown to oxidize the catechol groups of 2,3-dihydroxybenzoic acid, which is a precursor of enterobactin [24]. CueO has a structure similar to laccase and AO, but the conformation of the TNC makes it different from other MCOs. In addition, within the third domain, CueO contains methionine-rich regions which act as a copper ion sensor, in the presence of which the enzyme activity increases significantly [61, 72].

Another bacterial MCO is the copper resistance protein (CopA), consisting of three domains, described in *Pseudomonas syringae* or *Xanthomonas campestris* [26]. The activity of this enzyme was determined based on the ability to oxidize 2,6-dimethoxyphenol [84]. As reported by Nakamura and Go [61], in aerobic conditions CopA and CueO control copper metabolism by exporting the excess of Cu (I) from the cytoplasm and oxidation to Cu (II), which is less toxic.

The *B. subtilis* strain is capable of producing a thermostable protein – CotA, which coats endospores. CotA consists of more than 30 types of polypeptides and is resistant to both physical and chemical factors. It results from the function it performs, namely the production of melanin pigment, which protects against UV radiation and hydrogen peroxide [17, 29]. In addition, the protein is highly stable because the half-life of activity at 80°C was determined to be 2–4 hours [61]. According to Rajeswari [69], CotA laccase is similar to CueO in *E. coli* based on the construction of the catalytic centre, however, the cross-domain loop possesses sites at which allow for tighter packaging, which improves the stability of the entire structure and increases thermostability [61]. According to Enguit *et al.* [16], this segment contains only 4 of the 46 proline residues constituting the entire CotA. Therefore, it may suggest that the proline content both determines the thermostability of the protein and significantly increases it in combination with increased packaging [16].

MCO fungal pigments, found mainly in *Ascomycota*, including *Aspergillus nidulans* [90] are responsible for the oxidation of dihydroxyphenylalanine (DOPA) to dopaquinone along the melanin synthesis pathway. These enzymes differ significantly from other MCOs in the construction of the S2 region [44]. They oxidize typical laccase substrates, among others p-phenylenediamine, pyrogallol, gallic acid or ABTS [89].

MCO includes also ferroxidases, characterized by affinity to Fe (II), which is not shown by other multicopper oxidases. The most frequently described ferroxidases are the plasma membrane protein of *Saccha-*

romyces cerevisiae (Fet3p) and human ceruloplasmin (hCp) consisting of six domains. Based on X-ray examination of the crystal structure of ceruloplasmin, it was established that in the second, fourth and sixth domains there occurs a copper binding site, while the first and sixth domain divide the tri-nuclear, inter-domain copper binding site to form a pseudosymmetric C3 structure [61]. Cp exhibits the ability to oxidize aromatic diamines and other aromatic compounds [85]. Ferroxidases play an important role in iron homeostasis in yeasts and mammals [86]. Fet3p participates in the Fe (II) transport system with high affinity in yeast. Initially, Fe (III) is reduced to Fe (II) by ferroxidase, after which it is transported in the cell. Fet3p performs a protective role by suppressing the cytotoxic action of copper and iron [26, 85].

Another example of MCO is SLAC, a two-domain multicopper oxidase described in *Streptomyces coelicolor*, which exhibits the ability to oxidize aromatic and non-aromatic compounds containing amino and hydroxyl groups. Due to the similarity of the sequence to fungal laccases, but also smaller size, this enzyme has been defined as a small laccase. On the basis of the comparative analysis of protein sequences, the similarity of SLAC and other laccases in the position of metal ligands has been established. However, the occurrence of 24 histidine residues in the SLAC sequence has been established, which may indicate its role in binding the excess of intracellular copper ions in order to transfer them during export through the TAT secretory system [50]. SLAC is characterized by resistance to reducing compounds and thermal stability. In addition, this enzyme exhibits the highest activity in the environment with pH 9 [11, 50]. Owing to these features, SLAC have found their application in the pulp and paper industry for dye decolorization [11].

5. Application of multicopper oxidases

On account of the ability to oxidize many substrates, extracellular character and fairly high stability in a wide range of pH and temperatures, the MCO enzymes are characterized by a high application potential. So far enzymes from this group have found application in environmental protection, medicine, pharmaceutical industry, cosmetics and in the food industry. Laccases and laccase-like enzymes, which are the most common and characterized MCO group, are mainly used as biocatalysts in the synthesis reactions of new compounds, detection, biotransformation and biodegradation of toxic impurities (Table II).

MCO enzymes have been applied in many areas of the food industry, like baking, vegetable and fruit processing, winemaking and brewing. The bakery industry

commonly uses laccases to improve bread structure, as well as the flavour and durability of pastries [73]. However, it has been proven that other MCOs, such as bilirubin oxidase, can be used to cross-link biopolymers by improving the physicochemical properties of food products [98]. The process increases the durability and stability of dough, at the same time reducing its viscosity. This effect was noted especially when using lower quality flour [51]. MCO is used, e.g., for cross-linking arabinoxylans so that the created network of transverse polymer bonds has a positive effect on crumb and crustiness of bread [40]. Laccases can be used instead of physical adsorbents like SO₂ to eliminate undesirable phenol derivatives, causing darkening and clouding of fruit juices, beers and wines [13, 40]. New reports have appeared lately, indicating the possible use of MCOs isolated from cultures of lactic acid fermentation bacteria, among others for removing biogenic amines from wine and some oriental cuisine products [2, 8, 25]. The use of MCO in the food industry is quite common. Preparations available on the market, such as Falouvorstar, Suberase or LACCASE Y120, which are based on laccase activity, are successfully used in brewing, production of corks for the wine industry and improvement of the colour values of food products [40].

Enzymes from the MCO group are used as biocatalysts in the reactions yielding many active substances which are components in the composition of household chemistry, body care products and medicines characterized by antimicrobial and antioxidant activity [79, 93]. Such activity is exhibited by molecular iodine (I₂), whose preparation through oxidising I⁻ has been described for MCOs isolated from the culture of *Alphaproteobacterium* sp. Q-1 and *Roseovarius* sp. A-2 [81, 88]. Strong antifungal activity has also been proven for iodinated phenolic compounds obtained in the reaction catalysed by laccase [31, 78]. Laccases are also successfully used as biocatalysts in the synthesis of drugs, among others β -lactam antibiotics and anti-cancer agents, e.g. vinblastines or mitomycin [45, 56]. Since 2006, when antiproliferative activity of laccase was demonstrated for the first time, intensive research has been conducted on the use of this enzyme as an anti-cancer agent [92]. The ability to inhibit cell division of breast, liver, colon and prostate cancer has been proven for laccases from various species of basidiomycetes [68, 70]. The application of laccase manufactured by *Cerena unicolor* in the treatment of blood and cervical cancer has been demonstrated and covered by patent protection [37, 54]. This enzyme, added in the right concentration, had a strong cytotoxic effect on cervical cancer cells of the SiHa and CaSki line and did not affect adversely the fibroblast cells constituting the reference system [59]. The same enzyme exerted pro-apoptotic action on blood cancer cells of Jurkat and RPMI 8226 lines [55]. MCOs may

Table II
Reactions of synthesis, detection and biodegradation catalyzed by enzymes from the MCOs group

Appli- cation	Enzyme, activity	Organism	Substrate, catalyzed reaction	Reaction conditions, process efficiency	Refe- rences
Biodegradation of xenobiotics	Multicopper oxidase (1.5 U/mL)	<i>Spirulina platensis</i> CFTRI	Reactive Blue 4 (100 mg/L)	96%; 4 h	[1]
	Laccase	<i>Pleurotus ostreatus</i>	Atrazine, pentachlorophenol, naproxen, oksybenzone (0.5 mg/L)	60–99%; 24 h; vanillin	[3]
	Bilirubin oxidase	<i>Magnaporthe oryzae</i>	Remazol Brilliant Blue R (80 mg/L)	95%; 20 min.; ABTS	[14]
	Laccase cocktail (100 U/L)	<i>Pycnoporus sanguineus</i> CS43	Bisphenol A, 4-nonylphenol, tricolaan (10 mg/L)	89–100%; 5 h	[22]
	Laccase (3 U/mL)	<i>Phoma</i> sp. UHH 5-1-03	Sulfamethoxazole (0.25 mM)	87%; 22 h	[27]
	Laccase (0.05 U/mL)	<i>T. versicolor</i>	Chloropyrifos, atrazine, chlorothalonil, pyrimethanil (20 mg/L)	90–100%; 24 h – 8 days	[38]
	Bilirubin oxidase	<i>Myrothecium</i> sp. IMERI	Remazol Brilliant Blue R (80 mg/L)	91, 5%; 25 min.; ABTS	[48]
	Laccase-like multicopper oxidase (2 U/mL)	<i>Streptomyces</i> sp. C1	Indigo Carmine, Diamond Black PV	56–84%; 2 h; syringaldehyde	[49]
	Laccase (100 U/L)	<i>Pycnoporus sanguineus</i> CS43	2,4-dichlorophenol, β -nonylphenol (10 mg/L)	71–97%; 8 h	[75]
	Immobilized laccase (1 U/mL)	<i>Trametes pubescens</i> Cui 7571	Acid Black 172 (50 mg/L)	69%; 48 h	[100]
Synthesis and poly- merization reactions	Laccase	<i>Trametes versicolor</i>	4-methyl-3-hydroxyanthranilic acid	Actinocin synthesis, pH 5, immobilization in polyacrylamide gel	[63]
	Laccase	<i>Myceliophthora thermophila</i>	methyl-1,4-hydroquinone, 2,3-dimethyl-1,4-hydroquinone	Synthesis of β -lactam antibiotics	[56]
	Laccase (350 U/mg)	<i>Coriolus hirsutus</i>	Aniline (50 mM)	Polymerization of vanillin in lignosulfonate complex at pH 3.5–4.4	[42]
	Laccase	<i>Trametes pubescens</i>	Biosynthesis of totarol dimers	62.6% conversion of totarol after 24 h; pH 4.5–5, 30°C	[62]
Detection reactions	Laccase (0.29 U/mL)	<i>Aspergillus oryzae</i>	Detection of luteolin	Immobilized in chitosan (Chi) chemically cross-linked; with cyanuric chloride (CC)	[20]
	Laccase cocktail (31.5 U/mL)	<i>Pleurotus ostreatus</i>	Detection of adrenaline and dopamine	Adsorption on carbon paste; pH 7.0	[47]
	Bilirubin oxidase (50 U)	<i>Myrothecium verrucaria</i>	Detection of bilirubin	Immobilization in gold nanoparticles, pH 8.4	[41]
	Glucose dehydrogenase and laccase complex	<i>Coriolus hirsutus</i>	Detection of morphine	Immobilization in polyvinylalcohol (PVA); pH 6.5	[5]

also have antiviral effects. *Lentinus tigrinus*, a fungal laccase, inhibits the activity of HIV-1 reverse transcriptase, without which the virus is unable to transcribe genetic material from RNA to DNA [96].

MCOs can be used for analytical purposes, including biological, enzymatic and immunochemical tests [94]. Laccase is used to detect the presence of morphine [5]. In turn, bilirubin oxidase-based (BOD) biosensors, designed in order to precisely determine the level of bilirubin, whose excessively high concentration in human serum is lethal, are characterized by high sensitivity and efficacy [28]. Furthermore, bilirubin and ascorbate oxidases have been used for clinical trials aimed at eliminating the effect of ascorbate, which adversely affected the liver [76]. The MCO catalytic activity, which is accompanied by the reduction of oxygen to water molecules, is used to obtain electrons, i.e. the driving force of biocells [52, 77].

In the cosmetics industry laccase is used for the production of dyes and can be used in non-toxic hair dyes instead of the oxidizing agent – hydrogen peroxide [4]. Such preparations are more convenient to use and less irritating to the organism due to the replacement of the oxidizing agent, which weakens hair and destroys their structure [13]. Moreover, laccases can be used in skin lightening preparations by reducing the content of melanin [40].

Biosynthesis of dyes using laccase is an environmentally friendly alternative to chemical synthesis of textile dyes and allows for reducing process costs. Substances obtained in this way are characterized by a wide range of colours and durability comparable to synthetic dyes [33]. On the other hand, these enzymes can also be used for decolorization of fabrics (e.g. jeans) or transforming dye precursors into their active forms, increasing the efficiency of the dyeing process [66]. This allows for limiting the application of chemical bleaches and is particularly useful for fabrics sensitive to chemical compounds [94].

Due to the ability to remove toxic phenols formed during the degradation of lignin, laccase participates in the reactions of its depolymerization [93]. In addition, these enzymes may be useful in the modification of cellulose fibres [40]. In order to improve the oxidation of non-phenolic compounds, laccase activity is supported by mediators, e.g. ABTS, TEMPO or HBT. However, the cost of synthetic mediators is an important limiting factor [94].

Mostly laccases of fungal origin have been widely applied to the process of bioremediation of contaminated areas [36]. These enzymes are used both in a free and immobilized form to eliminate a wide spectrum of toxic compounds, such as: phenolic compounds, chlorophenols, cyclic aromatic hydrocarbons or alkenes being components of, among others, pesticides. MCO

enzymes can also be used for neutralization of compounds belonging to the group of the so-called hormone modulators (EDCs), i.e. compounds adversely affecting the function of the endocrine system of humans and animals [58]. The research carried out by Garcia-Morales *et al.* [22] demonstrated a high biocatalytic efficiency of the protein mixture (LacI and LacII) of the CS43 *Pycnoporus sanguineus* fungus, which was used in the biotransformation of EDCs such as bisphenol A, 4-nonylphenol, 17- α -ethinylestradiol and triclosan. The capabilities of different laccases, LMCO enzymes and bilirubin oxidase for decolorization of industrial dyes and their elimination from wastewater have also been widely explored [34, 94]. For example, a crude extract of the laccase derived from the fungus *P. nebrodensis* has shown effective decolorization (82.69%) of malachite green after just one hour of incubation [99].

6. Summary

MCO proteins are enzymes containing from one to six atoms of copper per molecule. Multicopper oxidases include laccases, ferroxidases, ascorbate oxidase, bilirubin oxidase, some fungal pigments with multicopper oxidase character and the so-called laccase-like enzymes. MCOs possess the ability to oxidize both organic and inorganic compounds. The reactions catalysed by MCO are accompanied by the reduction of molecular oxygen to water. These properties make them a valuable tool in bioremediation processes, medicine, pharmaceutical industry, cosmetics and food industry.

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References

1. Afreen S., Shamsi T.N., Baig M.A., Ahmad N., Fatima S., Qureshi M.I., Hassan M.I., Fatma T.: A novel multicopper oxidase (laccase) from cyanobacteria: Purification, characterization with potential in the decolorization of anthraquinonic dye. *PLoS One*, **12**, e0175144 (2017)
2. Alvarez M.A., Moreno-Arribas M.A.: The problem of biogenic amines in fermented foods and the use of potential biogenic amine-degrading microorganisms as a solution. *Trends Food Sci. Technol.* **39**, 146–155 (2014)
3. Ashe B., Nguyen L.N., Hai F.I., Lee D., Van De Merwe J.P., Leusch F.D.L., Price W.E., Nghiem L.D.: Impacts of redoxmediator type on trace organic contaminants degradation by laccase:

- Degradation efficiency, laccase stability and effluent toxicity. *Int. Biodeterior. Biodegradation*, **113**, 169–176 (2016)
4. Bartosiak M., Cieślak A.: Zastosowanie grzybowej lakazy w procesie bioremediacji (in) *Biotechnologia w analizie, ochronie środowiska, medycynie i przemyśle*, red. K. Kropiwek, M. Szala, Fundacja na rzecz promocji nauki i rozwoju TYGIEL, Lublin, 2015, p. 41–52
 5. Bauer C.G., Kühn A., Gajovic N., Skorobogatko O., Holt P.J., Neil C., Bruce N.C., Makower A., Lowe C.R., Scheller F.W.: New enzyme sensors for morphine and codeine based on morphine dehydrogenase and laccase. *Fresenius J. Anal. Chem.* **364**, 179–183 (1999)
 6. Brander S., Mikkelsen J.D., Kepp K.P.: Characterization of an Alkali- and Halide-Resistant Laccase Expressed in *E. coli*: CotA from *Bacillus clausii*. *PLoS One*, **9**, e99402 (2014)
 7. Brijwani K., Rigdon A., Vadlani P.V.: Fungal laccases: production, function, and applications in food processing. *Enzyme Res.* DOI:10.4061/2010/149748 (2010)
 8. Callejón S., Sendra R., Ferrer S., Pardo I.: Identification of a novel enzymatic activity from lactic acid bacteria able to degrade biogenic amines in wine. *Appl. Microbiol. Biotechnol.* **98**, 185–198 (2014)
 9. Chaurasia P.K., Bharati S.L., Singh S.K.: Comparative studies on the blue and yellow laccases. *Res. Plant Stud.* **1**, 32–37 (2013)
 10. Christenson A., Shleev S., Mano N., Heller A., Gorton L.: Redox potentials of the blue copper sites of bilirubin oxidases. *Biochim. Biophys. Acta*, **1757**, 1634–1641 (2006)
 11. Cook R., Hannon D., Southard J.N., Majumdar S.: Small laccase from *Streptomyces coelicolor*—an ideal model protein/enzyme for undergraduate laboratory experience. *Biochem. Mol. Biol. Educ.* DOI:10.1002/bmb.21102 (2017)
 12. Copete L.S., Chanagá X., Barriuso J., López-Lucendo M.F., Martínez M.J., Camarero S.: Identification and characterization of laccase-type multicopper oxidases involved in dye-decolorization by the fungus *Leptosphaerulina* sp. *BMC Biotechnology*, DOI:10.1186/s12896-015-0192-2 (2015)
 13. Desai S.S., Nityanand C.: Microbial laccases and their applications: a review. *Asian J. Biotechnol.* **3**, 98–124 (2011)
 14. Durand F., Gounel S., Kjaergaard C.H., Solomon E.L., Mano N.: Bilirubin oxidase from *Magnaporthe oryzae*: an attractive new enzyme for biotechnological applications. *Appl. Microbiol. Biotechnol.* **96**, 1489–1498 (2012)
 15. Dwivedi U.N., Singh P., Pandey V.P., Kumar A.: Structure-function relationship among bacterial, fungal and plant laccases. *J. Mol. Catal. B: Enzym.* **68**, 117–128 (2011)
 16. Enguita F.J., Martins L.O., Henriques A.O., Carrondo M.A.: Crystal structure of a bacterial endospore coat component. A laccase with enhanced thermostability properties. *J. Biol. Chem.* **278**, 19416–19425 (2003)
 17. Fernandes A.J.T.: Insight into the Multicopper Oxidases. Dissertation presented to obtain the PhD degree in Biochemistry at the Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa (2011)
 18. Fernandes T.A.R., da Silveira W.B., Passos F.M.L., Zucchi T.D.: Laccases from *Actinobacteria* – what we have and what to expect. *Adv. Microbiol.* **4**, 285–296 (2014)
 19. Forte S., Polak J., Valensina D., Taddei M., Basosi R., Vanhulle S., Jarosz-Wilkolazka A., Pogni R.: Synthesis and structural characterization of a novel phenoxazinone dye by use of a fungal laccase. *J. Mol. Catal. B: Enzym.* **63**, 116–120 (2010)
 20. Franzoi A.C., Vieira I.C., Dupont J., Scheeren C.W., de Oliveira L.F.: Biosensor for luteolin based on silver or gold nanoparticles in ionic liquid and laccase immobilized in chitosan modified with cyanuric chloride. *Analyst.* **134**, 2320–23528 (2009)
 21. Galhaup C., Goller S., Peterbauer C.K., Strauss J., Haltrich D.: Characterization of the major laccase isoenzyme from *Trametes pubescens* and regulation of its synthesis by metal ions. *Microbiology*, **148**, 2159–2169 (2002)
 22. Garcia-Morales R., Rodríguez-Delgado M., Gomez-Mariscal K., Orona-Navar C., Hernandez-Luna C., Parra R., Cárdenas-Chávez D., Mahlknecht J., Ornelas-Soto N.: Biotransformation of Endocrine-Disrupting Compounds in groundwater: bisphenol A, nonylphenol, ethynylestradiol and triclosan by a laccase cocktail from *Pycnoporus sanguineus* CS43. *Water Air Soil Pollut.* **226**, 251–265 (2015)
 23. Giardina P., Vincenza Faraco V., Pezzella C., Piscitelli A., Vanhulle S., Sannia G.: Laccases: a never-ending story. *Cell. Mol. Life Sci.* **67**, 369–385 (2010)
 24. Grass G., Thakali K., Klebba P.E., Thieme D., Müller A., Wildner G.F., Rensing Ch.: Linkage between catecholate siderophores and the multicopper oxidase CueO in *Escherichia coli*. *J. Bacteriol.* **186**, 5826–5833 (2004)
 25. Guarcello R., De Angelis M., Settanni L., Formiglio S., Gaglio R., Minervini F., Moschetti G., Gobetti M.: Selection of amine-oxidizing dairy lactic acid bacteria and identification of the enzyme and gene involved in the decrease of biogenic amines. *Appl. Environ. Microbiol.* **82**, 6870–6880 (2016)
 26. Hoegger P.J., Kilaru S., James T.Y., Thacker J.R., Kües U.: Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. *FEBS J.* **273**, 2308–2326 (2006)
 27. Hofmann U., Schlosser D.: Biochemical and physicochemical processes contributing to the removal of endocrine-disrupting chemicals and pharmaceuticals by the aquatic ascomycete *Phoma* sp. UHH 5-1-03. *Appl. Microbiol. Biotechnol.* **100**, 2381–2399 (2016)
 28. Hooda V., Gahlaut A., Gothwal A., Hooda V.: Bilirubin enzyme biosensor: potentiality and recent advances towards clinical bioanalysis. *Biotechnol. Lett.* **39**, 1453–1462 (2017)
 29. Hullo M.F., Moszer I., Danchin A., Verstraete I.M.: CotA of *Bacillus subtilis* is a copper-dependent laccase. *J. Bacteriol.* **183**, 5426–5430 (2001)
 30. Ihssen J., Reiss R., Luchsinger R., Thöny-Meyer L., Richter M.: Biochemical properties and yields of diverse bacterial laccase-like multicopper oxidases expressed in *Escherichia coli*. *Sci. Rep.* **12**, 10465 (2015)
 31. Ihssen J., Schubert M., Thöny-Meyer L., Richter M.: Laccase catalyzed synthesis of iodinated phenolic compounds with antifungal activity. *PLoS One*, **9**, e89924 (2014)
 32. Janusz G., Kucharzyk K.H., Pawlik A., Staszczak M., Paszczynski A.J.: Fungal laccase, manganese peroxidase and lignin peroxidase: gene expression and regulation. *Enzyme Microb. Technol.* **52**, 1–12 (2013)
 33. Jarosz-Wilkolazka A., Polak J., Olszewska A.: Method of obtaining synthetic dyes. Patent no. PL213247 (B1), Poland, 2012
 34. Jasińska A., Góralczyk A., Długoński J.: Dyes decolourisation and degradation by microorganisms (in) *Microbial biodegradation: from omics to function and application*, red. J. Długoński, Caister Academic Press, Norfolk, UK, 2016, p. 119–141
 35. Jasińska A., Góralczyk A., Soboń A., Długoński J.: Novel laccase-like multicopper oxidases from the *Myrothecium roridum* fungus – production enhancement, identification and application in the dye removal process. *Acta Biochim. Pol.* **65**, 287–295 (2018)
 36. Jasińska A., Różalska S., Bernat P., Paraszkiwicz K., Długoński J.: Malachite green decolorization by non-basidiomycete filamentous fungi of *Penicillium pinophilum* and *Myrothecium roridum*. *Int. Biodeter. Biodegr.* **73**, 33–40 (2012)
 37. Jaszek M., Matuszewska A., Osińska-Jaroszuk M., Janusz G., Sulej J., Stefaniuk D., Giannopoulos K., Karp.: Laccase enzyme

- isolated from *Cerrena unicolor* fungus to be applied in treatments of blood neoplastic diseases. Patent no. PL225934 (B1), Poland, 2016
38. Jin X., Yu X., Zhu G., Zheng Z., Feng F., Zhang Z.: Conditions optimizing and application of laccase-mediator system (LMS) for the laccase-catalyzed pesticide degradation. *Sci. Rep.* **24**, 35787 (2016)
 39. Jones S.M., Solomon E.I.: Electron transfer and reaction mechanism of laccases. *Cell. Mol. Life Sci.* **72**, 869–883 (2015)
 40. Kaczmarek M.B., Kwiatos N., Szczęsna-Antczak M., Bielecki S.: Laccases – enzymes with an unlimited potential. *Biotechnol. Food Sci.* **81**, 41–70 (2017)
 41. Kannan P., Chen H., Lee V.T., Kim D.H.: Highly sensitive amperometric detection of bilirubin using enzyme and gold nanoparticles on sol-gel film modified electrode. *Talanta*, **86**, 400–407 (2011)
 42. Karamyshev A., Sergey V.S., Koroleva O., Yaropolov A., Sakharov I.Y.: Laccase-catalyzed synthesis of conducting polyaniline. *Enzyme Microb. Technol.* **33**, 556–564 (2003)
 43. Kosman D.J.: Multicopper oxidases: a workshop on copper coordination chemistry, electron transfer, and metallophysiology. *J. Biol. Inorg. Chem.* **15**, 15–28 (2010)
 44. Kües U., Rühl M.: Multiple multi-copper oxidase gene families in *Basidiomycetes* – What for? *Curr. Genomics*, **12**, 72–94 (2011)
 45. Kunamneni A., Ballesteros A., Plou F.J., Alcalde M.: Fungal laccase – a versatile enzyme for biotechnological applications (in) communicating current research and educational topics and trends in applied microbiology, red. A. Mendez-Vilas, Formatex, Badajoz, 2007, p. 233–245
 46. Lang M., Braun C.L., Kanost M.R., Gorman M.J.: Multicopper oxidase-1 is a ferroxidase essential for iron homeostasis in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA*, **109**, 13337–13342 (2012)
 47. Leite O.D., Fatibello-Filho O., Barbosa A. de M.: Determination of catecholamines in pharmaceutical formulations using a biosensor modified with a crude extract of fungi laccase (*Pleurotus ostreatus*). *J. Braz. Chem. Soc.* **14**, 297–303 (2003)
 48. Liu Y., Huang J., Zhang X.: Decolorization and biodegradation of remazol brilliant blue R by bilirubin oxidase. *J. Biosci. Bioeng.* **108**, 496–500 (2009)
 49. Lu L., Zeng G., Fan Ch., Ren X., Wang C., Zhao Q., Zhang J., Chen M., Chen A., Jiang M.: Characterization of a laccase-like multicopper oxidase from newly isolated *Streptomyces* sp. C1 in agricultural waste compost and enzymatic decolorization of azo dyes. *Biochem. Eng. J.* **72**, 70–76 (2013)
 50. Machczyński M.C., Vijgenboom E., Samyn B., Canters G.W.: Characterization of SLAC: a small laccase from *Streptomyces coelicolor* with unprecedented activity. *Protein Sci.* **13**, 2388–2397 (2004)
 51. Madhavi V., Lele S.S.: Laccase properties and applications. *Bio-Resources*, **4**, 1694–1717 (2009)
 52. Mano N., Edembe L.: Bilirubin oxidases in bioelectrochemistry: features and recent findings. *Biosens. Bioelectron.* **15**, 478–485 (2013)
 53. Martins L.O., Paulo Durão P., Brissos V., Lindley P.F.: Laccases of prokaryotic origin: enzymes at the interface of protein science and protein technology. *Cell. Mol. Life Sci.* **72**, 911–922 (2015)
 54. Matuszewska A., Jaszek M., Janusz G., Osińska-Jaroszk M., Sulej J., Stefaniuk D., Rogalski J., Mizerska-Dudka M., Kandefer-Szerszeń M.: Laccase enzyme isolated from *Cerrena unicolor* fungus to be applied in the cervical carcinoma treatment. Patent no. PL225869 (B1), Poland, 2016
 55. Matuszewska A., Karp M., Jaszek M., Janusz G., Osińska-Jaroszk M., Sulej J., Stefaniuk D., Tomczak W., Giannopoulos K.: Laccase purified from *Cerrena unicolor* exerts antitumor activity against leukemic cells. *Oncol. Lett.* **11**, 2009–2018 (2016)
 56. Mikolasch A., Hildebrandt O., Schluter R., Hammer E., Witt S., Lindequist U.: Targeted synthesis of novel α -lactam antibiotics by laccase-catalyzed reaction of aromatic substrates selected by pre-testing for their antimicrobial and cytotoxic activity. *Appl. Microbiol. Biotechnol.* **100**, 4885–4899 (2016)
 57. Mikolasch A., Wurster M., Lalk M., Witt S., Seefekdt S., Hammer E., Schauer F., Julich W.D., Lindequist U.: Novel β -lactam antibiotics synthesized by amination of catechols using fungal laccase. *Chem Pharm. Bull.* **56**, 902–907 (2008)
 58. Minussi R.C., Miranda M.A., Silva J.A., Ferreira C.V., Aoyama H., Marangoni S., Rotilio D., Pastore G.M., Durán N.: Purification, characterization and application of laccase from *Trametes versicolor* for colour and phenolic removal of olive mill wastewater in the presence of 1-hydroxybenzotriazole. *Afr. J. Biotechnol.* **6**, 1248–1254 (2007)
 59. Mizerska-Dudka M., Jaszek M., Błachowicz A., Rejczak T.P., Matuszewska A., Osińska-Jaroszk M., Stefaniuk D., Janusz G., Sulej J., Kandefer-Szerszeń M.: Fungus *Cerrena unicolor* as an effective source of new antiviral, immunomodulatory, and anticancer compounds. *Int. J. Biol. Macromol.* **79**, 459–568 (2015)
 60. Murao S., Itoh H., Yajima T., Ozaki Y., Fukuyasu S., Shin T.: Isolation and purification of ascorbate oxidase from *Acremonium* sp. HI-25. *Biosci. Biotechnol. Biochem.* **56**, 847–852 (2014)
 61. Nakamura K., Go N.: Function and molecular evolution of multicopper blue proteins. *Cell. Mol. Life Sci.* **62**, 2050–2066 (2005)
 62. Ncanana S., Baratto L., Roncaglia L., Riva S., Burton S.G.: Laccase-mediated oxidation of totarol. *Adv. Synth. Catal.* **349**, 1507–1513 (2007)
 63. Osiaacz J., Al-Adhami A.J.H., Bajraszewska D., Fischer P., Peczyńska-Czoch W.: 1999. On the use of *Trametes versicolor* laccase for the conversion of 4-methyl-3-hydroxyanthranilic acid to actinocin chromophore. *J. Biotechnol.* **72**, 141–149 (1999)
 64. Perna A., Agger J.W., Holck J., Meyer A.S.: Multiple reaction monitoring for quantitative laccase kinetics by LC-MS. *Scientific Rep.* **8**, 8114 (2018)
 65. Perry C.R., Smith M., Britnell C.H., Wood D.A., Thurston C.F.: Identification of two laccase genes in the cultivated mushroom *Agaricus bisporus*. *J. Gen. Microbiol.* **139**, 1209–1218 (1993)
 66. Polak J., Jarosz-Wilkolazka A.: Reakcje katalizowane przez lakazę – mechanizm i zastosowanie w biotechnologii. *Biotechnologia*, **4**, 82–94 (2007)
 67. Potti R.B., Rajasekhar P., Subramanyam K.: Occurrences, physical and biochemical properties of laccase. *U.J.E.R.T.* **2**, 1–13 (2012)
 68. Qianqian M., Miao L., Hexiang W.: Purification and characterization of a laccase with antiproliferative activity from *Coriolus versicolor*. *Acta Edulis Fungi*, **17**, 43–47 (2010)
 69. Rajeswari M.: Characterization and optimization of bacterial laccase production and its application in the degradation of selected pollutants, 24.11.2015, <http://shodhganga.inflibnet.ac.in/handle/10603/93386> (20.10.2018)
 70. Rashid S., Unyayar A., Mazmanci MA., McKeown SR., Banat IM, Worthington J.: A study of anti-cancer effects of *Funalia trogii* in vitro and in vivo. *Food Chem. Toxicol.* **49**, 1477–1483 (2011)
 71. Reiss R., Ihssen J., Richter M., Eichhorn E., Schilling B., Thöny-Meyer L.: Laccase versus laccase-like multi-copper oxidase: a comparative study of similar enzymes with diverse substrate spectra. *PLoS One*, **8**, e65633 (2013)
 72. Roberts S.A., Weichsel A., Grass G., Thakali K., Hazzard J.T., Tollin G., Rensing Ch., Montfort W.R.: Crystal structure and electron transfer kinetics of CueO, a multicopper oxidase

- required for copper homeostasis in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*, **99**, 2766–2771 (2002)
73. Rodríguez-Couto S., Toca-Herrera J.L.: Inhibitors of laccases: a review. *Curr. Enzym. Inhib.* **2**, 343–352 (2006)
 74. Rodríguez-Couto S.: Fungal laccase in the textile industry (in) *Fungal biomolecules: sources, applications and recent developments*, red. V.K. Gupta, R.L. Mach, S. Sreenivasaprasad, John Wiley & Sons, Ltd, Chichester, UK, 2015, p. 63–72
 75. Rodríguez-Delgado M., Orona-Navar C., García-Morales R., Hernandez-Luna C., Parra R., Mahlknecht J., Ornelas-Soto N.: Biotransformation kinetics of pharmaceutical and industrial micropollutants in groundwaters by a laccase cocktail from *Pycnoporus sanguineus* CS43 fungi. *Int. Biodeterior. Biodegradation*, **108**, 34–41 (2016)
 76. Sakurai T., Kataoka K.: Basic and applied features of multicopper oxidases, CueO, bilirubin oxidase, and laccase. *Chem. Rec.* **7**, 220–229 (2007)
 77. Sané S., Richter K., Rubenwolf S., Matschke N.J., Jolival C., Madzak C., Zengerle R., Gescher J., Kerzenmacher S.: Using planktonic microorganisms to supply the unpurified multi-copper oxidases laccase and copper efflux oxidases at a biofuel cell cathode. *Bioresour. Technol.* **158**, 231–238 (2014)
 78. Schubert M., Ihssen J.: Wood protection against microorganisms by laccase-catalysed iodination. EP2871962A1EP, PCT/CH2013/000088 2013-11-28
 79. Senthivelan T., Kanagaraj J., Panda R.C.: Recent trends in fungal laccase for various industrial applications: an eco-friendly approach – A review. *Biotechnol. Bioproc. E.* **21**, 19–38 (2016)
 80. Shimada Y., Ko S.: Ascorbic acid and ascorbic acid oxidase in vegetables. *Chugokugakuen J.* **7**, 7–10 (2008)
 81. Shiroyama K., Kawasaki Y., Unno Y., Amachi S.: A putative multicopper oxidase, IoxA, is involved in iodide oxidation by *Roseovarius* sp. strain A-2. *Biosci. Biotechnol. Biochem.* **79**, 1898–1905 (2015)
 82. Shraddha, Shekher R., Sehgal S., Kamthania M., Kumar A.: Laccase: microbial sources, production, purification, and potential biotechnological applications. *Enzyme Res.* 217861 (2011)
 83. Sirim D., Wagner F., Wang L., Schmid R.D., Pleiss J.: The laccase engineering database: a classification and analysis system for laccases and related multicopper oxidases. Database (Oxford) bar006. Published online 2011-04-15 DOI: 10.1093/database/bar006
 84. Solano F., Lucas-Elio P., López-Serrano D., Fernández E., Sanchez-Amat A.: Dimethoxyphenol oxidase activity of different microbial blue multicopper proteins. *FEMS Microbiol. Lett.* **204**, 175–181 (2001)
 85. Solomon E.I., Sundaram U.M., Machonkin T.E.: Multicopper oxidases and oxygenases. *Chem. Rev.* **96**, 2563–2605 (1996)
 86. Stoj C., Kosman D.J.: Cuprous oxidase activity of yeast Fet3p and human ceruloplasmin: implication for function. *FEBS Lett.* **554**, 422–426 (2003)
 87. Subramanian J., Ramesh T., Kalaiselvam M.: Fungal laccases – properties and applications: A Review. *Int. J. Pharm. Biol. Arch.* **2**, 8–16 (2014)
 88. Suzuki M., Eda Y., Ohsawa S., Kanesaki Y., Yoshikawa H., Tanaka K., Muramatsu Y., Yoshikawa J., Sato I., Fujii T., Amachi S.: Iodide oxidation by a novel multicopper oxidase from the *Alphaproteobacterium* Strain Q-1. *Appl. Environ. Microbiol.* **78**, 3941–3949 (2012)
 89. Tamayo-Ramos J.A., Barends S., de Lange D., de Jel A., Verhaert R., de Graaff L.: Enhanced production of *Aspergillus niger* laccase-like multicopper oxidases through mRNA optimization of the glucoamylase expression system. *Biotechnol. Bioeng.* **2**, 543–551 (2013)
 90. Tamayo-Ramos J.A., van Berkel W.J.H., de Graaff L.: Biocatalytic potential of laccase-like multicopper oxidases from *Aspergillus niger*. *Microb. Cell Fact.* **11**, 165–176 (2012)
 91. Tasca F., Farias D., Castro C., Acuna-Rougier C., Antiochia R.: Bilirubin oxidase from *Myrothecium verrucaria* physically absorbed on graphite electrodes. Insights into the alternative resting form and the sources of activity loss. *PLoS One*, **10**, e0132181 (2015)
 92. Unyayar A., Demirbilek M., Turkoglu M., Celik A., Mazmanci M.A., Erkurt E.A., Unyayar S., Cekic O., Atacag H.: Evaluation of cytotoxic and mutagenic effects of *Coriolus versicolor* and *Funalia trogii* extracts on mammalian cells. *Drug. Chem. Toxicol.* **29**, 69–83 (2006)
 93. Upadhyay P., Shrivastava R., Agrawal P.K.: Bioprospecting and biotechnological applications of fungal laccase. *3 Biotech*, **6**, 1–12 (2016)
 94. Viswanath B., Rajesh B., Janardhan A., Kumar A.P., Narasimha G.: Fungal laccases and their applications in bioremediation. *Enzyme Res.* 163242 (2014)
 95. Xie N., Ruprich-Robert G., Silar P., Herbert E., Ferrari R., Chapeland-Leclerc F.: Characterization of three multicopper oxidases in the filamentous fungus *Podospora anserina*: A new role of an ABR1-like protein in fungal development? *Fungal Genet. Biol.* **116**, 1–13 (2018)
 96. Xu F., Shin W., Brown S.H., Wahleithner J.A., Sundaram U.M., Solomon E.I.: A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. *Biochim. Biophys. Acta*, **1292**, 303–311 (1996)
 97. Xu L.J., Wang H.X., Ng T.B.: A Laccase with HIV-1 reverse transcriptase inhibitory activity from the broth of mycelial culture of the mushroom *Lentinus tigrinus*. *J. Biomed. Biotechnol.* 536725 (2012)
 98. Yamaguchi S.: Method for cross-linking protein by using enzyme. No US6420148B2 US Grant 2002-01-24 Inventor
 99. Yuan X., Tian G., Zhao Y., Zhao L., Wang H., Ng T.B.: Degradation of dyes using crude extract and a thermostable and pH-stable laccase isolated from *Pleurotus nebrodensis*. *Biosci. Rep.* DOI:10.1042/BSR20160163 (2016)
 100. Zheng F., Cui B.-K., Wu X.-J., Meng G., Liu H.-X., Si J.: Immobilization of laccase onto chitosan beads to enhance its capability to degrade synthetic dyes. *Int. Biodeterior. Biodegradation*, **110**, 69–78 (2016).

CORRECTION

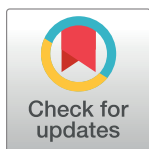
Correction: Laccase activity of the ascomycete fungus *Nectriella pironii* and innovative strategies for its production on leaf litter of an urban park

Aleksandra Góralczyk-Bińkowska, Anna Jasińska, Andrzej Długoński, Przemysław Płociński, Jerzy Długoński

There is an error in affiliation 2 for author Andrzej Długoński. The correct affiliation 2 is: Institute of Biological Sciences, Cardinal Stefan Wyszyński University, Warsaw, Warsaw, Poland.

Reference

1. Góralczyk-Bińkowska A, Jasińska A, Długoński A, Płociński P, Długoński J (2020) Laccase activity of the ascomycete fungus *Nectriella pironii* and innovative strategies for its production on leaf litter of an urban park. PLoS ONE 15(4): e0231453. <https://doi.org/10.1371/journal.pone.0231453> PMID: 32298310



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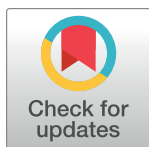
RESEARCH ARTICLE

Laccase activity of the ascomycete fungus *Nectriella pironii* and innovative strategies for its production on leaf litter of an urban park

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Abstract

A laccase-producing ascomycete fungus was isolated from soil collected around the premises of a textile dye factory and identified as *Nectriella pironii*. Efficient laccase production was achieved via the synergistic action of 1 mM copper sulfate and ferulic acid. Extracts of rapeseed oil cake, grass hay, and leaf litter collected in a pocket urban park were used for enzyme production. The highest laccase activity (3,330 U/L) was observed in the culture grown on the leaf litter extract. This is the first report on biosynthesis of laccase by *N. pironii*. This is also the first study on utilization of naturally fallen park leaves as a substrate for fungal laccase production. The extracellular enzyme possessing laccase activity was purified to homogeneity by ion-exchange and gel filtration chromatographic techniques. The amino acid sequence of the protein revealed highest similarity to the laccase enzyme produced by *Stachybotrys chartarum*—and considerable homology to those produced by other fungal species. The purified laccase possessed a molecular mass of 50 kDa. The enzyme had an optimum pH of 2.0 or 6.0 and retained more than 50% of residual activity after 3 hours of incubation at pH 3.0–10.6 or 4.0–9.0 when 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid or 2,6-dimethoxyphenol, respectively, were used. Dithiothreitol, β-mercaptoethanol, and sodium azide at 1 mM concentration strongly inhibited the laccase activity, while in the presence of 50 mM urea, the enzyme was found to retain 25% of its activity. The laccase was able to decolorize more than 80% of Indigo Carmine, Remazol Brilliant Blue R, Reactive Orange 16, and Acid Red 27 dyes within 1 h. The possibility of leaf litter use for the production of the laccase enzyme from *N. pironii* (IM 6443), exhibiting high pH stability and degradative potential, makes it a promising tool for use in different environmental and industrial operations.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Laccases (EC 1.10.3.2) are a group of copper-containing enzymes (multicopper oxidases, MCOs) commonly found in plants, bacteria, fungi, and insects. Fungal laccases are involved in sporulation, pigment production, fruiting body formation, and plant pathogenesis. These copper-containing enzymes catalyze the oxidation of a variety of phenolic and nonphenolic substrates with a simultaneous reduction of molecular oxygen to water [1, 2].

Due to their ability to oxidize a wide range of substrates without the requirement of any coenzyme factors and high efficiency, laccases have found application in many industries, medicine, environment protection, and various biotechnological processes [1, 3]. Laccases have been successfully used in delignification, paper pulping, and pretreatment of biomass for the production of biofuel [4, 5, 6]. They are also widely applied in wastewater treatment, degradation of xenobiotics, and as dye decolorizing agents. Water released from textile industries is polluted with dyes and is reported to be one of the top ten contaminating sources of water bodies. Due to the fact that traditional processes do not remove all dyes and are expensive, laccases provide a safe and efficient alternative for decolorizing and detoxification of dyes before their discharge into the environment [7, 8].

On the other hand, the absence of efficient expression system and high production costs hinder the large-scale production of laccases. Therefore, there is a need to identify new sources of laccases and find new methods of obtaining the enzyme in a rapid and economical way. Increase of enzyme biosynthesis through modification of the culture medium composition, changing the processing conditions, and supplementation with chemical inducers, such as metal ions and aromatic compounds, is a well-known solution for this problem. However, few studies indicate synergistic stimulation of laccase production by metal ions and aromatic compounds [9, 10]. Also, utilizing bio-waste to obtain an enzyme can reduce production cost while generating higher concentrations of products [11]. Till date, especially agricultural waste such as fruit peels, cereal bran, and straw or oil cakes have been successfully used for laccase production. Such residues contain polysaccharides and phenolic compounds that can stimulate both the fungal growth and subsequent laccase production [12]. Our previous study indicated rapeseed oil cake, hay, and sawdust as convenient substrates for laccase production by *Myrothecium roridum* [13]. High activity of the produced enzyme can be attributed to the presence of high content of reducing sugars and phenolic compounds, such as gallic or ferulic acid, in the media. Phenolic compounds are one of the plant secondary metabolites that play important roles in providing disease resistance, protection against pests, and species dissemination. Their presence has been reported in the leaves of trees such as *Betula pendula* [14], *Salix* sp. [15] and *Aesculus* sp. [16, 17] as well as the leaf litter of Central Europe's forests [18]. According to Chua and Hidayathulla [19], fallen senescent leaves contain more amounts of phenolic compounds compared to green leaves. Public green space including urban forests and parks form a significant portion of Central Europe cities, e.g. in Łódź [20] and Leipzig [21]. Nevertheless, the data with regard to the possibility of using naturally fallen leaves as a substrate for microbial enzymes production are scarce. Bio-waste collected from the recreational parks of urban agglomerations is mainly used for compost manufacture and rarely for energy production in biogas stations or incinerators [22]. The utilization of leaf litter (collected and gathered from urban parks during every autumn) for laccase biosynthesis could result in a more economical production process and additionally encourage self-sufficiency of the greenery.

This study was aimed to estimate possibility of leaf litter application for the newly identified fungal laccase with biodegradative potential. To the best of our knowledge, extract of leaf litter collected in an urban park during autumn season was for the first time utilized as a substrate for fungal laccase production. Subsequently, the characterization of the newly identified

enzyme has been performed. It included molecular weight determination, pH stability, and effect of inhibitors on the laccase activity. The capacity of the enzyme to decolorize the dyes was also determined.

Materials and methods

Chemicals

The compounds 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), 2,6-dimethoxyphenol (2,6-DMP), sodium azide (NaN_3), kojic acid, caffeic acid, ferulic acid, guaiacol, catechol, copper sulfate, manganese sulfate, syringaldazine, Indigo Carmine (IC), Remazol Brilliant Blue R (RBBR), Reactive Orange 16 (RO 16), and Amaranth (Acid Red 27 (AR 27)) were purchased from Sigma-Aldrich (high purity reagent grade).

Bio-waste components of fungal media

Rapeseed oil cake commonly used in pig nutrition [23] and previously utilized by Jasińska et al. [13] for laccase production by *M. roridum* IM 6482 was obtained from BIO-TECH Ltd (Gorzyn, Poland).

Commercial grass hay manufactured for rabbits and other small animals and previously used in our former study on laccase biosynthesis by *M. roridum* IM 6482 [13] was supplied by Eko-Sianko (Łask, Poland).

Naturally fallen foliage of trees (*Aesculus hippocastanum* L., *Populus simonii* "Fastigiata") and bushes (*Ribes alpinum* L.) was collected in the area of Plac Komuny Paryskiej pocket park (Łódź, Poland) with the surface area of 0.2 hectares at the end of autumn. This area is located within the internal Green Belt of Łódź (Fig 1) and is a valuable element of Łódź Green Infrastructure [22, 24, 25].

Media preparation for laccase production by fungal isolates

In this study, rapeseed press cake extract, grass hay, and leaf litter were used as substrates for the media preparation according to the protocol described by Atlas [26] with slight modifications. Before media preparation leaves have been washed with distilled water to remove soil residue and other contaminants. Rapeseed press cake extract (50 g), dry hay (20 g), dry leaf litter (25 g) were added separately to deionized water, and the final volume was made up to 1.0 L and mixed thoroughly. After boiling, solid residues have been removed *via* the filtration through 0.22 μm pore membrane filters and the extract was autoclaved at 117°C for 20 min. The pH of the extract was adjusted to 6.8 and supplemented with 2% (w/v) glucose and 1.0% (w/v) neopeptone. In the next step, 1 mM CuSO_4 was added to induce laccase production and the media were inoculated with 10% inoculum. The submerged cultures were prepared in Erlenmeyer flasks and incubated on a rotary shaker (120 rpm) at 28°C for 5 days.

Isolation of filamentous fungi from a postindustrial urban area and screening for laccase activity

Twenty-six fungal strains used in this study had been previously isolated from soil samples collected from the postindustrial textile green space of the Łódź Metropolitan Area (Poland) and stored at the strain collection center of the Department of Industrial Microbiology and Biotechnology, University of Łódź. The fungal strains were maintained on ZT slants [27] at 4°C and regularly transferred.

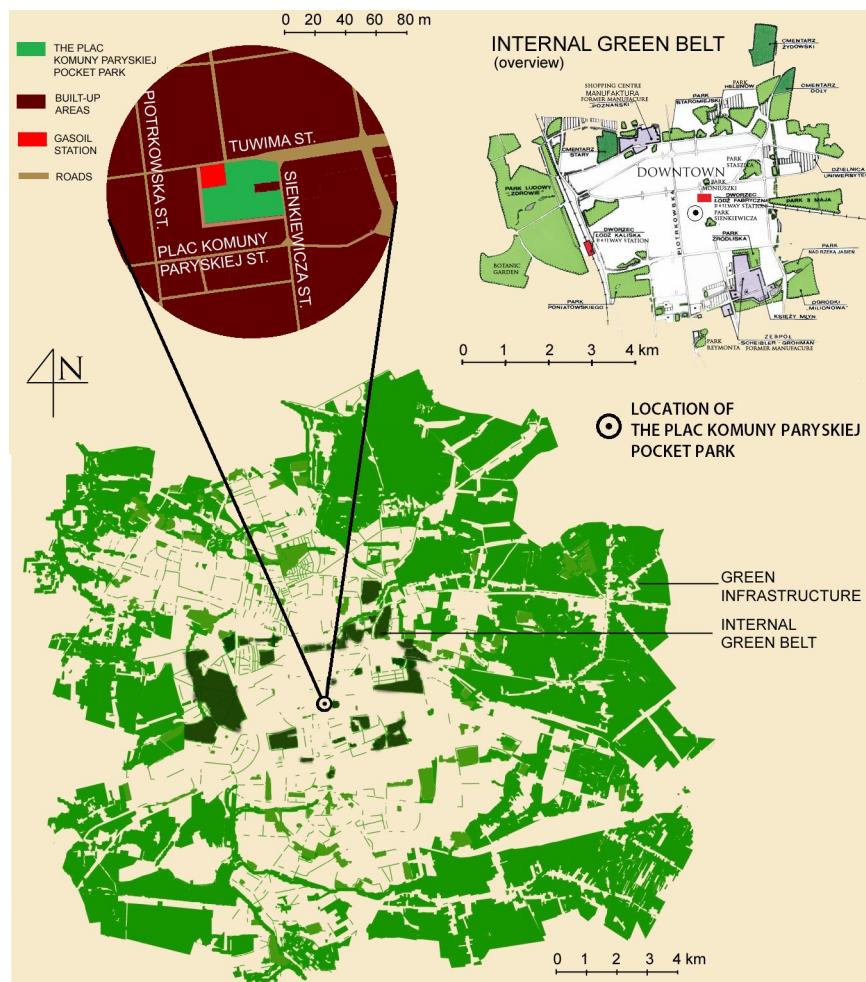


Fig 1. Distribution of the urban green spaces in Łódź.

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Qualitative analysis

Primary screening of filamentous fungi for laccase activity was carried out by inoculation of 1 cm diameter of mycelium from each strain on a Kirk and Farrell solid medium containing additionally 1 mM ABTS or 1 mM 2,6-DMP and then incubated at 28°C. The medium consisted of (g/L): glucose, 2.0; ammonium tartrate, 2.0; malt extract, 2.0; KH_2PO_4 , 0.26; Na_2HPO_4 , 0.26; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; agar, 2.0; ABTS, 0.35; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 10×10^{-3} ; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 6.6×10^{-3} ; FeSO_4 , 5×10^{-3} ; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5×10^{-3} ; Na_2MoO_4 , 0.02×10^{-3} ; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.09×10^{-3} ; and H_3BO_3 , 0.07×10^{-3} , pH 5.5 [28]. The formation of a dark green halo in the plates supplemented with ABTS and an orange halo in the plates supplemented with DMP indicated a positive laccase secretion. The diameters of the halo zones and the mycelium were measured at regular intervals of time.

Quantitative analysis

Quantitative examination of the fungal isolates that showed a positive laccase activity was carried out under submerged cultivation conditions. Inoculum (15 mL of 24-hour-old fungal culture) was introduced into 500 mL Erlenmeyer flasks containing 135 mL of modified Czapek-

Dox medium composed of (g/l): yeast extract, 3.0; KH_2PO_4 , 1.0; KCl, 0.5; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.5; $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.01; and glucose, 7.5, pH 6.8. The flasks were incubated on a rotary shaker (120 rpm) at 28°C. Sampling was done at regular intervals for determining the laccase activity and the whole experiment was carried out in triplicate.

Taxonomic identification of the IM 6443 fungal strain (DNA extraction, PCR amplification, and sequence data analysis)

Taxonomic identification was done by RDLS Heritage Ltd (Warsaw, Poland). Fungal genomic DNA was isolated using an Extract Me Genomic DNA Kit (Blirt S.A.). The gene fragment present in the 5.8S rRNA ITS region was amplified with the use of a Thermal Cycler (BioRad) using primer sets ITS1-F and ITS4-R. The PCR reaction was performed under the following conditions: initial denaturation (95°C for 3 min) and then denaturation (34 amplification cycles at 95°C for 30 s), annealing (54°C for 30 s), primer extension (72°C for 90 s), and final extension at 72°C for 10 min. Sequencing reactions were performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fischer). The obtained sequence was compared using a NCBI BLAST similarity search tool. The sequence was analyzed phylogenetically by comparing with closely related sequences of reference organisms from the BLAST network service followed by multiple sequence alignment by using the algorithm MUSCLE 3.7 [29].

Optimization of the laccase production

Fungal spores produced by 10-day-old cultures on ZT slants were used for the preparation of precultures in 25 mL of WHI medium [30] using 100 mL Erlenmeyer flasks. The incubation was conducted at 28°C on a rotary shaker (120 rpm). After 24 h, the precultures were transferred to fresh WHI medium in the ratio 1:4 and cultivated for subsequent 24 h. Next, 3 mL of the preculture was introduced into 27 mL of Sabouraud dextrose broth liquid medium (Bio-Maxima) supplemented with glucose (2%). Six different inducers (ABTS, 2,6-DMP, caffeic acid, ferulic acid, copper sulfate, and manganese sulfate) were added independently to Sabouraud fungal growth medium. The control flask was devoid of any inducer compound. The fungal isolates were incubated at 28°C for 6 days. Furthermore, the effect of combining copper ions with other inducers was evaluated. Sampling was done at regular intervals for measuring the laccase activity, and all the experiments were carried out in triplicate.

Assay for determining laccase activity and protein concentration

The laccase activity was determined by using ABTS as substrate and measuring the absorbance change caused by the action of enzyme on the substrate within 1 min using a Specord 200 spectrophotometer (Analytic Jena, Germany) and WinASPECT PLUS software [31]. Molar absorption coefficient (ϵ) for ABTS at 420 nm and for DMP at 470 nm was $36,000 \text{ M}^{-1} \text{ cm}^{-1}$ and $14,800 \text{ M}^{-1} \text{ cm}^{-1}$ [32]. The reaction mixture contained 250 μL of 10 mM ABTS or DMP, 740 μL of Mc Ilvaine Buffer (composed of 0.2 M dipotassium hydrogen phosphate and 0.1 M citric acid, buffered to pH 4.5), and 10 μL of enzyme extract (Jasińska et al. [33]). One unit of laccase activity (U) was defined as the concentration of the enzyme required to oxidize 1 μM of substrate per minute.

Enzyme activity was calculated according to the formula adopted from Leonowicz and Grzywnowicz [34] with slight modifications:

$$\text{Laccase activity [U/L]} = (\Delta\text{Abs} \times V \times 10^6) / (36,000 \times V_e \times \Delta t), \quad (1)$$

where ΔAbs is the difference in absorbance values at Abs_{60} and Abs_0 ;

V is the total volume of the sample [mL]; V_e is the volume of the enzyme [mL]; and Δt is the time [min].

The protein concentration was determined using the BCA test (bicinchoninic acid assay) according to the Pierce™ BCA Protein Assay Kit protocol (Thermo Fisher Scientific).

Protein isolation and purification

The crude laccase enzyme was precipitated from the culture media using ammonium sulfate precipitation technique. Briefly, the fungal culture was centrifuged at $8,500 \times g$ for 15 min at 4°C and the resulting supernatant was collected. Solid $(\text{NH}_4)_2\text{SO}_4$ up to 60% saturation was added slowly to the supernatant and incubated at 4°C overnight with constant stirring. The precipitated proteins were sedimented by centrifuging at $8,500 \times g$ for 15 min at 4°C , and the obtained pellet was resuspended in 100 mL of 0.05 M Tris buffer at pH 7.5. The enzyme solution was then concentrated using a 30 kDa ultrafiltration membrane (Amicon Ultra-15; Merck Millipore, USA) and purified on an FPLC System (ÄKTA Start; GE Healthcare). The buffered protein solution was first run on an ion-exchange HiTrap Q FF column (GE Healthcare) equilibrated with 0.05 M NaCl in 0.05 M Tris buffer at pH 7.5 and eluted in a linear gradient of salt at a flow rate of 2.5 mL/min. Both the wash and elution fractions were analyzed for laccase activity, as described in the previous section. Fractions with the highest laccase activity were pooled, concentrated by ultrafiltration, loaded onto a gel filtration HiPrep 16/60 Sephacryl S-200 column (GE Healthcare), and eluted at a flow rate of 1.0 mL/min. The resulting fractions containing purified laccase were pooled, concentrated, and used for further analyses.

Mass spectrometry analysis of the purified protein

Purified laccase was resolved on a native 12% Tris-glycine polyacrylamide gel. The gel was then developed as a zymogram using the ABTS solution, which was formulated exactly similar to that used for the laccase activity testing. A single protein band showing strong positive ABTS staining was excised from the gel and used for mass spectrometry analysis. The gel slice was processed by following the in-gel trypsin digestion protocol, as described in detail by Shevchenko et al. [35]. The resulting tryptic peptides were next analyzed using the ion source of the Q Exactive™ Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Electron Corp) coupled to a nano-HPLC system fitted with a RP-18 column (Waters). The mass spectrometer was operated in a data-dependent mode with a selected mass range of 300–2000 mass/charge (m/z). The raw result files were processed directly using the MaxQuant v.1.6.3.4 or PEAKS X Studio software. Initially, the fragmentation spectra were searched against a user-defined database, created by fetching the FASTA records for all proteins annotated as “multicopper oxidase” from the NCBI protein database. The secondary database search was performed against a user-defined database created by fetching 1000 proteins from NCBI BLAST, showing the best sequence similarity to the primary multicopper oxidase identified in the initial search. The PEAKS X Studio database search was performed with *de novo* identified peptide set to confirm the protein sequence similarity between the already annotated and the newly characterized proteins.

Laccase characterization

The pH-dependent activity of the purified enzyme was studied using ABTS (10 mM) at 420 nm and 2,6-DMP (10 mM) at 470 nm as substrates dissolved in the Mc Ilvaine buffer (pH 2.2–8.0) and Glycine-NaOH buffer (pH 8.6–10.6). The laccase activity after incubating the enzyme at 4°C for 3 h at varying pH values was estimated to determine the optimal pH at which the purified laccase showed maximum stability.

The inhibition effect of β -mercaptoethanol (β -ME), dithiothreitol (DTT), ethylenediamine-tetraacetic acid (EDTA), kojic acid, sodium dodecyl sulphate (SDS), NaN_3 , and urea on the purified laccase activity was studied in Mc Ilvaine buffer at pH 3.0 using 10 mM ABTS as a substrate. For control measurement, the enzyme activity was determined without any inhibitor. The inhibitors were premixed with the enzyme to acquire a final concentration from 0.1 to 500 mM and incubated for 3 h at room temperature. As far as possible, the STREND A guidelines were included in the description of the enzyme activity assays [36].

Decolorization studies

The capacity of purified laccase to decolorize IC, RBBR, RO 16, and AR 27 dyes was determined spectrophotometrically by measuring the decrease in absorbance at 611, 593, 521, and 499 nm, respectively. The reaction mixture contained 25 mg/L of dye dissolved in Mc Ilvaine buffer (pH 3.0) and 1 U/mL of laccase. The mixture was incubated at room temperature for 60 min.

The percentage of decolorization was calculated as follows:

$$D [\%] = [100 \times (A_0 - A_t/A_0)], \quad (2)$$

where A_0 is the absorbance of the abiotic control, A_t is the absorbance of culture supernatant, and D is the decolorization rate.

Statistical analysis

The obtained results were presented as the mean $\pm$ standard error of the mean (SEM) of three independent samples. Student's t -test was used to determine the statistical significance of differences between the mean values. P -value ≤ 0.05 was considered to indicate statistical significance. Statistical analyses were performed using Excel 2007 (Microsoft Corporation, USA).

Results and discussion

Screening of laccase-producing fungi

Due to their ability to oxidize both phenolic and nonphenolic compounds, laccases have been drawing the attention of researchers for the last few decades. Screening and selection of promising laccase producers from nature, followed by optimization of culture conditions, provide an effective approach to obtain organisms which have increased capacity to synthesize laccase.

In this study, 26 fungal cultures were isolated from a postindustrial textile green area and tested for laccase production on the medium containing phenolic as well as nonphenolic substrates (Table 1). Fungal cultures were checked for zone formation on agar plates containing ABTS or DMP, which are considered as standard substrates for laccase [2]. This allowed for the indication of laccase-producing fungi. Among all the tested isolates, strains marked as IM 6443, IM 6467, and IM 6482 formed a dark green halo in ABTS-supplemented plates, while 2,6-DMP oxidation expressed by the formation of an orange halo was shown by the strains IM 6443 and IM 6482. Screening of laccase-producing microorganisms on solid media containing coloured indicator compounds is known to enable quick visual detection of laccase production; however liquid cultivations are necessary for the measurement of enzyme activity [9]. The laccase activity of the tested strains was estimated over the period of 6 days at regular intervals of 24 h under submerged fermentation conditions (Fig 2). Among the isolates, the strain IM 6443 was observed to show the highest laccase activity (about 1,600 U/L on 4th day) and hence was selected for further experimental work and identification.

Table 1. Qualitative screening for laccase positive isolates using ABTS and 2,6-DMP after 4 days of cultivation.

Strain number	ABTS		2,6-DMP	
	Colony diameter [mm]	Oxidation	Colony diameter [mm]	Oxidation
6440	45	-	50	-
6443	22	+++	30	+++
6448	35	-	42	-
6449	70	-	65	-
6451	22	-	20	-
6452	30	-	30	-
6456	30	-	25	-
6457	40	-	35	+
6459	37	-	24	-
6460	37	-	30	-
6462	60	-	65	-
6463	70	-	70	-
6464	35	-	30	-
6467	45	++	40	+/-
6470	22	-	42	-
6473	58	-	40	-
6474	39	-	35	-
6480	40	-	45	-
6481	60	-	55	-
6482	20	+++	25	+++
6485	30	-	35	-
6486	27	-	30	+
6487	78	-	75	-
6488	78	-	65	-
6490	32	-	40	-
6493	20	-	20	-

Activity: +++ high; ++ medium; + low; +/- ambiguous;—none

<https://doi.org/10.1371/journal.pone.0231453.t001>

The molecular identification based on PCR amplification of the ITS r-DNA gene was carried out using the ITS1 and ITS4 primers. The phylogenetic analysis revealed that the IM 6443

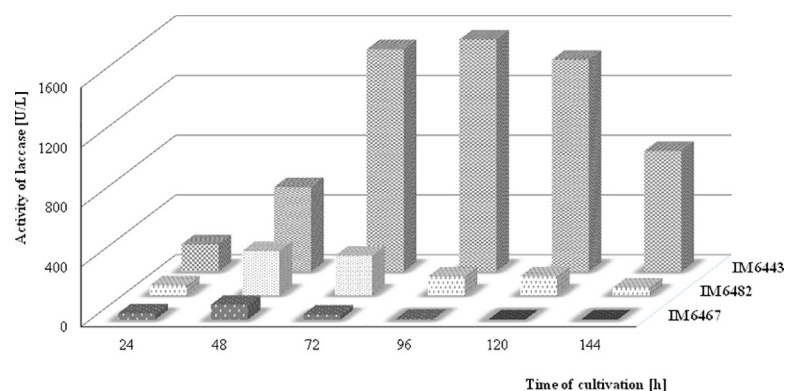


Fig 2. Time course of laccase production during IM 6443, IM 6467, and IM 6482 cultivation in modified Czapek-Dox medium containing 0.75% of glucose and 3.00% of yeast extract.

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strain showed 93% agreement with the *Nectriella pironii* strain CBS 264.80 (MH861261.1). The phylogenetic tree is presented in (S1 Fig).

Optimization of the laccase production

Large-scale applications of laccases are limited by the cost and efficiency of these enzymes [37, 38, 39]. Efforts have been made to produce large amounts of laccases at lower costs with the use of recombinant organisms or screening for naturally available hypersecretory strains. To enhance laccase production, different strategies can also be applied, such as optimization of culture conditions or use of lignocellulose materials as substrates [10, 13, 40]. Increased secretion of laccase by fungi when cultured in media supplemented with copper or different aromatic compounds has been well documented [41, 42]. In this study, the effect of ABTS, DMP, caffeic acid, ferulic acid, copper sulfate, and manganese sulfate on laccase secretion by *N. pironii* IM 6443 was determined. The induction of the enzyme in the cultures growing in Czapek-Dox medium was observed only in the presence of copper ions (data not shown), so further study was performed using Sabouraud medium. All the tested inducers increased the production of the enzyme (Fig 3A). Among them, the highest laccase induction was observed in the cultures enriched with copper sulfate and ferulic acid. After cultivating *N. pironii* IM 6443 in media containing these additives for 96 and 72 h, the enzyme activity reached 1,412 and 1,405 U/L, respectively, and was almost five fold higher than in the control sample cultivated in Sabuoraud medium. Copper has been reported to be a strong inducer of laccase in many fungal species, including *Aspergillus flavus* [43], *M. roridum* [44], and *Peniophora* sp. [45]. Copper not only regulates laccase gene expression but also positively affects the activity and stability of the enzyme by exerting an inhibitory effect on the activity of extracellular proteases [46]. On the other hand, the increased production of laccase in the presence of phenolic compounds may be a defensive reaction of the microorganism to oxidative stress, since these compounds are known to induce stress [47]. The surface of fungal hyphae contains specific receptors for small phenolic compounds that stimulate *de novo* synthesis of laccase [46]. Interestingly, in the present study, the combined supplementation of *N. pironii* IM 6443 cultures with copper sulfate and ferulic acid led to a significant increase in the production of laccase as compared to the cultures containing these compounds separately (Fig 3B). Activity of laccase determined after 120 hours of cultivation of the fungus in the media containing both mentioned inducers reached 3,347 U/L. The effect of the simultaneous use of copper and an aromatic compound on the laccase gene transcription was evaluated by Yang et al. [48]. They proved that copper ions and syringic acid exhibit a positive synergistic effect on the laccase production and laccase gene transcription in *Trametes velutina*. However, this is the first attempt involving simultaneous application of copper and ferulic acid for increasing laccase production.

Phenolic compounds are natural products of lignin degradation, thus lignocellulosic wastes can be utilized for the enhancement of low-cost laccase production [49, 50]. So far, various lignocellulosic materials, mainly constituting wastes of arable crops, have been used for the production of fungal laccases. For example, lavender straw [51], wheat bran [52], orange peel [53], and sesame oil cake [54] have been utilized as substrates for laccase production by *Pycnoporus cinnabarinus*, *Cyathus bulleri*, *Trametes polyzona*, and *Pleurotus* sp., respectively. In the presented study, extracts of grass hay, rapeseed oil cake, and litter of leaves collected in an urban park in autumn were used to prepare an alternative medium for laccase production. Grass hay and rapeseed oil cake have been previously applied for laccase production by *M. roridum* IM 6482 [13], while there have been no studies on the use of naturally fallen leaves as a substrate for fungal laccase production. However, under natural conditions, the decomposition of plant litter is associated with the secretion of extracellular enzymes like laccases whose concentration

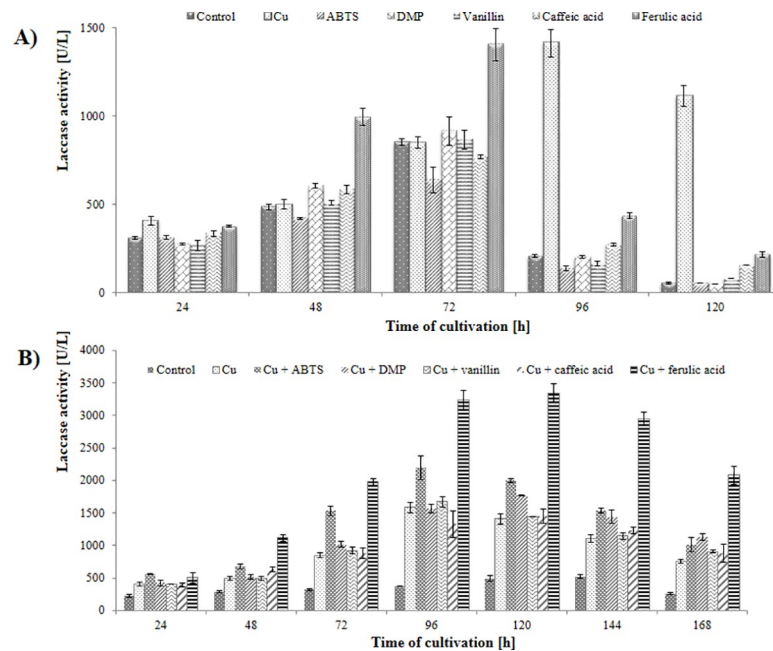


Fig 3. Laccase production by *Nectriella pironii* IM 6443 during the cultivation in Sabouraud medium supplemented with inducers (A) or their combination (B).

<https://doi.org/10.1371/journal.pone.0231453.g003>

and activity may change depending on the climate, litter quality, and the decomposer communities [18]. This finding suggests that litter of leaves might serve as a good substrate for enzyme production. According to the results presented in Fig 4, the nature of waste material used to prepare the medium strongly influenced the laccase production by *N. pironii* IM 6443. The highest activity of the enzyme was obtained after 96 hours of cultivation in growth medium based on the leaf litter. Laccase activity was found to be 3,330 U/L and was similar to those obtained in media containing copper ions and ferulic acid. To the best of our knowledge, this is the first study to report on laccase production from a *N. pironii* strain grown on the extract obtained from leaf litter.

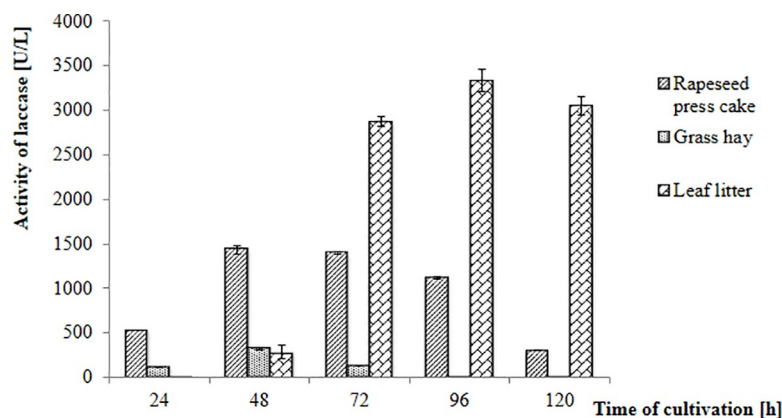


Fig 4. Laccase production by the *Nectriella pironii* IM 6443 strain during the culture in media containing bio-waste material.

<https://doi.org/10.1371/journal.pone.0231453.g004>



Fig 5. Mass spectrometry identification of the laccase preparations purified from *Nectriella pironii*.

<https://doi.org/10.1371/journal.pone.0231453.g005>

The presented results are promising as they not only indicate an economical method of obtaining laccase, but also demonstrate how to manage the previously unnecessary and decayed bio-waste. Considering the production scale of waste leaves, the possibility of using them in the process seems to be profitable. The studies of Hereźniak [55] and Długoński and Szumański [25] revealed that an estimated 0.8 Mg of leaf bio-waste (fresh matter) can be obtained from one adult urban tree on a yearly basis. By averaging these statistical data, it can be evaluated that about 5.4 Mg of wet leaf litter can be provided annually by adult trees of *A. hippocastanum* L. and *P. simonii* “Fastigiata” growing in the studied area (the Plac Komuny Paryskiej pocket park).

Identification of the protein with the laccase activity by mass spectrometry

The extracellular laccase produced on leaf extract medium by *N. pironii* was purified using a multistep purification strategy that included ammonium sulfate precipitation, ultrafiltration, ion-exchange chromatography on the HiTrap Q FF column, and a gel filtration step on the HiPrep 16/60 Sephacryl S-200 column. The purity and enzymatic activity of the obtained laccase enzyme was confirmed by the presence of a single band on a zymogram stained with ABTS solution and its molecular weight was found to be around 50 kDa, which is consistent with the typical weight of laccases obtained from white-rot fungi [56].

The purified protein possessing the laccase activity was visualized on an in-gel zymogram using ABTS as a substrate, and the ABTS-positive protein band was subsequently subjected to mass spectrometry identification (Fig 5). Searching the MS/MS fragmentation spectra against the initial “multicopper oxidase” database resulted in the identification of XP_018036634.1 multicopper oxidase from *Paraphaeosphaeria sporulosa* as the initial best hit. Since *P. sporulosa* is not closely related to *N. pironii*, the search was repeated against a secondary database containing FASTA records of proteins showing a significant similarity to the initial identification, but not necessarily annotated as multicopper oxidases. The results suggest that the laccase characterized in the current study belongs to a conserved family of laccases related to Laccase-2 from *Fusarium oxysporum* and Laccase-2 from *Colletotrichum trifolii* (S1 File). Several peptides were matched to the KFA51000.1 protein, a 61,845 Da hypothetical protein from *S. charitatum* IBT40293, by using both MaxQuant and PEAKS X Studio software suites. MaxQuant reported 19 unique peptides, whereas PEAKS X Studio reported 55 unique peptides matching the KFA51000.1 protein, with 16.1% and 62% protein sequence coverage, respectively. Importantly, all the regions of protein sequence match covered by MaxQuant were also covered by PEAKS. The KFA51000.1 protein, though being identified as hypothetical, shows high sequence conservation and belongs to the same protein family as the above-mentioned laccases (S1 File). The final identification of the *N. pironii* laccase as a protein most closely related to

KFA51000.1 is also one of the best possible matches with species phylogeny, as species of *Stachybotrys* are amongst the closest cousins of *Nectriella*.

Laccase characterization

The effect of the pH on the activity and stability of enzyme is presented in Fig 6. The optimum pH of the *N. pironii* IM 6443 laccase depends on the substrate employed. The optimum pH was observed in Mc Ilvaine buffer with pH 2.0 or 6.0 when 10 mM ABTS or DMP were, respectively, used as a substrate. Most fungal laccases are active at acidic pH values and lose activity under alkaline conditions [3]. The differences in pH optima between ABTS and DMP reflect the differences in the oxidation mechanism in various substrates [57]. In general, the catalytic activity of laccase shows a bell-shaped pH profile for the majority of substrates. The pH stability of *N. pironii* IM 6443 laccase was determined, and it retained almost 100% activity after 3 h at pH 6.0–8.0 for both the tested substrates. Moreover, it showed more than 50% residual activity after incubation at pH 4.0–9.0 or 3.0–10.6 when DMP or ABTS, respectively, were used. Zhao et al. [58] reported 40% activity of *M. verrucaria* NF-05 laccase at pH range 3–7 after 1 h of incubation. The stability in a wide pH range is a beneficial feature in different industrial and biotechnological applications [40]. The pH of industrial wastewater, depending on the process, can differ dramatically, thus supporting wastewater treatment opportunities for the *N. pironii* IM 6443 laccase enzyme.

The enzymatic activity of the *N. pironii* laccase was found to be completely inactivated at 0.1 mM concentration of sodium azide, DTT, and β -ME (Table 2). Similar results were obtained by Mukhopadhyay and Banerjee [59], who reported a total inhibition of *Lentinus squarrosulus* laccase by 0.1 mM DTT. Xu et al. [57] demonstrated that 12.5 mM concentration of β -ME caused total inhibition due to the reduction of disulfide bonds. The inhibitory effect rose with increasing concentrations of all the studied compounds. Kojic acid at 10 mM concentration caused 99% laccase inhibition, while the presence of urea did not show a significant inhibitory effect at concentrations below 100 mM.

Decolorization of synthetic dyes

The decolorization potential of laccase purified from *N. pironii* IM 6443 was evaluated for four dyes belonging to indigoid, anthraquinone, and azo classes. IC, RBBR, RO 16, and AR 27 are

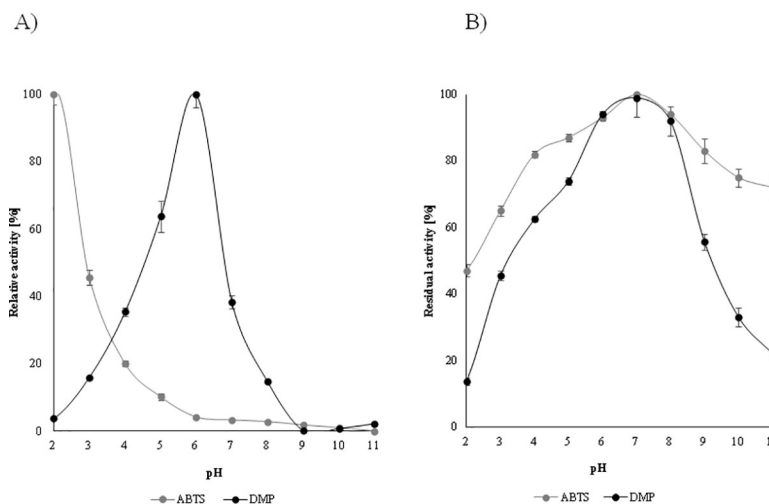


Fig 6. Effect of pH on the activity (A) and stability (B) of the purified laccase with ABTS and 2,6-DMP as substrates.

<https://doi.org/10.1371/journal.pone.0231453.g006>

Table 2. Effect of inhibitors on laccase activity.

Inhibitor	Concentration [mM]	Inhibition [%]
SDS	0.1	89.25 ± 0.12
	1	99.82 ± 0.08
	5	99.93 ± 0.11
Urea	50	76.41 ± 0.19
	100	89.14 ± 0.21
	500	94.75 ± 2.56
EDTA	1	95.60 ± 1.43
	100	99.99 ± 3.71
NaN ₃	0.1	100.00 ± 7.98
DTT	0.1	100.00 ± 5.54
β—mercaptoethanol	0.1	99.90 ± 0.17
Kojic acid	1	97.47 ± 0.52
	5	98.33 ± 0.78
	10	99.00 ± 0.09

<https://doi.org/10.1371/journal.pone.0231453.t002>

widely used as coloring agents in textile, leather, paper, and phenol-formaldehyde resin industries as well as food additives. However, there are increasing numbers of studies reporting about adverse effects of these compounds on the health of animals and humans. For example, since 1976, AR 27 has been banned in the United States by the Food and Drug Administration (FDA) as a suspected carcinogen. Nevertheless, its use is still legal in some countries. Till date, at industrial scale mainly physical and chemical methods have been applied for the removal of IC, RBBR, RO16, and AR 27 from wastewater [60]. However due to their shortcomings, biodegradation of synthetic dyes by different microbes has recently become an area of major scientific interest [61]. In the present study, 1 U/mL of the enzyme was incubated with 25 mg/L of the dye for 60 min. As shown in Fig 7, 69–92% of the dye was decolorized within 15 minutes of incubation with *N. pironii* IM 6443 laccase enzyme. Extending the incubation time to 1 hour slightly increased the level of dye decolorization. Similarly, Shobana and Thangam [62] obtained almost complete elimination of RO16 in a culture of *Nocardiopsis alba* isolated from acclimated sludge released from a textile wastewater treatment plant. On the other hand, laccase from the white-rot fungus *Marasmius scorodoni* was found to decolorize 48% and 61% of RO16 and RBBR, respectively, only in the presence of 1-hydroxybenzotriazole as a redox mediator [63]. The potential of bacteria and white rot-fungi has been applied for IC, RBBR,

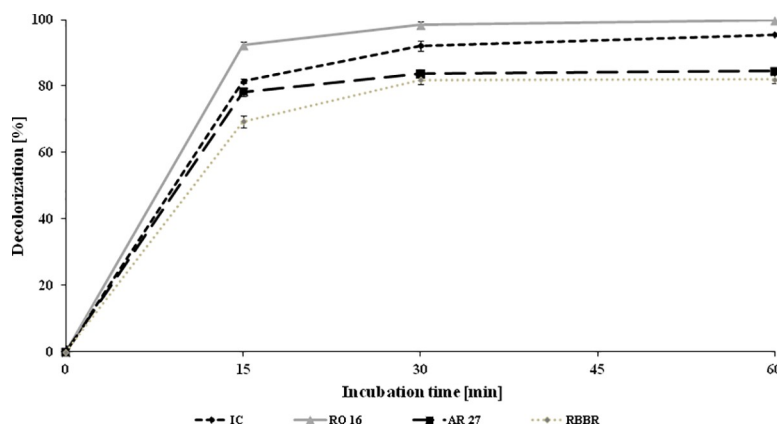


Fig 7. Decolorization of IC, RBBR, RO 16, and AR 27 (25 mg/L) by purified laccase (1 U/mL) at pH 3.0.

<https://doi.org/10.1371/journal.pone.0231453.g007>

RO 16, and AR 27 decolorization, while ascomycetous fungi and their laccases have not yet been sufficiently explored in this aspect.

Conclusion

This is the first study to report about the potential use of an ascomycetous fungus *N. pironii* IM 6443, isolated from a postindustrial textile green area, as a profitable laccase producer by utilizing extract of leaf litter as substrate. Efficient laccase production was achieved *via* the synergistic action of 1 mM copper sulfate and ferulic acid as well as through an environmentally friendly approach allowing for the management of decayed bio-waste of urban park leaves. After purification, the extracellular enzyme was identified *via* mass spectrometry as a laccase with the highest amino acid sequence similarity to *S. chartarum* laccase. The purified laccase exhibited high stability in a wide range of pH values and was found to effectively decolorize the dyes, which makes it a propitious implement for use in different environmental and industrial applications.

Supporting information

S1 Fig. The phylogenetic tree of *Nectriella pironii* IM 6443.
(TIF)

S1 File. Peptide identification details for MS experiments.
(XLSX)

S1 Raw image.
(TIF)

Acknowledgments

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References

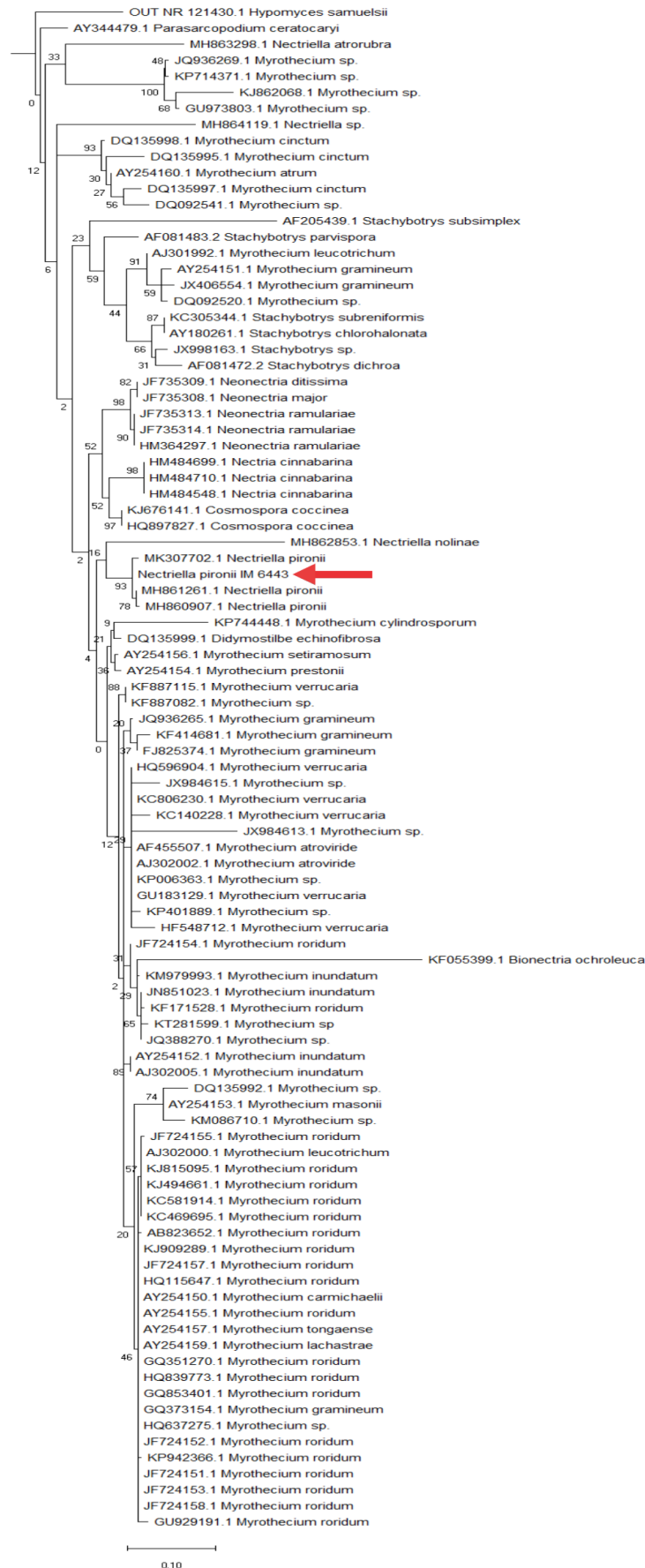
1. Hoegger PJ, Kilaru S, James TY, Thacker JR, Kües U. Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. *FEBS J.* 2006; 273: 2308–2326. <https://doi.org/10.1111/j.1742-4658.2006.05247.x> PMID: 16650005
2. Góralczyk-Bińkowska A, Jasińska A, Długoński J. Characteristics and use of multicopper oxidases enzymes. *Advancements of Microbiol.* 2019; 58: 7–18. <https://doi.org/10.21307/PM-2019.58.1.007>
3. Chaurasia PK, Bharati SL, Singh SK. Comparative studies on the blue and yellow laccases. *Res Pl Stud.* 2013; 1: 32–37. <https://doi.org/10.12691/plant-1-2-5>
4. Gygli G, van Berkel WJH. Oxizymes for biotechnology. *Curr Biotechnol.* 2015; 4: 100–110. <https://doi.org/10.2174/2211550104666150423202036>
5. Janusz G, Pawlik A, Sulej J, Swiderska-Burek U, Jarosz-Wilkolazka A, Paszczynski A. Lignin degradation: microorganisms, enzymes involved, genomes analysis and evolution. *FEMS Microbiol Rev.* 2017; 41: 941–962. <https://doi.org/10.1093/femsre/fux049> PMID: 29088355
6. Kaczmarek MB, Kwiatos N, Szczesna-Antczak M, Bielecki S. Laccases—enzymes with an unlimited potential. *Biotechnol. Food Sci.* 2017; 81: 41–70.
7. Jasińska A, Góralczyk A, Długoński J. Dye decolorization and degradation by microorganisms, in: Długoński J., (Ed.), *Microbial biodegradation, From omics to function and application*. Caister Academic Press, UK, Norfolk, pp. 119–142. 2016.
8. Ashrafi SD, Rezaei S, Forootanfar H, Mahvi AH, Faramarzi MA. The enzymatic decolorization and detoxification of synthetic dyes by the laccase from a soil-isolated ascomycete, *Paraconiothyrium variabile*. *Int Biodeter Biodegr.* 2013; 85: 173–181. <https://doi.org/10.1016/j.ibiod.2013.07.006>
9. Birhanli E, Yeşilada Ö. The effect of various inducers and their combinations with copper on laccase production of *Trametes versicolor* pellets in a repeated-batch process. *Turk J Biol.* 2017; 41: 587–599. <https://doi.org/10.3906/biy-1608-44>
10. Zhu Ch, Bao G, Huang S. Optimization of laccase production in the white-rot fungus *Pleurotus ostreatus* (ACCC 52857) induced through yeast extract and copper. *Biotechnol Biotec Equip.* 2016; 30: 270–276. <https://doi.org/10.1080/13102818.2015.1135081>
11. Panda SK, Mishra SS, Kayitesi E, Ray RC. Microbial-processing of fruit and vegetable wastes for production of vital enzymes and organic acids: Biotechnology and scopes. *Environ Res.* 2016; 146: 161–172. <https://doi.org/10.1016/j.envres.2015.12.035> PMID: 26761593
12. Ravindran R, Hassan SS, Williams GA, Jaiswal AK. A review on bioconversion of agro-industrial wastes to industrially important enzymes. *Bioengineering*, 2018; 5: 93–113. <https://doi.org/10.3390/bioengineering5040093>
13. Jasińska A, Góralczyk-Bińkowska A, Soboń A, Długoński J. Lignocellulose resources for the *Myrothecium roridum* laccase production and their integrated application for dyes removal. *Int J Environ Sci Te.* 2019; 16: 4811–4822. <https://doi.org/10.1007/s13762-019-02290-x>
14. Liimatainen J, Karonen M, Sinkkonen J, Helander M, Salminen JP. Phenolic compounds of the inner bark of *Betula pendula*: seasonal and genetic variation and induction by wounding. *J Chem Ecol.* 2012; 11: 1410–1418. <https://doi.org/10.1007/s10886-012-0199-2>
15. Gąsecka M, Mleczeck M, Jutrzenka A, Goliński P, Stuper-Szablewska K. Phenolic compounds in leaves of *Salix* species and hybrids growing under different soil conditions. *Chem Ecol.* 2017; 33: 196–212. <https://doi.org/10.1080/02757540.2017.1289186>
16. Dudek-Makuch M, Matławska I. Flavonoids from the flowers of *Aesculus hippocastanum*. *Acta Pol Pharm.* 2011; 68: 403–408. PMID: 21648195
17. Oszmiański J, Kalisz S, Aneta W. The content of phenolic compounds in leaf tissues of white (*Aesculus hippocastanum* L.) and red horse chestnut (*Aesculus carea* H.) colonized by the horse chestnut leaf miner (*Cameraria ohridella* Deschka & Dimić). *Molecules*, 2014; 19: 14625–14636. <https://doi.org/10.3390/molecules190914625> PMID: 25225723
18. Purahong W, Kapturska D, Pecyna MJ, Schulz E, Schlöter M, Buscot F. Influence of different forest system management practices on leaf litter decomposition rates, nutrient dynamics and the activity of lignolytic enzymes: a case study from Central European forests. *PLOS ONE*, 2014; 9: e93700. <https://doi.org/10.1371/journal.pone.0093700> PMID: 24699676
19. Chua LS, Hidayathulla S. Phytochemical profile of fresh and senescent leaves due to storage for *Ficus deltoidea*. *PI Biosystems.* 2017; 151: 74–83. <https://doi.org/10.1080/11263504.2015.1076085>
20. Feltynowski M, Kronenberg J, Bergier T, Kabisch N, Łaszkiewicz E, Strohbach MW. Challenges of urban green space management in the face of using inadequate data. *Urban For Urban Gree.* 2018; 31: 56–66. <https://doi.org/10.1016/j.ufug.2017.12.003>

21. Haase D, Jänicke C, Wellmann T. Front and back yard green analysis with subpixel vegetation fractions from earth observation data in a city. *Landsc Urban Plan.* 2019; 182: 44–54. <https://doi.org/10.1016/j.landurbplan.2018.10.010>
22. Długoński A, Szumański M. Use of recreational park bio-waste as locally available energy resource. *Ecol Chem Eng A.* 2016b; 3: 265–274. [https://doi.org/10.2428/ecea.2016.23\(3\)20](https://doi.org/10.2428/ecea.2016.23(3)20)
23. Kaczmarek P, Korniewicz D, Lipiński K, Mazur M. Chemical composition of rapeseed products and their use in pig nutrition. *Pol J Natur Sc.* 2016; 31: 545–562.
24. Długoński A, Szumański M. Analysis of green infrastructure in Lodz, Poland. *J. Urban Plan. Dev.* 2015; 141: A5014001–A5014009.
25. Długoński A, Szumański M. Eco-urban atlas of green infrastructure of Łódź city, Poland. Vol. Ia. Green areas in Łódź city. Parks in urban zone. Łódzkie Towarzystwo Naukowe [Lodz Scientific Society], Łódź. (in Polish with English abstract). 2016a.
26. Atlas RM. Handbook of microbiological media, 4th ed. ASM Press: Washington; 2010.
27. Nykiel-Szymańska J, Bernat P, Słaba M. Potential of *Trichoderma koningii* to eliminate alachlor in the presence of copper ions. *Ecotox Environ Safe.* 2018; 162: 1–9. <https://doi.org/10.1016/j.ecoenv.2018.06.060>
28. Saparrat MCN, Martínez MJ, Tournier HA, Cabello MN, Arambarri AM. Production of ligninolytic enzymes by *Fusarium solani* strains isolated from different substrata. *World J Microbiol Biotechnol.* 2000; 16: 799–803. <https://doi.org/10.1023/A:1008923804859>
29. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 2004; 32: 1792–1797. <https://doi.org/10.1093/nar/gkh340> PMID: 15034147
30. Paraszewicz K, Bernat P, Długoński J. Effect of nickel, copper, and zinc on emulsifier production and saturation of cellular fatty acids in the filamentous fungus *Curvularia lunata*. *Int. Biodeter. Biodegr.* 2009; 63: 100–105. <https://doi.org/10.1016/j.ibiod.2008.03.015>
31. Eggert C, Temp U, Dean JFD, Eriksson KEL. A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS Lett.* 1996; 391: 144–148. [https://doi.org/10.1016/0014-5793\(96\)00719-3](https://doi.org/10.1016/0014-5793(96)00719-3) PMID: 8706903
32. Sondhi S, Sharma P, Saini S, Puri N, Gupta N. Purification and characterization of an extracellular, thermo-alkali-stable, metal tolerant laccase from *Bacillus tequilensis* SN4 9. *PLOS ONE*, 2014; 9, e96951. <https://doi.org/10.1371/journal.pone.0096951> PMID: 24871763
33. Jasińska A, Różalska S, Bernat P, Paraszewicz K, Długoński J. Malachite green decolorization by non-basidiomycete filamentous fungi of *Penicillium pinophilum* and *Myrothecium roridum*. *Int. Biodeter. Biodegr.* 2012; 73: 33–40. <https://doi.org/10.1016/j.ibiod.2012.06.025>
34. Leonowicz A, Grzywnowicz K. Quantitative estimation of laccase forms in some white-rot fungi using syringaldazine as a substrate. *Enzyme Microb. Technol.* 1981; 3: 55–58. [https://doi.org/10.1016/0141-0229\(81\)90036-3](https://doi.org/10.1016/0141-0229(81)90036-3)
35. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc.* 2007; 1: 2856–2860. <https://doi.org/10.1038/nprot.2006.468>
36. <https://www.beilstein-institut.de/en/projects/strenda/guidelines>
37. Osma JF, Toca-Herrera JL, Rodríguez-Couto S. Uses of laccases in the food industry. *Enzyme Res.* 2010; 918761. <https://doi.org/10.4061/2010/918761>
38. Singh G, Kaur K, Puri S, Sharma P. Critical factors affecting laccase-mediated biobleaching of pulp in paper industry. *Appl Microbiol Biotechnol.* 2015; 99: 155–164. <https://doi.org/10.1007/s00253-014-6219-0> PMID: 25421562
39. Strong PJ, Claus H. Laccase: a review of its past and its future in bioremediation. *Crit Rev Environ Sci Technol.* 2011; 41: 373–434. <https://doi.org/10.1080/10643380902945706>
40. Fu K, Fu S, Zhan H, Zhou P, Liu M, Liu H. A newly isolated wood-rot fungus for laccase production in submerged cultures. *BioResources*, 2013; 8: 1385–1397. <https://doi.org/10.15376/biores.8.1.1385-1397>
41. Kuhar F, Papinutti L. Optimization of laccase production by two strains of *Ganoderma lucidum* using phenolic and metallic inducers. *Rev Argent Microbiol.* 2014; 46: 144–149. [https://doi.org/10.1016/S0325-7541\(14\)70063-X](https://doi.org/10.1016/S0325-7541(14)70063-X) PMID: 25011599
42. Piscitelli A, Giardina P, Lettera V, Pezzella C, Sannia G, Faraco V. Induction and transcriptional regulation of laccases in fungi. *Curr Genomics.* 2011; 12: 104–112. <https://doi.org/10.2174/138920211795564331> PMID: 21966248

43. Gomaa OM, Momtaz OA. Copper induction and differential expression of laccase in *Aspergillus flavus*. Braz J Microbiol. 2015; 46: 285–292. <https://doi.org/10.1590/S1517-838246120120118> PMID: 26221119
44. Jasińska A, Góralczyk A, Soboń A, Długoński J. Novel laccase-like multicopper oxidases from the *Myrothecium roridum* fungus-production enhancement, identification and application in the dye removal process. Acta Biochim Pol. 2018; 65: 287–295. https://doi.org/10.18388/abp.2017_2546 PMID: 29694448
45. Fonseca MI, Shimizu E, Zapata PD, Villalba L. Copper inducing effect on laccase production of white rot fungi native from Misiones (Argentina). Enzyme Microb Technol. 2010; 46: 534–539. <https://doi.org/10.1016/j.enzmictec.2009.12.017> PMID: 25919631
46. Haars A, Hüttermann A. Laccase induction in the white-rot fungus *Heterobasidion annosum* (Fr.) Bref. (*Fomes annosus* Fr. Cooke). Arch. Microbiol. 1983; 134: 309–313. <https://doi.org/10.1007/BF00407808>
47. Vrsanska M, Voberkova S, Langer V, Palovcikova D, Moulick A, Adam V et al. Induction of laccase, lignin peroxidase and manganese peroxidase activities in white-rot fungi using copper complexes. Molecules. 2016; 21: 1553–1564. <https://doi.org/10.3390/molecules21111553>
48. Yang Y, Wei F, Zhuo R, Fan F, Liu H, Zhang C et al. Enhancing the laccase production and laccase gene expression in the white-rot fungus *Trametes velutina* 5930 with great potential for biotechnological applications by different metal ions and aromatic compounds. PLOS ONE, 2013; 8: e79307. <https://doi.org/10.1371/journal.pone.0079307> PMID: 24244475
49. Elisashvili V, Kachlishvili E. Physiological regulation of laccase and manganese peroxidase production by white-rot *Basidiomycetes*. J Biotechnol. 2009; 144: 37–42. <https://doi.org/10.1016/j.jbiotec.2009.06.020> PMID: 19559737
50. Postemsky PD, Bidegain MA, Gonzalez-Matute R, Figlas ND, Cubitto MA. Pilot-scale bioconversion of rice and sunflower agro-residues into medicinal mushrooms and laccase enzymes through solid-state fermentation with *Ganoderma lucidum*. Bioresour Technol. 2017; 231: 85–93. <https://doi.org/10.1016/j.biortech.2017.01.064> PMID: 28199921
51. Lesage-Meessen L, Bou M, Ginies Ch, Chevret D, Navarro D, Drula E et al. Lavender- and lavandin-distilled straws: an untapped feedstock with great potential for the production of high-added value compounds and fungal enzymes. Biotechnol Biofuels, 2018; 11: 217–230. <https://doi.org/10.1186/s13068-018-1218-5> PMID: 30083230
52. Vats A, Mishra S. Identification and evaluation of bioremediation potential of laccase isoforms produced by *Cyathus bulleri* on wheat bran. J Hazard Mater. 2018; 344: 466–479. <https://doi.org/10.1016/j.jhazmat.2017.10.043> PMID: 29096258
53. Chairin T, Nitheranont T, Watanabe A, Asada Y, Khanongnuch C, Lumyong S. Purification and characterization of the extracellular laccase produced by *Trametes polyzona* WR710-1 under solid-state fermentation. J Basic Microbiol. 2014; 54: 35–43. <https://doi.org/10.1002/jobm.201200456> PMID: 23775771
54. Radhika R, Jebapriya GR, Gnanadoss JJ. Production of cellulase and laccase using *Pleurotus* sp. under submerged and solid-state fermentation. Int J Curr Sci. 2013; 6: 7–13.
55. Hereźniak J. Mocarze czasu. Pomnikowe drzewa w świecie i na ziemi łódzkiej. [Giants of the time. Monumental trees in the world and the Łódź region]. Łódzkie Towarzystwo Naukowe [Lodz Scientific Society], Łódź. (in Polish). 2012.
56. Baldrian P. Fungal laccases occurrence and properties. FEMS Microbiol Rev. 2006; 30: 215–242. <https://doi.org/10.1111/j.1574-4976.2005.00010.x> PMID: 16472305
57. Xu Y, Lu Y, Zhang R, Wang H, Liu Q. Characterization of a novel laccase purified from the fungus *Hohenbuehelia serotina* and its decolourisation of dyes. Acta Biochim Pol. 2016; 63: 273–279. https://doi.org/10.18388/abp.2015_1091 PMID: 26495441
58. Zhao D, Zhang X, Cui D, Zhao M. Characterisation of a novel white laccase from the *Deuteromycete* fungus *Myrothecium verrucaria* NF-05 and its decolourisation of dyes. PLOS ONE, 2012; 7: e38817. <https://doi.org/10.1371/journal.pone.0038817> PMID: 22715414
59. Mukhopadhyay M, Banerjee R. Purification and biochemical characterization of a newly produced yellow laccase from *Lentinus squarrosulus* MR13. 3 Biotech. 2015; 5: 227–236. <https://doi.org/10.1007/s13205-014-0219-8> PMID: 28324287
60. Anjaneyulu Y, Sreedhara Chary N, Samuel Suman Raj D. Decolourization of Industrial Effluents—Available Methods and Emerging Technologies—A Review. Rev. Environ. Sci. Biotechnol. 2005; 4: 245–273. <https://doi.org/10.1007/s11157-005-1246-z>
61. Viswanath B, Rajesh B, Janardhan A, Kumar AP, Narasimhan G. Fungal laccases and their applications in bioremediation. Enzyme Res. 2014; 163242. <https://doi.org/10.1155/2014/163242> PMID: 24959348

62. Shobana S, Thangam EB. Biodegradation and decolorization of Reactive Orange 16 by *Nocardiopsis alba* soil isolate. J Bioremed Biodeg. 2012; 3: 6–13.
63. Sung-Jong J, Su-Jin L. Purification and characterization of the laccase involved in dye decolorization by the white-rot fungus *Marasmius scorodonius*. J Microbiol Biotechnol. 2017; 27: 1120–1127. <https://doi.org/10.4014/jmb.1701.01004> PMID: 28376610

<https://doi.org/10.1371/journal.pone.0231453.s001>



S1 File. Peptide identification details for MS experiments.

<https://doi.org/10.1371/journal.pone.0231453.s002>

Settings: semispecific trypsin digest, fixed modifications: carbamidomethylation of cysteines; variable n

KFA51000.1	hypothetical protein S40293_07285	Stachybotrys chartarum IBT 40293
Sequence	N-term cleavage window	C-term cleavage window
AACDGNTASTR	AAGASAAPSPSIERRAACDGNTASTRSEWC	IERRAACDGNTASTRSEWCDYSIDTDWV
ADQADVADNFWMR	IIDMTMGQRYDVIVTADQADVADNFWMR	VTADQADVADNFWMRAIPQSACSDNDS
AIVYYGD	PQSACSDNDSTDNKAIVYYGDSASTPETT	NDSTDNIKAIVYYGDSASTPETTRYEFTDS
ATQYGSSWYH	QCPLAVGESMTYTWKATQYGSSWYHSHFATYTWKATQYGSSWYHSHFALQAYQGIFG	
ATQYGSSWYHSH	QCPLAVGESMTYTWKATQYGSSWYHSHFATWKATQYGSSWYHSHFALQAYQGIFGGI	
ATQYGSSWYHSHF	QCPLAVGESMTYTWKATQYGSSWYHSHFAWKATQYGSSWYHSHFALQAYQGIFGGI	
ATQYGSSWYHSHFAL	QCPLAVGESMTYTWKATQYGSSWYHSHFAATQYGSSWYHSHFALQAYQGIFGGI	
AVGESMTYTWK	TVQNDGVS AVTQCPLAVGESMTYTWKATCQCPLAVGESMTYTWKATQYGSSWYHSH	
DQADVADNFWMR	IDMTMGQRYDVIVTADQADVADNFWMR	VTADQADVADNFWMRAIPQSACSDNDS
DTALLPASGYLVM	YSSSVTLNTSNPPRRDTALLPASGYLVM	AW RRDTALLPASGYLVM AWETDNPGVWLM
DTALLPASGYLVMA	YSSSVTLNTSNPPRRDTALLPASGYLVM	AW RDTALLPASGYLVM AWETDNPGVWLM
QADVADNFWMR	DMTMGQRYDVIVTADQADVADNFWMR	VTADQADVADNFWMRAIPQSACSDNDS
RDTALLPASGY	TYSSSVTLNTSNPPRRDTALLPASGYLVM	AW NPPRRDTALLPASGYLVM AWETDNPGV
RDTALLPASGYLVM	TYSSSVTLNTSNPPRRDTALLPASGYLVM	AW RRDTALLPASGYLVM AWETDNPGVWLM
TDVTVSPDGVSR	EAVDTGVTREYWLELTDVTVSPDGVSR	SAMLELTDVTVSPDGVSR SAMAVNGTIPGPTL
TSGTSYR	DDDSTQTGSR LNIAFTSGTSYRM RIVNAAV	GSRLNIAFTSGTSYRM RIVNAAVDTNWK
VADNFWMR	MGQRYDVIVTADQADVADNFWMRAIPQS	VTADQADVADNFWMRAIPQSACSDNDS
VSPDGVSR	TGVTREYWLELTDVTVSPDGVSR	SAMAVNCELELTDVTVSPDGVSR SAMAVNGTIPGPTL
VTVSPDGVSR	VDTGVTREYWLELTDVTVSPDGVSR	SAMAVLELTDVTVSPDGVSR SAMAVNGTIPGPTL

other proteins

AAVDTHWK	VSFESGTSYRMRLVNAAVDTHWKFSIDNHTYRMRLVNAAVDTHWKFSIDNHTLTVITAI
ALQFLER	WLCHCHIGWHTSEGFALQFLERSSEIANVS GWHTSEGFALQFLERSSEIANVSTSSYVSE
EGFALQFLER	PGVWLCHCHIGWHTSEGFALQFLERSSEIA GWHTSEGFALQFLERSSEIANVSTSSYVSE
FALQFLER	VWLCHCHIGWHTSEGFALQFLERSSEIANV GWHTSEGFALQFLERSSEIANVSTSSYVSE
HTSEGFALQFLER	TDNPGVWLCHCHIGWHTSEGFALQFLERSSEGWHTSEGFALQFLERSSEIANVSTSSYVSE
IGWHTSEGFALQFLER	AWETDNPGVWLCHCHIGWHTSEGFALQFLGWHTSEGFALQFLERSSEIANVSTSSYVSE
LDLSMGQR	ITADLVPIEPYETTVDLSMGQRYDIIVTA EPYETTVDLSMGQRYDIIVTADQADVAC
LVNAAVDTH	RFNVSFESGTSYRMRLVNAAVDTHWKFSID TSYRMRLVNAAVDTHWKFSIDNHTLTVIT
TSEGFALQFLER	DNPGVWLCHCHIGWHTSEGFALQFLERSSEGWHTSEGFALQFLERSSEIANVSTSSYVSE
YDIIVTADQADVADNFW	EPYETTVDLSMGQRYDIIVTADQADVADN VTADQADVADNFWMRAIPQAACSDND
SLSSQLKPR	SLFASCFQHVRAASLSSQLKPRAACSGN QHVRAASLSSQLKPRAACSGNTASTRSE
SSGSFPRANLFP	AGALAGSLASALPGLSSGSFPRANLFPRAA GLSSGSFPRANLFPRAACSGNTASTRTAM
TEVPDTGVTR	TRTAWCDYSIDTDYATEVPDTGVTRREYWLE DTDYATEVPDTGVTRREYWLELTDVTVSP
AIPQSACSDNDNSDDIK	ITADQASVADNFWLRAIPQSACSDNDNSDE PQSACSDNDNSDDIKGIVYYGDSVGTPT

modifications: oxidation and protein N-terminal acetylation

Amino acid	First amino acid	Second amino acid	Third amino acid	Fourth amino acid	Fifth amino acid	A Count	R Count	N Count	D Count
R	A	A	T	R	S	3	1	1	1
T	A	D	M	R	A	3	1	1	3
K	A	I	G	D	S	1	0	0	1
K	A	T	Y	H	S	1	0	0	0
K	A	T	S	H	F	1	0	0	0
K	A	T	H	F	A	1	0	0	0
K	A	T	A	L	Q	2	0	0	0
L	A	V	W	K	A	1	0	0	0
A	D	Q	M	R	A	2	1	1	3
R	D	T	V	M	A	2	0	0	1
R	D	T	M	A	W	3	0	0	1
D	Q	A	M	R	A	2	1	1	2
R	R	D	G	Y	L	2	1	0	1
R	R	D	V	M	A	2	1	0	1
L	T	D	S	R	S	0	1	0	2
F	T	S	Y	R	M	0	1	0	0
D	V	A	M	R	A	1	1	1	1
T	V	S	S	R	S	0	1	0	1
D	V	T	S	R	S	0	1	0	1
N	A	A	W	K	F	2	0	0	1
F	A	L	E	R	S	1	1	0	0
S	E	G	E	R	S	1	1	0	0
G	F	A	E	R	S	1	1	0	0
W	H	T	E	R	S	1	1	0	0
H	I	G	E	R	S	1	1	0	0
V	L	D	Q	R	Y	0	1	0	1
R	L	V	T	H	W	2	0	1	1
H	T	S	E	R	S	1	1	0	0
R	Y	D	M	R	A	3	1	1	4
A	S	L	P	R	A	0	1	0	0
L	S	S	P	R	A	1	2	1	0
A	T	E	T	R	E	0	1	0	1
R	A	I	I	K	G	2	0	2	4

C Count	Q Count	E Count	G Count	H Count	I Count	L Count	K Count	M Count	F Count
1	0	0	1	0	0	0	0	0	0
0	1	0	0	0	0	0	0	0	1
0	0	0	1	0	1	0	0	0	0
0	1	0	1	1	0	0	0	0	0
0	1	0	1	2	0	0	0	0	0
0	1	0	1	2	0	0	0	0	1
0	1	0	1	2	0	1	0	0	1
0	0	1	1	0	0	0	1	1	0
0	1	0	0	0	0	0	0	1	1
0	0	0	1	0	0	0	3	0	1
0	0	0	1	0	0	0	3	0	1
0	1	0	0	0	0	0	0	1	1
0	0	0	1	0	0	2	0	0	0
0	0	0	1	0	0	3	0	1	0
0	0	0	1	0	0	0	0	0	0
0	0	0	1	0	0	0	0	0	0
0	0	0	0	0	0	0	0	1	1
0	0	0	1	0	0	0	0	0	0
0	0	0	1	0	0	0	0	0	0

0	0	0	0	1	0	0	1	0	0
0	1	1	0	0	0	2	0	0	1
0	1	2	1	0	0	2	0	0	2
0	1	1	0	0	0	2	0	0	2
0	1	2	1	1	0	2	0	0	2
0	1	2	2	1	1	2	0	0	2
0	1	0	1	0	0	2	0	1	0
0	0	0	0	1	0	1	0	0	0
0	1	2	1	0	0	2	0	0	2
0	1	0	0	0	2	0	0	1	1
0	1	0	0	0	0	2	1	0	0
0	0	0	1	0	0	1	0	0	2
0	0	1	1	0	0	0	0	0	0
1	1	0	0	0	2	0	1	0	0

P Count	S Count	T Count	W Count	Y Count	V Count	U Count	O Count	Length	Missed cle	
0	1	2	0	0	0	0	0	0	11	0
0	0	0	1	0	1	0	0	0	13	0
0	0	0	0	2	1	0	0	0	7	0
0	2	1	1	2	0	0	0	0	10	0
0	3	1	1	2	0	0	0	0	12	0
0	3	1	1	2	0	0	0	0	13	0
0	3	1	1	2	0	0	0	0	15	0
0	1	2	1	1	1	0	0	0	11	0
0	0	0	1	0	1	0	0	0	12	0
1	1	1	0	1	1	0	0	0	13	0
1	1	1	0	1	1	0	0	0	14	0
0	0	0	1	0	1	0	0	0	11	0
1	1	1	0	1	0	0	0	0	11	1
1	1	1	0	1	1	0	0	0	14	1
1	2	2	0	0	3	0	0	0	12	0
0	2	2	0	1	0	0	0	0	7	0
0	0	0	1	0	1	0	0	0	8	0
1	2	0	0	0	2	0	0	0	8	0
1	2	1	0	0	3	0	0	0	10	0

0	0	1	1	0	1	0	0	8	0
0	0	0	0	0	0	0	0	7	0
0	0	0	0	0	0	0	0	10	0
0	0	0	0	0	0	0	0	8	0
0	1	1	0	0	0	0	0	13	0
0	1	1	1	0	0	0	0	16	0
0	1	0	0	0	0	0	0	8	0
0	0	1	0	0	2	0	0	9	0
0	1	1	0	0	0	0	0	12	0
0	0	1	1	1	2	0	0	19	0
1	3	0	0	0	0	0	0	9	1
2	3	0	0	0	0	0	0	13	1
1	0	3	0	0	2	0	0	10	0
1	3	0	0	0	0	0	0	17	0

Mass	Proteins	Leading ra	Start posit	End positi	Unique (Groups)	Unique (Proteins)	Charges
1122.4724	KFA51000.KFA51000.		27	37	no	no	2
1537.662	RBQ79470KFA51000.		307	319	no	no	2
799.37522	KFA51000.KFA51000.		337	343	yes	no	1
1198.5043	KFA51000.KFA51000.		153	162	no	no	2
1422.5953	KFA51000.KFA51000.		153	164	no	no	2
1569.6637	KFA51000.KFA51000.		153	165	no	no	2;3
1753.7849	KFA51000.KFA51000.		153	167	no	no	2
1271.5856	KFA51000.KFA51000.		142	152	yes	no	2
1466.6249	RBQ79470KFA51000.		308	319	no	no	2
1349.6901	KFA51000.KFA51000.		485	497	no	no	2
1420.7272	KFA51000.KFA51000.		485	498	no	no	2
1351.5979	RBQ79470KFA51000.		309	319	no	no	2
1162.5982	KFA51000.KFA51000.		484	494	no	no	2
1505.7912	KFA51000.KFA51000.		484	497	no	no	2
1231.6044	KFA51000.KFA51000.		67	78	no	no	2
770.35588	KFA51000.KFA51000.		248	254	no	no	2
1037.4753	RBQ79470KFA51000.		312	319	no	no	2
815.41373	KFA51000.KFA51000.		71	78	no	no	2
1015.5298	KFA51000.KFA51000.		69	78	no	no	2

926.46102	RBQ79470RBQ79470		262	269	no	no	2
875.4865	RBQ79470RBQ79470		524	530	no	no	2
1208.619	RBQ79470RBQ79470		521	530	no	no	2
1022.5549	RBQ79470RBQ79470		523	530	no	no	2
1533.7576	RBQ79470RBQ79470		518	530	no	no	3
1889.9424	RBQ79470RBQ79470		515	530	no	no	3
918.4593	RBQ79470RBQ79470		295	302	no	no	2
938.48214	RBQ79470RBQ79470		259	267	no	no	2
1396.6987	RBQ79470RBQ79470		519	530	no	no	2
2242.0365	RBQ79470RBQ79470		303	321	no	no	2;3
1014.5822	RTE76960.RTE76960.		20	28	yes	no	2
1434.7368	TEA20253.TEA20253.		23	35	yes	yes	2
1073.5353	TEA20253.TEA20253.		60	69	no	no	2
1848.7796	XP_01803(XP_01803)		339	355	yes	yes	2

PEP	Score	Fraction A	Fraction St	Fraction 1	Experimen	Experimen	Experimen	Experimen	Intensity
1.4881E-2	138.98	1	0	1		1			15579000
4.0715E-1	102.52	1	0	1			1		14821000
0.11592	69.39	1	0	1			1		17560000
0.089444	60.76	1	0	1			1		27842000
0.01396	71.692	1	0	1			1		48520000
4.2434E-2	113.62	1	0	2			2		3,59E+08
0.05315	63.283	1	0	1			1		46995000
9.2043E-1	113.22	1	0	2			2		47248000
0.041707	62.303	1	0	1			1		2186100
4.8159E-2	132.76	1	0	1			1		1,36E+08
0.000161585	676	1	0	1		1			0
0.11559	55.567	1	0	1			1		11417000
0.000164589	0.08	1	0	1			1		95028000
3.8295E-1	105.2	1	0	2			2		1,48E+08
5.6676E-1	107.79	1	0	1		1			1390700
1.2827E-2	145.88	1	0	1		1			18250000
4.1257E-0	119.57	1	0	1			1		23350000
0.0028796	107.9	1	0	2		2			7024600
6.8014E-0	105.52	1	0	3		2	1		23637000

0.041763	89.55	1	0	1			1		13260000
6.1274E-1	143	1	0	4	1	1	2		7,08E+08
0.002013	87.308	1	0	1			1		12278000
0.001281	110.81	1	0	2		1	1		4379100
0.035681	46.069	1	0	1			1		2868600
0.08133	45.33	1	0	1			1		2564700
0.11176	53.166	1	0	1			1		0
7.7444E-1	120.62	1	0	2		2			1,07E+08
2.4922E-3	150.61	1	0	2		1	1		20650000
1.7331E-1	228.8	1	0	25			25		3,25E+08
0.268	34.824	1	0	1			1		0
0.02452	62.463	1	0	2		1		1	1 17803000
0.019384	79.128	1	0	2		1	1		95944000
3.7645E-0	62.14	1	0	1				1	0

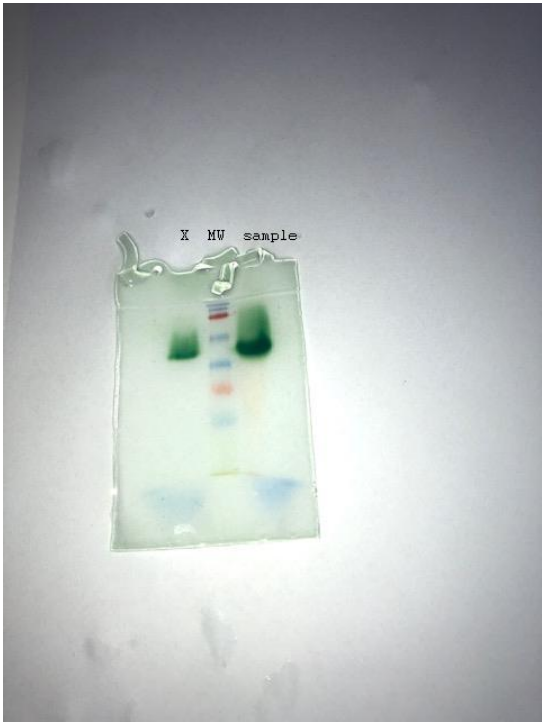
Intensity N	Intensity C	Intensity L	Intensity	Reverse	Potential	id	Protein gr	Mod. pept	Evidence I
0	15579000	0	0				0 39	0	0
0	0 14821000		0				6 39;40;41	7	10
0	0 17560000		0				17 39	18	35
0	0 27842000		0				29 39;43	30	61
0	0 48520000		0				30 39;43	31	62
0	0 3,59E+08		0				31 39;43	32 63;64	
0	0 46995000		0				32 39;43	33	65
0	0 47248000		0				33 39 34;35	66;67	
0	0 2186100		0				43 39;40;41	45	97
0	0 1,36E+08		0				45 39	47	102
0	0 0	0	0				46 39	48	103
0	0 11417000		0				241 39;40;41	254	712
0	0 95028000		0				259 39	273	780
0	0 1,48E+08		0				260 39 274;275	781;782	
0	1390700	0	0				313 39;41;42	332	955
0	18250000	0	0				330 39;43	350	1008
0	0 23350000		0				337 39;40;41	360	1036
0	7024600	0	0				351 39;41;42	374 1102;1103	
0	10927000	12710000	0				353 39;41;42	377 1109;1110	

0	0 13260000		0				2 40;41;42	2	2
8967300	2,07E+08	4,92E+08	0				23 40	24 46;47;48;4	
0	0 12278000		0				53 40	57	126
0	1086600	3292500	0				61 40	65 147;148	
0	0 2868600		0				123 40	129	295
0	0 2564700		0				127 40	133	307
0	0 0	0	0				166 40	174	436
0	1,07E+08	0	0				207 40;41;43;4	215 607;608	
0	4565600	16085000	0				329 40	349 1006;1007	
0	0 3,25E+08		0				359 40;41	383;384 1123;1124	
0	0 0	0	0				292 41	310	894
0	7665700	0 10137000					301 42	319 920;921	
0	27940000	68004000	0				316 42	335 961;962	
0	0 0	0	0				16 43	17	34

MS/MS ID:Best MS/MS Oxidation MS/MS Co			
0	0		1
13;14	14		2
45	45		1
79	79		1
80	80		1
81;82;83;8	83		4
85	85		1
86;87;88;8	89	21	5
137	137		1
142;143	142		2
144	144	22	1
909	909		1
981	981		1
982;983;9	983	22	3
1205	1205		1
1270	1270		1
1302	1302		1
1402;1403	1403		2
1409;1410	1411		3

2	2		1
60;61;62;6	61		7
167	167		1
191;192	191		2
365	365		1
379	379		1
564	564	23	1
786;787	786		2
1266;1267	1268		4
1424;1425	1434	24	29
1136	1136		1
1166;1167	1166		2
1211;1212	1211		2
44	44		1

S1 Raw image.
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Environmental and molecular approach to dye industry waste degradation by the ascomycete fungus *Nectriella pironii*

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Textile industry effluents and landfill leachate contain chemicals such as dyes, heavy metals and aromatic amines characterized by their mutagenicity, cytotoxicity and carcinogenicity. The aim of the present study was investigation of the ascomycete fungus *N. pironii* isolated from urban postindustrial textile green space for its ability to grow and retain metabolic activity in the presence of the dye industry waste. Research focused mainly on dyes, heavy metals and aromatic amines, which had been detected in landfill leachate via HPLC–MS/MS analysis. Presence of all tested compounds as well as leachate in the growth medium clearly favored the growth of fungal biomass. Only slight growth limitation was observed in the presence of 50 mg L⁻¹ *o*-toluidine. The fungus eliminated *o*-toluidine as well as dyes at all tested concentrations. The presence of metals slightly influenced the decolorization of the azo dyes; however, it was still similar to 90%. During fungal growth, *o*-toluidine was hydroxylated and/or converted to toluidine and its derivatives. Laccase and cytochrome P450 involvement in this process has been revealed. The results presented in the paper provide a valuable background for the development of a fungus-based system for the elimination of toxic pollutants generated by the textile industry.

Civilization development, extensive urbanization and progressive industrialization have contributed to a significant increase in industrial waste in recent years, including toxic pollutants of the dye industry. In 2019, the world dye and pigment market was valued at 33.2 billion USD¹. The textile industry and related synthetic dye production are also notable sectors of the Polish economy and are hallmarks of the Łódź region in Poland^{2–4}. However, it is estimated that approximately 10% of the 70 million tons of synthetic dyes produced annually worldwide are discharged into the environment in the form of process wastewater^{5–7}. Industry waste contains a number of deleterious substances and is a key problem both for the environment and for human life and health in numerous countries, including Poland^{8–11}. This applies not only to the current production but also to the industrial wastes created in the past. The threat to the environment arises especially there, where larger amounts of waste are improperly accumulated and stored, e.g., in Poland, postproduction waste landfills of the former “Boruta” Dye Industry Plant located in Zgierz near Łódź and the Textile Industry Factory “Wistom” in Tomaszów Mazowiecki (Fig. 1), which are on a list of “Waste collection places that pose a threat to human health and life”¹².

In the case of landfills for the textile industry, leachate frequently contains synthetic azo dyes, which are commonly used due to the widest scale of colours^{7,13,14} and their intermediates, e.g., potentially carcinogenic aromatic amines, which are formed by anaerobic reduction of these compounds underground. Aromatic amines are highly soluble in water and thus can easily penetrate through the soil and enter the water cycle. Next, the amino group of aromatic amines can be transformed to the reactive intermediate hydroxylamine, which can cause damage to proteins and DNA and thus has been categorized by the International Agency for Research on Cancer as a potential carcinogen¹⁵. Most important carcinogenic aromatic amines are benzidine and its derivatives, e.g., *o*-toluidine, 3,3'-dichlorobenzidine and 3,3'-dimethoxybenzidine, commonly used for azo dye production. A relationship has been shown between long-term exposure to these amines and the development of

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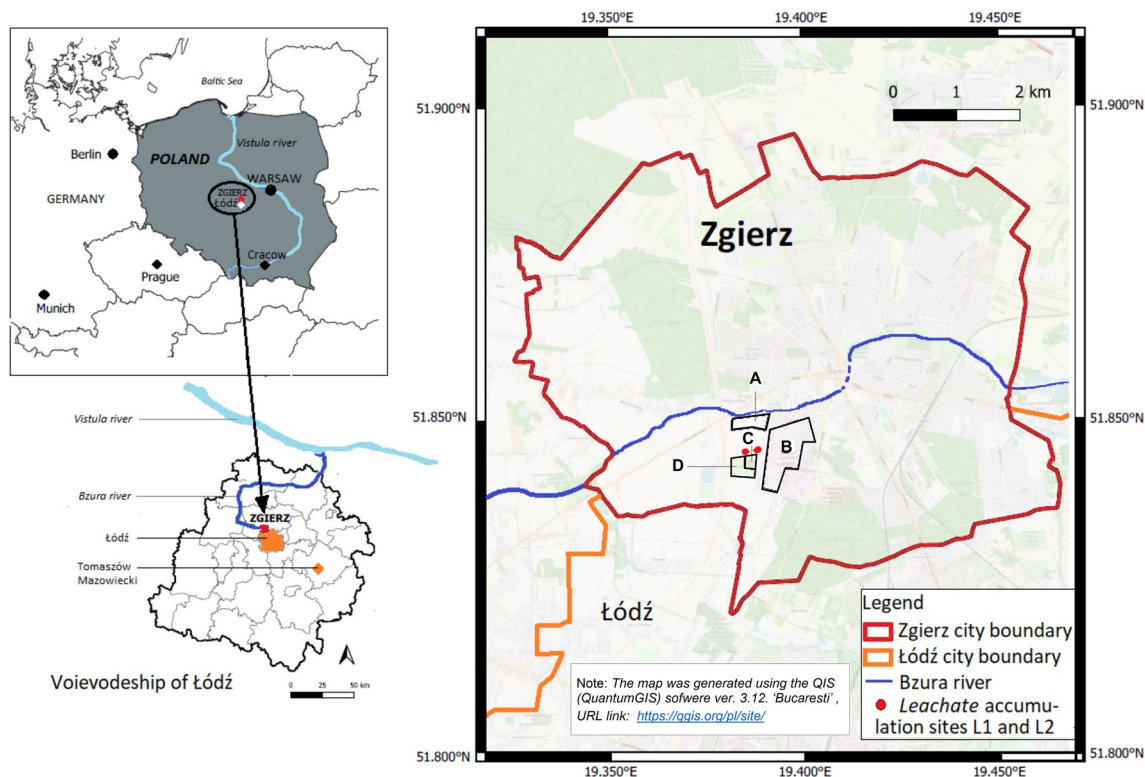


Figure 1. Location of the study region: the municipal and industrial wastewater treatment plant (A), the area of the former "Boruta" Dye Industry Plant (B), the closed landfill for hazardous waste of the former "Boruta" Dye Industry Plant (C), the closed energy ash and gypsum landfill (D) (author's own elaboration based on OpenStreetMap data and OSM Standard QuantumGIS).

bladder cancer¹⁶. The European Union has banned the usage of azo dyes that release one or more of 22 aromatic amines classified as potential carcinogens in textile materials above a threshold concentration of 30 mg kg⁻¹¹⁷.

Numerous physical and chemical methods as well as their combinations have been developed for deleterious components of textile wastewater elimination. However, these methods are often combined with high operation costs and low efficiency, which make them economically unsuitable, especially for small enterprises¹⁸. Additionally, during some chemical methods, e.g., oxidation or ozonation, hazardous intermediates are formed¹⁹. Methods based on biological degradation are more environmentally friendly and less expensive and can be an encouraging alternative for toxic compound elimination in polluted areas^{18,20–22}. However, dyeing processes involve not only a variety of colorants but also different alkalis, organic and inorganic salts, acids and heavy metals; hence, it is essential to seek microorganisms that not only decolorize dyes but also retain their properties in conditions unfavorable for the metabolic activity of most microorganisms.

Thus, the proper selection of microbial strains for hazardous waste elimination seems to be one of the most important factors influencing the biodegradation efficiency because only microorganisms adapted to the conditions specific for waste from the dye industry will be fully active in this habitat.

With this in mind, the ascomycete fungus *N. pironii*, previously isolated from urban postindustrial textile green space, was applied in the elimination of dyes and aromatic amines used for their production²³. The work focused on assessing the suitability of this fungus for the utilization of leachate from a waste landfill of the former "Boruta" Dye Industry Plant in Zgierz (Poland). The biodegradation of *o*-tolidine and azo dyes detected in the landfill leachate has also been demonstrated. Dye decolorization in the presence of heavy metals has been investigated. Preliminary identification of the mechanisms responsible for fungal degradation was carried out. Laccase and cytochrome P450 (CYP) involvement has been revealed, which may allow the design of efficient systems for the elimination of these deleterious contaminants from textile postindustrial areas.

Results and discussion

Characteristics of the landfill of the former "Boruta" Dye Industry Plant and the landfill leachate. The dye industry waste landfill is located within the town boundary of Zgierz (Fig. 1) near the former "Boruta" Dye Industry Plant (now a special economic zone with different industrial factories including dye houses). The landfill consists of one plot with an area of 0.81 ha and a cubature of 50,376 m³, limited by a dike²⁴. It is adjacent to the closed energy ash and gypsum landfill. The total surface of the area is approximately 10 ha. The slopes of the landfill are 4–6.5 m high (Fig. 2a). The bottom of the storage basin and the slopes are sealed. The waterproofing was made of native soil in the form of sandy loams with a thickness of 5 to 10 m, on which a levelling layer of medium-grained sand was laid. Above it, a 2 mm thick geomembrane and a filtering layer



Figure 2. The closed landfill for hazardous waste of the former "Boruta" Dye Industry Plant with visible plant succession. The slope of the landfill near the leachate accumulation site L1 (a) and the surface of the landfill with asbestos bags (b).

were installed. At the bottom of the landfill basin, there are drainage pipes for the discharge of leachate from the landfill area^{25–27}. Plant succession with grass predominance and growth of bushes and trees on the slopes of the landfill was observed on the surface of the landfill (Fig. 2a and b).

The dye industry landfill was explored in the years 1995–2015 mainly for the waste disposal of the former "Boruta" Dye Industry Plant in Zgierz. Postproduction waste of the former "Boruta" plant was stored in containers, barrels or bins and then covered with a layer of ash, sand or debris, as well as municipal waste with a pouring layer of ash, gypsum and bags with asbestos (Fig. 2b). The effluents from the landfill are introduced by the industrial sewage system of the former "Boruta" plant to the municipal and industrial wastewater plant in Zgierz. The plant receives (on average, per day) 10,000 m³ of urban wastewater and 1,500 m³ of industrial sludge, including liquid waste from azo dye production factories and landfill leachate. Cleaned wastewater is discharged to the Bzura River, which is a left tributary of the Vistula River that supplies water to the Baltic Sea (Fig. 1). The Baltic Sea is a relatively small (415,266 km²) semi-enclosed brackish water body, and the degree of contamination of the rivers flowing into it is of key importance for the biological balance of the sea²⁸.

Due to the potential risk of water and soil contamination by landfill leachate, the chemical composition of the leachate, groundwater (with 9 piezometer applications), cleaned plant wastewater and water of the Bzura River are monitored by The Voivodeship Inspectorate of Environmental Protection in Łódź^{19,20}. The results of the leachate study carried out in autumn 2020 are presented in Table 1.

The analysis of the composition of leachates L1 and L2 shows that both the high content of TOC (total organic carbon) exceed the permissible values²⁹ many times (799, 788 and 30 mg L⁻¹ C, respectively). The increase in the TOC concentration occurred due to anthropogenic activity is one of the indicators showing the degree of water pollution. In the present study, the chemical oxygen demand (COD) and biochemical oxygen demand (BOD₅) values were also high. Comparing the BOD₅ and COD values determined in the same samples, it is possible to assess the susceptibility of textile wastewater to biodegradation as well as biodecolorization³⁰. The BOD₅ values of 400 and 300 mg L⁻¹ O₂ (for L1 and L2, respectively) suggest that both leachates can be easily biodegradable. However, the high content of chlorides and iron may limit the ability of fungal degradation³¹. Additionally, the amount of volatile phenols was 3–4 times higher than the limit value.

Parameter	Unit	Limit values	Samples and exceeded values
COD _{Mang} ^A	mg L ⁻¹ O ₂	n.r	L1 462 ± 113 L2 428 ± 114
BOD ₅ ^B	mg L ⁻¹ O ₂	25	L1 400 ± 28.3 L2 300 ± 13.6
Chlorides ^A	mg L ⁻¹ Cl	1,000	L1 2,138 ± 274 L2 2,078 ± 78
Iron ^A	mg L ⁻¹ Fe	10	L1 23.8 ± 4.5 L2 23.1 ± 4.4
Volatile phenols ^A	mg L ⁻¹	0.1	L1 0.45 ± 0.111 L2 0.33 ± 0.082
<i>o</i> -tolidine ^B	mg L ⁻¹	n.r	L1 0.3 ± 0.01 L2 0.43 ± 0.02
4,4'-oxydianiline ^B	mg L ⁻¹	n.r	L1 0.23 ± 0.01 L2 0.03 ± 0.01
Electrolytic conductivity	μS cm ⁻¹	n.r	L1 10,150 ± 568 L2 10,145 ± 568

Table 1. Parameters of leachate situated below the landfill which exceeded the permissible values included in the Regulation of ME²⁴. n.r. – not regulated. ^AThe measurements were performed in the Main Research Laboratory in Łódź and presented in the Report no. 296/2020 prepared on behalf of the Voivodeship Inspectorate of Environmental Protection. ^BThe measurements were performed in the Department of Industrial Microbiology and Biotechnology, University of Lodz.

Attention should also be paid to the high values of electrolytic conductivity amounting to 10,150 and 10,145 μS cm⁻¹. Piekutin³² found that the electrolytic conductivity in groundwater samples around municipal waste landfills ranged from 373.4 μS cm⁻¹ to 998.8 μS cm⁻¹, and it was concluded that mineral substances originating from mineral wastes deposited in landfills caused the results.

Our chromatographic study additionally revealed the following toxic aromatic amines: *o*-tolidine (0.3 and 0.43 mg L⁻¹, respectively) and 3,3'-dioxylaniline (0.23 and 0.03 mg L⁻¹, respectively) in both leachate samples L1 and L2. According to information obtained from the state and local control units (see Acknowledgements), aromatic amines have not been detected in the effluents released by the treatment plant into the Bzura River. However, the high amount of iron in leachate samples L1 and L2 (Table 1) indicates corrosion of the metal containers stored in the landfill. For this reason, there is a high probability of releasing much larger amounts of toxic amines and other harmful chemicals that will be introduced to the sewage treatment plant. Jasim et al.³³ showed the presence of several aromatic amines (including benzidine and its derivatives, e.g., *o*-tolidine) in effluents collected from different stages of refinery industrial wastewater treatment plants and from the Tigris River around the station. The range of identified amines was from nondetected to almost 0.3 mg L⁻¹. Benzidine, *o*-toluidine, 3,3-dimethylbenzidine, and 3,3-dichlorobenzidine were detected by Mazzo et al.³⁴ in river receiving wastewater from a textile industry, which was previously treated by the industry using activated sludge. This clearly shows that currently used wastewater treatment methods have a limited capacity for aromatic amine removal. Therefore, it is necessary to search for additional environmentally friendly methods and/or microorganisms that efficiently support the elimination of these dangerous pollutants.

N. pironii growth in dye industry landfill leachate presence. The results of *N. pironii* growth on Sabouraud medium supplemented with 10, 20 and 40% v/v leachate are presented in Fig. 3. The presence of leachate in the growth environment clearly favors the growth of fungal biomass. In the presence of 10% of the leachate, the biomass content in the culture after 120 h of incubation was almost twice as high for leachate L1 (14.6 g L⁻¹) and more than twice as high for leachate L2 (19.6 g L⁻¹). In the sets containing 20 and 40% of the leachate, a greater increase in the biomass of the fungus was also observed than in the control; however, it was lower than in the culture with the addition of 10% of the leachate. These data seem to indicate the presence of inorganic contaminants in both leachates slow down the biosynthesis processes at higher concentrations and be the cause of the lower growth at 20 and 40% of the leachate in the fungal cultures (Fig. 3).

***o*-tolidine and azo dyes elimination.** The leachate analysis (Table 1) also revealed the presence of aromatic amine *o*-tolidine and volatile phenols, which are used for the production of soluble azo dyes and insoluble pigments, particularly in the textile industries. From the mid-seventies of the last century until the restructuring of the dye production plants in Zgierz at the beginning of this century, the leading activity of the factory was combined with the benzidine department and synthesis of many kinds of azo dyes. Additionally, the new enterprises established on the site of the “Boruta” plants are involved in the production of this group of dyes^{3,35}. The amount of *o*-tolidine in the effluents (Table 1) is relatively low (0.3 and 0.43 mg L⁻¹ for L1 and L2, respectively), but the high iron content (23.8 and 23.1 mg L⁻¹ Fe for L1 and L2, respectively) seems to indicate that this is corrosive to metal containers and that there may be a significant release of waste stored there, including aromatic

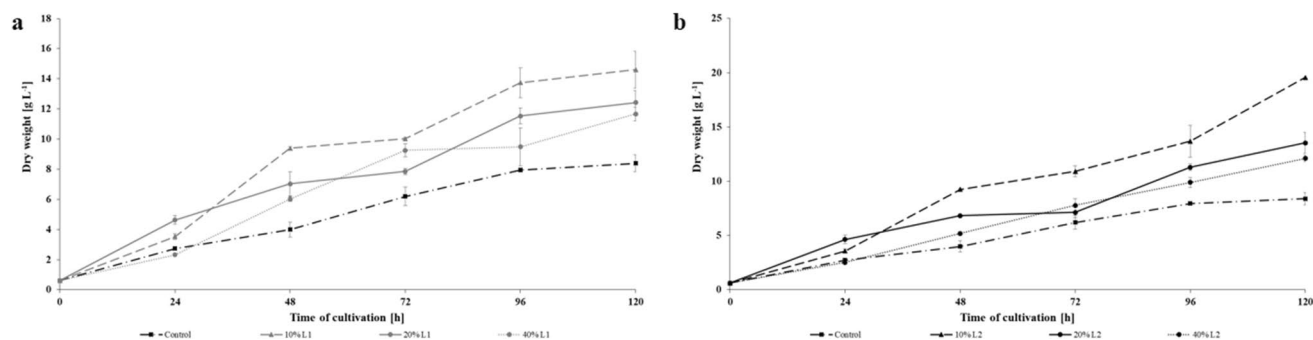


Figure 3. Dry weight of *N. pironii* cultivated in Sabouraud medium containing 10; 20 or 40% of L1 (a) or L2 (b) landfill leachate or without leachate supplementation (control). Data points represent the means $\pm$ s.d., $n = 3$, $P \leq 0.05$.

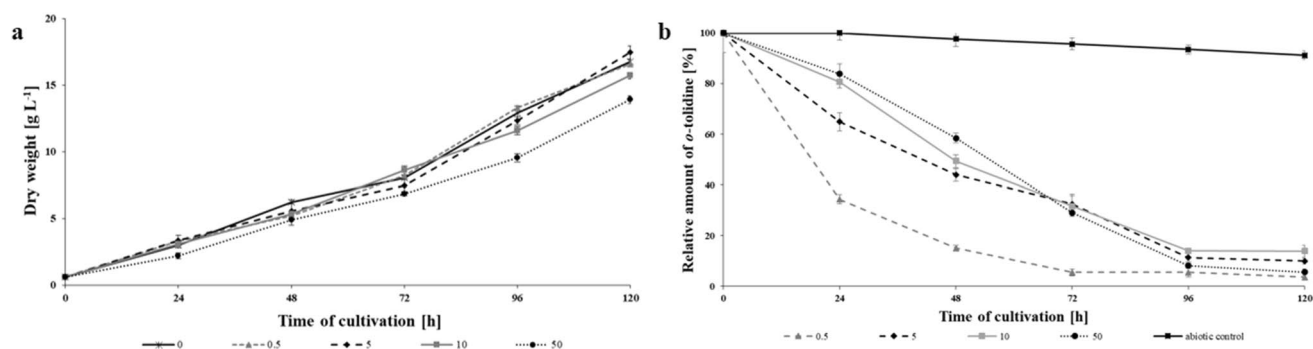


Figure 4. Dry weight of *N. pironii* (a) and *o*-tolidine residue (b) during the culture in Sabouraud medium with the addition of *o*-tolidine (0.5, 5, 10, 50 mg L⁻¹ and abiotic control at a concentration of 50 mg L⁻¹). Data points represent the means $\pm$ s.d., $n = 3$, $P \leq 0.05$.

amines, heavy metals and other components used for the synthesis of azo dyes. Therefore, the ability of *N. pironii* to grow and eliminate at higher concentrations of *o*-tolidine and following azo dyes was tested.

***o*-tolidine biodegradation.** Production of the dry mass of mycelium was assessed in cultures containing 0.5, 5, 10 or 50 mg L⁻¹ *o*-tolidine, and the elimination of this compound from the growth medium was assessed using HPLC–MS/MS analysis.

The microorganisms were able to grow in the presence of all tested *o*-tolidine concentrations (Fig. 4A). In the first 3 days of cultivation, the biomass determined in all test samples was similar and was in the range of 6–8 g L⁻¹. After 72 h, growth inhibition of the fungus appeared in the cultures containing the highest concentration tested. In samples containing 50 mg L⁻¹ *o*-tolidine taken after 96 h of cultivation, mycelium growth was limited by approx. 20%. At the same time, the time course of different concentrations of *o*-tolidine elimination was studied via HPLC–MS/MS analysis. It has been shown that microorganisms are able to eliminate *o*-tolidine from the growth medium at all tested concentrations (Fig. 4B). After 24 h of cultivation, almost 65% loss of the tested compound was found at the lowest of the tested concentrations. In cultures containing 5, 10 and 50 mg L⁻¹ *o*-tolidine, the elimination after 24 h of culture was 35, 19 and 16%, respectively. A significant increase in the rate of *o*-tolidine degradation in these systems was observed after 96 h of cultivation. The xenobiotic content in the cultures initially containing 5, 10 and 50 mg L⁻¹ decreased to 8–14% of the initial concentration and was 0.48, 1.34 and 4.11 mg L⁻¹, respectively.

There are limited studies on aromatic amine biodegradation. Removal of these compounds was described mainly for aromatic amines from pesticides, drugs and dyes^{36–39}. Various species of bacteria, e.g., *Bacillus* sp., *Pseudomonas* sp., *Proteus* sp., *Serratia* sp., *Enterobacter* sp. have been described as effective in aromatic amine biodegradation^{40,41}. Previously, de Lima et al.³⁷ reviewed papers describing “microbial bioremediation of aromatic amines” between 2015 and 2017 and found a lack of works employing fungal cells. Most of the work was on the biodegradation of primary or sulfonated amines.

In the present study, *o*-tolidine was transformed mainly to less toxic 3,3'-dihydroxybenzidine (m/z 217) (Fig. 5). According to Brüsweiler and Merlot¹⁵, this compound gave negative results in the Ames screening test at concentrations ranging from 3–5,000 μ g plate).

Additionally, in the cultures, acetyl *o*-toluidine (m/z 150.2) and *n*-hydroxy-*o*-toluidine (m/z 124) were identified. Mass spectra of the obtained metabolites were compared to the literature data. This suggests that *o*-tolidine may be hydroxylated (e.g., by extracellular laccase) and/or may be converted to toluidine and its derivatives. This is the first report on *o*-tolidine biotransformation by microscopic fungi. The obtained results may fill the gap in

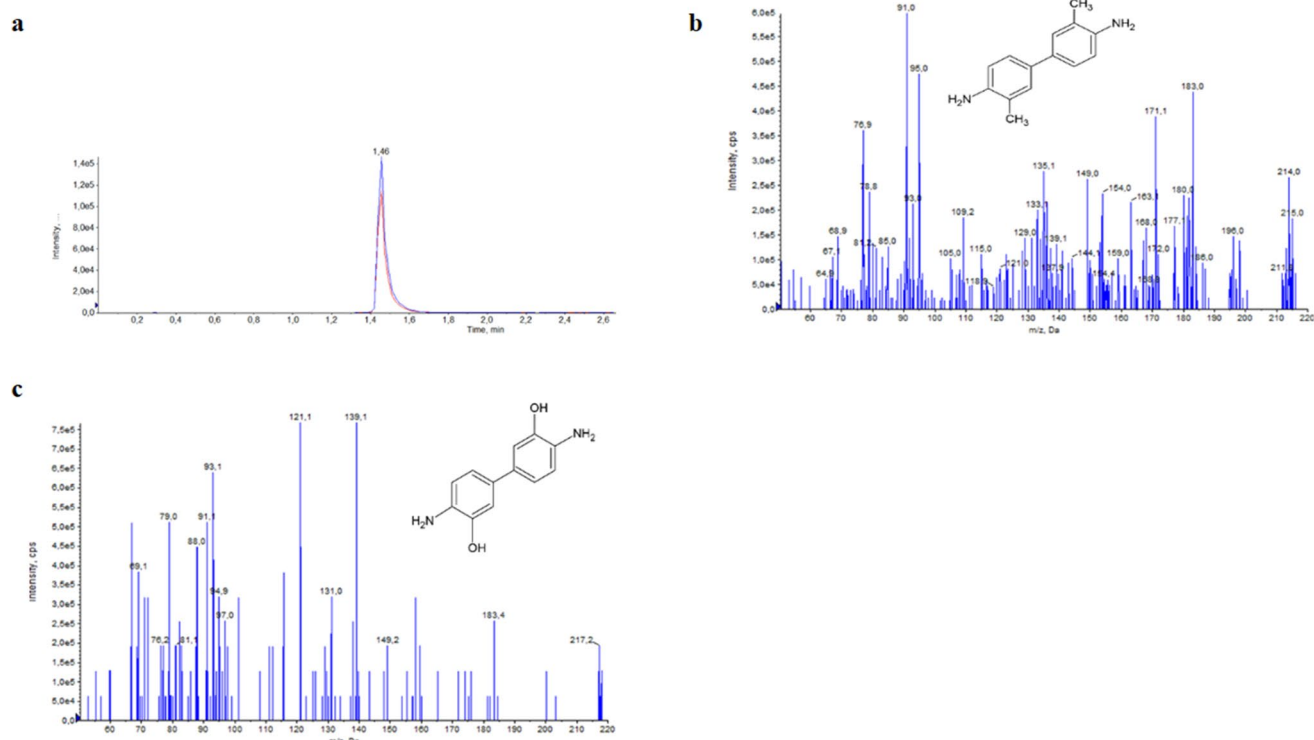


Figure 5. Chromatogram of *o*-tolidine (a), mass spectra of *o*-tolidine (b) and 3,3'-dihydroxybenzidine (c).

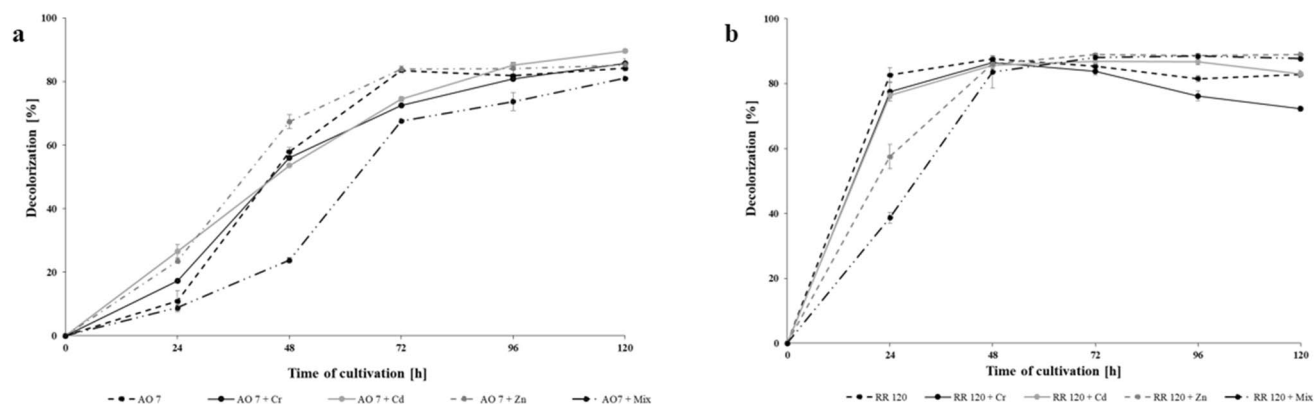


Figure 6. Decolorization of Acid Orange 7 (a) and Reactive Red 120 (b) at the concentration of 50 mg L⁻¹ by the *N. pironii* culture in the presence of heavy metals (Cr(VI) 0.1 mM; Cd(II) 0.75 mM and Zn(II) 1.75 mM). Data points represent the means $\pm$ s.d., n = 3, P $\leq$ 0.05.

the research on fungal elimination of aromatic amines and may help in the development of efficient systems for the removal of these compounds from the environment.

Azo dyes decolorization in the presence of heavy metals. The azo dye decolorization potential of the *N. pironii* fungus was assessed by examining its ability to degrade various dyes (Supplementary Table S1 online). The most efficient colour removal was observed for monoazo dye Acid Orange 7, followed by Reactive Red 120, a dye with a diazo bond. Just after 48 h of incubation, 79% of AO 7 and 75% of RR 120 were decolorized. Extending the cultivation time only slightly increased the level of decolorization, which indicates that dye elimination occurs mainly during the first 48 h of culture.

Considering that textile effluents are a mixture of pollutants containing dye residues, salts, heavy metals and many other chemicals used during dyeing, further study was carried out to examine the effect of selected metals on azo dye decolorization ability and *N. pironii* growth. The metals chosen were Cd(II), Cr(VI) and Zn(II), representing those commonly discharged in dye wastewaters⁴². The decolorization rate of RR 120 by *N. pironii* was affected mainly by Zn(II) added as a single metal (Fig. 6b). Decolorization noted in 24 h of cultivation in the presence of this metal was 24% lower than in the cultures conducted without metal addition. In turn, Cr(VI) and

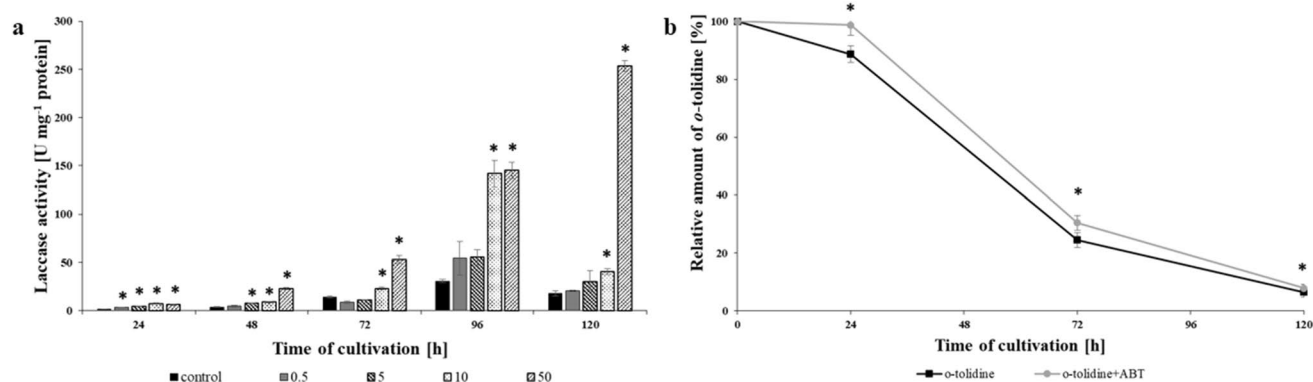


Figure 7. Laccase activity (U mg⁻¹ of protein) during the *N. pironii* culture in Sabouraud medium with the addition of *o*-tolidine (0.5, 5, 10, 50 mg L⁻¹) (a) and *o*-tolidine residue during the culture with *o*-tolidine (50 mg L⁻¹) and 1-Aminobenzotrazole (ABT, 0.25 mM) (b). Data points represent the means $\pm$ s.d., n = 3, * indicates values that differ significantly from the control at P < 0.001.

Cd(II) had only a very small or no effect on colour removal. When a metal mixture was used, decolorization was reduced by more than half. However, after 48 h of cultivation, no differences were observed in the tested systems. Initially, AO 7 decolorization was gently stimulated by the presence of the metals added individually (Fig. 6a). The presence of chromium, cadmium and zinc ions in the growth medium increased dye decolorization by 7%, 16% and 13%, respectively. However, these differences became less apparent in the next days of culturing. The addition of a mixture of all the tested metals significantly reduced the elimination of the dye by the fungus. This was particularly evident at 48 h of culture. The elimination of AO 7 in the presence of a metal mixture reached only 24%, while in the control system, the fungus removed 58% of the dye. Anwar et al.⁴³ tested 38 bacterial strains for their potential to decolorize RR 120 in the presence of 25 mg L⁻¹ Cr(VI). Only the strain identified as *Acinetobacter junii* showed the potential to simultaneously remove Cr(VI) and the selected azo dyes in the same medium. Similarly, the strain of *Pseudomonas* sp. showed good potential for decolorization of structurally diverse types of azo dyes in the presence of chromium, cadmium, zinc and lead ions. Furthermore, the bacterium reduced Cr(VI) by 41 to 95% along with concurrent decolorization of RR 120⁴⁴. High dye removal (88–97%) was observed during growth, while the removal percentage for heavy metals ranged from 58 to 75%.

In real sewage from the textile industry, dyes are accompanied by other harmful compounds, e.g., heavy metals or amines used as dye precursors⁴⁵. Therefore, the microorganisms used in dye biodegradation processes should also be resistant to the harmful effect of other substances present in sewage and should have the ability to perform biodegradation under such conditions⁴⁶.

Laccase and cytochrome P450 involvement in *o*-tolidine biotransformation. *Involvement of laccase in o-tolidine biotransformation* Previously, *N. pironii* was described as an efficient laccase producer²³. Thus, laccase activity was also estimated in the presence of *o*-tolidine at concentrations of 0.5, 5, 10 and 50 mg L⁻¹. The results are presented on Fig. 7a. During the first two days of incubation with *N. pironii*, the laccase activity was low. However, after 72 h of incubation in cultures with the addition of 10 and 50 mg L⁻¹ *o*-tolidine, the laccase activity was highest (22.8 and 52.7 U mg⁻¹ protein) compared to the control (14 U mg⁻¹ protein). Subsequently, laccase activity increased with increasing concentrations of *o*-tolidine. In the cultures with 10 and 50 mg L⁻¹ *o*-tolidine, similar levels of laccase activity were achieved and were found to be 141.8 and 145.3 U mg⁻¹ protein, respectively. The activity of the enzyme was the highest at 120 h in the presence of 50 mg L⁻¹ *o*-tolidine and reached almost 253.4 U mg⁻¹ protein.

In fungi, laccases are activated during the secondary metabolic phase of growth, which is caused by the depletion of nitrogen sources⁴⁷. Although laccases are generally produced in low concentrations, supplementation of the growth medium with appropriate inducers such as metal ions, amino acids or aromatic compounds may increase their production and cause the production of new isoforms of enzymes^{48,49}. Interestingly, an increased laccase activity in a *Trametes versicolor* culture was found to be supplemented with different compounds of industrial origin, such as nonylphenol or aniline⁵⁰. The highest laccase activity in the presence of *o*-tolidine (10 and 50 mg L⁻¹) obtained in this study may result from *o*-tolidine biotransformation and its metabolites, which affect enzyme production.

Laccase production is influenced by different factors (such as phenolic and nonphenolic inducer presence and carbon and nitrogen source depletion). Knowing that the biodegradation of *o*-tolidine involves laccase, it will be possible to control this process (increase its efficiency) by changing the environmental parameters and growth medium composition. In the future, the possibility of using pure *N. pironii* laccase for the elimination of *o*-tolidine and other toxic compounds derived from the textile industry will be tested.

Cytochrome P450 monooxygenase involvement in *o*-tolidine biodegradation. Fungal cytochrome P450 monooxygenases, due to their versatile catalytic properties, are involved in many various and essential cellular processes. They catalyse the conversion of hydrophobic intermediates of primary and secondary metabolic pathways, detoxify natural and environmental pollutants and permit fungi to grow under different

conditions^{51,52}. Several investigations were conducted to determine cytochrome P450 involvement in the decolorization of dyes⁵³ or elimination of xenobiotics. For example, our research team previously found involvement in the degradation of tributyltin (TBT) by *Cunninghamella elegans*⁵⁴, 2,4-dichlorophenoxyacetic acid (2,4-D) by *Umbelopsis isabellina*⁵⁵, 4-*n*-nonylphenol (4-*n*-NP) by *Metarhizium robertsii*⁵⁶ or chloroxyleneol (PCMX) by *C. elegans* IM1785/21GP⁵⁷. The involvement of cytochrome P450 monooxygenases of the *N. pironii* strain in *o*-toluidine biodegradation was verified using the competitive inhibitor 1-aminobenzotriazole (ABT). As shown in Fig. 7b, after the first 24 h of incubation with ABT, the removal of *o*-toluidine was partially inhibited and its content reached 88.7%, while in cultures without inhibitor, it was 98.8%. Additionally, after 72 h of cultivation in the presence of ABT, *o*-toluidine residue was decreased to 24.5%, while in the control, it reached 30.4%. However, after 120 h, the elimination of *o*-toluidine was almost at the same level. The results indicate that cytochrome P450 monooxygenases might participate in the initial stage of *o*-toluidine degradation, but later, this process was not dependent on CYP450. In the white rot fungus *Phanerochaete chrysosporium* cytochrome P450 monooxygenases catalyse the oxidation of phenanthrene⁵⁸ and pyrene⁵⁹. The involvement of cytochrome P450 and laccase was studied by Nykiel-Szymańska et al.⁶⁰. They proved that cytochrome P450 and laccase participate in the biotransformation of alachlor by *Trichoderma koningii*. The elimination of alachlor reached 90% after 72 h of incubation and even 80–60% in the presence of 1–5 mM copper.

Conclusions

The present study revealed the ability of the ascomycete fungus *Nectriella pironii* originating from an urban postindustrial area to grow and retain metabolic activity in the presence of the dye industry waste. The fungus efficiently removed Acid orange 7 and Reactive Red 120 and hazardous aromatic amine (*o*-toluidine) used in azo dye production. This study documented for the first time *o*-toluidine biotransformation by microscopic fungi. Moreover, fungal laccase and cytochrome P450 involvement in this process has been confirmed. Additionally, the decolorization of azo dyes with the addition of heavy metals by *N. pironii* was studied. The high tolerance of the fungus to the tested hazardous compounds connected with their removal makes it an attractive tool in wastewater treatment plants applied for the elimination of dye industry waste. The presented results may also contribute to a precise understanding of the mechanisms controlling this process and promote the creation of a fungus-based system for the elimination of toxic pollutants from textile industry areas.

Materials and methods

Chemicals and landfill leachate. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 1-aminobenzotriazole (ABT) were obtained from Sigma-Aldrich (Germany). Stock solutions of ABT were prepared in ethanol at a concentration of 50 mM.

o-toluidine was purchased from Chempur (Poland). A stock solution of *o*-toluidine (10 mg mL⁻¹) was prepared in ethanol. Liquid chromatography solvents were obtained from Avantor Performance Materials (Poland).

The azo dyes Reactive Orange 16 (CAS 12225–83-1), Acid Red 27 (CAS 915–67-3), Acid Orange 7 (CAS 633–96-5), Reactive Red 120 (CAS 61951–82-4), and Reactive Black 5 (CAS 17095–24-8) were purchased from Sigma-Aldrich (Germany). Stock solutions were prepared in water at a concentration of 50 mg mL⁻¹.

Cd(NO₃)₂ × 4 H₂O, K₂Cr₂O₇, and ZnSO₄ × 7 H₂O were purchased from Sigma-Aldrich (Germany). Metal stock solutions were prepared by dissolving in deionized water at a concentration of 1 M.

Samples of landfill leachate (L1 and L2) collected from the landfill of the former “Boruta” Dye Industry Plant in Zgierz, Poland (Fig. 1) were kindly supported by the Voivodeship Inspectorate of Environmental Protection in Łódź, Poland.

Strain and culture medium. The fungus *N. pironii* IM 6443 used in this study was previously isolated from a sample soil collected in the territory of the former “Boruta” Dye Industry Plant in Zgierz (a part of the Metropolitan Area of Łódź, Poland, Fig. 2) left to natural plant succession. The fungal strain was subcultured on ZT slants⁶¹ and maintained at 4 °C in the strain collection of the Department of Industrial Microbiology and Biotechnology, University of Łódź, Poland.

Experimental setup and design. *Fungal growth conditions.* Fungal spores from 10-day-old cultures incubated on ZT slants were used for the preparation of pre-cultures in 25 mL of WHI medium⁶² using 100-mL Erlenmeyer flasks. The incubation was conducted at 28 °C on a rotary shaker (120 rpm). After 24 h, the pre-cultures were transferred to fresh WHI medium at a ratio of 1:4 and cultivated for 24 h²³.

Growth of *N. pironii* in medium with landfill leachates. Landfill leachates were centrifuged (6,000 × g for 10 min at 4 °C) and filtered to remove suspended solids before measurement. The cultures were prepared by inoculating Sabouraud dextrose broth liquid medium (BioMaxima, Poland) with 10, 20 or 40% v/v landfill leachate and 10% v/v inoculum. In addition, control flasks without added landfill leachate were included. All of the experiments were conducted in triplicate. Flasks were incubated on a rotary shaker (120 rpm) at 28 °C for 120 h.

Analytical methods. *Dry weight estimation of fungal biomass.* The cultures in the flasks were filtered through pre-weighed Whatman filter paper No. 2 on a filtration set under slight vacuum, washed three times with distilled water and then dried at 105 °C in an oven until a constant weight⁶³. The difference between the weight of dried filter paper with biomass and empty filter paper represented the fungal biomass. The results are expressed as mg mL⁻¹, which corresponds to fungal biomass (mg) per volume of culture (mL).

***o*-tolidine extraction and analysis.** After incubation, the fungal cultures were transferred into 50 mL Falcon tubes with 10 mL of acetonitrile and glass beads and homogenized with a ball mill (MM 400, Retsch) for 2 min. Next, QuEChERS salts (2 g MgSO₄; 0.5 g NaCl; 0.5 g C₆H₅Na₃O₇ × 2 H₂O; 0.25 g C₆H₅Na₃O₇ × 1.5 H₂O) were added, and the tubes were vortexed for 2 min. Afterwards, the tubes were centrifuged for 10 min at 3,500 × g. After centrifugation, the upper layer was collected and transferred into an Eppendorf tube for chromatographic analysis.

Determination of *o*-tolidine and its metabolites by HPLC–MS/MS. Measurement was performed using an Agilent 1200 HPLC (Santa Clara CA, USA) system and a 4500 QTrap mass spectrometer (Sciex, Framingham, MA, USA) with an ESI source. For reversed-phase chromatographic analysis, 10 µl of the diluted sample was injected onto a Kinetex C18 column (50 mm × 2.1 mm, particle size: 5 µm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of 5 mM ammonium formate in water (A) and 5 mM ammonium formate in methanol (B). The solvent gradient was initiated at 20% B, increased to 80% B over 0.5 min, and maintained at 80% B for two additional minutes before returning to the initial solvent composition over 2 min. The column temperature was maintained at 40 °C, and the flow rate was 600 µl min^{−1}.

The instrumental settings were as follows: spray voltage 5500 V, curtain gas (CUR) 25, nebulizer gas (GS1) 50, turbo gas (GS2) 50, ion source temperature of 500 °C, and positive polarization. Data analysis was performed with Analyst version 1.6.2 software (<https://sciex.com/products/software/analyst-software>; Sciex, Framingham, MA, USA). The quantitative analysis of *o*-tolidine was performed using multiple reaction monitoring (MRM). The monitored MRM pairs were *m/z* 213.3–196 and 213.3–181.

Tandem mass spectrometry for the identification of *o*-tolidine metabolites was performed using an enhanced MS scan (EMS) and precursor ion scanning (Prec). An information-dependent acquisition method, Prec (*m/z* 94) → EPI and EMS → EPI, was used to search ions corresponding to the protonated molecules [M + H]⁺.

Landfill leachate analyses. The biochemical oxygen demand (BOD) of L1 and L2 was determined using the manometric respirometric BOD OxiTop (WTW, the Xylem Group, Germany). Samples were prepared according to European Norm⁶⁴ and incubated in the dark for five days at a temperature of 20 °C.

The measurements of the landfill leachate quality were performed in the Main Research Laboratory in Łódź on behalf of the Voivodeship Inspectorate of Environmental Protection and evaluated by the following physical–chemical parameters: temperature⁶⁵; pH⁶⁶; conductivity⁶⁷; COD_{Mang}⁶⁸; TOC⁶⁹; nitrites⁷⁰; nitrates, sulfates and chlorides⁷¹; antimony, arsenic, bar, beryl, boron, total chromium, zinc, aluminium, cadmium, cobalt, manganese, copper, molybdenum, nickel, lead, selenium, silver, thallium, titanium, vanadium and iron⁷²; mercury⁷³; free and bound cyanide⁷⁴; petroleum hydrocarbons⁷⁵; volatile phenols⁷⁶.

Assay of laccase activity and protein concentration. Laccase activity in the centrifuged supernatant was assayed by monitoring the oxidation of 10 mM ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) at 420 nm, as described previously^{23,77}. One unit of laccase activity (U) was determined as the concentration of the enzyme required to oxidize 1 µM substrate per minute.

The protein concentration was determined using a bicinchoninic acid assay (BCA) test according to the Pierce BCA Protein Assay Kit protocol (Thermo Fisher Scientific).

Cytochrome P450 inhibition study. 1-Aminobenzotriazole (ABT)—cytochrome 450 inhibiting compound was introduced to the *N. pironii* cultures with the addition of *o*-tolidine (50 mg L^{−1}) at the start of the cultivation to obtain a final concentration of 0.25 mM. Control flasks of *o*-tolidine (without inhibitor) were also prepared. All cultures were incubated on a rotary shaker (120 rpm) at 28 °C. The samples from 24, 72 and 120 h of cultivation were used in the experiment prepared in accordance with the method described in Section “*o*-tolidine extraction and analysis”.

Characterization of the decolorizing potential of the *N. pironii* strain. Assays for the decolorization of dyes (RO 16, AR 27, RR 120, RB 5 and AO 7) at the initial concentration of 25 mg L^{−1} were performed in 18 mL of Sabouraud medium and inoculated with 10% inoculum. The cultures were prepared in 100-mL Erlenmeyer flasks and incubated on a rotary shaker (120 rpm) at 28 °C for 5 days. The abiotic controls of dyes and samples tested were carried out in triplicate. One milliliter of culture broth was sampled in an Eppendorf tube, centrifuged at 6,000 × g for 5 min and measured at the dye maximum absorbance wavelength (RO 16 λ_{max} = 492 nm; AR 27 λ_{max} = 529 nm, RR 120 λ_{max} = 516 nm, RB 5 λ_{max} = 598 nm, AO 7 λ_{max} = 467 nm) in a FLUO-Star microplate reader (BMG Labtech, Germany) and Omega version 5.10 R2 software (<https://www.bmglabtech.com/microplate-reader-software/>)⁷⁸. The percentage decolorization was calculated as Eq. (1):

$$D [\%] = [100 \times (A_0 - A_t/A_0)], \quad (1)$$

where A₀ is the absorbance of the abiotic control, A_t is the absorbance of the culture supernatant, and D is the decolorization rate.

Additionally, the impact on decolorization rate of heavy metal compounds Cr(VI) 0.1 mM, Cd(II) 0.75 mM and Zn(II) 1.75 mM introduced to the *N. pironii* culture separately or as a mixture was measured as described previously.

Spatial data analysis. Spatial data on the characteristics and field parameters of a landfill were obtained by the method of mapping degraded areas⁷⁹ using GIS tools—Quantum GIS Bucuresti version 3.12 geoinformation

software (<https://qgis.org/pl/site/>), OpenStreetMap and OSM Standard online GIS sources. Data on vegetation and landfill management were sourced from the field observation of the area in period 2019–2021 by using the methods of eco-urban documentation of green infrastructure⁸⁰.

Statistical analysis. Experiments were conducted in triplicate. The data values are presented as the mean $\pm$ standard deviation (SD). The statistical significance of differences between the mean values was compared using an analysis of variance (ANOVA) with Tukey's post hoc test. The changes were considered significant at $P < 0.001$ in STATISTICA version 13.3 software (<https://statistica.software.informer.com/13.3/>; StatSoft).

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References

1. Dyes and pigments market - growth, trends, covid-19 impact, and forecasts (2021 - 2026). https://www.mordorintelligence.com/industry-reports/dyes-and-pigments-market?gclid=Cj0KCQjw5uWGBhCTARIsAL70sLLOVQUzpbXp6acFXmbpYQ7okD2h8qJaTaj9a0nXMeinPRP-C03hT_YaAhN0EALw_wcB. Accessed on 21 July 2021.
2. Jabłońska, M., Stawska, J. & Czechowska, D.I. Country-specific determinants of textile industry development in Poland: comparative analysis of the years 2007 and 2017. *Autex Res. J.* **20**(2), 186–193. <https://doi.org/10.2478/aut-2019-0064> (2020).
3. Hajdys, D., Jabłońska, M. & Ślebocka, M. Impact of textile industry restructuring on the financial condition of local government units for the example of the Łódź region in Poland. *Fibres Text. East. Eur.* **5**(143), 8–19. <https://doi.org/10.5604/01.3001.0014.2379> (2020).
4. Marszał, T. Łódź metropolitan area: delimitation, planning and development. *Geogr. Pol.* **90**(3), 281–300. <https://doi.org/10.7163/GPol.0096> (2017).
5. Bibi, I., Bhatti, H. N. & Asgher, M. Decolourisation of direct dyes with manganese peroxidase from white rot Basidiomycete *Ganoderma lucidum*-IBL-5. *Can. J. Chem. Eng.* **87**, 435–440. <https://doi.org/10.1002/cjce.20165> (2009).
6. Nouren, S. & Bhatti, H. N. Mechanistic study of degradation of basic violet 3 by Citrus limon peroxidase and phytotoxicity assessment of its degradation products. *Biochem. Eng. J.* **95**, 9–19. <https://doi.org/10.1016/j.bej.2014.11.021> (2015).
7. Benkhaya, S., M'rabbet, S. & El Harfi, A. A review on classifications, recent synthesis and applications of textile a review on classifications, recent synthesis and applications of textile dyes. *Inorg. Chem. Commun.* **115**, 107891 (2020).
8. Długoński, A. & Dushkova, D. The hidden potential of informal urban greenspace: an example of two former landfills in post-socialist cities (central Poland). *Sustainability* **13**, 3691. <https://doi.org/10.3390/su13073691> (2021).
9. Holkar, Ch. R., Jadhav, A. J., Pinjari, D. V., Mahamuni, N. M. & Pandit, A. B. A critical review on textile wastewater treatments: possible approaches. *J. Environ. Manage.* **182**, 351–366. <https://doi.org/10.1016/j.jenvman.2016.07.090> (2016).
10. Jędrzejczak, K. & Wojciechowski, M. A numerical method of analyzing the composition of colored wastewater from dyeing plant. *Int. J. Environ. Sci. Technol.* <https://doi.org/10.1007/s13762-021-03208-2> (2021).
11. Nai, C. *et al.* Potentially contamination and health risk to shallow groundwater caused by closed industrial solid waste landfills: Site reclamation evaluation strategies. *J. Clean. Prod.* **286**, 125402. <https://doi.org/10.1016/j.jclepro.2020.125402> (2021).
12. NIK Report. Hazard prevention from the landfills on the Lodz Voivodeship area. <https://www.nik.gov.pl/plik/id,23253,vp,25961.pdf>. Accessed on 21 July 2021.
13. Bibi, I. *et al.* Investigation of catalytic properties of Manganese Peroxidase (MnP) produced from *Agaricus bisporus* A21 and its potential application in the biotransformation of xenobiotic compound. *J. Chem. Soc. Pak.* **37**(05), 859–868 (2015).
14. Yeilagi, S., Rezapour, S. & Asadzadeh, F. Degradation of soil quality by the waste leachate in a Mediterranean semi-arid ecosystem. *Sci. Rep.* **11**, 11390. <https://doi.org/10.1038/s41598-021-90699-1> (2021).
15. Brüschweiler, B. J. & Merlot, C. Azo dyes in clothing textiles can be cleaved into a series of mutagenic aromatic amines which are not regulated yet. *Regul. Toxicol. Pharmacol.* **88**, 214–226. <https://doi.org/10.1016/j.yrtph.2017.06.012> (2017).
16. Özkan, B. Ç., Firat, M., Dotse, S. & Bakirdere, S. Accurate and sensitive determination of harmful aromatic amine products of azo dyes in wastewater and textile samples by GC – MS after multivariate optimization of binary solvent dispersive liquid-liquid microextraction. *Microchem. J.* **145**, 84–89. <https://doi.org/10.1016/j.microc.2018.10.023> (2019).
17. Piccinini, P., Senaldi, C. & Buriova, E. European Survey on the presence of banned azodyes in textiles. EUR 23447 EN. Luxembourg (Luxembourg): OPOCE; 2008. JRC44198. <https://publications.jrc.ec.europa.eu/repository/handle/JRC44198>. Accessed on 21 July 2021.
18. Jasińska, A., Góralczyk, A. & Długoński, J. Dyes decolourisation and degradation by microorganisms in *Microbial biodegradation: From omics to function and application* (ed. Długoński, J.) 119–141 (Caister Academic Press: Norfolk, UK, 2016) ISBN: 978-1-910190-45-6.
19. Noreen, R., Asgher, M., Bhatti, H. N., Batool, S. & Asad, M. J. *Phanerochaete chrysosporium* IBL-03 secretes high titers of manganese peroxidase during decolorization of Drimarine Blue K2RL textile dye. *Environ. Technol.* **32**(11–12), 1239–1246. <https://doi.org/10.1080/09593330.2010.534820> (2011).
20. Islam, M. A. *et al.* Microbial load in bio-slurry from different biogas plants in Bangladesh. *J. Adv. Vet. Anim. Res.* **6**(3), 376–383. <https://doi.org/10.5455/javar.2019.f357> (2019).
21. Kalsoom, U., Ashraf, S. S., Meetani, M. A., Rauf, M. A. & Bhatti, H. N. Mechanistic study of a diazo dye degradation by Soybean Peroxidase. *Chem. Cent. J.* **7**(1), 93. <https://doi.org/10.1186/1752-153X-7-93> (2013).
22. Szewczyk, R. & Kowalski, K. Metabolomics and crucial enzymes in microbial degradation of contaminants in *Microbial biodegradation: From omics to function and application, application* (ed. Długoński, J.) 43–65 (Caister Academic Press: Norfolk, UK, 2016) ISBN: 978-1-910190-45-6.
23. Góralczyk-Bińkowska, A., Jasińska, A., Długoński, A., Płociński, P. & Długoński, J. Laccase activity of the ascomycete fungus *Nectriella pironii* and innovative strategies for its production on leaf litter of an urban park. *PLoS ONE* **15**(4), e0231453. <https://doi.org/10.1371/journal.pone.0231453> (2020).
24. Janas, M. & Zawadzka, A. Assessment of the monitoring of an industrial waste landfill. *Ecol. Chem. Eng. S.* **25**(4), 659–669. <https://doi.org/10.1515/eces-2018-0044> (2018).
25. Kuźmierz, A., Krawczyńska, B. & Krawczyński, J. Ocena oddziaływania składowisk pozakładowych zlokalizowanych na terenie byłych zakładów "Boruta" w Zgierzu na życie i zdrowie mieszkańców Zgierza i okolic oraz dla środowiska [Assessment of the impact of off-site landfills located in the former "Boruta" plants in Zgierz on the life and health of the inhabitants of Zgierz and the surrounding area, and on the environment], Instytut Ochrony Środowiska – Państwowy Instytut Badawczy: Warsaw, Poland 2019. (in Polish).

26. Rysiukiewicz K. Dokumentacja określająca warunki hydrogeologiczne w rejonie osadników gipsów i popiołów na terenie Z.P.B. "Boruta" w Zgierzu w likwidacji, pow. zgierski, woj. łódzkie [Documentation specifying the hydrogeological conditions in the area of gypsum and ash settlers at the "Boruta" Dye Production Plant in Zgierz in liquidation, Zgierz powiat, Łódź voivodeship], Narodowe Archiwum Geologiczne, PIG-PIB; 2000. (in Polish).
27. Szadkowska M. Dokumentacja określająca warunki hydrogeologiczne w rejonie osadników gipsów i popiołów na terenie Z.P.B. "Boruta" w Zgierzu w likwidacji, pow. zgierski, woj. łódzkie [Documentation specifying the hydrogeological conditions in the area of gypsum and ash settlers at the "Boruta" Dye Production Plant in Zgierz in liquidation, Zgierz powiat, Łódź voivodeship], Narodowe Archiwum Geologiczne, PIG-PIB; 2000. (in Polish).
28. Svendsen, L. M. & Gustafsson, B. 2020. Waterborne nitrogen and phosphorus inputs and water flow to the Baltic Sea 1995–2018. HELCOM Baltic Sea Environment Fact Sheet 2020. <https://helcom.fi/media/documents/BSEFS-Waterborne-nitrogen-and-phosphorus-inputs-and-water-flow-to-the-Baltic-Sea.pdf>. Accessed on 21 July 2021.
29. Regulation of the Minister of Maritime Economy and Inland Navigation of 12 July 2019 on substances particularly harmful to the aquatic environment and the conditions to be met when discharging sewage into waters or ground, as well as when discharging rainwater or meltwater into waters or into devices aquatic. Dz.U. 2019 poz. 1311. <https://isap.sejm.gov.pl/isap.nsf/download.xsp/WDU20190001311/O/D20191311.pdf>. Accessed on 28 June 2021 (in Polish).
30. Sen, S. K., Patra, P., Das, C. R., Raut, S. & Raut, S. Pilot-scale evaluation of biodecolorization and biodegradation of reactive textile wastewater: an impact on its use in irrigation of wheat crop. *Water Resour. Ind.* **21**, 100106. <https://doi.org/10.1016/j.wri.2019.100106> (2019).
31. Różalska, S., Bernat, P., Michnicki, P. & Długoński, J. Fungal transformation of 17 α -ethinylestradiol in the presence of various concentrations of sodium chloride. *Int. Biodeter. Biodegr.* **103**, 77–84. <https://doi.org/10.1016/j.ibiod.2015.04.016> (2015).
32. Piekutin, J. Monitoring of groundwater in the area of a reclaimed municipal waste landfill. *J. Ecol. Eng.* **20**(8), 262–268 (2019). <https://doi.org/10.12911/22998993/111718>
33. Jasim, H. H., Altahir, B. M. & Sultan, M. S. Solid cartridges in determination of benzidines in river and wastewater by HPLC. *World Rural Observ.* **10**(1), 52–60. <https://doi.org/10.7537/marswro100118.07> (2018).
34. Mazzo, T. M., Saczk, A. A., Umbuzeiro, G. A. & Zanoni, M. V. B. Analysis of aromatic amines in surface waters receiving wastewater from a textile industry by liquid chromatographic with electrochemical detection. *Anal. Lett.* **39**(14), 2671–2685. <https://doi.org/10.1080/00032710600824797> (2006).
35. Przygucki, T. Sto lat koloru. Zakłady Przemysłu Barwników BORUTA S.A. [100 years of colour. BORUTA S.A. Dye Industry Plant] (Publishing House Pryzmat: Łódź, Poland 1994). (in Polish).
36. Castillo, J. C., Orrego-Hernández, J. & Portilla, J. Cs₂CO₃-Promoted direct *n*-alkylation: highly chemoselective synthesis of *n*-alkylated benzylamines and anilines. *Eur. J. Org. Chem.* **22**, 1–13. <https://doi.org/10.1002/efoc.201600549> (2014).
37. de Lima, D. P. *et al.* Fungal Bioremediation of pollutant aromatic amines. *Curr. Opin. Green Sustain. Chem.* **11**, 34–44. <https://doi.org/10.1016/j.cogsc.2018.03.012> (2018).
38. Singh, R. P., Singh, P. K. & Singh, R. L. Role of azoreductases in bacterial decolorization of azo dyes. *Curr. Trends Biomed. Eng. Biosci.* **9**(3), 50–52. <https://doi.org/10.19080/CTBEB.2017.09.555764> (2017).
39. Tong, Z., Yang, D., Xiao, T., Tian, Y. & Jiang, Z. Biomimetic fabrication of g-C₃N₄/TiO₂ nanosheets with enhanced photocatalytic activity toward organic pollutant degradation. *Chem. Eng. J.* **260**, 117–125. <https://doi.org/10.1016/j.cej.2014.08.072> (2015).
40. Barsing, P., Tiwari, A., Joshi, T. & Garg, S. Bioremediation technology application of a novel bacterial consortium for mineralization of sulfonated aromatic amines. *Bioresour. Technol.* **2011**(102), 765–771. <https://doi.org/10.1016/j.biortech.2010.08.098> (2011).
41. Fatima, M., Saeed, M., Aslam, M., Lindström, W. R. & Farooq, R. Application of novel bacterial consortium for biodegradation of aromatic amine 2-ABS using response surface methodology. *J. Microbiol. Methods* **174**, 105941. <https://doi.org/10.1016/j.mimet.2020.105941> (2019).
42. Gunatilake, S. K. Methods of removing heavy metals from industrial wastewater. *J. Multidiscip. Eng. Sci. Stud.* **1**, 12–18 (2015).
43. Anwar, F. *et al.* Characterization of Reactive Red-120 decolorizing bacterial strain *Acinetobacter junii* FA10 capable of simultaneous removal of azo dyes and hexavalent chromium. *Water Air Soil Pollut.* **2014**225, 1–16. <https://doi.org/10.1007/s11270-014-2017-7> (2017).
44. Hussain, S. *et al.* Simultaneous removal of reactive dyes and hexavalent chromium by a metal tolerant *Pseudomonas* sp. WS-D / 183 harboring plant growth promoting traits. *Int. J. Agric. Biol.* **23**(2), 241–252. <https://doi.org/10.17957/IJAB/15.1282> (2020).
45. Rezapour, S., Samadi, A., Kalavrouziotis, I. K. & Ghaemian, N. Impact of the uncontrolled leakage of leachate from a municipal solid waste landfill on soil in a cultivated-calcareous environment. *Waste Manage.* **82**, 51–61. <https://doi.org/10.1016/j.wasman.2018.10.013> (2018).
46. Bera, S. P. & Tank, S. K. Microbial degradation of Procion Red by *Pseudomonas stutzeri*. *Sci. Rep.* **11**, 3075. <https://doi.org/10.1038/s41598-021-82494-9> (2021).
47. Viswanath, B., Rajesh, B., Janardhan, A., Kumar, A. P. & Narasimha, G. Fungal laccases and their applications in bioremediation. *Enzyme Res.* **163**242, 1–21. <https://doi.org/10.1155/2014/163242> (2014).
48. Janusz, G. *et al.* Laccase properties, physiological functions, and evolution. *Int. J. Mol. Sci.* **21**, 966. <https://doi.org/10.3390/ijms21030966> (2020).
49. Piscitelli, A. *et al.* Induction and transcriptional regulation of laccases in fungi. *Curr. Genomics* **12**(2), 104–112. <https://doi.org/10.2174/138920211795564331> (2011).
50. Mougin, Ch., Kollmann, A. & Jolival, C. Enhanced production of laccase in the fungus *Trametes versicolor* by the addition of xenobiotics. *Biotechnol. Lett.* **24**, 139–142. <https://doi.org/10.1023/A:1013802713266> (2002).
51. Črešnar, B. & Petrič, Š. Cytochrome P450 enzymes in the fungal kingdom. *Biochim. Biophys. Acta - Proteins Proteomics* **1814**(1), 29–35. <https://doi.org/10.1016/j.bbapap.2010.06.020> (2011).
52. Hussain, R., Mushtaq, A., Tabreiz, A. K. & Yusuf, A. Fungal P450 monooxygenases - the diversity in catalysis and their promising roles in biocontrol activity. *Appl. Microbiol. Biotechnol.* **104**(3), 989–999. <https://doi.org/10.1007/s00253-019-10305-3> (2019).
53. Jasińska, A., Paraszkiewicz, K., Sip, A. & Długoński, J. Malachite Green decolorization by the filamentous fungus *Myrothecium roridum* - mechanistic study and process optimization. *Bioresour. Technol.* **194**, 43–48. <https://doi.org/10.1016/j.biortech.2015.07.008> (2015).
54. Bernat, P. & Długoński, J. Degradation of tributyltin by the filamentous fungus *Cunninghamella elegans*, with involvement of cytochrome P-450. *Biotechnol. Lett.* **24**(23), 1971–1974. <https://doi.org/10.1023/A:1021177716010> (2002).
55. Nykiel-Szymańska, J., Stolarek, P. & Bernat, P. Elimination and detoxification of 2,4-D by *Umbelopsis isabellina* with the involvement of cytochrome P450. *Environ. Sci. Pollut. Res. Int.* **25**(3), 2738–2743. <https://doi.org/10.1007/s11356-017-0571-4> (2018).
56. Nowak, M., Sobon, A., Litwin, A. & Różalska, S. 4-*n*-nonylphenol degradation by the genus *Metarhizium* with cytochrome P450 involvement. *Chemosphere* **220**, 324–334. <https://doi.org/10.1016/j.chemosphere.2018.12.114> (2019).
57. Nowak, M., Zawadzka, K., Szemraj, J., Góralczyk-Bińkowska, A. & Lisowska, K. Biodegradation of chloroxenol by *Cunninghamella elegans* IM 1785/21GP and *Trametes versicolor* IM 373: Insight into Ecotoxicity and Metabolic Pathways. *Int. J. Mol. Sci.* **22**, 4360. <https://doi.org/10.3390/ijms22094360> (2021).
58. Ning, D., Wang, H., Ding, Ch. & Lu, H. Novel evidence of cytochrome P450-catalyzed oxidation of phenanthrene in *Phanerochaete chrysosporium* under ligninolytic conditions. *Biodegradation* **21**(6), 889–901. <https://doi.org/10.1007/s10532-010-9349-9> (2010).
59. Syed, K. & Yadav, J. S. P450 monooxygenases (P450ome) of the model white rot fungus *Phanerochaete chrysosporium*. *Crit. Rev. Microbiol.* **38**(4), 339–363. <https://doi.org/10.3109/1040841X.2012.682050> (2012).

60. Nykiel-Szymańska, J., Bernat, P. & Ślaba, M. Potential of *Trichoderma koningii* to eliminate alachlor in the presence of copper ions. *Ecotoxicol. Environ. Saf.* **162**, 1–9. <https://doi.org/10.1016/j.ecoenv.2018.06.060> (2018).
61. Milczarek, K., Wrońska, N. & Felczak, A. Media in *Microbial biotechnology in the laboratory and in practice. Theory, exercises and specialist laboratories* (ed. Długoński, J.) 475 (Łódź University Press & Jagiellonian University Press: Łódź-Kraków, Poland, 2021) e-ISBN 978–83–8220–420–9.
62. Paraskiewicz, K., Bernat, P. & Długoński, J. Effect of nickel, copper, and zinc on emulsifier production and saturation of cellular fatty acids in the filamentous fungus *Curvularia lunata*. *Int. Biodegr. Biodegr.* **63**(1), 100–105. <https://doi.org/10.1016/j.ibiod.2008.03.015> (2009).
63. Siewiera, P., Różalska, S. & Bernat, P. Estrogen-mediated protection of the organotin-degrading strain *Metarhizium robertsii* against oxidative stress promoted by monobutyltin. *Chemosphere* **185**, 96–104. <https://doi.org/10.1016/j.chemosphere.2017.06.130> (2017).
64. PN-EN 1899–2:2002 Water Quality - Determination of Biological Oxygen Demand after n days (BOD_n)= Part 2: Method for undiluted samples. <https://www.iso.org/obp/ui/#iso:std:iso:5815:-2:ed-1:v1:en:ed1:v1>. Accessed on 21 July 2021.
65. Procedure PB-20, edition 1 of 06.05.2019 – Determination of temperature.
66. PN-EN ISO 10523:2012 - Water quality - Determination of pH. <https://sklep.pkn.pl/pn-en-iso-10523-2012e.html>. Accessed on 21 July 2021.
67. PN-EN 27888:1999 - Water quality - Determination of pH. https://infostore.saiglobal.com/en-gb/standards/pn-en-27888-1999-922966_saig_pkn_pkn_2178929/. Accessed on 21 July 2021.
68. PN-EN ISO 8467:2001 - Water quality - Determination of permanganate index. <https://www.iso.org/standard/15669.html>. Accessed on 21 July 2021.
69. PN-EN 1484:1999 - Water analysis - Guidelines for the determination of total organic carbon (TOC) and dissolved organic carbon (DOC). https://infostore.saiglobal.com/en-gb/standards/pn-en-1484-1999-934164_saig_pkn_pkn_2201325/. Accessed on 21 July 2021.
70. PN-EN ISO 13395:2001 - Water quality - Determination of orthophosphate and total phosphorus contents by flow analysis (FIA and CFA) — Part 1: Method by flow injection analysis (FIA). <https://www.iso.org/standard/35050.html>. Accessed on 21 July 2021.
71. PN-EN ISO 10304–1:2009+AC:2012 - Water quality - Determination of dissolved anions by liquid chromatography of ions - Part 1: Determination of bromide, chloride, fluoride, nitrate, nitrite, phosphate and sulphate. <https://standards.iteh.ai/catalog/standards/cen/b9f31761-ea7c-49b2-ba7d-29ea4ebca97c/en-iso-10304-1-2009-ac-2012>. Accessed on 21 July 2021.
72. PN-EN ISO 11885:2009 - Water quality - Determination of selected elements by inductively coupled plasma optical emission spectrometry (ICP-OES). <https://www.iso.org/obp/ui/#iso:std:iso:11885:ed-2:v1:en>. Accessed on 21 July 2021.
73. EPA Method 7473 02.2007 - Mercury in Solids and Solutions by Thermal Decomposition, Amalgamation, and Atomic Absorption Spectrophotometry. <https://www.epa.gov/sites/default/files/2015-12/documents/7473.pdf>. Accessed on 21 July 2021.
74. PN-EN ISO 14403–2:2012 - Water quality - Determination of total cyanide and free cyanide using flow analysis (FIA and CFA) — Part 2: Method using continuous flow analysis (CFA). <https://www.iso.org/obp/ui/#iso:std:iso:14403:-2:ed-1:v1:en>. Accessed on 21 July 2021.
75. PN-EN ISO 9377–2:2003 - Water quality - Determination of hydrocarbon oil index — Part 2: Method using solvent extraction and gas chromatography. <https://www.iso.org/obp/ui/#iso:std:iso:9377:-2:ed-1:v1:en>. Accessed on 21 July 2021.
76. PN-EN ISO 14402:2004 - Water quality - Determination of phenol index by flow analysis (FIA and CFA). <https://www.iso.org/obp/ui/#iso:std:iso:14402:ed-1:v1:en:en>. Accessed on 21 July 2021.
77. Jasińska, A., Góralczyk-Bińkowska, A. & Długoński, A. Characteristics and use of ligninolytic enzymes produced by fungi in environmental protection, industry and medicine in *Microbial biotechnology in the laboratory and in practice. Theory, exercises and specialist laboratories* (ed. Długoński, J.) 391–398 (Łódź University Press & Jagiellonian University Press: Łódź-Kraków, Poland, 2021) e-ISBN 978–83–8220–420–9.
78. Jasińska, A. & Góralczyk-Bińkowska, A. Dyes in *Microbial biotechnology in the laboratory and in practice. Theory, exercises and specialist laboratories* (ed. Długoński, J.) 357–361 (Łódź University Press & Jagiellonian University Press: Łódź-Kraków, Poland, 2021) e-ISBN 978–83–8220–420–9.
79. Pueffel, C., Haase, D. & Priess, J.A. Mapping ecosystem services on brownfields in Leipzig, Germany. *Ecosyst. Serv.* **30**, A, 73–85. <https://doi.org/10.1016/j.ecoser.2018.01.011> (2018).
80. Długoński, A. & Szumański, M. Atlas Ekourbanistyczny Zielonej Infrastruktury Miasta Łodzi. Tom, I. Tereny Zieleni. Część, A. Parki Strefy Śródmiejskiej; [Eco-Urban Atlas of Green Infrastructure. Vol. 1a. Green Areas of Downtown], Łódzkie Towarzystwo Naukowe: Łódź, Poland, 2016. (in Polish, with English Summary).

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Author contributions

A.G.-B., A.D., P.B., A.J. and J.D. wrote the main manuscript text. A.G.-B. prepared Figs. 3, 4, 6, 7; A.D. prepared Figs. 1–2; P.B. prepared Fig. 5. A.G.-B., P.B., A.J. conducted the experiments. A.J. and J.D. supervised this study. All authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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Environmental and molecular approach to dye industry waste degradation by the ascomycete fungus *Nectriella pironii*

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Supplementary Table S1. Decolorization of dyes (25 mg L⁻¹) in the submerged cultures of *N. pironii*.

Time of cultivation [h]	Decolorization [%]				
	RR 120	AO 7	RB 5	AR 27	RO 16
48	79.31 ± 1.29	76.14 ± 0.93	26.44 ± 1.84	4.68 ± 1.60	0.31 ± 0.01
96	79.74 ± 4.29	78.28 ± 0.28	45.66 ± 2.52	5.21 ± 1.80	8.70 ± 0.22
120	80.80 ± 2.36	78.98 ± 0.67	56.20 ± 3.59	6.80 ± 1.90	11.29 ± 1.01



Article

Accelerated PAH Transformation in the Presence of Dye Industry Landfill Leachate Combined with Fungal Membrane Lipid Changes

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Abstract: The ascomycete fungus *Nectriella pironii*, previously isolated from soil continuously contaminated by dye industry waste, was used for the biodegradation of phenanthrene (PHE), benz[a]anthracene (B[a]A), and benz[a]pyrene (B[a]P). The degradation of polycyclic aromatic hydrocarbons (PAHs) by *N. pironii* was accelerated in the presence of landfill leachate (LL) collected from the area of fungus isolation. The rate of cometabolic elimination of PHE and B[a]P in the presence of LL was, respectively, 75% and 94% higher than in its absence. LC-MS/MS analysis revealed that PAHs were converted to less-toxic derivatives. The parallel lipidomic study showed changes in membrane lipids, including a significant increase in the content of phosphatidylcholine (PC) (almost double) and saturated phospholipid fatty acids (PLFAs) and a simultaneous reduction (twofold) in the content of phosphatidylethanolamine (PE) and unsaturated PLFAs, which may have promoted the fungus to PHE + LL adaptation. In the presence of PHE, an intense lipid peroxidation (fivefold) was observed, confirming the stabilization of the cell membrane and its extended integrity. Determining the course of elimination and adaptation to harmful pollutants is essential for the design of efficient bioremediation systems in the future.

Keywords: *Nectriella pironii*; PAHs; leachates; biodegradation; lipidomics



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1. Introduction

Rapid industrial and commercial growth in countries around the world has led to an alarming increase in the generation of municipal and industrial solid waste. In the European Union, the total waste generated by economic and household activities in 2018 was 2337 million tonnes, of which 101.7 million (4.4% of the total) was hazardous waste (HW). Since 2010, the total amount of HW generated has increased by 11.9%. The main sources of HW are agricultural fields, industries, domestic facilities, mining, and mineral-processing sites, as well as the natural environment. Only half of the generated HW is recovered and reused as fuel for producing electricity, while the remaining is landfilled [1].

Inappropriate management and disposal of HW landfills can pose a serious threat to human health and the environment. Landfill waste undergoes several physicochemical and biological processes that result in leachate. The composition of leachate depends mainly on the types of waste and residual moisture content in the landfill, the water filtration rate, the degradation stage, and the landfilling technology. Most leachates have a high concentration of dissolved organic matter, inorganic macrocomponents, and heavy metals and organic xenobiotics [2]. When leachate reaches the soil, surface, and groundwater around the

landfill, the pollutants present in it can adversely affect ecosystems and public health. Leachate is often transported to the wastewater treatment plant (WWTP) for management; however, it is generally not treated adequately and poses a risk to the surface waters that receive the effluent.

Polycyclic aromatic hydrocarbons (PAHs) are a major group of contaminants in the leachate derived from HW landfills. These are present in coal and crude oil and are released during the combustion of fossil fuels. They are also found as a component in different chemicals, including textile dyes, resins, plastics, pesticides, and pharmaceuticals, or as intermediates during their transformation [3]. As a result, PAHs can be detected in various ecosystems, such as soil [4–6], surface and groundwater [7,8], and air [9]. It was reported that the content of 16 PAHs in the soils around the chemical plant in Shanxi (China) amounted to 42.5 mg kg^{-1} [10]. Torres-Farradá et al. [11] noted that the half-life of PAHs in soils is 5.7 to 9.1 years. As a result of their hydrophobic nature, PAHs easily bind to solids in wastewater and accumulate in the sludge. Their presence was observed in sludge released from textile dye plants [12,13] and leachate from landfills [14–17]. The concentration of PAHs in leachate samples could vary (between ng L^{-1} and $\mu\text{g L}^{-1}$) as these samples have undesirable dissolved organic matter [18].

The toxicity, mutagenicity, and carcinogenicity of PAHs have been confirmed by several authors and also previously reviewed by Idowu et al. [19] and Patel et al. [20]. Based on available data on the possible human exposure to PAHs, as well as their toxicity and frequency of occurrence at HW sites, the United States Environmental Protection Agency (USEPA) has classified 16 PAHs as priority pollutants. These compounds include benz[a]anthracene, benz[a]pyrene, and phenanthrene [21]. Because PAHs are relatively poorly soluble in water and highly lipophilic, they can easily bioaccumulate in lipid tissue and remain persistent and resistant to biodegradation [22]. The selection of appropriate methods for PAH remediation is crucial and strongly dependent on the kind of the polluted matrix and the state of the environment. It is noteworthy that particular PAHs are characterized by diverse physical and chemical properties, which complicate the development of remediation techniques for PAH-contaminated sites. Hence, the exploitation of real matrices (soil or sewage) in studies regarding the removal of PAHs contributes to better predictions of their action.

Currently, available approaches for PAH treatment are classified into three groups: physical, chemical, and biological. Due to the hydrophobicity of PAHs, the most suitable method for their removal (especially in the case of high-molecular-weight PAHs (HMW-PAHs)) seems to be soil washing with a solvent (e.g., acetone, alcohol, hexane, dichloromethane, methyl ethyl ketone, toluene) [23]. The addition of surfactants may enhance the efficiency of soil washing by changing the solubility of PAHs, which, on the other hand, is highly dependent on some factors such as soil composition, the characteristics of PAHs, and the structure of the surfactant [24]. Among the physical methods for PAH removal from water, different membrane filtration approaches such as ultrafiltration, micro-/nanofiltration, and reverse osmosis or the application of several adsorbents, e.g., activated carbon, charcoal, biochar, magnetic nanomaterials, and graphene oxide have been used [25,26]. The most frequently described chemical methods are oxidation processes through common oxidants such as the Fenton reagent, potassium permanganate, sodium persulfate, and hydrogen peroxide [27].

One of the biological approaches to PAH oxidation is bioremediation through microbial enzymes such as oxygenases, dehydrogenases, lignin peroxidases, manganese peroxidases, laccases, and phenol oxidases. The reactions catalyzed by these enzymes are characterized by extreme efficiency in a wide range of temperatures and pH. On the other hand, for the isolation and purification of these enzymes from bacteria, fungi, and other living organisms, specific conditions and apparatus are necessary, which generates costs [20]. Moreover, free cells (FC) and immobilized cells (IC) find application in PAH biodegradation. For example, Partovinia and Naeimpoor [28] showed the entire biodegradation of PHE (250 ppm) after 7 days using FC and IC of a microbial consortium (screened from the Tehran

Oil Refinery activated sludge) in polyvinyl alcohol cryogel beads. A promising biological method of PAH elimination from contaminated environments is microbial degradation, which is influenced by the number and type of the microorganisms, conditions of the environment, and chemical structure of degraded chemicals. In the literature, there are more studies regarding PAH elimination by bacteria than by fungi. Ligninolytic as well as nonligninolytic fungi show the ability to oxidize PAHs through the secretion of extracellular enzymes [29,30]. In many reviews, it can be found that the advantage of biological methods over physicochemical methods is, above all, their low cost and the fact that by-products characterized by higher toxicity than the parent compounds are produced during biodegradation [31]. Nevertheless, biological methods are ineffective at high concentrations of PAHs; hence, in the first stage of pollutant elimination, physical/chemical methods are used to reduce the concentration of chemicals to an optimal level for further biological processes and to increase the rate of biodegradation.

Several microbial species are known to degrade PAHs, and most of them—isolated and recovered from contaminated soil or sediments—show great potential against the contaminants. Although many investigations have focused on bacteria species, for example, *Mycobacterium* [32], *Nocardia* [33], and *Sphingomonas* [34] involved in the degradation of PAHs as sole sources of carbon and energy, it has also been found that fungi have the ability to mineralize toxic xenobiotics, especially to degrade HMW-PAHs in contaminated soils [35,36]. Besides the biosynthesis of enzymes, an advantage of using fungi is their tolerance to extreme and fluctuating environments [37,38]. According to Medura et al. [39], Ascomycete fungi belonging to the class Sordariomycetes, as well as the phylum Zygomycota, which are widely distributed in the environment and in polluted soils, have adapted to metabolize a broad spectrum of organic compounds, including PAHs.

This work demonstrates the ability of the ascomycete fungus *Nectriella pironii* IM 6443 to convert PHE, B[a]A, and B[a]P. The fungus was isolated from soil contaminated with deleterious industrial dye, which has previously been shown to retain its activity in the presence of landfill leachate (LL) and to eliminate azo dyes and aromatic amines [40,41]. Both the isolation of the fungus from contaminated areas and its application in biodegradation are based on the adaptation of the microorganism to unfavorable conditions. The present work showed that *N. pironii* IM 6443 can efficiently degrade particular PAHs in the presence of leachate obtained from a landfill where HW from dye industry plants was disposed of. The formed metabolites were identified by gas chromatography–tandem mass spectrometry analysis. A lipidomic study was conducted to understand the mechanisms that allow the fungus to survive in difficult environmental conditions and to remove PAHs from the growth environment. From the application point of view, an extremely important part of this work was the use of landfill leachates in the research on the removal of PAHs as real environmental matrices. Another significant aspect was the choice of microorganism, which, due to the growth in soil continuously contaminated by deleterious waste, has developed mechanisms of adaptation and survival. The results of this work are expected to make a significant contribution to solving the problem of hazardous waste landfill management.

2. Materials and Methods

2.1. Reagents and LL

LL samples (L1 and L2) collected (according to ISO 5667-10 standard) from the landfill of the former “Boruta” Dye Industry Plant in Zgierz (Zgierz, Poland), then transported and stored with accordance to the ISO 5667-3:2018 standard, were kindly provided by the Voivodeship Inspectorate of Environmental Protection in Łódź, Poland. PHE, B[a]A, B[a]P, butylated hydroxytoluene (BHT), and thiobarbituric acid were purchased from Merck (Darmstadt, Germany). A stock solution (20 mg mL^{−1}) of PHE was prepared in ethanol (POCH, Gliwice, Poland), and that of B[a]A and B[a]P in dimethyl sulfoxide (Merck, Germany). The phospholipid standards were obtained from Avanti Polar Lipids (Alabaster, AL, USA). The methanol, chloroform, and ethyl acetate solvents were purchased from Avantor (Gliwice, Poland). All chemicals were of high-purity grade.

2.2. Microorganism and Growth Conditions

The fungal strain *N. pironii* IM 6443 was isolated from a soil sample collected on the territory of the former “Boruta” Dye Industry Plant in Zgierz [40,41], and since that time, 20 passages were carried out. The strain was maintained on ZT slants (4 g of glucose, 4 g of Difco yeast extract, 25 g of agar, 0.5 L of wort (12° Blg), up to 1 L of H₂O; pH 6.6–7.0) at 4 °C in the strain collection of the Department of Industrial Microbiology and Biotechnology, University of Lodz (Poland) [42]. The spores obtained from 10-day-old cultures of *N. pironii* on ZT slants were inoculated into 25 mL of WHI medium [42]. Initial preculture was maintained under conditions previously described [41].

2.3. Growth of *N. pironii* in Medium with PAHs and LL

LL was centrifuged at 6000 × *g* for 10 min at 4 °C and filtered (0.22 µm). Then, 2 mL of the preculture was inoculated into Sabouraud dextrose broth liquid medium (BioMaxima, Lublin, Poland) supplemented with 20% (*v/v*) LL and 20 mg L^{−1} of PAHs (PHE, B[a]A, B[a]P). Biotic controls (containing only the medium and biomass) and abiotic controls (containing the medium and PAHs or LL) were also prepared. Samples containing the same volume of ethanol or DMSO were also prepared (to exclude the inhibitory effect of solvents on fungal growth). All the prepared cultures were incubated in a rotary shaker (120 rpm) at 28 °C. The biomass was separated from the cultures by filtration under a slight vacuum and then dried at 105 °C. The dry weight of the fungus was estimated as described by Góralczyk-Bińkowska et al. [41].

2.4. Analytical Methods

2.4.1. Extraction and Quantification of PAHs

After incubation, fungal culture (20 mL) was homogenized in Falcon tubes with 10 mL of ethyl acetate and glass beads using a ball mill (MM 400, Retsch, Haan, Germany) for 3 min. The samples were vigorously shaken and centrifuged at 6500 × *g* for 10 min. After centrifugation, the upper layer was collected and transferred to a new Falcon tube. Next, 10 mL of ethyl acetate was added to the bottom phase and the extraction procedure was repeated. The samples were shaken and centrifuged again as described above. Then, the upper phase was collected and combined with the already-collected phase. The resulting mixtures were dehydrated with anhydrous ammonium sulfate and evaporated to dryness under reduced pressure. Before chromatographic analysis, the solvent-free residues were dissolved in 1 mL of ultrapure ethyl acetate.

The PAHs in the samples were identified and quantified by gas chromatography–mass spectrometry (GC–MS). Analysis was carried out in an Agilent Model 7890 gas chromatograph, equipped with a 5975C mass detector. The compounds were separated using an HP 5 MS methyl polysiloxane capillary column (30 m × 0.25 mm i.d. × 0.25 mm ft). The column temperature was maintained at 70 °C for 3 min, then increased to 250 °C at a rate of 10 °C min^{−1} and finally to 280 °C at a rate of 20 °C min^{−1}, and maintained at 280 °C for 7 min. Helium was used as a carrier gas at a flow rate of 1 mL min^{−1}. Split injection was performed at a port temperature of 250 °C. Identification of PAHs in the samples was carried out based on the retention time and abundance of quantification/confirmation ions in the authentic PAH standards (Merck, Germany) or based on the literature data.

2.4.2. Phospholipid Extraction and Analysis

The phospholipids of *N. pironii* were extracted following the Folch method with some modifications suggested by Bernat et al. [43]. Briefly, 100 mg of fungal biomass was transferred to a 2 mL Eppendorf tube containing 0.66 mL of chloroform, 0.33 mL of methanol, and glass beads. The samples were homogenized using a ball mill (MM 400, Retsch, Germany) at 30 rpm for 3 min (each cycle for 1 min). The resulting mixture was transferred to another Eppendorf tube, in which 0.2 mL of deionized water was added. The sample was subsequently vortexed and centrifuged at 2000 × *g* for 5 min. After separation into two layers, the bottom phase was collected, transferred to a new Eppendorf tube,

evaporated, and stored at -20°C . Before analysis, the extracts were dissolved in 1 mL of methanol.

Phospholipid analysis was performed by liquid chromatography–tandem mass spectrometry (LC–MS/MS), as described by Bernat et al. [43]. Measurement was carried out in an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) and a 4500 QTrap mass spectrometer (Sciex, Framingham, MA, USA). For this, 10 μL of the diluted sample was injected into a Kinetex C18 column (50 mm $\times$ 2.1 mm, particle size: 5 μm ; Phenomenex, Torrance, CA, USA). Water (A) and methanol (B), both containing 5 mM ammonium formate, were used as mobile phases. The solvent gradient began at 70% B, and then after 0.25 min, reached 95% B in 1 min, and continued at 95% B for 7 min before returning to the initial solvent composition (70% B) in 2 min. The column temperature was maintained at 40°C and the flow rate at $500\ \mu\text{L min}^{-1}$. The settings applied to the mass spectrometer ion source were as follows: spray voltage $-4500\ \text{V}$, curtain gas 25 psi, nebulizer gas 60 psi, auxiliary gas 50 psi, and temperature 600°C . Data analysis was performed in Analyst™ version 1.6.2 software (<https://sciex.com/products/software/analyst-software> (accessed on 16 May 2022); Sciex, Framingham, MA, USA).

2.4.3. Lipid Peroxidation Assay

The degree of lipid peroxidation was determined as the content of thiobarbituric acid reactive substances (TBARS) using a spectrophotometric method described by Yagi with a few modifications suggested by Slaba et al. [44]. Briefly, 0.5 g of fresh wet biomass was homogenized with 9 mL of deionized water and 50 μL of 7.2% BHT (in ethanol) in a ball mill (MM 400, Retsch, Germany) at $30\ \text{m s}^{-1}$ for 3 min. Then, 1 mL of the mixture was transferred to a Falcon tube containing 2 mL of 15 mM thiobarbituric acid in 15% trichloroacetic acid. The mixture was vortexed and heated at 95°C in a water bath for 30 min. After cooling, the samples were centrifuged at $2000\times g$ for 15 min and incubated at room temperature for 10 min. The absorbance of the supernatant was measured using a SPECORD 200-222U313 spectrophotometer (Analytik Jena, Jena, Germany) at a wavelength (λ) of 531 nm, and the value of nonspecific absorption at $\lambda = 600\ \text{nm}$ was subtracted. The results of the lipid peroxidation analysis were presented as micromoles of TBARS calculated per gram of wet biomass.

2.5. Statistical Analysis

Data were analyzed in STATISTICA version 13.3 software (<https://statistica.software.informer.com/13.3/> (accessed on 25 May 2022); StatSoft, Tulsa, OK, USA). All samples were prepared in triplicate and the experiments were performed twice. The results are presented as the mean $\pm$ standard deviation. The normality of the data distribution was verified using the Shapiro–Wilk test. Normal distributed data were analyzed using one-way analysis of variance with Tukey’s post hoc test. For results with a nonnormal distribution, the nonparametric Mann–Whitney U test was performed and the means were compared based on the p values for multiple comparisons.

3. Results and Discussion

3.1. Characteristics of the Landfill of the Former “Boruta” Dye Industry Plant and the LL

The HW landfill of the former “Boruta” Dye Industry Plant in Zgierz (Figure 1A) is one of the four most dangerous sources (known as hotspots) of industrial pollution in Poland that affects the blue-green infrastructure and the Baltic Sea [45–47]. In addition to having a high amount of different toxic chemicals, including PAHs and their metabolites, these four hotspots are located near water courses. By polluting groundwater, toxic chemicals from these hotspots enter nearby local rivers and subsequently the main Polish rivers, the Vistula and Oder, accumulating in the Baltic Sea, which is a cramped inland ($415,266\ \text{km}^2$) sea [48–52].

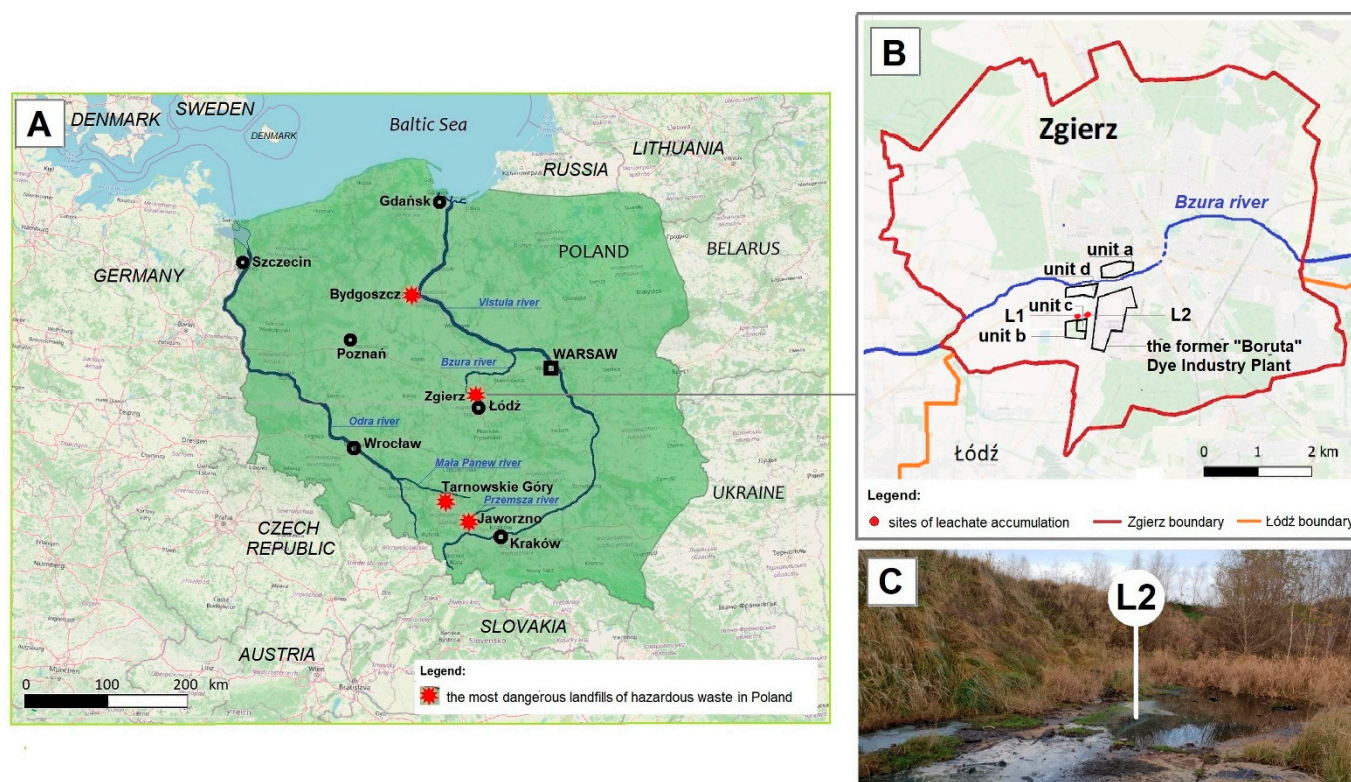


Figure 1. The most dangerous sources of industrial pollution in Poland (A) and location of the study region—the area of the former “Boruta” Dye Industry Plant and the landfill (B,C).

The total surface area of the landfill of the former “Boruta” Dye Industry Plant in Zgierz (Figure 1B) is 12 ha, which includes three units (marked as a, b, and c in Figure 1B). “Za Bzurą” which is the oldest landfill (Figure 1B, unit a), operating from the end of the 19th century up to 1995, has an area of 4.5 ha. This is followed by the ash, slag, and gypsum landfill (Figure 1B, unit b), with a surface area of 6.7 ha, which operated for about 10 years until 1996, and the last one “Kwatera I” (Figure 1B, unit c), with an area of 0.8 ha, in which a high amount of toxic industrial waste was deposited in the years 1995–2015 [41,53,54]. Although it occupies the smallest area, the last unit of the landfill poses the greatest threat to the environment. Due to improper slope protection, toxic leachates are not fully discharged into the municipal industrial WWTP (Figure 1B, unit d) and part of them accumulate in nearby areas (Figure 1C). Through groundwater, they pollute the Bzura and Vistula rivers one after another, and finally the Baltic Sea [54].

The main parameters of the leachates L1 and L2 that accumulate at the base of the landfill slope in the last years (2017–2021) are presented in Table 1. Throughout the period analyzed, the values of chemical oxygen demand (COD) and total organic carbon (TOC) were slightly and greatly higher, respectively, than the limits specified in the applicable standards, which is most likely due to the addition of municipal waste to the landfill [38].

Initially, petroleum hydrocarbon concentrations were at an acceptable level (below 15 mg L^{-1}) in both leachates. However, since autumn 2020, they have increased significantly in leachate L1, exceeding the allowed values. In addition to petroleum hydrocarbons, leachates also had an increased amount of iron, which is most likely related to corrosion of the metal containers used to store industrial waste [53,55].

Table 1. Summary of the most important results for 2017–2021 from the study of leachate from the Zgierz landfill based on the reports supported by the Voivodeship Inspectorate of Environmental Protection in Łódź (Poland) of 07 XI 2021.

Date of Sampling	COD _{Mang} (N = 125)		TOC (N = 30)		Iron (N = 10)		Petroleum Hydrocarbons (N = 15)	
	mg L ⁻¹ O ₂		mg L ⁻¹ C		mg L ⁻¹ Fe		mg L ⁻¹	
	L1	L2	L1	L2	L1	L2	L1	L2
17 X 2017	564 ± 150	568 ± 151	787 ± 118	753 ± 113	61.5 ± 11.7	58.4 ± 11.1	3.01 ± 0.81	3.80 ± 1.03
09 X 2018	247 ± 66	251 ± 67	540 ± 99	525 ± 101	27.3 ± 5.2	26.2 ± 5.0	5.40 ± 1.36	14.0 ± 3.5
09 IV 2019	460 ± 122	488 ± 130	725 ± 133	765 ± 140	22.3 ± 4.2	24.3 ± 4.6	1.02 ± 0.26	0.60 ± 0.15
11 VII 2019	520 ± 138	494 ± 131	660 ± 176	672 ± 179	13.2 ± 2.5	13.1 ± 2.5	6.13 ± 1.54	4.82 ± 1.21
21 V 2020	426 ± 113	428 ± 114	799 ± 146	798 ± 146	23.8 ± 4.5	23.1 ± 4.4	3.12 ± 0.79	2.08 ± 0.52
16 XI 2020	522.8 ± 138.5	348.8 ± 92.4	826 ± 151	1040.5 ± 190.4	28.86 ± 5.48	30.88 ± 5.87	23.7 ± 7.6	7.2 ± 2.3
09 III 2021	448 ± 119	460.8 ± 122.6	274.2 ± 50.2	294.7 ± 53.9	57 ± 11	31 ± 5.9	52.2 ± 16.7	1.91 ± 0.61
07 IX 2021	444 ± 118	392 ± 104	437 ± 80	372 ± 68	39.1 ± 7.4	32.9 ± 6.3	17.0 ± 5.4	14.8 ± 4.7

N—limit values, according to the Regulation of the Minister of Maritime Economy and Inland Navigation of 12 July 2019 on substances particularly harmful to the aquatic environment and the conditions to be met when discharged sewage into waters or ground, as well as when discharging rainwater or meltwater into waters or into aquatic devices. Dz. U. 2019 poz. 1311. <https://isap.sejm.gov.pl/isap.nsf/download.xsp/WDU20190001311/O/D20191311.pdf> (accessed on 21 May 2022).

3.2. Growth of *N. pironii* in the Presence of PAHs and Elimination of PAHs

The results of *N. pironii* growth in Sabouraud medium supplemented with 20 mg L⁻¹ of PHE, B[a]A, or B[a]P are presented in Figure 2A. In the presence of PHE, biomass production in the growth environment was reduced after 48 h. Due to the chemical structure of the PAHs used in this study and the fact that the resistance to biodegradation increases with the number of aromatic rings [56], it was expected that five-ring B[a]P would have the most toxic and inhibitory effect on fungal growth compared to three-ring PHE. However, the biomass content in the cultures containing B[a]A and B[a]P (10.07 ± 0.01 and 10.4 ± 0.24 g L⁻¹, respectively) was similar to that of the control cultures. Parallely, in cultures containing DMSO or ethanol (instead of PAH) to exclude their inhibitory effect, there were no significant differences in fungal growth.

Inhibition of *Penicillium* sp., *Talaromyces* sp., and *Hypoxylon* sp. growth in PHE-supplemented medium was previously reported by de Lima Souza et al. [57]. Lisowska et al. [58] also described that the spore germination of the filamentous fungus *Cunninghamella elegans* IM 1785/21Gp was limited by 70% after the addition of 25 mg L⁻¹ of PHE, and the growth of the fungal was completely inhibited in the presence of a higher concentration of PHE (50–100 mg L⁻¹). The data indicate that the tolerance of fungi to PAHs is influenced by various physical and chemical factors, including vapor pressure, solubility, and adsorption of these compounds [20]. Argumedo-Delira et al. [59] indicated a correlation between the growth of *Trichoderma* strains and factors such as vapor pressure and solubility of naphthalene, PHE, and B[a]P. According to Patel et al. [20] the vapor pressure of PHE, B[a]A, and B[a]P is equal to 6.8×10^{-4} , 2.5×10^{-6} , and 5.6×10^{-9} mmHg, respectively. Furthermore, the water solubility of PHE (1.1 mg L⁻¹) is significantly higher than that of B[a]A (0.011 mg L⁻¹) and B[a]P (0.0038 mg L⁻¹). These characteristics of PHE can promote its adsorption in growth medium and facilitate its better attachment to the mycelium during cultivation [20,59]. It should also be noted that the fungus *N. pironii* used in this study was isolated from soil contaminated with pollutants released from the textile industry, including dyes, heavy metals, aromatic amines, and PAHs. This microorganism may have adaptive mechanisms that allow it to survive in unfavorable environmental conditions and exhibit metabolic activity observed in studies on a laboratory scale. The application of environmental isolates has also been widely described in the literature. For example, the ascomycetous fungus *Myrothecium roridum* IM 6482 has been shown to have a high potential to eliminate bisphenol A (BPA) [60] and textile dyes [61]. Janicki et al. [62] described that the filamentous fungus *Umbelopsis isabellina* IM 833 adapted to

LL and degraded nonylphenol, 4-*tert*-octylphenol, 4-cumylphenol, and volatile phenols to less-toxic intermediates.

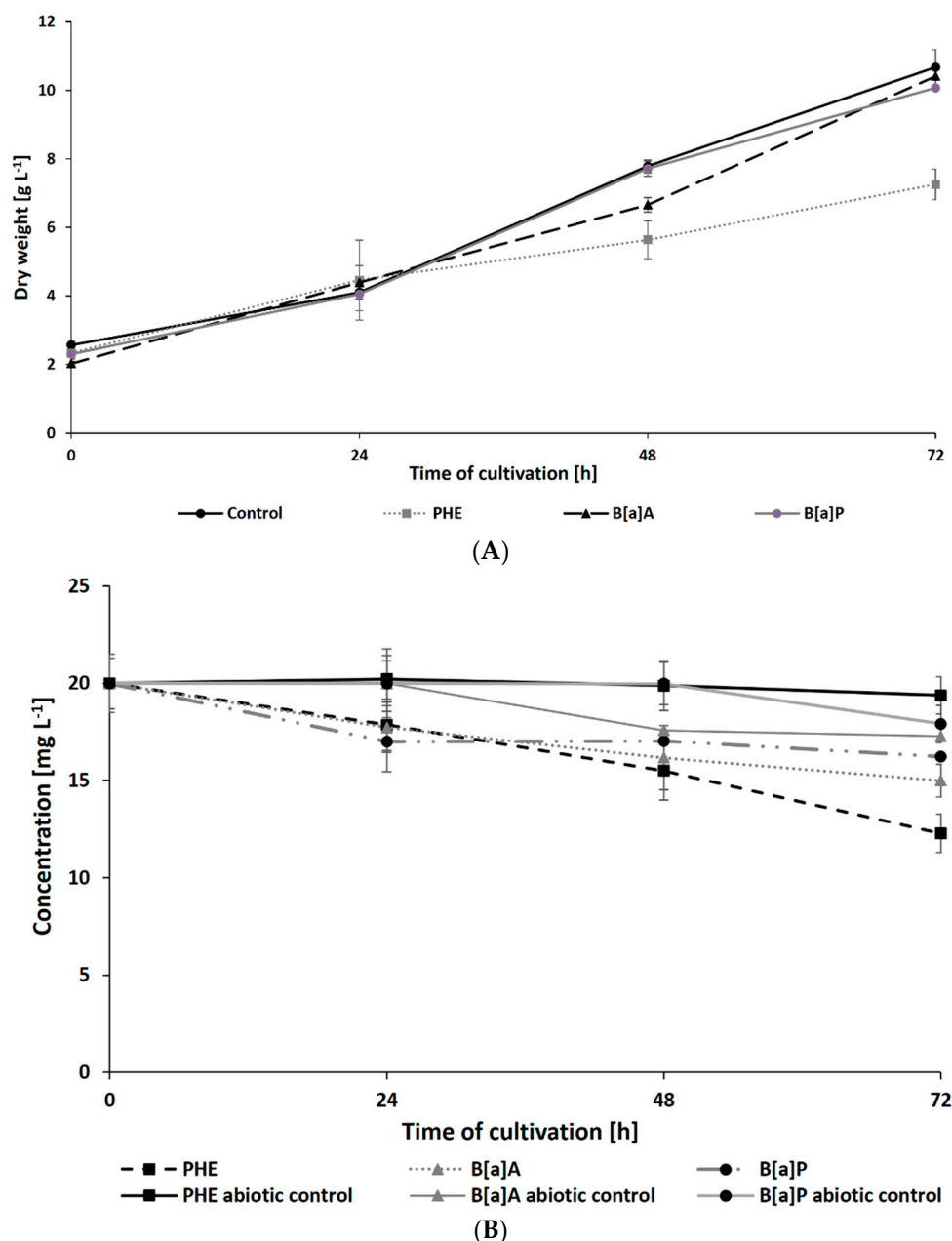
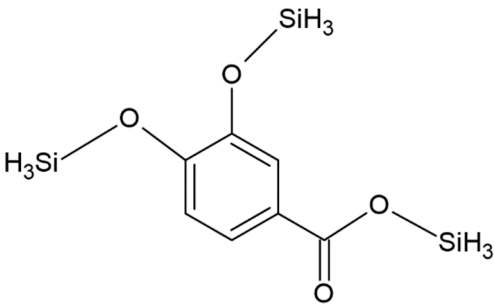
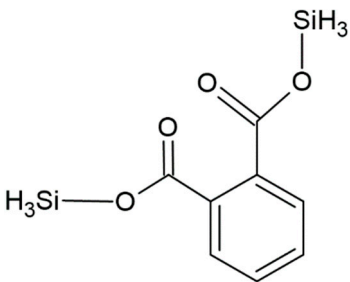
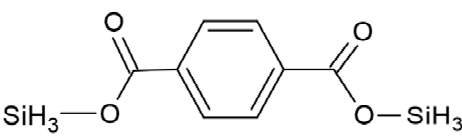
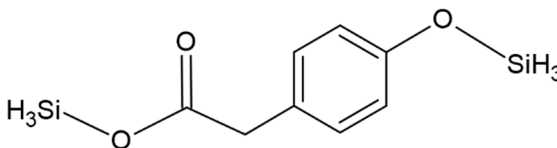


Figure 2. Dry weight of *N. pironii* (A) and PAH degradation of PAHs (B) during culture in Sabouraud medium containing PHE; B[a]A or B[a]P (20 mg L⁻¹).

The present study also analyzed the ability of *N. pironii* IM 6443 to attack PHE, B[a]A, and B[a]P. During the first 24 h of incubation, a decrease in the concentration of the tested PAHs (initial concentration: 20 mg L⁻¹) was observed (Figure 2B). After 72 h of incubation, the concentration of PHE, B[a]A, and B[a]P corresponded to 12.3 ± 0.99 , 14.9 ± 0.84 , and 16.2 ± 0.76 mg L⁻¹, respectively. As shown in Table 2, PAH metabolites formed during the reaction were identified by GC-MS analysis. The mass spectra of the obtained intermediates are shown in Figure S1, Supplementary Materials. According to previous studies, the main mechanism through which fungi degrade PAHs is the intracellular accumulation of chemicals followed by their transformation [29]. In the present study, the presence of a protocatechuic acid-TMS derivative (trimethylsilyl), which confirms the generation of protocatechuic acid, was observed in cultures of *N. pironii* containing PHE, B[a]A, and B[a]P.

Protocatechuic acid plays an important role in various metabolic pathways of PHE and substrate during ring-cleaving processes [63]. In addition, this phenolic acid has been found to occur naturally in most edible plants [64]. Furthermore, the study by Torres-Farradá et al. [11] revealed that this metabolite was the simplest and least toxic derivative, easily transformed to CO₂ and H₂O through biochemical reactions of microorganisms.

Table 2. Metabolites of degradation of PHE, B[a]A, and B[a]P obtained after *N. pironii* cultivation in the presence of leachate.

Compound Name	Chemical Structure	Fragment Ion (m/z)	Precursor Ion (m/z)
protocatechuic acid (tms)		193 281 355	370
phthalic acid (tms)		147 221 295	310
terephthalic acid (tms)		221 251 295	310
p-hydroxyphenylacetic acid (2 tms)		252 281	296

3.3. Influence of LL on *N. pironii* Growth and PAH Elimination

The growth of *N. pironii* in liquid Sabouraud medium containing PHE, B[a]A, or B[a]P was also studied in the presence of leachate collected from the HW landfill. As shown in Figure 3A,B, after 72 h of incubation, the biomass content in the culture containing 20% leachate and PHE ($7.06 \pm 0.35 \text{ g L}^{-1}$ for L1 and $7.34 \pm 0.17 \text{ g L}^{-1}$ for L2) was similar to that observed in the cultures containing PHE alone ($7.25 \pm 0.36 \text{ g L}^{-1}$). The addition of LL did not cause a significant decrease in fungal growth after 48 h of cultivation compared to the addition of PHE alone. Both alone and in combination with LL, PHE showed a more harmful effect on *N. pironii* than B[a]A or B[a]P.

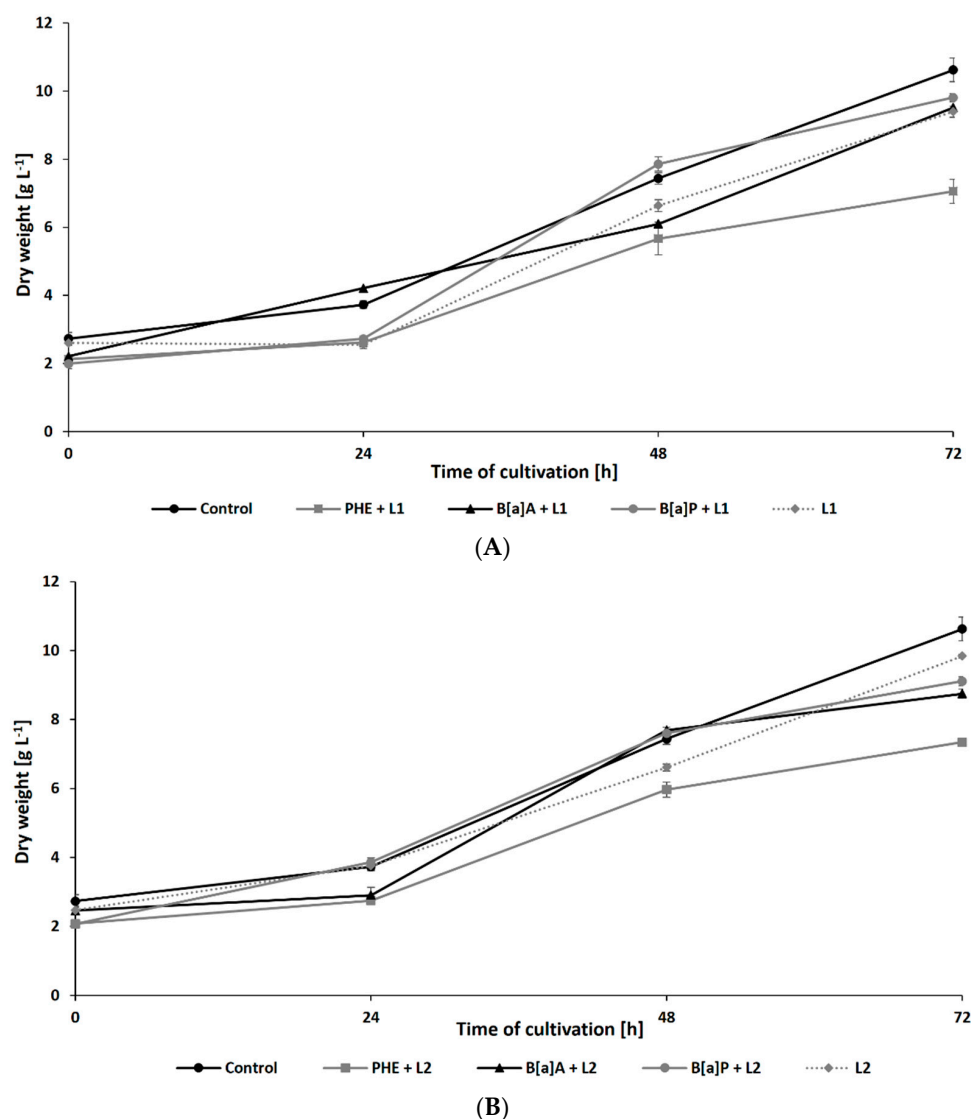


Figure 3. Dry weight of *N. pironii* during culture in Sabouraud medium containing PHE, B[a]A, or B[a]P (20 mg L⁻¹) with 20% of L1 (A) or L2 (B).

Surprisingly, the presence of leachates caused a significant increase in the rate of PAH degradation. After 72 h of cultivation, the residual concentration of PHE (initial concentration: 20 mg L⁻¹) in samples containing L1 and L2 reached 2.69 ± 0.21 and 2.99 ± 0.19 mg L⁻¹, respectively (Figure 4A). The addition of leachates also promoted the degradation of B[a]A and B[a]P by *N. pironii* (Figure 4B,C). The concentration of these compounds in cultures after 72 h of culture in the presence of L1 was 0.98 ± 0.02 and 0.65 ± 0.01 mg L⁻¹ and L2 was 3.09 ± 0.25 and 1.01 ± 0.08 mg L⁻¹, respectively. The higher removal efficiency of PAHs combined with LL compared to those of the individual compounds may suggest that leachates promote the transformation of these compounds. One of the possible explanations for this phenomenon is cometabolism. According to Acevedo et al. [65], cosubstrates could stimulate the secretion of catabolic enzymes and thus support the degradation of high-molecular-weight PAHs (HMW-PAHs). Janicki et al. [63] analyzed the toxicity of LL using the Phytotoxkit biotest based on the germination and growth of monocotyledonous *Sorghum saccharatum* and dicotyledonous *Lepidium sativum* and *Sinapsis alba*. In the present study, in addition to toxicity, the content of TOC was also determined. As shown in Table 1, the TOC content in L1 and L2 exceeded the permissible values. By comparing the biological oxygen demand (BOD₅) and the COD values determined in leachate and calculating their relationship, Lisowska et al. [66] stated that it is possible to

assess the susceptibility of wastewater to biodegradation. A BOD_5/COD ratio less than 0.2 means that the wastewater is practically nonbiodegradable, while values in the range of 0.5–0.7 indicate that the wastewater can be easily biodegraded. In the study by Janicki et al. [62], the BOD_5/COD ratio was 0.86, indicating that the leachate was readily biodegradable. LL also contains inorganic contaminants and petroleum hydrocarbons, which the fungus is known to be as a source of carbon and energy. This may stimulate catabolic processes as well as promote biodegradation and detoxication through cometabolism.

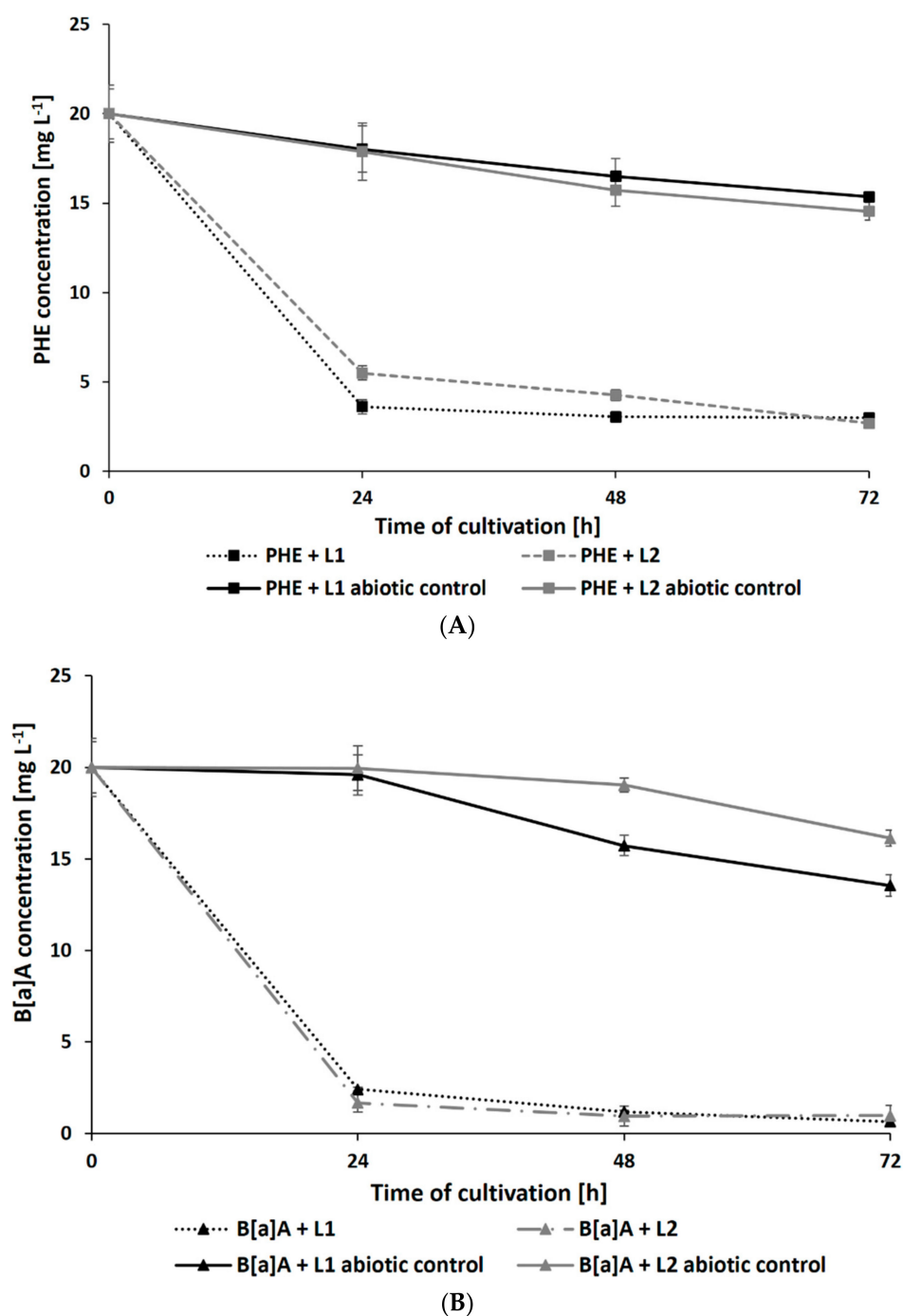


Figure 4. Cont.

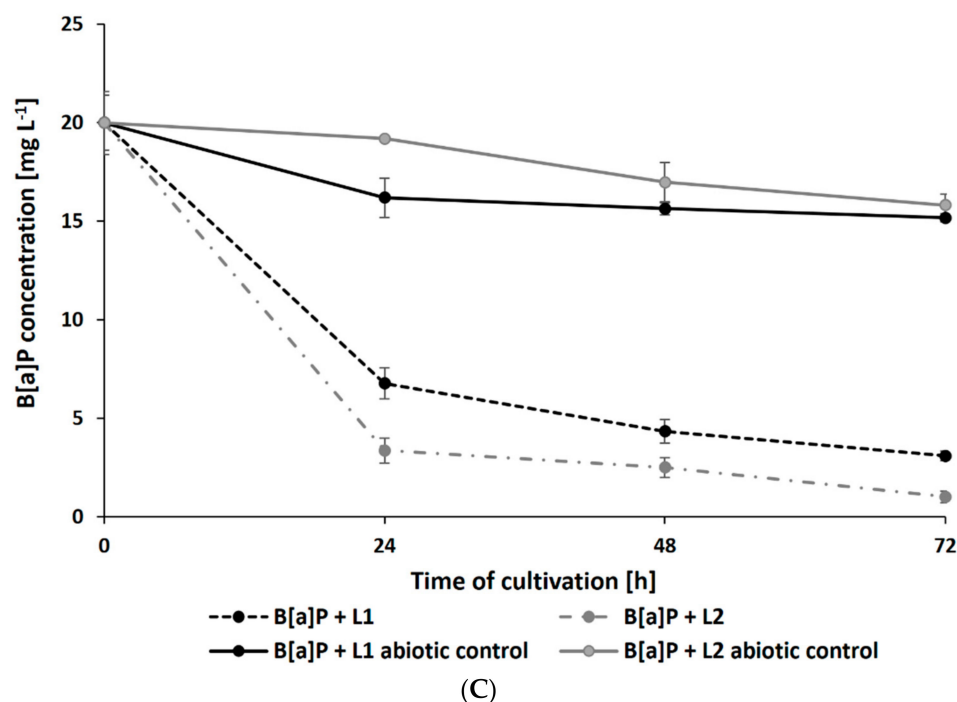


Figure 4. Residue of PHE (A), B[a]A (B), and B[a]P (C) during culture in Sabouraud medium with the addition of 20% leachate.

In this study, intermediate analysis after 48 and 72 h of *N. pironii* in the medium supplemented with PHE and LL revealed the presence of protocatechuic acid-TMS and phthalic acid-TMS derivatives. A protocatechuic acid-TMS derivative was also detected in cultures containing B[a]A + L1 and B[a]P + L2, indicating that *N. pironii* can convert B[a]P to less-toxic derivatives. This is an important finding because B[a]P, which consists of five fused aromatic rings, is highly stable and resistant to biodegradation. Furthermore, due to the high energy needed to break carbon–carbon bonds, B[a]P is characterized by low bioavailability [67].

3.4. Influence of PAHs on Membrane Condition—Lipidomic Study

The cell wall and cell membrane of microorganisms are the structures that come into direct contact with hazardous substances when they surround the cell. Microbial cell membranes play a dual role in microorganism–pollutant interactions. First, they transport pollutants inside the cell, after which the contaminants pass through the cell membranes via ion channels, self-diffusion, or transmembrane carriers and undergo intracellular changes, including complete degradation and detoxification. Second, membranes, together with the cell wall, act as a barrier that protects cells from the toxic effects of contaminants. By changing their structure, microbial membranes facilitate or limit the penetration of various substances into and out of the cell [68,69]. Membrane lipids are particularly sensitive to toxic chemicals in the surrounding environment. It has been well documented that toxic compounds induce qualitative and quantitative changes in the composition of membrane-forming lipids and thus disrupt the integrity of the cell membrane [70–72]. Omic methods, including lipidomics, have been used over the years to explore the mechanisms through which fungi adapt to stressful conditions.

In this study, an LC–MS/MS analysis was performed to investigate the changes that occur in the composition of membrane lipids due to the presence of PAHs in the growth medium. The analysis focused on determining the qualitative and quantitative composition of phospholipids, which are critical structural components of fungal cell membranes. Phospholipids can be divided into various classes, namely phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol

(PI), phosphatidylglycerol (PG), and phosphatidic acid (PA). The results of the analysis revealed that PC and PE are the primary components of the *N. pironii* membrane (Table S1, Supplementary Materials). However, their contribution to the total phospholipid pool varied depending on the culture conditions. At 24 h of cultivation, the PC content in the control cultures (containing neither PAH nor LL) was $36.15 \pm 2.18\%$ (Table 3), while it was slightly higher in the presence of leachate (20%), and was $59.98 \pm 1.75\%$ and $68.43 \pm 3.45\%$, respectively, in the cultures grown in the presence of PHE and PHE + leachate. Changes in PC and PE influenced the PC/PE ratio, which reflects the degree of integrity and permeability of the cell membrane and can be influenced by environmental conditions [69,73]. An increase in PC content in the lipid bilayer stabilizes it, while an increase in PE, a nonlayered lipid that forms a hexagonal phase, reduces the fluidity of the membrane and increases its permeability, leading to loss of cell integrity [73]. In *N. pironii* cultures incubated with PHE alone or in combination with leachate, the PC/PE ratio was, respectively, more than 2 and 4 times higher than that in the control cultures or in the cultures containing only leachate. Interestingly, such differences were observed only in cultures containing PHE. In cultures containing B[a]P or B[a]A instead of PHE, the PC/PE ratio only slightly differed from that of the control cultures. An increase in the PC/PE ratio was found in *C. elegans* cells cultured in the presence of lipophilic tributyltin (TBT) [74]. In another study, an increase in the PC/PE ratio was observed in *Metarhizium robertsii* cultures exposed to atrazine [75] or *Beauveria bassiana* cultures exposed to insecticides λ -cyhalothrin, α -cypermethrin, and deltamethrin [76]. On the other hand, a study showed a decrease in the PC/PE ratio, possibly leading to a decrease in membrane fluidity in the biomass of *Trichoderma harzianum* IM 0961 exposed to 2,4-D [72] or *M. robertsii* treated with TBT [70]. This suggests that microorganisms use different strategies to alter phospholipid membranes to maintain cell stability in the presence of various environmental contaminants. An increase in the PC/PE ratio has been observed in more-resistant fungi that are capable of maintaining the integrity of the lipid bilayer. In turn, the decrease in film fluidity resulting from the reduction in the PC/PE ratio allows for the gradual absorption of pollutants and their intracellular degradation, which is possible in the presence of low concentrations of pollutants or compounds of moderate toxicity [77]. In the study by Shon et al. [78], the bacterial strain *Sphingopyxis soli* KIT-001 cultivated in the presence of PHE showed an accelerated degradation of this compound under aerobic conditions. The level of PC and PG classes was also observed to increase, while that of PE and PA classes decreased significantly. However, data explaining the alteration occurring in the filamentous fungal membrane in response to PAHs are limited.

Table 3. The composition of phospholipids (PL) and PC/PE ratio determined in *N. pironii* cells after 24 h of cultivation with PHE, L1, or PHE + L1, or without the compounds tested. PA, phosphatidic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine. Data represent mean $\pm$ SE. * $p \leq 0.05$, ** $p \leq 0.01$.

PL Class	Control Biotic	L1	PHE	PHE + L1	B[a]A	B[a]A + L1	B[a]P	B[a]P + L1
PA	0.65 ± 0.02	$1.96 \pm 0.11^{**}$	$0.42 \pm 0.02^{*}$	$0.19 \pm 0.01^{**}$	0.71 ± 0.03	$1.20 \pm 0.04^{**}$	0.43 ± 0.02	0.88 ± 0.07
PC	36.15 ± 2.18	$40.60 \pm 2.54^{*}$	$59.98 \pm 1.75^{**}$	$68.43 \pm 3.45^{**}$	$45.11 \pm 1.45^{**}$	$42.06 \pm 3.36^{**}$	$40.51 \pm 3.54^{*}$	$47.67 \pm 2.39^{*}$
PE	58.08 ± 2.36	$52.45 \pm 3.17^{*}$	$38.48 \pm 2.14^{**}$	$24.51 \pm 1.19^{**}$	$51.26 \pm 3.36^{*}$	$48.09 \pm 1.56^{**}$	55.70 ± 4.25	$45.49 \pm 3.35^{*}$
PI	5.12 ± 0.41	4.99 ± 0.18	$1.12 \pm 0.04^{**}$	6.88 ± 0.23	2.92 ± 0.14	$8.65 \pm 0.37^{**}$	3.37 ± 0.21	5.96 ± 0.46
PC/PE	0.62 ± 0.03	0.77 ± 0.03	$1.56 \pm 0.07^{*}$	$2.79 \pm 0.25^{**}$	0.88 ± 0.05	0.87 ± 0.04	0.73 ± 0.04	1.05 ± 0.09

On the basis of the obtained results, it can be concluded that PHE influences the stability of the *N. pironii* membrane, which was manifested by an increase in the content of PC, enhancing the membrane integrity. These findings, together with observations regarding fungal growth (strong growth limitation in the presence of PHE), indicate that among the compounds tested, PHE caused the highest toxicity to the fungus. This was

confirmed by the analysis of individual phospholipid species. The properties of biological membranes depend not only on the relative proportions of phospholipids but also on the degree of unsaturation of fatty acids in the hydrophobic part of the lipid bilayer. Analysis of the distribution of hydrocarbon chains of phospholipid fatty acids (PLFAs) showed that palmitic acid (C16:0) was the predominant saturated fatty acid, and oleic (C18:1) and linoleic acid (C18:2) were the main unsaturated species (Figure 5). In the presence of PHE, a significant decrease was observed in the content of PC 18:2/18:2; PE 18:2/18:2, and PE 18:2/18:1, accompanied by an increase in saturated forms such as PC 16:0/18:2 and PC 16:0/18:1. This confirms that PHE improves the integrity of the fungal membrane. Bernat et al. [74] observed a similar effect in the mycelium of the zygomycete fungus *C. elegans* exposed to tributyltin. Heavy metal ions have been shown to change the saturation of phospholipids in fungal hyphae. For example, cadmium and nickel ions enhanced phospholipid saturation in the mycelium of *Paecilomyces marquandii*, representing the Ascomycota cluster [44]. It was concluded that increased saturation allowed the fungus to maintain adequate membrane fluidity while mitigating the stress induced by exposure to Cd and Ni ions. Similarly, in the present study, changes in the lipid composition may have favored the fungus to adapt to unfavorable conditions. This phenomenon seems to be a universal strategy for adapting fungi belonging to different phyla.

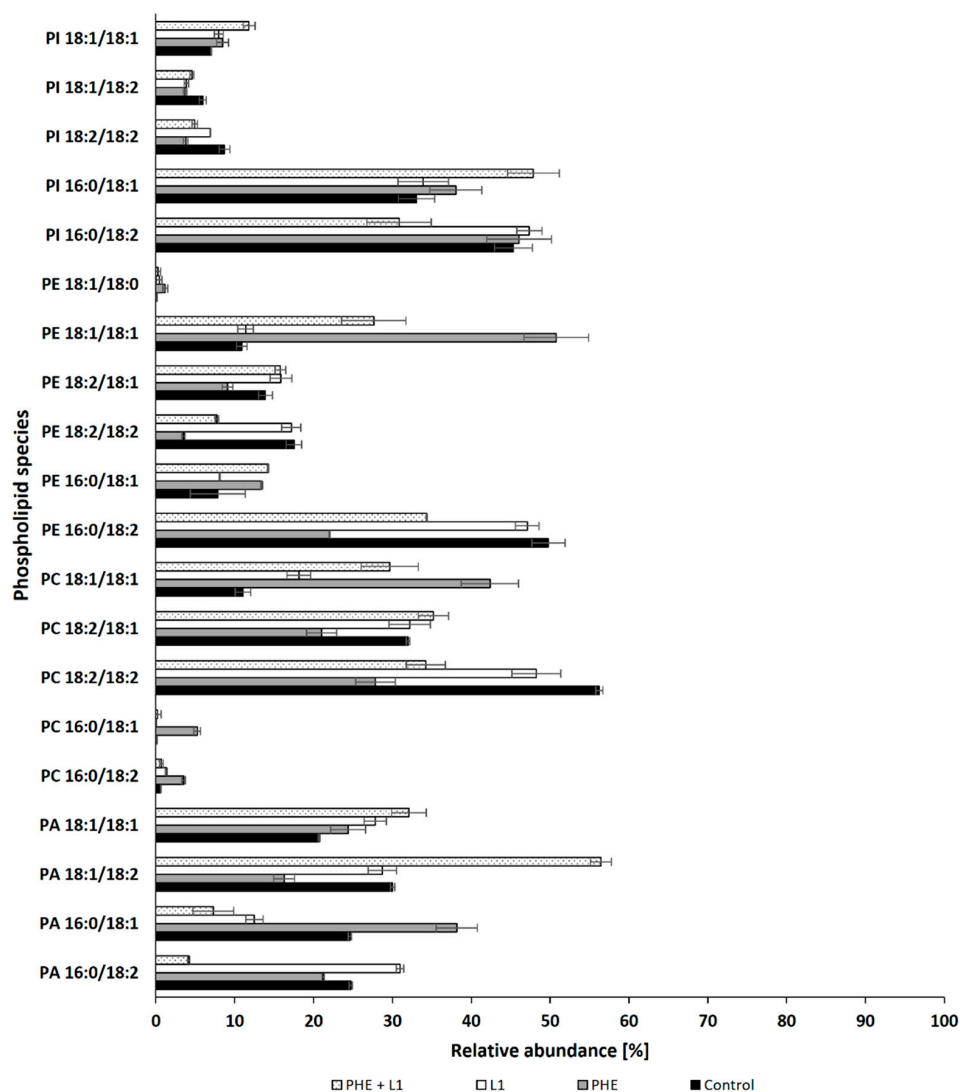


Figure 5. Relative abundances (%) for the main species of phospholipids in cultures of *N. pirionii* supplemented with PHE, L1, or PHE + L1.

Another parameter indicating the cellular changes resulting from exposure to PHE and/or leachate is the content of TBARS, which are markers of lipid peroxidation. As illustrated in Figure 6, exposure of the *N. pironii* mycelium to PHE (20 mg L⁻¹) caused a statistically significant increase in the level of TBARS ($p < 0.01$) from 2.34 ± 0.21 to $12.15 \pm 0.53 \mu\text{M g}^{-1}$ wet biomass during the first 24 h of cultivation. Surprisingly, the TBARS content in the biomass decreased after 48 h of incubation and was at a similar level in all tested samples (between 1.8 ± 0.03 and $2 \pm 0.08 \mu\text{M g}^{-1}$). The highest TBARS content was marked in the 2,4-D-treated mycelium of *T. harzianum* than in the untreated fungus [72]. Jasińska et al. [60] also observed an increase in the level of TBARS in the mycelium exposed to BPA compared to *M. roridum* biomass cultured without xenobiotics. In the present study, the level of lipid peroxidation initially increased under the influence of PHE and then decreased to the level of the control, indicating that *N. pironii* adapted to stress and/or detoxified the pollutant after its biotransformation to less-toxic intermediates.

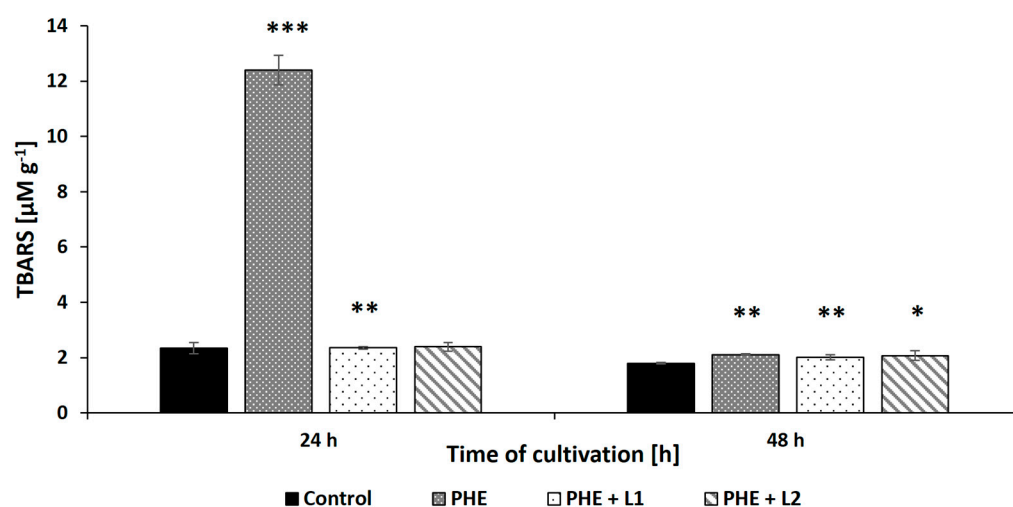


Figure 6. The TBARS content in the biomass of *N. pironii* exposed to PHE, L1, or PHE + L1. Data represent mean $\pm$ SE. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4. Conclusions

This study demonstrated that the ascomycete fungus *N. pironii*, isolated from a soil collected from the landfill of the former “Boruta” Dye Industry Plant in Zgierz (Poland), eliminated three-, four-, and five-ring PAHs from the growth medium. The fungal strain rapidly transformed the compounds tested in the presence of toxic LL collected from the area of the former “Boruta” Dye Industry Plant in Zgierz, which is also the site of fungal isolation. The observed findings confirmed the cometabolic nature of PAH elimination. LL is a mixture of organic and inorganic compounds and is characterized by high BOD₅/COD. Thus, the conversion of PHE, B[a]A, and B[a]P was substantially accelerated during the growth of the fungus in the liquid medium. Furthermore, the identified intermediates were less toxic than the parent compounds. Additionally, PHE influenced the stability of the fungal membrane, which was manifested by changes in the classes of phospholipids, including an increase in PC content that improves membrane integrity and a simultaneous decrease in PLFA unsaturation. The results also showed enhanced lipid peroxidation, indicated by an enhanced level of TBARS in the PHE-treated mycelium. Lipidomic analysis revealed that *N. pironii* modulates its membrane composition to stabilize the cell and adapt to PAH-induced stress conditions. The present study suggests that knowledge about the course of biodegradation, the nature of the intermediates formed, and the mechanism of fungal adaptation to unfavorable conditions can be beneficial in further research with the aim of developing methods for the elimination of pollutants of multiple components. Since social and economic development leads to an intensification in generating waste, it is extremely important to decrease the amount using properly selected methods. The

suitable selection of management and storage methods is also crucial to avoid releasing hazardous waste in landfills to the environment and thus posing the risk to society. The use of ascomycete fungus *N. pironii* belonging to Ascomycota in PAH elimination was also noteworthy in this work. The obtained results may contribute to broadening the knowledge regarding PAHs removal by fungi, especially Ascomycota. To sum up, the results presented in this work in combination with other conventional physical/chemical methods might find an application in PAH removal on a large scale.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijerph192113997/s1>, Figure S1: Mass spectra of qualitative analysis of PHE, B[a]A and B[a]P degradation by *N. pironii* cultivated with the presence of leachate; Table S1: The phospholipids composition and PC/PE ratio determined in the *N. pironii* cells after 24, 48, and 72 h of cultivation with PHE, L1 or PHE + L1, or without the tested compounds.

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References

1. Eurostat Statistics Explained—Waste Statistics. Available online: https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Waste_statistics#Hazardous_waste_generation (accessed on 10 May 2022).
2. Baderna, D.; Caloni, F.; Benfenati, E. Investigating landfill leachate toxicity in vitro: A review of cell models and endpoints. *Environ. Int.* **2019**, *122*, 21–30. [CrossRef] [PubMed]
3. Abdel-Shafy, H.I.; Mansour, M.S.M. A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation. *Egypt. J. Pet.* **2016**, *25*, 107–123. [CrossRef]
4. Cachada, A.; Pato, P.; Rocha-Santos, T.; Ferreira da Silva, E.; Duarte, A.C. Levels, sources and potential human health risks of organic pollutants in urban soils. *Sci. Total Environ.* **2012**, *430*, 184–192. [CrossRef]
5. Riccardi, C.; Di Filippo, P.; Pomata, D.; Di Basilio, M.; Spicaglia, S.; Buiaelli, F. Identification of hydrocarbon sources in contaminated soils of three industrial areas. *Sci. Total Environ.* **2013**, *450*, 13–21. [CrossRef] [PubMed]
6. Jia, T.; Guo, W.; Xing, Y.; Lei, R.; Wu, X.; Sun, S.; He, Y.; Liu, W. Spatial distributions and sources of PAHs in soil in chemical industry parks in the Yangtze River Delta, China. *Environ. Pollut.* **2021**, *283*, 117121. [CrossRef]
7. Liu, B.; Liu, Y.; Bu, Q.; Cao, H.; Zhang, H.; Liu, C.; He, X.; Yun, M. Polycyclic Aromatic Hydrocarbons in surface water from Wuhai and Lingwu Sections of the Yellow River: Concentrations, sources, and ecological risk. *J. Chem.* **2020**, *2020*, 8458257. [CrossRef]
8. Montuori, P.; De Rosa, E.; Di Duca, F.; Provisiero, D.P.; Sarnacchiaro, P.; Nardone, A.; Triassi, M. Estimation of Polycyclic Aromatic Hydrocarbons pollution in Mediterranean Sea from Volturno River, Southern Italy: Distribution, Risk Assessment and Loads. *Int. J. Environ. Res. Public Health* **2021**, *18*, 1383. [CrossRef] [PubMed]
9. Pietrogrande, M.C.; Demaria, G.; Russo, M. Determination of particulate polycyclic aromatic hydrocarbons in ambient air by gas chromatography-mass spectrometry after molecularly imprinted polymer extraction. *J. Environ. Sci.* **2023**, *124*, 644–654. [CrossRef] [PubMed]
10. Jiao, H.; Wang, Q.; Zhao, N.; Jin, B.; Zhuang, X.; Bai, Z. Distributions and Sources of Polycyclic Aromatic Hydrocarbons (PAHs) in Soils around a Chemical Plant in Shanxi, China. *Int. J. Environ. Res. Public Health* **2017**, *14*, 1198. [CrossRef] [PubMed]
11. Torres-Farradá, G.; Manzano-León, A.M.; Rineau, F.; Ramos Leal, M.; Thijs, S.; Jambon, I.; Put, J.; Czech, J.; Guerra Rivera, G.; Carleer, R.; et al. Biodegradation of polycyclic aromatic hydrocarbons by native *Ganoderma* sp. strains: Identification of metabolites and proposed degradation pathways. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 7203–7215. [CrossRef]

12. Salihoglu, N.K.; Salihoglu, G.; Tasdemir, Y.; Cindoruk, S.S.; Yolsal, D.; Ogulmus, R.; Karaca, G. Comparison of Polycyclic Aromatic Hydrocarbons levels in sludges from municipal and industrial wastewater treatment plants. *Arch. Environ. Contam. Toxicol.* **2010**, *58*, 523–534. [\[CrossRef\]](#)
13. Ning, X.A.; Lin, M.Q.; Shen, L.Z.; Zhang, J.H.; Wang, J.Y.; Wang, Y.J.; Yang, Z.Y.; Liu, J.Y. Levels, composition profiles and risk assessment of polycyclic aromatic hydrocarbons (PAHs) in sludge from ten textile dyeing plants. *Environ. Res.* **2014**, *132*, 112–118. [\[CrossRef\]](#)
14. Xu, Y.; Zhou, Y.; Wang, D.; Chen, S.; Liu, J.; Wang, Z. Occurrence and removal of organic micropollutants in the treatment of landfill leachate by combined anaerobic-membrane bioreactor technology. *J. Environ. Sci.* **2008**, *20*, 1281–1287. [\[CrossRef\]](#)
15. Wojciechowska, E. Removal of persistent organic pollutants from landfill leachates treated in three constructed wetland systems. *Water Sci. Technol.* **2013**, *68*, 1164–1172. [\[CrossRef\]](#)
16. Ghosh, P.; Gupta, A.; Thakur, I.S. Combined chemical and toxicological evaluation of leachate from municipal solid waste landfill sites of Delhi, India. *Environ. Sci. Pollut. Res. Int.* **2015**, *22*, 9148–9158. [\[CrossRef\]](#)
17. Smol, M.; Włodarczyk-Makuła, M.; Mielczarek, K.; Bohdziewicz, J.; Włóka, D. The use of reverse osmosis in the removal of PAHs from municipal landfill leachate. *Polycycl. Aromat. Compd.* **2016**, *36*, 20–39. [\[CrossRef\]](#)
18. Pisharody, L.; Gopinath, A.; Malhotra, M.; Nidheesh, P.V.; Kumar, M.S. Occurrence of organic micropollutants in municipal landfill leachate and its effective treatment by advanced oxidation processes. *Chemosphere* **2022**, *287*, 132216. [\[CrossRef\]](#)
19. Idowu, O.; Semple, K.T.; Ramadass, K.; O'Connor, W.; Hansbro, P.; Thavamani, P. Beyond the obvious: Environmental health implications of polar polycyclic aromatic hydrocarbons. *Environ. Int.* **2019**, *123*, 543–557. [\[CrossRef\]](#)
20. Patel, A.B.; Shaikh, S.; Jain, K.R.; Desai, C.; Madamwar, D. Polycyclic Aromatic Hydrocarbons: Sources, toxicity, and remediation approaches. *Front. Microbiol.* **2020**, *11*, 562813. [\[CrossRef\]](#)
21. Bojes, H.K.; Pope, P.G. Characterization of EPA's 16 priority pollutant polycyclic aromatic hydrocarbons (PAHs) in tank bottom solids and associated contaminated soils at oil exploration and production sites in Texas. *Regul. Toxicol. Pharmacol.* **2007**, *47*, 288–295. [\[CrossRef\]](#)
22. Ghosal, D.; Ghosh, S.; Dutta, T.K.; Ahn, Y. Current State of Knowledge in Microbial Degradation of Polycyclic Aromatic Hydrocarbons (PAHs): A Review. *Front. Microbiol.* **2016**, *7*, 1369. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Peng, T.; Luo, A.; Kan, J.; Liang, L.; Huang, T.; Hu, Z. Identification of a ring-hydroxylating dioxygenases capable of anthracene and benz[a]anthracene oxidation from *Rhodococcus* sp. P14. *J. Mol. Microbiol. Biotechnol.* **2018**, *28*, 183–189. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Gitipour, S.; Sorial, G.A.; Ghasemi, S.; Bazyari, M. Treatment technologies for PAH-contaminated sites: A critical review. *Environ. Monit. Assess.* **2018**, *190*, 1–17. [\[CrossRef\]](#)
25. Cevallos-Mendoza, J.; Amorim, C.G.; Rodríguez-Díaz, J.M.; Montenegro, M.D.C.B.S.M. Removal of Contaminants from Water by Membrane Filtration: A Review. *Membranes* **2022**, *12*, 570. [\[CrossRef\]](#)
26. Xinhong, G.; Ying, T.; Wenjie, R.; Jun, M.; Christie, P.; Yongming, L. Optimization of ex-situ washing removal of polycyclic aromatic hydrocarbons from a contaminated soil using nano-sulfonated graphene. *Pedosphere* **2017**, *27*, 527–536. [\[CrossRef\]](#)
27. Shih, Y.J.; Binh, N.T.; Chen, C.W.; Chen, C.F.; Dong, C.D. Treatability assessment of polycyclic aromatic hydrocarbons contaminated marine sediments using permanganate, persulfate and Fenton oxidation processes. *Chemosphere* **2016**, *150*, 294–303. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Partovinia, A.; Naeimpoor, F. Comparison of phenanthrene biodegradation by free and immobilized cell systems: Formation of hydroxylated compounds. *Environ. Sci. Pollut. Res. Int.* **2014**, *21*, 5889–5898. [\[CrossRef\]](#)
29. Marco-Urrea, E.; García-Romera, I.; Aranda, E. Potential of non-ligninolytic fungi in bioremediation of chlorinated and polycyclic aromatic hydrocarbons. *N. Biotechnol.* **2015**, *32*, 620–628. [\[CrossRef\]](#) [\[PubMed\]](#)
30. Pozdnyakova, N.N. Involvement of the ligninolytic system of white-rot and litter-decomposing fungi in the degradation of polycyclic aromatic hydrocarbons. *Biotechnol. Res. Int.* **2012**, *2012*, 243217. [\[CrossRef\]](#)
31. Dhar, K.; Panneerselvan, L.; Venkateswarlu, K.; Megharaj, M. Efficient bioremediation of PAHs-contaminated soils by a methy-lotrophic enrichment culture. *Biodegradation* **2022**, *33*, 575–591. [\[CrossRef\]](#)
32. Kweon, O.; Kim, S.J.; Sutherland, J.B.; Cerniglia, C.E. Novel insights into Polycyclic Aromatic Hydrocarbon biodegradation pathways using systems biology and bioinformatics approaches. In *Microbial Biodegradation from Omics to Function and Application*, 1st ed.; Długoński, J., Ed.; Caister Academic Press: Norfolk, UK, 2016; pp. 143–166.
33. Azadi, D.; Shojaei, H. Biodegradation of polycyclic aromatic hydrocarbons, phenol and sodium sulfate by *Nocardia* species isolated and characterized from Iranian ecosystems. *Sci. Rep.* **2020**, *10*, 21860. [\[CrossRef\]](#)
34. Hesham, A.; Mawad, A.M.; Mostafa, Y.M.; Shoreit, A. Biodegradation ability and catabolic genes of petroleum-degrading *Sphingomonas koreensis* strain ASU-06 isolated from Egyptian oily soil. *BioMed Res. Int.* **2014**, *2014*, 127674. [\[CrossRef\]](#) [\[PubMed\]](#)
35. Aranda, E.; Godoy, P.; Reina, R.; Badia-Fabregat, M.; Rosell, M.; Marco-Urrea, E.; García-Romera, I. Isolation of Ascomycota fungi with capability to transform PAHs: Insights into the biodegradation mechanisms of *Penicillium oxalicum*. *Int. Biodeter. Biodegr.* **2017**, *122*, 141–150. [\[CrossRef\]](#)
36. Prenafeta-Boldú, F.X.; Kuhn, A.; Luykx, D.M.A.M.; Anke, H.; van Groenestijn, J.W.; de Bont, J.A.M. Isolation and characterisation of fungi growing on volatile aromatic hydrocarbons as their sole carbon and energy source. *Mycolog. Res.* **2001**, *105*, 477–484. [\[CrossRef\]](#)

37. Worrich, A.; Stryhanyuk, H.; Musat, N.; König, S.; Banitz, T.; Centler, F.; Frank, K.; Thullner, M.; Harms, H.; Richnow, H.H. Mycelium-mediated transfer of water and nutrients stimulates bacterial activity in dry and oligotrophic environments. *Nat. Commun.* **2017**, *8*, 15472. [CrossRef]
38. González-Abradelo, D.; Pérez-Llano, Y.; Peidro-Guzmán, H.; Sánchez-Carbente, M.D.R.; Folch-Mallol, J.L.; Aranda, E.; Vaidyanathan, V.K.; Cabana, H.; Gunde-Cimerman, N.; Batista-García, R.A. First demonstration that ascomycetous halophilic fungi (*Aspergillus sydowii* and *Aspergillus destruens*) are useful in xenobiotic mycoremediation under high salinity conditions. *Bioresour. Technol.* **2019**, *279*, 287. [CrossRef]
39. Medaura, M.C.; Guivernau, M.; Moreno-Ventas, X.; Prenafeta-Boldú, F.X.; Viñas, M. Bioaugmentation of native fungi, an efficient strategy for the bioremediation of an aged industrially polluted soil with heavy hydrocarbons. *Front. Microbiol.* **2021**, *12*, 626436. [CrossRef]
40. Góralczyk-Bińkowska, A.; Jasińska, A.; Długoński, A.; Płociński, P.; Długoński, J. Laccase activity of the ascomycete fungus *Nectriella pironii* and innovative strategies for its production on leaf litter of an urban park. *PLoS ONE* **2020**, *15*, e0231453; Erratum in *PLoS ONE* **2020**, *15*, e0233553. [CrossRef]
41. Góralczyk-Bińkowska, A.; Długoński, A.; Bernat, P.; Długoński, J.; Jasińska, A. Environmental and molecular approach to dye industry waste degradation by the ascomycete fungus *Nectriella pironii*. *Sci. Rep.* **2021**, *11*, 23829. [CrossRef]
42. Milczarek, K.; Wrońska, N.; Felczak, A. Media. In *Microbial Biotechnology in the Laboratory and in Practice. Theory, Exercises and Specialist Laboratories*, 1st ed.; Długoński, J., Ed.; Łódź University Press & Jagiellonian University Press: Łódź-Kraków, Poland, 2021; pp. 475–496.
43. Bernat, P.; Nykiel-Szymańska, J.; Stolarek, P.; Słaba, M.; Szewczyk, R.; Różalska, S. 2,4-dichlorophenoxyacetic acid-induced oxidative stress: Metabolome and membrane modifications in *Umbelopsis isabellina*, a herbicide degrader. *PLoS ONE* **2018**, *13*, e0199677. [CrossRef]
44. Słaba, M.; Bernat, P.; Różalska, S.; Nykiel, J.; Długoński, J. Comparative study of metal induced phospholipid modifications in the heavy metal tolerant filamentous fungus *Paecilomyces marquandii* and implications for the fungal membrane integrity. *Acta Biochim. Pol.* **2013**, *60*, 695–700. [CrossRef]
45. Długoński, A.; Szumański, M. Analysis of green infrastructure in Lodz, Poland. *J. Urban Plan. Dev.* **2014**, *141*, A5014001. [CrossRef]
46. Długoński, A.; Szumański, M. *Eco-Urban Atlas of Green Infrastructure of Lodz City*; Łódzkie Towarzystwo Naukowe: Łódź, Poland, 2016; (In Polish with English Summary). Available online: https://www.ltn.lodz.pl/index.php?option=com_content&view=article&id=693&Itemid=170 (accessed on 16 September 2022).
47. MKiS. Projekt Ustawy o Terenach Zdegradowanych Nr UD312 [Draft Act on Large-Scale Degraded Areas]. 2022. Available online: <https://www.gov.pl/web/premier/projekt-ustawy-o-wielkoobszarowych-terenach-zdegradowanych2> (accessed on 25 May 2022).
48. HELCOM. State of the Baltic Sea—Second HELCOM Holistic Assessment 2011–2016. Baltic Sea Environment Proceedings 155. 2018. Available online: <https://www.eea.europa.eu/data-and-maps/indicators/chlorophyll-in-transitional-coastal-and-3/helcom-2018-state-of-the> (accessed on 11 August 2022).
49. HELCOM. Thematic Assessment of Hazardous Substances 2011–2016. Supplementary Report to the HELCOM “State of the Baltic Sea” Report. 2018. Available online: https://www.helcom.fi/wp-content/uploads/2019/08/HELCOM_Thematic-assessment-of-hazardous-substances-2011-2016_pre-publication.pdf (accessed on 11 August 2022).
50. HELCOM. Polyaromatic Hydrocarbons (PAHs) and Their Metabolites. HELCOM Core Indicator Report. 2018. Available online: <https://helcom.fi/wp-content/uploads/2019/08/Polyaromatic-hydrocarbons-PAHs-and-their-metabolites-HELCOM-core-indicator-2018.pdf> (accessed on 11 August 2022).
51. Kanwischer, M.; Asker, N.; Wernersson, A.S.; Wirth, M.A.; Fisch, K.; Dahlgren, E.; Osterholz, H.; Habedank, F.; Naumann, M.; Mannio, J.; et al. Substances of emerging concern in Baltic Sea water: Review on methodological advances for the environmental assessment and proposal for future monitoring. *Ambio* **2022**, *51*, 1588–1608. [CrossRef] [PubMed]
52. OSS. Ocena Stanu Środowiska Polskich Obszarów Morskich Bałtyku na Podstawie Danych Monitoringowych z Roku 2019 na Tle Dziesięciolecia 2009–2018 [Assessment of the Environmental Status of the Polish Baltic Sea Regions Based on Monitoring Data from 2019 in the Context of the Decade 2009–2018]. Ed. Narodowy Fundusz Ochrony Środowiska: Warsaw, Poland. 2020. Available online: https://www.gios.gov.pl/images/dokumenty/pms/monitoring_wod/ocena_stanu_2019_2009-2018.pdf (accessed on 10 May 2022).
53. Janas, M.; Zawadzka, A. Assessment of the Monitoring of an Industrial Waste Landfill. *Ecol. Chem. Eng. S* **2018**, *25*, 659–669. [CrossRef]
54. NIK Report. Plan Pracy Najwyższej Izby Kontroli na 2022 rok. Work Plan of the Supreme Audit Office for 2022. Nr 30; p. 71. 2021. Available online: <https://www.nik.gov.pl/kontrola/plan-pracy-nik/> (accessed on 10 May 2022).
55. Kuśmierz, A.; Krawczyńska, B.; Krawczyński, J. *Ocena Oddziaływania Składowisk Pozakładowych Zlokalizowanych na Terenie Byłych Zakładów “Boruta” w Zgierzu na Życie i Zdrowie Mieszkańców Zgierza i Okolic Oraz Dla Środowiska* [Assessment of the Impact of the Off-Site Landfills on the Site of the Former “Boruta” Industrial Area in Zgierz on the Life and Health of the Inhabitants of Zgierz and Its Surroundings and on the Environment]; IOS-PIB: Warsaw, Poland, 2019.

56. Fayeulle, A.; Veignie, E.; Schroll, R.; Munch, J.C.; Rafin, C. PAH biodegradation by telluric saprotrophic fungi isolated from aged PAH-contaminated soils in mineral medium and historically contaminated soil microcosms. *J. Soil Sediments* **2019**, *19*, 3056–3067. [\[CrossRef\]](#)
57. de Souza, H.M.L.; Barreto, L.R.; da Mota, A.J.; de Oliveira, L.A.; dos Barroso, H.S.; Zanutto, S.P. Tolerance to Polycyclic Aromatic Hydrocarbons (PAHs) by filamentous fungi isolated from contaminated sediment in the Amazon region. *Acta Sci. Biol. Sci.* **2017**, *39*, 481–488. [\[CrossRef\]](#)
58. Lisowska, K.; Pałecz, B.; Długoński, J. Microcalorimetry as a possible tool for phenanthrene toxicity evaluation to eukaryotic cells. *Thermochim. Acta* **2004**, *411*, 181–186. [\[CrossRef\]](#)
59. Argumedo-Delira, R.; Alarcón, A.; Ferrera-Cerrato, R.; Almaraz, J.J.; Peña-Cabriales, J.J. Tolerance and growth of 11 *Trichoderma* strains to crude oil, naphthalene, phenanthrene and benzo[a]pyrene. *J. Environ. Manag.* **2012**, *9*, S291–S299. [\[CrossRef\]](#)
60. Jasińska, A.; Soboń, A.; Różalska, S.; Średnicka, P. Bisphenol A removal by the fungus *Myrothecium roridum* IM 6482—Analysis of the cellular and subcellular level. *Int. J. Mol. Sci.* **2021**, *22*, 10676. [\[CrossRef\]](#)
61. Jasińska, A.; Góralczyk-Bińkowska, A.; Soboń, A.; Długoński, J. Lignocellulose resources for the *Myrothecium roridum* laccase production and their integrated application for dyes removal. *Int. J. Environ. Sci. Technol.* **2019**, *16*, 4811–4822. [\[CrossRef\]](#)
62. Janicki, T.; Długoński, A.; Felczak, A.; Długoński, J.; Krupiński, M. Ecotoxicological estimation of 4-cumylphenol, 4-*t*-octylphenol, nonylphenol, and volatile leachate phenol degradation by the microscopic fungus *Umbelopsis isabellina* using a battery of biotests. *Int. J. Environ. Res. Public Health* **2022**, *19*, 4093. [\[CrossRef\]](#) [\[PubMed\]](#)
63. Liao, S.G.; Zhang, L.J.; Sun, F.; Wang, Z.; He, X.; Wang, A.M.; Li, Y.J.; Huang, Y.; Lan, Y.Y.; Zhang, B.L.; et al. Identification and characterisation of phenolics in *Polygonum capitatum* by ultrahigh-performance liquid chromatography with photodiode array detection and tandem mass spectrometry. *Phytochem. Anal.* **2013**, *24*, 556–568. [\[CrossRef\]](#)
64. Kakkar, S.; Bais, S. A review on protocatechuic acid and its pharmacological potential. *ISRN Pharmacol.* **2014**, *2014*, 952943. [\[CrossRef\]](#) [\[PubMed\]](#)
65. Acevedo, F.; Pizzul, L.; del Pilar Castillo, M.; Cuevas, R.; Diez, M.C. Degradation of polycyclic aromatic hydrocarbons by the Chilean white-rot fungus *Anthrachyllum discolor*. *J. Hazard. Mater.* **2011**, *185*, 212–219. [\[CrossRef\]](#)
66. Lisowska, K.; Zawadzka, K.; Długoński, A. Biological wastewater treatment processes. In *Microbial Biotechnology in the Laboratory and in Practice. Theory, Exercises and Specialist Laboratories*, 1st ed.; Długoński, J., Ed.; Łódź University Press & Jagiellonian University Press: Łódź-Kraków, Poland, 2021; pp. 317–325.
67. Nzila, A.; Musa, M.M. Current status of and future perspectives in bacterial degradation of benzo[a]pyrene. *Int. J. Environ. Res. Public Health* **2021**, *18*, 262. [\[CrossRef\]](#) [\[PubMed\]](#)
68. Bernat, P. Application of lipidomics in the study of detoxification processes in microorganisms. In *Microbial Biotechnology in the Laboratory and Practice. Theory, Exercises and Specialist Laboratories*, 1st ed.; Długoński, J., Ed.; Łódź University Press & Jagiellonian University Press: Łódź-Kraków, Poland, 2021; pp. 452–459.
69. de Kroon, A.I.; Rijken, P.J.; De Smet, C.H. Checks and balances in membrane phospholipid class and acyl chain homeostasis, the yeast perspective. *Prog. Lipid. Res.* **2013**, *52*, 374–394. [\[CrossRef\]](#) [\[PubMed\]](#)
70. Siewiera, P.; Bernat, P.; Różalska, S.; Długoński, J. Estradiol improves tributyltin degradation by the filamentous fungus *Metarhizium robertsii*. *Int. Biodeterior. Biodegr.* **2015**, *104*, 258–263. [\[CrossRef\]](#)
71. Nykiel-Szymańska, J.; Różalska, S.; Bernat, P.; Słaba, M. Assessment of oxidative stress and phospholipids alterations in chloroacetanilides-degrading *Trichoderma* spp. *Ecotoxicol. Environ. Saf.* **2019**, *184*, 109629. [\[CrossRef\]](#)
72. Mironenka, J.; Różalska, S.; Soboń, A.; Bernat, P. Lipids, proteins and extracellular metabolites of *Trichoderma harzianum* modifications caused by 2,4-dichlorophenoxyacetic acid as a plant growth stimulator. *Ecotoxicol. Environ. Saf.* **2020**, *194*, 110383. [\[CrossRef\]](#)
73. Li, Z.; Agellon, L.B.; Allen, T.M.; Umeda, M.; Jewell, L.; Mason, A.; Vance, D.E. The ratio of phosphatidylcholine to phosphatidylethanolamine influences membrane integrity and steatohepatitis. *Cell Metab.* **2006**, *3*, 321–331. [\[CrossRef\]](#)
74. Bernat, P.; Gajewska, E.; Szewczyk, R.; Słaba, M.; Długoński, J. Tributyltin (TBT) induces oxidative stress and modifies lipid profile in the filamentous fungus *Cunninghamella elegans*. *Environ. Sci. Pollut. Res. Int.* **2014**, *21*, 4228–4235. [\[CrossRef\]](#) [\[PubMed\]](#)
75. Szewczyk, R.; Różalska, S.; Mironenka, J.; Bernat, P. Atrazine biodegradation by mycoinsecticide *Metarhizium robertsii*: Insights into its amino acids and lipids profile. *J. Environ. Manag.* **2020**, *262*, 110304. [\[CrossRef\]](#) [\[PubMed\]](#)
76. Litwin, A.; Bernat, P.; Nowak, M.; Słaba, M.; Różalska, S. Lipidomic response of the entomopathogenic fungus *Beauveria bassiana* to pyrethroids. *Sci. Rep.* **2021**, *11*, 21319. [\[CrossRef\]](#)
77. Fedoseeva, E.V.; Danilova, O.A.; Ianutsevich, E.A.; Terekhova, V.A.; Tereshina, V.M. Micromycete lipids and stress. *Microbiology* **2021**, *90*, 37–55. [\[CrossRef\]](#)
78. Shon, J.C.; Noh, Y.J.; Kwon, Y.S.; Kim, J.H.; Wu, Z.; Seo, J.S. The impact of phenanthrene on membrane phospholipids and its biodegradation by *Sphingopyxis soli*. *Ecotoxicol. Environ. Saf.* **2020**, *192*, 110254. [\[CrossRef\]](#)

Fig. S1. Mass spectra of qualitative analysis of PHE, B[a]A and B[a]P degradation by *N. pironii* cultivated with the presence of leachate.
<https://www.mdpi.com/article/10.3390/ijerph192113997/s1>

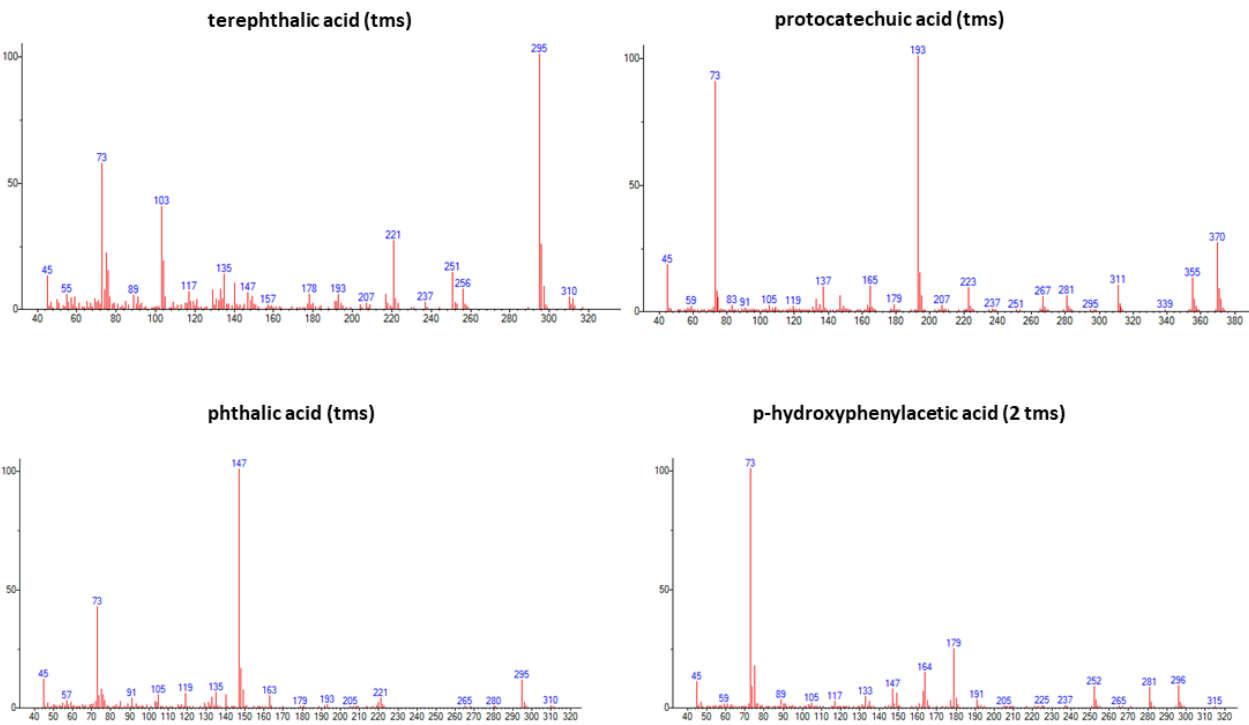


Table S1. The phospholipids composition and PC/PE ratio determined in the *N. pironii* cells after 24, 48, and 72 h of cultivation with PHE, L1 or PHE + L1, or without the tested compounds.

	Phospholipid class	Control biotic	L1	PHE	PHE + L1	B[a]A	B[a]A + L1	B[a]P	B[a]P + L1
24 h	PA	0.65±0.02	1.96±0.11	0.42±0.02	0.19±0.01	0.71±0.03	1.20±0.04	0.43±0.02	0.88±0.07
	PC	36.15±2.18	40.60±2.54	59.98±1.75	68.43±3.45	45.11±1.45	42.06±3.36	40.51±3.54	47.67±2.39
	PE	58.08±2.36	52.45±3.17	38.48±2.14	24.51±41.19	51.26±3.36	48.09±1.56	55.70±4.25	45.49±3.35
	PI	5.12±0.41	4.99±0.18	1.12±0.04	6.88±0.23	2.92±0.14	8.65±0.37	3.37±0.21	5.96±0.46
	PC/PE	0.62±0.03	0.77±0.03	1.56±0.07	2.79±0.25	0.88±0.05	0.87±0.04	0.73±0.04	1.05±0.09
48 h	PA	0.43±0.02	11.60±0.91	0.53±0.04	0.11±0.01	0.46±0.03	1.05±0.58	0.34±0.02	1.21±0.11
	PC	40.27±3.24	36.39±2.27	68.28±5.54	70.24±6.37	48.66±3.54	48.49±3.69	47.72±2.24	57.71±5.31
	PE	55.25±4.98	46.56±3.39	29.06±2.68	24.68±2.21	47.97±4.11	41.18±2.98	49.42±3.87	35.57±3.36
	PI	4.04±0.36	5.48±0.44	2.13±0.19	4.97±0.37	2.91±0.26	9.27±0.87	2.52±0.19	5.51±0.45
	PC/PE	0.73±0.06	0.78±	2.35±0.19	2.85±0.21	1.01±0.04	1.18±0.14	0.97±0.06	1.62±0.13
72 h	PA	0.34±0.02	0.80±0.63	0.82±0.07	0.18±0.01	0.43±0.02	1.23±0.11	0.71±0.05	1.05±0.09
	PC	35.04±2.7	39.90±2.11	58.35±4.9	73.64±6.54	41.44±3.37	34.90±2.94	32.49±2.74	37.10±2.97
	PE	59.74±5.69	54.65±2.87	38.51±2.25	21.19±1.98	55.10±4.65	55.64±4.98	63.34±5.79	54.15±4.36
	PI	4.88±3.61	4.65±0.06	2.32±0.28	4.99±3.56	3.03±0.28	8.23±0.44	3.46±0.28	7.70±0.69
	PC/PE	0.59±0.48	0.73±0.01	1.52±0.14	3.48±0.27	0.75±0.04	0.63±0.03	0.51±0.03	0.69±0.05

Oświadczenia współautorów o udziale w publikacjach

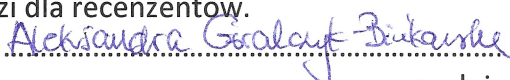
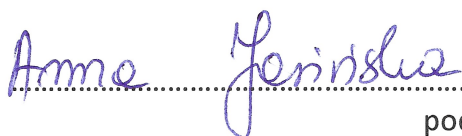
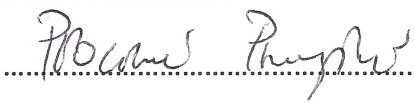
P1 - Góralczyk-Bińkowska A., Jasińska A., Długoński J. 2019. Characteristics and use of multicopper oxidases enzymes. *Advancements of Microbiology*. 58, 1, 7–18. <https://doi.org/10.21307/PM-2019.58.1.007>

Imię i nazwisko	Udział [%]	Opis działań i podpis
mgr Aleksandra Góralczyk-Bińkowska	70	Przygotowanie podstawowej wersji manuskryptu, wykonanie tabeli i rycin. Edycja końcowa tekstu manuskryptu. Udział w przygotowaniu odpowiedzi dla recenzentów.  podpis
dr Anna Jasińska	15	Opracowanie koncepcji pracy. Przygotowanie podstawowej wersji manuskryptu. Edycja końcowa tekstu manuskryptu. Udział w przygotowaniu odpowiedzi dla recenzentów.  podpis
prof. dr hab. Jerzy Długoński	15	Udział w przygotowaniu odpowiedzi dla recenzentów.  podpis

IF₂₀₂₁ = 1,106; IF_{5-letni} = 1,955; punkty MEiN = 20; 3 cytowania (dane z bazy Journal Citation Reports™, stan na dzień 07.11.2022 r.)

Wartość IF oraz punktację MEiN podano zgodnie z listą obowiązującą w 2022 roku

P2 - Góralczyk-Bińkowska A., Jasińska A., Długoński A., Płociński P., Długoński J. 2020. Laccase activity of the ascomycete fungus *Nectriella pironii* and innovative strategies for its production on leaf litter of an urban park. PLoS ONE, 15, 4, e0231453; Errata w PLoS ONE 2020, 15, e0233553. <https://doi.org/10.1371/journal.pone.0231453>

Imię i nazwisko	Udział [%]	Opis działań i podpis
mgr Aleksandra Góralczyk-Bińkowska	60	Zaplanowanie i realizacja doświadczeń obejmująca skринing grzybów zdolnych do produkcji enzymu o aktywności lakazy, prowadzenie hodowli z dodatkiem induktorów. Przygotowanie podłoży na bazie odpadów ligninocelulozowych. Izolacja i oczyszczanie białek grzyba. Analizy biochemiczne oczyszczonego białka. Ocena dekoloryzacji barwników tekstylnych z wykorzystaniem oczyszczonego białka. Analiza statystyczna otrzymanych wyników. Przygotowanie podstawowej wersji manuskryptu, opracowanie graficzne wyników. Udział w przygotowaniu odpowiedzi dla recenzentów.  podpis
dr Anna Jasińska	10	Przygotowanie manuskryptu. Edycja końcowa tekstu manuskryptu. Udział w przygotowaniu odpowiedzi dla recenzentów.  podpis
dr inż. Andrzej Długoński	10	Przygotowanie podstawowej wersji manuskryptu i ryciny. Udział w przygotowaniu odpowiedzi dla recenzentów.  podpis
dr Przemysław Płociński	10	Realizacja eksperymentów dotyczących oczyszczania białek i identyfikacji lakazy. Przygotowanie manuskryptu. Udział w przygotowaniu odpowiedzi dla recenzentów.  podpis
prof. dr hab. Jerzy Długoński	10	Przygotowanie manuskryptu. Edycja końcowa tekstu manuskryptu. Udział w przygotowaniu odpowiedzi dla recenzentów.  podpis

IF₂₀₂₁ = 3,752; IF_{5-letni} = 4,069; punkty MEiN = 100; 18 cytowań (dane z bazy Journal Citation Reports™, stan na dzień 07.11.2022 r.)

Wartość IF oraz punktację MEiN podano zgodnie z listą obowiązującą w 2022 roku

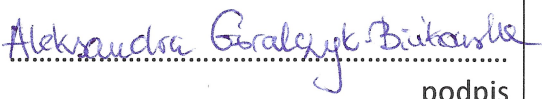
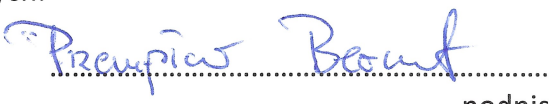
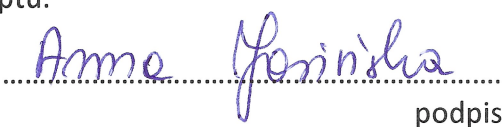
P3 - Góralczyk-Bińkowska A., Długoński A., Bernat P., Długoński J., Jasińska A. 2021. Environmental and molecular approach to dye industry waste degradation by the ascomycete fungus *Nectriella pironii*. Scientific Reports, 11, 23829. <https://doi.org/10.1038/s41598-021-03446-x>

Imię i nazwisko	Udział [%]	Opis działań i podpis
mgr Aleksandra Góralczyk-Bińkowska	60	Zaplanowanie i realizacja doświadczeń dotyczących oceny zdolności grzyba do wzrostu w obecności odcieków i amin aromatycznych. Przygotowanie prób do analizy HPLC. Analiza mechanizmów enzymatycznych zaangażowanych w eliminację amin aromatycznych. Ocena stopnia dekoloryzacji barwników azowych w hodowlach. Analiza statystyczna otrzymanych wyników. Przygotowanie podstawowej wersji manuskryptu, opracowanie graficzne wyników. Udział w przygotowaniu odpowiedzi dla recenzentów.  podpis
dr inż. Andrzej Długoński	10	Przygotowanie podstawowej wersji manuskryptu, opracowanie ryciny, wykonanie fotografii. Przygotowanie podstawowej wersji manuskryptu.  podpis
dr hab. Przemysław Bernat, prof. UŁ	10	Współudział w wykonywaniu eksperymentów dotyczących ilościowej i jakościowej analizy biodegradacji amin aromatycznych.  podpis
prof. dr hab. Jerzy Długoński	10	Opracowanie koncepcji badań związanych z odciekami i aminami aromatycznymi. Przygotowanie podstawowej wersji manuskryptu. Udział w przygotowaniu odpowiedzi dla recenzentów.  podpis
dr Anna Jasińska	10	Opracowanie koncepcji badań związanych z odciekami i aminami aromatycznymi. Przygotowanie podstawowej wersji manuskryptu. Edycja końcowa tekstu manuskryptu. Udział w przygotowaniu odpowiedzi dla recenzentów.  podpis

IF₂₀₂₁ = 4,997; IF_{5-letni} = 5,516;; punkty MEiN = 140; 3 cytowania (dane z bazy Journal Citation Reports™, stan na dzień 07.11.2022 r.)

Wartość IF oraz punktację MEiN podano zgodnie z listą obowiązującą w 2022 roku

P4 - Góralczyk-Bińkowska A., Długoński A., Bernat P., Długoński J., Jasińska A. 2022. Accelerated PAHs transformation in the presence of dye industry landfill leachate combined with fungal membrane lipids changes. International Journal of Environmental Research and Public Health, 19, 13997. <https://doi.org/10.3390/ijerph192113997>

Imię i nazwisko	Udział [%]	Opis działań i podpis
mgr Aleksandra Góralczyk-Bińkowska	60	Zaplanowanie i realizacja doświadczeń dotyczących oceny zdolności grzyba do wzrostu w obecności odcieków składowiskowych i badanych WWA. Przygotowanie prób do analiz chromatograficznych oraz lipidomicznych. Analiza zmian w przepuszczalności błony komórkowej badanego grzyba oraz stopnia peroksydacji lipidów. Analiza statystyczna otrzymanych wyników. Przygotowanie podstawowej wersji manuskryptu, opracowanie graficzne wyników. Udział w przygotowaniu odpowiedzi dla recenzentów. Edycja końcowa tekstu manuskryptu.  podpis
dr inż. Andrzej Długoński	10	Przygotowanie podstawowej wersji manuskryptu, tabeli oraz ryciny.  podpis
dr hab. Przemysław Bernat, prof. UŁ	10	Współudział w wykonywaniu eksperymentów dotyczących: ilościowej i jakościowej analizy biodegradacji WWA, zmian profilu fosfolipidowego grzyba pod wpływem odcieków i WWA, konsultacjach naukowych.  podpis
prof. dr hab. Jerzy Długoński	10	Opracowanie koncepcji badań związanych z odciekami składowiskowymi i WWA. Przygotowanie podstawowej wersji manuskryptu.  podpis
dr Anna Jasińska	10	Przygotowanie podstawowej wersji manuskryptu. Edycja końcowa tekstu manuskryptu.  podpis

IF₂₀₂₁ = 4,614; IF_{5-letni} = 4,799; punkty MEiN = 140, brak cytowań (dane z bazy Journal Citation Reports™, stan na dzień 07.11.2022 r.)

Wartość IF oraz punktację MEiN podano zgodnie z listą obowiązującą w 2022 roku