Charakterystyka wybranych melanin pochodzących z biomasy grzybowej i ich zastosowanie do poprawy właściwości użytkowych materiałów opakowaniowych

Characterisation of selected melanins derived from fungal biomass and their application to improve the properties of packaging materials

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Rozprawa doktorska wykonana

w Centrum Bioimmobilizacji i Innowacyjnych Materiałów Opakowaniowych

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za cenne wskazówki oraz życzliwą pomoc,

składam wyrazy serdecznego podziękowania

Mojej kochanej Mamie

Za zrozumienie, wsparcie

i siłę jaką mi daje,

oraz nieustającą wiarę we mnie

Streszczenie rozprawy doktorskiej pt. „Charakterystyka wybranych melanin pochodzących z biomasy grzybowej i ich zastosowanie do poprawy właściwości użytkowych materiałów opakowaniowych"

Modyfikacja materiałów opakowaniowych w kierunku poprawienia ich właściwości użytkowych i nadania im m.in. właściwości przeciwutleniających, przeciwdrobnoustrojowych i barierowych względem światła, tlenu i pary wodnej jest jednym z kierunków rozwoju nowoczesnego opakowalnictwa. Wieloma unikalnymi właściwościami charakteryzują się melaniny (m.in. pochodzące z biomasy grzybowej), które mogą znaleźć zastosowanie w modyfikacji materiałów opakowaniowych.

Celem pracy było określenie możliwości zastosowania melanin pochodzących z biomasy grzybowej do poprawy właściwości użytkowych materiałów opakowaniowych. Założono, że biomasa grzybowa (odpad z produkcji pieczarki dwuzarodnikowej *Agaricus bisporus*, ryzomorfy opieńki miodowej *Armillaria mellea* oraz owocniki tęgoskóra cytrynowego *Scleroderma citrinum*) może stanowić źródło melanin o właściwościach przeciwutleniających, barierowych względem promieniowania UV, przeciwdrobnoustrojowych.

W pracy dokonano izolacji i charakterystyki fizykochemicznej melanin pochodzących z odpadu z *A. bisporus*, ryzomorf *A. mellea* oraz owocników *S. citrinum*. Ponadto określono właściwości przeciwutleniające, barierowe względem promieniowania UV-Vis oraz przeciwdrobnoustrojowe oczyszczonych i nieoczyszczonych barwników. Uzyskane barwniki wykorzystano jako dodatek do powłok z chitozanu i skrobi nanoszonych na folie PLA [poli(kwas mlekowy)], PE (polietylen), BOPP (polipropylen orientowany dwuosiowo) i PET [poli(tereftalan etylenu)]. Analizowano wpływ dodatku melanin na właściwości przeciwutleniające powłok oraz ich barwę. W przypadku folii BOPP i PET dokonano analizy właściwości optycznych folii. Odpad z produkcji *A. bisporus* wykorzystano jako dodatek do folii PLA uzyskując kompozytowy materiał w postaci folii. Wykonano badania mechaniczne obejmujące określenie sił rozerwania i przebicia, oraz siły potrzebnej do rozerwania zgrzewu. Określono również dynamiczne mechaniczne właściwości folii. Dokonano analizy barierowości kompozytowych materiałów względem pary wodnej metodą grawimetryczną, właściwości powierzchniowych (pomiar kąta zwilżania) oraz wpływu dodatku melanin na krystaliczność. Przeprowadzono analizy spektroskopowe modyfikowanych folii z żelatyny obejmujące analizę w zakresie promieniowania UV-Vis, FT-IR oraz Ramana. Dokonano analizy wpływu dodatku melaniny w różnych stężeniach na wartości składowych barwy w systemie CIE Lab. Badaniom poddane zostały również właściwości przeciwutleniające, ilość grup polifenolowych dostępnych na powierzchni materiału oraz właściwości antymikrobiologiczne. Melaninę z odpadu *A. bisporus* wykorzystano także jako dodatek do folii z żelatyny. Analizowano rozpuszczalność folii, właściwości mechaniczne, przepuszczalność tlenu oraz pary wodnej, właściwości powierzchniowe (pomiar kąta zwilżania). Dodatkowo przeprowadzono badania zawartości grup polifenolowych w foliach, właściwości przeciwutleniających, oraz przeciwdrobnoustrojowych. Dokonano analizy wpływu dodatku melaniny w różnych stężeniach na wartości składowych barwy w systemie CIE Lab. Przeprowadzono analizy spektroskopowe obejmujące analizę w zakresie promieniowania UV-Vis (pomiar transmitancji folii), FT-IR oraz Ramana. Aby określić możliwość zastosowania modyfikowanego melaninami materiału z żelatyny na modelowym produkcie spożywczym wybrano smalec. Smalec firmy „Nasze Smaki" pokrywano roztworami powłokotwórczymi, które wcześniej wykorzystywano do otrzymywania folii z żelatyny (0.1%; 0.5%; 1%) z dodatkiem melaniny z odpadu

A. bisporus. Smalec przechowywano przez okres 21 dni w temperaturze 25°C, wilgotności 50% RH, bez dostępu światła. Po 7, 14 i 21 dniach przechowywania oznaczano liczby: ponadtlenkową (POV), jodową (IV) oraz kwasową (AV).

W przedstawionej rozprawie doktorskiej otrzymano barwniki z trzech rodzajów biomasy grzybowej: odpadu powstającego przy produkcji pieczarki dwuzarodnikowej (*A. bisporus*), ryzomorf opieńki miodowej (*A. mellea*) oraz owocników tęgoskóra cytrynowego (*S. citrinum*). Zaobserowowano, że badane melaniny charakteryzują się zdolnością do pochłaniania promieniowania UV-Vis, szczególnie w zakresie promieniowania ultrafioletowego oraz właściwościami przeciwutleniającymi. Stwierdzono, że oczyszczone melaniny mają lepsze niż nieoczyszczone formy właściwości przeciwutleniające, barierowe względem promieniowania UV i charakteryzują się niższym parametrem *L* (jasność) oraz wyższymi parametrami *a* (w kierunku barwy czerwonej) i *b* (w kierunku barwy żółtej), wyższą zawartością grup polifenolowych, wykazują także różnice w obecnych w cząsteczkach grupach funkcyjnych. Analizowane melaniny charakteryzowały się również właściwościami przeciwdrobnoustrojowymi.

Na podstawie przeprowadzonych doświadczeń stwierdzono, że dodatek melanin grzybowych do powłok chitozanowych i skrobiowych nanoszonych na powierzchnię folii PE, PLA, BOPP i PET pozwala nadać im właściwości przeciwutleniające. Właściwości te zależą nie tylko od źródła pochodzenia melanin, ale również od nośników wykorzystanych do tworzenia powłok. Wzrastające stężenie melanin w roztworach powłokotwórczych spowodowało wzrost właściwości przeciwutleniających. Nie odnotowano znaczącej poprawy właściwości barierowych względem promieniowania UV-Vis w przypadku modyfikowanych powłokami zawierającymi melaniny folii BOPP i PET. Stwierdzono, że dodatek melanin wpływa na barwę folii.

Na podstawie przeprowadzonych badań stwierdzono, że możliwe jest uzyskanie kompozytowego materiału opakowaniowego z poli(kwasu mlekowego) i melaniny z odpadu *A. bisporus* o polepszonych właściwościach mechanicznych i barierowych względem pary wodnej (przy udziale 0.025% i 0.05% melaniny). Zaobserwowano niewielką poprawę właściwości barierowych względem promieniowania UV-Vis. Stwierdzono, że dodatek melanin nie miał istotnego wpływu na właściwości powierzchniowe folii. Analiza DSC wykazała, że pomimo zwiększenia krystaliczności materiału w porównaniu do próby kontrolnej, dodatek melanin nie wpłynął istotnie na wartości temperatur zeszklenia (T_g), zimnej krystalizacji (T_c) oraz topnienia (T_m). Ponadto wszystkie badanie folie charakteryzowały się wysoką amorficznością. Badania kolorymetryczne wykazały, że dodatek melanin obniżył jasność folii (*L*) oraz zwiększył wartości parametrów *a* (w kierunku barwy czerwonej) i *b* (w kierunku barwy żółtej), a także obniżył wartość parametru YI w porównianiu do folii kontrolnej. Niemodyfikowane folie nie wykazywały właściwości przeciwutleniających. Zaobserowano, że wraz ze wzrostem udziału melanin w matrycy polimeru wzrastały zarówno właściwości przeciwutleniające folii jak i ilość grup polifenolowych na powierzchnii materiału. Modyfikowane folie charakteryzowały się właściwościami antybakteryjnymi. Były aktywne przeciwko *Enterococcus faecalis*, *Pseudomonas aeruginosa* i *Pseudomonas putida*.

Przeprowadzone badania wykazały, że możliwe jest uzyskanie folii z żelatyny modyfikowanych melaniną z odpadu *A. bisporus*. Stwierdzono, że dodatek melanin istotnie obniżył rozpuszczalność folii. Odnotowano znaczący wzrost kąta zwilżania dla folii modyfikowanych melaniną (53.3° dla folii niemodyfikowanych, 72.9° dla folii z dodatkiem 1% melaniny), co świadczy o zmianie właściwości powierzchniowch folii i wzroście hydrofobowości materiału. Zaobserowano nieznaczy spadek przepuszczalności względem pary wodnej (nieistotny statystycznie), natomiast właściwości barierowe względem tlenu uległy poprawie. Nie stwierdzono istotnego wpływu melanin

na właściwości mechaniczne folii. Odnotowano znaczącą poprawę właściwości barierowych względem promieniowania UV-Vis. Dodatek melaniny do folii z żelatyny znacząco poprawił właściwości przeciwutleniające. Zarówno modyfikowane folie jak i folia bez dodatku melanin nie wykazywały właściwości przeciwdrobnoustrojowych. Zaobserwowano, że dodatek melaniny do folii zmniejszył wartość parametru *L*, oraz zwiększył wartość parametrów *a* i *b*.

Na podstawie przeprowadzonych badań stwierdzono, że liczby ponadtlenkowa (POV) i kwasowa (AV) niepokrytego smalcu wzrastały w szybki sposób w czasie przechowywania. Zaobserowano również spadek liczby jodowej (IV), spowodowany zmniejszeniem zawartości nienasyconych kwasów tłuszczowych. Odnotowano, że obecność powłok z żelatyny znacząco spowalnia wzrost POV i AV, oraz spadek IV. W całym okresie inkubacji POV i AV smalcu pokrytego powłokami zawierającymi melaninę były niższe niż smalcu pokrytego powłoką bez dodatku melaniny, zaś IV wyższe.

Uzyskane rezultaty badań wskazują, że melaniny pochodzące z biomasy grzybowej mogą znaleźć zastosowanie w modyfikacji właściwości materiałów opakowaniowych.

Summary of the PhD thesis "Characterisation of selected melanins derived from fungal biomass and their application to improve the properties of packaging materials"

Modification of packaging materials in the direction of improving their properties and giving them antioxidant, antimicrobial and barrier properties against light, oxygen and water vapor is one of the directions of development of modern packaging. Melanins (including those from fungal biomass) are characterizad by many unique properties, that may be used in the modification of packaging materials.

The aim of the PhD thesis was to determine the possibility of using melanins derived from fungal biomass to improve the properties of packaging materials. It was assumed that the fungal biomass (waste from the production of *Agaricus bisporus*, the *Armillaria mellea* rhizomorphs and the fruiting bodies of *Scleroderma citrinum*) can be a source of melanins (with antioxidant, UV-Vis light barrier and antimicrobial properties).

The work included the isolation, purification and physicochemical characteristics of melanins derived from *A. bisporus* waste, *A. mellea* rhizomorphs and *S. citrinum* fruiting bodies. In addition, the antioxidant, UV-Vis light barrier and antimicrobial properties of native and purified pigments were determined. Obtained pigments were used as an additive to coatings from chitosan and starch applied on PLA, PE, BOPP and PET films. The effect of melanins addition on the antioxidant properties of coatings and their color was analyzed. In the case of BOPP and PET films, the optical properties of the films were analyzed. The waste from the *A. bisporus* production was used as an additive to the PLA to form a composite film materials. Mechanical tests were carried out, including the determination of breakage and breakdown forces, and the force required to break the seal. The dynamic mechanical properties of the films were also determined. Analysis of the barrier properties of composite materials against water vapor using the gravimetric method, surface properties (measurement of contact angle) and the effect of melanins addition on crystallinity were performed. Spectroscopic analyzes including UV-Vis, FT-IR and Raman were carried out. The analysis of the effect of melanins addition in various concentrations on the values of colour in the CIE Lab system was performed. Antioxidant properties, amount of polyphenol groups available on the surface of the material and antimicrobial properties were also analyzed. The melanin from *A. bisporus* was also used as an additive to the gelatin films. The films solubility, mechanical properties, oxygen and water vapor permeability, surface properties (measurement of contact angle) were analyzed. Additionally, the content of polyphenolic groups in films, antioxidant properties and antimicrobial properties were investigated. The analysis of the effect of melanin addition in various concentrations on the values of colour in the CIE Lab system was performed. Spectroscopic analyzes were carried out, including analysis in the UV-Vis, FT-IR and Raman. To determine the possibility of using gelatin-modified material from gelatin on a model food product, lard was chosen. Lard from "Nasze smaki" company was coated with coating solutions that were previously used to obtain a gelatin film (0.1%, 0.5%, 1% with the addition of melanin from *A. bisporus* waste). Lard was stored for 21 days at 25 ° C, 50% RH, without light. After 7, 14 and 21 days of storage, the following values were determined: superoxide (POV), iodine (IV) and acid (AV).

In the presented PhD thesis melanins have been obtained from three fungal biomass sources: waste from harvesting of *A. bisporus*, rhizomorphs of *A. mellea* and fruiting bodies of *S. citrinum*. It was noted, that analysed melanins have UV-Vis light barrier (especially in UV range) as well as antioxidant properties. It has been found that purified melanins have better antioxidant properties, barrier to UV radiation and are characterized by a lower *L* parameter (brightness) and higher

parameters *a* (in the red direction) and *b* (in the yellow direction), higher content of polyphenol groups and also show differences in functional groups present in molecules. It was noted, that the analyzed melanins have antimicrobial properties.

Based on the experiments conducted, it was found that the addition of fungal melanins to chitosan and starch coatings applied to the surface of PE, PLA, BOPP and PET films allows to give them antioxidant properties. These properties depend not only on the source of the melanins, but also on the carriers used to form the coatings. Increasing concentrations of melanins in coating-forming solutions resulted in an increase in antioxidant properties. There was no significant improvement in UV-Vis light barrier properties of BOPP and PET films. It was found that the addition of melanin affects the colour of the films.

On the basis of the conducted tests, it was found that it is possible to obtain a composite packaging material from poly(lactic acid) and melanin from *A. bisporus* with improved mechanical and barrier properties against water vapor (with melanin participation of 0.025% and 0.05%). A slight improvement in the UV-Vis barrier properties was observed. It was found that the addition of melanin had no significant effect on the surface properties of the film. DSC analysis showed that despite the increased crystallinity of the material compared to the control sample, melanin addition did not significantly affect the glass transition temperature (T_g), cold crystallization (T_c) and melting temperature (T_m), furthermore all foils were higly amorphic. Colorimetric studies showed that the addition of melanin reduced the lightness of the films (*L*) and increased the values of parameters *a* (in the red direction) and *b* (in the yellow direction), as well as decreased the YI parameter in comparison to the control film. The unmodified films did not exhibit antioxidant properties, it was observed that as the melanin content increased in the polymer matrix, both the antioxidant properties of the films and the amount of polyphenol groups on the surface of the material increased. Modified were active against *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Pseudomonas putida*.

The tests have shown that it is possible to obtain gelatin films modified with *A. bisporus* melanin. It was found that melanin addition significantly reduced films solubility. There was a significant increase in the contact angle for melanin modified films (53.3° for unmodified films, 72.9° for films with 1% melanin), indicating a change in surface film properties and hydrophobicity of the material. The water vapor permeability decreased slighlty (not statistically significant), oxygen barrier properties were improved. There was no significant influence of melanin on the mechanical properties of the films. There has been a significant improvement in UV barrier properties. The addition of melanin to the gelatin films significantly increased antioxidant properties. Both the modified films and the film without the addition of melanin did not show antimicrobial properties. It was observed that the addition of melanin to the film decreased the values of colour parameter *L*, and increased the values of parameters *a* and *b*.

Based on the conducted tests, it was found that the peroxide (POV) and acid (AV) values of uncoated lard increased quickly during storage in controlled conditions. A decrease in the iodine value (IV) was also observed, due to the decrease in the content of unsaturated fatty acids. It was observed that the presence of gelatin coatings significantly retarded the growth of POV and AV, and decrease IV. During the incubation period, lard coated with melanin-containing coatings had lower POV and AV, and higher IV than that of lard coated with gelatin devoid of melanin, and without coating.

The obtained results indicate that melanins from fungal biomass can be used to modify the properties of packaging materials.

Wyniki przedstawionej rozprawy doktorskiej zawarte są w następujących oryginalnych pracach naukowych:

P1. Łopusiewicz Ł. Waste from the harvesting of button mushroom (*Agaricus bisporus*) as a source of natural melanin. Artykuł przyjęty do druku: *Folia Pommeranae Universitatis Technologiae Stetinensis Aglicultura, Alimentaria, Piscaria et Zootechnica.*

P2. Łopusiewicz Ł. *Scleroderma citrinum* melanin: isolation, purification, spectroscopic studies with characterization of antioxidant, antibacterial and light barrier properties. *World Scientific News* 2018, 94(2): 114-129.

P3. Łopusiewicz Ł. The isolation, purification and analysis of the melanin pigment extracted from *Armillaria mellea* rhizomorphs. *World Scientific News* 2018 100: 135-153.

P4. Łopusiewicz Ł., Lisiecki S., Mizielińska M. Aktywność przeciwutleniająca folii PE i PLA modyfikowanych powłokami zawierającymi melaniny grzybowe. *Opakowanie* 2017, 3: 81-85.

P5. Łopusiewicz Ł., Lisiecki S., Mizielińska M. Właściwości przeciwutleniające i optyczne folii BOPP i PET modyfikowanych powłokami zawierającymi melaniny grzybowe. *Opakowanie* 2017, 7: 48-55.

P6. Łopusiewicz Ł., Jędra F., Mizielińska M. New poly(lactic acid) active packaging composite films incorporated with fungal melanin. *Polymers* 2018, 10(4): 386.

P7. Łopusiewicz Ł., Jędra F., Bartkowiak A. New active packaging films made from gelatin modified with fungal melanin. *World Scientific News* 2018, 101: 1-30.

P8. Łopusiewicz Ł., Jędra F., Bartkowiak A. The application of melanin modified gelatin coatings for packaging and oxidative stability of pork lard. *World Scientific News* 2018, 101: 108-119.

Spis treści

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1. Wstęp i uzasadnienie wyboru tematu

Rozwój gospodarczy, zmiany trybu życia i trendów żywieniowych spowodowały lawinowy wzrost zapotrzebowania na opakowania. Rosnąca konkurencja wymusza na producentach dążenie do ich produkcji w coraz lepszych i innowacyjnych formach, spełniających wymagania wszystkich ich użytkowników, w tym konsumentów. Pełniąc wciąż swoją pierwotną funkcję zabezpieczania, opakowania odgrywają również rolę przyciągania uwagi klienta do danego produktu i – pomimo że spełniają szereg różnych zadań, przez co często bardzo różnią się między sobą – posiadają wiele cech wspólnych. Powyższe pozwala zdefiniować opakowanie jako „wyrób zapewniający utrzymanie określonej jakości pakowanych produktów, przystosowanie ich do transportu i składowania oraz do prezentacji, a także chroniący środowisko przed szkodliwym działaniem niektórych produktów"(PN-0-79000:97).

Opakowanie powinno zatem bezpośrednio i skutecznie chronić produkt, przedłużać jego trwałość, a przy tym posiadać wszystkie niezbędne informacje o zawartości, zastosowanych dodatkach, wartości odżywczej, okresie przydatności oraz warunkach przechowywania. Musi być również elementem strategii marketingowej i usprawniać zarządzanie w fizycznym przepływie towarów. Współcześnie stosowane opakowania spełniają zatem wiele funkcji, ukierunkowanych bezpośrednio na produkt, dystrybutorów i handlowców, konsumentów czy środowisko naturalne, które wynikają ze świadomego oraz celowego procesu projektowania i rozwoju (Stewart 2014).

Mając na uwadze powyższe, podkreślić należy fakt, iż opakowanie jest formą zabezpieczania umieszczonego w nim produktu przed uszkodzeniami w czasie transportu i składowania, powinno ono także umożliwiać zachowanie jakości higienicznej towaru oraz ułatwić jego użytkowanie (Panfil-Kuncewicz 1993). Materiały opakowaniowe winny cechować się więc odpowiednią barierowością w stosunku do wody, gazów, tłuszczów i innych labilnych substancji, jak również chronić produkt przed zmianami chemicznymi oraz mikrobiologicznymi i zapewnić odbiorcy (konsumentowi) dostarczenie wyrobu w atrakcyjnej dla niego postaci. Co również istotne, opakowania powinny charakteryzować się obojętnością chemiczną w stosunku do pakowanego artykułu, tym samym nie jest dopuszczalna migracja składników materiału opakowaniowego do żywności w ilości zagrażającej zdrowiu człowieka (wyjątek stanowią tzw. opakowania aktywne). Wpływ na jakość zapakowanych wyrobów może być pośredni bądź bezpośredni. Pierwszy z nich występuje, gdy opakowanie zabezpiecza w wystarczającym stopniu przed zmianami jakie zachodzą w produkcie w wyniku działania tlenu, przenikania promieni słonecznych i pary wodnej. Natomiast wpływ bezpośredni polega na migracji składników opakowania do wyrobu lub odwrotnie.

Wśród materiałów opakowaniowych wykorzystywanych w przetwórstwie żywności dominują te z tworzyw sztucznych (Janicki i Ćwiek-Ludwicka 1999). Liczba odmian dostępnych tworzyw sztucznych stale wzrasta, a ilość wzajemnych kombinacji oraz możliwości ich łączenia z tworzywami klasycznymi jest niemal nieograniczona (Hadryjańska 2004). Produkcja tworzyw sztucznych, w związku ze swoją dynamiką, stała się więc we współczesnych czasach jednym z najbardziej istotnych problemów ekologicznych.

Wszechobecne i wszechstronnie stosowane polimery syntetyczne nie ulegają biorozkładowi lub są trudno rozkładalne przy zastosowaniu skomplikowanych procesów technologicznych. Większość produktów opakowaniowych cechuje stosunkowo krótki „czas życia" i zazwyczaj przystosowane są one do jednorazowego użytku. W związku z tym, istnieje potrzeba ciągłego pozyskiwania surowców niezbędnych do ich wytwarzania (np. ropy naftowej, gazu ziemnego, aluminium, drewna itd.) co dodatkowo obciąża środowisko naturalne.

Jednym z proponowanych rozwiązań jest wprowadzenie zasad gospodarki o obiegu zamkniętym (cyrkularnej), która ma na celu minimalizację zużycia surowców i wielkości odpadów oraz emisji i utraty energii poprzez tworzenie zamkniętej pętli procesów. Polega ona na tym, że odpady z jednych procesów są wykorzystywane jako surowce dla innych, co maksymalnie zmniejsza ilość odpadów produkcyjnych. Należy podkreślić, że obecnie opakowania są nieodzownym elementem obrotu towarowego, a stosowanie ich jest wymagane nie tylko ze względów logistycznych ale również higieniczno-sanitarnych. Rezygnacja z produkcji opakowań wydaje się zatem niemożliwa. Istnieje natomiast nagląca potrzeba znalezienia odpowiednich rozwiązań, pozwalających na częściową likwidację zagrożenia dla środowiska naturalnego, jakie stanowią zajmujące coraz większe obszary składowania odpadów opakowaniowych. Sytuacja ta doprowadziła do zwrócenia uwagi świata naukowego i producentów na poszukiwanie alternatywnego, a tym samym przyjaznego dla środowiska, naukowego rozwiązania, polegającego na rozwoju produkcji opakowań opartych na biodegradowalnych materiałach. Opakowania biodegradowalne są wymieniane jako jeden z alternatywnych kierunków rozwoju opakowań w wytycznych gospodarki o obiegu zamkniętym. Biodegradowalne materiały polimerowe wytwarzane są z surowców naturalnych lub syntetycznych. Ze względu na sposób otrzymywania mogą one zostać zaliczone do jednej z czterech grup (Macyszyn i Kozłowski 2012):

1. Polimery uzyskiwane przez bezpośrednią ekstrakcję z biomasy. Należą do nich polisacharydy (skrobia, celuloza, chityna, chitozan) i białka (pochodzenia zwierzęcego lub roślinnego);

2. Tworzywa syntetyzowane z monomerów pochodzących z przyrody. Jednym z przykładów jest poli(kwas mlekowy) (PLA) syntetyzowany w wyniku procesu polikondensacji z kwasu mlekowego, otrzymywanego w procesie fermentacji surowców cukrowych należących do zasobów odnawialnych;

3. Polimery produkowane przez mikroorganizmy. Jednymi z najczęściej stosowanych w przemyśle opakowaniowym polimerów pochodzenia mikrobiologicznego są polimery i kopolimery kwasu 3-hydroksymasłowego (HB) oraz 3-hydroksywalerianowego (HV);

4. Materiały otrzymywane przez syntezę z surowców petrochemicznych. Otrzymuje się tak m.in.: polialkohol winylowy (PVA), polikwas glikolowy (PGA), polikaprolakton (PCL) (Kozłowski 2008).

Materiały biodegradowalne znajdują zastosowanie w wielu sektorach przemysłu. Do celów opakowaniowych produkowane są one w formie m.in. pojemników, tacek, folii, kubków czy butelek. Znajdują również zastosowanie jako materiały do bezpośredniego

powlekania produktów spożywczych bądź innych materiałów opakowaniowych (np. papieru, tektury).

Niezbędność stosowania opakowań należy uznać za niezaprzeczalną, ze względu na ich wiele, różnie definiowanych funkcji, wśród których można wyróżnić: funkcję ochronną, marketingową, informacyjną oraz zarządzającą (Karpiel i Skrzypek 2000), przy czym szczególne zainteresowanie należy poświecić pierwszej z nich. Funkcja ochronna polega bowiem na zachowaniu wszelkich cech fizycznych, chemicznych i sensorycznych zapakowanego produktu i ochronie przed negatywnym oddziaływaniem czynników zewnętrznych i wewnętrznych, w całym cyklu życia opakowania, ze szczególnym uwzględnieniem przemieszczania towarów do konsumenta. Czynniki te mogą być zlikwidowane bądź zminimalizowane poprzez dobór odpowiedniego materiału opakowaniowego jak i jego formy konstrukcyjnej. Ważna jest również technologia i technika pakowania np. pakowanie w kontrolowanej lub modyfikowanej atmosferze MAP (ang. *modified atmosphere packaging*) (Singh 2010), umieszczanie absorberów tlenu (Mizielińska i współaut. 2015), wilgoci (Sumińska i Kowalska 2014), pakowanie aseptyczne i utrwalanie (fizyczne, chemiczne, biologiczne).

Istotnym trendem w rozwoju opakowań jest zastosowanie do ich modyfikacji substancji pochodzenia naturalnego. Substancje te mogą wpływać zarówno na materiał opakowaniowy (na jego właściwości mechaniczne, termiczne, barierowe, optyczne i powierzchniowe) ale również na zawarty w nim produkt (opakowania aktywne), chroniąc przed niekorzystnymi zmianami, które mogą zachodzić w wyrobie w wyniku rozwoju niepożądanych drobnoustrojów czy procesów oksydacyjnych. Obecnie najczęściej wykorzystywanymi w opakowalnictwie związkami aktywnymi są: bakteriocyny, ekstrakty z roślin, olejki eteryczne, enzymy oraz kwasy organiczne (Łopusiewicz i Mizielińska 2016). W środowisku naukowym pojawiła się również koncepcja wykorzystania w ten sposób melanin.

Terminem „melaniny" określa się grupę wielkocząsteczkowych barwników (czarnych, brązowych, żółtoczerwonych i o barwach pośrednich) odpowiedzialnych za ciemną pigmentację organizmów. Powstają one w wyniku oksydacyjnej polimeryzacji związków fenolowych i indolowych. Melaniny są najprawdopodobniej najbardziej powszechnymi, odpornymi, heterogenicznymi i najstarszymi ewolucyjnie pigmentami obecnymi w przyrodzie (Solano 2014; Łopusiewicz i Lisiecki 2016). Są syntetyzowane przez rośliny, zwierzęta, grzyby oraz bakterie, wśród których pełnią istotną rolę w procesach termoregulacji, chemoprotekcji, kamuflażu oraz procesach rozmnażania. Nazwa „melanina" pochodzi od greckiego terminu *melanos*, który oznacza „czarny/ciemny" i najprawdopodobniej została użyta po raz pierwszy przez szwedzkiego chemika Jönsa Joacoba Berzeliusa w 1840 roku do opisu ciemnego pigmentu wyizolowanego z oka (Solano 2014).

Melaniny to związki o nieregularnej i trójwymiarowej strukturze amorficznej, obdarzone ładunkiem ujemnym, zbudowane z monomerów związków fenolowych (przy czym najczęstszym substratem jest aminokwas tyrozyna) oraz indolowych, które łącząc się w sposób losowy tworzą strukturę polimeru (Solano 2014; Łopusiewicz i Lisiecki 2016). Ich

struktura i skład zależą nie tylko od budujących ich monomerów ale również od warunków środowiska w jakich zachodzi ich polimeryzacja (Henson 2005).

Wyróżnia się trzy grupy melanin: eumelaniny, feomelaniny i allomelaniny (Plonka i Grabacka 2006). Eumelaniny są polimerami złożonymi z pochodnych tyrozyny, głównie kwasu 5,6-dihydroksyindolo-2-karboksylowego (DHICA) oraz dihydroksyindolu (DHI). Wzajemny stosunek składowych eumelaniny decyduje o ostatecznej intensywności jej barwy. *In vivo* eumelaniny tworzą złożone kompleksy z białkami oraz metalami (głównie miedzią, cynkiem, żelazem oraz wapniem). Feomelaniny to żółto-czerwone barwniki, bogate w aminokwasy siarkowe (głównie cysteinę), posiadają w swojej strukturze podjednostki benzotiazyny oraz benzotiazolu. Allomelaniny natomiast są zróżnicowaną grupą barwników, niezawierających albo zawierających małe ilości azotu, które występują głównie w roślinach i grzybach, są syntetyzowane ze związków fenolowych (Socaciu 2008).

Szczególnym rodzajem melanin jest neuromelanina, specyficzna dla naczelnych, która stanowi kompleks zmodyfikowanej eumelaniny nawiniętej na rdzeń utworzony z feomelaniny. Rola neuromelaniny w mózgu polega prawdopodobnie na usuwaniu jonów organicznych i nieorganicznych, oraz ochronie przez wolnymi rodnikami (Zecca i współaut. 2001).

Warto wspomnieć, iż melaniny znalazły szerokie zastosowanie m.in. w syntezie nanocząstek metali o właściwościach przeciwdrobnoustrojowych (Apte i współaut. 2013), w diagnostyce medycznej (obrazowanie optoakustyczne) (Liopo i współaut. 2015), formowaniu powłok półprzewodnikowych, soczewek chroniących przed promieniowaniem UV, modyfikacji powierzchni materiałów w celu zwiększenia ich odporności na korozję, hydrofobizacji powierzchni materiałów oraz zwiększania ich zdolności adhezyjnych (Bernsman i współaut. 2010; Moressi i współaut. 2010; Sono i współaut. 2012). Obecne są one również w produktach i surowcach wykorzystywanych przez człowieka w przemyśle spożywczym (Łopusiewicz i Lisiecki 2016).

Melaniny mają zazwyczaj ciemny kolor jednak prezentują one szeroką paletę barw od czarnobrązowej (eumelanina, melaniny roślinne, bakteryjne i grzybowe) do żółtoczerwonej (feomelanina). Ta różnorodność kolorów i odcieni wynika z różnej zdolności do absorbcji i rozpraszania światła. Przyjmuje się, że im mniejsze są granule melaniny, tym jaśniejszy mają kolor (Prota 1992). Spektrum absorbcji światła przez melaniny nie ma jednego charakterystycznego maksimum pochłaniania i jest dość szeroko rozłożone w zakresie długości fal odpowiadających promieniowaniu UV, widzialnemu oraz częściowo podczerwonemu, jednak lepiej pochłania fale o krótszej długości. Ogółem, melaniny mają ciemną barwę, ponieważ ich cząsteczki nie wypromieniowują z powrotem zaabsorbowanego widzialnego lub niewidzialnego dla oka ludzkiego światła, ale przekształcają jego energię, rozpraszając ją w postaci ciepła (Łopusiewicz i Lisiecki 2016).

Co więcej, melaniny wykazują specyficzne właściwości fizykochemiczne, cechując się silnymi właściwościami przeciwutleniającymi. Wpływa na to obecność stabilnej populacji organicznych wolnych rodników *o*-semichinonowych, a także grup utleniających lub redukujących, np. *o*-chinonów oraz hydrochinonów (Prota 1992). Dzięki temu posiadają

właściwości przeciwutleniające, chroniące komórki przed cytotoksycznymi reaktywnymi formami tlenu (ROS – ang. *reactive oxygen species*) i wolnymi rodnikami, takimi jak: tlen singletowy, rodnik hydroksylowy oraz anionorodnik ponadtlenkowy (Różanowska i współaut. 1999; Łopusiewicz i Lisiecki 2016). Melaniny wykazują silne właściwości przeciwutleniające, już w stężeniach rzędu µg/ml, czyli porównywalne lub lepsze do właściwości powszechnie stosowanych przeciwutleniaczy takich jak butylowany hydroksytoluen (BHT) czy butylowany hydroksyanizol (BHA). Związki te mogą być szkodliwe dla zdrowia człowieka, dlatego wiele uwagi poświęca się przeciwutleniaczom pochodzenia naturalnego. Wykazano, że melaniny roślinne obecne m.in. w herbacie, orzechach kasztana chińskiego, nasionach arbuza, owocach roślin *Vitex mollis*, *Randia echinocarpa* i *Crescentia alata* (endemicznych dla Meksyku) wykazują silne właściwości przeciwutleniające oraz chelatujące jony metali np. Fe^{2+} (Sava i współaut. 2001; Yao i współaut. 2012; Cuevaz-Juárez i współaut. 2014; Łopusiewicz 2018b). Wiele melanin grzybowych również wykazuje właściwości przeciwutleniające (Eisenman i Casadevall 2012; Toledo i współaut. 2017; Łopusiewicz 2018a).

Potwierdzono, że niektóre melaniny charakteryzują się właściwościami przeciwdrobnoustrojowymi. Stwierdzono, że melaniny z owocników kisielnicy kędzierzawej (*Exidia nigricans*) oraz z nasion arbuza (*Citrullus lanatus*) wykazują aktywność przeciwko *Enterococcus faecalis* i *Pseudomonas aeruginosa* (Łopusiewicz 2018a,b). Laxmi i współaut. (2016) zaobserwowali aktywność melaniny z bakterii *Providencia rettgeri* przeciwko *Pseudomonas aeruginosa*, zaś Helan Soundra Rani i współaut. (2013) stwierdzili aktywność przeciwdrobnoustrojową melaniny z drożdży *Hortaea werneckii*. Xu i współaut. (2017) zwrócili natomiast uwagę na szeroką aktywność przeciwdrobnoustrojową melaniny pochodzącej ze szczepu grzyba *Lachnum* YM30. Autorzy ci sugerują, że aktywność przeciwdrobnoustrojowa melanin może wynikać z mechanizmu uszkodzenia ściany komórkowej bakterii a tym samym zaburzenia jej funkcjonowania. Różnorodne spektrum aktywności przeciwdrobnoustrojowej może natomiast wynikać z różnic w budowie i składzie melanin (Correra i współaut. 2017). W piśmiennictwie obecne są również doniesienia, że melaniny mogą wpływać na bakteryjny system *quorum sensing* („porozumiewanie się" bakterii poprzez cząsteczki sygnałowe), zaburzając tym samym mechanizm tworzenia biofilmu m. in. przez patogeniczne szczepy *P. aeruginosa* (Zhu i współaut. 2011; Bin i współaut. 2012)

Dotychczas dostępne piśmiennictwo dotyczące zastosowania melanin do modyfikacji materiałów opakowaniowych nie jest szerokie i z pewnością nie wyczerpuje tego tematu. Dong i współaut. (2014) zastosowali melaninę pochodzącą z sepii do modyfikacji poli(alkoholu winylowego). Stwierdzili, że melaniny mogą zwiększać termostabilność materiału, nawet w niskich stężeniach (0.5%), z uwagi na ich zdolność do wyłapywania wolnych rodników, które odpowiedzialne są za procesy degradacji. Shanmuganathan i współaut. (2011) dodając do PMMA (polimetylmetakrylanu) syntetyczną L-DOPA melaninę w ilościach 0.5-5% uzyskali znaczny wzrost temperatury rozkładu takiego polimeru (o 50-90°C, w porównaniu do niemodyfikowanego polimeru), spowodowany wyłapywaniem przez melaninę wolnych rodników powodujących depolimeryzację materiału. Dodatkowo, 1%

udziału melaniny w folii z PMMA pozwolił na zatrzymanie 80% przechodzącego przez nią światła. Kiran i współaut. (2017) opracowali kompozytowy materiał składający się z nanomelaniny i polihydroksymaślanu, który charakteryzował się silnymi właściwościami przeciwutleniającymi i hamował wzrost wieloopornego szczepu *Staphylococcus aureus*. Kuang i współaut. (2014) wykorzystali zaś melaniny do pokrywania poliwęglanu, a następnie do wzbogacania takiego układu antybiotykiem – gentamycyną. Tak przygotowane układy były zdolne do kontrolowanego uwalniania antybiotyku i hamowania wzrostu niepożądanych drobnoustrojów. Warto również zaznaczyć, że same melaniny mogą być wykorzystane do wytwarzania cienkich powłok (Da Silva i współaut. 2004; Dezidério i współaut. 2004; Bernsman i współaut. 2010; Moressi i współaut. 2010; Sono i współaut. 2012; Wu i Hong 2015).

Powyższe publikacje niewątpliwie wskazują na potencjał wykorzystania melanin w produkcji materiałów opakowaniowych a co za tym idzie, wzbudzają zainteresowanie tym tematem, także w zakresie dotyczącym sposobu pozyskiwania naturalnej melaniny na szeroką, przemysłową skalę. Wydaje się, iż na rynku polskim, odpowiednim źródłem tej substancji może być, w szczególności, hodowla pieczarek.

Produkcja pieczarki dwuzarodnikowej (*Agaricus bisporus*) w Polsce charakteryzuje się od lat dynamicznym wzrostem ilościowym, który wynosi nawet 320 tys. ton na rok. Polska jest głównym producentem *A. bisporus* w Europie i jednym z największych na świecie (pierwsze miejsce zajmują Chiny) (Xu i współaut. 2016). Produkcja ta wprowadza do środowiska naturalnego duże ilości odpadów, nie tylko w postaci podłoża do hodowli pieczarki, ale również biomasy grzybowej, które muszą być zagospodarowane.

Rolnicze i pozarolnicze wykorzystanie zużytych odpadów grzybowych jest możliwe, gdy jest w odpowiedni sposób zabezpieczone, poprzez minimalizację ryzyka przenoszenia szkodników, patogennych mikroorganizmów i nasion chwastów. Zużyta materia organiczna, powstająca przy produkcji grzybów, zawiera znaczne ilości węgla i azotu, przy czym stosunek tych pierwiastków jest podobny do poziomu w humusie, obecnym w glebie mineralnej i biologicznie aktywnej (Kalembasa i współaut. 2012). Według Majchrowskiej-Safaryan i Tkaczuka (2013) zastosowanie zużytego substratu grzybowego do gleby poprawia jej właściwości fizyczne i zwiększa zawartość makro- i mikroelementów, co sprawia, że może być on wykorzystany jako nawóz. Po zakończeniu procesu uprawy, grzyby (kapelusze wraz z częściami trzonów) są odcinane i przekazywane do dalszego przetwórstwa lub na sprzedaż. Podstawy trzonów są traktowane jako odpad i używane wraz z substratem do wzrostu jako nawóz. W dostępnym piśmiennictwie nie znaleziono informacji na temat innych zastosowań odpadów z produkcji pieczarki. Biorąc pod uwagę skalę produkcji pieczarek i ilość powstających odpadów, zasadnym wydaje się poszukiwanie innych sposobów ich wykorzystania, takich jak przetwarzanie ich do cennych bioaktywnych produktów, np. melanin.

Pieczarki bogate są w związki fenolowe, które mogą być substratem dla naturalnie w nich występującego enzymu, tyrozynazy. Tyrozynaza przekształca związki fenolowe do *o*-difenoli, które następnie przekształcane są do *o*-chinonów, wysoce reaktywnych związków,

które w warunkach tlenowych mogą polimeryzować do melanin (w warunkach naturalnych to zjawisko zwane jest jako ciemnienie enzymatyczne) (Jolivet i współaut. 1998; Weijn i współaut. 2013; Łopusiewicz i Lisiecki 2016). Opierając się więc na naturalnej obecności związków fenolowych i tyrozynazy w pieczarkach, można – w znaczący sposób – obniżyć koszty pozyskiwania melanin.

Przytoczone powyżej informacje dały asumpt do podjęcia badań na temat możliwości pozyskania z biomasy grzybowej melanin, które mogą stanowić obiecującą grupę związków o wielu pożądanych właściwościach, mogących znaleźć zastosowanie do modyfikacji materiałów opakowaniowych. Podjęta tematyka rozprawy doktorskiej porusza również zagadnienia z zakresu ekologii, ponieważ wskazuje kierunek zagospodarowania odpadu przemysłu rolno-spożywczego za pomocą procesów biokonwersji do uzyskania cennego produktu.

2. Cel pracy i hipotezy badawcze

Celem pracy było określenie możliwości zastosowania melanin z biomasy grzybowej do poprawy właściwości użytkowych materiałów opakowaniowych.

Hipotezy badawcze:

Założono, że biomasa grzybowa (odpad z produkcji pieczarki dwuzarodnikowej *Agaricus bisporus*, ryzomorfy opieńki miodowej *Armillaria mellea* oraz owocniki tęgoskóra cytrynowego *Scleroderma citrinum*) może stanowić źródło melanin o właściwościach przeciwutleniających, barierowych względem promieniowania UV oraz przeciwdrobnoustrojowych.

Równocześnie założono, że:

- melaniny z biomasy grzybowej mogą stanowić dodatek do powłok nanoszonych na folie opakowaniowe i mieć wpływ na ich właściwości przeciwutleniające oraz optyczne;

- melaniny z biomasy grzybowej mogą stanowić dodatek do folii z poli(kwasu mlekowego) (PLA) i mieć wpływ na ich właściwości mechaniczne, termomechaniczne, barierowe, powierzchniowe, optyczne, przeciwutleniające i przeciwdrobnoustrojowe;

- melaniny z biomasy grzybowej mogą stanowić dodatek do folii z żelatyny i mieć wpływ na ich właściwości mechaniczne, termomechaniczne, barierowe, powierzchniowe, optyczne, przeciwutleniające i przeciwdrobnoustrojowe;

- powłoki z żelatyny modyfikowanej melaniną mogą być zastosowane na modelowym produkcie spożywczym (smalec wieprzowy), chroniąc go przed procesami utleniania.

3. Materiały i metody

Do pozyskiwania melanin wykorzystano 3 rodzaje biomasy grzybowej:

a) odpad powstający przy produkcji pieczarki dwuzarodnikowej (*Agaricus bisporus*) w postaci trzonów owocników (ABW – *Agaricus bisporus* waste), pozyskany od lokalnego producenta w Wolsztynie (województwo wielkopolskie), oraz owocniki pieczarek zakupione w supermarkecie „Tesco";

b) owocniki tęgoskóra cytrynowego (*Scleroderma citrinum*);

c) ryzomorfy opieńki miodowej (*Armilaria mellea*).

Wybór materiału w postaci ABW podyktowany był jego dużą ilością oraz brakiem dostępnej literatury dotyczącej jego zagospodarowania w kierunku pozyskiwania substancji aktywnych. Ryzomorfy *A. mellea* i owocniki *S. citrinum* zostały wybrane z uwagi na dostępność materiału na stanowiskach naturalnych.

Aby oznaczyć suchą masę zawartą w odpadzie z produkcji pieczarki dwuzarodnikowej posłużono się metodą grawitmetryczną (3-6 pojedynczych trzonów suszono w temperaturze 105°C do stałej wagi). Całkowitą zawartość rozpuszczalnych polifenoli (TPC – *total polyphenolic content*), tj. prekursorów do syntezy melaniny, oznaczono metodą Folina-Ciocalteau w 80% ekstrakcie etanolowym z ABW. Reakcję pozyskiwania melaniny z ABW oparto na dwuetapowym procesie. W pierwszym wykorzystano naturalną zawartość enzymu tyrozynazy, odpowiedzialnej za hydroksylację monofenoli to *o*-difenoli, oraz *o*-difenoli do chinonów, inkubując zhomogenizowany odpad przez 24 godziny w temperaturze 37°C (optimum temperaturowe dla tyrozynazy). W drugim etapie homogenat alkalizowano do pH 10 przy pomocy 1 M NaOH i inkubowano przez kolejne 24 godziny w temperaturze 65°C (w warunkach alkalicznych i tlenowych zachodziła spontaniczna polimeryzacja chinonów do melanin). Po okresie inkubacji mieszaninę reakcyjną filtrowano, odwirowywano (6000 obr/min, 10 min), uzyskując alkaliczny roztwór nieoczyszczonej melaniny ABW (ABW-RM – *Agaricus bisporus* waste – raw melanin).

Natywne melaniny z ryzomorf *A. mellea* i owocników *S. citrinum* pozyskiwano poprzez bezpośrednią ekstrakcję ze zhomogenizowanej biomasy w warunkach alkalicznych, podobnie jak w przypadku ABW uzyskując zasadowe roztwory AM-RM (*Armillaria mellea* – raw melanin) i SC-RM (*Scleroderma citrinum* – raw melanin). Kolejne etapy badań były wspólne dla wszystkich trzech rodzajów melanin.

W celu oczyszczenia melanin, ich zasadowe roztwory zakwaszano 1 M roztworem HCl do pH 2 (w warunkach kwasowych następuje wytrącanie melanin). Osad każdej melaniny odwirowano (6000 obr/min, 10 min), a następnie przeprowadzano hydrolizę w 6 M HCl (90°C, 2 godziny) w celu usunięcia związanych z melaninami polischacharydów, białek i innych składników. Po hydrolizie osady odwirowano i przemywano kilkukrotnie wodą destylowaną, aż do usunięcia kwasu. Następnie osady przemywano trzykrotnie chloroformem, octanem etylu i etanolem, w celu usunięcia lipidów i produktów hydrolizy.

Osady suszono, rozdrabniano w moździerzu na drobny proszek i przechowywano w temp. -20°C do dalszych badań. W ten sposób uzyskano oczyszczone melaniny określane jako ABW-PM (*Agaricus bisporus* waste – pure melanin), AM-PM (*Armillaria mellea* – pure melanin) oraz SC-PM (*Scleroderma citrinum* – pure melanin).

Dla każdej melaniny natywnej i oczyszczonej przeprowadzono badania ich rozpuszczalności (w wodzie i rozpuszczalnikach organicznych: acetonie, chloroformie, dimetylosulfotlenku, etanolu i octanie etylu; ponadto przeprowadzono testy zachowania się melanin w obecności czynnika utleniającego – H_2O_2), właściwości redukujących (reakcja z amoniakalnym roztworem $AgNO_3$) oraz na obecność polifenoli (reakcja z $FeCl_3$). Oceniano również wizualnie barwę. Jako standard melaniny we wszystkich badaniach wykorzystywano syntetyczną L-DOPA melaninę.

Przeprowadzono analizę spektroskopową uzyskanych melanin, określając ich widma absorpcji i transmitancji w zakresie promieniowania UV-Vis (200-800 nm), na podstawie których wyznaczano wartości A300/A600 (wartości absorbancji przy długościach fal 300 i 600 nm, informujące o stopniu oksydacji i rozmiarze cząstek melanin) oraz wykresy log absorbancji w funkcji długości fali.

Przeprowadzono również analizę spektroskopii FT-IR oraz Ramana w celu uzyskania informacji o grupach funkcyjnych obecnych w melaninach.

Pomiary kolorymetryczne przeprowadzono przy pomocy kolorymetru określając na podstawie modelu CIE Lab wartości parametrów *L*, *a*, *b* opisujących jasność oraz barwy chromatyczne. Określono również zawartość grup polifenolowych w cząsteczkach melanin oraz ich właściwości przeciwutleniające za pomocą odczynnika ABTS. Właściwości przeciwdrobnoustrojowe (względem bakterii Gram+ i Gram-) melanin oznaczano metodą dyfuzyjno-dołkową.

Przygotowano roztwory powłokotwórcze z chitozanu oraz skrobi hydroksypropylowanej A4b, do których dodawano roztwory oczyszczonych melanin ABW-PM, AM-PM oraz SC-PM rozpuszczonych w DMSO. Próbę kontrolną stanowiły układy powłokotwórcze chitozanu i skrobi z dodatkiem DMSO, pozbawione melanin. Następnie nanoszono układy powłokotwórcze na aktywowane koronowo folie PE (polietylen), PLA [poli(kwas mlekowy)], BOPP (polipropylen orientowany dwuosiowo) i PET [poli(tereftalan etylenu)] za pomocą powlekarki laboratoryjnej, które następnie suszono 20 min w temp. 45°C. Dla wszystkich folii oznaczono zawartość polifenoli w powłokach (metoda Folina-Ciocalteau) oraz właściwości przeciwutleniające (metoda DPPH). Dla folii BOPP i PET przeprowadzono pomiary parametrów *L*, *a*, *b* i na ich podstawie wyznaczono wartości parametrów ΔE (różnica koloru w odniesieniu do standardowej białej płytki), YI (yellownes index) oraz WI (whiteness index). Oznaczono również właściwości barierowe folii względem promieniowania UV-Vis poprzez pomiar transmitancji.

Poli(kwas mlekowy) w postaci granulatu typ 4043D pozyskano od firmy NatureWorks – dostawca Resinex. Melaninę ABW-PM dodawano do granulatu PLA w ilościach (w/w): 0.025%; 0.05% i 0.2%. Kompozytowe materiały modyfikowane z dodatkiem melaniny

wytłaczano na wytłaczarce dwuślimakowej w postaci wstęg folii o grubości 75-80 μm. Próbę kontrolną stanowiła folia bez dodatku melaniny. Wykonano badania mechaniczne uzyskanych materiałów obejmujące określenie maksymalnych sił zerwania i przebicia, oraz siły potrzebnej do rozerwania zgrzewu. Określono również dynamiczne mechaniczne właściwości kopozytowych materiałów przy pomocy urządzenia DMA, wyznaczając wartości E', E'', tan δ oraz temperatury zeszklenia T_g. Dokonano również analizy barierowości kompozytowych materiałów względem pary wodnej (WVTR – *Water Vapour Transmission Rate*) metodą grawimetryczną, właściwości powierzchniowych (pomiar kąta zwilżania) oraz wpływu dodatku melanin na krystaliczność metodą skaningowej kalorymetrii różnicowej. Przeprowadzono analizy spektroskopowe obejmujące analizę w zakresie promieniowania UV-Vis, FT-IR oraz Ramana. Dokonano analizy wpływu dodatku melaniny w różnych stężeniach na wartości składowych barwy w systemie CIE Lab. Dokonano pomiarów właściwości przeciwutleniających (ABTS), ilości grup polifenolowych dostępnych na powierzchni materiału (metoda Folina-Ciocalteu) oraz właściwości antymikrobiologicznych (ISO22196:2007(E)).

Do badań weryfikacji możliwości zastosowania melanin do modyfikacji folii z żelatyny wybrano żelatynę firmy Rousselot SAS (Saint-Michel, Francja). Melaninę ABW-PM rozpuszczano przez 24 h (50°C) w wodzie destylowanej o pH 10 (uzyskanym przy pomocy wody amoniakalnej) w stężeniach 0.1%; 0.5% i 1% wag. Uzyskane roztwory przefiltrowan i dodawano do nich żelatynę (8 g na 100 mL roztworu). Roztwory inkubowano 2 godziny w temperaturze 60°C, po czym dodawano glicerolu jako plastyfikatora – 10% w stosunku do masy żelatyny. Uzyskane roztwory filmotwórcze wylewano na polistyrenowe szalki Petriego o średnicy 90 mm, po czym suszono je przez 48 godzin w temperaturze 25°C w komorze klimatyczej (50% RH). W tym czasie następowało odparowanie rozpuszczalnika i amoniaku oraz formowanie się folii. Folie bez dodatku melaniny stanowiły próbę kontrolną. Wszystkie folie przed badaniami kondycjonowano 24 godziny w temperaturze 25°C i wilgotności 50% RH. Analizowano rozpuszczalność folii, właściwości mechaniczne (maksymalna siła zerwania, przebicia oraz wydłużenie procentowe przy zerwaniu), przepuszczalność tlenu OTR (metodą kulometryczną) oraz pary wodnej WVTR (metodą grawimetryczną) a także właściwości powierzchniowe (pomiar kąta zwilżania). Dodatkowo przeprowadzono badania zawartości grup polifenolowych w foliach (metoda Folina-Ciocalteu), właściwości przeciwutleniających (ABTS) oraz przeciwdrobnoustrojowych. Dokonano analizy wpływu dodatku melaniny w różnych stężeniach na wartości składowych barwy w systemie CIE Lab. Przeprowadzono analizy spektroskopowe obejmujące analizę w zakresie promieniowania UV-Vis (pomiar transmitancji), FT-IR oraz Ramana.

Aby określić możliwość zastosowania modyfikowanego melaninami materiału z żelatyny do pakowania modelowego produktu spożywczego wybrano smalec. Smalec wieprzowy firmy „Nasze Smaki" powlekano roztworami powłokotwórczymi, które wcześniej wykorzystywano do otrzymywania folii z żelatyny (0.1%; 0.5%; 1% z dodatkiem melaniny ABW-PM). Układy przechowywano przez okres 21 dni w temperaturze 25°C, 50% RH, bez dostępu światła. Po 7, 14 i 21 dniach przechowywania oznaczano liczby; kolejno ponadtlenkową (POV), jodową (IV) oraz kwasową (AV). Wyniki odnoszono do smalcu niepowlekanego oraz pokrytego roztworem powłokotwórczym bez dodatku melaniny.

4. Wyniki badań

4.1. Biomasa grzybowa jako źródło melanin

W przedstawionej rozprawie doktorskiej otrzymano barwniki z trzech rodzajów biomasy grzybowej: odpadu powstającego przy produkcji pieczarki dwuzarodnikowej (*A. bisporus*), ryzomorf opieńki miodowej (*A. mellea*) oraz owocników tęgoskóra cytrynowego (*S. citrinum*). W wyniku przeprowadzonych analiz i porównania z syntetyczną L-DOPA melaniną stwierdzono, że wszystkie pozyskane związki to melaniny. Stwierdzono, że w zaproponowanym procesie (wykorzystującym naturalną zawartość enzymu tyrozynazy) można uzyskać 5.19±0.24% oczyszczonej melaniny w stosunku do suchej masy odpadu *A. bisporus*.

Wszystkie uzyskane melaniny były nierozpuszczalne w wodzie i większości rozpuszczalników organicznych (aceton, chloroform, octan etylu, metanol, etanol). Rozpuszczały się jedynie w alkalicznych roztworach wodnych oraz DMSO, zaś w roztworach kwaśnych wytrącały się w postaci osadu. Wszystkie analizowane melaniny w obecności czynnika utleniającego (H_2O_2) odbarwiały się, co świadczy o ich degradacji. Wykazały one również właściwości redukujące (osad srebra w wyniku reakcji z amoniakalnym roztworem $AgNO_3$) oraz pozytywny wynik reakcji na polifenole. Zaobserwowano, że wszystkie melaniny charakteryzowały się ciemną barwą.

Stwierdzono, że badane melaniny charakteryzują się zdolnością do pochłaniania promieniowania UV-Vis, szczególnie w zakresie promieniowania ultrafioletowego, osiągając maksimum absorbcji ok. 220 nm. Stwierdzono, że melaniny oczyszczone posiadają lepsze właściwości barierowe względem promieniowania UV-Vis niż melaniny nieoczyszczone, a także wyższą zawartość grup polifenolowych oraz wyższe właściwości przeciwutleniające. Analiza FT-IR wykazała różnice ilościowe i rodzajowe w badanych próbkach nieoczyszczonych i oczyszczonych melanin, które wynikają z obecności polisacharydów i białek związanych z nieoczyszczonymi melaninami. Melaniny ABW-RM, ABW-PM, SC-RM i SC-PM charakteryzowały się aktywnością przeciwbakteryjną w stosunku do *Enteroccoccus facealis* i *Pseudomonas aeruginosa*. Najszerszym spektrum aktywności przeciwbakteryjnej charakteryzowała się melanina AM-RM, która wykazywala aktywność przeciwko *Staphyloccocus aureus*, *Enterococcus facealis*, *Escherichia coli*, *Micrococcus luteus*, *Bacillus atrophaeus*, *Bacillus cereus*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Pseudomonas putida*), zaś oczyszczona forma AM-PM była aktywna przeciw *B. cereus*, *B. subtilis*, *E. faecalis*, *P. aeruginosa* i *P. putida*. Szczegółowe wyniki wraz z dyskusją zawarte są kolejno w pracach **P1**, **P2** i **P3**.

4.2. Melaniny grzybowe jako dodatek do powłok nanoszonych na folie opakowaniowe

Na podstawie przeprowadzonych doświadczeń stwierdzono, że dodatek melanin grzybowych do powłok chitozanowych i skrobiowych nanoszonych na powierzchnię folii PE, PLA, BOPP i PET pozwala nadać im właściwości przeciwutleniające. Właściwości te zależą

nie tylko od źródła pochodzenia melanin, ale również od nośników wykorzystanych do tworzenia powłok. Wzrastające stężenie melanin w roztworach powłokotwórczych spowodowało wzrost właściwości przeciwutleniających. W przypadku zarówno powłok z chitozanu, jak i ze skrobi zaobserwowano, że wzrastająca zawartość melanin spowodowała wzrost zawartości polifenoli w powłokach. Odnotowano również niewielką aktywność przeciwutleniającą powłok bez dodatku melanin, wynikającą z obecności grup aminowych lub hydroksylowych w chitozanie i skrobi. Nie odnotowano znaczącej poprawy właściwości barierowych względem promieniowania UV-Vis w przypadku folii BOPP i PET modyfikowanych powłokami zawierającymi melaniny. Duży wpływ na właściwości barierowe miały same powłoki niezawierające melanin, szczególnie ze skrobi. Największy wpływ melanin na właściwości barierowe powłok względem promieniowania wśród folii BOPP, odnotowano dla folii powleczonej chitozanem zawierającym 500 µg/mL melaniny ABW-PM, w przypadku której transmitancja w zakresie długości fal 250-300 nm była o ok. 4% niższa, niż w przypadku folii powleczonej jedynie chitozanem, natomiast przy długości fali 225 nm, była o 16.61% niższa. Brak znaczącej poprawy właściwości barierowych względem promieniowania UV-Vis odnotowano także w przypadku modyfikowanych folii PET. Zaobserwowano jedynie nieznaczną poprawę właściwości barierowych względem promieniowania UV-Vis w przypadku folii z powłokami skrobiowymi, przy długości fali 325 nm folie charakteryzowały się transmitancją o ok. 4% niższą niż folie powleczone powłokami skrobiowymi bez dodatku melanin. Najprawdopodobniej wykorzystane w badaniach własnych stężenia melanin były zbyt niskie dla uzyskania satysfakcjonującej poprawy barierowości względem promieniowania UV-Vis. Zaobserwowano wpływ dodatku melanin grzybowych w powłokach na barwę modyfikowanych folii BOPP i PET. Wzrastające stężenia melanin grzybowych w powłokach spowodowały wzrost parametru *a* (w kierunku barwy czerwonej) w obu rodzajach folii. Odnotowano również w przypadku wszystkich folii BOPP i PET, że wzrastające stężenia melanin powodowały wzrost parametru *b* (w kierunku barwy żółtej). Obliczone na podstawie uzyskanych wyników parametry ΔE, w przypadku wszystkich modyfikowanych folii przyjmowały wartość większą od 3.5, co świadczy o tym, że różnica barw dostrzegalna przez obserwatora była wyraźna. Zaobserwowano również wzrost parametru YI, co wiązało się ze zmianą parametru *b* w kierunku barwy żółtej. Szczegółowe wyniki wraz z dyskusją zawarte są w pracach **P4 i P5.**

4.3. Modyfikacja melaninami folii z poli(kwasu mlekowego)

Na podstawie przeprowadzonych badań stwierdzono, że możliwe jest uzyskanie kompozytowego materiału opakowaniowego z poli(kwasu mlekowego) i melaniny. Kompozytowy materiał z PLA z dodatkiem 0.025%; 0.05% i 0.2% melaniny ABW-PM wytłoczono w postaci wstęgi folii. Analiza mikroskopowa wykazała, że granule melaniny (wielkości poniżej 1 µm) są jednorodnie rozmieszczone w materiale. Stwierdzono, że dodatek melaniny wypłynął na właściwości mechaniczne i barierowe folii, zależnie od stężenia. Dodatek 0.025% i 0.05% spowodował poprawienie zarówno właściwości mechanicznych (wytrzymałość na rozerwanie, siła przebicia) i barierowych względem pary wodnej, lecz co ciekawe większy udział 0.2% wag. melaniny wpłynął na nie negatywnie. Zaobserwowano

niewielką poprawę właściwości barierowych względem promieniowania UV-Vis, folie z dodatkiem 0.2% melaniny charakteryzowały się transmitancją ok. 7-8% niższą w zakresie promieniowania UV-A i UV-B. Stwierdzono, że dodatek melanin nie miał istotnego wpływu na właściwości powierzchniowe folii. Analiza DSC wykazała, że pomimo zwiększenia krystaliczności materiału w porównaniu do próby kontrolnej, dodatek melanin nie wpłynął istotnie na wartości temperatur zeszklenia (T_g), temperatur zimnej krystalizacji (T_c) oraz topnienia (T_m). Ponadto wszystkie badanie folie charakteryzowały się wysoką amorficznością. Na podstawie analizy FT-IR jedynie w przypadku folii z największym (0.2%) dodatkiem melaniny zaobserwowano różnice w intensywności niektórych pasm widma. Zauważalne różnice zaobserwowano na widmach Ramanowskich, uzyskując wyraźne i intensywne pasma pochodzące głównie od pierścieni aromatycznych i grup –CH obecnych w cząsteczkach melanin. Badania kolorymetryczne wykazały, że dodatek melanin obniżył jasność folii (*L*) oraz zwiększył wartości parametrów *a* (w kierunku barwy czerwonej) i *b* (w kierunku barwy żółtej), a także obniżył wartość parametru YI w porównaniu do folii kontrolnej. W przypadku folii z największym dodatkiem melaniny (0.2%) wartość parametru ΔE wynosiła 1.33, co oznacza, że różnica w barwie w odniesieniu do niemodyfikowanej folii była dostrzegalna. Niemodyfikowane folie nie wykazywały właściwości przeciwutleniających, natomiast zaobserowano, że wraz ze wzrostem melanin w matrycy polimeru wzrastały zarówno właściwości przeciwutleniające folii jak i ilość grup polifenolowych występujących na powierzchnii materiału. Modyfikowane folie charakteryzowały się właściwościami antybakteryjnymi. Były aktywne przeciwko *Enterococcus faecalis*, *Pseudomonas aeruginosa* i *Pseudomonas putida*, nie działały jednak na *Escherichia coli* i *Staphylococcus aureus*. Szczegółowe wyniki wraz z dyskusją zawarte są w publikacji **P6.**

4.4. Modyfikacja melaninami folii z żelatyny

Przeprowadzone badania wykazały, że możliwe jest uzyskanie folii z żelatyny modyfikowanej melaniną ABW-PM. Folie z udziałem 0.1%; 0.5% i 1% melaniny, plastyfikowane glicerolem formowano metodą wylewania wodnej mieszaniny foliotwórczej na szalki Petriego i odparowywania rozpuszczalnika. Stwierdzono, że dodatek melanin znacząco obniżył rozpuszczalność folii (72.16±3.24% dla folii niemodyfikowanych, 31.05±2.75% dla folii z dodatkiem 1% melaniny). Odnotowano znaczący wzrost kąta zwilżania dla folii modyfikowanych melaniną (53.3° dla folii niemodyfikowanych oraz 72.9° dla folii z dodatkiem 1% melaniny), co świadczy o zmianie właściwości powierzchniowych folii i wzroście hydrofobowości materiału. Zaobserwowano nieznaczny spadek przepuszczalności względem pary wodnej (nieistotny statystycznie), właściwości barierowe względem tlenu uległy poprawie. Nie odnotowano istotnego wpływu melanin na właściwości wytrzymałości mechanicznej folii. Odnotowano znaczącą poprawę właściwości barierowych względem promieniowania UV-Vis. Dodatek melaniny do folii z żelatyny znacząco zwiększył właściwości przeciwutleniające. Zarówno modyfikowane folie jak i folia bez dodatku melanin nie wykazywały właściwości przeciwdrobnoustrojowych. Zaobserwowano, że dodatek melaniny do folii zmniejszył wartość parametru *L* (jasność), oraz zwiększył wartość

parametrów *a* (w kierunku barwy czerwonej) i *b* (w kierunku barwy żółtej). Szczegółowe wyniki wraz z dyskusją zawarte są w pracy **P7**.

4.5. Powłoki z żelatyny modyfikowanej melaniną i ich wpływ na procesy utleniania w modelowym produkcie spożywczym, w postaci smalcu wieprzowego

Na podstawie przeprowadzonych badań stwierdzono, że liczby ponadtlenkowa (POV) oraz kwasowa (AV) niepowlekanego smalcu wzrastały w szybkim tempie w czasie przechowywania. Zaobserwowano również spadek liczby jodowej (IV), spowodowany spadkiem zawartości nienasyconych kwasów tłuszczowych. Odnotowano, że obecność powłok z żelatyny znacząco spowalnia wzrost POV i AV, oraz spadek IV. W całym okresie inkubacji POV i AV smalcu powleczonego powłokami zawierającymi melaninę były niższe niż smalcu pokrytego powłoką bez dodatku melaniny, zaś IV wyższe. Sugeruje się, że efekt ten można przypisać działaniu przeciwutleniającemu melaniny, oraz obniżonej barierowości względem tlenu modyfikowanych folii, co wykazano w pracy P7. Szczegółowe wyniki wraz z dyskusją zawarte są w pracy **P8.**

5. Podsumowanie

Celem przedstawionej rozprawy doktorskiej było uzyskanie odpowiedzi na pytanie czy melaniny pozyskane z biomasy grzybowej mogą być zastosowane do poprawy właściwości użytkowych materiałów opakowaniowych. W szczególności skupiono się na odpadzie powstającym przy produkcji pieczarki, z uwagi na jego dostępność i szczególny potencjał produkcji tego rodzaju surowca, ponieważ Polska znajduje się na 4-5 miejscu pod względem produkcji pieczarek na świecie. Uzyskane wyniki pozwoliły na otrzymanie cennego produktu konkurującego cenowo z dostępnymi na rynku syntetycznymi melaninami i to z odpadu, który był dotychczas postrzegany jako nieużyteczny lub stosowany jako nawóz. Koszt otrzymania 1 grama melaniny z odpadu jest około tysiąc razy mniejszy niż koszt 1 grama syntetycznej melaniny. Zaproponowane rozwiązanie może przyczynić się w istotnym stopniu do ochrony środowiska poprzez zagospodarowanie odpadu powstającego w znacznych ilościach w pieczarkarniach. Ponadto wykazano, że biomasa grzybowa pozyskana ze stanowisk naturalnych w postaci ryzomorf oraz owocników również może stanowić źródło melanin. Melaniny, charakteryzując się szeregiem pożądanych właściwości takich jak właściwości przeciwutleniające, barierowe względem światła oraz przeciwdrobnoustrojowe, mogą znaleźć zastosowanie do modyfikacji materiałów opakowaniowych. W pracy doktorskiej wykazano, że można je zastosować zarówno jako bezpośredni dodatek do matrycy polimeru (PLA), dodatek modyfikujący polimer w roztworze (żelatyna) oraz jako dodatek do powłok. Wykazano również, że dodatek melanin może wpływać na szereg właściwości użytkowych modyfikowanych materiałów, tj. właściwości mechanicznych, barierowych, optycznych, powierzchniowych oraz antybakteryjnych. Ma to szczególne znaczenie z punktu widzenia zastosowania modyfikowanych materiałów opakowaniowych należących do grupy biotworzyw, gdyż polimery biodegradowalne, np. polilaktyd, są postrzegane jako alternatywa dla poliolefin i innych polimerów pochodzących z surowców nieodnawialnych, znajdując szerokie zastosowanie nie tylko w opakowalnictwie, ale także w innych dziedzinach np. biomedycynie. Uzyskane w przedstawionej pracy wyniki pozwalają stawiać kolejne pytania i tezy dotyczące m. in. możliwości zastosowania melanin grzybowych do modyfikacji innych materiałów opakowaniowych (np. folii i powłok z białek roślinnych lub zwierzęcych, polisacharydów, polimerów syntetycznych), ale także pozyskania i zastosowania melanin innego pochodzenia np. syntetycznych, bakteryjnych i roślinnych.

6. Wnioski

1. Biomasa grzybowa w postaci odpadu powstającego przy produkcji pieczarki dwuzarodnikowej (*Agaricus bisporus*) może stanowić bogate źródło melanin, pozyskiwanych dzięki naturalnej zawartości związków fenolowych oraz obecności enzymu tyrozynazy, zdolnej do modyfikacji chemicznej w kierunku melanin, które następnie mogą być ekstrahowane z biomasy w warunkach alkalicznych.

2. Zastosowanie odpadu z produkcji pieczarki do pozyskiwania melaniny pozwoliło na wskazanie alternatywnego kierunku zagospodarowania tego odpadu, oraz obniżenia kosztów pozyskiwania produktu finalnego.

3. Biomasa grzybowa w postaci ryzomorf opieńki miodowej (*Armillaria mellea*) i tęgoskóra cytrynowego (*Scleroderma citrinum*) może stanowić źródło melanin, które mogą być ekstrahowane z biomasy w warunkach alkalicznych.

4. Melaniny z biomasy grzybowej charakteryzowały się właściwościami przeciwutleniającymi, barierowymi względem promieniowania UV-Vis oraz przeciwbakteryjnymi.

5. Oczyszczone melaniny mają lepsze niż formy nieoczyszczone właściwości przeciwutleniające, barierowe względem promieniowania UV-Vis i charakteryzują się niższym parametrem *L* (jasność) oraz wyższymi parametrami *a* i *b*, wyższą zawartością grup polifenolowych. Melaniny nieoczyszczone i oczyszczone wykazują także różnice w obecnych w cząsteczkach grupach funkcyjnych.

6. Dodatek melanin do powłok z chitozanu i skrobi nanoszonych na folie PE, PLA, BOPP i PET pozwala nadać im właściwości przeciwutleniające.

7. Dodatek melanin do powłok z chitozanu i skrobi nanoszonych na folie BOPP i PET nie poprawił znacząco właściwości barierowych folii względem promieniowania UV-Vis.

8. Dodatek melanin do powłok z chitozanu i skrobi nanoszonych na folie BOPP i PET znacząco wpłynął na barwę folii.

9. Możliwe jest zastosowanie melaniny jako bezpośredniego dodatku podczas modyfikacji „w masie" folii PLA, co wpływa na właściwości przeciwutleniające, przeciwbakteryjne, barwę, krystaliczność oraz – zależnie od udziału melaniny – na właściwości mechaniczne i barierowe względem pary wodnej.

10. Możliwe jest zastosowanie melaniny jako dodatku do folii z żelatyny, co wpływa na obniżenie jej rozpuszczalności, zwiększenie właściwości przeciwutleniających, hydrofobowości, barierowości względem tlenu, barierowości względem promieniowania UV-Vis oraz na barwę materiału. Folie z żelatyny nie wykazywały właściwości przeciwbakteryjnych.

11. Możliwe jest zastosowanie powłok z żelatyny na smalcu wieprzowym, które działają ochronnie, spowalniając procesy jełczenia.

7. Literatura

1. Apte M., Girme G., Bankar A., Ravikumar A., Zinjarde S. 2013. 3,4-dihydoxy-L-phenylalanine-derived melanin from *Yarrowia lipolytica* mediates the synthesis of silver and gold nanostructures. *Journal of Nanobiotechnology* 11.
2. Bernsman F., Ersen O., Voegel J.C., Jan E., Kotov N.A., Ball V. 2010. Melanin-containing films: growth from dopamine solution versus layer-by-layer deposition. *ChemPhysChem* 11(15): 3299-305.
3. Bin L., Wei L., Xiaohong C., Mei J., Mingsheng D. 2012. In vitro antibiofilm activity of the melanin from *Auricularia auricula*, an edible jelly mushroom. *Annals of Microbiology* 62(4): 1523-1530.
4. Correa N., Covarrubias C., Rodas P.I., Hermosilla G., Olate V.R., Valdés C., Meyer W., Magne F., Tapia C.V. 2017. Differential Antifungal Activity of Human and Cryptococcal Melanins with Structural Discrepancies. *Frontiers in Microbiology* 8: 1–13.
5. Cuevas-Juárez E., Yuriar-Arredondo K.Y., Pío-León J.F., Montes-Avila J., López-Angulo G., Díaz-Camacho S.P., Delgado-Vargas F. 2014. Antioxidant and α-glucosidase inhibitory properties of soluble melanins from the fruits of *Vitex mollis* Kunth, *Randia echinocarpa* Sessé et Mociño and *Crescentia alata* Kunth. *Journal of Functional Foods* 9: 78-88.
6. Da Silva M.I.N., Dezidério S.N., Gonzalez J.C., Graeff C.F.O., Cotta M.A. 2004. Synthetic melanin thin films: Structural and electrical properties. *Journal of Applied Physics* 96: 5803–5807.
7. Dezidério S.N., Brunello D.A., da Silva M.I.N., Cotta M.A., Graeff C.F.O. 2004. Thin films of synthetic melanin. *Journal of Non-Crystalline Solids* 338-340: 634-638.
8. Dong W., Wang Y., Huang C., Xiang S., Ma P., Ni Z., Chen M. 2014. Enhanced thermal stability of poly(vinyl alcohol) in presence of melanin. *Journal of Thermal Analysis and Calorimetry* 115: 1661-1668.
9. Eisenman H.C., Casadevall A. 2012. Synthesis and assembly of fungal melanin. *Applied Microbiology and Biotechnology* 93: 931-940.
10. Hadryjańska B. 2004. Korzyści ekologiczne wynikające z wprowadzenia systemu zagospodarowania zużytych opakowań. *Opakowanie* 5: 10-15.
11. Helan Soundra Rani M., Ramesh T., Subramanian J., Kalaiselvam M. 2013. Production and Characterization of Melanin Pigment from Halophilic Black Yeast *Hortaea werneckii*. *International Journal of Pharma Research & Review* 2: 9–17.
12. Henson J.M, 2005. *Melanin. Biopolymers* Online. http://onlinelibrary.wiley.com/doi/10.1002/3527600035.bpol1008/full (dostęp 21.05.2018).
13. Janicki A., Ćwiek-Ludwicka K. *Towaroznawstwo żywność przetworzonej*, Wydawnictwo SGGW 1999.
14. Jolivet S., Arpin N., Wichers H.J., Pellon G. 1998. *Agaricus bisporus* browning: a review. *Mycological Research* 102(12): 1459-1483.
15. Kalembasa D., Becher M., Bik B., Makolewski A. 2012. Właściwości materii organicznej podłoża po uprawie pieczarki. *Acta Agrophysica* 19(4): 713-723.
16. Karpiel Ł., Skrzypek M. *Towaroznawstwo ogólne*. Wydawnictwo Akademii Ekonomicznej w Krakowie, 2000.
17. Kiran G.S., Jackson S.A., Priyadharsini S., Dobson A.D.W., Selvin J. 2017. Synthesis of Nm-PHB (nanomelanin-polyhydroxybutyrate) nanocomposite film and its protective effect against biofilm-forming multi drug resistant *Staphylococcus aureus*. *Scientific Reports* 7: 9167.
18. Kozłowski M. Materiały z surowców odnawialnych. *Recykling i Odzysk Materiałów Polimerowych* 2008.

19. Kuang J., Guo J.L., Messersmith P.B. 2014. High ionic strength formation of DOPA-melanin coating for loading and release of cationic microbial compounds. *Advanced Materials Interfaces* 1: 1–12.
20. Laxmi M., Kurian N.K., Smitha S., Bhat S.G. 2016. Melanin and bacteriocin from marine bacteria inhibit biofilms of foodborne pathogens. *Indian Journal of Biotechnology 15*: 392–399.
21. Liopo A., Su R., Oraevsky A.A. 2015. Melanin nanoparticles as a novel contrast agent for optoacoustic tomography. *Photoacoustics* 3(1): 35-43.
22. Łopusiewicz Ł. 2018a. Isolation, characterisation and biological activity of melanin from *Exidia nigricans*. *World Scientific News 91*.:111–129.
23. Łopusiewicz Ł. 2018b. Antioxidant, antibacterial properties and the light barrier assessment of of raw and purified melanins isolated from *Citrullus lanatus* (watermelon) seeds. *Herba Polonica* 64(2).
24. Łopusiewicz Ł., Lisiecki S. 2016. „Czarne złoto" – melaniny w życiu człowieka. *Kosmos* 65(4): 621-629.
25. Łopusiewicz Ł., Mizielińska M. 2016. Aktywność antymikrobiologiczna folii PLA powlekanych ekstraktami z *Macrolepiota konradii*. *Opakowanie* 4: 57-60.
26. Macyszyn J., Kozłowski M. Spienianie polilaktydu. *Materiały opakowaniowe z kompostowalnych tworzyw polimerowych*, 2012.
27. Majchrowska-Safaryan A., Tkaczuk C. 2013. Possibility to use the spent mushroom substrate in soil fertilization as one of its disposal methods. *Journal of Research and Application in Agricultural Engineering* 58(4): 57-62.
28. Mizielińska M., Lisiecki S., Czyżo I., Bartkowiak A. 2015. The attempt to obtain the sachets for producing a biologically modified active atmosphere in the package. Part 1. *Opakowanie*: 1: 54-58.
29. Morresi L., Ficcadenti M., Pinto N., Murri R., Cuccilioni M., Angeletti M., Tombesi P. 2010. Optical and electrical behavior of synthetic melanin thin films spray-coated. *Energy Procedia 2*: 177–182.
30. Panfil-Kuncewicz H. 1993. Opakowanie a jakość produktów mlecznych. *Przegląd mleczarski* 2: 44-46.
31. Plonka P.M., Grabacka M. 2006. Melanin synthesis in microorganisms-biotechnological and medical aspects. *Acta Biochimica Polonica* 53: 429-443.
32. Polska norma PN-0-79000:97
33. Prota G. 1992. *Melanins and melanogenesis*. San-Diego:Academic Press.
34. Różanowska M., Sanra T., Land E., Truscott G. 1999. Free radical scavenging properties of melanin interaction of eu- and pheomelanin models with reducing and oxidising radicals. *Free Radical Biology & Medicine* 26: 518-525.
35. Sava V., Galkin B., Hung M.Y., Yang P.C., Huang G.S. 2001. A novel melanin-like pigment derived from black tea leaves with immuno-stimulating activity. *Food Research International* 34: 337-343
36. Shanmuganathan K., Cho J.H., Iyer P., Baranowitz S., Ellison C.J. 2011. Thermooxidative Stabilization of Polymers Using Natural and Synthetic Melanins. *Macromolecules 44*: 9499–9507.
37. Singh S. 2010. Modified atmosphere packaging of fresh produce: Current status and future needs. *LWT- Food Science and Technology* 43(3): 381-392.
38. Socaciu C. 2008. *Food Colorants: Chemical and Functional Properties*. CRC Press, Cluj-Napoca.
39. Solano F. 2014. Melanins: skin pigments and much more – types, structural models, biological functions, and formation routes. *New Journal of Science*: 1-28.
40. Sono K., Lye D., Moore Ch.A., Boyd W.Ch., Gorlin T.A., Belitsky J.M. 2012. Melanin-based coatings as lead-binding agents. *Bioinorganic Chemistry and Applications*: 1-10.

41. Stewart B. Projektowanie i rozwój opakowań w: *Technika opakowań. podstawy, materiały, procesy wytwarzania*. Wydawnictwo PWN, 2014.
42. Sumińska P., Kowalska U. Opakowania do warzyw i owoców: stan obecny i perspektywy w: *Kierunki rozwoju opakowań*. 2014.
43. Toledo A.V., Franco M.E.E., Lopez S.M.Y., Troncozo M.I., Saparrat M.C.N., Balatti P.A. 2017. Melanins in fungi: Types, localization and putative biological roles. *Physiological and Molecular Plant Biology* 99: 2-6.
44. Weijn A., Bastiaan-Net S., Wichers H.J., Mes J.J. 2013. Melanin biosynthesis pathway in *Agaricus bisporus*. *Fungal Genetics and Biology* 55: 42-53.
45. Wu T., Hong J. 2015. Dopamine-Melanin Nanofilms for Biomimetic Structural Coloration. *Biomacromolecules* 16(2): 660-666.
46. Xu C., Li J., Yang L., Shi F., Yang L., Ye M. 2017. Antibacterial activity and a membrane damage mechanism of *Lachnum* YM30 melanin against *Vibrio parahaemolyticus* and *Staphylococcus aureus*. *Food Control* 73: 1445–1451.
47. Xu Y., Tian Y., Ma R., Liu Q., Zhang J. 2016. Effect of plasma activated water on the postharvest quality of button mushrooms, *Agaricus bisporus*. *Food Chemistry* 197: 436-444.
48. Yao Z., Qi J., Wang L. 2012. Isolation, Fractionation and Characterization of Melanin-like Pigments from Chestnut (*Castanea mollissima*) Shells. *Journal of Food Science* 77: 671-676.
49. Zecca L., Tampellini D., Gerlach M., Riederer P., Fariello R.G., Sulzer D. 2001. Substantia nigra neuromelanin: structure, synthesis, and molecular behaviour. *Journal of Clinical Pathology—Molecular Pathology* 54: 414–418.
50. Zhu H., He C-C., Chu Q-H. 2011. Inhibition of quorum sensing in *Chromobacterium violaceum* by pigments extracted from *Auricularia auricular*. *Letters in Applied Microbiology* 52: 269–274.

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Łukasz ŁOPUSIEWICZ
„ WASTE FROM THE HARVESTING OF BUTTON MUSHROOM (*Agaricus bisporus*) AS A SOURCE OF NATURAL MELANIN"

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WASTE FROM THE HARVESTING OF BUTTON MUSHROOM (*Agaricus bisporus*) AS A SOURCE OF NATURAL MELANIN

ODPAD Z PRODUKCJI PIECZARKI DWUZARODNIKOWEJ (*Agaricus bisporus*) JAKO ŹRÓDŁO NATURALNEJ MELANINY

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ABSTRACT

Natural melanins are of great potential value and application in the fields of pharmacology, cosmetics and functional foods. In the present study, natural melanin was reclaimed from waste after the harvesting of button mushroom (*Agaricus bisporus*). The physiochemical properties of raw and purified melanin were determined, including their ultraviolet-visible (UV-Vis), infrared (IR) and Raman spectra. Colour values, polyphenolic content, antioxidant and antibacterial activity were also evaluated. The result showed that the characteristics of the obtained pigments were similar to synthetic L-DOPA melanin. Raw and purified melanins showed differences in chemical composition, antioxidant activity and light barrier properties. Isolated melanins showed antibacterial activity against *Enterococcus faecalis and Pseudomonas aeruginosa* but were inactive against *Bacillus cereus*, *Escherichia coli* and *Staphylococcus aureus*. This is the first research on the character of melanin reclaimed from waste after the harvesting of *A. bisporus*.

STRESZCZENIE

Naturalne melaniny mają duży potencjał aplikacyjny w dziedzinach farmakologii, kosmetologii oraz żywności funkcjonalnej. W pracy otrzymano naturalną melaninę z odpadu powstającego przy produkcji pieczarki dwuzarodnikowej (*Agaricus bisporus*). Dokonano analizy fizykochemicznej natywnych i oczyszczonych melanin, w tym spektroskopię UV-Vis, IR oraz Ramana. Dokonano również analizy wartości składowych barwy, zawartości polifenoli oraz właściwości antyoksydacyjnych i antybakteryjnych. W wyniku przeprowadzonych analiz stwierdzono, że uzyskane barwniki były podobne do syntetycznej L-DOPA melaniny. Natywne i oczyszczone melaniny wykazywały różnice w budowie chemicznej, aktywności antyoksydacyjnej oraz właściwościach barierowych względem światła. Otrzymane melaniny charakteryzowały się właściwościami przeciwbakteryjnymi względem *Enterococcus faecalis* i *Pseudomonas aeruginosa*, nie działały względem *Bacillus cereus*, *Escherichia coli* i *Staphylococcus aureus*. Są to pierwsze badania na temat melanin otrzymanych z odpadu powstającego przy produkcji *A. bisporus*.

KEYWORDS: melanin, *Agaricus bisporus*, antioxidants, antibacterial properties, UV-protection

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SŁOWA KLUCZOWE: melanina, *Agaricus bisporus*, antyoksydanty, właściwości antybakteryjne, ochrona przed UV

INTRODUCTION

Among edible fungi, the white button mushroom, *Agaricus bisporus* (J.E.Lange) Imbach holds a unique position. Sporocarps of this basidiomycete are cultivated in large quantities and used as a vegetable crop. *A. bisporus* is widely recognised for its nutritional, organoleptic and medicinal properties, and is highly popular with consumers (De Groot et al. 1998, Gao et al. 2014). Mushrooms contain proteins, vitamins, lipids, carbohydrates and minerals. The protein concentration in mushrooms accounts for 19-35% of their dry weight. In addition, mushrooms contain 1.1-8.2% lipids, 4-8.1% carbohydrates and 3-32% fibre in their dry state. Button mushrooms are also a good source of vitamins (vitamin B complexes, niacin and foliates) as well as mineral elements (potassium, phosphorus, copper and zinc) (Coşkuner and Özdemir 2000, Xu et al. 2016).

The cultivation of *A. bisporus* began in France over 200 years ago and in the last few decades mushroom production has grown to be a sizeable crop within agriculture, and expanded all over the world (De Groot et al. 1998). The production of *A. bisporus* in Poland is characterized by high dynamic development. Intensive growth in mushrooms cultivation in Poland has caused the production of these products exceed 200 thousand ton in recent years (Olewnicki and Jabłońska 2012). Poland is the main producer of *A. bisporus* in Europe and one of the biggest in the world (China is the leader) (Xu et al. 2016). This production introduces large quantities of spent mushroom waste into the natural environment that must be dealth with. The agricultural and non-agricultural use of spent mushroom waste, is possible when it is secured in a suitable manner, minimizing the risk of transmission of pests, pathogenic microorganisms and weed seeds. The substrates of spent mushroom organic matter contain sizeable amount of carbon and nitrogen, with the ratio of these elements being similar to the level of humus in mineral and biologically active soil (Kalembasa et al. 2012). According to Majchrowska-Safaryan and Tkaczuk (2013) the application of spent mushroom substrate into the soil improves its physical properties and increases the content of macro- and microelements and thus may be used as a fertilizer. Another use for the *A. bisporus* waste ways may be its processing into valuable bioactive products such as melanin.

After the cultivation process mushrooms (caps together with mid-stipes) are cut off and passed on for further processing or for sale. Stipe bases are considered waste and used together with spent mushroom substrate as fertilizer. No data on the disposal of stipe bases for other applications was found in literature. After harvesting, mushrooms lose their whiteness and become increasingly brown in appearance. This change in colour, called mushroom browning, causing a deterioration in quality, is a result of the enzymatic oxidation of the polyphenols in the fungi tissues by polyphenol oxidase to create quinones, which ultimately condense to form melanin (Coşkuner and Özdemir 2000). *A. bisporus* contain polyphenols which can be oxidized into melanins through two distinct mechanisms: the activation of tyrosinase (an enzyme belonging to the polyphenoloxidase family) and/or spontaneous oxidation (Jolivet et al. 1998).

In recent years there has been a revival interest in the development of natural colorants for use as food additives, and in the cosmetic and pharmaceutical industries. This has been encouraged by strong consumer demand, as synthetic colorants are frequently perceived as undesirable or harmful (Yao et al. 2012). Owing to the high toxicity of synthetic compounds, the search for new natural colorants with antiradical as well as antimicrobial properties still remains a challenge for modern science.

Melanins have been isolated from a variety of phylogenic sources: animals (Mbonyiryivuze et al. 2015, Polidori et al. 2017, Sun et al. 2017), plants (Wang et al. 2006, Kannan and Ganjewala 2009, Cuevas-Juárez et al. 2014), bacteria (Kurian et al. 2014, Laxmi et al. 2016, El-Naggar and El-Ewasy 2017) and fungi (Harki et al. 1997, Suryanarayanan et al. 2004, Ye et al. 2011, Łopusiewicz 2018). Melanins are commonly represented as black and brown pigments, high molecular weight heterogenous polymers derived from the oxidation of monophenols and the subsequent polymeryzation of intermediate o-diphenols and their resulting quinones (Solano 2014). Melanins are types of pigments, possessing broad biological activities including antioxidant, radioprotective, thermoregulative, chemoprotective, antitumor, anti-viral, antimicrobial, immunostimulating and anti-inflammatory. Based on these features, natural melanin has the potential to be of great value and application in the fields of pharmacology, cosmetics, and functional foods (Zhang et al. 2015). Melanins have also application in modern science including modification of polymer materials (Shanmuganathan et al. 2011, Kiran et al. 2017), synthetis of nanoparticles (Apte et al. 2013), developing of semiconducting coatings (Lee at el. 2007), optoacoustic imaging (Liopo et al. 2015), modification of coatings for packaging materials (Łopusiewicz et al. 2017a, Łopusiewicz et al. 2017b).

Considering the growth of interest in new sources of melanin the purpose of this study was to investigate the possible use of waste, in the form of spent button mushrooms, in the production of natural melanin.

MATERIAL AND METHODS

Material

Waste from the production of *A. bisporus* (ABW - Agaricus Bisporus Waste) in the form of stipes was obtained from a local producer in Wolsztyn (Wielkopolskie voivodeship, Poland).

Chemicals

NaOH, HCl, $AgNO_3$, H_2O_2, $FeCl_3$, acetone, ethanol, ethyl acetate, chloroform, DMSO and methanol (Chempur, Poland) were used to extract, purify and offer up a characterisation of the active substances from the ABW. ABTS and KBr (Sigma Aldrich) were also used in this study.

To verify the antimicrobial properties of any melanin, Mueller-Hinton broth and Mueller-Hinton agar media (Merck, Germany) were used. All media were prepared according to the Merck protocol.

Analysis of ABW

Determination of dry matter

The moisture content of ABW was determined gravimetrically. 3-6 representative stipes were dried at 105°C to constant weight to determine their dry matter content. After drying the residues were weighten and dry matter content was calculated (Wang et al. 2010).

The polyphenolic content of ABW

Total soluble phenolic compounds (TPC) in the ethanolic extract from the ABW were measured according to Soares et al. (2009) and Gao et al. (2014) and expressed as gallic acid equivalents. Five grams of ABW were homogenised with 20 ml of 80% ethanol for 24 h. The homogenised mixture was filtered, and the liquid obtained was centrifuged at 6000 rpm for 15 min. 1 mL of the supernatant liquid was mixed with 1 mL of Folin-Ciocalteu reagent and 10 mL of saturated solution of Na_2CO_3. This was topped up to 25 ml with distilled water and left to settle for 30 min. The absorbance was then read at 765 nm. A standard curve of gallic acid (0-1 mg/mL) was used for quantification, and TPC was calculated as milligrams of gallic acid equivalents (GAE)/gram of melanin (mg GAE/g).

Melanin production process

The isolation and purification of melanin

500 g of ABW was first homogenised (Heidolph Brinkmann Homogenizer Silent Crusher) in 500 mL of distilled water, and incubated (24 h, 37°C) to allow acting of tyrosinase. After incubation the homogenate mixture was adjusted to pH=10 by 1 M NaOH, and incubated (24 h, 65°C) to allow spontaneous polymerization of resulting o-diphenols and quinones to form melanin. Then, the mixture was filtered, centrufiged (6000 rpm, 10 min), and alkaline ABW-RM mixture was used to purify melanin.

The purification of melanin was performed as described by Harki et al. (1997) with minor modifications. Alkaline ABW-RM mixture was first adjusted to pH 2.0 with 1 M HCl to precipitate melanin, followed by centrifugation at 6000 rpm for 10 min and a pellet was collected. Then, the pellet was hydrolyzed in 6 M HCl (90°C, 2 h), centrifuged (6000 rpm, 10 min) and washed by distilled water five times to remove acid. The pellet was washed with chloroform, ethyl acetate and ethanol three times to wash away lipids and other residues. Finally, the purified melanin was dried, ground to a fine powder in a mortar and stored at -20°C until testing.

The physiochemical characteristics of melanins

Diagnostic tests

Different diagnostic tests as described by Selvakumar et al. (2008) and Rajagopal et al. (2011) were conducted on the melanins isolated from ABW. The testing organic solvents included acetone, ethanol, ethyl acetate, chloroform, methanol and DMSO.

Ultraviolet-visible absorption and transmittance spectra

Melanin solutions were prepared at concentration 0.1 mg/mL and the UV-Vis absorption and transmitance spectra were measured between 200 and 800 nm. The absorbance ratio (A300/A600) values of melanins were calculated as well (Cuevas-Juárez et al. 2014). Transmittance values were measured between 200 and 800 nm at 0.01; 0.05; 0.1; 0.5 and 1 mg/mL for ABW-RM and ABW-PM; for L-DOPA melanin 0.01; 0.05; 0.1; 1 mg/mL concentrations were measured. All spectrophotometric assays were conducted in the Thermo Scientific Evolution 220 spectrophotometer.

IR spectroscopy

The IR spectra of melanins solid samples were obtained at room temperature by attenuated total reflection with a Fourier transform infrared spectrometer (Spectrum 100, FT-

IR Spectrometer, Perkin Elmer). The samples were evenly mixed with KBr, and pressed into tablets, then, scanned at a range between 650 cm^{-1} and 4000 cm^{-1} (64 scans and 1 cm^{-1} resolution) (Cuevas-Juárez et al. 2014).

Raman spectroscopy

Melanin samples were analysed using a Raman station (RamanStation 400F, Perkin Elemer) with point-and-shot capability using an excitation laser source at 785 nm, 100 micron spot size.

The visual colour of melanins

Visual colour of melanin solutions (0.1 mg/mL) values were measured by a Konica Minolta CR-5 colorimeted with the Hunter LAB colour system. The colour values were expressed as L* (brightness/darkness), a* (redness/greenness) and b* (yellowness/blueness) as an averages of five measurements.

Antioxidant activity (ABTS assay)

An ABTS assay was performed according to Cuevas-Juárez et al. (2014) with minor modifications. Radical 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS·+) was produced by mixing 7 mM ABTS with 2.45 mM potassium persulfate (5 mL of ABTS + 5 mL of potassium persulphate 4.9 mM). The mixture was incubated in darkness at room temperature for 16 h, diluted with 7 mM phosphate buffer (pH 7.4) to reach an absorbance of between 1.0 and 1.2 at 734 nm. For the ABTS assay, 50 µL of melanin (RM or PM), or dissolvent as control, were mixed with 1.95 mL of ABTS·+ solution, incubated in darkness for 10 min at 37 °C and then absorbance was measured at 734 nm and antioxidant activity (%AA) was calculated as $\%AA=[(A_c-A_m)/A_c]x100$; where A_c and A_m are the absorbances for the control and melanin, respectively.

Determination of the polyphenolic content of melanins

The total phenolics of the melanins were determined by the Folin-Ciocalteu reaction according to Cuevas-Juárez et al. (2014) with minor modifications. Melanin dissolved in DMSO (20 µl) was mixed with 1.58 ml of deionized water and 100 µl of the Folin-Ciocalteu reagent, stirred gently for 5 min and added with 300 µl of saturated solution of Na_2CO_3. The mixture was allowed to stand in darkness for 30 min at 40°C, the absorbance was then measured at 765 nm.

The antibacterial activity of isolated melanins

Test microorganisms, including *Bacillus cereus* ATCC14579, *Enterococcus faecalis* ATCC29212, *Escherichia coli* DSMZ1576, *Pseudomonas aeruginosa* ATCC27853 and *Staphylococcus aureus* DSMZ346 were separately cultivated in Mueller-Hinton broth. The antibacterial activity was tested through a well diffusion method (Helan Sounra Rani et al. 2013). 50 mL of Mueller-Hinton broth was inoculated by a single bacterial strain and incubated at 37°C for 24 h. Mueller-Hinton agar was autoclaved and on reaching approx. 45°C, 200 µL of bacterial suspension was added to 20 mL of medium, vigorously vortexed and poured on 90 mm Petri dishes. Wells were cut out by sterile tips (9 mm diameter) in triplicate on each plate and 100 µL of melanin solutions at 0.1 mg/mL in DMSO were placed

in the wells. DMSO served as a control. Plates were incubated at 37°C for 24 h. After incubation the inhibition zones were measured.

RESULTS AND DISCUSSION

Melanins from *A. bisporus* waste (ABW) were obtained. To the best of our knowledge this is the first report of natural melanin production from button mushroom waste. The raw melanin (ABW-RM) was obtained through a two-step process which consisted of an enzymatic reaction, oxidative polymerization of products in alkaline conditions and acid precipitation. Purified melanin (ABW-PM) was obtained by acid hydrolysis, repeated precipitation and purification by the use of organic solvents. Figure 1 shows the scheme of obtaining ABW-RM and ABW-PM. Melanins are insoluble in acid solution, therefore, acid hydrolysis classically used to purify them is, probably insufficient to remove all associated macromolecules. Further purification was achieved by washing the melanin with chloroform, ethyl acetate and ethanol. Since this technique removes only small quantities of macromolecules, it is necessary to repeat the process several times (Harki et al. 1997).

ABW-RM was obtained based on the natural presence of tyrosinase in the fruiting bodies of *A. bisporus*. The PPO family includes catechol oxidase and laccase, both of which oxidize diphenols to corresponding quinones, but the former can also oxidize some monophenols into o-diphenols. This activity is also referred to as „phenolase" or „tyrosinase", which has become a common name for catechol oxidase (Nerya et al. 2006). The monophenolase activity of tyrosinase is usually much lower than diphenolase activity, and this ratio, which can vary from 1:40 to 1:1 is regarded as a pivotal step in browning biosynthesis (Vamos-Vigyazo 1981). Several authors have described tyrosinase activity in button mushroom fruiting bodies during their development and there is consensus that a latent form of tyrosinase occurs, though its ectent is not clear. Latent tyrosinase can become active after damage, coming into contact with bacteria or a toxin such a tolaasin produced by *Pseudomonas tolaasii* (Soler-Rivas et al. 1997). Four pathways for natural melanin synthesis in *A. bisporus* have been proposed by Weijn et al. (2013) started from chorismate and catechol. Melanin is deposited mainly in spores. In the present research, during the first step of the process naturally occurring ABW phenolic compounds are oxidized to o-diphenols and subsequently into corresponding o-quinones. O-quinones are highly reactive compounds which may spontaneously polymerize into melanins, especially in alkaline conditions (Solano 2014).

It was noted that ABW contains polyphenolics, which may be precursors for melanin production. The total polyphenolic content in ABW was 6.22 mg GAE/g of fresh stipes. The richness of phenolics in mushrooms is probably associated with a basidospore discharging function, even under circumstances of wounding/infection, since phenolics are known to act as defence substances. High phenolic content in the button mushroom is probably linked to the epigeal growth of the fruiting body. Even under circumstances serious microbial attack, the releasing function of the basidiospores produced is their main task (Rajarathnam et al. 2003). Alispahić et al. (2015) found that total phenolic content in fresh fruiting bodies of *A. bisporus* was 6.43 mg GAE/g of fresh mushrooms. According to Palacios et al. (2011) and Muszyńska et al. (2017) *A. bisporus* fruiting bodies contain total phenolic compunds level of 3.5 mg GAE/g of dry mushrooms and individual phenolics such as caffeic, chlorogenic, p-coumaric, ferulic, gallic, p-hydroxybenzoic, homogenistic, protocatechuic acids and catechin, myricetin and pyrogallol too, which may be pottentially polymerized into melanin.

The ABW contained 12.75±0.18% of dry mass. The extraction yield obtained for ABW was 8.24±0.13% of ABW dry mass for ABW-RM and 5.19±0.24% AWB-PM after acid hydrolysis and purification. The amount of melanin in various natural sources is diverse and

was reported for impure soluble melanins from fungus *Inonotus obliquus* (20%) (Mazurkiewicz 2006), pure melanins from Mexican fruits (3.63-19.03%) (Cuevas-Juárez et al. 2014), pure melanins from black tea (Sava et al. 2001), fruits and seeds of *Nyctanthes arbor-tristis* (0.05%) (Kannan and Ganjewala 2009) obtained by alkaline extraction. The alkaline extraction method followed by purification through strong acid hydrolysis and washing with suitable solvents (to remove proteins and lipid residues) has been reported in literature (Harki et al. 1997, Suryanarayanan et al. 2004, Zhan et al. 2011), and is the most popular method. However, a number of other methods such as, gradient ultracentrifugation, enzymatic procedure, reprecipitation, gel filtration, adsorption on anion exchange resin, and direct rinsing with solvents without acid hydrolysis have been developed and have been noted in literature (Yao et al. 2012).

The structure of melanin polymers is little understood and an accurate definition of melanin is still required. However, the following criteria indicate melanin is: black/brown in colour, insoluble in water and most other organic solvents, resistant to degradation by hot or cold acids, bleached by oxidizing agents and solubilised by alkali solutions (Zhan et al. 2011). The ABW-RM and ABW-PM pigments presented all of the physical and chemical properties common to natural melanins and the experimental data within this work were found to be comparable to those reported in literature. ABW-RM and ABW-PM gave a positive reaction for polyphenols by producing flocculent brown precipitates with $FeCl_3$, and reduced the ammonia solution of $AgNO_3$. The results are summarized in Table 1 which also shows the properties of the L-DOPA melanin sample used for calibration. Melanins generally displayed, the same behaviour, except in the time required to bleach their alkaline solutions by the oxidizing agent used (10 min, 17 min, 19 min, for ABW-RM, ABW-PM and L-DOPA melanin, respectively. According to Harki et al. (1997) this difference is of importance, because it depends on the type and structure of each melanin. Different reaction rates with these reagents were also obtained with L-DOPA, chatechol and DHN melanin. Melanins are commonly oxidized easly by strong oxidants such as $KMnO_4$, H_2O_2, $K_2Cr_2O_7$, NaOCl, and reduced by reducing agents such as Na_2SO_3, thus changing their chemical structures (Hung et al. 2002). In the presence of H_2O_2 the colour of ABW-RM and ABW-PM solution gradually faded, indicating that melanins were easly oxidized. Similar observations were made by Ye et al. (2011) using melanin isolated from *Lachnum singerianum*.

As shown in the Table 1 the solubility experiments indicate that ABW-RM and ABW-PM were insoluble in both water and common organic solvents (such as ethanol, chloroform, ethyl acetate, acetone). They dissolved in alkali solution and DMSO, precipitated in acidic aqueous solution (pH<3). The solubility of melanins was very similar to those of natural melanin previously reported, as well as synthetic melanin (Zhang et al. 2015). Lowering the pH of a melanin solution causes the formation of large agglomerates and melanin sedimentation. Additionaly, an increase in the pH value rapidly produced disaggregation into smaller sized clusters from less polymerized oligomers. This behaviour is connected to the presence of ionisable groups and hydrophobic interactions within the molecule (Prota 1992).

Figure 2 showed ABW-RM and ABW-PM have maximum absorption peaks at 226 nm and 223 nm, respectively, and exhibited an exponential decrease in the visible region. This behaviour in ABW-RM and ABW-PM were similar to the melanin synthetized from L-DOPA, used as a melanin standard. There was no absorption peak between 260-280 nm in the ABW-PM UV-Vis spectrum, indicating, that melanin do not contain proteins and nucleic acids. ABW-RM UV-Vis spectrum shows weak absorbtion peak in 260-280 nm region, suggesting that ABW-RM molecules may contain some amounts of linked proteins, peptides or nucleic acids (Ye et al. 2012, Łopusiewicz 2018). The UV-Vis absorption spectra of the impure (RM) and purified (PM) melanins were similar to those reported in other literature. Melanins obtained from black tea (Sava et al. 2001), plants (Wang et al. 2006), Mexican fruits (Cuevas-Juárez et al. 2014), fungi (Harki et al. 1997) and bacteria (Kurian et al. 2014) showed λ_{max} values in the region from 200 to 300 nm and lower absorption at higher wavelenghts. This phenomenon is characteristic of melanins and has been associated with

their complex structure, the presence of phenolic groups, as the main chromophores and the formation of complexes with compounds of low and no absorption in the visible region (Cuevas-Juárez et al. 2014). The spectral behaviour of ABW-RM and ABW-PM was very similar. Just as ABW-RM, the UV-Vis absorption spectrum of the ABW-PM melanin showed hyperchromic and bathochromic effects that could be explained by the elimination of components during the purification process. Purer melanin must induce an additive effect on chromophores characterized by a higher molar absorption (hyperchromic effect), as well as higher interactions among these chomophores (the λ_{max} were higher - bathochromic effect). Similar observations were obtained by Cuevas-Juárez et al. (2014) on melanin isolates from Mexican fruits.

The broadband absorption of melanins is considered typical for organic chromophores (Meredith and Sarna, 2006). Three possible explanations are given for this characteristic, featureless absorption spectrum of melanic pigments: a) that it represents a scattering phenomenon and not electronic absorption, b) that it is due to the amorphous semiconducting nature of the pigment, and c) that the spectrum is formed by the superposition of the spectra of many chemically different structures that make up the melanin molecule (Riesz, 2007).

A decrease in absorption with increasing wavelength is almost linear in the case of melanins. Hence, the slopes of linear plots are often used to identify melanin. The log of optical density of a melanin solution when plotted against the wavelength produces a linear curve with negative slopes. Such characteristic straight lines with negative slopes have been obtained in some terrestian and marine fungi (Ravishankar et al. 1995, Suryanarayanan et al. 2004, Selvakumar et al. 2008, Zhan et al. 2011, Zhang et al. 2015). ABW-RM and ABW-PM had straight lines with negative slopes of -0.0049 and -0.0044, respectively, indicating that the black pigments are melanins (Figure 3).

The A300/A600 ratios offer information about the oxidation state and the range size of melanin molecules (Cuevas-Juárez et al. 2014). Melanin oxidation induces lower absorbance values at 600 nm (A600), and the A300/A600 absorbance ratio was proposed as a measure of the oxidation extent, high values corresponded to greater oxidized melanin molecules. Also, it was argued that during the melanin oxidation, phenolics are converted to semiquinones or quinones, which produce more oxidized (higher A300/A600 absorbance ratios) and smaller melanins (molecular weight < 1000 Da) (Haywood et al. 2006, Cuevas-Juárez et al. 2014). ABW-RM showed a higher value (26.80) than its corresponding pure ABW-PM (17.59) and L-DOPA melanin (16.00). This data supports the fact than ABW-RM are a more complex mixture of melanin molecules that that of ABW-PM, with a variability in size and degree of oxidation. These data are consistent with the results of Cuevas-Juárez et al. (2014) and also with observations made by Hung et al. (2002), who noted that oxidized and reduced melanins obtained from black tea have differences in their absorption spectra. Reduced forms of melanin have phenolic form prevalence, which when oxidized, forms show preponderance for quinone forms. Differences were also observed in the FT-IR spectra of oxidized and reduced melanin, showing changes in wavelengths at 3450 cm^{-1}, this corresponding to the hydroxyl stretching vibrations that were significantly weaker in oxidized melanin (Hung et al. 2002).

The light barrier properties of ABW-RM, ABW-PM and L-DOPA melanin are shown in Figure 4. It was noted that in all analysed concentrations, the ABW-RM transmittance values were higher than those of the corresponding ABW-PM, which suggets that in purified form, melanin has better light barrier properties, even when the transmittance values of ABW-PM were smaller than synthetic melanin.

The colour values of ABW-RM, ABW-PM and synthetic melanin are shown in Table 2. Results from the colorimeter indicated that ABW-PM presented lower L* value, higher a* and b* values than ABW-RM in Hunter Lab colour system. This might be caused by the high conjugation degree of melanin which resulted in weak spectral absorbance. Similar

observations were made by Zou et al. (2014). In general, melanins are dark because they do not re-radiate the absorbed visible or invisible light, but transform the energy into rotational and vibrational activity within the molecule and then dissipate it as heat. According to Nicolaus (1968), when the oxidation of diphenols such as L-DOPA, 5,6-dihydroxyindole, catechol and 1,8-dihydroxynaphtalene produce quinones with many active centres for polymerization, the resulting compound is generally a black pigment, whereas when the number of active centres is limited, the resulting pigment is brown, reddish-brown or yellowish-brown. Melanins with high levels of indole quinones (such as L-DOPA derived) appear darker due to the strong absorbance in the red portion of the spectrum. This low frequency light absorption is largely through carbonyls and/or oxygen-containing groups; melanins with fewer carbonyl groups are paler and appear more yellow or red (Riley, 1997).

The TPC values for melanins were 0.14±0.03; 0.21±0.07; 0.29±0.05 mg GAE/g for ABW-RM, ABW-PM and L-DOPA melanin, recpectively. The TPC of ABW-PM was higher than the TPC of ABW-RM. The L-DOPA melanin showed the highest TPC value. The phenolic hydroxyl groups have a strong proton donating power. The more the hydroxyl groups are present within the melanin molecule, the more hydrogen ions are provided to combine with more active free radicals (Ye et al. 2011).

In general, the %AA values of ABW-PM were higher than those of ABW-RM, and the %AA of both melanins were lower than corresponded concentrations of L-DOPA melanin as shown in Table 3. The high antioxidant activity of melanins was expected because the protection against UV-radiation and free radical scavenging are their main functions (Huang et al. 2004, Solano 2014, Cuevas-Juárez et al. 2014). The ABTS method measures both electron- and proton-transfer reactions, sensing lipophilic and hydrophilic compounds (Cuevas-Juárez et al. 2014). The ability of melanin to scavenge reactive oxygen species (ROS), such as singlet oxygen, hydroxyl radical and superoxide anion, has been firmly established in model systems, suggesting that melanin could protect pigment cells against oxidative stress that may accompany the formation of ROS in cells. Even though critical damage to oxidatively stressed cells may result from the reaction of crucial cellular constituents with ROS, an efficient antioxidant may protect the cells by scavenging other oxidizing radicals such as peroxyl radical, and by interacting with molecular oxygen (Różanowska et al. 1999). In melanins synthetized by L-DOPA oxidation, free protons found in their intermolecular spaces mediate the equilibrium between semiquinones, indolquinones, and hydroquinones forms (Goncalvez and Pombeiro-Sponchiado 2005, Cuevas-Juárez et al. 2014). It was found, that the antioxidant activity of melanin depends on its oxidative state. The possibility of melanin existing in various degrees of oxidation is one of its fundamental properties. This is caused phenol-quinine structure of melanin. However, the role of such structural peculiarities in the antioxidant activity of melanin is not clear (Hung et al. 2002). It was noted, that there was positive correlation between %AA and TPC of analysed melanins, and their %AA and TPC can be ordered as L-DOPA melanin > ABW-PM > ABW-RM. The positive correlation of the %AA and TPC for melanins could be due to their structures. Similar observations were made by (Cuevas-Juárez et al. 2014). Melanin molecules contain phenolic groups and their quantification could estimate the pigment concentration in the sample.

Infrared spectroscopy has been used in the chemical structure study of many melanins. It has been suggested that identical melanin structures do not exist in nature and their chemical characterization is a complicated task. Their composition depends not only on their different monomeric units, but also on enviornmental conditions during polymerization. Infrared spectrometric techniques offer information on the main functional groups in the melanin structure (Harki et al. 1997, Selvakumar et al. 2008, Zhan et al. 2011, Yao et al. 2012, Cuevas-Juárez et al. 2014, Zhang et al. 2015). A detailed comparative analysis of the infrared spectra of the melanins studied may supply valuable information on the effect of each treatment step used to purify the melanin and the distinct functional gropus prevailing in the various samples. Figure 5 shows the IR-spectra of ABW-RM, ABW-PM and L-DOPA melanin. Extra display broad absorption bands at 3600-3000 cm^{-1} were noted, attributed to

stretching vibrations of C-H, N-H and/or O-H groups. The C-H could be due to the presence of aromatic rings, with strong bands at 1634 cm^{-1} and 1629 cm^{-1}, for ABW-RM and ABW-PM, respectively, which corresponds to the vibration of aromatic C=C, more intense in ABW-PM. Two small peaks at 2921 cm^{-1} to 2851 cm^{-1} in both melanins may result from the oscillation of aliphatic CH_2 and CH_3 groups. The bands at 1228 cm^{-1} and 1224 cm^{-1} due to C-N and C-O, would support the presence of phenols and aromatic amines. It is difficult to state whether there is an amide group, as the C=O group that complements it might be joined in the band corresponding to aromatic C=C. There are differences between the ABW-RM and ABW-PM spectra which - may result from the purification process.

Figure 6 shows the Raman spectra of ABW-RM, ABW-PM and synthetic melanin. ABW-RM and ABW-PM Raman spectra were similar to spectrum of L-DOPA melanin. The melanin Raman spectrum of ABW-RM is dominated by two intense and broad peaks at about 1635 cm^{-1} and 1248 cm^{-1}, while ABW-PM spectrum peaks at 1620 cm^{-1} and 1235 cm^{-1} were noted. According to Huang et al. (2004) the peaks can be interrelated as originating from the in-plane stretching of the aromatic rings and the linear stretching of the C-C bonds within the rings, along with some contributions from the C-H vibrations in the methyl and methylene groups. A peak at 2000 cm^{-1} from both melanins is similar to those obtained by Galvan et al. (2013) from eumelanin and may be caused by the stretching of three of the six C-C bonds within the melanin rings. It was noted, that on both melanin Raman spectra peaks (385 cm^{-1} and 395 cm^{-1}, for ABW-RM and ABW-PM, respectively) are present, which are thought to correspond to peaks obtained from pheomelanin and eumelanin and are caused by an out-of-plane deformation of phenyl rings. Peaks 2010 cm^{-1} and 1990 cm^{-1}, for ABW-RM and ABW-PM, respectively, these are also similar to peaks seen in pheomelanin and are probably due to overtone or combination bands (Galván et al. 2013).

The results of an antibacterial activity assessment of ABW-RM and ABW-PM is illustrated in Table 4. The zones of growth inhibition of *E. faecalis* and *P.* aeruginosa were 12.2±0.2 mm and 13.7±0.1 mm for ABW-RM, respectively, while ABW-PM, were 11.3±0.1 mm and 12.8±0.2 mm. No inhibition on *B. cereus*, *E. coli* and *S. aureus* was observed. Those results are supported by results of Łopusiewicz (2018), who noted similar antymicrobial activity of melanins isolated from *Exidia nigricans* and are partially consistent with results found by other authors. Helan Soundra Rani et al. (2013) noted antimicrobial activity of melanin isolated from halophilic black yeast *Hortaea werneckii*. Laxmi et al. (2016) observed that growth of *P. aeruginosa* was inhibited on the presence of melanin obtained from *Providencia rettgeri*, but in their study some *Bacillus* species were sensitive for melanin. Xu et al. (2017) analysed the antimicrobial activity of melanin from *Lachnum* YM30 and noted that it was active against a wide spectrum of bacteria including *S. aureus*. The authors suggest that melanin antibacterial activity might result due from damage of the cell membrane and affect the bacteria membrane function. A discrepancy in melanin antimicrobial activity may result from differences within molecule structure and composition (Correa et al. 2017). From the other hand there are some reports that melanins have antibiofilm activity agains pathogenic bacteria including *P. aeruginosa* and could interfere with bacterial quorum-sensing system, regulate its associate functions and prevent bacterial pathogenesis (Zhu et al. 2011, Bin et al. 2012, Laxmi et al. 2016, Xu et al. 2017).

Natural antioxidants (e.g. plant extracts) are important in the food industry to avoid oxidative reactions in diverse products (e.g. meat, poultry, emulsions, beverages and snacks) and to increase their shelf-life. Some desirable characteristics of antioxidants are the ease with they can be incorporated to diverse food matrices, heat stability, low toxicity and cost. In particular, heat stability is important in the use of such antioxidants in cooked foods (Cuevas-Juárez et al. 2014). The melanins reported in this research have the aformentioned characteristics, but non-toxicity must be corroborated in order for them to be recommended as a food additive.

CONCLUSIONS

1. The present study revealed that waste from the harvesting of button mushroom may be considered as a promising source of natural melanin.

2. Isolated pigments presented all the physical and chemical properties common to natural and synthetic melanins.

3. Raw (ABW-RM) and purified (ABW-PM) melanins showed differences in chemical composition, antioxidant activity and light barrier properties.

4. Both melanins showed antibacterial activity against *P. aeruginosa* and *E. faecalis*. No antibacterial activity towards *B. cereus*, *E. coli* and *S. aureus* was observed.

REFERENCES

Alişpahić A., Šapčanin A., Salihović M., Ramić E., Dedić A., Pazalja M. 2015. Phenolic content and antioxidant activity of mushroom extracts from Bosnian market. Bulletin of the Chemists and Technologists of Bosnia and Herzegovina 44, 5-8.

Apte M., Girme G., Bankar A., Ravikumar A., Zinjarde S. 2013. 3,4-dihydroxy-L-phenylalanine-derived melanin from *Yarrowia lipolytica* mediates the synthesis of silver and gold nanostructures. J. Nanobiotechnology 11(2), 1-9.

Bin L., Wei L., Xiaohong Ch., Mei J., Mingsheng D. 2012. In vitro antibiofilm activity of the melanin from *Auricularia auricula*, an edible jelly mushroom. Ann. Microbiol. 62(4), 1523-1530.

Correa N., Covarrubias C., Rodas P.I., Hermosilla G., Olate V.R., Valdés C., Meyer W., Magne F., Tapia C.V. 2017. Differential Antifungal Activity of Human and Cryptococcal Melanins with Structural Discrepancies. Front. Microbiol. 8, 1-13.

Coşkuner Y., Özdemir Y. 2000. Acid and EDTA blanching effects on the essential element content of mushrooms (*Agaricus bisporus*). J. Sci. Food Agric. 80, 2074-2076.

Cuevas-Juárez E., Yuriar-Arredondo K.Y., Pío-León J.F., Montes-Avila J., López-Angulo G., Díaz-Camacho S.P., Delgado-Vargas F. 2014. Antioxidant and α-glucosidase inhibitory properties of soluble melanins from the fruits of *Vitex mollis* Kunth, *Randia echinocarpa* Sessé et Mociño and *Crescentia alata* Kunth. J. Funct. Foods 9, 78-88.

De Groot P. W., Visser J., Van Griensven L. J. L. D., Shaap P. J. 1998. Biochemical and molecular aspects of growth and fruiting of the edible mushroom *Agaricus bisporus*. Mycol. Res. 102(11), 1297-1308.

El-Naggar E-N. N., El-Ewasy S. M. 2017. Bioproduction, characterization, anticancer and antioxidant activities of extracellular melanin pigment producet by newly isolated microbial cell factories *Streptomyces glaucescens* NEAE-H. Sci. Rep. 7:42129, 1-19.

Galván I., Jorge A., Ito K., Tabuchi K., Solano F., Wakamatsu K. 2013. Raman spectroscopy as non-invasive technique for quantification of melanins in feathers and hairs. Pigment Cell Melanoma Res. 26, 1-7.

Gao M., Feng L., Jiang T. 2014. Browning inhibition and quality preservation of button mushroom (*Agaricus bisporus*) by essential oil fumigation treatment. Food Chem. 149, 107-113.

Goncalvez R., Pombeiro-Sponchiado S. R. 2005. Antioxidant activity of the melanin pigment extracted from Aspergillus nidulans. Biol. Pharmaceut. Bull. 28, 1129-1131.

Harki E., Talou T., Dargent R. 1997. Purification, characterisation and analysis of melanin extracted from Tuber melanosporum Vitt. Food Chem. 58(1-2), 69-73.

Hayawood R. M., Lee M., Linge C. 2006. Synthetic melanin is a model for soluble natural eumelanin in UVA-photosensitised superperoxide production. J. Photochem. Photobiol. B 82, 224-235.

Helan Soundra Rani M., Ramesh T., Subramanian J., Kalaiselvam M. 2013. Production and Characterization of Melanin Pigment from Halophilic Black Yeast *Hortaea werneckii*. IJPRR 2(8), 9-17.

Huang Z., Lui H., Chen M.X.K. Alajlan A., McLean D.I., Zeng H. 2004. Raman spectroscopy of in vivo cutaneous melanin. J.Biomed. Opt. 9(6), 1198-1205.

Hung Y-Ch., Sava V., Makan S., Chen Tz-H. J., Hong M-Y., Huang G. S. 2002. Antioxidant activity of melanins derived from tea: comparision beteen different oxidative states. Food Chem. 78, 233-240.

Jolivet S., Arpin N., Wichers H.J., Pellon G. 1998. *Agaricus bisporus* browning: a review. Mycol. Res. 102(12),1459-1483.

Kalembasa D., Becher M., Bik B., Makolewski A. 2012. Właściwości materii organicznej podłoża po uprawie pieczarki [Organic matter properties of spent mushroom substrate]. Acta Agrophys. 19(4), 713-723. [in Polish.]

Kannan P., Ganjewala D. 2009. Preliminary characterization of melanin isolated form frutis and seeds of *Nyctanthes arbor-tristis*. J. Sci. Res. 1(3), 655-661.

Kiran G.S., Jackson S.A., Priyadharsini S., Dobson A.D.W., Selvin J. 2017. Synthesis of Nm-PHB (nanomelanin-polyhydroxybutyrate) nanocomposite film and its protective effect against biofilm-forming multi drug resistant Staphylococcus aureus. Sci. Rep. 7(1), 9167.

Kurian N.K., Nair H.P., Bhat S.G. 2014. Melanin producing *Pseudomonas stutzeri* BTCZ10 from marine sediment at 96 m depth (Sagar Sampada cruise #305). Int. J. Curr. Biotechnol. 2(5), 6-11.

Laxmi M., Kurian N.K., Smitha S., Bhat S.G. 2016. Melanin and bacteriocin from marine bacteria inhibit biofilms of foodborne pathogens. Indian J. Biotechnol. 15(3), 392-399.

Lee H., Dellatore S.M., Miller W.M., Messersmith P.B. 2007. Mussel-inspired surface chemistry for multifunctional coatings. Science 318, 426-430.

Liopo A., Su R., Oraevsky A. 2015. Melanin nanoparticles as a novel contrast agent for photoacoustic tomography. Photoacoustics 3, 35-43.

Łopusiewicz Ł. 2018. Isolation, characterisation and biological activity of melanin from *Exidia nigricans*. World Scientific News 91,11-129.

Łopusiewicz Ł., Lisiecki S., Mizielińska M. 2017 b. Właściwości przeciwutleniające i optyczne folii BOPP i PET modyfikowanych powłokami zawierającymi melaniny grzybowe [Antioxidant and optical properties of BOPP and PET foils modified by coatings containing fungal melanins]. Opakowanie 7, 48-56. [in Polish].

Łopusiewicz Ł., Lisiecki S., Mizielińska M. 2017a. Aktywność przeciwutleniająca folii PE i PLA modyfkowanych powłokami zawierającymi melaniny grzybowe [Antioxidant activity of polyethylene and polylactide foils modified by coating containing fungal melanins]. Opakowanie 3, 81-85. [in Polish].

Majchrowska-Safaryan A., Tkaczuk C. 2013. Możliwość wykorzystanie podłoża po produkcji pieczarki w nawożeniu gleb jako jeden ze sposobów jego utylizacji [Possibility to uuse the spent ushroom substrate in soil fertilization as one of its disposal methods]. Journal of Research and Applications in Agricultural Engineering 58(4), 57-62. [in Polish.]

Mazurkiewicz W. 2006. Analysis of aqueous extract of *Inonotus abliquus*. Acta Pol. Pharm.63, 497-501.

Mbonyiryivuze A., Nuru Z.Y., Ngom B.D., Mwakikunga B., Dhlamini S.M., Park E., Maaza M. 2015. Morphological and Chemical Composition Characterization of Commercial Sepia Melanin. AJN 3(1), 22-27.

Meredith P., Sarna T. 2006. The physical and chemical properties of eumelanin. Pigment Cell Res. 19, 572-594.

Muszyńska B., Kała K., Rojowski J., Grzywacz A., Opoka W. 2017. Composition and Biological Properties of *Agaricus bisporus* Fruiting Bodies – a Review. Pol. J. Food Nutr. Sci. 67(3), 173-181.

Nerya A., Ben-Arie R., Luzzatto T., Musa R., Khativ S., Vaya J. 2006. Prevention of *Agaricus bisporus* postharvest browning with tyrosinase inhibitors. Postharvest Biol.Technol.y 39, 272-277.

Nicolaus R.A. 1968. Melanins. Paris: Hermann.

Olewnicki D., Jabłońska L. 2012. Długookresowa analiza rozwoju sektora pieczarkarskiego w Polsce [Long term analysis of the mushrooms production development in Poland]. Roczniki Ekonomii Rolnictwa i Rozwoju Obszarów Wiejskich 99(4), 127-132. [in Polish].

Palacios I., Lozano M., Moro C., D'Arrigo M., Rostagno M.A., Martínez J.A. García-Laufente A., Guillamón E., Villares A. 2011. Antioxidant properties of phenolic compounds occuring in edible mushrooms. Food Chem. 128, 674-678.
Polidori C., Jorge A., Ornosa C. 2017. Eumelanin and pheomelanin are predominant pigments in bumblebee (*Apidae*: *Bombus*) pubescence. PeerJ 5, 1-21.

Prota G. 1992. Melanins and melanogenesis. San Diego, CA: Academic Press.

Rajagopal K., Kathiravan G., Karthikeyan S. 2011. Extraction and characterization of melanin from *Phomopsis*: A phellophytic fungi isolated form *Azadirachta indica* A. Juss. Afr. J. Microbiol. Res. 5(7), 762 -766.

Rajarathnam S., Shashirekha M., Rashmi S. 2003. Biochemial changes associated with mushroom browning in *Agaricus bisporus* (Lange) Imbach and *Pleurotus florida* (Block & Tsao): commercial implications. J. Sci. Food Agric. 83, 1531-1537.

Ravishankar J.P., Muruganandam V., Suryanarayanan T.S. 1995. Isolation and characterization of melanin from a marine fungus. Bot. Mar. 38, 413-416.

Riesz J.J. 2007. The spectroscopic properties of melanin. PhD Thesis, School of Physical Sciences, University of Queensland.

Riley P. A. 1997. Melanin. International Journal of Biochemistry & Cell Biology 29, 1235-1239.

Różanowska M., Sarna T., Land E.J. Truscott T.G. 1999. Free radical scavenging properties of melanin: interaction of eu- and pheo-melanin models with reducing and oxidizing radicals. Free Radic. Biol. Med. 26, 518-525.

Sava V.M., Yang S-M., Hong M-Y., Yang P-C., Huang G.S. 2001. Isolation and characterisation of melanic pigments derived from tea and tea polyphenols. Food Chem. 73, 177-184.

Selvakumar P., Rajasekar S., Periasamy K., Raaman N. 2008. Isolation and characterization of melanin pigment from *Pleurotus cystidiosus* (telomorph of *Antromycopsis macrocarpa*). World J. Microbiol. Biotechnol. 24, 2125-2131.

Shanmuganathan K., Cho J.H., Iyer P., Baranowitz S., Ellison Ch.J. 2011. Thermooxidative stabilization of Polymers Using Natural and Synthetic Melanins. Macromolecules 44, 9499-9507.

Soares A. A., de Souza C. G. M., Daniel F. M., Ferrari G. P., da Costa S. M. G., Peralta R. M. 2009. Antioxidant activity and total phenolic content of Agaricus brasiliensis (*Agaricus blazei* Murril) in two stages of maturity. Food Chem. 112, 775-781.

Solano F. 2014. Melanins: skin pigments and much more – types, structural models, biological functions, and formation routes. New J. Sci, 1-28.

Soler-Rivas C., Arpin N., Olivier J.M., Wichers H.J. 1997. Activation of tyrosinase in *Agaricus bisporus* strains followin infection by *Pseudomonas tolaasii* with tolaasin-containing preparation. Mycol. Res. 101, 375-382.

Sun X., Wu B., Zhou L., Liu Z., Dong Y., Yang A. 2017. Isolation and characterization of melanin pigment from yesso scallop *Patinopecten yessoensis.* J. Ocean Univ. China 16(2), 279-284.

Suryanarayanan T., Ravishankar J., Venkatesan G., Murali T. 2004. Characterization of the melanin pigment of a cosmopolitan fungal endophyte. Myc. Res. 108(8), 974-978.

Vamos-Vigyazo L. 1981. Polyphenol oxidase and peroxidase in fruits and vegetables. Crit. Rev. Food Sci. Nutr. 15, 49-127.

Wang H., Pan Y., Tang X., Huang Z. 2006. Isolation and characterization of melanin from *Osmanthus fragrans'* seeds. LWT 39, 496-502.

Wang Q., Li B.B., Li H., Han J.R. 2010. Yield, dry matter and polysaccharides content of the mushroom Agaricus blazei produced on asparagus straw substrate. Sci. Hortic.125, 16-18.

Weijn A., Bastiaan-Net S., Wichers H.J., Mes J.J. 2013. Melanin biosynthesis pathway in Agaricus bisporus. Fungal Genet. Biol. 55, 42-53.

Xu C., Li J., Yang L., Shi F., Yang L., Ye M. 2017. Antibacterial activity and a membrane damage mechanism of *Lachnum* YM30 melanin against *Vibrio parahaemolyticus* and *Staphylococcus aureus.* Food Control 73, 1445-1451.

Xu Y., Tian Y., Ma R., Liu Q., Zhang J. 2016. Effect of plasma activated water on the postharvest quality of button mushrooms, *Agaricus bisporus.* Food Chem. 197, 436-444.

Yao Z., Qi J., Wang L. 2012. Isolation, Fractionation and Characterization of Melanin-like Pigments from Chestnut (*Castanea mollissima*) Shells. J. Food Sci. 77(6), 671-676.

Ye M., Guo G., He Y., Lu Y., Ye Y., Yang Q., Yang P. 2012. Physiochemical characteristics and antioxidant activity of arginine-modified melanin from *Lachnum* YM-346. Food Chem. 135, 2490-2497.

Ye M., Wang Y., Qian M., Chen X., Hu X. 2011. Preparation and properties of the melanin from *Lachnum singerianum*. IJBAS-IJENS 11(3), 51-58.

Zhan F., He Y., Zu Y., Li T., Zhao Z. 2011. Characterisation of melanin isolated form a dark septate endpophyte (DSE), *Exophiala pisciphila*. World J. Microbiol. Biotechnol. 27, 2483-2489.

Zhang M., Xiao G., Thring R.W., Chen W., Zhou H., Yang H. 2015. Production and Characterization of melanin by submerged culture of culinary and medicinal fungi *Auricularia auricula*. Appl. Biochem. Biotechnol. 176, 253-266.

Zhu H., He C-C., Chu Q-H. 2011. Inhibition of quorum sensing in *Chromobacterium violaceum* by pigments extracted from Auricularia auricular. Lett. Apll. Microbio.l 52, 269-274.

Zou Y., Wen Y., She W., Hu W. 2014. Physiochemical properties of melanin from *A. auricula* fruiting bodies. Adv. J. Food Sci. Technol. 6(8), 1002-1004.

Table 1. Diagnostic tests for melanins

Tabela 1. Testy diagnostyczne melanin

	Test Test	Result Wynik		
		ABW-RM	ABW-PM	L-DOPA melanin
1.	Solubility in water Rozpuszczalność w wodzie	Insoluble Nierozpuszczalny		
2.	Solubility in organic solvents (acetone, chloroform, ethanol, ethyl acetate, methanol, DMSO) Rozpuszczalność w rozpuszczalnikach organicznych (aceton, chloroform, etanol, octan etylu, metanol, DMSO)	Insoluble (Soluble only in DMSO) Nierozpuszczalny (Rozpuszczalność jedynie w DMSO)		
3.	Solubility in 1 M NaOH Rozpuszczalność w 1 M NaOH	Soluble Rozpuszczalny		
4.	Precipitation in acidic conditions Wytrącanie się w kwaśnym środowisku	Readily precipitation Szybka precypitacja		
5.	Reaction with oxidizing agents (H_2O_2) Reakcja z czynnikami utleniającymi (H_2O_2)	Decolorized Odbarwienie		
6.	Reaction with ammoniacal $AgNO_3$ solution Reakcja z amoniakalnym roztworem $AgNO_3$	Gray coloured silver precipitate on tube side Szary osad srebra na ściankach probówki		
7.	Reaction for polyphenols ($FeCl_3$ test) Reakcja na polifenole (test z $FeCl_3$)	Brown precipitate Brązowy osad		

8.	Colour Barwa	Black Czarna

Table 2. The visual colour values of ABW-RM, ABW-PM and L-DOPA melanin (mean±SD, n=5)

Tabela 2. Wartości barwy melanin ABW-RM, ABW-PM i L-DOPA (średnia±SD, n=5)

	L*	a*	b*
ABW-RM	95.53±0.00	-0.26±0.00	12.73±0.00
ABW-PM	89.10±0.00	1.52±0.00	26.81±0.00
L-DOPA melanin	74.87±0.01	10.56±0.00	47.74±0.01

L* - lightness, a* - green/red, b* - blue/yellow

L* - jasność, a* - zielony/czerwony, b* - niebieski/żółty

Table 3. The antioxidant activity (%AA values) of ABW-RM, ABW-PM and L-DOPA melanin (mean±SD, n=3)

Tabela 3. Aktywność antyoksydacyjna (%AA) melanin ABW-RM, ABW-PM i L-DOPA (średnia±SD, n=3)

ABW-RM	%AA	ABW-PM	%AA	L-DOPA melanin	%AA
0.0625 mg/mL	0.60±0.04	0.0625 mg/mL	7.18±0.32	0.0625 mg/mL	20.31±0.18
0.125 mg/mL	1.45±0.05	0.125 mg/mL	11.58±0.11	0.125 mg/mL	31.51±0.43
0.25 mg/mL	5.39±0.15	0.25 mg/mL	13.19±0.23	0.25 mg/mL	50.75±0.16
0.5 mg/mL	16.76±0.13	0.5 mg/mL	30.64±0.07	0.5 mg/mL	95.91±0.29
1 mg/mL	48.40±0.08	1 mg/mL	75.05±0.44	1 mg/mL	97.16±0.03

Table 4. The antibacterial activity of ABW-RM, ABW-PM and L-DOPA melanin (zones of growth inhibition, mm), (mean±SD, n=3)

Tabela 4. Aktywność antymikrobiologiczna melanin ABW-RM, ABW-PM i L-DOPA (strefy zahamowanego wzrostu, mm) (średnia±SD, n=3)

	BC	EC	EF	PA	SA
ABW-RM	-	-	12.2±0.2	13.7±0.1	-
ABW-PM	-	-	11.3±0.1	12.8±0.2	-
L-DOPA melanin	-	-	11.4±0.2	13.1±0.1	-

"-" – no inhibition zone, "BC" - *B. cereus* ATCC14579, "EC" - *E. coli* DSMZ1576, "EF" - *E. faecalis* ATCC29212, "PA" - *P. aeruginosa* ATCC2753, "SA" - *S. aureus* DSMZ346

"-" – brak strefy zahamowanego wzrostu, "BC" - *B. cereus* ATCC14579, "EC" - *E. coli* DSMZ1576, "EF" - *E. faecalis* ATCC29212, "PA" - *P. aeruginosa* ATCC2753, "SA" - *S. aureus* DSMZ346

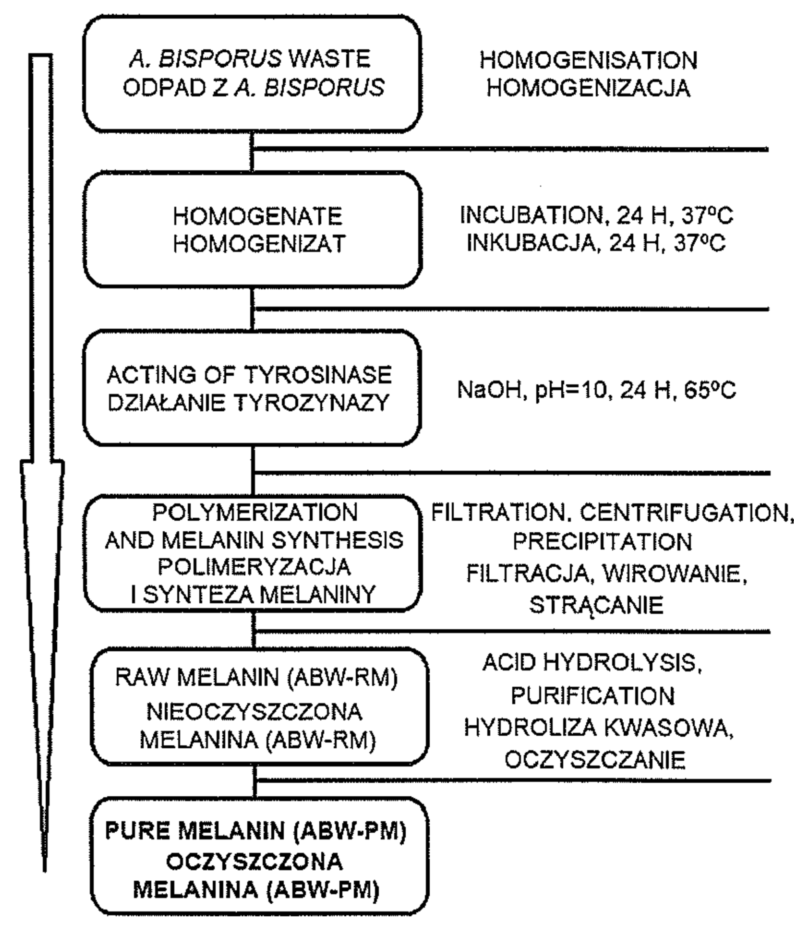

Fig. 1 The isolation and purification of melanin scheme

Ryc. 1 Schemat izolacji i oczyszczania melaniny

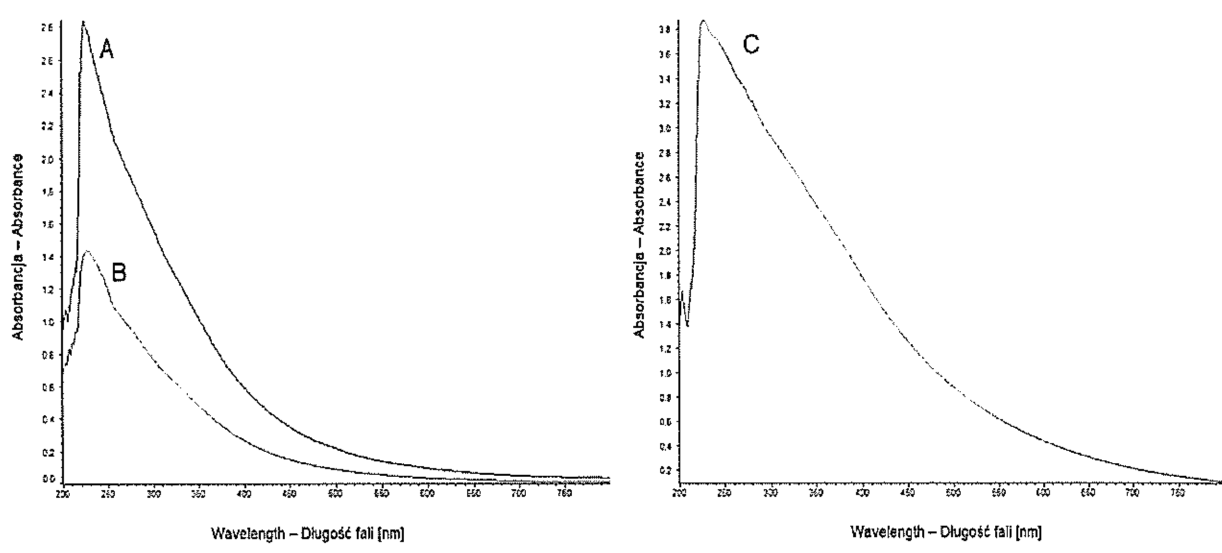

Fig. 2 The absorbance of ABW-RM (A), ABW-PM (B) and L-DOPA melanin (C)

Ryc. 2 Absorbancja ABW-RM (A), ABW-PM (B) i L-DOPA melaniny (C)

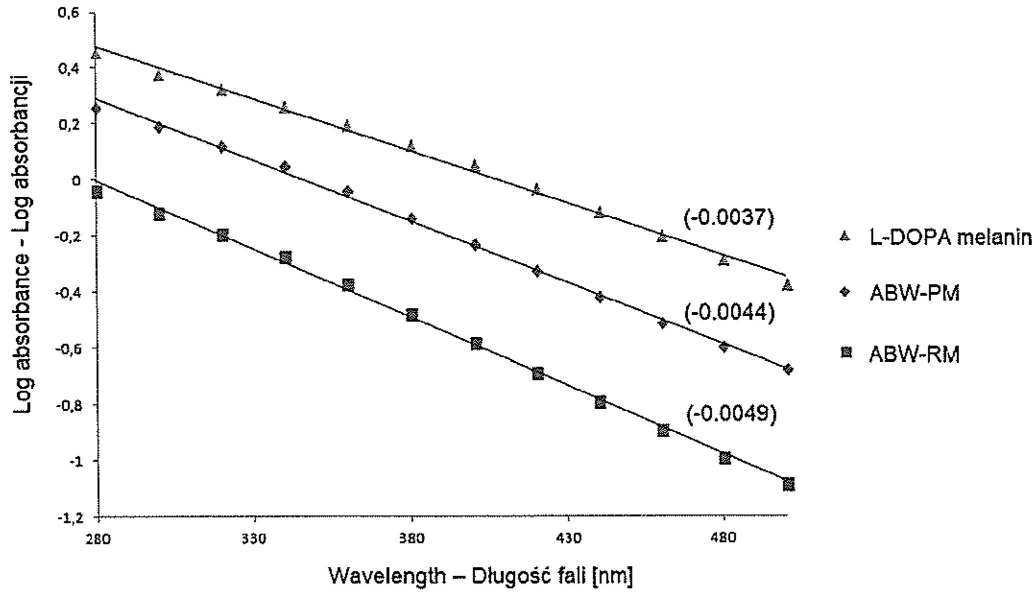

Fig. 3 A plots of log of absorbance of ABW-RM, ABW-PM and L-DOPA melanin against wavelength

Ryc. 3 Wykres log absorbancji ABW-RM, ABW-PM i L-DOPA melaniny względem długości fali

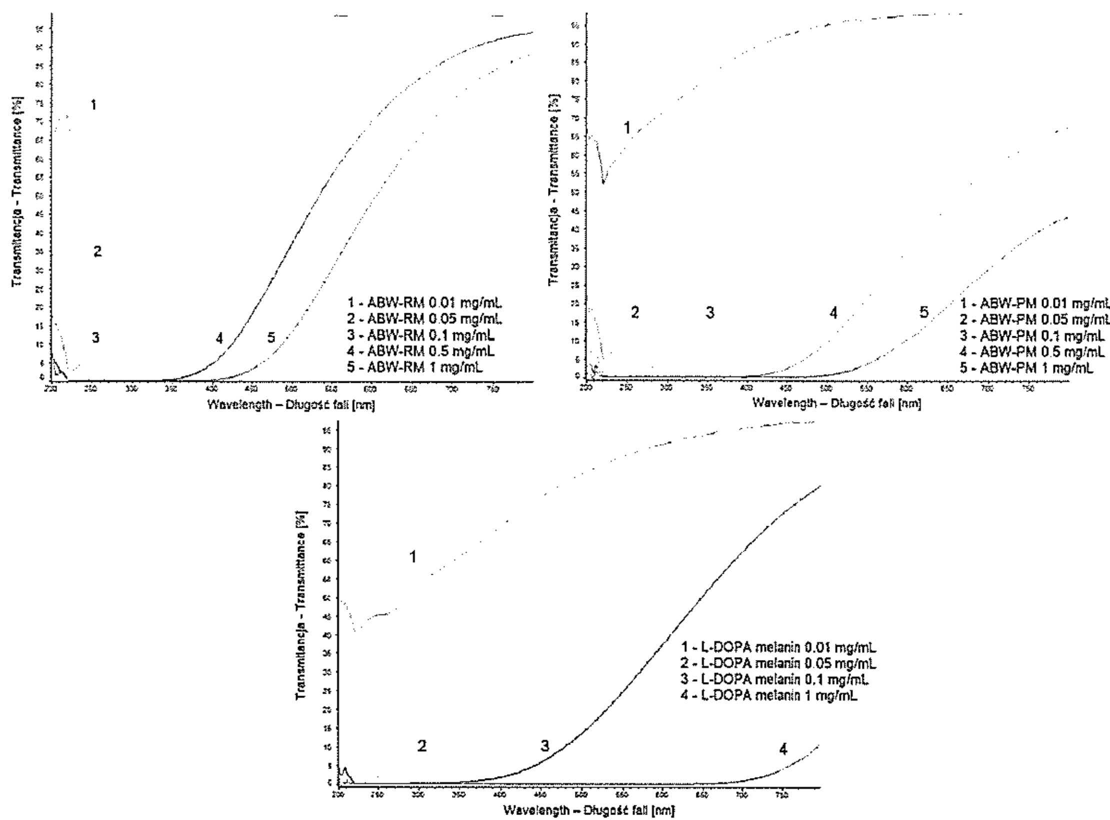

Fig. 4 Transmittance values of SC-RM (A), SC-PM (B) and L-DOPA melanin (C)

Ryc. 4 Wartości transmitancji ABW-RM (A), ABW-PM (B) i L-DOPA melaniny (C)

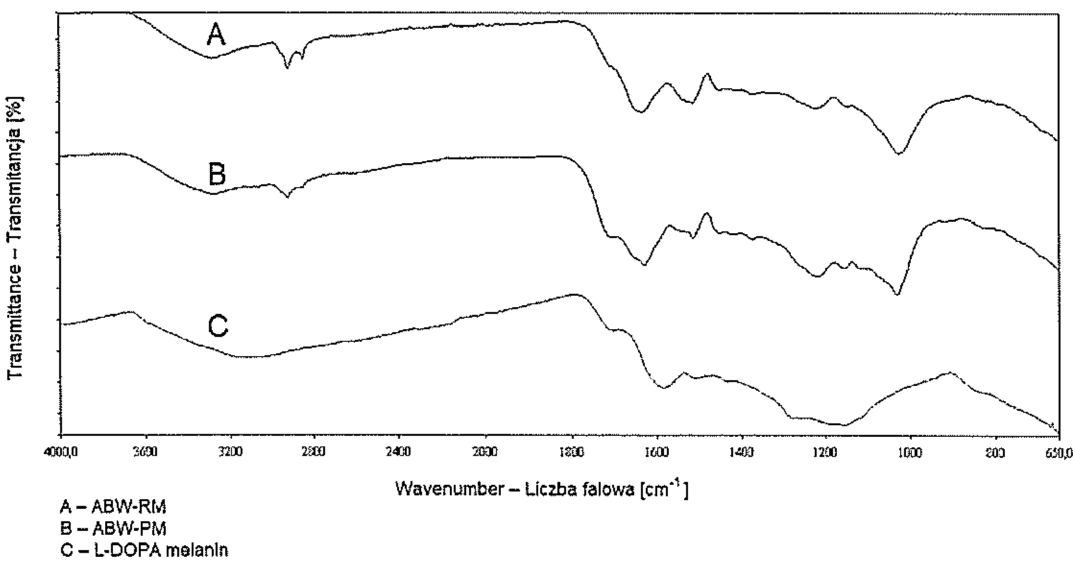

Fig. 5 Infrared spectra of L-DOPA melanin, ABW-RM and ABW-PM

Ryc. 5 Widmo FT-IR L-DOPA melaniny, ABW-RM i ABW-PM

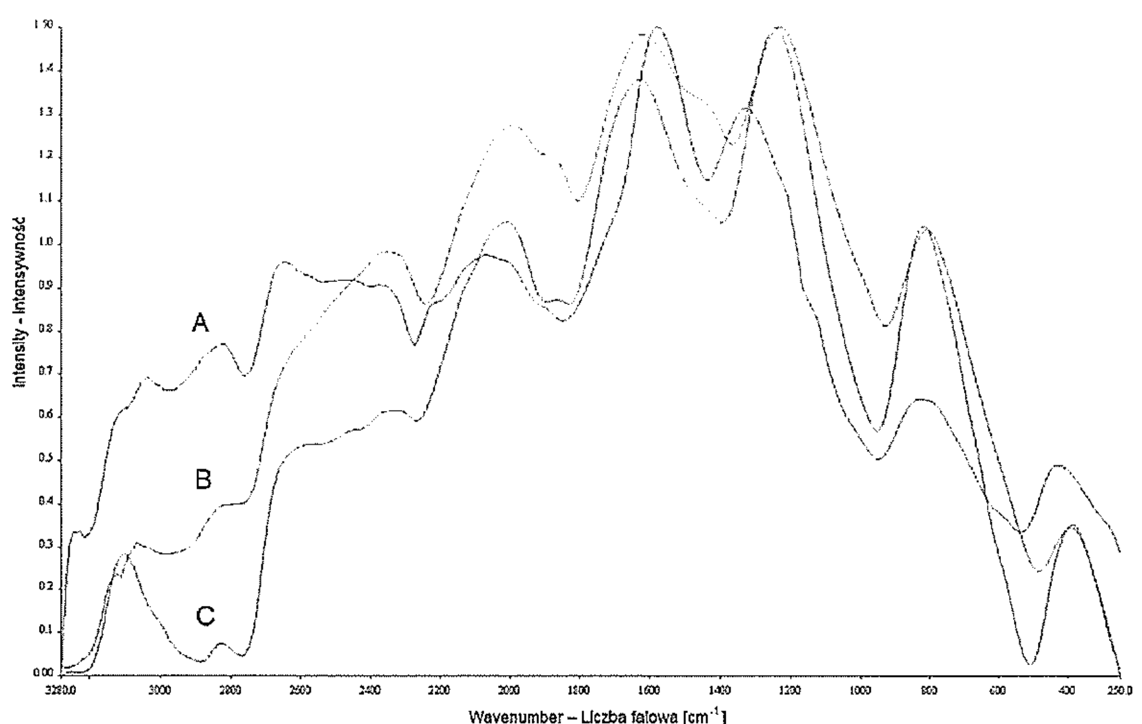

Fig. 6 Raman spectra of L-DOPA melanin (A), ABW-PM (B) and ABW-RM (C)

Ryc. 6 Widmo Ramana L-DOPA melaniny (A), ABW-PM (B) i ABW-RM (C)

P2

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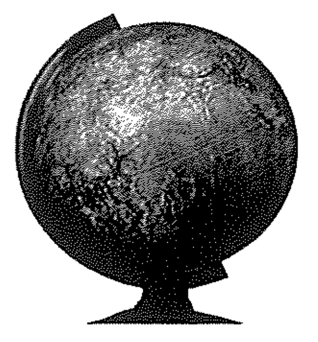

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Scleroderma citrinum melanin: isolation, purification, spectroscopic studies with characterization of antioxidant, antibacterial and light barrier properties

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ABSTRACT

The aim of this study was to isolate and evaluate biological properties of raw and purified melanins isolated from *Scleroderma citrinum*. Native melanin was isolated from the gleba of fresh *S. citrinum* fruiting bodies by alkaline extraction. Obtained pigment was purifed by acid hydrolysis and washed by organic solvents. Chemical tests, FT-IR and Raman spectroscopy analysis were conducted to determine the melanin nature of the isolated pigments. UV-Vis, transmittance, total polyphenolic content and colour properties were evaluated. Antioxidant activity was determined using ABTS and antibacterial activity by a well diffusion method. The results of the study demonstrated that melanins isolated from *S. citrinum* had antioxidant, light barrier and antibacterial properties. A purified form of melanin offered better light properties and higher antioxidant activity than the raw form. Both melanins inhibited the growth of *E. facealis* and *P. aeruginosa*. This study revealed that *S. citrinum* may be considered as a promising source of natural melanin. Isolated pigments presented all the physical and chemical properties common to natural and synthetic melanins. Raw and purified melanins showed differences in chemical composition, antioxidant activity and light barrier properties. Results suggest that, melanins from *S. citrinum* possess remarkable therapeutic action and could be applied in the food, cosmetics and pharmaceutical industries.

Keywords: melanin, *Scleroderma citrinum*, earthball, antioxidant, antibacterial, light barrier

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1. INTRODUCTION

The genus *Scleroderma* comprises of gesteroid ectomycorrhizal (ECM) basidiomycetes with reticulate to echinulate globose spores, usually known as earthballs [1,2]. *Scleroderma* taxa are distributed worldwide in tropical, temperate and subtropical ecosystems [3], and have been reported from Asia [4-9], Africa [10], Australia [11], Europe [12-15], North America [13] and South America [2,3,16]. Several morphological and molecular studies have confirmed the systematic position of the *Scleroderma* genus, placing it in the suborder *Sclerodermataceae* within the *Boletales* order. This genus of fungi with hard-skinned basidiomata can be recognized by its epigenous and single-layered peridium opening by irregular dehiscence and gleba without capillitium [1,16].

Scleroderma citrinum Pers., occurs as a saprotroph on soil or humus with mosses, sometimes on rotting wood but has also been demonstrated to form typical ECM and is common in coniferous and mixed forest [13]. When mature, brown, leathery, rounded structures become dry and crack at maturity, and the dry, powdery, dark basidiospores are dispersed by the wind blowing through fissures in the upper surface of the basidiome. The dark colour of basidiospores is caused by melanin. Several studies describe the biological activities of *S. citrinum* including insecticidal [17], antibacterial [4], antifungal [18,19] and antiviral [20]. The known chemical compounds from *S. citrinum* have been reported including lanostane-type steroids [18], lanostane-type terpenoids [20], pulvinic acid derivatives [4,21], carbonyl reducatases [22] and pigments such as sclerocitrin and norbadione A [23].

Melanins have been isolated from a variety of phylogenic sources: animals [24], plants [25], bacteria [26,27] and fungi [28,29]. Melanins are commonly represented as black and brown pigments, high molecular weight heterogenous polymers derived from the oxidation of monophenols and the subsequent polymeryzation of intermediate o-diphenols and their resulting quinones [30]. Melanins are types of pigments, possessing broad biological activities including; antioxidant, radioprotective, thermoregulative, chemoprotective, antitumor, antiviral, antimicrobial, immunostimulating and anti-inflammatory properties [24-30]. Based on these features, natural melanin has the potential to be of great value and application in the fields of pharmacology, cosmetics, and functional foods [28,31]. However, knowledge relating to pharmacological and the biological activities of melanins from *S. citrinum* is highly limited. In recent years there has been a revival of interest in the development of natural colorants as food additives, and also in the cosmetic and pharmaceutical industries. This has been encouraged by strong consumer demand, as synthetic colorants are frequently perceived as undesirable or harmful [28,32]. Owing to the high toxicity of synthetic compounds, the search for new natural colorants with antiradical, light barrier as well as antimicrobial properties still remains a challenge for modern science.

The aim of present study was to isolate, characterize and investigate the antioxidant, antimicrobial and light barrier properties of raw and purified melanins form *S. citrinum*. This represents a first report on the isolation and biological activities of melanins from *S. citrinum*.

2. MATERIALS AND METHODS

2. 1. Test fungus

The tests were made up of fresh mature fruiting bodies of *S. citrinum* within this study.

The fruiting bodies were collected in September 2017 in a mixed forest near Szczecin, Poland (53° 20' N, 14° 49' E).

2. 2. Chemicals

NaOH, HCl, $FeCl_3$, H_2O_2, $AgNO_3$, acetone, ethanol, ethyl acetate, chloroform, DMSO and methanol (Chempur, Poland) were used to extract, purify and offer up a characterisation of the active substances from the *S. citrinum* fruiting bodies. ABTS and KBr (Sigma Aldrich) were also used in this study. To verify the antimicrobial properties of any melanin, Mueller-Hinton broth and Mueller-Hinton agar media (Merck, Germany) were used. All media were prepared according to the Merck protocol.

2. 3. Extraction and purification

The isolation and purification of melanin was performed as described by Łopusiewicz [28]. External layers of peridium were removed, and only gleba was used for melanin extraction. To summarise, 5 g of gleba were homogenised in 50 ml of 1 M NaOH, extracted in orbital shaker (150 rpm, 50 °C, 24 h) and centrifuged at 6000 rpm for 10 min to remove fungal tissue. Alkaline SC-RM (Scleroderma citrinum raw melanin) mixture was first adjusted to pH 2.0 with 1 M HCl to precipitate melanin, followed by centrifugation at 6000 rpm for 10 min and a pellet was collected. Then, the pellet was hydrolyzed in 6 M HCl (90 °C, 2 h), centrifuged (6000 rpm, 10 min) and washed by distilled water five times to remove acid. The pellet was washed with chloroform, ethyl acetate and ethanol three times to wash away lipids and other residues. Finally, the purified melanin (SC-PM – Scleroderma citrinum pure melanin) was dried, ground to a fine powder in a mortar and stored at -20°C until testing.

2. 4. Chemical tests

Different diagnostic tests, as described by Selvakumar et al. [33], were conducted on the SC-RM and SC-PM isolated melanins in comparision with L-DOPA melanin used as a melanin standard. The testing organic solvents included ethanol, methanol, chloroform, ethyl acetate, acetone and DMSO.

2. 5. Ultraviolet-visible absorption and transmittance spectra

Melanin solutions were prepared at concentration 0.1 mg/mL and UV-Vis absorption spectra were measured between 200 and 800 nm. The absorbance ratio (A300/A600) values and plots of optical densities against wavelenghts of melanins were also calculated [25,28]. Transmittance values were measured between 200 and 800 nm at 0.01; 0.05; 0.1; 0.5 and 1 mg/mL for SC-RM and SC-PM; for L-DOPA melanin 0.01; 0.05; 0.1; 1 mg/mL concentrations were measured. All spectrophotometric assays were conducted in a Thermo Scientific Evolution 220 spectrophotometer.

2. 6. IR spectroscopy

The IR spectra of melanins solid samples were obtained at room temperature by attenuated total reflection with a Fourier transform infrared spectrometer (Perkin Elmer). The samples were evenly mixed with KBr, and pressed into tablets, then scanned at a range

between 650 cm^{-1} and 4000 cm^{-1} (64 scans and 1 cm^{-1} resolution) [28]. Obtained spectra have been normalized, baseline corrected and analysed using SPECTRUM software.

2. 7. Raman spectroscopy

Melanin samples were analysed using a Raman station (RamanStation 400F, Perkin Elemer) with point-and-shot capability using an excitation laser source at 785 nm, 100 micron spot size, 25% laser power, 4 shots, 8 second exposition time. Obtained spectra have been normalized, baseline corrected and analysed using SPECTRUM software.

2. 8. The antioxidant activity (ABTS assay)

An ABTS assay was performed according to Łopusiewicz [28]. Radical 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS·+) was produced by mixing 7 mM ABTS with 2.45 mM potassium persulfate (5 mL of ABTS + 5 mL of potassium persulphate 4.9 mM). The mixture was incubated in darkness at room temperature for 16 h, diluted with 7 mM phosphate buffer (pH 7.4) to reach an absorbance of between 1.0 and 1.2 at 734 nm. For the ABTS assay, 50 μL of melanin (SC-RM, SC-PM, L-DOPA melanin; 0.0625; 0.125; 0.25, 0.5; 1 mg/mL), or dissolvent as control, were mixed with 1.95 mL of ABTS·+ solution, incubated in darkness for 10 min at 37 °C, and then the absorbance was measured at 734 nm and antioxidant activity (%AA) was calculated as $\%AA = [(A_m*100)/A_c]$; where A_c and A_m are absorbances for the control and melanin sample, respectively.

2. 9. Determination of the total phenolics content (TPC) of melanins

The total phenolics content (TPC) of the melanins were determined by the Folin-Ciocalteu reaction according to Cuevas-Juárez et al. [16]. Melanin dissolved in DMSO (20 μl) was mixed with 1.58 ml of deionized water and 100 μl of the Folin-Ciocalteu reagent, stirred gently for 5 min and added with 300 μl of saturated solution of Na_2CO_3. The mixture was allowed to stand in darkness for 30 min at 40 °C, the absorbance was then measured at 765 nm. A calibration curve of gallic acid in water-methanol (1:1, v/v) (0, 50, 100, 200, 400 and 500 μg/mL) was prepared and TPC was calculated as miligrams of gallic acid equivalents (GAE)/gram of melanin (mg GAE/g). The results were presented as an average of three samples with standard deviation.

2. 10. The visual colour of melanins

The visual colour of melanin solution (0.1 mg/mL) values were measured by a Konica Minolta CR-5 colorimeter with the Hunter LAB colour system. The colour values were expressed as L* (brightness/darkness), a* (redness/greenness) and b* (yellowness/blueness) as an averages of five measurements.

2. 11. The antibacterial activity of isolated melanins

Test microorganisms, including *Bacillus cereus* ATCC14579, *Enterococcus faecalis* ATCC29212, *Escherichia coli* DSMZ1576, *Pseudomonas aeruginosa* ATCC2753 and *Staphylococcus aureus* DSMZ346 were separately cultivated in Mueller-Hinton broth. The antibacterial activity was tested through a well diffusion method. 50 mL of Mueller-Hinton broth was inoculated by a single bacterial strain and incubated at 37 °C for 24 h. Mueller-

Hinton agar was autoclaved and on reaching approx. 45°C, 200 µL of bacterial suspension was added to 20 mL of the medium, vigorously vortexed and poured on 90 mm Petri dishes. Wells were cut out by sterile tips (9 mm diameter) in triplicate on each plate and 100 µL of melanin solutions at 0.1 mg/mL in DMSO were placed in the wells. DMSO served as a control. Plates were incubated at 37°C for 24 h. The inhibition zones were measured after incubation. The results were presented as an average of three samples with standard deviation.

3. RESULTS

The results of the study demonstrated that raw and purified black pigments from *S. citrinum* had antioxidant, antibacterial and light barrier properties. The SC-RM and SC-PM pigments presented all of the physical and chemical properties common to natural melanins and the experimental data within this work were found to be comparable to those reported in literature. The results are summarized in Table 1, which also shows the properties of the L-DOPA melanin sample used for calibration.

Table 1. Diagnostic tests for melanins.

No	Test	Result		
		SC-RM	SC-PM	L-DOPA melanin
1.	Solubility in water	Insoluble		
2.	Solubility in organic solvents (acetone, chloroform, ethanol, ethyl acetate, methanol)	Insoluble		
3.	Solubility in 1 M NaOH	Soluble		
4.	Precpitation in acidic conditions	Readily precipitation		
5.	Reaction with oxidizing agents (H_2O_2)	Decolorized		
6.	Reaction with ammoniacal $AgNO_3$ solution	Gray coloured silver precipitate on tube side		
7.	Reaction for polyphenols ($FeCl_3$ test)	Brown precipitate		
8.	Colour	Black		

Figure 1 shows SC-RM and SC-PM have maximum absorption peaks at 222 nm and 225 nm, respectively, and exhibited an exponential decrease in the visible region. This behaviour in SC-RM and SC-PM were similar to the melanin synthetized from L-DOPA, which is used as a melanin standard.

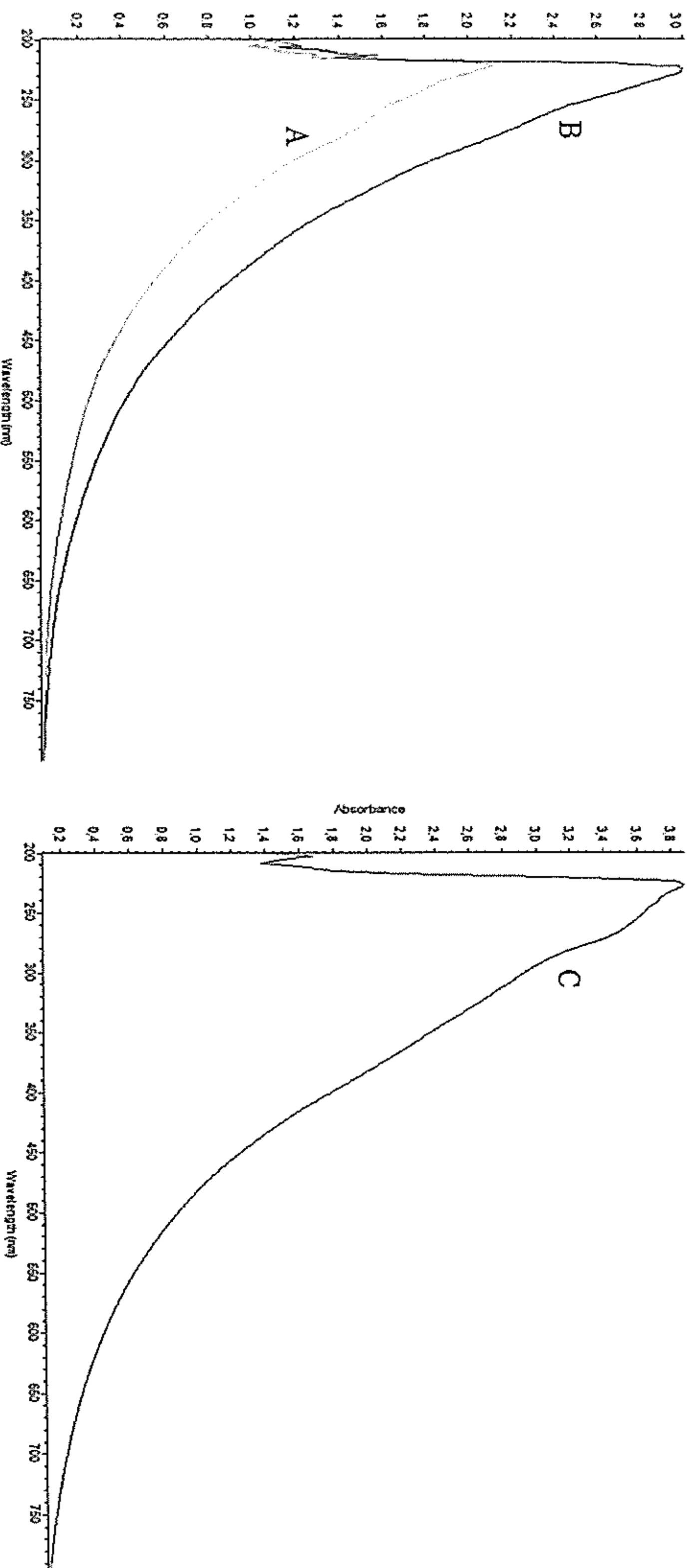


Fig. 1. The absorbance of SC-RM (A), SC-PM (B) and L-DOPA melanin (C)

Figure 2 shows the log of optical density of a melanin solutions when plotted against the wavelength of SC-RM, SC-PM and L-DOPA melanin. SC-RM, SC-PM and L-DOPA melanin had straight lines with negative slopes of -0.003464, -0.003222 and -0.003741, respectively. The light barrier properties of SC-RM, SC-PM and L-DOPA melanin are shown in Figure 3. It was noted that in all analysed concentrations, the SC-RM transmittance values were higher than those of the corresponding SC-PM, which suggests that in purified form, melanin had better light barrier properties, even when the transmittance values of SC-PM were smaller than the synthetic melanin.

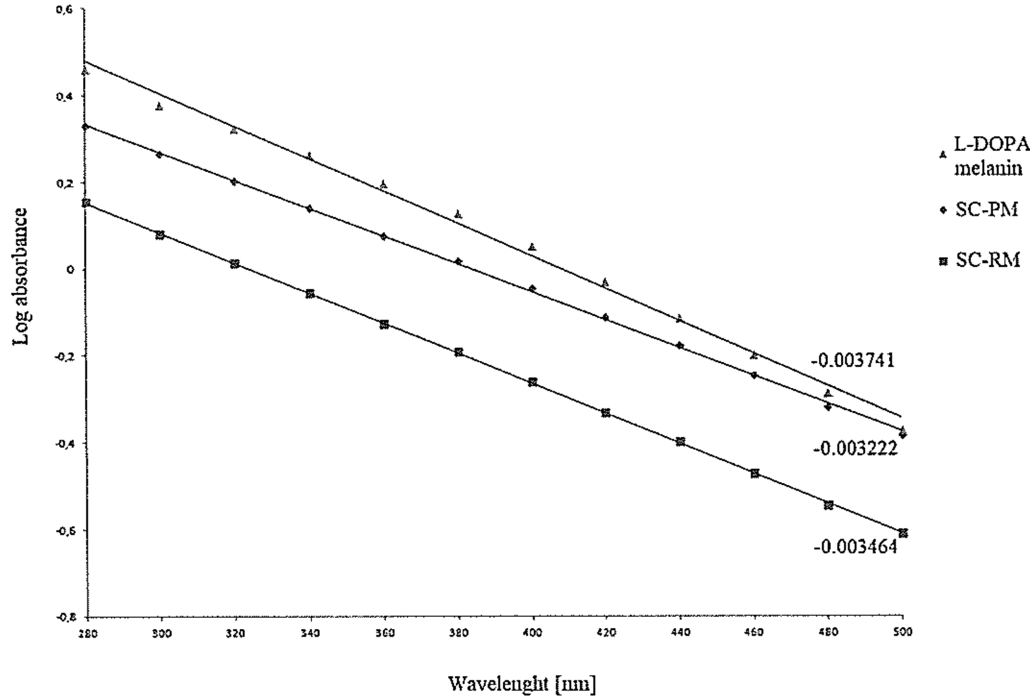


Fig. 2. A plots of optical densities against wavelenght for L-DOPA melanin, SC-PM and SC-RM

The colour values of SC-RM, SC-PM and synthetic melanin are shown in Table 2. Results from the colorimeter indicated that SC-PM presented lower L* value, and higher a* and b* values than SC-RM in Hunter Lab colour system.

Table 2. The visual colour values of SC-RM, SC-PM and L-DOPA melanin (mean±SD, n=5)

	L*	a*	b*
SC-RM	88.17±0.01	2.58±0.00	22.23±0.01
SC-PM	78.34±0.00	7.14±0.02	34.94±0.00
L-DOPA melanin	74.87±0.00	10.56±0.00	47.74±0.02

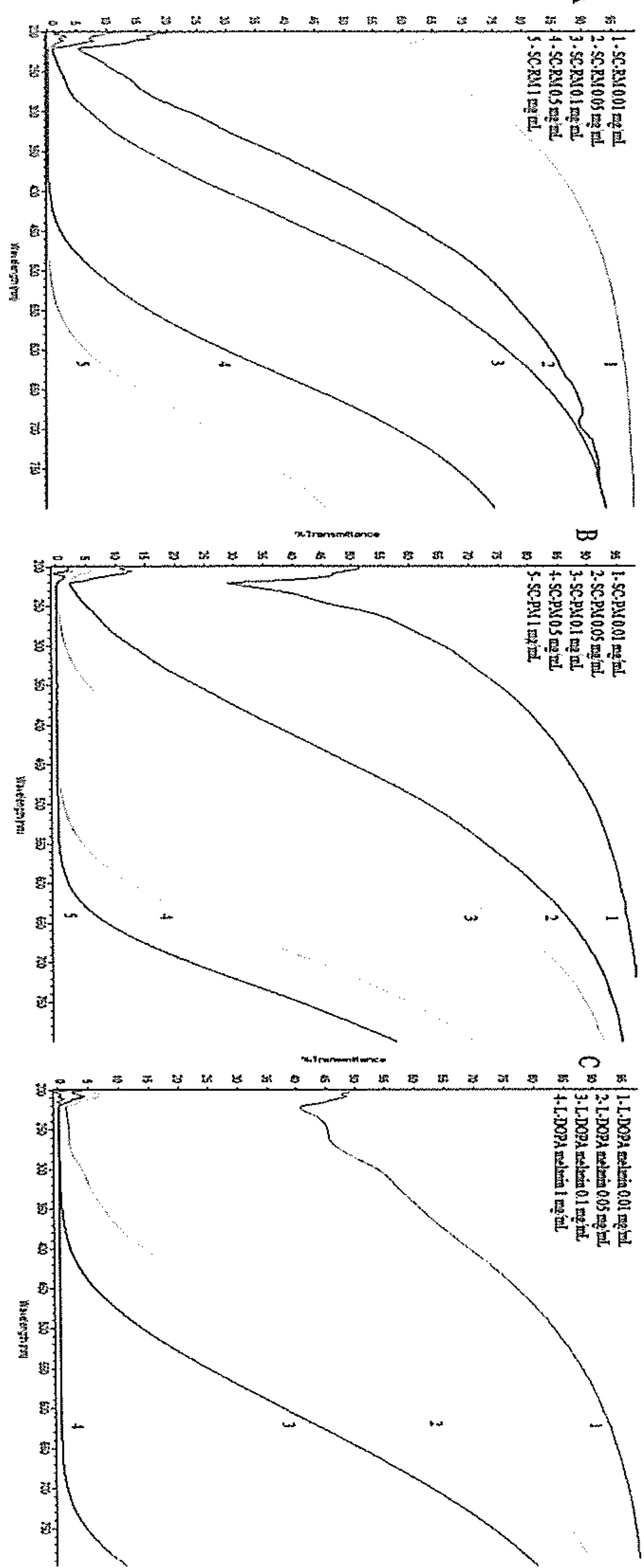
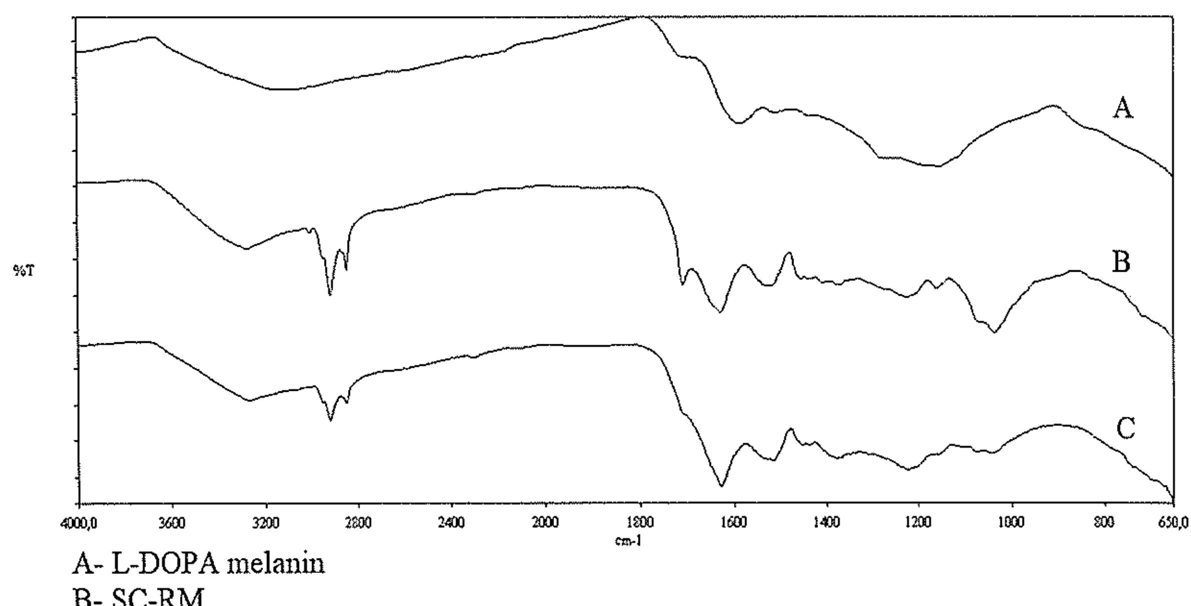


Fig. 3. Transmittance values of SC-RM (A), SC-PM (B) and L-DOPA melanin (C)

The TPC values for melanins were 0.18±0.01; 0.26±0.03; 0.29±0.05 mg GAE/g for SC-RM, SC-PM and L-DOPA melanin, recpectively. In general, the %AA values of SC-PM were higher than those of SC-RM, and the %AA of both melanins were lower than the corresponding concentrations of L-DOPA melanin, as shown in Table 3.

Table 3. The antioxidant activity (%AA values) of SC-RM, SC-PM and L-DOPA melanin (mean±SD, n = 3)

	%AA	SC-PM	%AA	L-DOPA melanin	%AA
0.0625 mg/mL	12.68±0.57	0.0625 mg/mL	13.99±0.31	0.0625 mg/mL	20.31±0.26
0.125 mg/mL	21.05±1.11	0.125 mg/mL	30.77±0.08	0.125 mg/mL	31.51±1.04
0.25 mg/mL	23.76±0.25	0.25 mg/mL	40.98±0.44	0.25 mg/mL	50.75±0.18
0.5 mg/mL	40.27±0.23	0.5 mg/mL	50.37±0.19	0.5 mg/mL	95.91±0.33
1 mg/mL	75.66±0.13	1 mg/mL	86.71±1.23	1 mg/mL	97.16±0.05



A- L-DOPA melanin
B- SC-RM
C- SC-PM

Fig. 4. Infrared spectra of L-DOPA melanin, SC-RM and SC-PM

Figure 4 shows the IR-spectra of SC-RM, SC-PM and L-DOPA melanin. Extra display broad absorption bands at 3600-3000 cm^{-1} were noted, attributed to stretching vibrations of C-

H, N-H and/or O-H groups. The C-H could be due to the presence of aromatic rings, with strong bands at 1630 cm^{-1} and 1627 cm^{-1}, for SC-RM and SC-PM, respectively, which corresponds to the vibration of aromatic C=C, and more intense in SC-PM. Two peaks at 2921 cm^{-1} to 2851 cm^{-1} in both melanins may result from the oscillation of aliphatic CH_2 and CH_3 groups. The bands at 1228 cm^{-1} and 1224 cm^{-1} due to C-N and C-O, would support the presence of phenols and aromatic amines. It is difficult to state whether there is an amide group, as the C=O group that it complements might be joined in the band corresponding to the aromatic C=C. There are differences between the SC-RM and SC-PM spectra which may be a result of the purification process.

Figure 5 shows the Raman spectra of synthetic melanin (A), SC-PM (B) and SC-RM (C). SC-RM and SC-PM Raman spectra were similar to spectrum of L-DOPA melanin. The Raman spectrum of SC-RM is dominated by two intense and broad peaks at about 1612 cm^{-1} and 1238 cm^{-1}, while at SC-PM spectrum peaks at 1625 cm^{-1} and 1245 cm^{-1} were observed. A peak at 2000 cm^{-1} from both melanins is noticeable. Peaks 395 $cm^{-1}$and 400 $cm^{-1}$for SC-RM and SC-PM, respectively, are present.

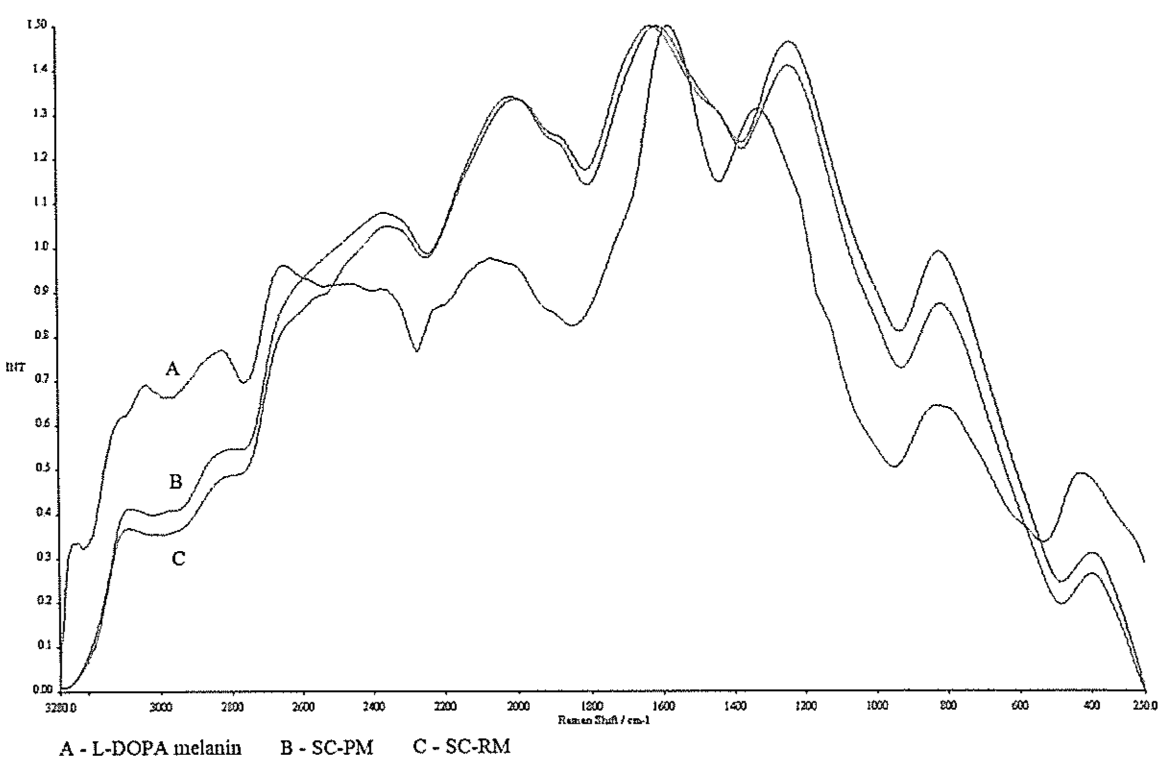


Fig. 5. The Raman spectra of L-DOPA melanin (A), SC-PM (B) and SC-RM (C)

The results of an antibacterial activity assessment of SC-RM and SC-PM are illustrated in Table 4. The zones of growth inhibition of *E. faecalis* and *P. aeruginosa* were 12.6±0.2 mm and 13.5±0.1 mm for SC-RM, respectively, while SC-PM, were 10.3±0.3 mm and 11.7±0.2 mm. No inhibition on *B. cereus*, *E. coli* and *S. aureus* was observed.

Table 4. The antibacterial activity of SC-RM, SC-PM and L-DOPA melanin (zones of growth inhibition, mm), (mean±SD, n = 3)

	BC	EC	EF	PA	SA
SC-RM	-	-	12.6±0.2	13.5±0.1	-
SC-PM	-	-	10.3±0.1	11.7±0.2	-
L-DOPA melanin	-	-	11.4±0.2	13.1±0.1	-

"-" – no inhibition zone, "BC" - *B. cereus* ATCC14579, "EC" - *E. coli* DSMZ1576, "EF" - *E. faecalis* ATCC29212, "PA" - *P. aeruginosa* ATCC2753, "SA" - *S. aureus* DSMZ346

4. DISCUSSION & CONCLUSIONS

Through the work of this study, it is clear that the melanins isolated from *S. citrinum* possess promising antioxidant, light barrier and antibacterial properties.

Chemical tests and FT-IR conducted on isolated pigments in comparison to the synthetic L-DOPA melanin clearly demonstrated that they are melanins. Purified melanin (SC-PM) was obtained by acid hydrolysis, repeated precipitation and purification through the use of organic solvents. The structure of melanin polymers is poorly understood and an accurate definition of melanin is still required. However, the following criteria indicate melanin is: black/brown in colour, insoluble in water and most other organic solvents, resistant to degradation by hot or cold acids, bleached by oxidizing agents and solubilised by alkali solutions [28,31].

Infrared spectroscopy has been used in the chemical structure study of many melanins. It has been suggested that identical melanin structures do not exist in nature and their chemical characterization is a complicated task. Their composition depends not only on their different monomeric units, but also on environmental conditions during polymerization. Infrared spectrometric techniques offer information on the main functional groups in the melanin structure [24,25,28]. A detailed comparative analysis of the infrared spectra of the melanins studied may supply valuable information on the effect of each treatment step used to purify the melanin and the distinct functional groups prevailing in the various samples.

The Raman spectrum of SC-RM is dominated by two intense and broad peaks at about 1612 cm^{-1} and 1238 cm^{-1}, while SC-PM spectrum peaks at 1625 cm^{-1} and 1245 cm^{-1} were noted. The peaks can be interrelated as originating from the in-plane stretching of the aromatic rings and the linear stretching of the C-C bonds within the rings, along with some contributions from the C-H vibrations in the methyl and methylene groups [34]. The peaks at about 2000 cm^{-1} from both melanins are similar to those obtained by Galvan et al. [35] from eumelanin and may be caused by the stretching of three of the six C-C bonds within the melanin aromatic rings. It was noted, that on both melanin Raman spectra peaks (395 cm^{-1} and 400 cm^{-1}, for SC-RM and SC-PM, respectively) are present, which are thought to correspond to peaks obtained from pheomelanin and eumelanin and are caused by an out-of-

plane deformation of phenyl rings. Obtained spectra of *S. citrinum* melanins are similar to *Exidia nigricans* melanins obtained in previous study [28].

There was no absorption peak between 260-280 nm in the UV-Vis spectra, indicating, that melanins do not contain proteins and nucleic acids [28]. The UV-Vis absorption spectra of the impure (RM) and purified (PM) melanins were similar to those reported in other literature. Also light barrier properties are in accorandce with previosus studies, showed that purified melanin form from *E. nigricans* offered better light prperties than the raw form [28].

In general, melanins are dark because they do not re-radiate the absorbed visible or invisible light, but transform the energy into rotational and vibrational activity within the molecule and then dissipate it as heat. This phenomenon protects melanised tissues against light-induced damage [28,34]. In previous study it has been also observed that purified melanin showed lower L* and higher a* and b* values [28]. The high antioxidant activity of the melanins was expected due to the protection against UV-radiation and free radical scavenging being their main functions [36-39]. The ability of melanin to scavenge reactive oxygen species (ROS), such as singlet oxygen, hydroxyl radical and superoxide anion, has been firmly established in model systems, suggesting that melanin could protect pigmented cells against oxidative stress that may accompany the formation of ROS in cells. Even though critical damage to oxidatively stressed cells may result from the reaction of crucial cellular constituents with ROS, an efficient antioxidant may protect the cells by scavenging other oxidizing radicals such as the peroxyl radical, and by interacting with molecular oxygen [40]. The high antioxidant activity of melanin isolated from various sources has been reported by other authors [24-40]. Antioxidant activity of *S. citrinum* melanins is higher than those analyzed in previous study, also polyphenolic content is higher, which suggest that, presence of higher polyphenolics in melanin molecules is linked with higher antioxidant activity [28].

The A300/A600 ratios offer information about the oxidation state and the range size of melanin molecules [25,28]. Melanin oxidation induces lower absorbance values at 600 nm (A600), and the A300/A600 absorbance ratio was proposed as a measure of oxidation extent, with high values corresponding to greater oxidized melanin molecules. Also, it was argued that during the melanin oxidation, phenolics are converted to semiquinones or quinones, which produce more oxidized (higher A300/A600 absorbance ratios) and smaller melanins (molecular weight < 1000 Da) [28]. SC-RM showed a higher value (10.23) than its corresponding pure SC-PM (9.72) and L-DOPA melanin (16.00). This data supports the fact than SC-RM are a more complex mixture of melanin molecules than that of SC-PM, with a variability in size and degree of oxidation. In constrast, for *E. nigricans* melanins, opposite observations have been made, showed that pure form of melanin had higher A300/A600 value being more complex than the raw form [28]. These data are also consistent with the results of Cuevaz-Juárez et al. [25] and also with observations made by Hung et al. [41] who noted that oxidized and reduced melanins obtained from black tea have variances in their absorption spectra. Reduced forms of melanin have phenolic form prevalence, which when oxidized, forms show preponderance for quinone forms.

Many fungi contain melanins in vegetative as well reproductive structures (e. g. spores). Melanins such as 1,8-dihydroxynaphtalene and L-3,4-dihydroxyphenylalanine (L-DOPA) types have been mostly associated to ascomycetous and basidiomycetous forms, respectively. Some fungi have the ability to synthetize a type of melanin according to the environmental conditions, as well as at the developmental stage. The presence of melanins in fungi adapted to different environments and the fact that the same melanin is synthetized by a fungus,

indecently of environmental conditions, suggests that this molecule plays different crucial biological roles in fungal physiology [36-38]. Melanins enhance the tolerance of fungi to environmental stresses, improving their survival. Melanins protect fungal structures from UV radiation, temperature, desiccation, oxidizing agents and toxic compounds, such as antibiotics or heavy metals, due to their chelating ability [28,36-39].

Both melanins showed antibacterial activity against *P. aeruginosa* and *E. faecalis*. No antibacterial activity towards *B. cereus*, *E. coli* and *S. aureus* was observed. This data are supported by previous study showed that melanins from *E. nigricans* have been active against *P. aeruginosa* and *E. faecalis* [28]. Antimicrobial assessment results are partially consistent with results found by other authors. Helan Soundra Rani et al. [42] noted the antimicrobial activity of melanin isolated from halophilic black yeast *Hortaea werneckii*. Laxmi et al. [43] observed that growth of *P. aeruginosa* was inhibited on the presence of melanin obtained from *Providencia rettgeri*, but in their study some *Bacillus* species were sensitive to melanin. Xu et al. [44] analysed the antimicrobial activity of melanin from *Lachnum* YM30 and noted that it was active against a wide spectrum of bacteria, including *S. aureus*. The authors suggest that melanin antibacterial activity might result from damage of the cell membrane and affect bacteria membrane function. A discrepancy in melanin antimicrobial activity may result in differences within the molecule structure and composition [46]. From the other hand there are some reports that melanins have antibiofilm activity agains pathogenic bacteria including *P. aeruginosa* and could interfere with bacterial quorum-sensing system, regulate its associate functions and prevent bacterial pathogenesis [43,44,46,47].

References

[1] Gurgel FE, Silva BDB, Baseia IG. New records of *Scleroderma* from Northeastern Brazil. *Mycotaxon* 2008, 105, 399-405.

[2] Sims KP, Watling R, Jeffries P. A revised key to the genus *Scleroderma*. *Mycotaxon* 1995, 56, 403-420.

[3] Montager DF, Coelho G, Silveira AO, Baldoni DB, Antoniolli ZI. Morphological and molecular analyses in *Scleroderma* (Basidiomycota) associated with exotic forests in Pampa biome, southern Brazil. *Mycosphere* 2015, 6(3), 337-344, DOI: 10.5943/mycosphere/6/3/9.

[4] Ghate SD, Sridhar KR, Karun NC. Macrofungi of the coastal sand dunes of south-western India. *Mycosphere* 2014, 5(1), 144-151, DOI: 10.5943/mycosphere/5/1/6.

[5] Pandey N, Budhathoki U. Protein determination through Bradford's method of nepalese mushroom. *Scientific World* 2007, 5(5), 85-88, DOI: 10.3126/sw.v5i5.2662.

[6] Kumla J, Suwannarach N, Bussaban B, Lumyong S. *Scleroderma suthepense*, a new ectomycorrhizal fungus from Thailand. *Mycotaxon* 2013, 123, 1-7, DOI: 10.5248/123.1.

[7] Yousaf N, Khald AN, Niazi AR. New records of *Scleroderma* species (*Sclerodermataceae, Agaricomycetes*) from Pakistan. *Mycotaxon* 2012, 122, 43-50, DOI: 10.5248/122.43.

[8] Zhang C, Xu X, Liu J, He M, Wang W, Wang Y, Ji K. *Scleroderma yunnanense*, a new species from South China. *Mycotaxon* 2013, 125, 193-200, DOI: 10.5248/125.193.

[9] Van der Sar SA, Blunt JW, Cole ALJ, Din LB, Munro MHG. Dichlorinated pulvinic acid derivative from a Malaysian *Scleroderma* sp. *J Nat Prod* 2005, 68(12), 1799-1801, DOI: 10.1021/np0503395.

[10] Mahmoud YAG, Amer SM, El-Souod SM. Cadmium, lead and zinc contents of sporocarps of some basidiomycetous fungi within gharbia governorate, Egypt. *New Egyptian Journal of Microbiology* 2007, 17(2), 89-94, DOI: 10.4314/nejmi.v17i1.40317.

[11] Hubregtse J. Fungi in Australia, Rev 2.0; 2017.

[12] Falandysz J. Mercury in mushrooms and soil of the Tarnobrzeska Plain, south-eastern Poland. *Journal of Enviornmental Science and Health, Part A* 2002, 37(3), 343-352, DOI: 10.1081/ESE-120002833.

[13] Mrak T, Kühdorf K, Grebenc T, Štraus I, Münzenberger B, Kraigher H. *Scleroderma areolatum* ectomycorrhiza on *Fagus sylvatica* L. *Mycorrhiza* 2017, 27(3), 283-293, DOI: 10.1007/s00572-016-0748-6.

[14] Guzmán G, Cortés-Pérez A, Guzmán-Dávalos L, Ramírez-Guillén F, del Refugio Sánchez-Jácome M. An emendation of *Scleroderma*, new species, and review of the known species in Mexico. *Revista Mexicana de Biodiversidad* 2013, 173-191, DOI: 10.7550/rmb.31979.

[15] Rusevska K, Karadelev M, Phosri C, Dueñas M, Watling R, Martín F. Rechecking of the genus *Scleroderma* (*Gasteromycetes*) from Macedonia using barcoding approach. *Turk J Botany* 2014, 38, 375-385, DOI: 10.3906/bot-1301-36.

[16] Nouhra ER, Caffot MLH, Pastor N, Crespo EM. The species of *Scleroderma* from Argentina, including a new species from the *Nothofagus* forest. *Mycologia* 2012, 104, 488-495, DOI: 10.3852/11-082.

[17] Wei Y, Gao JM, Hao SH, Zhang X. Insecticidal activity of basidiomycete *Scleroderma citrinum*. *Acta Botanica Boreali Occidentalica Sinica* 2005, 25(2), 382-385.

[18] Soytong K, Sibounnavong P, Kanokmedhakul K, Kanokmedhakul S. Biological active compounds of *Scleroderma citrinum* that inhibit plant pathogenic fungi. *International Journal of Agricultural Technology* 2014, 10(1), 79-86.

[19] Mohan V, Nivea R, Menon S. Evaluation of ectomycorrhizal fungi as potential bio-control agents against selected plant pathogenic fungi. *JAIR* 2015, 3(9), 408-412.

[20] Kanokmedhakul S, Kanokmedhakul K, Prajuabsuk T, Soytong K, Kongsaeree P, Suksamrarn A. A bioactive triterpenoid and vulpinic acid derivatives from the mushroom *Scleroderma citrinum*. *Planta Med* 2003, 69(6), 568-571, DOI: 10.1055/s-2003-40639.

[21] Winner M, Giménez A, Schmidt H, Sontag B, Steffan B, Steglich W. Unusual pulvinic acid dimers from the common fungi *Scleroderma citrinum* (common earthball) and *Chalciporus piperatus* (peppery bolete). *Angew Chem Int Ed Engl* 2004, 43(14), 1883-1886, DOI: 10.1002/anie.200352529.

[22] Loderer Ch, Ansorge-Schumacher MB. Magic mushrooms: screening for novel biocatalyst in the phylum Basidiomycota. *Advances in Bioscience and Biotechnology* 2016, 7, 446-453, DOI: 10.4236/abb.2016.711043.

[23] Velíšek J, Cejpek K. Pigments of higher fungi: a review. *Czech J Food Sci* 2011, 29(2), 87-102.

[24] Mbonyiryivuze A, Nuru ZY, Ngom BD, Mwakikunga B, Dhlamini SM, Park E, Maaza M. Morphological and Chemical Composition Characterization of Commercial Sepia Melanin. *AJN* 2015, 3(1), 22-27, DOI: 10.12691/ajn-3-1-3.

[25] Cuevas-Juárez E, Yuriar-Arredondo KY, Pío-León JF, Montes-Avila J, López-Angulo G, Díaz-Camacho SP, Delgado-Vargas F. Antioxidant and α-glucosidase inhibitory properties of soluble melanins from the fruits of *Vitex mollis* Kunth, *Randia echinocarpa* Sessé et Mociño and *Crescentia alata* Kunth. *J Funct Foods* 2014, 9, 78-88, DOI: 10.1016/j.jff.2014.04.016.

[26] Kurian NK, Nair HP, Bhat SG. Melanin producing *Pseudomonas stutzeri* BTCZ10 from marine sediment at 96 m depth (Sagar Sampada cruise #305). *Int J Curr Biotechnol* 2014; 2(5): 6-11.

[27] El-Naggar E-N, El-Ewasy SM. Bioproduction, characterization, anticancer and antioxidant activities of extracellular melanin pigment produced by newly isolated microbial cell factories *Streptomyces glaucescens* NEAE-H. *Scientific Reports* 2017, 7, 1-19, DOI: 10.1038/srep42129.

[28] Łopusiewicz Ł. Isolation, characterisation and biological activity from *Exidia nigricans*. *World Scientific News* 2018, 91, 111-129.

[29] Suryanarayanan T, Ravishankar J, Venkatesan G, Murali T. Characterization of the melanin pigment of a cosmopolitan fungal endophyte. *Myc Res* 2004, 108(8), 974-978, DOI: 10.1017/S0953756204000619.

[30] Solano F. Melanins: skin pigments and much more – types, structural models, biological functions, and formation routes. *New Journal of Science* 2014, DOI: 10.1155/2014/498276.

[31] Zhan F, He Y, Zu Y, Li T, Zhao Z. Characterisation of melanin isolated form a dark septate endophyte (DSE), *Exophiala pisciphila*. *World J Microbiol Biotechnol* 2011, 27, 2483-2489, DOI: 10.1007/s11274-011-0712-8.

[32] Yao Z, Qi J, Wang L. Isolation, Fractionation and Characterization of Melanin-like Pigments from Chestnut (*Castanea mollissima*) Shells. *J Food Sci* 2012, 77, 671-676, DOI: 10.1111/j.1750-3841.2012.02714.x.

[33] Selvakumar P, Rajasekar S, Periasamy K, Raaman N. Isolation and characterization of melanin pigment from *Pleurotus cystidiosus* (teleomorph of *Antromycopsis macrocarpa*). *World J Microbiol Biotechnol* 2008, 24, 2125-2131, DOI: 10.1007/s11274-008-9718-2.

[34] Huang Z, Lui H, Chen MXK, Alajlan A, McLean DI, Zeng H. Raman spectroscopy of in vivo cutaneous melanin. *J Biomed Opt* 2004, 9, 1198-1205, DOI: 10.1117/1.1805553.

[35] Galván I, Jorge A, Ito K, Tabuchi K, Solano F, Wakamatsu K. Raman spectroscopy as non-invasive technique for quantification of melanins in feathers and hairs. *Pigment Cell Melanoma Res* 2013, 26, 1-7, DOI: 10.1111/pcmr.12140.

[36] Toledo AV, Franco MEE, Lopez SMY, Troncozo MI, Saparrat MCN, Balatti PA. Melanins in fungi: Types, localization and putative biological roles. *Physiol Mol Plant Pathol* 2017, 99, 2-6, DOI: 10.1016/j.pmpp.2017.04.004.

[37] Fogarty RV, Tobin JM. Fungal melanins and their interactions with metals. *Enzyme Microb Technol* 1996, 19, 311-317, DOI: 10.1016/0141-0229(96)00002-6.

[38] Eisenman HC, Casadevall A. Synthesis and assembly of fungal melanin. *Appl Microbiol Biotechnol* 2012, 93, 931-940, DOI: 10.1007/s00253-011-3777-2.

[39] d'Ischia M, Wakamatsu K, Napolitano A, Briganti S, Garcia-Borron J-C, Kovacs D, Meredith P, Pezzella A, Picardo M, Sarna T, Simon JD, Ito S. Melanins and melanogenesis: methods, standards, protocols. *Pigment Cell Melanoma Res* 2013, 26, 616-633, DOI: 10.1111/pcmr.12121.

[40] Różanowska M, Sarna T, Land EJ, Truscott TG. Free radical scavenging properties of melanin: interaction of eu- and pheo-melanin models with reducing and oxidizing radicals. *Free Radic Biol Med* 1999, 26, 518-525, DOI: 10.1016/S0891-5849(98)00234-2.

[41] Hung Y-Ch, Sava V, Makan S, Chen Tz-HJ, Hong M-Y, Huang GS. Antioxidant activity of melanins derived from tea: comparision beteen different oxidative states. *Food Chem* 2002, 78, 233-240, DOI: 10.1016/S0308-8146(01)00403-4.

[42] Helan Soundra Rani M, Ramesh T, Subramanian J, Kalaiselvam M. Production and Characterization of Melanin Pigment from Halophilic Black Yeast *Hortaea werneckii*. *IJPRR* 2013, 2, 9-17.

[43] Laxmi M, Kurian NK, Smitha S, Bhat SG. Melanin and bacteriocin from marine bacteria inhibit biofilms of foodborne pathogens. *Indian J Biotechnol* 2016, 15, 392-399.

[44] Xu C, Li J, Yang L, Shi F, Yang L, Ye M. Antibacterial activity and a membrane damage mechanism of *Lachnum* YM30 melanin against *Vibrio parahaemolyticus* and *Staphylococcus aureus*. *Food Control* 2017, 73, 1445-1451, DOI: 10.1016/j.foodcont.2016.10.048.

[45] Correa N, Covarrubias C, Rodas PI, Hermosilla G, Olate VR, Valdés C, Meyer W, Magne F, Tapia CV. Differential Antifungal Activity of Human and Cryptococcal Melanins with Structural Discrepancies. *Front Microbiol* 2017, 8, 1-13, DOI: 10.3389/fmicb.2017.01292.

[46] Bin L, Wei L, Xiaohong Ch, Mei J, Mingsheng D. In vitro antibiofilm activity of the melanin from *Auricularia auricula*, an edible jelly mushroom. *Annals of Microbiology* 2012, 62(4), 1523-1530, DOI: 10.1007/s13213-011-0406-3.

[47] Zhu H, He C-C, Chu Q-H. Inhibition of quorum sensing in *Chromobacterium violaceum* by pigments extracted from *Auricularia auricular*. *Letters in Applied Microbiology* 2011, 52(3), 269-274, DOI: 10.1111/j.1472-765X.2010.02993.x.

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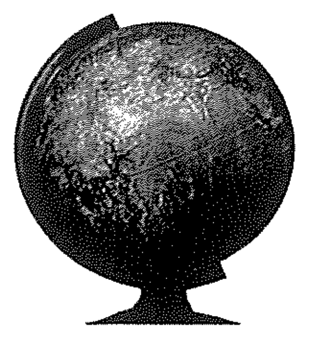

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The isolation, purification and analysis of the melanin pigment extracted from *Armillaria mellea* rhizomorphs

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ABSTRACT

The aim of present study was isolation and characteriation of raw and purified melanin from *Armillaria mellea* rhizomorphs. Native melanin was isolated from the rhizomorphs of *A. mellea* by alkaline extraction. Obtained pigment was purifed by acid hydrolysis and washed by organic solvents. Chemical tests, FT-IR and Raman spectroscopy analysis were conducted to determine the melanin nature of the isolated pigment. UV-Vis, transmittance and colour properties were evaluated. Antioxidant activity was determined using ABTS and antibacterial activity by a well diffusion method. The results of the study demonstrated that melanins isolated from *A. mellea* rhizomorphs had antioxidant, light barrier and antibacterial properties. A purified form of melanin offered better light properties and higher antioxidant activity than the raw form. Both melanins showed antimicrobial activity, raw melanin form had broader activity compared to the pure form. This study revealed that *A. mellea* rhizomorphs may be considered as a promising source of natural melanin. Isolated pigments presented all the physical and chemical properties common to natural and synthetic melanins. Raw and purified melanins showed differences in chemical composition, antioxidant activity and light barrier properties. Results of this study suggest that, melanins from *A. mellea* could be applied in the food, cosmetics and pharmaceutical industries.

Keywords: melanin, *Armillaria mellea*, rhizomorphs, antioxidant, antibacterial, light barrier

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1. INTRODUCTION

The genus *Armillaria* comprises of soil borne fungi that play an important role in the decomposition of wood. Fungi belonging to the genus *Armillaria* cause root disease of deciduous and coniferous trees and shrubs in forests, plantations, orchards and gardens in boreal, temperate and tropical habitats worldwide [1-8]. The root disease-causing capabilities of different *Armillaria* spp. on a given host species can be thought of as points along a continuum with strict saprotrophism at one extreme and relatively virulent parasitism at the other. Saprotrophic and parasitic *Armillaria* spp. are frequently sympatric [6,9]. It is well known that fruiting bodies of basidiomycetes of *Armillaria* genus are not able to emit visible light. Luminescence occurs only in mycelium, growing on the natural substrate (wood) or artificial substrates (nutrient medium) [10]. Most *Armillaria* species have the ability to spread in the soil by rhizomorphs to reach new food bases or potential hosts. Rhizomorphs (also called mycelial cords) are root-like fungal structures with diameters between 0.5 and 3 mm, that consist of an outer melanised cortex and an inner core, called medulla. Due to the melanin content, the cortex protects the rhizomorphs from environmental stress including attacks by antagonistic fungi and bacteria. The medulla is the main active structure of the rhizomorphs responsible for transport of water, nutrients, and oxygen. Rhizomorphs normally are growing out from a woody food base and can produce extensive networks in the soil [4,5,11,12].

Armillaria mellea (Vahl) P. Kumm is a basidiomycete fungus in the genus *Armillaria* in the Physalacriaceae family which grows on the stumps and roots of a wide variety of woody and herbaceous plants. It is also called honey mushroom and is a widespread fungus in Asia, Europe, North America and Africa [11-17]. In moderate climatic zones, the fruiting bodies are usually formed from July to November, with the most abundantly in October [17]. *A. mellea* is a fungus symbiotic with the Chinese medicinal herb "Tianma" (*Gastrodia elata* Blume). The fruiting bodies of *A. mellea* have been used in traditional Chinese medicine for the treatment of hypertension, headache, insomnia, dizziness and vertigo. Recently, the cultured mycelium of *A. mellea* became a health food in Taiwan and China and its tablets are used to treat geriatric patients with palsy, headache, insomnia, dizziness, and neurasthenia [2,13,16,18]. Although the fungus is considered a weak parasite or a saprophyte on forest trees, it can cause serious damage to a wide range of tree species, including *Abies* spp., *Quercus* spp., *Fagus sylvatica*, *Castanea sativa*, *Populus* spp. and *Ostrya carpinifolia*. In addition, *A. mellea* was commonly found on cultivated woody plants, causing damage, especially on apple, pear and in vineyards. *A. mellea* is known to occur in central and southern Europe, but is only common in the southern and western parts of this area. In Europe and North America, *A. mellea* occurs mainly in broadleaved forests, though less commonly in coniferous forests. The fungus is considered thermophilic, and in central Europe is restricted to low altitudes. In central and western Europe, *A. mellea* was not reported above 1000 m, whilst in the Mediterranean and Balkans regions, it has been found in altitudes up to 1750 m [7].

The known chemical compounds from *A. mellea* fruiting bodies and mycelial cultures have been reported include: carbohydrates (glucose, xylose, trehalose, D-mannitol, D-erythritol, glucans), peptides (prosomatostatin), sphingolipids (armillaramide), sterols (ergosterol, ergosterol peroxide), fatty acids (oleic, linoleic, palmitic and stearic acids), sesquiterpenoids (proto-illudan derivatives such as armillarin, melleolid, melledonal A, B and

C), phenolics (*p*-hydroxybenzoic and cinnamic acids), indole compounds (serotonin, tryptamine, tryptophan), ascorbic acid, enzymes and polysaccharides (exopolysaccharides and intracellular polysaccharides). Several studies describe the biological activities of *A. mellea* including immuno-modulating, anti-cancer, antioxidant, anti-inflammatory, antibacterial and fibrinolytic [2,13,16-22].

Melanins have been isolated from a variety of phylogenic sources: animals [24], plants [25], bacteria [26,57] and fungi [27,28,56]. Melanins are commonly represented as black and brown pigments, high molecular weight heterogenous polymers derived from the oxidation of monophenols and the subsequent polymeryzation of intermediate o-diphenols and their resulting quinones [29,30]. Melanins are types of pigments, possessing broad biological activities including; antioxidant, radioprotective, thermoregulative, chemoprotective, antitumor, antiviral, antimicrobial, immunostimulating and anti-inflammatory properties [24-30]. Based on these features, natural melanin has the potential to be of great value and application in the fields of pharmacology, cosmetics, functional foods and material modifications [30-33]. However, knowledge relating to the physiochemical properties and biological activities of melanins from *A. mellea* rhizomorphs including antioxidant, antimicrobial, light barrier properties is highly limited. In recent years there has been a revival of interest in the development of natural colorants as food additives, and also in the cosmetic and pharmaceutical industries. This has been encouraged by strong consumer demand, as synthetic colorants are frequently perceived as undesirable or harmful [27,28]. Owing to the high toxicity of synthetic compounds, the search for new natural colorants with antiradical, light barrier as well as antimicrobial properties still remains a challenge for modern science.

The aim of present study was to isolate, characterize and investigate the antioxidant, antimicrobial and light barrier properties of raw and purified melanins form *A. mellea* rhizomorphs.

2. MATERIALS AND METHODS

2. 1. Test fungus

The tests were made up of rhizomorphs of *A. mellea* within this study. The rhizomorphs were collected in October 2017 in a mixed forest near Szczecin, Poland (53° 20' N, 14° 49' E).

2. 2. Extraction and purification

The isolation and purification of melanin was performed as described by Łopusiewicz [27,28]. The rhizomorphs were washed with distilled water to remove any impurities, powdered in blender and used for melanin extraction. To summarise, 5 g of the rhizomorphs powder were homogenised in 50 ml of 1 M NaOH, extracted in orbital shaker (150 rpm, 50 °C, 24 h) and centrifuged at 6000 rpm for 10 min to remove fungal tissue. Alkaline AM-RM (*Armillaria mellea* raw melanin) mixture was first adjusted to pH 2.0 with 1 M HCl to precipitate melanin, followed by centrifugation at 6000 rpm for 10 min and a pellet was collected. Then, the pellet was hydrolyzed in 6 M HCl (90 °C, 2 h), centrifuged (6000 rpm, 10 min) and washed by distilled water five times to remove acid. The pellet was washed with chloroform, ethyl acetate and ethanol three times to wash away lipids and other residues. Finally, the purified melanin (AM-PM – *Armillaria mellea* pure melanin) was dried, ground to a fine powder in a mortar and stored at –20 °C until testing.

2. 3. Chemical tests

Different diagnostic tests, as described by Selvakumar et al. [34], were conducted on the AM-RM and AM-PM isolated melanins in comparison with L-DOPA melanin used as a melanin standard. The testing organic solvents included ethanol, methanol, chloroform, ethyl acetate, acetone and DMSO.

2. 4. Ultraviolet-visible absorption and transmittance spectra

Melanin solutions were prepared at concentration 0.1 mg/mL and UV-Vis absorption spectra were measured between 200 and 800 nm. The absorbance ratio (A300/A600) values and plots of optical densities against wavelengths of melanins were also calculated [25,27,28]. Transmittance values were measured between 200 and 800 nm at 0.01; 0.05; 0.1; 0.5 and 1 mg/mL for AM-RM and AM-PM; for L-DOPA melanin 0.01; 0.05; 0.1; 1 mg/mL concentrations were measured. All spectrophotometric assays were conducted in a Thermo Scientific Evolution 220 spectrophotometer.

2. 5. IR spectroscopy

The IR spectra of melanins solid samples were obtained at room temperature by attenuated total reflection with a Fourier transform infrared spectrometer (Perkin Elmer). The samples were evenly mixed with KBr, and pressed into tablets, then scanned at a range between 650 cm^{-1} and 4000 cm^{-1} (64 scans and 1 cm^{-1} resolution) [27,28]. Obtained spectra have been normalized, baseline corrected and analysed using SPECTRUM software.

2. 6. Raman spectroscopy

Melanin samples were analysed using a Raman station (RamanStation 400F, Perkin Elemer) with point-and-shot capability using an excitation laser source at 785 nm, 100 micron spot size, 25% laser power, 4 shots, 8 second exposition time. Obtained spectra have been normalized, baseline corrected and analysed using SPECTRUM software.

2. 7. The antioxidant activity (ABTS assay)

An ABTS assay was performed according to Łopusiewicz [27,28]. Radical 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS·+) was produced by mixing 7 mM ABTS with 2.45 mM potassium persulfate (5 mL of ABTS + 5 mL of potassium persulphate 4.9 mM). The mixture was incubated in darkness at room temperature for 16 h, diluted with 7 mM phosphate buffer (pH 7.4) to reach an absorbance of between 1.0 and 1.2 at 734 nm. For the ABTS assay, 50 μL of melanin (AM-RM, AM-PM, L-DOPA melanin; 0.0625; 0.125; 0.25, 0.5; 1 mg/mL), or dissolvent as control, were mixed with 1.95 mL of ABTS·+ solution, incubated in darkness for 10 min at 37 °C, and then the absorbance was measured at 734 nm and antioxidant activity (%AA) was calculated as $\%AA = [(A_c-A_m)/A_c]\times 100$; where A_c and A_m are absorbances for the control and melanin sample, respectively.

2. 8. Determination of the total phenolics content (TPC) of melanins

The total phenolics content (TPC) of the melanins were determined by the Folin-Ciocalteu reaction. Melanin dissolved in DMSO (20 μl) was mixed with 1.58 ml of deionized water and 100 μl of the Folin-Ciocalteu reagent, stirred gently for 5 min and added with 300

μl of saturated solution of Na_2CO_3. The mixture was allowed to stand in darkness for 30 min at 40 °C, the absorbance was then measured at 765 nm. A calibration curve of gallic acid in water-methanol (1:1, v/v) (0, 50, 100, 200, 400 and 500 μg/mL) was prepared and TPC was calculated as miligrams of gallic acid equivalents (GAE)/gram of melanin (mg GAE/g). The results were presented as an average of three samples with standard deviation.

2. 9. The visual colour of melanins

The visual colour of melanin solution (0.1 mg/mL) values were measured by a Konica Minolta CR-5 colorimeter with the Hunter LAB colour system. The colour values were expressed as L* (brightness/darkness), a* (redness/greenness) and b* (yellowness/blueness) as an averages of five measurements.

2. 10. The antibacterial activity of isolated melanins

Test microorganisms, including *Bacillus athrophaeus* ATCC49337, *Bacillus cereus* ATCC14579, *Bacillus subtilis* ATCC6633, *Enterococcus faecalis* ATCC29212, *Escherichia coli* DSMZ1576, *Micrococcus luteus* ATCC4698, *Pseudomonas aeruginosa* ATCC2753, *Pseudomonas putida* ATCC12633 and *Staphylococcus aureus* DSMZ346 were separately cultivated in Mueller-Hinton broth. The antibacterial activity was tested through a well diffusion method. 50 mL of Mueller-Hinton broth was inoculated by a single bacterial strain and incubated at 37 °C for 24 h. Mueller-Hinton agar was autoclaved and on reaching approx. 45 °C, 200 μL of bacterial suspension was added to 20 mL of the medium, vigorously vortexed and poured on 90 mm Petri dishes. Wells were cut out by sterile tips (9 mm diameter) in triplicate on each plate and 100 μL of melanin solutions at 0.1 mg/mL in DMSO were placed in the wells. DMSO served as a control. Plates were incubated at 37 °C for 24 h. The inhibition zones were measured after incubation. The results were presented as an average of three samples with standard deviation.

3. RESULTS

Table 1. The results are summarized, which also shows the properties of the L-DOPA melanin sample used for calibration.

No	Test	Result		
		AM-RM	AM-PM	L-DOPA melanin
1.	Solubility in water	Insoluble	Insoluble	Insoluble
2.	Solubility in organic solvents (acetone, chloroform, ethanol, ethyl acetate, methanol)	Insoluble	Insoluble	Insoluble

3.	Solubility in 1 M NaOH	Soluble	Soluble	Soluble
4.	Precipitation in acidic conditions	Precipitation	Precipitation	Precipitation
5.	Reaction with oxidizing agents (H_2O_2)	Decolorized	Decolorized	Decolorized
6.	Reaction with ammoniacal $AgNO_3$ solution	Positive*	Positive*	Positive*
7.	Reaction for polyphenols ($FeCl_3$ test)	Brown precipitate	Brown precipitate	Brown precipitate
8.	Colour	Black	Black	Black

*Positive – gray-coloured silver precipitate on tube side

The results of the study demonstrated that raw and purified black pigments from *A. mellea* had antioxidant, antibacterial and light barrier properties. The AM-RM and AM-PM pigments presented all of the physical and chemical properties common to natural melanins and the experimental data within this work were found to be comparable to those reported in literature (Table 1). Figure 1 shows the log of optical density of a melanins solutions when plotted against the wavelength of AM-RM, AM-PM and L-DOPA melanin. AM-RM, AM-PM and L-DOPA melanin had straight lines with negative slopes of -0.004695, -0.003644 and -0.003741, respectively.

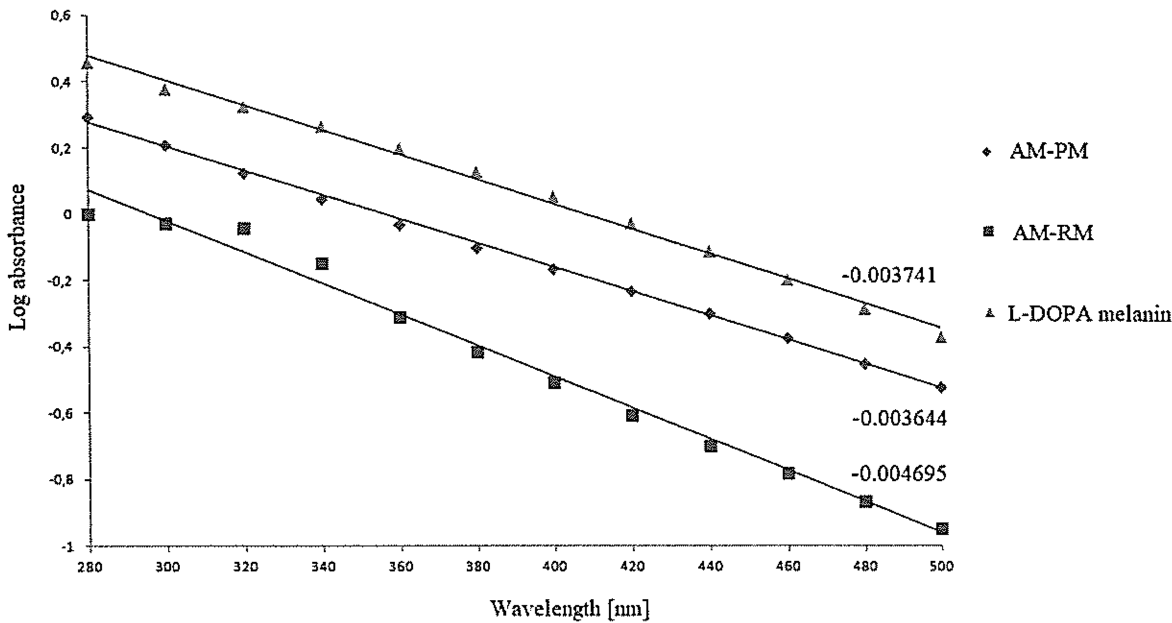

Figure 1. A plots of log of optical denisity of AM-PM, AM-RM and L-DOPA melanin against wavelength

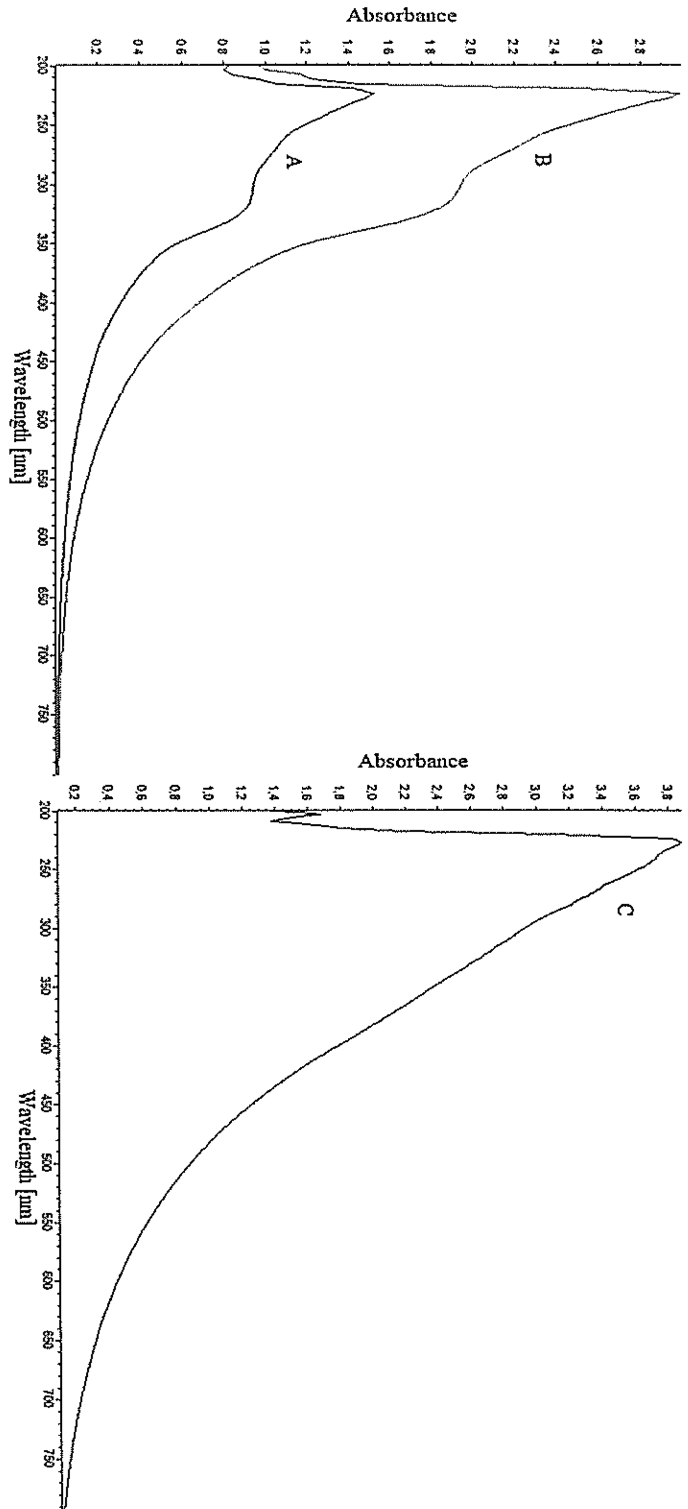

Figure 2. The absorbance of AM-RM (A), AM-PM (B) and L-DOPA melanin (C)

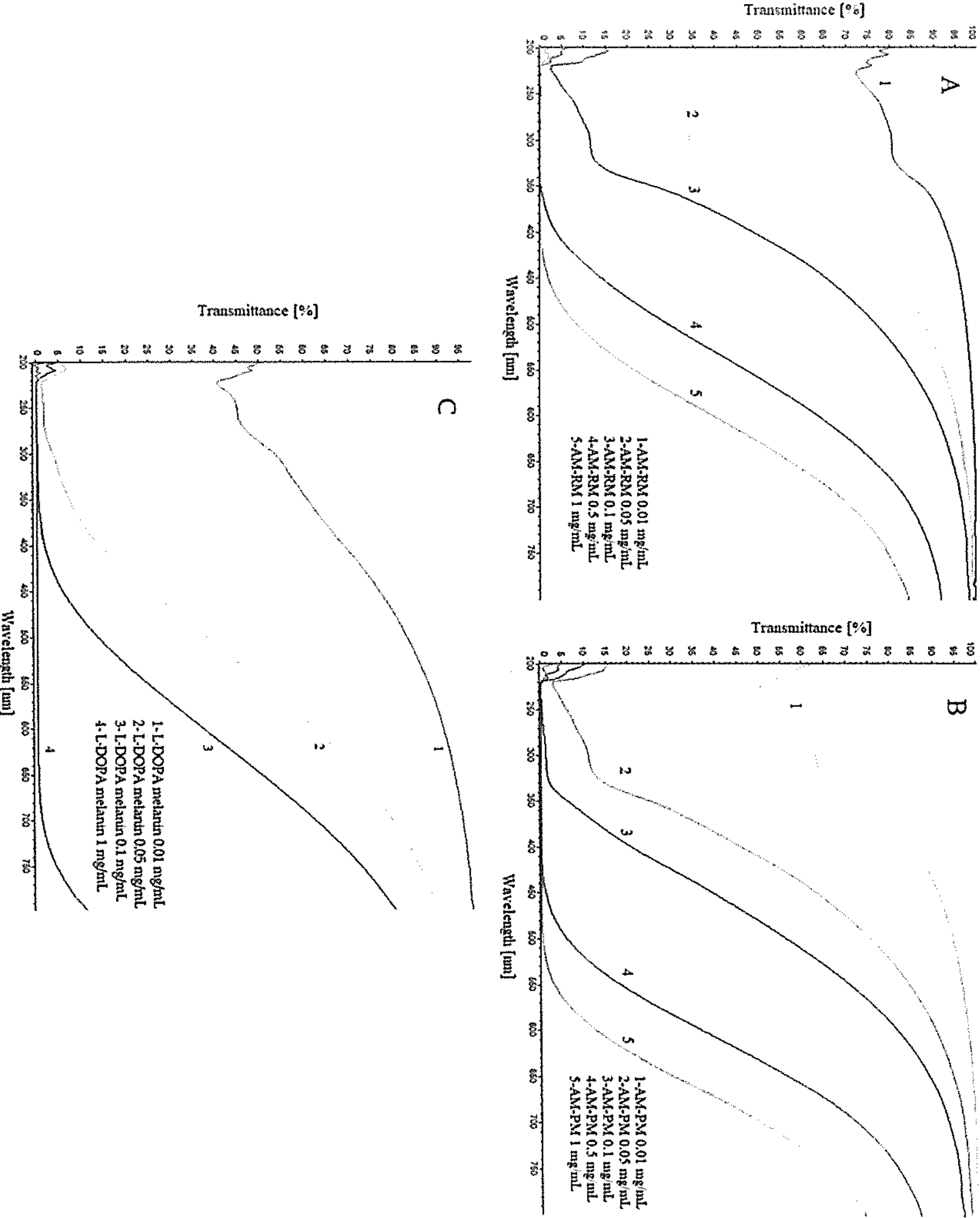

Figure 3. Transmittance values of AM-RM (A), AM-PM (B) and L-DOPA melanin (C)

Figure 2 shows AM-RM (A) and AM-PM (B) have maximum absorption peaks at 224 nm and 227 nm, respectively, and exhibited an exponential decrease in the visible region. This behaviour in AM-RM and AM-PM were similar to the melanin synthesized from L-DOPA (C), which is used as a melanin standard.

The light barrier properties of AM-RM (A), AM-PM (B) and L-DOPA melanin (C) are shown in Figure 3. It was noted that in all analysed concentrations, the AM-RM transmittance values were higher than those of the corresponding AM-PM, which suggests that in purified form, melanin had better light barrier properties, even when the transmittance values of AM-PM were smaller than the synthetic melanin.

The colour values of AM-RM, AM-PM and synthetic melanin are shown in Table 2. Results from the colorimeter indicated that AM-PM presented lower L* value, and higher a* and b* values than AM-RM in Hunter Lab colour system.

Table 2. The visual colour values of AM-RM, AM-PM and L-DOPA melanin (mean ± SD, n = 5)

	L*	a*	b*
AM-RM	94.99±0.00	0.58±0.00	11.39±0.01
AM-PM	89.37±0.01	3.30±0.00	29.90±0.00
L-DOPA melanin	74.87±0.02	10.56±0.01	47.74±0.01

The TPC values for melanins were 0.12±0.02; 0.25±0.01; 0.29±0.05 mg GAE/g for AM-RM, AM-PM and L-DOPA melanin, respectively. In general, the %AA values of AM-PM were higher than those of AM-RM, and the %AA of both melanins were lower than the corresponding concentrations of L-DOPA melanin, as shown in Table 3.

Table 3. The antioxidant activity (%AA values, [%]) of AM-RM, AM-PM and L-DOPA melanin at various concentrations [mg/ml] (mean ± SD, n = 3)

AM-RM [mg/mL]	%AA [%]	AM-PM [mg/mL]	%AA [%]	L-DOPA melanin [mg/mL]	%AA [%]
0.0625	9.67.1±0.14	0.0625	16.09±0.45	0.0625	20.31±0.26
0.125	15.05±0.34	0.125	28.02±0.19	0.125	31.51±1.04
0.25	27.22±0.89	0.25	44.88±0.22	0.25	50.75±0.18
0.5	39.78±0.33	0.5	61.33±0.56	0.5	95.91±0.33
1	68.12±0.51	1	89.17±0.12	1	97.16±0.05

Figure 4 shows the IR-spectra of AM-RM, AM-PM and L-DOPA melanin. Extra display broad absorption bands at 3600-3000 cm^{-1} were noted, attributed to stretching vibrations of C-H, N-H and/or O-H groups. The C-H could be due to the presence of aromatic rings, with strong bands at 1629 cm^{-1} and 1628 cm^{-1}, for AM-RM and AM-PM, respectively, which corresponds to the vibration of aromatic C=C, and more intense in AM-PM. Two peaks at 2915 cm^{-1} to 2847 cm^{-1} in both melanins may result from the oscillation of aliphatic CH_2 and CH_3 groups. The bands at 1212 cm^{-1} and 1236 cm^{-1} due to C-N and C-O, would support the presence of phenols and aromatic amines. It is difficult to state whether there is an amide group, as the C=O group that it complements might be joined in the band corresponding to the aromatic C=C. Noticeable intense peak at 1024 cm^{-1} in AM-RM spectrum could be due to the presence of glucose residues, suggesting polysaccharides components linked with AM-RM. There are differences between the AM-RM and AM-PM spectra which may be a result of the purification process.

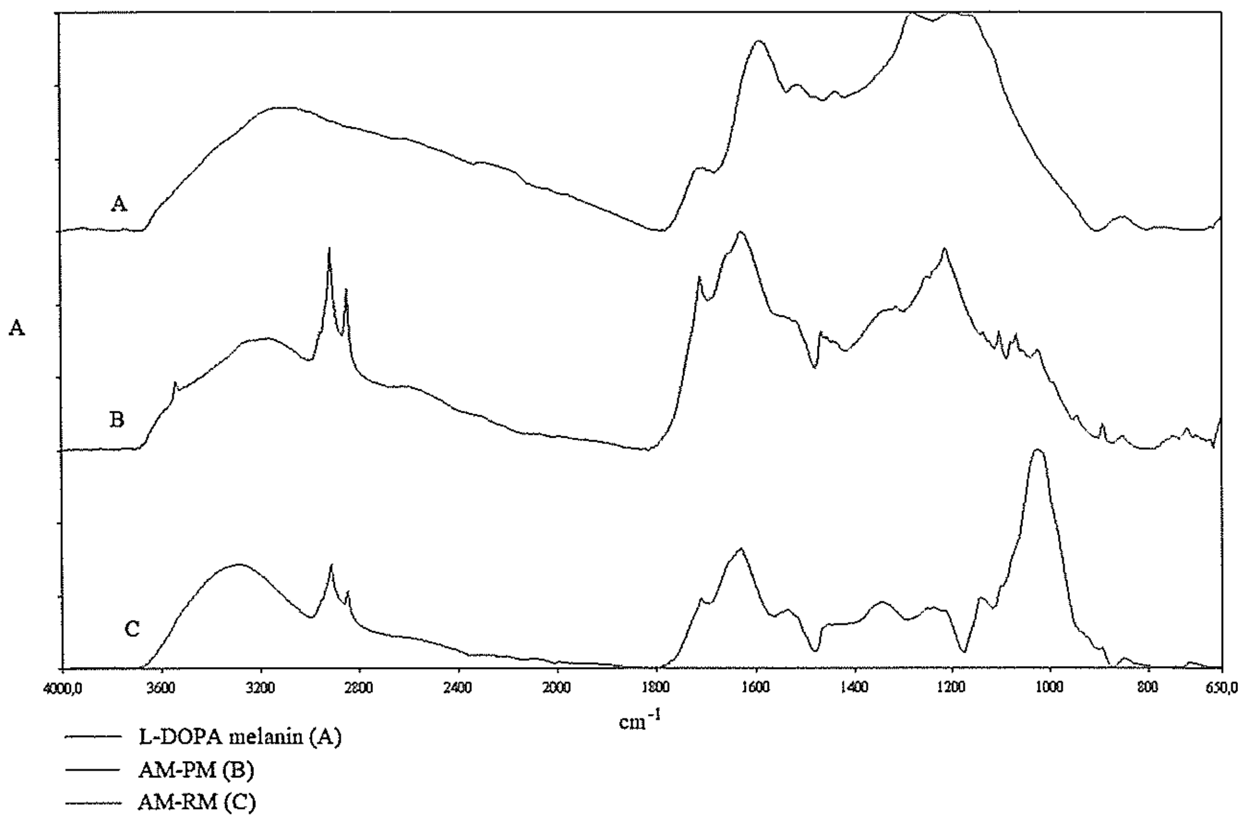

Figure 4. FT-IR spectra of AM-RM, AM-PM and L-DOPA melanin

Figure 5 shows the Raman spectra of synthetic melanin (A), AM-PM (B) and AM-RM (C). AM-RM and AM-PM Raman spectra were similar to spectrum of L-DOPA melanin. The Raman spectrum of AM-RM is dominated by two intense and broad peaks at about 1618 cm^{-1} and 1239 cm^{-1}, while at AM-PM spectrum peaks at 1622 cm^{-1} and 1241 cm^{-1} were observed. A peak at 1970 cm^{-1} from both melanins is noticeable. Peaks 391 cm^{-1}and 398 cm^{-1}for AM-RM and AM-PM, respectively, are present.

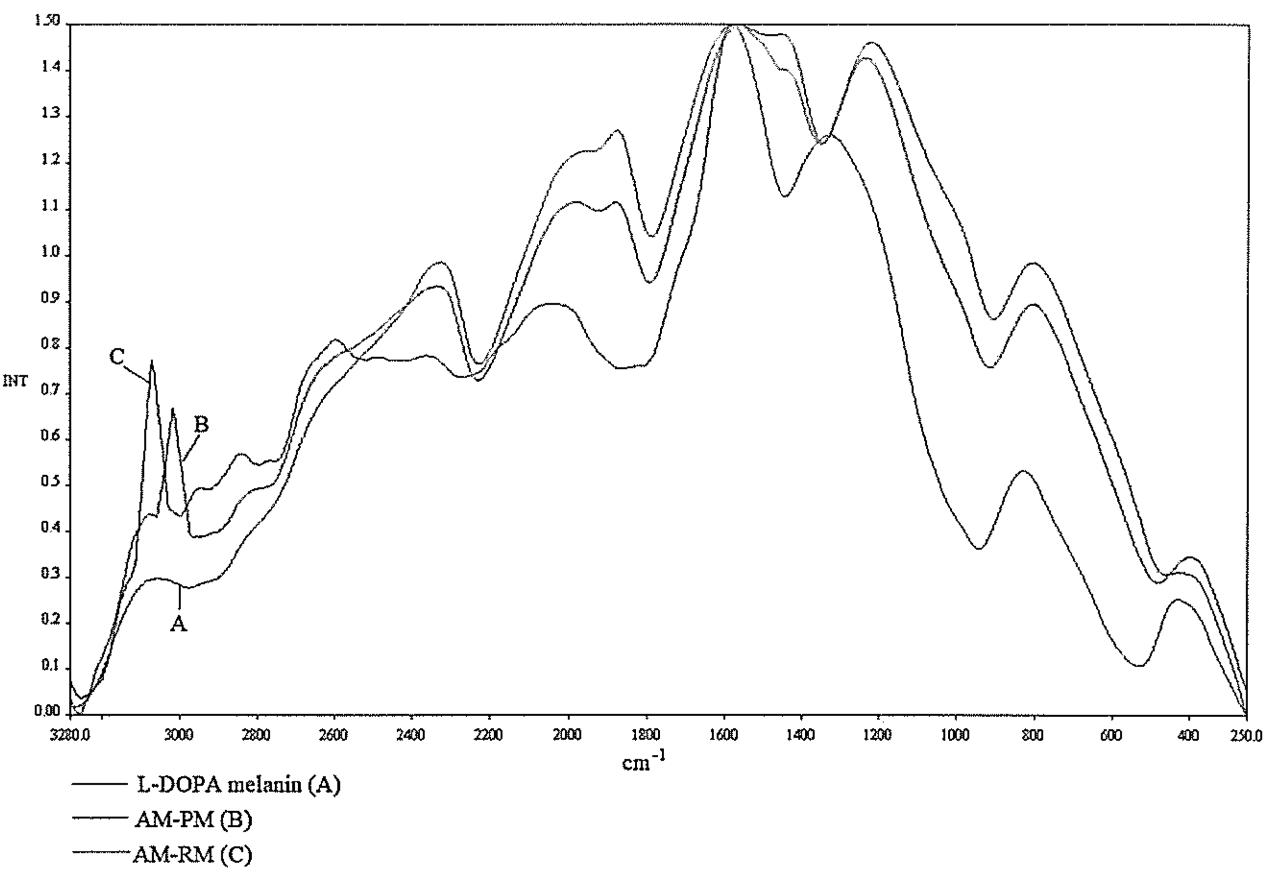

Figure 5. Raman spectra of L-DOPA melanin (A), AM-PM (B), AM-RM (C)

Table 4. The antibacterial activity of EN-RM, EN-PM and L-DOPA melanin (zones of growth inhibition, mm), (mean ± SD, n = 3)

	BA	BC	BS	EC	EF	ML	PA	PP	SA
AM-RM	12.1 ±0.1	11.5 ±0.3	13.8 ±0.2	10.3 ±0.1	14.4 ±0.2	11.1 ±0.5	14.5 ±0.2	11.3 ±0.3	10.4 ±0.2
AM-PM	-	10.7 ±0.2	12.4 ±0.4	-	12.2 ±0.1	-	12.2 ±0.3	-	-
L-DOPA melanin	-	-	-	-	11.4 ±0.2	-	13.1 ±0.1	-	-

"-" – no inhibition zone, "BA" – *B. athrophaeus* ATCC49337, "BC" - *B. cereus* ATCC14579, "BS" – *B. subtilis* ATCC6633, "EC" - *E. coli* DSMZ1576, "EF" - *E. faecalis* ATCC29212, "ML" – *M. luteus* ATCC4698, "PA" - *P. aeruginosa* ATCC2753, "PP"- *P. putida* ATCC12633, "SA" - *S. aureus* DSMZ346

The results of an antibacterial activity assessment of AM-RM and AM-PM are presented in Table 4. It was noted that AM-RM melanin was active agains all selected strains. AM-PM was active against *B. cereus*, *B. subtilis*, *E. faecalis* and *P. aeruginosa*, and showed no activity against *B. atrophaeus*, *E. coli*, *M. luteus*, *P. putida* and *S. aureus*.

4. DISCUSSION & CONCLUSIONS

Through the work of this study, it is clear that the melanins isolated from *A. mellea* rhizomorphs possess promising antioxidant, light barrier and antibacterial properties.

Chemical tests and FT-IR conducted on isolated pigments in comparison to the synthetic L-DOPA melanin clearly demonstrated that they are melanins. Purified melanin (AM-PM) was obtained by acid hydrolysis, repeated precipitation and purification through the use of organic solvents. The structure of melanin polymers is poorly understood and an accurate definition of melanin is still required. However, the following criteria indicate melanin is: black/brown in colour, insoluble in water and most other organic solvents, resistant to degradation by hot or cold acids, bleached by oxidizing agents and solubilised by alkali solutions [27,28,35].

There was no absorption peak between 260-280 nm in the UV-Vis spectra, indicating, that melanins do not contain proteins and nucleic acids [27,28]. The UV-Vis absorption spectra of the impure (RM) and purified (PM) melanins were similar to those reported in other literature. Also light barrier properties are in accordance with previous studies, showed that purified melanins form *E. nigricans* and *S. citrinum* offered better light properties than the raw form [27,28].

In general, melanins are dark because they do not re-radiate the absorbed visible or invisible light, but transform the energy into rotational and vibrational activity within the molecule and then dissipate it as heat. This phenomenon protects melanised tissues against light-induced damage [23-30]. In previous study it has been also observed that purified melanins showed lower L* and higher a* and b* values [25,27,28]. The high antioxidant activity of the melanins was expected due to the protection against UV-radiation and free radical scavenging being their main functions [23-30]. The ability of melanin to scavenge reactive oxygen species (ROS), such as singlet oxygen, hydroxyl radical and superoxide anion, has been firmly established in model systems, suggesting that melanin could protect pigmented cells against oxidative stress that may accompany the formation of ROS in cells. Even though critical damage to oxidatively stressed cells may result from the reaction of crucial cellular constituents with ROS, an efficient antioxidant may protect the cells by scavenging other oxidizing radicals such as the peroxyl radical, and by interacting with molecular oxygen [36]. The high antioxidant activity of melanin isolated from various sources has been reported by other authors [23-28]. Antioxidant activity of *A. mellea* melanins is comparable to those analyzed in previous studies, also polyphenolic content, which suggest that presence of polyphenolics in melanin molecules is linked with antioxidant activity [27,28].

The A300/A600 ratios offer information about the oxidation state and the range size of melanin molecules [27,28]. Melanin oxidation induces lower absorbance values at 600 nm (A600), and the A300/A600 absorbance ratio was proposed as a measure of oxidation extent, with high values corresponding to greater oxidized melanin molecules. Also, it was argued

that during the melanin oxidation, phenolics are converted to semiquinones or quinones, which produce more oxidized (higher A300/A600 absorbance ratios) and smaller melanins (molecular weight < 1000 Da) [27,28]. AM-RM showed a higher value (22.45) than its corresponding pure AM-PM (11.56) and L-DOPA melanin (16.00). This data supports the fact than AM-RM are a more complex mixture of melanin molecules than that of AM-PM, with a variability in size and degree of oxidation. These data are consistent with previous study showed that raw form of *S. citrinum* melanins are more complex that the pure form [28]. In contrast, for *E. nigricans* melanins, opposite observations have been made, showed that pure form of melanin had higher A300/A600 value being more complex than the raw form [27]. These data are also consistent with the results of Cuevaz-Juárez et al. [37] and also with observations made by Hung et al. [38] who noted that oxidized and reduced melanins obtained from black tea have variances in their absorption spectra. Reduced forms of melanin have phenolic form prevalence, which when oxidized, forms show preponderance for quinone forms.

It was noted that AM-RM melanin was active against all selected strains. AM-PM was active against *B. cereus*, *B. subtilis*, *E. faecalis* and *P. aeruginosa*, and showed no activity against *B. atrophaeus*, *E. coli*, *M. luteus*, *P. putida* and *S. aureus*. This data are partially supported by previous study showed that melanins from *E. nigricans* and *S. citrinum* have been active against *P. aeruginosa* and *E. faecalis* [27,28]. Antimicrobial assessment results are also partially consistent with results found by other authors. Helan Soundra Rani et al. [39] noted the antimicrobial activity of melanin isolated from halophilic black yeast *Hortaea werneckii*. Laxmi et al. [40] observed that growth of *P. aeruginosa* was inhibited on the presence of melanin obtained from *Providencia rettgeri*, but in their study some *Bacillus* species were sensitive to melanin. Xu et al. [41] analysed the antimicrobial activity of melanin from *Lachnum* YM30 and noted that it was active against a wide spectrum of bacteria, including *S. aureus*. The authors suggest that melanin antibacterial activity might result from damage of the cell membrane and affect bacteria membrane function. A discrepancy in melanin antimicrobial activity may result in differences within the molecule structure and composition [42]. In fact, FT-IR spectra showed that AM-RM melanins are probably linked with high content of polysaccharides. Some fungal polyssacharides are known from their antimicrobial activity [43], and it is tempting to suggest that broad antimicrobial activity of AM-RM may result from the presence of polysaccharides linked with melanin molecules. From the other hand there are some reports that melanins have antibiofilm activity against pathogenic bacteria including *P. aeruginosa* and could interfere with bacterial quorum-sensing system, regulate its associate functions and prevent bacterial pathogenesis [44-46].

Members of the *Armillaria* genus are among the most damaging root pathogens of forest and orchard trees worldwide. Rhizomorphs, similar to roots in external appearance, are produced by these fungi on infected root systems. They grow through the soil, where they may contact roots of neighbouring trees [47]. *Armillaria* infects trees either by the rhizomorph penetration of healthy roots or through physical contact of a susceptible root with a diseased root. Rhizomorphs are important in the dissemination and survival of the pathogen, also playing pivotal role in their aeration [4]. During infection rhizomorphs penetrate the bark and form mycelial fans, which spread within the inner bark and cambium of the host root [48]. The black pigment found in rhizomorphs is a melanin type pigment, and has a protective role against unfavourable environmental conditions [4,47,49]. It is commonly observed that rhizomorph tips that are in contact the atmosphere become brown. Worral *et al.* suggest that

enzyme laccase participates in the formation of melanin in rhizomorphs [47]. This observation is accordance with fact that the formation of melanin occurs mainly in aerobic conditions [30]. Many fungi contain melanins in vegetative as well reproductive structures (e. g. spores). Melanins such as 1,8-dihydroxynaphtalene and L-3,4-dihydroxyphenylalanine (L-DOPA) types have been mostly associated to ascomycetous and basidiomycetous forms, respectively. Some fungi have the ability to synthetize a type of melanin according to environmental conditions, as well as at a developmental stage. The presence of melanins in fungi adapted to different environments and the fact that the same melanin is synthesized by a fungus, independent of environmental conditions, suggests that this molecule plays various crucial biological roles in fungal physiology [50-52]. Melanins enhance the tolerance of fungi to environmental stresses, improving their survival. Melanins protect fungal structures from UV radiation, temperature, desiccation, oxidizing agents and toxic compounds, such as antibiotics or heavy metals, due to their chelating ability [4,50-52]. In several plant pathogens melanin a plays pivotal role in generating osmotic pressure within the appresorium, when hyphae penetrate cell walls. They also may protect rhizomorphic structures from microbial lysis in the soil [47].

During their growth through soils, rhizomorphs can take up mineral nutrients at their growing tips [6]. The cortex of the rhizomorphs have the ability to absorb metal ions from natural soils [11,53]. The chemical structure of melanin presents many oxygen-containing groups, including carboxyl, phenolic and alcoholic hydroxyl, carbonyl, and methoxy groups, which have the ability to bind to a broad spectrum of substances. In literature, studies have confirmed that fungal melanin acts as a metal chelator, enhancing biomass-metal interaction, and consequently its biosorption capacity [54]. Rizzo *et al.* reported a binding of metals to the mycelial melanin of the *Armillaria* spp. finding that the melanized rhizomorph mycelia concentrated Al, Zn, Fe and Cu ions. Through their ability to bind metal ions and to produce extensive networks in the soil *Armillaria* rhizomorphs may contribute to the stabilization of heavy metals in contaminated soils. Fungal melanins contain various functional (chemical) groups, which provide binding sites for metal ions and microorganisms find them rather difficult to decompose [51-52]. Therefore, rhizomorphic melanin might still bind heavy metals as part of the soil organic matter after the death of the rhizomorphs. In fungi the amount of melanin produced is associated with the level of resistance to radiation. Fungi living on rocks, exposed surfaces or in extreme environments are often heavily pigmented and able to resist elevated temperatures and UV radiation [55]. *A. mellea* rhizomorphs often grow on wood surfaces and bark, which are often exposed on direct sun radiation. Thus, it is obvious, that fungi evolved panoply of adaptations in rhizomoprhs, which include adaptations for overcoming host barriers, successfully compete with other microorganisms and are able to survive harsh environmental conditions. Melanins are also responsible for dark lines formed in the inhabited wood. Fungal melanin deposition in spatial demarcation could be triggered by limited water availability. The anticipation of desiccation might lead to the development of an effective strategy to ensure the survival of the colonies. They produce high resistance, melanin-type pigment that surrounds the fungal community like a barrier, blocking water exchange within the wood substrate. This may appear as fine delimitation lines in sections. The formation of melanised mycelium in zone lines, in inter- and intraspecific antagonistic reactions, offer a perspective on substrate and environmental conditions that influence such formations in natural settings. Such lines have been also reported for *A. mellea*, which occupies wood moister than ambient [49].

References

[1] Asef M.R., Mohammadi Goltapeh E., Alizadeh A. 2003. Identification of Armillaria species in Iran. *Fungal Diversity* 14: 51-60.

[2] Gao J-M., Yang X., Wang C-Y., Liu J-K. 2001. Armillaramide, a new sphingolipid from the fungus Armillaria mellea. *Fitoterapia* 72: 858-864. DOI: 10.1016/S0367-326X(01)00319-7

[3] Mwenje E., Wingfield B.D., Coetzee M.P.A. Wingfield M.J. 2003. Molecular characterisation of Armillaria species from Zimbabwe. *Mycological Research* 107(3): 291-296. DOI: 10.1017/S0953756203007408

[4] Pareek M., Cole L., Ashford A.E. 2001. Variations in structure of aerial and sumberged rhizomorphs of Armillaria luteobubalina indicate that they may be organs of absorption. *Mycological Research* 105(11): 1377-1387. DOI: 10.1017/S0953756201004622

[5] Mihail J.D., Bruhn J.N. 2007. Dynamics of bioluminescence by Armillaria gallica, A. mellea and A. tabescens. *Mycologia* 99(3): 341-350.

[6] Morrison D.J. 2004. Rhizomorph growth habit, saprophytic ability and virulence of 15 Armillaria species. *Forest Pathology* 34: 15-26. DOI: 10.1046/j.1439-0329.2003.00345.x

[7] Lushaj B.M., Woodward S., Keča N., Intini M. 2010. Distribution, ecology and host range of Armillaria species in Albania. *Forest Pathology* 40: 485-499. DOI: 10.1111/j.1439-0329.2009.00624.x

[8] Guillaumin J.J., Mohammed C., Anselmi N., Courtecuisse R., Gregory S.C., Holdenrieder O., Intini M., Lung B., Marxmüller H., Morrison D., Risbeth J., Termorshuizen A.J., Tirró A., van Dam B. 1993. Geographical distribution and ecology of the Armillaria species in western Europe. *Forest Pathology* 23: 321-341. DOI: doi.org/10.1111/j.1439-0329.1993.tb00814.x

[9] Mihail J.D., Bruhn J.N. 2005. Foraging behaviour of Armillaria rhizomorph systems. *Mycological Research* 109(11): 1195-1207. DOI: 10.1017/S0953756205003606

[10] Puzyr A.P., Medvedeva S.E., Bondar V.S. 2017. Biochemical changes causes lack of bioluminescence in fruiting bodies of Armillaria. *Mycosphere* 8(1): 9-17. DOI: 10.5943/mycosphere/8/1/2

[11] Rigling D., Günthardt-Goerg M.S., Blauenstein H., Frey B. 2006. Accumulation of heavy metals into Armillaria rhizomorphs from contaminated soil. *Forest Snow and Landscape Research* 80(2): 213-220.

[12] Yafetto L., Davis D.J., Money N.P. 2009. Biomechanic of invasive growth by Armillaria rhizomorphs. *Fungal Genetics and Biology* 46: 688-694. DOI: 10.1016/j.fgb.2009.04.005

[13] Li Z., Wang Y., Jiang B., Li W., Zheng L., Yang X., Bao Y., Sun L., Huang Y., Li Y. 2016. Structure, cytotoxic activity and mechanism of protoilludane sesquiterpene aryl esters from the mycelium of Armillaria mellea. *Journal of Ethnopharmacology* 184: 119-127. DOI: 10.1016/j.jep.2016.02.044

[14] Putrov K.V., Petushkov V.N., Rodionova N.S., Gitelson J.I. 2017. Why Does the Bioluminescent Fungus Armillaria mellea Have Liminous Mycelium but Nonluminous Fruiting Body? *Doklady Biochemistry and Biophysics* 474: 217-219. DOI: 10.1134/S1607672917030176

[15] Lung M-Y., Chang Y-C. 2011. In vitro antioxidant properties of polysaccharides from Armillaria mellea in batch fermentation. *African Journal of Biotechnology* 10(36): 7048-7057.

[16] Zavastin D.E., Mircea C., Aprotosoaie A.C., Gherman S., Hancianu M., Mron A. 2015. Armillaria mellea: phenolic content, in vitro antioxidant and antihyperglicemic effects. *Revista medico-chirurgicala a Societatii de Medici si Naturalisti din Iasi* 119(1): 273-280.

[17] Muszyńska B., Maślanka A., Ekiert H., Sułkowska-Ziaja K. 2011. Analysis of indole compounds in Armillaria mellea fruiting bodies. *Acta Poloniae Pharmaceutica – Drug Research* 68(1): 93-97.

[18] Chen C-C., Kuo Y-H., Cheng J-J., Sung P-J., Ni C-L., Chen C-C., Shen C-C. 2015. Three new Sesquiterpene Aryl Esters from the Mycelim of Armillaria mella. *Molecules* 20: 9994-10003. DOI: 10.3390/molecules20069994

[19] Gao L.W., Li W.Y., Zhao Y.L., Wang J.W. 2009. The cultivation, bioactive components and pharmacological effects of Armillaria mellea. *African Journal of Biotechnology* 8(25): 7383-7390.

[20] Chen Y-J., Chen C-C., Huang H-L. 2016. Induction of apoptosis by Armillaria mellea constituent armillarikin in human hepatocellular carcinoma. *OncoTargets and Therapy* 9: 4773-4783. DOI: 10.2147/OTT.S103940

[21] Lung M-Y., Chang Y-C. 2011. Antioxidant Properties of the Edible Basidiomycete Armillaria mellea in Submerged Cultures. *International Journal of Molecular Sciences* 12: 6367-6384. DOI: 10.3390/ijms12106367

[22] Lung M-Y., Huang P-C. 2009. Optimization of exopolysaccharide production from Armillaria mellea in submerged cultures. *Letters in Applied Microbiology* 50: 198-204. DOI: 10.1111/j.1472-765X.2009.02777.x

[23] Sun X., Wu B., Zhou L., Liu Z., Dong Y., Yang A. 2017. Isolation and characterization of melanin pigment from yesso scallop Patinopecten yessoensis. *Journal of Ocean University China* 16(2): 279-284. DOI: 10.1007/s11802-017-3162-6

[24] Kannan P., Ganjewala D. 2009. Preliminary characterization of melanin isolated form frutis and seeds of Nyctanthes arbor-tristis. *Journal of Scientific Research* 1(3): 655-661.

[25] Łopusiewicz Ł. 2018. Antioxidant, antibacterial properties and the light barrier assessment of of raw and purified melanins isolated from Citrullus lanatus (watermelon) seeds. *Herba Polonica* 64(2).

[26] Kurian N.K., Nair H.P., Bhat S.G. 2014. Melanin producing Pseudomonas stutzeri BTCZ10 from marine sediment at 96 m depth (Sagar Sampada cruise #305). *International Journal of Current Biotechnology* 2(5): 6-11.

[27] Łopusiewicz Ł. 2018. Isolation, characterisation and biological activity of melanin from Exidia nigricans. *World Scientific News* 91, 111–129.

[28] Łopusiewicz Ł. 2018. Scleroderma citrinum melanin: isolation, purification, spectroscopic studies with characterization of antioxidant, antibacterial and light barrier properties. *World Scientific News* 94(2), 114-129.

[29] Solano F. Melanins: skin pigments and much more – types, structural models, biological functions, and formation routes. *New Journal of Science* 2014.

[30] Łopusiewicz Ł., Lisiecki S. 2016. „Czarne złoto" – melaniny w życiu człowieka [In Polish]. *Kosmos* 65(4): 621-629.

[31] Łopusiewicz Ł., Jędra F., Mizielińska M. 2018. New Poly(lactic acid) Active Packaging Composite Films Incorporated with Fungal Melanin. *Polymers* 10(4): 386. DOI: 10.3390/polym10040386

[32] Łopusiewicz Ł., Lisiecki S., Mizielińska M. 2017. Aktywność przeciwutleniające folii PE i PLA modyfikowanych powłokami zawierającymi melaniny grzybowe [In Polish]. *Opakowanie* 3: 81-85.

[33] Łopusiewicz Ł., Lisiecki S., Mizielińska M. 2017. Właściwości przeciwutleniające i optyczne folii BOPP i PET modyfikowanych powłokami zawierającymi melaniny grzybowe [In Polish]. *Opakowanie* 7: 48-55.

[34] Selvakumar P., Rajasekar S., Periasamy K., Raaman N. 2008. Isolation and characterization of melanin pigment from Pleurotus cystidiosus (telomorph of Antromycopsis macrocarpa). *World Journal of Microbiology and Biotechnology* 24: 2125-2131. DOI: 10.1007/s11274-008-9718-2

[35] Zhan F., He Y., Zu Y., Li T., Zhao Z. 2011. Characterisation of melanin isolated form a dark septate endpophyte (DSE), Exophiala pisciphila. *World Journal of Microbiology and Biotechnology* 27: 2483-2489. DOI: 10.1007/s11274-011-0712-8

[36] Różanowska M., Sarna T., Land E.J., Truscott TG. 1999. Free radical scavenging properties of melanin: interaction of eu- and pheo-melanin models with reducing and oxidizing radicals. *Free Radical Biology and Medicine* 26: 518-525. DOI: 10.1016/S0891-5849(98)00234-2

[37] Cuevas-Juárez E., Yuriar-Arredondo K.Y., Pío-León J.F., Montes-Avila J., López-Angulo G., Díaz-Camacho S.P., Delgado-Vargas F. 2014. Antioxidant and α-glucosidase inhibitory properties of soluble melanins from the fruits of Vitex mollis Kunth, Randia echinocarpa Sessé et Mociño and Crescentia alata Kunth. *Journal of Functional Foods* 9: 78-88. DOI: 10.1016/j.jff.2014.04.016

[38] Hung Y-C., Sava V., Makan S., Chen T-HJ., Hong M-Y., Huang GS. 2002. Antioxidant activity of melanins derived from tea: comparison between different oxidative states. *Food Chemistry* 78: 233-240. DOI: 10.1016/S0308-8146(01)00403-4

[39] Helan Soundra Rani M., Ramesh T., Subramanian J., Kalaiselvam M. 2013. Production and Characterization of Melanin Pigment from Halophilic Black Yeast Hortaea werneckii. *International Journal of Pharma Research & Review* 2(8): 9-17.

[40] Laxmi M., Kurian N.K., Smitha S., Bhat S.G. 2016. Melanin and bacteriocin from marine bacteria inhibit biofilms of foodborne pathogens. *Indian Journal of Biotechnology* 15(3): 392-399.

[41] Xu C., Li J., Yang L., Shi F., Yang L., Ye M. 2017. Antibacterial activity and a membrane damage mechanism of Lachnum YM30 melanin against Vibrio parahaemolyticus and Staphylococcus aureus. *Food Control* 73: 1445-1451. DOI: 10.1016/j.foodcont.2016.10.048

[42] Correa N., Covarrubias C., Rodas P.I., Hermosilla G., Olate V.R., Valdés C., Meyer W., Magne F., Tapia C.V. 2017. Differential Antifungal Activity of Human and Cryptococcal Melanins with Structural Discrepancies. *Frontiers in Microbiology* 8: 1-13. DOI: 10.3389/fmicb.2017.01292

[43] Friedman M. 2016. Mushroom Polysaccharides: Chemistry and Antiobesity, Antidiabetes, Anticancer, and Antibiotic Properties in Cells, Rodents, and Humans. *Foods* 5(4): 80. DOI: 10.3390/foods5040080

[44] Bin L., Wei L., Xiaohong C., Mei J., Mingsheng D. 2012. In vitro antibiofilm activity of the melanin from Auricularia auricula, an edible jelly mushroom. *Annals of Microbiology* 62(4): 1523-1530. DOI: 10.1007/s13213-011-0406-3

[45] Zhu H., He C-C., Chu Q-H. 2011. Inhibition of quorum sensing in Chromobacterium violaceum by pigments extracted from Auricularia auricular. *Letters in Apllied Microbiology* 52: 269-274. DOI: 10.1111/j.1472-765X.2010.02993.x

[46] Valeru S.P., Rompikuntal P.K., Ishikawa T., Vaitkevicius K., Sjöling A., Dolganov N., Zhu J., Schoolnik G., Wai S.N. 2009. Role of melanin pigment in expression of Vibrio cholerae virulence factors. *Infection and Immunity* 77: 935–942. DOI: 10.1128/IAI.00929-08

[47] Worrall J.J., Chet I., Hüttermann A. 1986. Association of Rhizomorph Formation With Laccase Activity in Armillaria spp. *Journal of General Microbiology* 132: 2527-2533. DOI: 10.1099/00221287-132-9-2527

[48] Kwaśna H., Kotyńska U., Łakomy P., Mallet K. 2001. Stimulation of Armillaria rhizomorph growth by oak root fungi. *Acta Mycologica* 36(2): 257-272.

[49] Tudor D., Robinson S.C., Cooper P.A. 2012. The influence of moisture content variation on fungal pigment formation in spalted wood. *AMB Express* 2: 69. DOI: 10.1186/2191-0855-2-69

[50] Toledo A.V., Franco M.E.E., Lopez S.M.Y., Troncozo M.I., Saparrat M.C.N., Balatti P.A. 2017. Melanins in fungi: Types, localization and putative biological roles. *Physiological and Molecular Plant Pathology* 99: 2-6. DOI: 10.1016/j.pmpp.2017.04.004

[51] Fogarty R.V., Tobin J.N. 1996. Fungal melanins and their interactions with metals. *Enzyme and Microbial Technology* 19(4): 311-317. DOI: 10.1016/0141-0229(96)00002-6

[52] Eisenman H.C., Casadevall A. 2012. Synthesis and assembly of fungal melanin. *Applied Microbiology and Biotechnology* 93(3): 931-940. DOI: 10.1007/s00253-011-3777-2

[53] Rizzo D.M., Blanchette R.A., Palmer M.A. 1992. Biosorption of metal compounds by Armillaria rhizomorphs. *Canadian Journal of Botany* 70(8): 1515-1520. DOI: 10.1139/b92-190

[54] Pombeiro-Sponchiado S.R, Sousa G.S., Andrade J.C.R., Lisboa H.F., Gonçalves R.C.R. 2017. Production of Melanin Pigment by Fungi and its Biotechnological Applications. DOI: 10.5772/67375

[55] Onofri S. 1999. Antarctic Microfungi. In: Seckbach J. (eds) Enigmatic Microorganisms and Life in Extreme Environments. Cellular Origin and Life in Extreme Habitats, Vol. 1. Springer, Dordrecht. DOI: 10.1007/978-94-011-4838-2_26

[56] Łukasz Łopusiewicz, Małgorzata Mizielińska, Antifungal activity of PLA foils covered with ethylocelulose containing essential oils. *World News of Natural Sciences* 12 (2017) 27-32

[57] Michał Jarosz, Patrycja Sumińska, Urszula Kowalska, Małgorzata Mizielińska, Antibacterial activity of covered paper after storage. *World News of Natural Sciences* 17 (2018) 141-146

P4

Łukasz Łopusiewicz, Sławomir Lisiecki, Małgorzata Mizielińska:
Antioxidant activity of polyethylene and polylactide foils modified by coating containing fungal melanins
The aim of the study was evaluation of antioxidant properties of PE and PLA foils modified by coatings contaning fungal melanins. Melanins used in the study were obtained from waste from production of button mushroom *(Agaricus bisporus)*, rhizomorphs of honey fungus *(Armillaria mellea)* and fruiting bodies of common earthball *(Scleroderma citrinum)*. Chitosan and starch A4b were choosen as a coatings. Antioxidant activity of films was evaluated by DPPH and poliphenolic content by Folin-Ciocalteau's reagent. The findings of the study point out that fungal melanins may be used in modification of foils to goal their antioxidant properties.

Aktywność przeciwutleniająca folii PE i PLA modyfikowanych powłokami zawierającymi melaniny grzybowe

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Wstęp

Innowacyjnym rozwiązaniem dotyczącym ochrony produk-ów spożywczych przed psuciem się jest stosowanie opakowań ktywnych bądź odpowiednich systemów pakowania żywno-ci. Jednym z rozwiązań decydujących o tym, czy opakowanie ostanie zakwalifikowane do opakowań aktywnych, jest pokry-vanie materiałów opakowaniowych powłokami zawierającymi ubstancje aktywne, m.in. antymikrobiologiczne bądź przeciw-itleniające.

Przeciwutleniacze są powszechnie dodawane do produktów pożywczych w celu opóźnienia bądź zahamowania niekorzyst-ıych zmian wynikających z procesów utleniania. Najczęściej vykorzystywanymi w przemyśle spożywczym przeciwutlenia-:zami są: kwas askorbinowy (E300), BHA (butylohydroksyani-ol, E320), BHT (butylohydroksytoluen, E321), galusan propylu E310) oraz tokoferole (E306-E309) [1].

Jednym z kierunków rozwoju opakowań aktywnych jest mo-lyfikacja ich powierzchni za pomocą nośników zawierających :ubstancje o właściwościach przeciwutleniających bądź bezpo-średnie włączanie takich substancji w strukturę materiału opa-kowaniowego, np. folii. Duże nadzieje wiąże się z substancjami pochodzenia naturalnego. W piśmiennictwie obecne są donie-sienia o modyfikacji powierzchni/struktury materiałów opako-waniowych w kierunku nadania właściwości przeciwutleniają-cych przez zastosowanie m.in. β-karotenu, tokoferolu, olejków eterycznych, kwasu askorbinowego [1].

Melaniny to ogólna nazwa grupy wielkocząsteczkowych pig-mentów odpowiedzialnych za ciemną pigmentację organizmów, powstających w wyniku oksydacyjnej polimeryzacji związków fenolowych i indolowych. Melaniny wykazują specyficzne wła-ściwości fizykochemiczne. Wpływa na to obecność stabilnej po-pulacji organicznych wolnych rodników o-semichinonowych, a także grup utleniających lub redukujących, np. o-chinonów oraz hydrochinonów. Dzięki temu posiadają właściwości anty-utleniające, chroniące komórki przed cytotoksycznymi reaktyw-nymi formami tlenu (RFT) i wolnymi rodnikami takimi jak tlen singletowy, rodnik hydroksylowy oraz anionorodnik ponadtlen-kowy [2]. Znalazły zastosowanie w medycynie i technologii,

m.in. w tomografii optoakustycznej, w syntezie nanocząstek o właściwościach antymikrobiologicznych czy do tworzenia soczewek pochłaniających promieniowanie UV [3]. W związku z tymi właściwościami zasadna wydaje się próba ich zastosowania do modyfikacji materiałów opakowaniowych w celu nadania im właściwości przeciwutleniających.

Celem pracy była próba modyfikacji folii PE (polietylen) oraz PLA (polilaktyd) poprzez ich powlekanie nośnikami zawierającymi melaniny grzybowe w kierunku uzyskania właściwości przeciwutleniających. Założono, że do uzyskania powłok aktywnych zostaną wykorzystane: melaniny pochodzące z odpadu powstałego przy produkcji pieczarki dwuzarodnikowej *(Agaricus bisporus)*, z ryzomorf opieńki miodowej *(Armillaria mellea)* oraz owocników tęgoskóra cytrynowego *(Scleroderma citrinum)*.

Materiał i metody

Materiał badawczy stanowiły: odpad z produkcji pieczarki dwuzarodnikowej *(Agaricus bisporus)* w postaci trzonów owocników pozyskany z pieczarkarni na terenie Wolsztyna (województwo wielkopolskie), ryzomorfy opieńki miodowej *(Armilaria mellea)* oraz owocniki tęgoskóra cytrynowego *(Scleroderma citrinum)* zebrane w lesie mieszanym w okolicach Kobylanki (województwo zachodniopomorskie) w październiku 2016 roku.

Materiał do badań stanowiły także wytłoczone w CBiMO (Centrum Bioimmobilizacji i Innowacyjnych Materiałów Opakowaniowych) folie PE o grubości 80 µm oraz PLA o grubości 20 µm.

Wszystkie wykorzystane w badaniach odczynniki pochodziły z firmy Sigma-Aldrich.

Otrzymywanie melanin. W związku ze specyfiką materiału badawczego zastosowano dwie procedury pozyskania melanin. Odpad z produkcji *A. bisporus* zawiera niewiele melaniny, natomiast jest bogaty w jej prekursory oraz zawiera tyrozynazę (EC 1.14.18.1), dzięki czemu można przekształcić zawarte w odpadzie związki polifenolowe w melaninę. Ryzomorfy *A. mellea* oraz owocniki *S. citrinum* zawierają natomiast natywne melaniny.

W celu uzyskania jak największej ilości melaniny z odpadu *A. bisporus* jest on w pierwszej kolejności oczyszczany z resztek substratu do wzrostu wykorzystywanego w pieczarkarni oraz innych zanieczyszczeń. Następnie oczyszczony odpad homogenizowano mechanicznie (mielenie) w celu zwiększenia dostępności tyrozynazy do substratów polifenolowych, po czym inkubowano 24 h w temp. 37°C (optimum temperaturowe dla tyrozynazy) (IKA KS 4000i). Po 24 h do mieszaniny dodano 1 M NaOH do uzyskania pH=10 (w celu umożliwienia spontanicznej polimeryzacji w warunkach tlenowych pozostałych związków polifenolowych do melaniny) i inkubowano kolejne 24 h w temp. 65°C. Po okresie inkubacji mieszaninę filtrowano. Oddzielony supernatant doprowadzono do pH 2 za pomocą 1 M HCl (w celu wytrącenia melaniny). Wytrąconą melaninę odwirowywano (6000 rpm, 10 min, MPW-352R, MPW MED. INSTRUMENTS), po czym inkubowano 1 h w 6 M HCl, w cieplarce (Binder, 90°C) w celu oczyszczenia z komponentów białkowych i węglowodanowych. Osad melaniny odwirowano i oczyszczano za pomocą octanu etylu, etanolu oraz chloroformu w celu usunięcia lipidów. Oczyszczoną melaninę odwirowano i w celu usunięcia resztek rozpuszczalników umieszczono w szklanym krystalizatorze w cieplarce w temperaturze 60°C aż do całkowitego odparowania.

Ryzomorfy *A. mellea* oczyszczano i homogenizowano. Owocniki *S. citrinum* oczyszczano i pozbawiano perydrium (osłonki otaczającej owocnik). Zawartą we wnętrzu owocnika warstwę hymenialną (gleba) homogenizowano. Następne etapy postępowania były wspólne dla ryzomorf *A. mellea* i gleby *S. citrinum*. 50 g materiału umieszczano w 200 ml 1 M NaOH, po czym ekstrahowano melaniny w wytrząsarce (200 rpm, 50°C, 24 h). Po tym czasie mieszaninę filtrowano. Supernatant zakwaszano do pH=2 za pomocą 1 M HCl. Kolejne etapy postępowania były identyczne jak w przypadku oczyszczania melaniny z odpadu *A. bisporus*.

Przygotowanie układów powłokotwórczych oraz nanoszenie powłok. Przygotowano 18 układów powłokotwórczych zawierających melaniny oraz 6 układów kontrolnych. Jako nośniki wybrano: chitozan (Zhejiang Golden-Shell Pharmaceutical CO. Ltd., Chiny) oraz skrobię hydroksypropylowaną A4b (Dow, USA).

Melaniny otrzymane z odpadu *A. bisporus*, ryzomorf *A. mellea* oraz owocników *S. citrinum* oznaczono odpowiednio: „Ab", „R", „Sc". Każdą melaninę rozpuszczono w DMSO (dimetylosulfotlenku) do uzyskania stężenia 5 mg/ml.

W celu wytworzenia powłoki na bazie chitozanu (CH) do 1% roztworu kwasu mlekowego w wodzie dodano chitozan do uzyskania stężenia 2%. Całość mieszano za pomocą mieszadła magnetycznego (Ika) z prędkością 600 rpm przez 24 h.

W celu uzyskania powłoki na bazie skrobi (SK) przeprowadzono proces kleikowania. 8% wodny roztwór skrobi A4b umieszczono w łaźni wodnej (Julabo) i podgrzewano do 90°C jednocześnie mieszając za pomocą mieszadła mechanicznego (400 rpm, IkaT18 Ultra Turrax). Roztwór inkubowano w łaźni przez 30 min w celu dokładnego skleikowania i uzyskania jednorodnego roztworu.

Układy powłokotwórcze przygotowano w ten sposób, że do 10 ml roztworów nośników dodawano odpowiednio 200 µl, 500 µl, 1000 µl roztworów melanin w DMSO, tak aby uzyskać stężenia: (1) 100 µg/ml, (2) 250 µg/ml, (3) 500 µg/ml. Po dodaniu melaniny roztwory dokładnie worteksowano. Przygotowano również układy kontrolne (K), do których dodano DMSO bez melanin, odpowiednio 200 µl, 500 µl, 1000 µl. Przygotowane układy powłokotwórcze ilustruje tab. 1.

Folie przed procesem powlekania poddano aktywacji koronowej. Folie powlekano za pomocą powlekarki (Unicoater 409, Erichsen, Niemcy) z wykorzystaniem wałka o średnicy drutu

0,76 mm i grubości warstwy osadzanej 60 µm. Następnie arkusze folii były suszone w cieplarce (Binder) przez 20 minut w temperaturze 45°C.

Oznaczanie właściwości przeciwutleniających folii. Do oznaczenia właściwości przeciwutleniających folii posłużono się metodą z użyciem syntetycznego rodnika DPPH (1,1-difenylo-2-pikrylohydrazyl) [4,5,6]. 3 ml roztworu powlekającego (RP) mieszano z 1 ml 1mM roztworu rodnika DPPH w metanolu. Następnie mieszaninę worteksowano i inkubowano w ciemności, w temperaturze 25°C przez 30 minut. Po okresie inkubacji absorbancję roztworu oznaczano spektrofotometrycznie (Evolution 220, Thermo Scientific) przy długości fali 517 nm. Każdego pomiaru dokonano w trzech powtórzeniach, wyniki uśredniono. Procentową wartość właściwości przeciwutleniających roztworów powlekających obliczano z wzoru:

$$\%inhibicji\ DPPH = \frac{AbsDPPH - AbsRP}{AbsDPPH} \times 100\%$$

gdzie: AbsDPPH – wartość absorbancji 1mM roztworu DPPH w metanolu przy długości fali 517 nm, AbsRP – wartość absorbancji roztworu powlekającego przy długości fali 517 nm

Oznaczenie zawartości polifenoli w powłokach. W celu oznaczenia zawartości polifenoli w powłokach z każdej folii wycięto 10 kwadratów o powierzchni 1 cm^2. Kwadraty umieszczono w probówkach typu Falcon, do których dodano 2,4 ml wody destylowanej oraz 0,15 ml odczynnika Folina-Ciocalteau. Mieszaninę inkubowano 8 minut w temperaturze 25°C, po czym dodawano 0,45 ml nasyconego roztworu Na_2CO_3. Całość inkubowano w temp. 40°C przez 30 minut w ciemności. Po tym czasie mierzono spektrofotometrycznie (Evolution 220, Thermo Scientific) wartość absorbancji roztworu przy długości fali 765 nm [4]. Pomiaru dokonano 3 razy, a wyniki uśredniono. Przygotowano roztwór kalibracyjny kwasu galusowego w metanolu. Zawartość polifenoli w powłokach wyrażono w przeliczeniu na µg kwasu galusowego zawarte w 1 cm^2 folii, korzystając z równania krzywej kalibracyjnej ($y = 1,1672x + 0,0053$, $R^2 = 0,9985$).

Tabela 1. Wykorzystane w badaniach układy powłokotwórcze oraz stężenie melanin grzybowych [µg/ml]

SYMBOL UKŁADU POWLEKAJĄCEGO	STĘŻENIE MELANINY [µg/ml]
CHAb1	100
CHAb2	250
CHAb3	500
CHR1	100
CHR2	250
CHR3	500
CHSc1	100
CHSc2	250
CHSc3	500
SKAb1	100
SKAb2	250
SKAb3	500
SKR1	100
SKR2	250
SKR3	500
SKSc1	100
SKSc2	250
SKSc3	500
KCH1	0
KCH2	0
KCH3	0
KSK1	0
KSK2	0
KSK3	0

Wyniki i dyskusja

Na podstawie przeprowadzonych doświadczeń stwierdzono, że dodatek melanin grzybowych do powłok nanoszonych na powierzchnię folii PE oraz PLA pozwala nadać im właściwości przeciwutleniające.

Folie PE przed powlekaniem poddano procesowi aktywacji koronowej, procesowi technologicznemu, w wyniku którego otrzymuje się powierzchnie zwilżalne farbami drukarskimi, klejami oraz mieszankami powlekającymi. Powierzchnię folii PE charakteryzuje stosunkowo niska energia powierzchniowa i dopiero aktywacja umożliwia drukowanie, klejenie czy powlekanie [5].

W piśmiennictwie można znaleźć wiele przykładów modyfikacji folii PE i PLA. Trzcińska i wsp. [5] modyfikowali folie polietylenowe powłoką z chitozanu, w której immobilizowano galusan propylu, dzięki czemu uzyskano powłokę o właściwościach przeciwutleniających. Kwiatkowski i wsp. [7] modyfikowali powierzchnię folii PLA powłokami z etylocelulozy zawierającymi olejki eteryczne z fenkułu, rozmarynu i kminku, natomiast Łopusiewicz i Mizielińska [8] modyfikowali powierzchnię folii PLA powłokami z etylocelulozy i antymikrobiologicznego ekstraktu z grzyba *Macrolepiota konradii*.

W celu określenia właściwości przeciwutleniających powłok posłużono się metodą z użyciem syntetycznego rodnika DPPH. DPPH jest stabilnym wolnym rodnikiem i ma niesparowany elektron na powłoce walencyjnej na jednym z atomów azotu tworzących mostek azowy. Jego alkoholowy roztwór ma ciemnofioletową barwę. W reakcji z substancją, która może być donorem atomu wodoru, DPPH tworzy żółtą formę zredukowaną; wówczas zanika ciemnofioletowe zabarwienie, a zmiany te można monitorować spektrofotometrycznie. Stopień zmiany barwy roztworu DPPH po dodaniu roztworu zawierającego substancje przeciw-

Tabela 2. Właściwości przeciwutleniające i zawartość polifenoli w modyfikowanych foliach PE i PLA

FOLIA	INHIBICJA DPPH, %	POLIFENOLE, µg/cm²	FOLIA	INHIBICJA DPPH, %	POLIFENOLE, µg/cm²
CZYSTY PE	0,00±0,00	0,00±0,00	CZYSTY PLA	0,00±0,00	0,00±0,00
PE-KCH1	6,67±0,02	1,08±0,02	PLA-KCH1	6,67±0,03	1,09±0,02
PE-KCH2	6,55±0,04	1,08±0,03	PLA-KCH2	6,57±0,04	1,08±0,04
PE-KCH3	6,59±0,03	1,01±0,02	PLA-KCH3	6,6±0,04	1,03±0,03
PE-KSK1	5,00±0,05	0,48±0,03	PLA-KSK1	5,01±0,05	0,47±0,04
PE-KSK2	5,02±0,03	0,57±0,02	PLA-KSK2	5,02±0,04	0,57±0,05
PE-KSK3	5,01±0,02	0,48±0,04	PLA-KSK3	5,00±0,04	0,48±0,01
PE-CHAb1	8,61±0,08	3,28±0,05	PLA-CHAb1	8,62±0,04	3,26±0,05
PE-CHAb2	10,59±0,04	4,68±0,03	PLA-CHAb2	10,59±0,07	4,68±0,03
PE-CHAb3	16,53±0,02	6,71±0,08	PLA-CHAb3	17,53±0,04	6,71±0,04
PE-CHR1	9,31±0,06	3,4±0,04	PLA-CHR1	9,31±0,03	3,4±0,10
PE-CHR2	10,47±0,07	4,11±0,07	PLA-CHR2	10,47±0,10	4,12±0,05
PE-CHR3	10,71±0,09	4,43±0,04	PLA-CHR3	10,81±0,04	4,43±0,04
PE-CHSc1	11,52±0,07	4,91±0,03	PLA-CHSc1	11,52±0,08	4,91±0,05
PE-CHSc2	20,83±0,02	7,68±0,07	PLA-CHSc2	20,63±0,03	7,66±0,07
PE-CHSc3	25,49±0,07	9,22±0,05	PLA-CHSc3	25,49±0,14	9,23±0,10
PE-SKAb1	13,27±0,24	5,60±0,01	PLA-SKAb1	13,27±0,09	5,60±0,04
PE-SKAb2	20,6±0,12	7,51±0,08	PLA-SKAb2	20,6±0,11	7,50±0,04
PE-SKAb3	21,88±0,08	8,20±0,04	PLA-SKAb3	21,88±0,04	8,20±0,13
PE-SKR1	12,45±0,06	5,11±0,04	PLA-SKR1	12,46±0,01	5,11±0,04
PE-SKR2	21,53±0,03	8,17±0,03	PLA-SKR2	21,43±0,05	8,17±0,08
PE-SKR3	23,28±0,04	8,62±0,05	PLA-SKR3	23,28±0,07	8,62±0,01
PE-SKSc1	14,39±0,11	6,05±0,02	PLA-SKSc1	14,39±0,60	6,04±0,02
PE-SKSc2	16,88±0,09	6,85±0,01	PLA-SKSc2	16,88±0,14	6,85±0,04
PE-SKSc3	22,93±0,14	8,54±0,07	PLA-SKSc3	22,93±0,05	8,54±0,07

utleniające jest miarą ich zdolności do zmiatania wolnych rodników [4,5,6]. Odnotowano, że wszystkie modyfikowane folie – zarówno PE, jak i PLA – wykazywały właściwości przeciwutleniające. Wśród powłok na foliach PE (tab. 2) najwyższą wartość inhibicji rodnika DPPH zaobserwowano w przypadku folii PE-CHSc3 (25,49±0,07%); była to również najwyższa wartość wśród wszystkich powłok z chitozanu na foliach PE, najmniejszą zaś wartość odnotowano dla folii PE-CHAb1 (8,61±0,08%). Wśród powłok ze skrobi A4b największą zdolnością do inhibicji rodnika DPPH charakteryzowała się folia PE-SKR3 (23,28±0,04%), najmniejszą PE-SKR1 (12,36±0,06%). Wśród powłok chitozanowych na foliach PLA (tab. 2) najwyższą wartość inhibicji rodnika DPPH zanotowano dla folii PLA-CHSc3 (25,49±0,14%), najniższą dla folii PLA-CHAb1 (8,62±0,04%). Na powłokach ze skrobi A4b najwyższą wartość zaobserwowano dla folii PLA-SKR3 (23,28±0,07%), najmniejszą zaś dla PLA-SKR1 (12,46±0,01%). Mehdizadeh i wsp. [4] dodawali do kompozytowych folii skrobiowo-chitozanowych olejek eteryczny z rośliny *Thymus kotschyanus*. Uzyskane przez nich folie charakteryzowały się nie tylko właściwościami przeciwutleniającymi, ale także antybakteryjnymi. Dodatek 0,50% olejku eterycznego do roztworu filmotwórczego pozwalał uzyskać inhibicję rodnika DPPH na poziomie ok. 17%, natomiast 2% dodatek olejku powodował ok. 40% redukcję rodnika DPPH. Mikusanti i Masril [6] dodawali olejek imbirowy do folii ze skrobi uzyskanej z pochrzynu skrzydlatego *(Dioscorea alata)*. Dodatek 0,5% olejku do folii powodował 5,68% redukcję rodnika DPPH, natomiast dodatek 3% o 31,5%. Należy zwrócić uwagę, że udział melanin w najwyższym stężeniu wykorzystanym w badaniach własnych wynosi 0,05% roztworów powlekających, przy którym uzyskano inhibicję rodnika DPPH na poziomie 25,49%.

Powłoki chitozanowe i skrobiowe, do których nie dodano melanin, również charakteryzowały się właściwościami przeciwutleniającymi, jednakże znacznie mniejszymi w porównaniu z powłokami zawierającymi melaniny. Wynika to z faktu, że wolne rodniki DPPH mogą reagować z grupami aminowymi (NH_2) chitozanu, tworząc formę zredukowaną, grupy aminowe zaś, przyjmując atom wodoru, mogą tworzyć jony amonowe (NH_3^+). Podobnie w przypadku skrobi: wolne rodniki DPPH mogą reago-

wać z grupami hydroksylowymi (-OH), które w wyniku przyłączenia atomu wodoru tworzą grupę (OH^{2+}) [4,6].

W celu oznaczenia zawartości polifenoli w powłokach posłużono się metodą Folina-Ciocalteau. Podstawą oznaczania jest odwracalna reakcja redukcji przez fenole w środowisku alkalicznym molibdenu (VI) do molibdenu (V) zawartego w odczynniku Folina-Ciocalteau. W wyniku reakcji powstaje niebieski związek, który wykazuje maksimum absorpcji przy długości fali 765 nm. Intensywność absorpcji przy tej długości fali jest proporcjonalna do stężenia związków fenolowych w badanej próbie. Melaniny zawierają w swojej budowie grupy fenolowe [3]. W badaniach własnych za pomocą metody Folina-Ciocalteau oznaczono zawartość polifenoli przypadającą na 1 cm^2 powłok na foliach PE i PLA. Zaobserwowano, że w przypadku powłok chitozanowych i skrobiowych wraz ze wzrastającym stężeniem melanin w roztworze powłokotwórczym wzrastała zawartość polifenoli na 1 cm^2 powłok. Najwyższą zawartość polifenoli wśród folii PE (tab. 2) odnotowano w przypadku folii PE-CHSc3 (9,22±0,05 $\mu g/cm^2$), dla folii tej uzyskano również najwyższy stopień inhibicji rodnika DPPH. Podobnie najwyższą zawartość polifenoli (9,23±0,10 $\mu g/cm^2$) wśród folii PLA (tab. 2) odnotowano w przypadku folii PLA-CHSc3. Folie pokryte jedynie nośnikami bez melanin również dały dodatni wynik na polifenole w powłoce. Wynika to najprawdopodobniej z faktu, że zarówno chitozan, jak i skrobia zawierają w cząsteczkach grupy -OH, które mogą powodować interferencje w uzyskanym wyniku [4,6].

W celu nadania właściwości przeciwutleniających powłokom nanoszonym na folie wykorzystano melaniny pozyskane z grzybów: odpadu powstającego przy produkcji *A. bisporus*, ryzomorf *A. mellea* oraz owocników *S. citrinum*. Melaniny pełnią istotną funkcję w fizjologii grzybów. Przyjmuje się, że – jako metabolity wtórne – nie są one niezbędne dla wzrostu i rozwoju komórek grzybowych, ale stanowią ich swoisty „system obronny". Wpływ obecności melanin na zwiększenie zdolności przeżywania grzybów w niekorzystnych dla nich warunkach wynika głównie z pełnionej przez nie funkcji zewnątrzkomórkowego układu buforowego, neutralizującego czynniki utleniające [9]. Chronią komórki grzybów przed niekorzystnym działaniem promieniowania UV, promieniowaniem jonizującym oraz wolnymi rodnikami, mogącymi uszkadzać komórki i zawarty w nich materiał genetyczny. Odgrywają również istotną rolę w patogenezie niektórych gatunków grzybów patogenicznych dla roślin i zwierząt, wpływając m.in. immunomodulująco na organizm gospodarza lub wzmacniając struktury uczestniczące w tkanki roślinne (appresoria). Mogą być syntetyzowane w różnych szlakach metabolicznych: szlaku DHN (1,8-dihydroksynaftalen) lub szlaku L-DOPA (3,4-dihydroksyfenyloalanina). Melaniny powstałe w szlaku DHN zawierają tylko węgiel oraz tlen, natomiast melaniny powstałe w szlaku L-DOPA zwierają również azot. W strukturze melanin mogą być także obecne komponenty białkowe, węglowodanowe oraz jony; stąd wynikają ich różnorodność i niekiedy odmienne właściwości [10]. Melaniny obecne są w komórkach grzybów w postaci granul (np. u *Aureobasidium pullulans* [11]), mogą być wbudowane w ścianę komórkową (w jej zewnętrzną lub wewnętrzną stronę, np. u *Cryptococcus neoformans*, zarodnikach np. *Aspergillus*, *A. bisporus* [9,12]) lub zewnętrzne warstwy struktur ponadkomórkowych (np. w ryzomorfach *A. mellea*) [13].

Podsumowując, należy zauważyć, że melaniny grzybowe mogą być wykorzystane w celu modyfikacji folii z polimerów syntetycznych (PE) i naturalnych (PLA) w kierunku nadania im właściwości przeciwutleniających. Właściwości te zależą nie tylko od źródła pochodzenia melanin, ale również od nośników wykorzystanych do tworzenia powłok. Wzrastające stężenie melanin w roztworach powłokotwórczych powoduje wzrost właściwości przeciwutleniających. W przypadku zarówno powłok z chitozanu, jak i ze skrobi zaobserwowano zależność pomiędzy wzrastającą zawartością polifenoli a wzrastającymi właściwościami przeciwutleniającymi.

Wnioski

Melaniny grzybowe mogą znaleźć zastosowanie w modyfikacji folii w kierunku nadania im właściwości przeciwutleniających. ■

Literatura:

[1] Brody A., Strupinsky E., Kline L. 2001. Active Packaging for Food Applications
[2] Różanowska M., Sarna T., Land E., Truscott G., 1999. Free radical scavening properties of melanin interaction of eu- and pheo-melanin models with reducing and oxidising radicals. Free Radical Biology & Medicine 26, 518-525
[3] Łopusiewicz Ł., Lisiecki S., 2016. "Czarne złoto"- melaniny w życiu człowieka. Kosmos Problemy Nauk Biologicznych 65, 4(313), 621-629
[4] Mehdizadeh T., Tajik H., Rohani S.M.R., Oromiehie A.R., 2012. Antibacterial, antioxidant and optical properties of edible starch-chitosan composite film containing Thymus kotschyanus essential oil. Veterinary Research Forum 3(3), 167-173
[5] Trzcińska M., Sieliwanowicz B., Hałasińska A., Czupryński B., 2007. Application of physiochemically modified polymeric foil to develop antioxidant packaging. Polish Journal of Food and Nutrition Sciences 57(2), 173-176
[6] Mikusanti H., Masnil K.I. 2013. Antibacterial and Antioxidant of Uwi (Dioscorea alata L) Starch Edible Film Incorporated with Ginger Essential Oil. International Journal of Bioscience, Biochemistry and Bioinformatics 3(4), 354-356
[7] Kwiatkowski P., Giedrys-Kalemba S., Mizielińska M., Bartkowiak A. 2016. Modification of PLA foil surface by ethylcellulose and essential oils. Journal of Microbiology, Biotechnology and Food Sciences 5(5), 440-444
[8] Łopusiewicz Ł., Mizielińska M. 2016. Aktywność antymikrobiologiczna folii PLA powlekanych ekstraktami z Macrolepiota konradii. Opakowanie 4, 57-60
[9] Goncalvez R., Pombeiro-Sponchiado S.R., 2005. Antioxidant activity of the melanin pigment extracted from Aspergillus nidulans. Biological and Pharmaceutical Bulletin 28, 1129-1131
[10] Eisenman H., Casadevall A. 2011. Synthesis and assembly of fungal melanin. Appl Microbiol Biotechnol 93(3), 931-40
[11] Gniewosz M., Duszkiewicz-Reinhard 2008. Comparative studies on pullulan synthesis, melanin synthesis and morphology of white mutant Aureobasidium pullulans B-1 and parent strain A.p.-3. Carbohydrate Polymers 72, 431-438
[12] Wang Y., Casadevall A., 1994. Decreased susceptibility of melanized Cryptococcus neoformans to UV light. Applied Environmental Microbiology 60, 3865-3866
[13] Rigling D., Grünthardt-Goerg M., Blaustein H., Frey B. 2006. Accumulation of heavy metals into Armillaria rhizomorphs from contaminated soils. For. Snow Landsc. Res. 80 (2), 213-220

P5

Antioxidant and optical properties of BOPP and PET foils modified by coatings containing fungal melanins
BOPP and PET foils were coated with coatings containg fungal melanins. Antioxidant properties, polyphenolic content, UV-Vis barier properties and changes of colour were analyzed. The result suggested that incorporation of fungal melanins into foil coatings may improve their antioxidant properties, increasing of polyphenolic content and changes in colour. No improvement of UV-Vis barier properties was observed.

Właściwości przeciwutleniające i optyczne folii BOPP i PET modyfikowanych powłokami zawierającymi melaniny grzybowe

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Innowacyjnym rozwiązaniem dotyczącym ochrony produktów spożywczych przed psuciem się jest stosowanie opakowań aktywnych bądź odpowiednich systemów pakowania żywności. Jednym z rozwiązań decydujących o tym, czy opakowanie zostanie zakwalifikowane do opakowań aktywnych, jest pokrywanie materiałów opakowaniowych powłokami zawierającymi substancje aktywne, m.in. antymikrobiologiczne bądź przeciwutleniające. Przeciwutleniacze są powszechnie dodawane do produktów spożywczych w celu opóźnienia bądź zahamowania niekorzystnych zmian wynikających z procesów utleniania. Najczęściej wykorzystywanymi w przemyśle spożywczym przeciwutleniaczami są: kwas askorbinowy (E300), BHA (butylohydroksyanizol, E320), BHT (butylohydroksytoluen, E321), galusan propylu (E310) oraz tokoferole (E306-E309) [1].

Jednym z kierunków rozwoju opakowań aktywnych jest modyfikacja ich powierzchni za pomocą nośników zawierających substancje o właściwościach przeciwutleniających oraz barierowych względem promieniowania UV, lub też bezpośrednie włączanie takich substancji w strukturę materiału opakowaniowego, np. folii. Substancje promieniochronne ze względu na różny mechanizm działania można podzielić na filtry chemiczne (które rozpraszają padające na nie światło, np. TiO_2, ZnO) oraz filtry chemiczne (które absorbują promieniowanie o określonej długości fali) [2]. Substancje barierowe względem promieniowania UV mogą chronić nie tylko produkt umieszczony w opakowaniu, ale również materiał opakowaniowy. Ważną cechą materiałów polimerowych jest ich odporność na działanie promieniowania UV, naturalnie występującego w środowisku i wpływającego na właściwości fizyczne oraz mechaniczne materiałów polimerowych, a także na trwałość ich wybarwienia. Materiały polimerowe poddane działaniu czynników środowiska naturalnego ulegają przede wszystkim fotodegradacji, biodegradacji oraz degradacji hydrolitycznej. Zwłaszcza istotna jest podatność na fotodegradację, gdyż wyroby z tworzyw polimerowych znajdujące się w powszechnym użyciu są narażone zwykle na działanie światła słonecznego [3]. Dlatego poszukuje się skutecznych rozwiązań mających na celu przeciwdziałanie tym problemom. Duże nadzieje wiąże się z substancjami pochodzenia naturalnego. W piśmiennictwie obecne są doniesienia o modyfikacji powierzchni/struktury materiałów opakowaniowych w kierunku nadania właściwości przeciwutleniających i barierowych w stosunku do promieniowania UV przez zastosowanie m.in. β-karote-

nu, tokoferolu, olejków eterycznych, kwasu askorbinowego, pochodnych kwasu cynamonowego oraz salicylowego, wyciągów roślinnych [1,4,5,6,7,8].

Melaniny to ogólna nazwa grupy wielkocząsteczkowych pigmentów odpowiedzialnych za ciemną pigmentację organizmów, powstających w wyniku oksydacyjnej polimeryzacji związków fenolowych i indolowych. Melaniny wykazują specyficzne właściwości fizykochemiczne. Wpływa na to obecność stabilnej populacji organicznych wolnych rodników o-semichinonowych, a także grup utleniających lub redukujących, np. o-chinonów oraz hydrochinonów. Dzięki temu posiadają właściwości antyutleniające, chroniące komórki przed cytotoksycznymi reaktywnymi formami tlenu (RFT) i wolnymi rodnikami takimi jak: tlen singletowy, rodnik hydroksylowy oraz anionorodnik ponadtlenkowy [9]. Melaniny są jednym z naturalnych środków ochronnych przed promieniowaniem UV (UVB i UVA). Znalazły zastosowanie w medycynie i technologii, m.in. w tomografii optoakustycznej, w syntezie nanocząstek o właściwościach antymikrobiologicznych czy do tworzenia soczewek pochłaniających promieniowanie UV [10]. W związku z tymi właściwościami zasadna wydaje się próba ich zastosowania do modyfikacji materiałów opakowaniowych w celu nadania im właściwości przeciwutleniających oraz ochronnych względem promieniowania UV.

Celem pracy była próba modyfikacji folii BOPP (polipropylenowa folia dwuosiowo orientowana) oraz PET (poli(tereftalan etylenu)) poprzez ich powlekanie nośnikami zawierającymi melaniny grzybowe w kierunku uzyskania właściwości przeciwutleniających oraz ochronnych względem promieniowania UV, a także określenie wypływu powłok oraz dodatku melanin na właściwości optyczne i barwę folii. Założono, że do uzyskania powłok aktywnych zostaną wykorzystane: melaniny pochodzące z owocników pieczarki dwuzarodnikowej *(Agaricus bisporus)*, z ryzomorf opieńki miodowej *(Armillaria mellea)* oraz owocników tęgoskóra cytrynowego *(Scleroderma citrinum)*.

Materiał i metody

Materiał badawczy stanowiły: owocniki pieczarki dwuzarodnikowej *(Agaricus bisporus)* zakupione w sklepie Tesco w Szczecinie, ryzomorfy opieńki miodowej *(Armilaria mellea)* oraz owocniki tęgoskóra cytrynowego *(Scleroderma citrinum)* zebrane w lesie mieszanym w okolicach Kobylanki, województwo zachodniopomorskie, w październiku 2016 roku. Materiał do badań stanowiły także wytłoczone w CBiMO (Centrum Bioimmobilizacji i Innowacyjnych Materiałów Opakowaniowych) folie BOPP o grubości 30 μm oraz PLA o grubości 20 μm. Wszystkie wykorzystane w badaniach odczynniki pochodziły z firmy Sigma-Aldrich.

Otrzymywanie melanin. W związku ze specyfiką materiału badawczego zastosowano dwie procedury pozyskania melanin. Owocniki *A. bisporus* zawierają niewiele melaniny, natomiast są bogate w jej prekursory oraz zawierają tyrozynazę (EC 1.14.18.1), dzięki czemu można przekształcić zawarte w owocnikach związki polifenolowe w melaninę. Ryzomorfy *A. mellea* oraz owocniki *S. citrinum* zawierają natomiast natywne melaniny.

W celu uzyskania jak największej ilości melaniny z owocników *A. bisporus* są one w pierwszej kolejności oczyszczane z resztek substratu do wzrostu wykorzystywanego w pieczarkarni oraz innych zanieczyszczeń. Następnie oczyszczony materiał homogenizowano mechanicznie (mielenie) w celu zwiększenia dostępności tyrozynazy do substratów polifenolowych, po czym inkubowano 24 h w temp. 37°C (optimum temperaturowe dla tyrozynazy) (IKA KS 4000i). Po 24 h do mieszaniny dodano 1 M NaOH do uzyskania pH=10 (w celu umożliwienia spontanicznej polimeryzacji w warunkach tlenowych pozostałych związków polifenolowych do melaniny) i inkubowano kolejne 24 h w temp. 65°C. Po okresie inkubacji mieszaninę filtrowano. Oddzielony supernatant doprowadzono do pH 2 za pomocą 1 M HCl (w celu wytrącenia melaniny) [11]. Wytrąconą melaninę odwirowywano (6000 rpm, 10 min, MPW-352R, MPW MED. INSTRUMENTS), po czym inkubowano 1 h w 6 M HCl, w cieplarce (Binder, 90°C) w celu oczyszczenia z komponentów białkowych i węglowodanowych. Osad melaniny odwirowano i oczyszczano za pomocą octanu etylu, etanolu oraz chloroformu w celu usunięcia lipidów. Oczyszczoną melaninę odwirowano i w celu usunięcia resztek rozpuszczalników umieszczono w szklanym krystalizatorze w cieplarce w temperaturze 60°C aż do całkowitego odparowania.

Ryzomorfy *A. mellea* oczyszczano i homogenizowano. Owocniki *S. citrinum* oczyszczano i pozbawiano *perydrium* (osłonki otaczającej owocnik). Zawartą we wnętrzu owocnika warstwę hymenialną (gleba) homogenizowano. Następne etapy postępowania były wspólne dla ryzomorf *A. mellea* i gleby *S. citrinum*. 50 g materiału umieszczano w 200 ml 1 M NaOH, po czym ekstrahowano melaniny w wytrząsarce (200 rpm, 50°C, 24 h). Po tym czasie mieszaninę filtrowano. Supernatant zakwaszano do pH=2 za pomocą 1 M HCl. Kolejne etapy postępowania były identyczne jak w przypadku oczyszczania melaniny z *A. bisporus*.

Przygotowanie układów powłokotwórczych oraz nanoszenie powłok. Przygotowano 18 układów powłokotwórczych zawierających melaniny oraz 6 układów kontrolnych. Jako nośniki wybrano: chitozan (Zhejiang Golden-Shell Pharmaceutical CO. Ltd., Chiny) oraz skrobię hydroksypropylowaną A4b (Dow, USA).

Melaniny otrzymane z odpadu *A. bisporus*, ryzomorf *A. mellea* oraz owocników *S. citrinum* oznaczono odpowiednio: „Ab", „R", „Sc". Każdą melaninę rozpuszczono w DMSO (dimetylosulfotlenku) do uzyskania stężenia 5 mg/ml.

W celu wytworzenia powłoki na bazie chitozanu (CH) do 1% roztworu kwasu mlekowego w wodzie dodano chitozan do uzyskania stężenia 2%. Całość mieszano za pomocą mieszadła magnetycznego (Ika) z prędkością 600 rpm przez 24 h.

W celu uzyskania powłoki na bazie skrobi (SK) przeprowadzono proces kleikowania. 8% wodny roztwór skrobi A4b umieszczono w łaźni wodnej (Julabo) i podgrzewano do 90°C jednocześnie mieszając za pomocą mieszadła mechanicznego (400 rpm, IkaT18 Ultra Turrax). Roztwór inkubowano w łaźni przez 30 min w celu dokładnego skleikowania i uzyskania jednorodnego roztworu.

Układy powłokotwórcze przygotowano w ten sposób, że do 10 ml roztworów nośników dodawano odpowiednio 200 µl, 500 µl, 1000 µl roztworów melanin w DMSO, tak aby uzyskać stężenia: (1) 100 µg/ml, (2) 250 µg/ml, (3) 500 µg/ml. Po dodaniu melaniny roztwory dokładnie worteksowano. Przygotowano również układy kontrolne (K), do których dodano DMSO bez melanin, odpowiednio 200 µl, 500 µl, 1000 µl. Przygotowane układy powłokotwórcze ilustruje tab. 1.

Folie przed procesem powlekania poddano aktywacji koronowej. Folie powlekano za pomocą powlekarki (Unicoater 409, Erichsen, Niemcy) z wykorzystaniem wałka o średnicy drutu 0,76 mm i grubości warstwy osadzanej 60 µm. Następnie arkusze folii były suszone w cieplarce (Binder) przez 20 minut w temperaturze 45°C.

Tabela 1. Wykorzystane w badaniach układy powłokotwórcze oraz stężenie melanin grzybowych [µg/ml]

SYMBOL UKŁADU POWLEKAJĄCEGO	STĘŻENIE MELANINY [µg/ml]
CHAb1	100
CHAb2	250
CHAb3	500
CHR1	100
CHR2	250
CHR3	500
CHSc1	100
CHSc2	250
CHSc3	500
SKAb1	100
SKAb2	250
SKAb3	500
SKR1	100
SKR2	250
SKR3	500
SKSc1	100
SKSc2	250
SKSc3	500
KCH1	0
KCH2	0
KCH3	0
KSK1	0
KSK2	0
KSK3	0

Metodyka badań

Oznaczanie grubości folii. Grubość folii po procesie powlekania i suszenia mierzono za pomocą grubościomierza (Sylvac µS229, Szwajcaria) w 5 losowo wybranych miejscach zarówno na foliach z powłokami, jak i niemodyfikowanych foliach BOPP i PET; wyniki uśredniono.

Oznaczanie właściwości przeciwutleniających folii. Do oznaczenia właściwości przeciwutleniających folii posłużono się metodą z użyciem syntetycznego rodnika DPPH (1,1-difenylo-2-pikrylohydrazyl) [4,5,12]. 3 ml roztworu powlekającego (RP) mieszano z 1 ml 1mM roztworu rodnika DPPH w metanolu. Następnie mieszaninę worteksowano i inkubowano w ciemności, w temperaturze 25°C przez 30 minut. Po okresie inkubacji absorbancję roztworu oznaczano spektrofotometrycznie (Evolution 220, Thermo Scientific) przy długości fali 517 nm. Każdy pomiar wykonano w trzech powtórzeniach, wyniki uśredniono. Procentową wartość właściwości antyutleniających roztworów powlekających obliczano z wzoru:

$$\%inhibicji\ DPPH = \frac{AbsDPPH - AbsRP}{AbsDPPH} \times 100\%$$

gdzie: AbsDPPH – wartość absorbancji 1mM roztworu DPPH w metanolu przy długości fali 517 nm, AbsRP – wartość absorbancji roztworu powlekającego przy długości fali 517 nm

Oznaczanie zawartości polifenoli w powłokach. W celu oznaczenia zawartości polifenoli w powłokach z każdej folii wycięto 10 kwadratów o powierzchni 1 cm^2. Kwadraty umieszczono w probówkach typu Falcon, do których dodano 2,4 ml wody destylowanej oraz 0,15 ml odczynnika Folina-Ciocalteau. Mieszaninę inkubowano 8 minut w temperaturze 25°C, po czym dodawano 0,45 ml nasyconego roztworu Na_2CO_3. Całość inkubowano w temp. 40°C przez 30 minut w ciemności. Po tym czasie mierzono spektrofotometrycznie (Evolution 220, Thermo Scientific) wartość absorbancji roztworu przy długości fali 765 nm[4)]. Każdy pomiar wykonano w trzech powtórzeniach, wyniki uśredniono. Przygotowano roztwór kalibracyjny kwasu galusowego w metanolu. Zawartość polifenoli w powłokach wyrażono w przeliczeniu na µg kwasu galusowego zawarte w 1 cm^2 folii, korzystając z równania krzywej kalibracyjnej ($y = 1{,}1672x + 0{,}0053$, $R^2 = 0{,}9985$).

Oznaczanie właściwości barierowych folii względem promieniowania UV-Vis. Właściwości barierowe względem promieniowania UV-Vis oznaczano poprzez pomiar transmitancji folii (Evolution 220, Thermo Scientific). Prostokątne kawałki folii o wym. 2 cm x 4 cm umieszczano bezpośrednio na kwarcowej

Tabela 2. Właściwości przeciwutleniające i zawartość polifenoli w modyfikowanych foliach BOPP i PET

FOLIA	INHIBICJA DPPH [%]	POLIFENOLE [μg/cm2]	FOLIA	INHIBICJA DPPH [%]	POLIFENOLE [μg/cm2]
CZYSTY BOPP	0,00±0,00	0,00±0,00	CZYSTY PET	0,00±0,00	0,00±0,00
BOPP-KCH1	6,67±0,02	1,08±0,02	PET-KCH1	6,67±0,03	1,09±0,02
BOPP-KCH2	6,55±0,04	1,08±0,03	PET-KCH2	6,57±0,04	1,08±0,04
BOPP-KCH3	6,59±0,03	1,01±0,02	PET-KCH3	6,6±0,04	1,03±0,03
BOPP-KSK1	5,00±0,05	0,48±0,03	PET-KSK1	5,01±0,05	0,47±0,04
BOPP-KSK2	5,02±0,03	0,57±0,02	PET-KSK2	5,02±0,04	0,57±0,05
BOPP-KSK3	5,01±0,02	0,48±0,04	PET-KSK3	5,00±0,04	0,48±0,01
BOPP-CHAb1	8,61±0,08	3,28±0,05	PET-CHAb1	8,62±0,04	3,26±0,05
BOPP-CHAb2	10,59±0,04	4,68±0,03	PET-CHAb2	10,59±0,07	4,68±0,03
BOPP-CHAb3	16,53±0,02	6,71±0,08	PET-CHAb3	17,53±0,04	6,71±0,04
BOPP-CHR1	9,31±0,06	3,4±0,04	PET-CHR1	9,31±0,03	3,4±0,10
BOPP-CHR2	10,47±0,07	4,11±0,07	PET-CHR2	10,47±0,10	4,12±0,05
BOPP-CHR3	10,71±0,09	4,43±0,04	PET-CHR3	10,81±0,04	4,43±0,04
BOPP-CHSc1	11,52±0,07	4,91±0,03	PET-CHSc1	11,52±0,08	4,91±0,05
BOPP-CHSc2	20,83±0,02	7,68±0,07	PET-CHSc2	20,63±0,03	7,66±0,07
BOPP-CHSc3	25,49±0,07	9,22±0,05	PET-CHSc3	25,49±0,14	9,23±0,10
BOPP-SKAb1	13,27±0,24	5,60±0,01	PET-SKAb1	13,27±0,09	5,60±0,04
BOPP-SKAb2	20,6±0,12	7,51±0,08	PET-SKAb2	20,6±0,11	7,50±0,04
BOPP-SKAb3	21,88±0,08	8,20±0,04	PET-SKAb3	21,88±0,04	8,20±0,13
BOPP-SKR1	12,45±0,06	5,11±0,04	PET-SKR1	12,46±0,01	5,11±0,04
BOPP-SKR2	21,53±0,03	8,17±0,03	PET-SKR2	21,43±0,05	8,17±0,08
BOPP-SKR3	23,28±0,04	8,62±0,05	PET-SKR3	23,28±0,07	8,62±0,01
BOPP-SKSc1	14,39±0,11	6,05±0,02	PET-SKSc1	14,39±0,60	6,04±0,02
BOPP-SKSc2	16,88±0,09	6,85±0,01	PET-SKSc2	16,88±0,14	6,85±0,04
BOPP-SKSc3	22,93±0,14	8,54±0,07	PET-SKSc3	22,93±0,05	8,54±0,07

kuwecie, próbę odniesienia stanowiła pusta kuweta. Transmitancję folii mierzono w zakresie od 200 nm do 800 nm [7].

Przeźroczystość folii (T, *transparency*) obliczano z wzoru:

$T = -\log T_{600}/X$

gdzie: T_{600} – wartość transmitancji folii przy długości fali 600 nm, X – grubość folii, mm. Im wyższa wartość T, tym niższa przezroczystość folii [7]

Oznaczanie barwy folii. Pomiaru barwy folii dokonano za pomocą spektrodensytometru (FD-5, Konica Minolta, Japonia). Na podstawie modelu CIE L*a*b dokonano obliczeń parametrów L, *a, *b, opisujących jasność próbek oraz parametry chromatyczne. Folie umieszczano nad dołączoną do zestawu standardową białą płytką (L = 92,99, a = - 0,15, b = 2,74). Pomiarów dokonano w 5 powtórzeniach, wyniki zaś uśredniono. Wyznaczono również parametry ΔE (różnicę koloru w odniesieniu do standardowej białej płytki), YI (*yellowness index*), WI (*whiteness index*) na podstawie wzorów [4]:

$\Delta E = [(L_{standard} - L_{folii})^2 + (a_{standard} - a_{folii})^2 + (b_{standard} - b_{folii})^2]^{0,5}$

$YI = 142,86b \times L^{-1}$

$WI = 100 - [(100 - L)^2 + a^2 + b^2]^{0,5}$

Wyniki i dyskusja

Na podstawie przeprowadzonych doświadczeń stwierdzono, że dodatek melanin grzybowych do powłok nanoszonych na powierzchnię folii BOPP oraz PET pozwala nadać im właściwości przeciwutleniające (tab. 2.).

Odnotowano, że wszystkie folie – zarówno BOPP, jak i PET – wykazywały właściwości przeciwutleniające. Wśród powłok na foliach BOPP najwyższą wartość inhibicji rodnika DPPH zaobserwowano w przypadku folii BOPP-CHSc3 (25,49±0,07%), była to również najwyższa wartość wśród wszystkich powłok z chitozanu na foliach BOPP, najmniejszą zaś wartość odnotowano dla folii BOPP-CHAb1 (8,61±0,08%). Wśród powłok ze skrobi A4b największą zdolnością do inhibicji rodnika DPPH charakteryzowała się folia BOPP-SKR3 (23,28±0,04%), najmniejszą BOPP-SKR1 (12,45±0,06%). Na powłokach z lakieru „Varnish" największą wartość odnotowano dla folii BOPP-VR3 (20,72%), najmniejszą dla folii BOPP-VAb1 (8,03%). Wśród powłok chitozanowych na foliach PET najwyższą wartość inhibicji rodnika DPPH zanotowano dla folii PET-CHSc3 (25,49±0,14%), najniższą dla folii PET-CHAb1 (8,62±0,04%). Na powłokach ze skrobi A4b najwyższą

Tabela 3. Właściwości barierowe [%T] modyfikowanych folii BOPP względem promieniowania UV-Vis

FOLIA	200 nm	225 nm	250 nm	275 nm	300 nm	325 nm	350 nm	375 nm	400 nm	500 nm	600 nm	700 nm	800 nm
czysty BOPP	13,00	57,99	80,08	80,16	84,33	85,22	86,04	86,65	87,02	87,94	88,59	89,00	89,70
BOPP-KCH1	7,77	56,25	79,00	79,28	83,58	84,80	85,86	86,75	87,14	88,07	88,71	89,15	89,47
BOPP-KCH2	7,74	56,18	78,98	79,14	83,42	84,56	85,60	85,07	85,90	87,00	87,47	87,73	88,71
BOPP-KCH3	7,69	56,19	78,95	79,25	83,36	84,22	85,33	86,56	87,07	88,15	88,61	88,66	89,64
BOPP-KSK1	4,51	44,81	71,34	72,00	76,69	76,69	78,01	79,94	80,47	81,85	82,63	83,34	83,78
BOPP-KSK2	3,76	44,68	70,13	70,86	75,32	76,62	77,75	78,57	79,10	80,56	81,52	82,32	83,01
BOPP-KSK3	3,32	43,62	68,84	69,78	74,11	75,44	76,58	77,39	77,83	78,93	80,15	80,93	81,39
BOPP-CHAb1	7,59	55,66	78,90	79,20	83,49	84,75	86,12	86,90	86,80	88,16	88,78	88,83	89,46
BOPP-CHAb2	6,34	52,62	76,08	76,91	81,44	82,34	84,32	85,88	87,50	88,44	88,95	89,32	89,55
BOPP-CHAb3	1,37	46,86	74,44	76,05	80,73	82,99	84,62	85,75	86,52	88,03	88,77	89,45	89,22
BOPP-CHR1	8,81	55,91	79,42	79,59	84,03	85,15	86,31	87,01	87,44	88,51	88,89	89,35	89,13
BOPP-CHR2	8,15	55,51	78,47	78,87	83,26	84,50	85,87	86,62	87,25	88,23	88,92	89,46	88,77
BOPP-CHR3	7,47	52,84	75,41	76,31	81,21	82,87	84,42	85,44	86,10	87,65	88,39	89,22	89,30
BOPP-CHSc1	8,38	55,25	78,28	78,72	83,33	84,53	85,66	86,67	87,16	87,99	88,90	89,09	89,74
BOPP-CHSc2	7,94	54,91	77,33	78,13	82,58	84,10	85,57	86,38	87,12	88,59	89,28	89,27	90,12
BOPP-CHSc3	7,90	54,22	76,58	77,27	81,83	83,65	84,90	86,04	86,58	87,83	88,99	89,44	89,60
BOPP-SKAb1	4,84	46,94	72,61	73,58	78,67	80,41	81,92	83,06	83,82	85,59	86,44	86,96	87,39
BOPP-SKAb2	4,57	44,50	68,29	69,25	73,78	75,23	76,49	77,39	78,00	79,60	80,66	81,40	82,01
BOPP-SKAb3	4,01	43,58	68,32	68,32	74,14	75,78	77,16	78,20	78,84	80,40	81,61	82,33	82,96
BOPP-SKR1	4,14	44,14	68,31	69,35	74,18	75,59	76,80	77,70	78,30	79,92	80,64	81,32	81,98
BOPP-SKR2	6,02	47,72	47,72	71,26	76,69	78,07	79,30	80,20	80,78	82,17	83,11	83,79	84,38
BOPP-SKR3	4,09	44,49	69,18	70,19	74,81	76,17	77,38	78,30	78,85	80,26	81,17	81,76	82,25
BOPP-SKSc1	5,90	46,61	69,74	70,71	75,60	77,23	78,53	79,49	80,09	81,68	82,61	83,32	83,87
BOPP-SKSc2	5,29	46,30	69,90	70,81	75,52	77,03	78,28	79,15	79,68	81,03	82,10	82,78	83,16
BOPP-SKSc3	4,04	44,61	69,63	70,49	75,31	76,76	77,96	78,85	79,42	80,92	81,80	82,33	83,01

wartość zaobserwowano dla folii PET-SKR3 (23,28±0,07%), najmniejszą zaś dla PET-SKR1 (12,46±0,01%). Powłoki chitozanowe i skrobiowe, do których nie dodano melanin, również charakteryzowały się właściwościami przeciwutleniającymi, jednakże znacznie mniejszymi w porównaniu z powłokami zawierającymi melaniny. Wynika to z faktu, że wolne rodniki DPPH mogą reagować z grupami aminowymi (NH_2) chitozanu, tworząc formę zredukowaną, grupy aminowe zaś, przyjmując atom wodoru, mogą tworzyć jony amonowe (NH^{3+}). Podobnie w przypadku skrobi: wolne rodniki DPPH mogą reagować z grupami hydroksylowymi (-OH), które w wyniku przyłączenia atomu wodoru tworzą grupę (OH^{2+}) [4,5].

W badaniach własnych za pomocą metody Folina-Ciocalteau oznaczono zawartość polifenoli przypadającą na 1 cm^2 powłok na foliach BOPP i PET. Zaobserwowano, że w przypadku powłok chitozanowych i skrobiowych wraz ze wzrastającym stężeniem melanin w roztworze powłokotwórczym wzrastała zawartość polifenoli na 1 cm^2 powłok (tab. 2.). Najwyższą zawartość polifenoli wśród folii BOPP odnotowano w przypadku folii BOPP-CHSc3 (9,22±0,05 µg/cm^2), dla folii tej uzyskano również najwyższy stopień inhibicji rodnika DPPH. Podobnie najwyższą zawartość polifenoli (9,23±0,10 µg/cm^2) wśród folii PET odnotowano w przypadku folii PET-CHSc3. Folie pokryte jedynie nośnikami bez melanin również dały dodatni wynik na polifenole w powłoce. Wynika to najprawdopodobniej z faktu, że zarówno chitozan, jak i skrobia zawierają w cząsteczkach grupy -OH, które mogą powodować interferencje w uzyskanym wyniku [4,6].

Nie odnotowano znaczącej poprawy właściwości barierowych względem promieniowania UV-Vis w przypadku modyfikowanych folii BOPP (tab. 3.) oraz PET (tab. 4.). Duży wpływ na właściwości barierowe miały same powłoki niezawierające melanin, szczególnie powłoki ze skrobi. Największy wpływ melanin na właściwości barierowe powłok względem promieniowania UV wśród folii BOPP zaobserwowano dla folii BOPP-CHAb3, w przypadku której transmitancja przy długości fali w zakresie 250-300 nm była o ok. 4% niższa niż w przypadku folii powleczonej chitozanem, natomiast przy długości fali 225 nm była niższa o ok. 16,61%. Wynika to z faktu, że wiele melanin grzybowych wykazuje maksimum absorbancji w zakresie 210--230 nm [11,13,14,15]. Brak znaczącej poprawy właściwości barie-

Tabela 4. Właściwości barierowe [%T] modyfikowanych folii PET względem promieniowania UV-Vis

FOLIA	200 nm	225 nm	250 nm	275 nm	300 nm	325 nm	350 nm	375 nm	400 nm	500 nm	600 nm	700 nm	800 nm
czysty PET	0,02	0,01	0,01	0,01	0,02	76,90	80,97	82,99	83,44	86,85	88,70	87,22	88,76
PET-KCH1	0,02	0,01	0,01	0,01	0,02	78,68	84,27	84,98	86,65	87,09	89.13	87,07	87,07
PET-KCH2	0,02	0,01	0,01	0,01	0,02	79,19	83,16	85,26	85,79	87,09	88,04	87,20	86,81
PET-KCH3	0,02	0,02	0,01	0,01	0,01	78,90	78,64	84,44	84,89	86,21	87,57	87,59	87,40
PET-KSK1	0,02	0,01	0,01	0,02	0,01	74,96	77,82	77,81	78,42	80,47	80,26	79,80	78,44
PET-KSK2	0,02	0,01	0,01	0,02	0,02	75,95	77,84	77,84	78,59	80,30	80,30	79,81	78,77
PET-KSK3	0,02	0,01	0,01	0,01	0,02	73,48	77,69	79,71	80,10	82,07	83,20	82,73	82,07
PET-CHAb1	0,02	0,01	0,01	0,01	0,02	77,37	81,70	84,13	84,54	85,97	86,89	86,64	86,25
PET-CHAb2	0,02	0,01	0,01	0,01	0,01	77,33	81,99	83,57	85,35	86,62	88,28	87,06	87,96
PET-CHAb3	0,02	0,01	0,01	0,01	0,02	76,38	80,78	83,48	84,16	86,19	87,60	86,81	87,17
PET-CHR1	0,02	0,01	0,01	0,01	0,01	78,15	82,17	85,19	84,36	86,87	86,83	88,02	83,84
PET-CHR2	0,02	0,01	0,01	0,01	0,02	78,11	82,86	84,55	85,17	86,59	87,59	88,20	88,62
PET-CHR3	0,02	0,01	0,01	0,01	0,02	77,12	82,87	83,58	84,06	85,88	88,31	87,79	89,26
PET-CHSc1	0,02	0,01	0,01	0,01	0,01	77,20	77,20	82,33	84,20	83,75	85,54	87,97	86,49
PET-CHSc2	0,02	0,01	0,01	0,01	0,02	67,48	73,18	76,98	78,46	82,78	85,61	85,39	85,61
PET-CHSc3	0,02	0,01	0,01	0,01	0,02	67,47	73,17	76,96	78,43	82,78	85,64	85,37	85,60
PET-SKAb1	0,02	0,01	0,01	0,01	0,02	70,33	73,92	76,51	76,95	78,89	80,70	80,29	80,80
PET-SKAb2	0,02	0,01	0,01	0,01	0,02	69,59	73,76	75,71	76,37	78,50	79,64	79,34	79,99
PET-SKAb3	0,02	0,01	0,01	0,01	0,02	71,03	75,23	77,51	78,20	80,69	82,38	81,51	82,63
PET-SKR1	0,02	0,01	0,01	0,01	0,02	71,52	75,40	77,52	78,07	79,89	81,02	80,98	81,78
PET-SKR2	0,02	0,01	0,01	0,01	0,02	71,32	75,26	77,58	78,00	79,12	79,98	80,71	81,00
PET-SKR3	0,02	0,01	0,01	0,01	0,02	72,77	76,61	78,83	79,43	80,72	82,53	82,69	81,78
PET-SKSc1	0,02	0,01	0,01	0,01	0,02	72,12	75,86	77,99	78,50	80,50	81,97	81,22	81,88
PET-SKSc2	0,02	0,01	0,01	0,01	0,02	72,27	76,15	78,24	78,89	80,62	81,86	82,18	82,78
PET-SKSc3	0,02	0,01	0,01	0,01	0,02	70,57	74,54	76,83	77,46	81,26	81,26	80,88	81,47

owych względem promieniowania UV-Vis odnotowano także v przypadku modyfikowanych folii PET. Widoczna jest wysoka barierowość niemodyfikowanej folii PET w zakresie 200-300 nm. Zaobserwowano jedynie nieznaczną poprawę w przypadku folii : powłokami skrobiowymi zawierających melaniny grzybowe, ıp. przy długości fali 325 nm folie charakteryzowały się transmitancją o ok. 4% niższą niż folie modyfikowane samymi powłokami skrobiowymi. Najprawdopodobniej wykorzystane w badaniach własnych stężenia melanin były zbyt niskie dla uzyskania satysfakcjonującej poprawy właściwości barierowych względem promieniowania UV-Vis. Wykorzystując wyniki pomiarów grubości folii oraz wartości transmitancji przy długości ali 600 nm obliczono wartości T (przeźroczystości) folii. Odnotowano, że folie pokryte powłokami ze skrobi były mniej przeźroczyste w porównaniu do czystych, niemodyfikowanych folii BOPP (tab. 5.) i PET (tab. 6.).

Zaobserwowano wpływ dodatku melanin grzybowych v powłokach na parametry chromatyczne modyfikowanych folii. Wyniki przedstawione zostały w postaci zmiennych *a, *b oraz L, gdzie *a określa zmianę barwy od zielonej (dla wartości ujemnych) do czerwonej (dla wartości dodatnich), natomiast *b od niebieskiej (dla wartości ujemnych) do żółtej (dla wartości dodatnich). L oznacza wartości luminancji (jasność) i zmienia się w zakresie od 0 dla barwy czarnej do 100 dla barwy białej. Zaobserwowano, że wzrastające stężenia melanin grzybowych w powłokach powodowały wzrost wartości parametru *a (w kierunku czerwonej) zarówno w przypadku modyfikowanych folii BOPP (tab. 5.), jak i PET (tab. 6.). Zanotowano również w przypadku wszystkich folii zawierających melaniny grzybowe wzrastające wraz ze stężeniem melanin w powłoce wartości parametru *b (w kierunku barwy żółtej). Parametry chromatyczne w kierunku barw czerwonej i żółtej są charakterystyczne dla melanin [15]. Na podstawie pomiarów wartości chromatycznych oraz jasności obliczono również zmianę barwy folii ΔE w stosunku do standardowej białej płytki. Przyjmuje się, że niedoświadczony obserwator zauważa już różnicę odpowiadającą $\Delta E>2$, natomiast gdy $\Delta E>3,5$, różnica barw jest wyraźna. W przypadku wszystkich folii zaobserwowano $\Delta E>3,5$, tak więc różnica barw była wyraźna w odniesieniu do standardowej białej płytki. Zanotowano, że wraz ze wzrostem stężenia melanin w powłokach

Tabela 5. Parametry chromatyczne (L*a*b), zmiana barwy (ΔE), parametry WI, YI oraz grubość i przeźroczystość modyfikowanych folii BOPP

FOLIA	L	*a	*b	ΔE	YI	WI	G [mm]	T
czysty BOPP	88,85±0,99	-0,2±0,02	2,63±0,28	4,15±0,99	4,22±0,40	88,53±0,90	0,041	1,27
BOPP-KCH1	88,05±0,81	-0,2±0,03	2,54±0,10	4,95±0,82	4,11±0,12	87,01±0,65	0,045	1,16
BOPP-KCH2	87,85±1,54	-0,18±0,02	2,59±0,33	5,71±1,86	4,20±0,46	87,38±1,05	0,044	1,32
BOPP-KCH3	86,43±2,04	-0,18±0,03	2,49±0,38	6,72±1,99	4,11±0,54	86,77±1,48	0,045	1,17
BOPP-KSK1	87,01±1,09	-0,21±0,02	2,41±0,11	5,80±1,22	3,96±0,14	86,39±0,90	0,045	1,84
BOPP-KSK2	85,74±1,84	-0,23±0,03	2,36±0,18	6,52±1,70	3,94±0,23	86,47±1,22	0,048	1,85
BOPP-KSK3	86,63±1,08	-0,23±0,01	2,25±0,10	5,87±1,17	3,71±0,11	86,67±0,27	0,047	2,06
BOPP-CHAb1	86,34±3,27	-0,19±0,03	2,77±0,29	5,04±1,11	4,57±0,35	86,77±0,85	0,048	1,08
BOPP-CHAb2	87,94±1,72	-0,17±0,03	2,94±0,14	5,83±1,63	4,97±0,54	87,48±1,03	0,047	1,08
BOPP-CHAb3	85,42±2,86	-0,12±0,05	3,07±0,39	7,68±2,78	4,91±0,13	85,00±2,71	0,046	1,12
BOPP-CHR1	87,52±1,96	-0,23±0,03	2,58±0,17	5,26±0,47	4,43±0,39	86,96±0,97	0,042	1,22
BOPP-CHR2	87,08±1,32	-0,16±0,04	2,73±0,09	5,71±1,86	4,24±0,23	85,09±0,54	0,042	1,22
BOPP-CHR3	86,10±2,24	-0,07±0,02	2,88±0,09	6,54±2,46	4,78±0,08	85,35±1,77	0,042	1,28
BOPP-CHSc1	87,88±1,10	-0,17±0,05	2,66±0,19	4,68±0,46	4,41±0,47	87,01±0,65	0,045	1,14
BOPP-CHSc2	88,98±2,20	-0,16±0,03	2,71±0,32	5,15±0,53	4,27±0,38	87,10±0,69	0,042	1,18
BOPP-CHSc3	88,24±0,55	-0,02±0,05	3,09±0,19	5,35±1,02	5,00±0,28	87,36±0,84	0,049	1,04
BOPP-SKAb1	85,38±1,20	-0,18±0,01	2,35±0,12	6,17±0,63	3,92±0,14	85,88±1,20	0,046	1,37
BOPP-SKAb2	87,35±1,18	-0,16±0,01	2,69±0,15	7,67±1,15	4,65±0,41	86,77±0,48	0,047	1,97
BOPP-SKAb3	84,51±1,71	-0,11±0,07	2,84±0,29	8,42±1,70	4,54±0,16	86,41±0,40	0,049	1,81
BOPP-SKR1	86,56±2,42	-0,20±0,02	2,46±0,11	5,71±1,86	4,23±0,53	86,67±1,23	0,050	1,88
BOPP-SKR2	83,47±5,23	-0,18±0,03	2,49±0,27	8,14±1,90	4,26±0,43	83,87±5,22	0,049	1,64
BOPP-SKR3	84,94±1,90	-0,14±0,01	2,57±0,39	10,55±4,53	4,13±0,10	86,56±0,27	0,049	1,86
BOPP-SKSc1	85,99±2,55	-0,18±0,02	2,46±0,06	5,36±1,43	4,09±0,11	85,56±0,27	0,046	1,79
BOPP-SKSc2	87,16±2,09	-0,11±0,02	2,73±0,31	5,82±2,10	4,46±0,40	85,82±1,18	0,048	1,80
BOPP-SKSc3	87,37±1,22	-0,04±0,01	2,97±0,37	7,20±2,40	4,85±0,85	86,63±0,36	0,048	1,83

wzrastała wartość YI, co wiąże się ze zmianą parametru *b w kierunku barwy żółtej.

Podsumowanie i wnioski

Powlekanie folii BOPP i PET powłokami zawierającymi melaniny grzybowe może służyć modyfikacji folii w kierunku nadania im właściwości przeciwutleniających. Wraz ze zwiększającym się udziałem melanin grzybowych w powłokach wzrastały ich właściwości przeciwutleniające, co najprawdopodobniej wiąże się ze wzrastaniem ilości grup hydroksylowych zdolnych do inhibicji wolnych rodników. Mimo wzrastającego udziału melanin w powłokach nie zaobserwowano znaczącej poprawy właściwości barierowych względem promieniowania UV zarówno folii BOPP, jak i PET. Dodatek melanin do powłok wpłynął na parametry chromatyczne folii. Wraz ze wzrastającym stężeniem melanin w powłokach zaobserwowano wzrastającą zmianę barwy folii w kierunku barw czerwonej i żółtej. ■

Literatura

[1] Brody A., Strupinsky E., Kline L., 2001, Active Packaging for Food Applications

[2] Nałęcz-Jawecki G., Zawadzki T., Skrzypczak A., 2012, Substancje promieniochronne a środowisko przyrodnicze. Biuletyn Wydziału Farmaceutycznego Warszawskiego Uniwersytetu Medycznego, 5, 32-39

[3] Stepczyńska M., 2015. Wpływ promieniowania UV-Vis na właściwości termomechaniczne i strukturę barwionej folii PLA. Polimery, 60 (6), 385-390

[4] Mehdizadeh T., Tajik H., Rohani S. M. R., Oromiehie A. R., 2012. Antibacterial, antioxidant and optical properties of edible starch-chitosan composite film containing Thymus kotschyanus essential oil. Veterinary Research Forum 3 (3), 167-173

[5] Mikusanti H., Masril K. I. 2013. Antibacterial and Antioxidant of Uwi (Dioscorea alata L) Starch Edible Film Incorporated with Ginger Essential Oil. International Journal of Bioscience, Biochemistry and Bioinformatics 3 (4), 354-356

Tabela 6. Parametry chromatyczne (L*a*b), zmiana barwy (ΔE), parametry WI, YI oraz grubość i przeźroczystość modyfikowanych folii PET

FOLIA	L	*a	*b	ΔE	YI	WI	G [mm]	T
czysty PET	87,79±0,27	-0,08±0,01	3,31±0,12	5,24±0,29	5,38±0,22	86,93±0,30	0,012	5,03
PET-KCH1	88,36±0,10	-0,10±0,07	3,55±0,17	4,68±0,06	5,73±0,28	87,16±0,58	0,014	3,66
PET-KCH2	88,47±0,04	-0,08±0,04	3,53±0,11	4,58±0,53	5,70±0,18	87,20±0,63	0,014	4,05
PET-KCH3	88,45±0,12	-0,05±0,07	3,51±0,12	4,66±0,12	5,67±0,19	87,20±0,64	0,014	4,22
PET-KSK1	88,83±0,10	-0,12±0,03	3,28±0,19	4,18±0,11	5,28±0,32	87,36±0,84	0,020	4,86
PET-KSK2	88,91±0,51	-0,15±0,05	3,18±0,23	4,30±0,20	5,10±0,37	87,28±0,73	0,019	5,10
PET-KSK3	88,73±0,18	-0,07±0,04	3,26±0,18	4,35±0,21	5,26±0,30	87,33±0,79	0,018	4,52
PET-CHAb1	88,24±0,20	-0,03±0,03	3,67±0,13	4,85±0,19	5,95±0,20	84,14±0,55	0,014	4,47
PET-CHAb2	88,13±0,06	-0,01±0,05	3,74±0,11	4,94±0,03	6,07±0,17	87,05±0,45	0,014	3,96
PET-CHAb3	88,06±0,20	0,06±0,06	4,09±0,26	5,02±0,13	6,63±0,44	87,04±0,44	0,013	4,31
PET-CHR1	88,3±0,25	-0,04±0,12	3,49±0,20	4,69±0,19	5,65±0,32	87,09±0,50	0,013	4,60
PET-CHR2	88,42±0,07	-0,05±0,21	3,66±0,07	4,67±0,06	5,92±0,10	87,15±0,57	0,013	4,32
PET-CHR3	88,02±0,14	0,21±0,08	4,01±0,39	5,10±0,13	6,51±0,65	86,99±0,38	0,013	4,05
PET-CHSc1	88,23±0,31	-0,12±0,08	3,42±0,28	4,96±0,16	5,53±0,44	87,04±0,44	0,014	4,96
PET-CHSc2	88,23±0,14	-0,20±0,31	3,67±0,21	4,95±0,11	5,94±0,33	87,08±0,49	0,013	5,06
PET-CHSc3	87,23±1,88	-0,12±0,26	3,92±0,20	5,93±1,82	6,42±0,29	86,13±1,57	0,013	5,05
PET-SKAb1	88,65±0,26	-0,06±0,02	3,49±0,06	4,36±0,27	5,62±0,10	87,19±0,64	0,019	4,90
PET-SKAb2	88,06±0,15	-0,04±0,03	3,53±0,21	4,39±0,13	5,69±0,33	87,23±0,67	0,019	5,30
PET-SKAb3	88,26±0,09	0,03±0,01	4,04±0,06	4,86±0,07	6,53±0,10	87,04±0,43	0,018	4,59
PET-SKR1	88,71±0,09	-0,08±0,02	3,19±0,36	4,34±0,11	5,22±0,40	87,36±0,82	0,019	4,90
PET-SKR2	88,41±0,76	-0,07±0,02	3,24±0,25	4,32±0,40	5,16±0,54	87,29±0,74	0,019	5,11
PET-SKR3	88,58±0,16	-0,02±0,03	3,67±0,15	4,57±0,18	5,92±0,24	87,06±0,71	0,018	4,55
PET-SKSc1	88,67±0,14	-0,06±0,02	3,29±0,24	4,38±0,14	5,31±0,39	87,27±0,72	0,018	4,71
PET-SKSc2	88,49±0,11	-0,03±0,01	3,51±0,15	4,56±0,13	5,66±0,24	87,19±0,62	0,018	4,92
PET-SKSc3	88,35±0,24	0,12±0,01	3,75±0,23	4,82±0,16	6,06±0,37	87,12±0,54	0,018	5,01

6] Siripatrawan U., Harte B. R., 2010. Physical properties and antioxidant activity of an active film from chitosan incorporated with green tea extract. Food Hydrocolloids, 24, 77-775

7] Wu J., Chen S., Ge S., Miao J., Li J., Zhang Q., 2013. Preparation, properties, and antioxidant activity of an active film from silver carp (Hypophthalamichthys molitrix) skin gelatin with green tea extract. Food Hydrocolloids, 32, 42-51

8] Norajit K., Kim K. M., Ryu G. H., 2010. Comparative studies on the characterization and antioxidant properties of biodegradable alginate films containing ginseng extract. Journal of Food Engineering, 98, 377-384

9] Różanowska M., Sarna T., Land E., Truscott G., 1999. Free radical scavening properties of melanin interaction of eu- and pheo-melanin models with reducing and oxidising radicals. Free Radical Biology & Medicine 26, 518-525

10] Łopusiewicz Ł., Lisiecki S., 2016 „Czarne złoto" – melaniny w życiu człowieka. Kosmos Problemy Nauk Biologicznych 65, 4 (313), 621-629. Ł. Łopusiewicz, S. Lisiecki, Kosmos 2016, 4 (313), 621-629

[11] Harki E., Talou T., Dargent R., 1997. Purification, characterisation and analysis of melanin extracted from Tuber melanosporum Vitt. Food Chemistry, 58 (1-2), 69-73

[12] Trzcińska M., Sieliwanowicz B., Hałasińska A., Czupryński B., 2007. Application of physiochemically modified polymeric foil to develop antioxidant packaging. Polish Journal of Food and Nutrition Sciences 57 (2), 173-176

[13] Zhan F., He Y., Zu Y., Li T., Zhao Z., 2011. Characterisation of melanin isolated from a dark septate endophyte (DSE), Exophiala pisciphila. World J. Microbiol Biotechnol, 27, 2483-2489

[14] Ye M., Wang Y., Qian M., Chen X., Hu X., 2011. Preparation and Properties of Melanin from Lachnum singerianum. International Journal of Basic & Applied Sciences, 11 (3), 51-58

[15] Zou Y., Wen Y., She W., Hu W., 2014. Physiochemical Properties of Melanin from A. auricula Fruiting Bodies. Advance Journal of Food Science and Technology, 6 (8), 1002-1004

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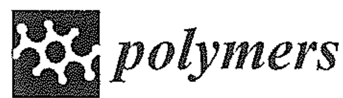

Article

New Poly(lactic acid) Active Packaging Composite Films Incorporated with Fungal Melanin

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Abstract: In this work, fungal melanin was used for the first time to prepare poly(lactic acid)-based composites. The films of various melanin concentrations (0.025%, 0.05% and 0.2% *w/w*) were prepared using an extrusion method. The mechanical, antioxidant, antimicrobial, water vapor and UV-Vis barrier properties, as well as available polyphenolics on the surface, were studied. FT-IR and Raman spectroscopy studies were carried out to analyze the chemical composition of the resulting films. The hydrophobicity, color response, thermal, optical properties, and opacity values were also determined. The results of this study show that the addition of fungal melanin to poly(lactic acid) (PLA) as a modifier influenced mechanical and water vapor barrier properties depending on melanin concentration. In low concentration, melanin enhanced the mechanical and barrier properties of the modified films, but in larger amounts, the properties were decreased. The UV-Vis barrier properties of PLA/melanin composites were marginally improved. Differential Scanning Calorimetry (DSC) analysis indicated that crystallinity of PLA increased by the addition of melanin, but this did not affect the thermal stability of the films. Modified PLA/melanin films showed good antioxidant activity and were active against *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Pseudomonas putida*. The addition of melanin caused changes in color values, decreasing lightness and increasing the redness and yellowness of films. Based on the results of this study, fungal melanin has good potential to be exploited as a value-added modifier that can improve the overall properties of PLA.

Keywords: poly(lactic acid); biopolymer; melanin; packaging; polymer blends; antioxidant; barrier properties; mechanical properties

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1. Introduction

Natural polymers, biopolymers, and synthetic polymers based on annually renewable resources are the basis of a 21st-century portfolio of sustainable, eco-efficient plastics. These biosourced materials are hoped to gradually replace the currently existing family of petroleum-based polymers as they become less competitive regarding cost performance [1,2]. Biodegradable polymers from renewable resources have attracted a large amount of attention in research. They are defined as polymers that undergo microbially-induced chain scission leading to mineralization [3,4]. Polylactide or poly(lactic acid) (PLA) is the most promising bio-based polymer in the emerging bioplastics market, with the high availability and a more attractive cost structure. PLA has a panoply of advantages: it is a thermoplastic material with a rigidity and clarity similar to polystyrene (PS) or poly(ethylene terephthalate) (PET), it is bio-based, resorbable and biodegradable under industrial composting conditions, but also processable in most standard equipment (injection molding, blow molding, thermoforming, extrusion and casting [1,3,5–8]. PLA has a wide spectrum of applications: packaging, medical, agricultural and engineering materials, as well as textile preparation [1].

The end uses of PLA within the packaging industry include rigid packaging, flexible film packaging, cold drinks cups, cutlery, apparel and stable fibers, bottles, injection molded products, as well as extrusion coatings [1,8]. PLA also has a wide spectrum of medical applications, such as surgical implant materials, dental materials, drug delivery systems, guided tissue and bone regeneration platforms, porous scaffolds for tissues growth and fracture fixation devices [4,7,9].

As a packaging material PLA is attractive because it exhibits a tensile strength comparable to that of petroleum-derived thermoplastics, is biodegradable and can be sealed at low temperatures [1]. The properties of PLA, such as thermal stability and impact resistance, medium gas barrier properties and low solvent resistance of pure PLA are inferior to those of conventional polymers used for thermoplastic applications. Therefore, PLA is not ideally suited to compete against conventional polymers and limits its use for food packaging applications [10]. In order to improve the properties of PLA and increase its potential application, PLA modification, copolymerization with other monomers, and PLA composites are still being developed to improve its properties, regarding stiffness, permeability, crystallinity, and thermal stability [1].

A wide spectrum of additives (nano or microsized) have been used for the preparation of PLA composites including: clays and organoclays [11,12], starch [5], carbon nanotubes [13], metal nanoparticles [14–16], graphene [7,13,17], as well as cellulose and glass fibers [18], aromatic compounds and essential oils [19,20], chitosan [21,22], epoxidized vegetable oils [10], plant fibers (wood [23], bamboo [24], banana [25], cotton and flax [26], hemp [27] and artichoke [28]), collagen [9] and tannins [29]. Those additives may not only influence the mechanical, optical properties and thermal stability of the blends but also enhance the microbial stability of the packaged foods by using active packaging developed by the incorporation of antimicrobial compounds into the polymer matrix. Contact between the active materials and food, which has the ability to change food composition or the atmosphere around it, represents an active packaging system that inhibits the growth of microorganisms present on the surface of food products [30,31]. PLA could also be modified by active coatings that contain antimicrobials [32].

Melanins have been isolated and characterized from a variety of phylogenic sources, such as animal [33], plant [34], bacteria [35] and fungi [36,37]. Melanins are commonly represented as black and brown pigments, high molecular weight heterogeneous polymers derived from the oxidation of monophenols and the subsequent polymerization of intermediate o-diphenols and their resulting quinones [38]. Melanins are types of pigments, possessing broad biological properties including antioxidant, radioprotective, thermoregulative, chemoprotective, antitumor, antiviral, antimicrobial, immunostimulating and anti-inflammatory [33–38]. Potentially, these melanin attributes could also be imparted to plastics, and in the case of bioplastics, potentially enhancing performance as well as sustainability credentials, explore its other nonconventional applications such as crosslinking during polymerization, antioxidant and antimicrobial activity, radioprotective ability and improving the biological properties of the polymer. The use of melanins remains relatively unexplored with few examples of these compounds blended with plastics [39–41], and no studies on PLA/melanin composites are available in the scientific literature.

The aim of this study was to investigate the influence of fungal melanin on the properties of modified poly(lactic acid) films. To evaluate the potentiality of the developed films for different industrial and biomedical applications, the mechanical, barrier, antioxidant and antimicrobial properties were all evaluated. Spectroscopic studies were performed to elucidate melanin addition in the chemical composition of modified blends. The goal of the study was also to evaluate the influence of fungal melanin on the color, opacity and optical properties of the films.

2. Materials and Methods

2.1. Poly(lactic acid)

Poly(lactic acid) (PLA), 4043D, was purchased in pellet form from Resinex (a Polish supplier of Natureworks resins)—dedicated to extruding thin films through a casting method.

2.2. The Isolation of Melanin from A. bisporus Waste

Agricultural waste from the production of *A. bisporus* (ABW—Agaricus Bisporus Waste) in the form of fruiting bodies stipes was obtained from a local producer in Wolsztyn (Wielkopolskie voivodeship, Poland). 500 g of ABW was first homogenized (Heidolph Brinkmann Homogenizer Silent Crusher, Schwabach, Germany) in 500 mL of distilled water and incubated (24 h, 37 °C) to allow enzyme tyrosinase action (hydroxylation of monophenols to *o*-diphenols). After incubation, the homogenate mixture was adjusted to pH = 10 by 1 M NaOH, and incubated (24 h, 65 °C) to allow a spontaneous oxidative polymerization of the resulting *o*-diphenols and quinones to form melanin. Afterward, the mixture was filtered, centrifuged (6000 rpm, 10 min), and an alkaline ABW raw melanin mixture was used to purify the melanin. An alkaline ABW raw melanin mixture was first adjusted to pH 2.0 with 1 M HCl to precipitate melanin, followed by centrifugation at 6000 rpm for 10 min and a resulting pellet was collected. The pellet was then hydrolyzed in 6 M HCl (90 °C, 2 h), centrifuged (6000 rpm, 10 min) and washed in distilled water five times to remove acid. The pellet was washed with chloroform, ethyl acetate and ethanol three times to wash away lipids and other residues. Finally, the purified melanin was dried, ground to a fine powder in a mortar and stored at −20 °C until testing.

2.3. The Preparation of PLA/Melanin Films

The blends of PLA and melanin were obtained by means of a twin-screw extruder combined with an air-cooling system and a side-cut pelletizer which feature a co-rotating twin-screw extruder (screws of 20 mm diameter, a length/diameter ratio (L/D): 40/1, LabTech Engineering, Samut Prakan, Thailand), cooling conveyor (equipped with 8 fans, LabTech Engineering, Samut Prakan, Thailand), side-cut pelletizer (with 8 rotary knives producing pellets with dimensions of 5 × Ø3 mm, LabTech Engineering, Samut Prakan, Thailand). An average of 75–80 micron thick films were obtained by means of a laboratory cast film extrusion line (LabTech Engineering, Samut Prakan, Thailand), with following equipment parameters: screw diameter—20 mm; length/diameter (L/D) screw ratio of: 30/1; output of extruder (each): approx. 5 kg/h of LDPE; screw type: transporting-mixing/ transporting; screw speed (rpm) during the experiment: 60. "0"—pure PLA film (devoid of melanin, served as a control sample) and three PLA/melanin composites with various concentrations of melanin were prepared: "1"—0.025%; "2"—0.05% and "3"—0.2%.

2.4. The Mechanical Properties of PLA/Melanin Films

Mechanical measurements were tested by the use of Zwick/Roell 2,5 Z equipment (Zwick/Roell, Ulm, Germany) and they included tensile strength (the gap between tensile clamps was 25 mm and tensile speed was 100 mm/min), and burst strength (transducer diameter 0.75 mm, speed 50 mm/min). The dynamic mechanical analyses of the pure PLA and modified PLA/melanin films were performed by the use of DMA analyzer (Q800, TA Instruments, New Castle, DE, USA). Films were prepared as a rectangular (30 mm × 5 mm × 0.08 mm) samples. Samples were located between clamps and analyzed with following parameters: temperature range 30–80 °C; constant heating rate 3 °C/min; amplitude 15 μm; force track 125%; oscillation frequency 1 Hz. The storage modulus (E'), loss modulus (E''), loss factor (tan δ), and glass transition temperature (T_g) of each specimen were obtained as a function of temperature.

2.5. The Water Vapor Transmission Rate of the Films

The Water Vapor Transmission Rate (WVTR) was measured by means of a gravimetric method that is based on the sorption of humidity by calcium chloride and a comparison of sample weight gain. Initially, the amount of dry $CaCl_2$ inside the container was 9 g. The area of film was 8.86 cm^2. Measurement was carried out for a period of 4 days, each day the containers were weighed to determine the amount of absorbed water vapor through the films. The result was expressed as average values from each day of measurement and each container. Analyses were carried out at ten independent containers for each type of films, calculated as a standard unit $g/(m^2 \times day)$ and presented as a mean ± standard deviation.

2.6. The Contact Angle (CA)

The surface properties of modified and pure PLA films were measured through a contact angle analyzer. The following measurement was carried out by means of a laboratory goniometer (Haas μL), drop of distilled water was placed on the surface of the film using a microsyringe. Analyses were carried out at three independent times and presented as mean ± standard deviation.

2.7. Spectral Analysis

2.7.1. UV-Vis Spectroscopy

The UV-Vis spectra of the films samples were measured by the use of a UV-Vis Thermo Scientific Evolution 220 spectrophotometer at 200–800 nm.

2.7.2. FTIR Spectroscopy

Fourier transform infrared (FT-IR) spectra of the unmodified and modified film samples were measured using a FT-IR spectroscopy (Perkin Elmer Spectrophotometer, Spectrum 100, Waltham, MA, USA), operated at a resolution of 4 cm^{-1}, over 64 scans. Film samples were cut into square shapes (2 cm × 2 cm) and placed directly at the ray-exposing stage. The spectra were recorded at a wavelength of 650–4000 cm^{-1}. The spectra were normalized, baseline corrected and analyzed using SPECTRUM software.

2.7.3. Raman Spectroscopy

Pure and modified films were analyzed using a Raman station (RamanStation 400F, Perkin Elemer, Waltham, MA, USA) with point-and-shot capability using an excitation laser source at 785 nm, 100 micron spot size. Film samples were cut into square shapes (2 cm × 2 cm) and placed directly at the ray-exposing stage. The spectra were recorded at a wavelength of 250–3300cm^{-1}. The spectra were normalized, baseline corrected and analyzed using SPECTRUM software (v10, PerkinElmer, Waltham, MA, USA).

2.8. Color Response Analysis

The color changes of the films were measured by using a colorimeter (CR-5, Konica Minolta, Tokyo, Japan). The results were expressed as L^* (lightness), a^* (red to green), and b^* (yellow to blue) parameters to evaluate color changes in the modified PLA/melanin films. All of the measurements were determined at three random points on both sides of each film, and the experiments were performed five times and presented as a mean ± standard deviation.

To determine other color properties of the films, ΔE (color difference), YI (yellowness index) and WI (whiteness index) values were calculated using following equations (where pure PLA film served as a standard):

$$\Delta E = [(L_{standard} - L_{sample})^2 + (a_{standard} - a_{sample})^2 + (b_{standard} - b_{sample})]^{0.5} \quad (1)$$

$$YI = 142.86b^{*}L^{-1} \quad (2)$$

$$WI = 100 - [(100 - L)^2 + a^2 + b^2]^{0.5} \quad (3)$$

2.9. Opacity Measurements

The opacity of modified PLA/melanin films and pure PLA was carried out in Opacimeter EE Model 12 (Diffusion Systems Ltd., London, UK). The opacimeter was initially calibrated using standard white plate (value 100 ± 1, Diffusion Systems Ltd.) and measurements were performed on each film six times, and presented as mean ± standard deviation.

2.10. The Antioxidant Activity of PLA/Melanin Blends

2.10.1. Determination of Available Phenolic Groups on the Modified Films Surface

The method for the determination of available phenolic groups (APG) on the modified PLA/melanin films was carried out according to Bishai et al. [42]. 100 mg of PLA/melanin films or pure PLA was taken in a volumetric flask. Sequentially, 1 mL of 10% Folin-Ciocalteu reagent and 4 mL of 2% sodium carbonate solution were added to the flask. Finally, the volume was made up to 25 mL with distilled water and mixed well. The reaction mixture was kept at room temperature for 48 h and the resultant absorbance was determined at 760 nm. A control absorbance was also measured where the aforesaid reaction mixture, devoid of any film, was kept under the same reaction conditions. To determine the available phenolic groups on the modified films surface, a calibration curve was prepared using gallic acid standard solutions and the results were expressed as μmoles of gallic acid equivalents (GAE) per gram of dry film. All experiments were performed in triplicate and presented as mean ± standard deviation.

2.10.2. A Determination of the Free Radical Scavenging Activity of Modified Films

The free radical scavenging property determination of melanin incorporated PLA films was carried out using ABTS and DPPH reagents according to Bishai et al. [42]. Radical 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid ($ABTS^+$) was produced by mixing 7 mM ABTS with 2.45 mM potassium persulfate (5 mL of ABTS + 5 mL of potassium persulphate 4.9 mM). The mixture was then incubated for 16 h in the dark, at room temperature and subsequently diluted with water to an absorbance of maximum 1.00 at 734 nm. To determine the antioxidant capacity of PLA/melanin films, 1 g of the film was put into 25 mL of $ABTS^+$ solution and incubated up to 24 h at room temperature. Control sets without the film were also kept under identical conditions. After incubation period, the film samples were removed from the $ABTS^+$ solution. Absorbance for both sets was taken and antioxidant activity ($AA\%$) was calculated using the equation:

$$AA\% = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100 \quad (4)$$

1 g of modified PLA/melanin films was taken in 25 mL of 0.004% (w/v) methanolic DPPH solution. A set of controls was also placed where 25 mL of the same solution was taken, excluding the addition of the film. Both samples were incubated up to 24 h, at room temperature. After incubation period, the film samples were removed from the DPPH solution. Sample and control set absorbance were measured at 517 nm and the free radical activity of the polymeric films was calculated according to the same equation as the ABTS method.

2.11. The Antimicrobial Activity of Films

The test microorganisms used in this study were obtained from the American Type Culture Collection (ATCC). The strains used in this study were *Escherichia coli* ATCC8739, *Enterococcus faecalis* ATCC29212, *Pseudomonas aeruginosa* ATCC2783, *Pseudomonas putida* ATCC31753 and *Staphylococcus aureus* ATCC12600. To verify the antimicrobial properties of films Mueller-Hinton medium (Merck,

Darmstadt, Germany) was used. The medium was prepared according to the Merck protocol (medium was weighted according to the manufacturer's instructions, suspended in 1000 mL of distilled water, and autoclaved at 121 °C for 15 min). The film samples were cut into square shapes (5 cm × 5 cm). The antimicrobial properties of non-modified and modified films were carried out according to ISO 22196:2007(E) standard [43].

2.12. Microscopic Examination of Films

To determine the melanin particles distribution in resulted films the samples were examined under the light microscope Zeiss SteREO Discovery.V20 (Carl Zeiss Microscopy GmbH, Jena, Germany)equipped with PlanApo S lens (2.3×, FWD 10 mm) and AxioCam MRc 5 camera, with magnitude 17.2× and 3.7 μm resolution.

2.13. Differential Scanning Calorimetry (DSC)

DSC measurements were performed using a DSC calorimeter (204 F1 Phoenix, NETZSCH, Selb, Germany) in a temperature range from −25 °C to 210 °C at $\varphi = 5$ °C/min, performing two heating and one cooling scans. Melting and cold crystallization temperatures and enthalpies (T_m, T_c, ΔH_m, ΔH_c) were determined from the second heating scan and glass transition temperatures (T_g) were also measured. The crystallinity degree (χ) was calculated according to the following Equation:

$$\chi(\%) = (\Delta H_m / W \Delta H_{m0}) \times 100 \quad (5)$$

where ΔH_m is the enthalpy for melting, ΔH_{m0} is enthalpy of melting for a 100% crystalline PLA sample (taken as 93 J/g) and W is weight fraction of PLA in the sample [16].

All the experiments were carried out under nitrogen flow. The results were visualized and analyzed using NETZSCH Proteus Software (v6.0, NETZSCH-Gerätebau GmbH, Selb, Germany).

2.14. Statistical Analyses

All determinations were carried out in triplicate as a minimum. Statistical significance was determined using an analysis of variance (ANOVA) followed by Duncans's test. The values were considered as significantly different when $p < 0.05$. All analyses were performed with Statistica version 10 (StatSoft Polska, Kraków, Poland).

3. Results

3.1. Material Processing

The process of extrusion was even and stable and no "disturbing" effects regarding the addition of melanin in a form of powder were observed. All parameters, such as pressure and motor current remained at typical values for PLA. The process of compounding the composite was preceded by drying according to the technical data sheet.

3.2. Mechanical Properties

Table 1 shows that the ratios 0.025% and 0.05% were able to slightly improve the tensile strength of the PLA-based films in a transversal direction ($p < 0.05$). It was observed that the highest concentration of melanin (0.2%) led to a decrease in tensile strength in both directions, as well as a burst strength in the PLA-based films ($p < 0.05$). The results obtained for the other two concentrations were comparable to the results of the reference film. With regards to the burst strength of the samples, the lowest (sample "1") addition of melanin to the polymer matrix increased the maximum force value slightly—from 23.50 ± 2.41 MPa to 27.45 ± 1.45 MPa. Sample "3" exhibited the lowest value, whereas sample "2" had an almost identical value to the reference sample "0" ($p > 0.05$). All the other differences between the results were statistically significant as proved by the Duncan test that $p < 0.05$.

Table 1. Tensile strength in machine direction (TS MD), tensile strength in transversal direction (TS TD), burst strength (BS), seal strength (SS) and Water Vapor Transmission Rate (WVTR) of pure PLA and PLA/melanin modified films.

Sample	TS MD (MPa)	TS TD (MPa)	BS (MPa)	SS (MPa)	WVTR (g/(m^2 × Day))
0	59.47 ± 8.86	45.87 ± 1.01	23.50 ± 2.41	10.18 ± 1.68	24.60 ± 0.33
1	63.40 ± 4.30	61.55 ± 3.23	27.45 ± 1.45	8.75 ± 2.35	21.30 ± 2.56
2	63.78 ± 5.16	54.87 ± 2.56	23.78 ± 1.59	8.92 ± 0.92	23.60 ± 0.98
3	45.42 ± 2.84	40.78 ± 1.04	16.10 ± 2.92	7.12 ± 0.77	28.20 ± 1.31

Due to the powder-like, non-thermoplastic characteristics of melanin it had to be taken into consideration that the additive could have deteriorated the sealing behavior of the PLA-based composite films. However, only the greatest addition (0.2%) of melanin resulted in a significant decrease in the sealing strength—from 10.18 ± 1.68 MPa to 7.12 ± 0.77 MPa ($p < 0.05$). The differences between the reference value and those obtained for samples "1" and "2" were not statistically significant ($p > 0.05$).

As concerns the dynamic mechanical analysis (DMA), the temperature curves of the storage modulus (E'), loss modulus (E'') and of the loss factor (tan δ) are shown in Figure 1. The storage modulus of the sample "1" and "2" at medium temperatures is higher than that of the pure PLA film, probably because of the reinforcement effect of melanin particles. These data are in agreement with the tensile strength results, as discussed above: the addition of melanin at concentrations 0.025% and 0.05% reinforce the PLA matrix in contrast to concentration 0.2%. As shown in Figure 1B, the storage modulus decreases by increasing the temperature for all of the samples, with a significant drop in the range between 60 °C and 70 °C, corresponding to the glass transition region. Figure 1C shows also the loss modulus of pure PLA and PLA/melanin modified films. The glass transition temperature (T_g) evaluated as the temperature at which the damping attains its maximum value is shown in Figure 1. In particular, the addition of melanin influenced slightly the T_g temperature and those results were not statistically significant ($p > 0.05$).

3.3. *Surface Properties—Contact Angle*

Three repetition tests were performed for each sample of the PLA-based films. The average values of the contact angle obtained for distilled water were as follows: 66.96°; 67.67°; 67.00°; 67.25° for samples "0", "1", "2", and "3", respectively. The Duncan's test was applied to demonstrate that these differences of averaged values were statistically insignificant ($p > 0.05$).

3.4. *Barrier Properties—WVTR*

Polylactide itself is not a high barrier material, both in terms of transmission of oxygen and water vapor. The water vapor transmission rate of all four samples was measured by means of a gravimetric method, which is based on the sorption of humidity by calcium chloride and a weight gain comparison of the samples. As reported in the Table 1 sample "1" exhibited the lowest values of WVTR which was 21.30 ± 2.56 g/(m^2 × day), whereas the reference value was 24.60 ± 0.33 g/(m^2 × day). However, after considering standard deviations and statistical analysis, these differences are not statistically significant. Sample "2" had a comparable value with the reference value and sample "3" exhibited the highest value of 28.20 ± 1.31 g/(m^2 × day). As before, Duncan test was carried out in order to verify the differences between WVTR values. This was calculated by Statistica software that following differences: "0" vs. "1", "0" vs. "2" and "0" vs. "3" were statistically significant ($p < 0.05$). All of the other pairs of average values were not statistically significant ($p > 0.05$).

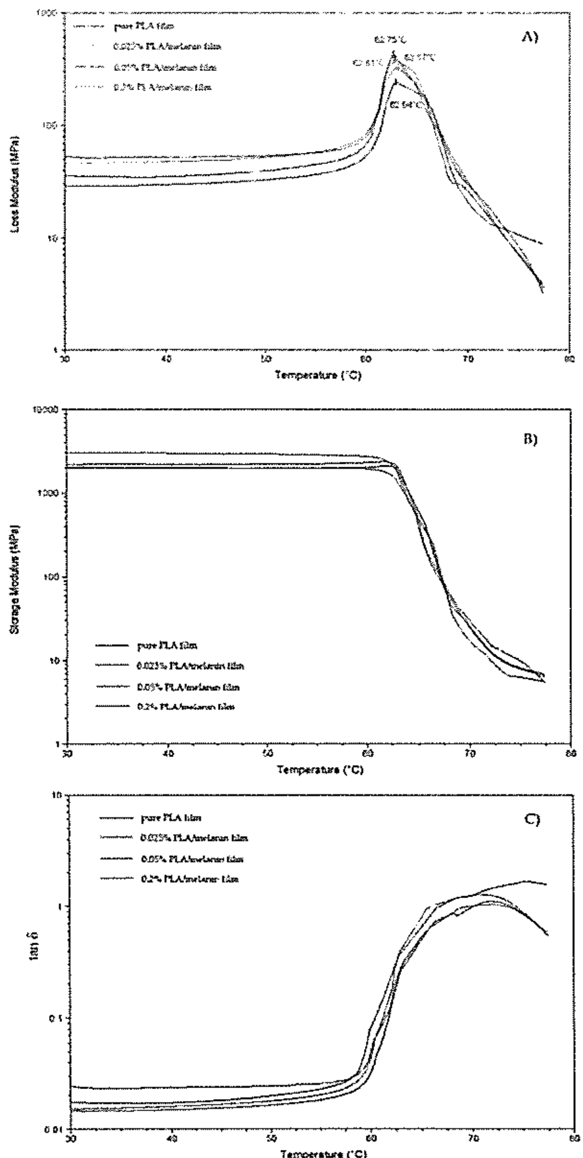

Figure 1. Loss modulus (A); storage modulus (B) and tan δ (C) of pure poly(lactic acid) (PLA) and PLA/melanin modified films.

3.5. The Spectral Analysis of Modified Films

3.5.1. UV-Vis Spectra

UV-Vis spectra of pure PLA and PLA/melanin films are shown in Figure 2. The addition of melanin caused moderate improvement of the light barrier properties. Sample "3" showed approximately 7–8% lower transmittance values at UV-A and UV-B regions. From 250 nm all samples showed the same transmittance pattern.

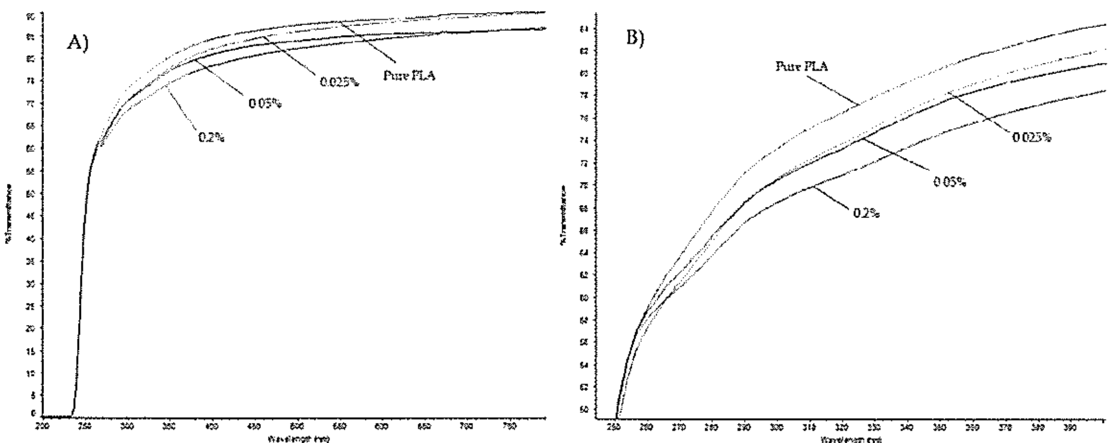

Figure 2. The UV-Vis spectra of pure PLA and PLA/melanin modified films at 200–800 nm (**A**) and 250–400 nm (**B**).

3.5.2. The FT-IR Spectra

The FT-IR spectra of pure PLA and PLA/melanin films are shown in Figure 3. The strong IR bands at 2997.69 cm^{-1}; 2947.48 cm^{-1} are assigned to the -CH stretching region ($-CH_{3(asymm)}$, $-CH_{3(symm)}$, and –CH modes). The C=O stretching region appeared in IR spectra at about 1747.21 cm^{-1} as a broad asymmetric band mainly due to A and E_1 active modes. $-CH_3$ was responsible for the appearance of the band at 1454.49 cm^{-1}. –CH deformation and asymmetric bands appeared at 1382.12 cm^{-1} and 1360.76 cm^{-1}. Moreover, the –CH bending modes resulted in bands at 1315 cm^{-1} and 1300 cm^{-1}. The C–O stretching modes of the ester group appeared at 1266.81 cm^{-1} and the C–O–C asymmetric mode appeared at 1080.13 cm^{-1}. At 956.26 cm^{-1}, bands characteristic of helical backbone vibrations with CH_3 rocking modes were visible. At 867.67 cm^{-1} and 754.23 cm^{-1} two bands appeared that could be attributed to the amorphous and crystalline phases of PLA, respectively [1]. These peaks can be also assigned to –C–C– stretching and C=O stretching, respectively [16]. Only the spectrum of PLA/melanin film with the highest melanin content (sample "3") offered noticeable differences, mainly at wavelengths: 1454.49 cm^{-1}; 1382.12 cm^{-1}; 1360.76 cm^{-1}; 1127.94 cm^{-1}; 1042.93 cm^{-1}; 956.26 cm^{-1}; 867.67 cm^{-1}; 754.23 cm^{-1} and 705.41 cm^{-1}. Those differences were the result of melanin addition into PLA.

3.5.3. The Raman Spectra

Raman spectroscopy of pure PLA and modified PLA melanin films can be seen in Figure 4. Pure PLA spectra can be characterized with stretching –C=O groups which are present at many wave number values. Weak –C=O groups are present at a frequency range from 675 cm^{-1} to 711 cm^{-1} while moderate groups can be found between 736 cm^{-1} and 760 cm^{-1}. Strong –C=O groups in PLA polymer can be found at 1773 cm^{-1}. –C=O groups broadened at a frequency range from 1700 to 1800 cm^{-1}. Specific peak group of PLA polymer appeared at 1450 cm^{-1} which is attributed to asymmetric –CH groups. $-CH_3$ symmetric group used to appear at a frequency range between 1384 and 1388 cm^{-1} shifted to 1390 cm^{-1}. Then, $-CH_3$ asymmetric groups are found at 1128 cm^{-1}. Raman spectra at a frequency range of 1179 and 1216 cm^{-1} were moderate –C–O–C asymmetric groups.

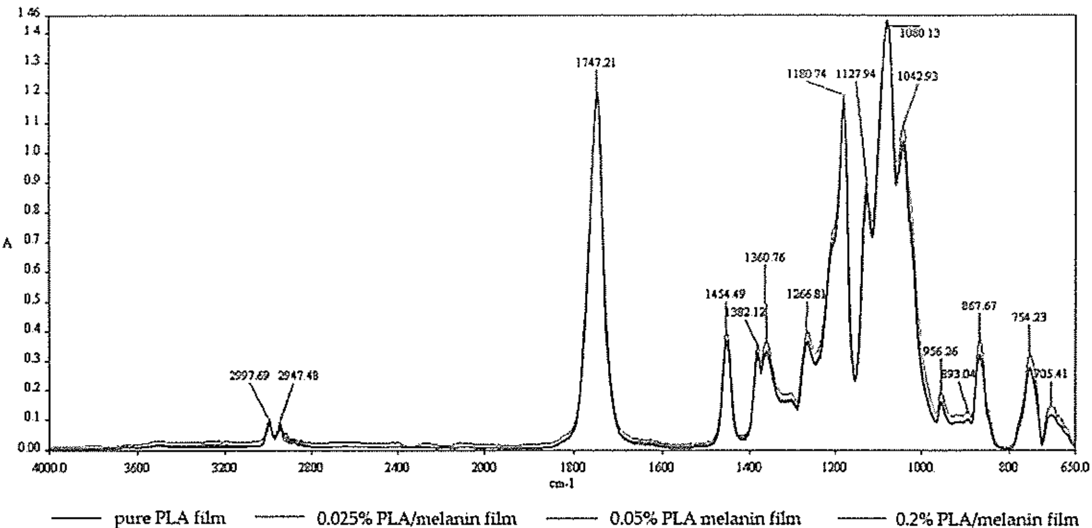

Figure 3. The FT-IR spectra of pure PLA and PLA/melanin modified films.

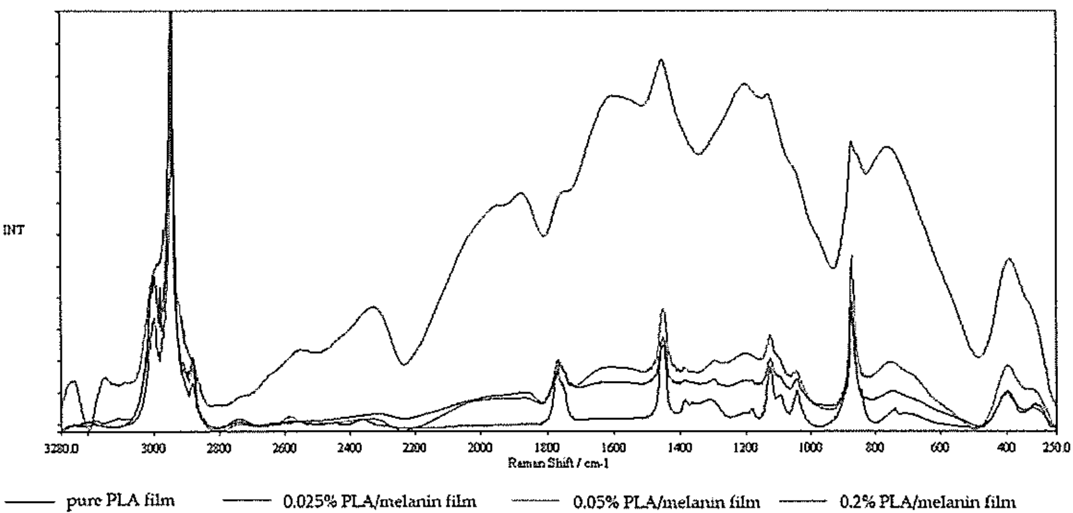

Figure 4. The Raman spectra of pure PLA and PLA/melanin modified films.

3.6. The Visual Appearance and Color

The visual appearance of pure PLA and PLA/melanin modified films is shown in Figure 5 while the results of microscopic examination are shown in Figure 6. As can be seen in Figure 6 the distribution of melanin particles in polymer matrix was homogenous. The majority of melanin particles size was less than 1 μm. The color, ΔE, YI and WI values are presented in Table 2. The growing addition of melanin influenced the color values in comparison to pure PLA film, causing a reduction in the lightness (L^*) and an increase in the redness (a^*) and yellowness (b^*) values. Color differences were statistically significant ($p < 0.05$). ΔE values ranged from 0.60 (sample "1") to 1.33 (sample "3"). The yellowness (YI) increased with increasing melanin amount, in contrast, the whitening index (WI) decreased when the melanin content was increasing.

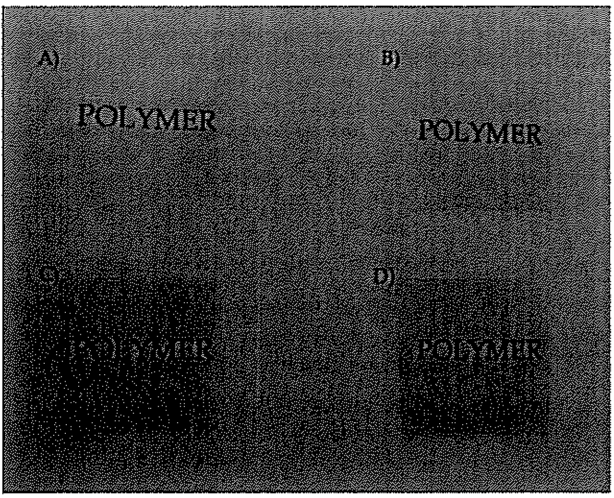

Figure 5. The visual appearance of the films: (A) pure PLA film (B) 0.025% PLA/melanin film (C) 0.05% PLA/melanin film (D) 0.2% PLA/melanin film.

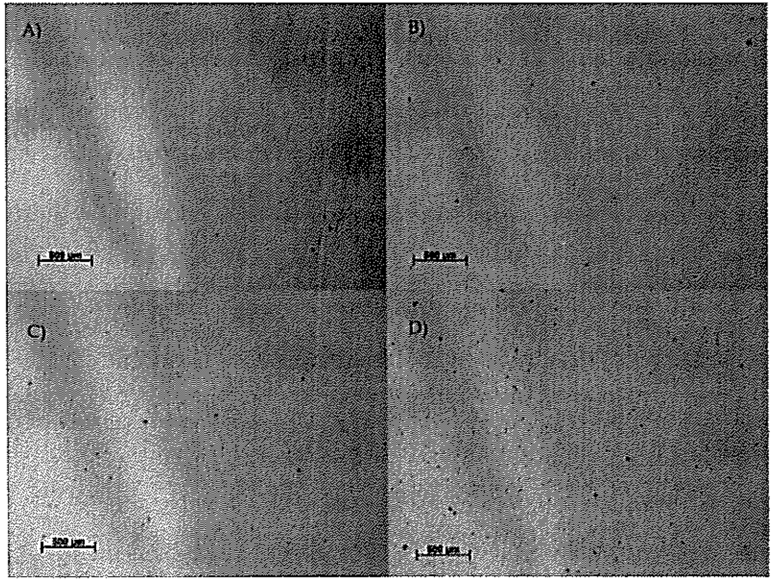

Figure 6. The results of microscopic examination of the films: (A) pure PLA film (B) 0.025% PLA/melanin film (C) 0.05% PLA/melanin film (D) 0.2% PLA/melanin film.

Table 2. Color parameters (L^*, a^*, b^*), ΔE, yellowness index (*YI*), whitening index (*WI*) and opacity of pure PLA and PLA/melanin modified films.

Sample	L^*	a^*	b^*	ΔE	*YI*	*WI*	Opacity
0	97.41 ± 0.00	0.01 ± 0.00	0.21 ± 0.01	used as standard	0.31	97.40	6.90 ± 0.09
1	97.03 ± 0.00	0.05 ± 0.00	0.67 ± 0.00	0.60	0.99	96.95	6.67 ± 0.04
2	97.02 ± 0.00	0.07 ± 0.00	0.78 ± 0.00	0.69	1.15	96.92	6.53 ± 0.07
3	96.51 ± 0.01	0.18 ± 0.00	1.18 ± 0.00	1.33	1.75	96.31	6.41 ± 0.12

3.7. Opacity

The opacity of pure PLA and PLA/melanin modified films is shown in Table 2. The opacity values of modified PLA/melanin films were lower than the pure PLA film. The opacity of PLA/melanin

films decreased after the addition of melanin particles (from 6.9 ± 0.09 of sample "0" to 6.41 ± 0.12 of sample "3"). This may have been due to the color and the content of the melanin particles. Those differences were not statistically significant ($p > 0.05$).

3.8. Antioxidant Activity

Table 3 presents results of an assessment of the available phenolic groups on the films surface and antioxidant activity of pure PLA and PLA/melanin modified films. The total available phenolics were determined to be 0.018; 0.020 and 0.033 μmole GAE/g film for samples "1", "2" and "3", respectively. No polyphenolics were detected in the pure PLA film (sample "0"). The antioxidant activity of modified PLA/melanin films grew with the increasing content of melanin, reaching 21.66% and 23.20%, which was determined by DPPH and ABTS methods, respectively. No antioxidant activity of the unmodified PLA film was observed. Differences between the modified films and pure PLA were statistically significant ($p < 0.05$).

Table 3. The antioxidant activity determined by ABTS (*AA*% ABTS) and DPPH (*AA*% DPPH) methods and available phenolic groups (APG) of pure PLA and PLA/melanin modified films.

Sample	*AA*% ABTS (%)	*AA*% DPPH (%)	APG (μmole GAE/g)
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
1	5.83 ± 0.11	5.69 ± 0.04	0.0181 ± 0.006
2	11.41 ± 0.23	7.43 ± 0.12	0.0205 ± 0.013
3	23.20 ± 0.09	21.66 ± 0.15	0.0234 ± 0.009

3.9. The Crystallization Characterization—DSC

In this study, DSC measurements were carried out to investigate the thermal characteristics of the films. The glass transition temperatures (T_g), cold crystallization temperatures (T_c), melting temperatures (T_m) and crystallinity degrees (χ) of the tested materials at φ = 5 °C/min are summarized in Table 4. The thermograms corresponding to the second heating scan (A) and cooling (B) of pure PLA and PLA/melanin modified films are shown in Figure 7. Compared to the pure PLA film, the addition of melanin particles did not significantly affect the T_g and T_m ($p > 0.05$) of the modified PLA/melanin films. T_g of films resulted from DSC are comparable to the results of DMA analysis. The PLA/melanin films displayed double melting behavior. It can be seen that the degree of crystallinity (χ) of pure PLA was 0.23%, while crystallinity degrees of samples "1", "2" and "3" were 0.92%; 2.43%; 1.61% respectively.

Table 4. Thermal characteristics of pure PLA and PLA/melanin films.

Sample	T_g (°C)	T_c (°C)	T_m (°C)	χ (%)
0	61.4	112.9	148.7	0.23
1	61.2	107.1	147.4	0.92
2	61.5	108.3	147.9	2.43
3	61.5	107.3	147.5	1.61

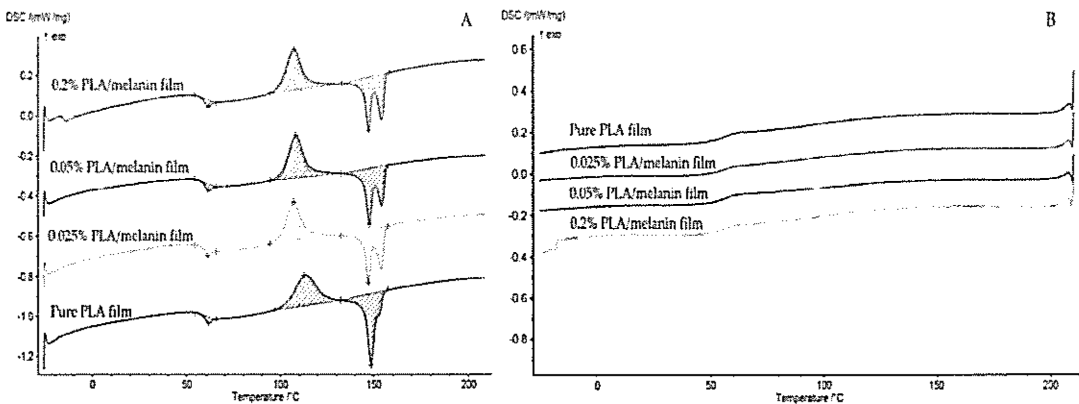

Figure 7. The DSC curves of pure PLA and PLA/melanin modified films (**A**) second heating scan (**B**) cooling.

3.10. *Antimicrobial Activity*

The susceptibility assay of *E. coli, E. faecalis, P. aeruginosa, P. putida* and *S. aureus* with respect to the pure PLA and modified PLA/melanin films is shown in Figure 8. The results of this research determined that neither pure PLA nor modified films were found to be active against *E. coli* and *S. aureus*. As indicated by statistical analysis, the differences between numbers of *E. coli* and *S. aureus* cells exposed to pure PLA and modified PLA/melanin films were not significant ($p > 0.05$). The results of this research demonstrated that pure PLA films had no influence on the growth of *E. faecalis, P. aeruginosa* and *P. putida* cells but the cells exhibited sensitivity towards modified films. A log reduction of the number of cells was noted when bacterial cells were exposed to highest melanin concentration in modified films. As can be seen in Figure 8 the growth of *E. faecalis, P. aeruginosa* and *P. putida* were observed after contact with samples "1" and "2", but the number of bacterial cells was reduced than in comparison to control film, devoid of melanin. The differences between the number of viable cells were significant, as confirmed by a Duncan test ($p < 0.05$).

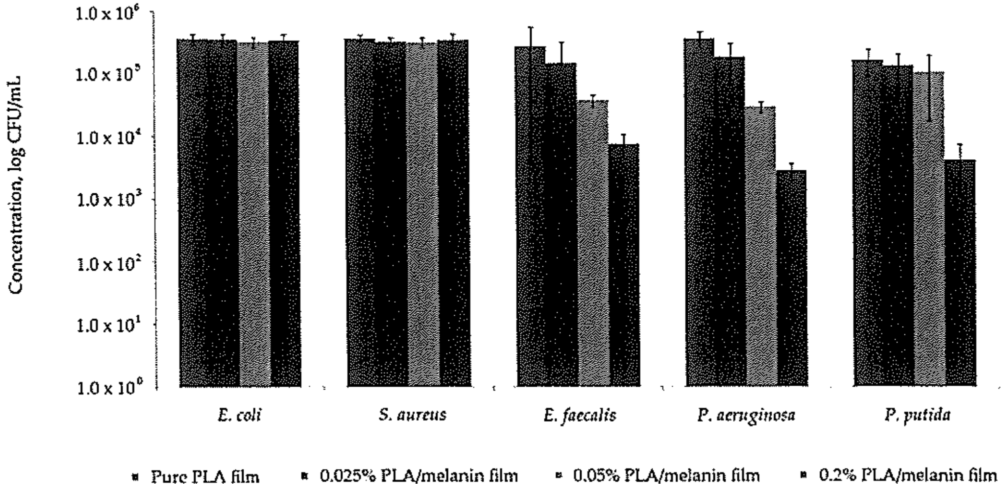

Figure 8. The influence of pure PLA and PLA/melanin modified films on *E. coli, S. aureus, E. faecalis, P. aeruginosa* and *P. putida* growth.

4. Discussion

The results of this study presented that the melanin isolated from ABW used as an additive for PLA at various concentrations may influence the properties of modified films depending on concentration. The increased availability of PLA has stimulated increased research and development activities, which can also be partly attributed to the escalating "green" movement that is encouraging the use of bio-polymers. Since the packaging industry plays a dominant role in the short-term use of cheap non-biodegradable petroleum-based materials, their replacement with PLA could provide a significant step to eco-friendly solutions [1,5,44]. Hence, the incorporation of melanin has opened up new avenues to discover its applicability in the packaging industry, such as packaging material for avoiding oxidation of sensitive food, thus expanding the spectrum of its uses.

The application of melanins for the modification of packaging polymers known from literature is relatively limited. Dong et al. modified poly(vinyl alcohol) with natural melanin from cuttlefish ink and synthetic melanin from dopamine hydrochloride autoxidation. Their results revealed that melanins may enhance the thermal stability of polymer, even in low content (0.5 mass%) due to their ability to scavenge radicals responsible for the degradation process [40]. Shanmuganathan et al. have reported that synthetic melanins derived from the oxidation of L-Dopa significantly improve the onset decomposition temperature of PMMA by 50–90 °C, when the addition of melanins amounts are low (0.5–5 mass%) [39]. Kiran et al. synthetized nanomelanin-polyhydroxybutyrate nanocomposite film which showed antioxidant activity and a strong protective effect against multidrug-resistant *Staphylococcus aureus* [41]. It is noteworthy that melanin itself may be used to prepare thin films [45–47].

In order to adequately preserve the quality of food goods, the packaging materials have to provide efficient barriers against light, water vapor, atmospheric gases and volatile organic compounds, preventing food spoilage. When the modified blend film is applied to preserve food, its integrity has to be maintained and external stress withstood, so these mechanical properties are vitally important characteristics of the film [1]. A relatively wide-ranging tensile strength (14–70 MPa) and deformation at break (1–8%) has been found, depending on the type of PLA and process [5]. Due to the lack of literature relating to the effect of melanin on the mechanical properties of PLA-based films, other non-thermoplastic substances were considered in order to discuss the results described above in this work. Rhim et al. reported that the addition of nanoclay (Cloisite 20A) had a negative impact on both, the tensile strength of the PLA-based films and elongation at break when the additive represented more than 3% (*w*/*w*) of the PLA/nanoclay composite film. Both parameters decreased explicitly with an increase of nanoclay content and eventually, when the PLA/Cloisite 20A ratio was 95:5, the values of tensile strength and elongation at break dropped by approximately 10% [11]. Jamishidan et al. described the influence of selected antioxidants on the mechanical and surface properties of PLA based bio-composites. Both α-tocopherol and ascorbyl palmitate reduced PLA tensile strength by about 8% and 43% respectively. α-tocopherol as a plasticizer, increased elongation at break by about 16% [48]. Our results suggest, that melanin in low and moderate concentrations may enhance the mechanical properties of PLA such as tensile and burst strength, whereas high melanin content may result in a deterioration of PLA properties.

Dynamic mechanical analysis is a method based on oscillating loading of a sample and an elastic and viscous response monitored as a function of temperature. The results are expressed by three parameters: the storage modulus (E'), the loss modulus (E') and the loss factor—tan δ (which is ratio E''/E'). The storage modulus usually strongly decreases when temperature crosses the dynamic glass transition (when heating at constant frequency), when the loss modulus (E'') and tan δ exhibit a peaked shape [10]. The DMA analysis for the films was carried out to determine the effect of the melanin on their thermomechanical properties. PLA is a semi-crystalline material, and its storage modulus decrease rapidly when the material enters its glass transition. Thus, it displays a region of relative stability before its modulus plummets rapidly as PLA structure approaches the melting point [10]. As can be seen in Figure 1B sample "0" and "2" shown a similar pattern, while the peak of sample "1" was more intense. On the contrary, the presence of the highest melanin content in sample "3"

hinders the polymer chains mobility, resulting in a broader and larger peak in comparison to other samples. The different shape of the tan δ peak (Figure 1C) can be explained taking into account the random placement of the melanin particles within the PLA matrix. Indeed, this arrangement leads to the overlapping of the melanin particles, thus creating some matrix-poor areas, which can influence the PLA chain mobility [10,28].

Food products are very susceptible to rancidity caused by oxidation of lipids that contain unsaturated fatty acids that can be attacked by oxygen free radicals. Antioxidants are added to foods to intercept and react with these free radicals at a faster rate than the lipid substrate. Nevertheless, the current incorporation of antioxidants throughout the entire food matrix in one large initial dose is not an efficient process due to the oxidation occurring at the surface and high initial doses of antioxidant having a pro-oxidant effect. Therefore, one emerging technology is the use of antioxidant active packaging, where the antioxidant is incorporated to a packaging material with the purpose of being delivered to the food surface during commercialization, at an appropriate rate. Most of the active packaging developments base their work on the mass transportation properties of plastic materials (sorption, migration, and permeation), and the release of the active agents depends on several factors, such as the type of polymer and type of food [49]. However, the presence of synthetic antioxidants in food is questionable, owing to the potential risks. This has been encouraged by strong consumer demand, as synthetic compounds are frequently perceived as undesirable or harmful. Natural antioxidants are preferred to artificial substances, especially by consumers [36,37]. Moreover, the use of active antioxidant packaging that incorporates natural antioxidants presents important advantages. The addition of a natural compound to the packaging may reduce the need to use synthetic antioxidants in the plastic, reducing the risk of potential toxicity by migration [49]. Several reports of PLA modifications by antioxidants are known from the literature. A wide spectrum of additives was used including humic acids [42], α-tocopherol and ascorbyl palmitate [48,50], as well as nano-lignin [51]. Some other natural additives have also been used to modify other polymers to develop antioxidant activity, such as green tea extract [49], extracts from pine bark and grapes, carotenoid-containing oleoresin from tomato processing [52], extracts of red and white grape seeds and tomato [53].

Phenolic structures present in melanin molecules are considered to be major electron donating moieties and responsible for antioxidant activity [36,37]. A standard Folin-Ciocalteu method was utilized for this purpose, which is a simple technique extensively used in the quantification of phenolic compound content in various substances. Following the methodology described by Bishai et al. [42] the total available phenolics have been determined to be 0.018; 0.020 and 0.033 μmole GAE/g film for samples "1", "2" and "3", respectively. No polyphenolics were detected in pure PLA film (sample "0"), which is evident as PLA does not contain polyphenolics. It is comparable to the results of Bishai et al. who modified PLA with the incorporation of humic acid, and in their study, the total available phenolic was determined to be a 0.075 μmole GAE/g film [42]. Ambrogi et al. stated that the addition of polyphenolics containing compounds, such as extracts from pine bark and grapes and carotenoid-containing oleoresin from tomato processing increased the oxidative stability of polypropylene films [52]. Cerruti et al. also demonstrated that natural additives, such as extracts from red and white grape seeds and tomato effectively stabilize the polymer by acting as an antioxidant [53].

To evaluate the antioxidant properties of the films, methods based on the color quenching of synthetic radical ABTS and DPPH were performed in aqueous and methanol medium, respectively. Within this reaction charged ABTS chromophore is decolorized by electrons donated by hydroxyl/phenolic groups of melanin and a decrease in absorbance was measured at 734 nm. The DPPH method was based on the presence of its odd electron, DPPH offers strong absorption maxima at 517 nm (purple) with a visible spectroscopy. As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, i.e., a free radical scavenging antioxidant, the absorption intensity is decreased. The resulting decolorization is stochiometric with respect to the number of electrons captured [36,37,42]. Modified PLA/melanin films have shown good radical scavenging activity. Our

results are comparable to results obtained by Bishai et al. who used humic acid to develop antioxidant activity in PLA films [42].

Sensitive components of food such as lipids, flavors, vitamins and pigments may undergo photodegradation reactions. The spectrum and the intensity of the light source, the conditions of light exposure, and the degree of packaging material light transmittance are factors that can significantly affect food quality. Thus, packaging plays a pivotal role in the prevention of the photodegradation of food components during storage [1]. The design of the packaging for a specific food product involves not only the choice of appropriate packaging material, but also the addition of the right additives or stabilizers to the packaging in order to provide a more efficient UV-Vis light barrier, and thus a significant improvement in the protection of food quality after storage. The absorption and transmission of light by polymers is particularly important in the food packaging industry where the packaged goods are light sensitive. Another issue in fresh food packaging is the effect of irradiation in the package, since ultraviolet light irradiation is a common method used for lowering microbial population in foods [1]. PLA has reasonably good optical properties compared to existing petroleum-based polymers [1,54]. PLA reinforced with bentonite, layered silicate, and microcrystalline cellulose has shown promising results in terms of a reduction in UV transmission and visual radiation, which can be advantageous in packaging applications [1]. Shanmuganathan et al. have reported that the incorporation of melanins at very low levels (0.5–5 mass%) to PMMA, decreased the transmittance of material by 80% [39]. At 225 nm PLA shows a significant increase in UV light transmitted, reaching about 85% at 250 nm and 95% at 300 nm. Thus, most of the UV-B and UV-A radiation passes through the PLA films. No UV radiation transmission was found in the lower range of UV, in the 190–220 nm wavelength region. As shown in Figure 2 the addition of melanin into PLA produced approximate 8% improvement in UV-Vis barrier properties at 400 nm and approximately 7% at 280 nm. PLA the maximum absorbance wavelength is 240 nm and can be attributed to the ester group present in the polymer [1]. The melanin transmittance pattern is very similar to that of PLA, thus the UV-Vis barrier properties were not strongly enhanced, but nevertheless, some improvement was observed.

FT-IR and Raman spectroscopies are useful and highly important tools to characterize the physicochemical nature of the polymers [1,9,10,15]. Due to the high sensitivity of FT-IR spectroscopy to changes in the dipole moment of a given vibrating group, this technique was used to identify polar groups. In contrast to Raman spectroscopy, it is especially useful in the characterization of the homonuclear polymer backbone due to its sensitivity to changes and polarizability [1]. Peaks of 867.67 cm^{-1} and 754.23 cm^{-1} appear as two bands that can be attributed to the amorphous and crystalline phases of PLA, respectively, suggesting, that the films obtained may have both phases in their structure [1]. No evident differences between samples 0, 1 and 2 were observed. This may be caused by fact that FT-IR spectroscopy is a rather shallow penetrative technique (approximately 0.5 μm at 4000 cm^{-1} up to 5 μm at 400 cm^{-1}) whereas film thickness was 75–80 μm. Consequently, the more penetrative Raman spectroscopy was used [55,56]. Results of the Raman spectroscopy analysis showed noticeable differences in the obtained spectra. With higher melanin content peaks were observed with greater insensitivity. The peaks can be interrelated as originating from the in-plane stretching of the aromatic rings and the linear stretching of the C–C bonds within the rings, along with some contributions from the C–H vibrations in the methyl and methylene groups in the melanin molecules [57]. A peak at 2000 cm^{-1} is similar to those obtained by Galvan et al. from eumelanin and may be caused by the stretching of three of the six C–C bonds within the melanin aromatic rings [58]. It was noted, that on all modified films, Raman spectra peaks at 395 cm^{-1} are present, which are thought to correspond to peaks obtained from pheomelanin and eumelanin and are caused by an out-of-plane deformation of the phenyl rings. Peaks at 2010 cm^{-1} are also similar to peaks seen in pheomelanin and are probably due to overtone or combination bands [57,58].

The water contact angle of the material is associated with its hydrophilicity. In general, the smaller the water contact angle, the higher the hydrophilicity. In the work of Cui et al. the water angle of pure PLA was approximately 72° [9]. In our work, the contact angle of pure PLA was approximately 66.96°.

This discrepancy may be a result of the PLA compositions from various manufacturers. The addition of melanin into PLA at all concentrations did not significantly ($p > 0.05$) affected the surface properties of the polymer. In contrast, in a study by Jamishidan et al. the authors observed that ascorbyl palmitate decreased the PLA film contact angle (of a water droplet) and increased the polarity and wettability of the material [48].

One of the most important properties of bio-based films for the application of packaging is to minimize moisture transfer from the environment to the packed goods. Water vapor permeability (WVP) is one of the most important properties in food packaging due to the noticeable role water has in deteriorative reactions and microbial growth. For this purpose, the WVP of packaging materials should be as low as possible [1,16,20,59]. Jamishidan et al. observed that pure PLA films had parameters that were minimally lower than compared to the films containing either 2% of α-tocopherol or 1% of ascorbyl palmitate. However, no significant differences between the values were reported [48]. Rhim et al. proved that the incorporation of Cloisite 20A nanoclay decreased the values by less than 3% [11]. In our study, the addition of 0.025% of melanin into the PLA matrix resulted in improved barrier properties. This may be caused by the intramolecular interactions of the melanin particles and PLA chains. However, at higher concentrations (0.05% and 0.2%), the WVTR of modified blends was exacerbated than compared to neat PLA. This might be attributed to the fact that the addition of some compounds in high concentrations may increase the average pore size of the films, thus facilitating water molecule penetration into the polymer matrix [16].

Some additives for PLA composites may influence the color values of the modified blends. Li et al. developed antimicrobial packaging film made from PLA with TiO_2 and Ag nanoparticles. The addition of TiO_2 increased the L^* (lightness) value of the modified films, due to the white color of nano-TiO_2 powder, but in contrast, the addition of Ag nanoparticles resulted in a dark color explained by the argentous sheen of the nano-Ag powder [16]. Liu et al. noted that the addition of oregano essential oil had a clear influence on the color values of the PLA films [20]. Shi et al. observed that the co-polymerization of PLA with 3,4-dihydroxyphenylalanine, an amino acid being a precursor of DOPA melanin resulted in a yellow-brown color in the modified films [60]. Also Kiran et al. observed that synthetized nanomelanin-polyhydroxybutyrate nanocomposite film is characterized by a yellowish-brownish color [41]. Our results indicate that the increasing addition of melanin influences the color values in comparison to pure PLA film, leading to reduction in lightness (L^*), as well as an increase in the redness (a^*) and yellowness (b^*) values. ΔE values ranged from 0.60 to 1.33. $\Delta E > 1$ is considered perceptible to the human eye, so the highest melanin content caused noticeable color changes. The yellowness (*YI*) increased with increasing melanin amount, while the whitening index (*WI*) decreased when the melanin content was increased. The yellowness index or a change in the degree of yellowness is a number calculated from spectrophotometric data that describes the change in color of a test sample from clear or white to yellow. The opacity of PLA/melanin films decreased with the addition of melanin particles (from 6.90 ± 0.09 of sample "0" to 6.41 ± 0.12 of sample "3"). This was probably due to the color of the melanin powder. The changes of film transparency as a consequence of the addition of nanoparticles had been reported with PLA films [15]. However, the difference in opacity among film samples was not perceptible to the human eye and not statistically significant ($p > 0.05$). PLA/melanin films in all melanin concentrations still had good transparency, even at high melanin content. This result suggested high PLA/melanin film transparency, meaning that packaging film could be transparent, which an important requirement for consumers and this would have a clear influence on customer choice [16].

Compared to the neat PLA film, the addition of melanin particles into polymer matrix did not affect the T_g and T_m of PLA. This might be because the addition of the melanin did not change the mobility of the PLA macromolecular chains [16,20]. On the other hand, it can be seen in Figure 7, that the cold crystallization temperature (T_c) was changed by the introduction of melanin and led to an obvious decrease in comparison to pure PLA. Similar observations were made by Li et al. [16] who found that the addition of titanium dioxide and silver nanoparticles did not result in obvious changes

in thermal transitions of PLA, but introduction of nano-TiO_2 decreased the T_c values of modified films. As can be seen in Figure 7, the addition of melanin changed the melting behavior of resulted films in comparison to pure PLA. Under this heating conditions, the amorphous PLA chains do not have enough time to self-adjust, resulting in reduced cold crystallization and the subsequent multi-melting behavior, which is indicative of polymorphism, melt recrystallization [61] or to lamellar populations with different perfection degrees [62]. As can be seen in Figure 7 the enthalpy of crystallite formation in cold crystallization temperature is in fact almost identical to the melting enthalphy of crystallites at the melting point, which means that all crystallites in the polymer matrix rise above approximately 100 °C, which is confirmed by the cooling curve, where there is no peak of crystallization, only the glass transition temperature. As listed in Table 4, the addition of melanin led to a significant increase of the χ values of modified PLA/melanin films in comparison to the PLA film devoid of melanin. Crystallization degrees of pure PLA and PLA/melanin films values are relatively low indicating, that all the tested samples presented high amorphic structure. According to Li et al. this result can be explained by the phenomenon of heterogeneous nucleation [16]. Considering the increase in χ and decrease in the cold crystallization peaks as a result of melanin addition it is tempting to suggest that melanin is acting as a nucleating agent. Sullivan et al. observed that cellulose nanocrystals also can act as nucleating agent when added to PLA [61], whereas Bishai et al. [42] noticed that incorporation of humic acid into PLA matrix reduced its crystallinity.

Modified PLA/melanin films showed antibacterial activity against *E. faecalis*, *P. aeruginosa* and *P. putida*. No antibacterial activity towards, *E. coli* and *S. aureus* was observed. This data are supported by previous study showed that melanins from some fungi have been active against *P. aeruginosa* and *E. faecalis* [36,37]. The literature on melanin applications to develop antimicrobial properties of polymer film is relatively limited. Kiran et al. synthetized nanomelanin-polyhydroxybutyrate nanocomposite film which showed a strong protective effect against multidrug-resistant *Staphylococcus aureus* [41]. Kuang et al. developed antimicrobial DOPA-melanin coatings that were able to load and release of a cationic aminoglycoside active against *S. aureus* [45]. Some authors noted antimicrobial activity of melanins from various microbial sources. Helan Soundra Rani et al. [63] noted the antimicrobial activity of melanin isolated from halophilic black yeast *Hortaea werneckii*. Laxmi et al. [64] observed that growth of *P. aeruginosa* was inhibited on the presence of melanin obtained from *Providencia rettgeri*. Xu et al. [65] analyzed the antimicrobial activity of melanin from *Lachnum* YM30 and noted that it was active against a wide spectrum of bacteria, including *S. aureus*. The authors suggest that melanin antibacterial activity might result from damage of the cell membrane and affect bacteria membrane function. A discrepancy in melanin antimicrobial activity may result in differences within the molecule structure and composition but also particles size [41,66]. On the other hand, there are some reports that melanins have antibiofilm activity against pathogenic bacteria including *P. aeruginosa* and could interfere with bacterial quorum-sensing system, regulate its associated functions, and prevent bacterial pathogenesis [64,65,67,68].

5. Conclusions

This article explored the properties of modified PLA films incorporated with fungal melanin. The properties of PLA/melanin composites were compared to pure PLA. Melanin played a vital role in mechanical, antioxidant, antimicrobial and barrier properties. The UV-Vis barrier properties of modified PLA were slightly improved by the incorporation of melanin. However, tensile and burst strength, water vapor transmission rate, color and the antioxidant properties of PLA based films varied depending on the presence and amount of melanin. An improvement in the antioxidant activities of modified PLA film is worth mentioning as an important aspect of the work. Considerable improvement in these properties has been observed in comparison to pure PLA. The results described here are particularly interesting if one considers that the additives used have a natural origin and are extracted from bio-waste, providing added value in the development of sustainable alternatives to traditional synthetic antioxidants.

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References

1. Auras, R.; Lim, L.-T.; Selke, S.E.M.; Tsuji, H. *Poly(lactic acid). Synthesis, Structures, Properties, Processing, and Applications*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2010; ISBN 978-0-470-29366-9.
2. Al-Itry, R.; Lamnawar, K.; Maazouz, A. Improvement of thermal stability, rheological and mechanical properties of PLA, PBAT and their blends by reactive extrusion with functionalized epoxy. *Polym. Degrad. Stab.* **2012**, *97*, 1898–1914. [CrossRef]
3. Garrison, T.F.; Murawski, A.; Quirino, R.L. Bio-Based Polymers with Potential for Biodegradability. *Polymers* **2016**, *8*, 262. [CrossRef]
4. Hamad, K.; Kaseem, M.; Yang, H.W.; Deri, F.; Ko, Y.G. Properties and medical applications of polylactic acid: A review. *Express Polym. Lett.* **2015**, *9*, 435–455. [CrossRef]
5. Muller, J.; González-Martínez, C.; Chiralt, A. Combination of Poly(lactic) Acid and Starch for Biodegradable Food Packaging. *Materials* **2017**, *10*, 952. [CrossRef] [PubMed]
6. Siracusa, V.; Rosa, M.D.; Iordanskii, A.L. Performance of Poly(lactic acid) Surface Modified Films for Food Packaging Application. *Materials* **2017**, *10*, 850. [CrossRef] [PubMed]
7. Bayer, I.S. Thermomechanical Properties of Polylactic Acid-Graphene Composites: A State-of-the-Art Review for Biomedical Applications. *Materials* **2017**, *10*, 748. [CrossRef] [PubMed]
8. Aldana, D.S.; Villa, E.D.; Hernández, M.D.D.; Sánchez, G.G.; Cruz, Q.R.; Gallardo, S.F.; Castillo, H.P.; Cassarubias, L.B. Barrier Properties of Polylactic Acid in Cellulose Based Packages Using Montmorillonite as Filler. *Polymers* **2014**, *6*, 2386–2403. [CrossRef]
9. Cui, M.; Liu, L.; Guo, N.; Su, R.; Ma, F. Preparation, cell compatibility and degradability of collagen-modified poly(lactic acid). *Molecules* **2015**, *20*, 595–607. [CrossRef] [PubMed]
10. Chieng, B.W.; Ibrahim, N.A.; Then, Y.Y.; Loo, Y.Y. Epoxidized vegetable oils plasticized poly(lactic acid) biocomposites: Mechanical, thermal and morphology properties. *Molecules* **2014**, *19*, 16024–16038. [CrossRef] [PubMed]
11. Rhim, J.-W.; Hong, S.-I.; Ha, C.-S. Tensile, water vapor barrier and antimicrobial properties of PLA/nanoclay composite films. *LWT Food Sci. Technol.* **2009**, *42*, 612–617. [CrossRef]
12. Yourdkhani, M.; Mousavand, T.; Chapleau, N.; Hubert, P. Thermal, oxygen barrier and mechanical properties of polylactide-organoclay composites. *Compos. Sci. Technol.* **2013**, *82*, 47–53. [CrossRef]
13. Gonçalvez, C.; Gonçalvez, I.C.; Magalhães, F.D.; Pinto, A.M. Poly(lactic acid) Composites Containing Carbon-Based Nanomaterials: A Review. *Polymers* **2017**, *9*, 269. [CrossRef]
14. Fortunati, E.; Rinaldi, S.; Peltzer, M.; Bloise, N.; Visai, L.; Armentano, I.; Jiménez, A.; Latterini, L.; Kenny, J.M. Nano-biocomposite films with modified cellulose nanocrystals and synthesized silver nanoparticles. *Carbohydr. Polym.* **2014**, *101*, 1122–1133. [CrossRef] [PubMed]
15. Chu, Z.; Zhao, T.; Li, L.; Fan, J.; Qin, Y. Characterisation of Antimicrobial Poly(Lactic Acid)/Nano-Composite Films with Silver and Zinc Oxide Nanoparticles. *Materials* **2017**, *10*, 659. [CrossRef] [PubMed]
16. Li, W.; Zhang, C.; Chi, H.; Li, L.; Lan, T.; Han, P.; Chen, H.; Qin, Y. Development of Antimicrobial Packaging Film Made from Poly(lactic acid) Incorporating Titanium Dioxide and Silver Nanoparticles. *Molecules* **2017**, *22*, 1170. [CrossRef] [PubMed]
17. Scaffaro, R.; Botta, L.; Maio, A.; Gallo, G. PLA graphene nanoplatelets nanocomposites: Physical properties an release kinetics of an antimicrobial agent. *Compos. Part B Eng.* **2017**, *109*, 138–146. [CrossRef]
18. Jaszkiewicz, A.; Bledzki, A.K.; Franciszczak, P. Improving the mechanical performance of PLA composites with natural, man-made cellulose and glass fibers—A comparison to PP counterparts. *Polimery* **2013**, *58*, 435–442. [CrossRef]

19. Arrieta, M.P.; Samper, M.D.; Aldas, M.; López, J. On the Use of PLA-PHB Blends for Sustainable Food Packaging Applications. *Materials* 2017, *10*, 1008. [CrossRef] [PubMed]
20. Liu, D.; Li, H.; Jiang, L.; Chuan, Y.; Yuan, M.; Chen, H. Characterization of Active Packaging Films Made from Poly(lactic acid)/Poly(Trimethylene Carbonate) Incorporated with Oregano Essential Oil. *Molecules* 2016, *21*, 695. [CrossRef] [PubMed]
21. Bonilla, J.; Fortunati, E.; Vargas, M.; Chiralt, A.; Kenny, J.M. Effects of chitosan on the physiochemical and antimicrobial properties of PLA films. *J. Food Eng.* 2013, *119*, 236–243. [CrossRef]
22. Wan, Y.; Wu, H.; Yu, A.; Wen, D. Biodegradable polylactide/chitosan blend membranes. *Biomacromolecules* 2006, *7*, 1362–1372. [CrossRef] [PubMed]
23. Peltola, H.; Pääkkönen, E.; Jetsu, P.; Heinemann, S. Wood based PLA and PP composites: Effect of fibre type and matrix polymer on fibre morphology, dispersion and composite properties. *Compos. Part A Appl. Sci. Manuf.* 2014, *61*, 13–22. [CrossRef]
24. Morales, A.P.; Güemes, A.; Fernandez-Lopez, A.; Carcelen Valero, V.; De La Rosa Llano, S. Bamboo-Polylactic Acid (PLA) Composite Material for Structural Applications. *Materials* 2017, *10*, 1286. [CrossRef] [PubMed]
25. Shih, Y.-F.; Huang, C.-C. Polylactic acid (PLA)/banana fiber (BF) biodegradable green composties. *J. Polym. Res.* 2011, *18*, 2335–2340. [CrossRef]
26. Bajracharya, R.M.; Bajwa, D.S.; Bajwa, S.G. Mechanical properties of polylactic acid composites reinforced with cotton gin waste and flax fibers. *Procedia Eng.* 2017, *200*, 370–376. [CrossRef]
27. Sawpan, M.A.; Pickering, K.L.; Fernyhough, A. Flexural properties of hemp fibre reinforced polylactide and unsaturated polyester composites. *Compos. Part A Appl. Sci. Manuf.* 2012, *43*, 519–526. [CrossRef]
28. Botta, L.; Fiore, V.; Scalici, T.; Valenza, A.; Scaffaro, R. New Polylactic Acid Composites Reinforced with Artichoke Fibers. *Materials* 2015, *8*, 7770–7779. [CrossRef]
29. Grigsby, W.J.; Bridson, J.H.; Lomas, C.; Elliot, J.-A. Esterification of Condensed Tannins and Their Impact on the Properties of Poly(Lactic Acid). *Polymers* 2013, *5*, 344–360. [CrossRef]
30. Mizielińska, M.; Łopusiewicz, Ł.; Mężyńska, M.; Bartkowiak, A. The Influence of Accelerated UV-A and Q-SUN Irradiation on the Antimicrobial Properties of Coatings Containing ZnO Nanoparticles. *Molecules* 2017, *22*, 1556. [CrossRef] [PubMed]
31. Walczak, M.; Swiontek Brzezinska, M.; Richert, A.; Kalwasińska, A. The effect of polyhexamethylene guanidine hydrochloride on biofilm formation on polylactide and polyhydroxybutyrate composites. *Int. Biodeterior. Biodegrad.* 2015, *98*, 1–5. [CrossRef]
32. Łopusiewicz, Ł.; Mizielińska, M. Antifungal activity of PLA foils covered with ethylocelulose containing essential oils. *World News Nat. Sci.* 2017, *12*, 27–32.
33. Sun, X.; Wu, B.; Zhou, L.; Liu, Z.; Dong, Y.; Yang, A. Isolation and characterization of melanin pigment from yesso scallop *Patinopecten yessoensis*. *J. Ocean Univ. China* 2017, *16*, 279–284. [CrossRef]
34. Xiao, X.; Zhang, J.; Cheng, L.; Liu, X.; Jiang, Y.; Zhang, T. Isolation and characterization of melanin from palm fiber. *J. Nat. Fibers* 2018, 1–13. [CrossRef]
35. Kurian, N.K.; Bhat, S.G. Photoprotection and Anti-inflammatory Properties of Non-cytotoxic Melanin from Marine Isolate *Providencia rettgeri* strain BTKKS1. *Biosci. Biotechnol. Res. Asia* 2017, *14*, 1475–1484. [CrossRef]
36. Łopusiewicz, Ł. Isolation, characterisation and biological activity of melanin from *Exidia nigricans*. *World Sci. News* 2018, *91*, 111–129.
37. Łopusiewicz, Ł. *Scleroderma citrinum* melanin: Isolation, purification, spectroscopic studies with characterization of antioxidant, antibacterial and light barrier properties. *World Sci. News* 2018, *94*, 115–130.
38. Solano, F. Melanins: Skin pigments and much more—Types, structural models, biological functions, and formation routes. *New J. Sci.* 2014, *2014*, 498276. [CrossRef]
39. Shanmuganathan, K.; Cho, J.H.; Iyer, P.; Baranowitz, S.; Ellison, C.J. Thermooxidative Stabilization of Polymers Using Natural and Synthetic Melanins. *Macromolecules* 2011, *44*, 9499–9507. [CrossRef]
40. Dong, W.; Wang, Y.; Huang, C.; Xiang, S.; Ma, P.; Ni, Z.; Chen, M. Enhanced thermal stability of poly(vinyl alcohol) in presence of melanin. *J. Therm. Anal. Calorim.* 2014, *115*, 1661–1668. [CrossRef]
41. Kiran, G.S.; Jackson, S.A.; Priyadharsini, S.; Dobson, A.D.W.; Selvin, J. Synthesis of Nm-PHB (nanomelanin-polyhydroxybutyrate) nanocomposite film and its protective effect against biofilm-forming multi drug resistant *Staphylococcus aureus*. *Sci. Rep.* 2017, *7*, 9167. [CrossRef] [PubMed]
42. Bishai, M.; De, S.; Adhikari, B.; Banerjee, R. A comprehensive study on enhanced characteristics of modified polylactic acid based versatile polymer. *Eur. Polym. J.* 2014, *54*, 52–61. [CrossRef]

43. International Standard ISO22196:2007(E). Plastics-Measurement of Antibacterials Activity on Plastics Surfaces. Available online: http://www.poudrox.com/poudrox/wp-content/uploads/norme_ISO221961.pdf (accessed on 11 February 2018).
44. Song, X.; Wang, M.; Weng, Y.; Huang, Z. Effect of Bamboo Flour Grafted on the Interfacial Compatibility of Polylactic Acid/Bamboo Flour Composites. *Polymers* 2017, *9*, 323. [CrossRef]
45. Kuang, J.; Guo, J.L.; Messersmith, P.B. High ionic strength formation of DOPA-melanin coating for loading and release of cationic microbial compounds. *Adv. Mater. Interfaces* 2014, *1*, 1–12. [CrossRef] [PubMed]
46. Morresi, L.; Ficcadenti, M.; Pinto, N.; Murri, R.; Cuccilioni, M.; Angeletti, M.; Tombesi, P. Optical and electrical behavior of synthetic melanin thin films spray-coated. *Energy Procedia* 2010, *2*, 177–182. [CrossRef]
47. Da Silva, M.I.N.; Dezidério, S.N.; Gonzalez, J.C.; Graeff, C.F.O.; Cotta, M.A. Synthetic melanin thin films: Structural and electrical properties. *J. Appl. Phys.* 2004, *96*, 5803–5807. [CrossRef]
48. Jamshidian, M.; Tehrany, E.A.; Imran, M.; Akhtar, M.J.; Cleymand, F.; Desobry, S. Structural, mechanical and barrier properties of active PLA-antioxidant films. *J. Food Eng.* 2012, *110*, 380–389. [CrossRef]
49. De Castillo, C.L.; del Mar Castro-López, M.; López-Vilariño, J.M.; González-Rodríguez, M.V. Immobilization of green tea extract on polypropylene films to control the antioxidant activity in food packaging. *Food Res. Int.* 2013, *53*, 522–528. [CrossRef]
50. Munteanu, B.S.; Aytac, Z.; Pricope, G.M.; Uyar, T.; Vasile, C. Polylactic Acid (PLA)/Silver-NP/Vitamin E nanocomposite electrospun nanofibers with antibacterial and antioxidant activity. *J. Nanopart. Res.* 2014, *16*, 2643. [CrossRef]
51. Kai, D.; Ren, W.; Tian, L.; Chee, P.L.; Liu, Y.; Ramakrishna, S.; Loh, X.J. Engineering Poly(lactide)-Lignin Nanofibers with Antioxidant Activity for Biomedical Applcation. *ASC Sustain. Chem. Eng.* 2016, *4*, 5268–5276. [CrossRef]
52. Ambrogi, V.; Cerruti, P.; Carfagna, C.; Malinconico, M.; Marturano, V.; Perrotti, M.; Persico, P. Natural antioxidants for polypropylene stabilization. *Polym. Degrad. Stab.* 2011, *96*, 2152–2158. [CrossRef]
53. Cerruti, P.; Malinconico, M.; Rychly, J.; Matisova-Rychla, L.; Carfagna, C. Effect of natural antioxidants on the stability of polypropylene films. *Polym. Degrad. Stab.* 2009, *94*, 2095–2100. [CrossRef]
54. Peinado, V.; Castell, P.; García, L.; Fernández, Á. Effect of Extrusion on the Mechanical and Rheological Properties of a Reinforced Poly(lactic acid): Reprocessing and Recycling of Biobased Materials. *Materials* 2015, *8*, 7106–7117. [CrossRef] [PubMed]
55. Yuniarto, K.; Purwanto, Y.A.; Purwanto, S.; Welt, B.A.; Purwadaria, H.K.; Sunarti, T.C. Infrared and Raman Studies on Polylactide Acid and Polyethylene Glycol-400 Blend. *AIP Conf. Proc.* 2016, *1725*, 020101. [CrossRef]
56. Smith, P.B.; Leugers, A.; Kang, S.; Yang, X.; Hsu, S.L. Raman characterization of orientation in poly(lactic acid) films. *Macromol. Symp.* 2001, *175*, 81–94. [CrossRef]
57. Huang, Z.; Lui, H.; Chen, M.X.K.; Alajlan, A.; McLean, D.I.; Zeng, H. Raman spectroscopy of in vivo cutaneous melanin. *J. Biomed. Opt.* 2004, *9*, 1198–1205. [CrossRef] [PubMed]
58. Galván, I.; Jorge, A.; Ito, K.; Tabuchi, K.; Solano, F.; Wakamatsu, K. Raman spectroscopy as non-invasive technique for quantification of melanins in feathers and hairs. *Pigment Cell Melanoma Res.* 2013, *26*. [CrossRef] [PubMed]
59. Arrieta, M.P.; López, J.; Ferrándiz, S.; Peltzer, M.A. Characterization of PLA-limonene blends for food packaging. *Polym. Test.* 2013, *32*, 760–768. [CrossRef]
60. Shi, D.; Shen, J.; Zhao, Z.; Shi, C.; Chen, M. Studies on Preparation of Poly(3,4-Dihydroxyphenylalanine)-Polylactide Copolymers and the Effect of the Structure of the Copolymers on Their Properties. *Polymers* 2016, *8*, 92. [CrossRef]
61. Sullivan, E.M.; Moon, R.J.; Kalaitzidou, K. Processing and Characterization of Cellulose Nanocrystals/Polylactic Acid Nanocomposite Films. *Materials* 2015, *8*, 8106–8116. [CrossRef] [PubMed]
62. Fortunati, E.; Armenato, I.; Zhou, Q.; Puglia, D.; Terenzi, A.; Berglund, L.A.; Kenny, J.M. Microstructure and nonisothermal cold crystallization of PLA composites based on silver nanoparticles and nanocrystalline cellulose. *Polym. Degrad. Stab.* 2012, *97*, 2027–2036. [CrossRef]
63. Helan Soundra Rani, M.; Ramesh, T.; Subramanian, J.; Kalaiselvam, M. Production and Characterization of Melanin Pigment from Halophilic Black Yeast *Hortaea werneckii*. *Int. J. Pharm. Res. Rev.* 2013, *2*, 9–17.
64. Laxmi, M.; Kurian, N.K.; Smitha, S.; Bhat, S.G. Melanin and bacteriocin from marine bacteria inhibit biofilms of foodborne pathogens. *Indian J. Biotechnol.* 2016, *15*, 392–399.

65. Xu, C.; Li, J.; Yang, L.; Shi, F.; Yang, L.; Ye, M. Antibacterial activity and a membrane damage mechanism of *Lachnum* YM30 melanin against *Vibrio parahaemolyticus* and *Staphylococcus aureus*. *Food Control* 2017, *73*, 1445–1451. [CrossRef]
66. Correa, N.; Covarrubias, C.; Rodas, P.I.; Hermosilla, G.; Olate, V.R.; Valdés, C.; Meyer, W.; Magne, F.; Tapia, C.V. Differential Antifungal Activity of Human and Cryptococcal Melanins with Structural Discrepancies. *Front. Microbiol.* 2017, *8*, 1–13. [CrossRef] [PubMed]
67. Bin, L.; Wei, L.; Xiaohong, C.; Mei, J.; Mingsheng, D. In vitro antibiofilm activity of the melanin from *Auricularia auricula*, an edible jelly mushroom. *Ann. Microbiol.* 2012, *62*, 1523–1530. [CrossRef]
68. Zhu, H.; He, C.-C.; Chu, Q.-H. Inhibition of quorum sensing in *Chromobacterium violaceum* by pigments extracted from *Auricularia auricular*. *Lett. Appl. Microbiol.* 2011, *52*, 269–274. [CrossRef] [PubMed]

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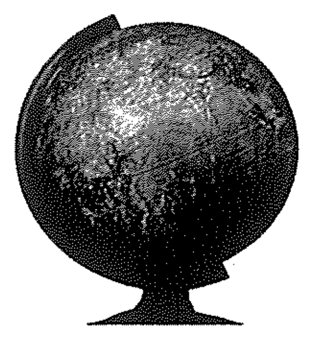

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New Active Packaging Films Made from Gelatin Modified with Fungal Melanin

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ABSTRACT

Fungal melanin was used to prepare gelatin-based composite films. The films of various melanin concentrations (0.1%, 0.5% and 1% w/w) were prepared using a solution casting method. The mechanical, antioxidant, antimicrobial, water vapor, oxygen and UV-Vis barrier properties, as well as any available surface polyphenolics were studied. FT-IR and Raman spectroscopy studies were carried out to analyse the chemical composition of the resulting films. The hydrophobocity, solubility, colour response, optical properties and opacity were also determined. The results of this study showed that the modification of gelatin with fungal melanin had no influence on mechanical and water vapor barrier properties, oxygen barrier properties were improved. The UV-Vis barrier properties of modified films were significantly improved. Modified gelatin films showed good antioxidant activity but were inactive against microorganisms. The modification with melanin caused changes in colour values, decreasing lightness and increasing the redness and yellowness of the films. Based on the results of this study, fungal melanin has good potential to be utilised as a value-added modifier that can improve the properties of gelatin films.

Keywords: gelatin, melanin, packaging, antioxidant, barrier properties, mechanical properties, *Agaricus bisporus*

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1. INTRODUCTION

Natural polymers, biopolymers, and synthetic polymers based on annually renewable resources are the basis of a 21st-century portfolio of sustainable, eco-efficient plastics. These biosourced materials are hoped to gradually replace the currently existing family of petroleum-based polymers as they become less competitive in regards to cost performance. Biodegradable polymers from renewable resources have attracted a great deal of attention in research. They are defined as polymers that undergo microbially-induced chain scission leading to mineralization [1]. The development of biodegradable packaging materials is an effective alternative to synthetic packaging material from petrochemical products, which are non-biodegradable and have a negative impact on the environment. Moreover, biodegradable materials are eco-friendly, non-toxic and have been shown to have many desirable physico-chemical characteristics over their synthetic counterparts [2-4]. In the last few decades, there has been a marked increase in the use of natural polymer-based film materials and coatings in packaging for the food industry, which protect food from external contamination, impeding its deterioration by extending shelf life and maintaining quality and safety [4]. In addition to consumer requirements and in order to substitute petroleum-based plastic packaging, a wide variety of biopolymers that come from agro-food industrial wastes and renewable low cost natural resources have emerged [5]. Packaging is widely used for the protection of food quality, thereby ensuring hygiene and extending the shelf life of perishable items, especially those susceptible to oxidative and microbiological deterioration [6]. In this context, the formulation of films and coatings for food packaging applications must include at least one component capable of forming a cohesive three-dimensional matrix [4]. Biopolymers directly extracted from biomass mainly used in edible films for food packaging are proteins, polysaccharides, lipids and their combinations. The physical and chemical properties of the biopolymer used determine the final properties of the developed films [2-4,6,7]. The protein-based films from various sources have excellent oxygen, carbon dioxide and volatile compound barrier properties, than compared with synthetic films under low relative humidity conditions [2,4,5].

Gelatin is a natural water soluble protein characterized by the absence of an appreciable odour and the random configuration of the polypeptide chains in aqueous solution. It is obtained from the denaturation and partial hydrolysis of collagen, a fibrous protein found mainly in certain parts of vertebrate and invertebrate animals as bones, skins, connective tissues and tendons. Its structure consists of rigid bar-like molecules that are arranged in fibres inter-connected by covalent bonds [6,8-10]. It has a triple-helix structure mainly stabilized by the formation of inter-chain hydrogen bonds between carbonyl and amines [4,9-12]. With respect to collagen, it does not express antigenicity in physiological conditions, it is completely resorbable in vivo, and its physicochemical properties can be suitably modulated. Furthermore, it is much cheaper and easier to obtain in concentrate solutions [13]. The abundance, availability and low cost of gelatin promote its use in a wide variety of applications [12]. It is unique among hydrocolloids as its melting point is so close to body temperature [8]. At a temperature of about 40°C, gelatin aqueous solutions are in a sol state and change into gels when they are cooled at room temperature, provided that their concentration is high enough [13,14]. The sol–gel transformation is due to a conformational

disorder–order transition of the gelatin chains which form thermo-reversible networks by associating helices in junction zones stabilized by hydrogen bonds [13].

Gelatin is accepted as a "Generally Recognized as Safe" (GRAS) substance by the U.S. Food and Drug Administration (FDA) [11]. Gelatin is widely used in the manufacture of edible films due to its excellent film forming ability, low gelling and melting point and biodegradability [4,8]. Gelatin films exhibit good oxygen barrier properties at low or intermediate relative humidity (RH) and satisfactory mechanical properties, making them suitable for use as coating or food packaging materials [4,11,12]. Depending on the processing method, gelatin can be classified into two types: (1) type A: with an isoelectric point at pH ~8–9, obtained from acid treated collagen; and (2) type B: with an isoelectric point at pH ~4–5, derived from an alkali treated precursor which converts asparagine and glutamine residues into their respective acids, resulting in higher viscosity. Gelatin derived from pig skin is normally referred as type A, and it is derived from beef skin or pig or cattle hides, as well as bones, and is referred to as type B [4].

The food, pharmaceutical, and photographic industries are the main users of gelatin, which has several other technical applications. Its most frequent uses in the biomedical field include hard and soft capsules, microspheres, sealants for vascular prostheses, wound dressing and adsorbent pads for surgical use, as well as three-dimensional tissue regeneration [4,14]. In particular, gelatin is used to provide gelling, stabilization, texturization and emulsification for bakery, beverages, confectionary and dairy products for the food industry. However, the limited thermal stability and mechanical properties of gelatin, especially during processing, limit its potential application [4].

A number of recent studies have dealt with extending the functional properties of biodegradable films by adding different compounds with antioxidant or antimicrobial activities in order to yield a biodegradable active packaging material. In order to reduce the use of synthetic chemical additives in the food industry, the use of natural food additives without any negative effects on human health has increased in recent years [4,15]. Those additives may not only influence the mechanical, barrier, optical properties and thermal stability of the blends but also enhance the microbial stability of the packaged foods by using active packaging developed by the incorporation of antimicrobial compounds into the polymer matrix. Contact between the active materials and food, which has the ability to change the food composition or the atmosphere around it, represents an active packaging system that inhibits the growth of microorganisms present on the surface of food products [1].

Several properties of gelatin films, such as mechanical, permeability, light absorption, transparency, antimicrobial activity and antioxidant ability, are not only influenced by the addition of active substances, but also by physical methods [4,6,12,16-18]. A wide spectrum of additives (nano or microsized) have been used for the preparation of gelatin composites including: hydroxyapatite [19], polysaccharides [20,21], rice flour [22], vegetable carbon black [23], carbon nanotubes [24], metal nanoparticles [25,26], fatty acid sucrose esters [27] and genipin [13].

Additionally, gelatin films can be used as vehicles for the release of antioxidant compounds such as: curcumin and its derivatives [12], essential oils [2,5,8,28-36], esculine [37], butylated hydroxytoluene and α-tocopherol [38], lignin [9,39], liquid smoke [40], carvacrol [41], tannin [11,42] vanillin [43], riboflavin [44], gallic acid [45], aloe vera gel [46,47], tea polyphenols and green tea extracts loaded into chitosan nanoparticles [15,47-50], grapefruit seed extract [51] and tomato pulp [52].

Currently, naturally occurring bioactive compounds are preferred by both consumers and companies due to concerns over the potential risks of synthetic compounds.

In this context, fungi and plants are a valuable source of active compounds, such as antioxidants and antimicrobials, used for pharmaceutical, medical and food applications [4,12,53]. Melanins have been isolated and characterized from a variety of phylogenic sources, such as animal [54], plant [55], bacteria [56] and fungi [57-59]. Melanins are commonly represented as black and brown pigments, high molecular weight heterogeneous polymers derived from the oxidation of monophenols and the subsequent polymerization of intermediate *o*-diphenols and their resulting quinones [60,61]. Melanins are types of pigments, possessing broad biological properties including antioxidant, radioprotective, thermoregulative, chemoprotective, antitumor, antiviral, antimicrobial, immunostimulating and anti-inflammatory [54-61]. Potentially, these melanin attributes could also be imparted to polymers, and in the case of biopolymers, potentially enhancing performance as well as sustainability credentials, explore its other nonconventional applications such as cross-linking during polymerization, antioxidant and antimicrobial activity, radioprotective ability and improving the biological properties of the polymer. The use of melanins remains relatively unexplored with few examples of these compounds blended with packaging polymers or use them to modify coatings [1,62-66], and no studies on gelatin/melanin composites are available in the scientific literature.

The aim of this study was to investigate the influence of fungal melanin on the properties of modified gelatin films. To evaluate the potential functionality in food packaging applications, the mechanical, barrier, antioxidant and antimicrobial properties were all evaluated. Spectroscopic studies were performed to elucidate melanin addition in the chemical composition of modified blends. Additionally, the goal of the study was also to evaluate the influence of fungal melanin on the colour, solubility, opacity and optical properties of the films.

2. MATERIAL AND METHODS

2. 1. The Isolation of Melanin from *A. bisporus* Waste

Agricultural waste from the production of *A. bisporus* (ABW—Agaricus Bisporus Waste) in the form of fruiting bodies stipes was obtained from a local producer in Wolsztyn (Wielkopolskie voivodeship, Poland). 500 g of ABW was first homogenized (Heidolph Brinkmann Homogenizer Silent Crusher, Germany) in 500 mL of distilled water and incubated (24 h, 37 °C) to allow enzyme tyrosinase action (hydroxylation of monophenols to *o*-diphenols). After incubation, the homogenate mixture was adjusted to pH = 10 by 1 M NaOH, and incubated (24 h, 65 °C) to allow a spontaneous oxidative polymerization of the resulting *o*-diphenols and quinones to form melanin. Afterward, the mixture was filtered, centrifuged (6000 rpm, 10 min), and an alkaline ABW raw melanin mixture was used to purify the melanin. An alkaline ABW raw melanin mixture was first adjusted to pH 2.0 with 1 M HCl to precipitate melanin, followed by centrifugation at 6000 rpm for 10 min and a resulting pellet was collected. The pellet was then hydrolyzed in 6 M HCl (90 °C, 2 h), centrifuged (6000 rpm, 10 min) and washed in distilled water five times to remove acid. The pellet was washed with chloroform, ethyl acetate and ethanol three times to wash away lipids

and other residues. Finally, the purified melanin was dried, ground to a fine powder in a mortar and stored at −20 °C until testing.

2. 2. The Preparation of Modified Gelatin Films

Gelatin films were prepared by the use of a solution casting method. Commercial bovine gelatin was purchaed from Rousselot SAS (Saint-Michel, France). Firstly, ammonia water was added to the distilled water and the pH was adjusted to 10 (to enable the solubilization of melanin). Next, fungal melanin was added to alkaline solutions to obtain a 0.1% (sample "1"), 0.5% (sample "2") and 1% (sample "3") (on gelatin dry basis), and the solutions were placed in a stirrer (120 rpm), at 50 °C for 24 h. The solution devoid of melanin addition served as a control film forming solution (sample "0"). When melanin was dissolved, the solutions were filtered through Whatman filter paper under vacuum. 8 g of gelatin were added to 100 mL of filtered alkaline solutions and kept for 2 h at 60 °C under continuous stirring (120 rpm) to obtain a homogenous solution. Then, 10 % w/w (based on gelatin dry mass) of glycerol was added (this was used as a plasticizer). The film forming solutions were stirred for 15 min and finally poured into polypropylene Petri dishes (90 mm diameter), and kept at 25 °C, 50% relative humidity (RH) in controlled chamber during 48 h to evaporate water and ammonia, and form the films. Before all experiments all films were conditioned in a chamber 48 h, at 25 °C and 50% RH.

2. 3. Water solubility of the films

Preweighted (W_0) dried films (2 cm × 2 cm) were immersed in 15 mL of distilled water with the addition of 0.01% of sodium azide (as antimicrobial agent) at 30 °C under constant agitation (50 rpm) for 24 h. The undissolved matter was separated by centrifugation at 1000 rpm for 5 min and the pellet was dried at 105 °C to determine the weight (W_1) of the insoluble contents. All tests were carried out in triplicate and water solubility was calculated using the following Equation:

$$\text{Water solubility (\%)} = [(W_0 - W_1) / W_0] \times 100\% \quad (1)$$

2. 4. The Mechanical Properties of the Films

Mechanical measurements were tested by the use of Zwick/Roell 2,5 Z equipment (Zwick/Roell, Germany) and they included tensile strength (the gap between tensile clamps was 25 mm and tensile speed was 100 mm/min), elongation at break, and burst strength (transducer diameter 0.75 mm, speed 50 mm/min).

2. 5. The Water Vapor Transmission Rate and Oxygen Transmission Rate of the Films

The Water Vapor Transmission Rate (WVTR) was measured by means of a gravimetric method that is based on the sorption of humidity by calcium chloride and a comparison of sample weight gain. Initially, the amount of dry $CaCl_2$ inside the container was 9 g. The area of film was 8.86 cm^2. Measurement was carried out for a period of 4 days, each day the containers were weighed to determine the amount of absorbed water vapor through the films.

The results were expressed as average values from each day of measurement and each container. Analyses were carried out at ten independent containers for each type of films, calculated as a standard unit g/(m^2 × day) and presented as a mean ± standard deviation.

The Oxygen Transmission Rate (OTR) was measured by means of Ox-Tran 2/10 instrument (Mocon, USA) equipped with a culometric sensor. The method is based on a standard ASTM D3985 – appropriate for films and laminates.

2. 6. The Contact Angle (CA)

The surface properties of modified and pure gelatin films were measured through a contact angle analyzer. The following measurement was carried out by means of a laboratory goniometer (Haas µL). Film samples were cut into 3 cm x 9 cm and fitted on a sample stage and leveled horizontally. A drop of distilled water was placed on the surface of the film using a microsyringe. Analyses were carried out at three independent times and presented as mean ± standard deviation.

2. 7. Spectral Analysis

2. 7. 1. UV-Vis spectroscopy

The UV-Vis spectra of the films samples were measured by the use of a UV-Vis Thermo Scientific Evolution 220 spectrophotometer at 200–1100 nm.

2. 7. 2. FT-IR Spectroscopy

Fourier transform infrared (FT-IR) spectra of the unmodified and modified film samples were measured using a FT-IR spectroscopy (Perkin Elmer Spectrophotometer, Spectrum 100, USA), operated at a resolution of 4 cm^{-1}, over 64 scans. Film samples were cut into square shapes (2 cm × 2 cm) and placed directly at the ray-exposing stage. The spectra were recorded at a wavelength of 650–4000 cm^{-1}. The spectra were normalized, baseline corrected and analyzed using SPECTRUM software.

2. 7. 3. Raman spectroscopy

Pure and modified films were analyzed using a Raman station (RamanStation 400F, Perkin Elemer, USA) with point-and-shot capability using an excitation laser source at 785 nm, 100 micron spot size. Film samples were cut into square shapes (2 cm × 2 cm) and placed directly at the ray-exposing stage. The spectra were recorded at a wavelength of 250–3300 cm^{-1}. The spectra were normalized, baseline corrected and analyzed using SPECTRUM software (10, PerkinElmer, USA).

2. 8. Color response analysis

The color changes of the films were measured by using a colorimeter (CR-5, Konica Minolta, Japan). The results were expressed as L (lightness), a (red to green), and b (yellow to blue) parameters to evaluate color changes in the modified films. All of the measurements were determined at three random points on both sides of each film, and the experiments were performed five times and presented as a mean ± standard deviation.

To determine other color properties of the films, ΔE (color difference), YI (yellowness index), WI (whiteness index), hue angle (h*ab) and chroma (C*ab) values were calculated using following Equations (where pure gelatin film served as a standard) [1,34]:

$$\Delta E = [(L_{\text{standard}} - L_{\text{sample}})^2 + (a_{\text{standard}} - a_{\text{sample}})^2 + (b_{\text{standard}} - b_{\text{sample}})]^{0.5} \quad (2)$$

$$\text{YI} = 142.86 b \times L^{-1} \quad (3)$$

$$\text{WI} = 100 - [(100 - L)^2 + a^2 + b^2]^{0.5} \quad (4)$$

$$\text{h*ab} = arctg \frac{b_{sample}}{a_{sample}} \quad (5)$$

$$\text{C*ab} = [(\text{a}_{\text{sample}})^2 + (\text{b}_{\text{sample}})^2]^{0.5} \quad (6)$$

2. 9. Opacity measurements

The opacity of modified gelatin/melanin and pure gelatin films was carried out in Opacimeter EE Model 12 (Diffusion Systems LTD, UK). The opacimeter was initially calibrated using standard white plate (value 100 ± 1, Diffusion Systems LTD) and measurements were performed on each film six times, and presented as mean ± standard deviation.

2. 10. The Antioxidant Activity of Gelatin/Melanin Blends

2. 10. 1. Determination of Available Phenolic Groups on the Films Surface

The method for the determination of available phenolic groups (APG) on the non modified and modified gelatin/melanin films was carried out according to Łopusiewicz *et al.* [1]. 100 mg of gelatin/melanin or pure gelatin films was taken in a volumetric flask. Sequentially, 1 mL of 10% Folin-Ciocalteu reagent and 4 mL of 2% sodium carbonate solution were added to the flask. Finally, the volume was made up to 25 mL with distilled water and mixed well. The reaction mixture was kept at room temperature for 48 h and the resultant absorbance was determined at 760 nm. A control absorbance was also measured where the aforesaid reaction mixture, devoid of any film, was kept under the same reaction conditions. To determine the available phenolic groups on the modified films surface, a calibration curve was prepared using gallic acid standard solutions and the results were expressed as μmoles of gallic acid equivalents (GAE) per gram of dry film. All experiments were performed in triplicate and presented as mean ± standard deviation.

2. 10. 2. A Determination of the Free Radical Scavenging Activity of the Films

The free radical scavenging property determination of melanin incorporated gelatin films was carried out using ABTS according to Łopusiewicz *et al.* [1]. Radical 2,2′-azino-bis(3-ethylbenzothiazoline)-6-sulphonic acid ($ABTS^+$) was produced by mixing 7 mM ABTS with 2.45 mM potassium persulfate (5 mL of ABTS + 5 mL of potassium persulphate 4.9 mM). The mixture was then incubated for 16 h in the dark, at room temperature and subsequently diluted with water to an absorbance of maximum 1.00 at 734 nm. To determine

the antioxidant capacity of the films, 1 g of the film was put into 25 mL of $ABTS^+$ solution and incubated up to 24 h at room temperature. Control sets without the film were also kept under identical conditions. After incubation period, the film samples were removed from the $ABTS^+$ solution. Absorbance for both sets was taken and antioxidant activity (AA%) was calculated using the Equation:

$$AA\% = [(A_{control} - A_{sample})/A_{control}] \times 100 \quad (7)$$

2. 11. The Antimicrobial Activity of Films

The test microorganisms used in this study were obtained from the American Type Culture Collection (ATCC). The strains used in this study were *Enterococcus faecalis* ATCC29212, *Pseudomonas aeruginosa* ATCC2783, *Pseudomonas putida* ATCC31753. To verify the antimicrobial properties of films Mueller-Hinton agar and broth (Merck, Germany) were used. The media were prepared according to the Merck protocol (medium was weighted according to the manufacturer's instructions, suspended in 1000 mL of distilled water, and autoclaved at 121 °C for 15 min). Antibacterial activity of films was evaluated by the use of two methods. Firstly, 50 mL of Mueller-Hinton broth was inoculated by a single bacterial strain and incubated at 37 °C for 24 h and after incubation time 200 μL of bacterial suspension was added to agar surface by a glass spreader. The film samples were cut into square shapes (2 cm × 2 cm), and put directly on the bacteria. The plates were incubated at 30 °C for 24 h. The positive antimicrobial activity was considered as growth inhibition zones around the films. Second test was performed to evaluate the influence of the films of bacterial growth. The film samples (1 g) were incubated with single bacterial strain suspensions (1.0×10^6 CFU/mL) in sterile physiological saline (0.9% NaCl) for 24 h at 30°. After incubation period the bacterial cells concentration was evaluated on solid Mueller-Hinton agar.

2. 12. Statistical Analyses

All determinations were carried out in triplicate as a minimum. Statistical significance was determined using an analysis of variance (ANOVA) followed by Duncans's test. The values were considered as significantly different when $p < 0.05$. All analyses were performed with Statistica version 10 (StatSoft Polska, Kraków, Poland).

3. RESULTS

3. 1. Mechanical Properties

In order to evaluate the potential influence of melanin on the mechanical features of non –modified and modified gelatin-based films, three measurements were carried out: tensile strength, elongation at break and burst strength. The results of tensile strength, elongation at break and burst strength of the films are shown in Table 1. No significant differences ($p>0.05$) in the results of tensile strength were observed for the samples, the reference sample and those containing 0.1%; 0.5% and 1% of melanin, respectively. This confirms any lack of impact of melanin on the tensile strength. Regarding the brittle/elastic behaviour of the samples, the lowest (0.1%) addition of melanin to the gelatin matrix increased elongation at break value – from 19.31% (reference sample) to 35.65%. The other samples (0.5% and 1%) exhibited

lower values: 23.8% and 24.14%, respectively. Nevertheless, after considering statistical analysis, no significant differences between all of the results were detected, and this was confirmed by the Duncan test (p>0.05). As shown in Table 1, no significant influence of melanin was indicated in the results of maximum burst strength (p>0.05).

Table 1. Tensile strength (TS), elongation at break (EB), burst strength (BS), Water Vapor Transmission Rate (WVTR), Oxygen Transmission Rate (OTR) and solubility (%) of pure gelatin and gelatin/melanin modified films.

Sample	TS (MPa)	EB (%)	BS (MPa)	WVTR $\left(\frac{g}{m^2 \times day}\right)$	OTR $\left(\frac{cm^3}{m^2 \times day}\right)$	Solubility (%)
0	111.92 ±16.01	19.31 ±7.89	20.95 ±3.96	36.22 ±8.20	5.44 ±0.41	72.16 ±3.24
1	111.00 ±4.35	35.65 ±9.25	16.76 ±3.60	28.14 ±3.95	4.88 ±0.25	66.25 ±2.15
2	107.17 ±7.10	23.80 ±8.33	18.22 ±1.79	30.47 ±5.41	4.09 ±0.11	43.56 ±4.08
3	118.00 ±12.40	24.14 ±7.61	18.35 ±1.68	30.71 ±7.53	3.85 ±0.16	31.05 ±2.75

3. 2. Solubility of the films

Table 1 shows films solubility. In the present study, the control film showed the highest solubility (72.16±3.24%) among all of the films and the addition of 0.1%; 0.5% and 1% of melanin decreased the solubility of the films significantly (p<0.05) to 66.25±2.15; 43.56±4.08 and 31.05±2.75%, respectively.

3.3 . Surface Properties—Contact Angle

Three repetition tests were performed for each sample of the gelatin-based films. The average values of the contact angle obtained for distilled water were as follows: 53.3°; 69.9°; 71.5°; 72.9° for samples "0", "1", "2", and "3", respectively. The Duncan's test was applied to demonstrate that these differences of averaged values were statistically significant (p < 0.05).

3. 4. Barrier Properties—WVTR and OTR

The water vapor transmission rate of all four samples was measured by means of a gravimetric method, which is based on the sorption of humidity by calcium chloride and a weight gain comparison of the samples.

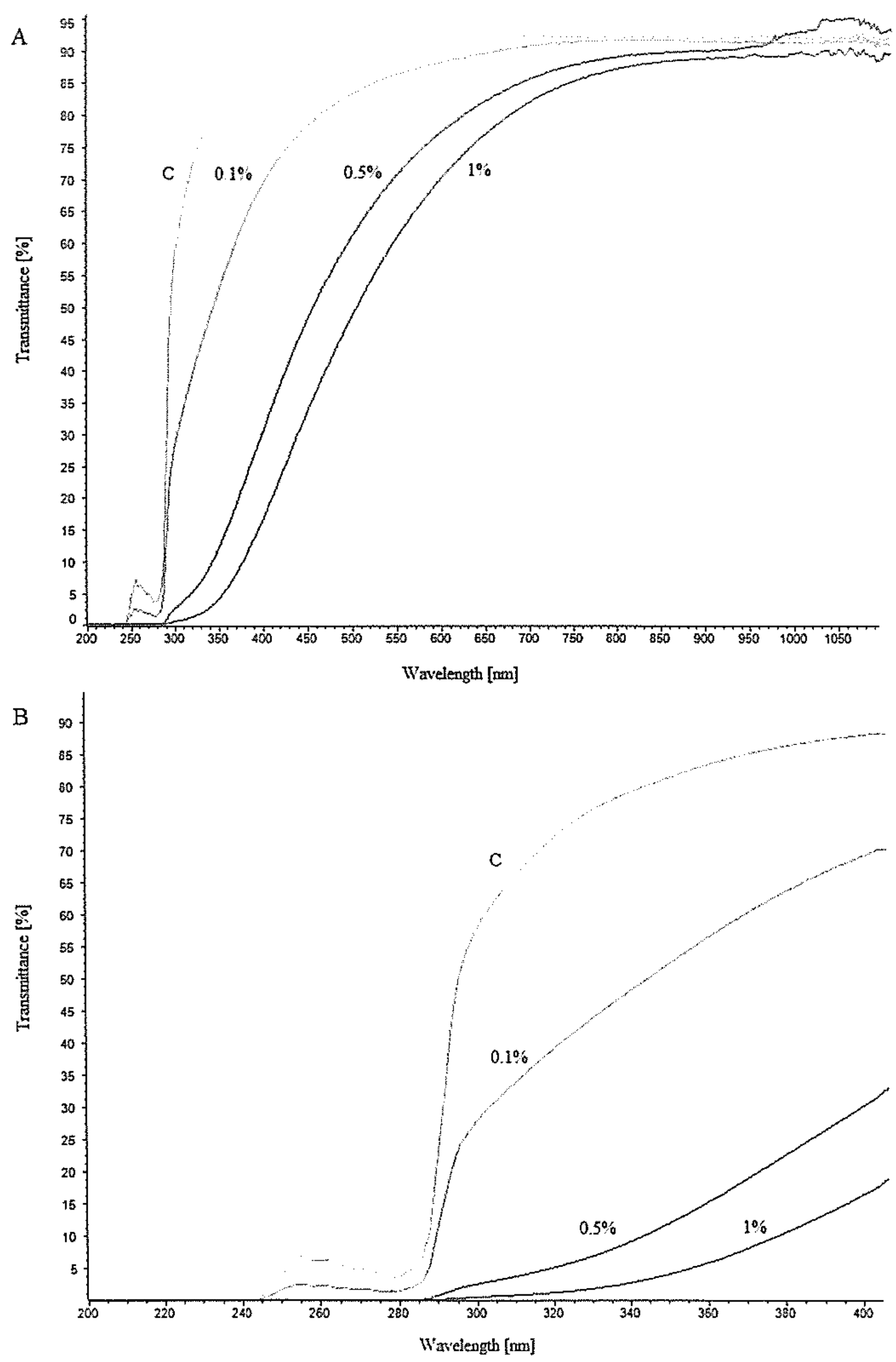

Figure 1. The UV-Vis spectra of pure gelatin and gelatin/melanin films (A)200-1100 nm (B) 200-400 nm

As reported in the Table 1 sample "0" exhibited the highest values of WVTR, 36.22±8.20 g/(m^2×day), whereas the values obtained for the modified samples (with increasing melanin content) were 28.14±3.95; 30.47±5.41 and 30.71±7.53 g/(m^2×day) respectively. However, after considering the statistical analysis, the differences between all the samples were not statistically significant (p>0.05). As shown in Table 1 the oxygen transmission rate values of the films were influenced by the addition of melanin in comparison to the control sample. Sample "0" (devoid of melanin) exhibited the highest value of OTR ((5.44 ± 0.41 (cm^3/(m^2×day))), whereas the values obtained for the modified samples were growing with increasing melanin content and were 4.88 ± 0.25; 4.09 ± 0.11 and 3.85 ± 0.16 cm^3/(m^2×day), respectively. The Duncan's test was applied to demonstrate that these differences of averaged values were statistically significant ($p < 0.05$).

3. 5. The Spectral Analysis of Modified Films

3. 5. 1. The UV-Vis spectra

UV-Vis spectra of pure gelatin and gelatin/melanin films in selected wavelengths from 200 to 1100 nm in UV and visible ranges are shown in Figure 1a and 1b. The addition of melanin caused noticeable improvement of the light barrier properties. Decreases in light transmission of films modified with melanin were observed at all wavelengths, compared with control film. A noticeable peak at 280 nm in control sample is resulted from aromatic amino acids. The results indicated that melanin was able to impede the light transmission through the films.

3. 5. 2. The FT-IR spectra

FT-IR spectra of pure gelatin and gelatin/melanin films are shown in Figure 2. The addition of melanin caused noticeable changes in intensity of Amide I band at 1633.38 cm^{-1}, Amide II band at 1538.39 cm^{-1}, and Amide III band at 1237.36 cm^{-1} than compared with pure gelatin film. In addition, absorption bands at the wavenumbers of 1033.28 cm^{-1} were found. Moreover, amide-A band, arising from the stretching vibration of NH group appeared at wave numbers of 3294.26 cm^{-1}. The amide-B bands were observed at wavenumber 3074.88 cm^{-1}. The asymmetric and symmetric CH_2 vibrations at 2937.05 cm^{-1} and 2877.50 cm^{-1}, respectively are noticeable.

3. 5. 3. The Raman spectra

The Raman spectra of pure gelatin and gelatin/melanin films are shown in Figure 3. The addition of melanin caused noticeable signal increasing at wavenumbers ranges 2400-2200 cm^{-1}, 2200-1800 cm^{-1}, 178-1380 cm^{-1}, 1380-900 cm^{-1}, 870-250 cm^{-1}

3. 6. The Visual Appearance and Color

The visual appearance of pure gelatin and gelatin/melanin modified films is shown in Figure 4. As can be seen in Figure 4 the polymer matrix was homogenous. The color, chroma, hue angle, ΔE, YI and WI values are presented in Table 2. The growing addition of melanin influenced the color values in comparison to pure gelatin film, causing a reduction in the lightness (L) and an increase in the redness (a), yellowness (b), chroma (C^*ab) and hue angle (h^*ab) values. Color differences were statistically significant ($p < 0.05$). ΔE values ranged

from 4.67 (sample "1") to 32.39 (sample "3"). The yellowness (YI) increased with increasing melanin amount, in contrast, the whitening index (WI) decreased when the melanin content was increasing.

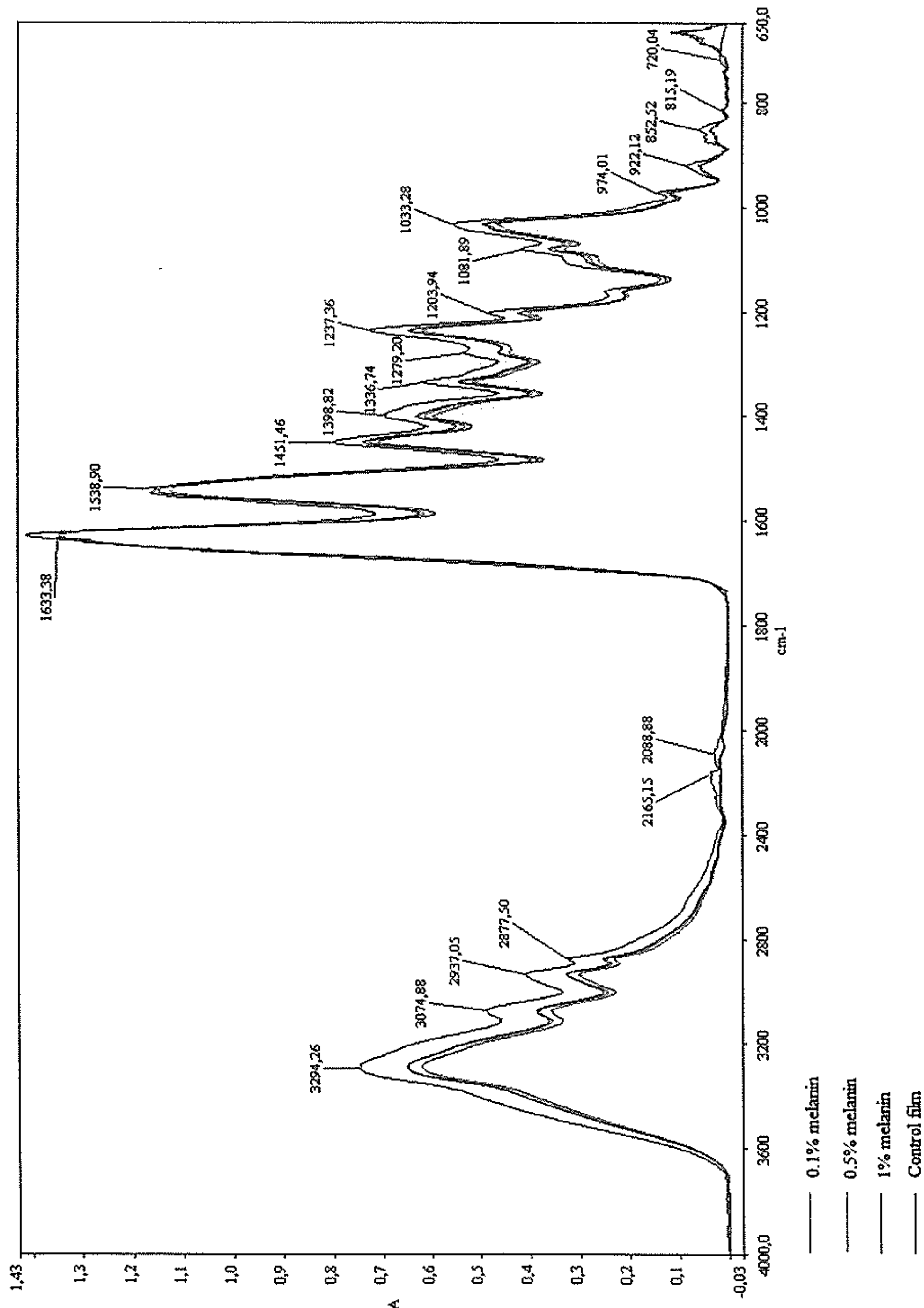

Figure 2. The FT-IR spectra of pure gelatin and gelatin/melanin films

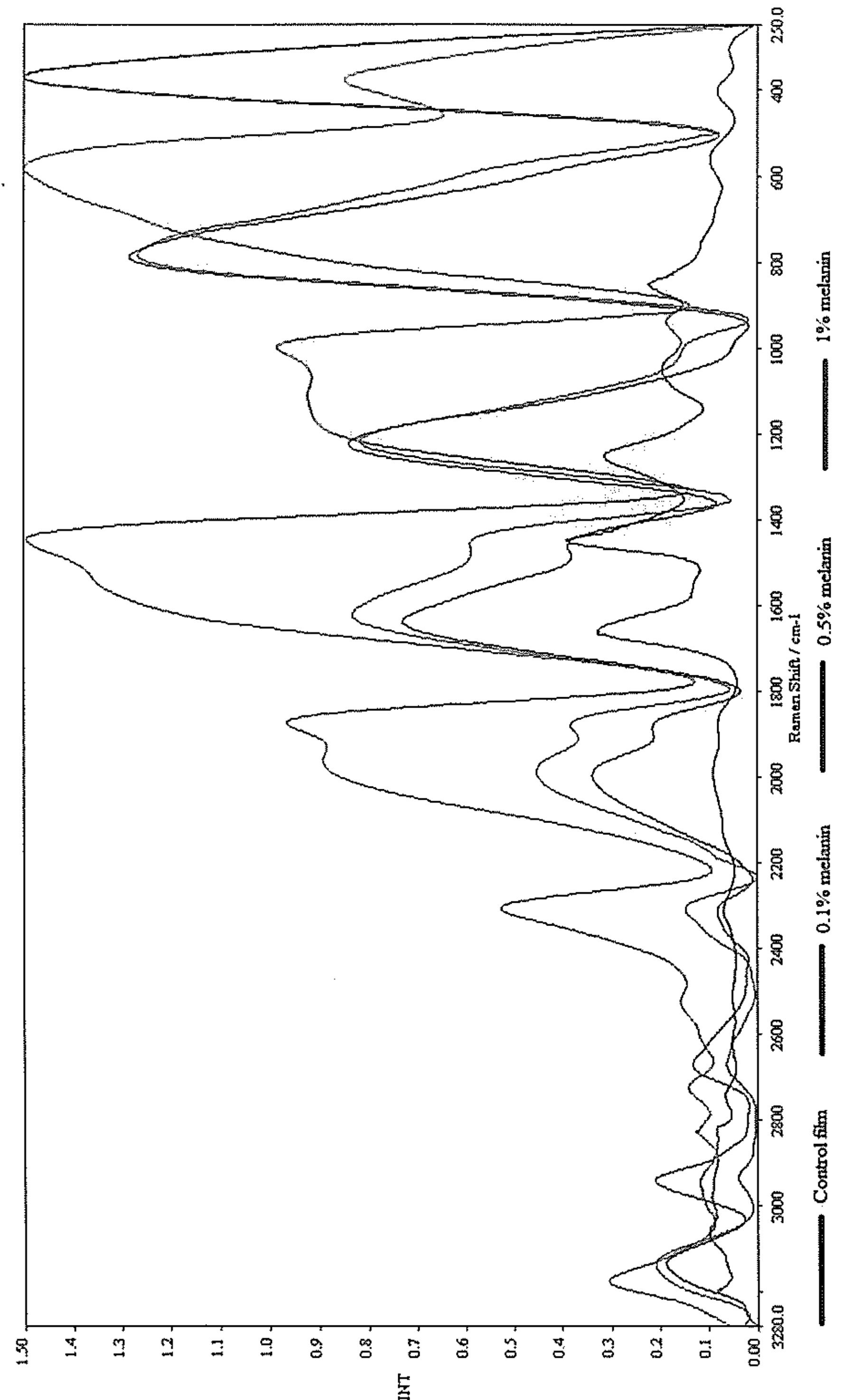

Figure 3. The Raman spectra of pure gelatin and gelatin/melanin films

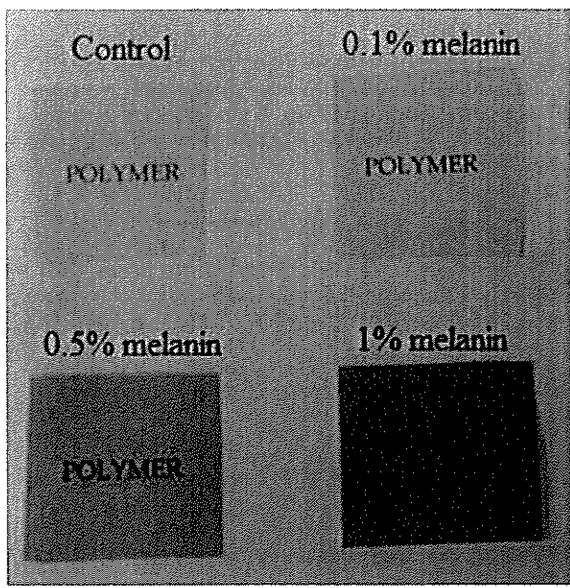

Figure 4. The visual appearance of pure gelatin and gelatin/melanin films

Table 2. Color parameters (L^*, a^*, b^*), chroma (C*ab), hue angle (h*ab), ΔE, yellownes index (YI), whitening index (WI) and opacity of pure gelatin and gelatin/melanin modified films.

Sample	*L*	*a*	*b*	*C*ab*	*h*ab*	ΔE	YI	WI	Opacity
0	96.47 ±0.01	-0.12 ±0.00	0.88 ±0.0	0.89	-1.44	used as standard	1.30	96.36	9.08 ±0.02
1	94.48 ±0.00	-0.09 ±0.00	5.11 ±0.00	5.11	-1.55	4.67	7.72	92.47	8.40 ±0.08
2	87.88 ±0.01	1.03 ±0.01	17.52 ±0.00	17.55	1.51	18.76	28.48	78.67	7.23 ±0.05
3	81.58 ±0.00	3.23 ±0.00	29.46 ±0.01	29.63	1.46	32.39	51.58	65.11	6.71 ±0.06

3. 7. Opacity

The opacity of pure gelatin and gelatin/melanin modified films is shown in Table 2. The opacity values of modified gelatin/melanin films were lower than the pure gelatin film. The opacity of gelatin/melanin films decreased after the addition of melanin (from 9.08± 0.02 of sample "0" to 6.71 ± 0.0.06 of sample "3"). This may have been due to the color and the content of the melanin. Those differences were statistically significant ($p < 0.05$).

3. 8. Antioxidant Activity

Table 3 presents results of an assessment of the available phenolic groups on the films surface and antioxidant activity of pure gelatin and gelatin/melanin modified films. The total available phenolics were determined to be 0.0134; 0.0199 and 0.0245 μmole GAE/g film for samples "1", "2" and "3", respectively. Pure gelatin film (sample "0") also reacted with Folin-Ciocalteu reagent due to high proline content and showed antioxidant activity (the result was considered as no polyphenolics). The antioxidant activity of modified gelatin/melanin films grew with the increasing content of melanin, reaching 34.95± 0.21%. Differences between the modified films and pure gelatin film were statistically significant ($p < 0.05$).

Table 3. The antioxidant activity (AA%) and available phenolic groups (APG) of pure gelatin and gelatin/melanin modified films.

Sample	AA% (%)	APG (μmole GAE/g)
0	0.14 ± 0.02	-
1	11.44 ± 0.23	0.0134 ± 0.011
2	17.78 ± 0.18	0.0199 ± 0.021
3	34.95 ± 0.21	0.0245 ± 0.013

3. 9. Antimicrobial activity

Table 4. Antimicrobial activity of the pure gelatin and gelatin/melanin films

Strain	Film sample	Inhibition zone	Number of cells after incubation with films [CFU/mL]
E. faecalis	0	-	3.24×10^7
	1	-	1.98×10^8
	2	-	2.02×10^7
	3	-	2.17×10^7
P. aeruginosa	0	-	3.22×10^7
	1	-	2.56×10^7
	2	-	1.56×10^7
	3	-	3.89×10^7
P. putida	0	-	2.26×10^7
	1	-	1.12×10^8
	2	-	3.34×10^7
	3	-	2.74×10^7

The susceptibility assay of *E. faecalis*, *P. aeruginosa* and *P. putida* with respect to the pure gelatin and modified gelatin/melanin films is shown in Table 4. The results of this research determined that neither pure gelatin nor modified films were found to be active against all strains (no growth inhibition zones). The results of this research demonstrated that pure gelatin and modified films had influence on the growth of *E. faecalis*, *P. aeruginosa* and *P. putida*, a 1-2 log increase of the number of cells was noted when bacterial cells were incubated with the films.

4. DISCUSSION & CONCLUSIONS

The results of this study indicate that melanin isolated from ABW used as an additive for gelatin at various concentrations may influence the properties of modified films, depending on concentration. The increased availability of biopolymers has stimulated increased research and development of activities, which can also be partly attributed to the escalating "green" movement that is encouraging the use of biopolymers. Since the packaging industry plays a dominant role in the short-term use of cheap non-biodegradable petroleum-based materials, their replacement with biopolymers could provide a significant step to eco-friendly solutions [1]. Nowadays, agricultural by-products are usually incinerated or dumped, causing environmental problems such as pollution, soil erosion and decreasing biological activity in soil. The incorporation of agricultural residues into polymer matrices is currently a trending topic in research [4]. Thus, the application of melanin from ABW to modify gelatin matches the current trend for bio-based composite films. Gelatin is unique among hydrocolloids in forming thermo-reversible systems with a melting point close to the body temperature, which is particularly significant in packaging and pharmaceutical applications [67]. Hence, the incorporation of melanin has opened up new avenues to discover its applicability in the packaging industry, such as packaging material for avoiding oxidation of sensitive food, thus expanding the spectrum of its uses.

In order to adequately preserve the quality of food goods, the packaging materials have to provide efficient barriers against light, water vapor, atmospheric gases and volatile organic compounds, preventing food spoilage. When the modified blend film is applied to preserve food, its integrity has to be maintained and all external stress withstood, so these mechanical properties are vitally important characteristics for the film. Gelatin forms a three-dimensional network with zones of intermolecular microcrystalline junctions and the dehydration of this system may produce brittle films. Gelatin films are fragile and susceptible to cracking due to the high cohesive energy density of proteins [50]. So, the addition of a plasticizer is necessary to overcome the brittleness of the films, to reduce inter-chain interactions during the dehydration which can improve flow and flexibility, and to increase toughness and impact resistance of the film coating, to prevent them from cracking during packing and transportation [45,67,68]. The composition, size and form of the plasticizer molecule has an influence on its ability to interact with protein chains and bind with the molecules of water, causing more plasticization owing to the fact that water is an effective film plasticizer based on hydrophilic biopolymers [68]. In this study glycerol was used as a plasticizer, it has hygroscopic character and therefore attracts more water into the structure of the films, thus promoting greater flexibility.

When the modified blend film is applied to preserve food, its integrity has to be maintained and external stress withstood, so these mechanical properties are vitally important characteristics of the film [1]. The mechanical properties of protein films could provide an indication of expected film integrity under conditions of stress that would occur during processing, handling, and storage [3]. Gelatin can be blended with different compounds and/or polymers to obtain bio-composite films and coatings that combine the advantages of each component [4,71-74]. Tensile strength (TS) plays an important role in determining the mechanical properties of edible or packaging films developed for use in many food applications. TS are an indication of film strength, whereas elongation at break is an indicator of the stretchability of films prior to breakage [74]. According to Liang *et al.* stable aromatic ring, may hinder the rotation of intramolecular hydrogen bonds in the films [37]. Melanin has ring structures within its molecules that hinder conformational variations. The observed mechanical properties indicate that the incorporation of melanin is likely to result in the development of a heterogenous film structure, nevertheless the results suggest that melanin did not significantly influence ($p>0.05$) the mechanical properties of the films. Some compounds may enhance the TS of gelatin, which was noted by Liang *et al.*, who used esculin to modify gelatin [37]. These results may be attributed to the supramolecular interactions between gelatin and additives, including bonding between their chemical moieties with gelatin amino acids, as well as hydrophobic or π-π interactions. Ahmad *et al.* noted that moderate amounts of essential oils enhanced the mechanical properties of modified gelatin films, but when the concentration of essential oils was high, the mechanical properties worsened [6]. Tongnuanchan *et al.* observed that the addition of bergamot essential oil decreased the tensile strength of modified films [2], similarly Rawdkuen *et al.*, noted the negative influence of catechin on gelatin film tensile strength [75].

The solubility of edible film in water is an essential asset, and water resistance is typically required for possible commercial applications of these films [9,15]. When a film is placed over the food surface, its solubility largely determines the release of active compounds [75]. The solubility of the melanin-added films decreased as the concentration of melanin increased from 0.1% to 1% ($p<0.05$). In general, high water solubility may indicate lower water resistance, and the lower water solubility of melanin modified films might result from the stronger structure of the film network via strong interactions between the protein and hydroxyl groups of melanin. The incorporation of melanin might be associated with its hydrophobic moietes. Non-polar moietes of melanin interacted favourably with the hydrophobic domains of gelatin, leading to an increase in the hydrophobocity of the resulting film. As a result, the solubility of films was lowered. Similar mechanism has been proposed by Ahmad *et al.* [6]. Another mechanism has been proposed by Nafchi *et al.* [76] who reported that increasing the nanoparticles (ZnO) content of films results in the formation of more hydrogen bonds in the ZnO and the matrix components. Thus, free water molecules do not interact as strongly with nanocomposite films than compared with composite films alone. Our results are in line with the results of Liang *et al.* [37], who observed that the solubility of esculine-incorporated gelatin films was lower that of the film comprising of gelatin alone. Other authors also noted the influence of some additives on gelatin film solubility, such as tannic acid [42], tannin [77], catechin [75], ribose [74], formaldehyde and glyoxal (as cross-linking agents) [78].

Food products are very susceptible to rancidity caused by the oxidation of lipids that contain unsaturated fatty acids that can be attacked by oxygen free radicals. Antioxidants are

added to foods to intercept and react with these free radicals at a faster rate than the lipid substrate. Nevertheless, the current incorporation of antioxidants throughout the entire food matrix in one large initial dose is not an efficient process due to the oxidation occurring at the surface and high initial doses of antioxidant having a pro-oxidant effect. Therefore, one emerging technology is the use of antioxidant active packaging, where the antioxidant is incorporated to a packaging material with the purpose of being delivered to the food surface during commercialization, at an appropriate rate. Most of the active packaging developments have been based on the mass transportation properties of plastic materials (sorption, migration, and permeation), and the release of the active agents depends on several factors, such as the type of polymer and type of food. However, the presence of synthetic antioxidants in food is questionable, owing to the potential risks. This has been encouraged by strong consumer demand, as synthetic compounds are frequently perceived as undesirable or harmful. Natural antioxidants are preferred to artificial substances, especially by consumers. Moreover, the use of active antioxidant packaging that incorporates natural antioxidants presents important advantages. The addition of a natural compound to the packaging may reduce the need of using synthetic antioxidants in the plastic, reducing the risk of potential toxicity by migration [1]. There are many reports of gelatin modifications by antioxidants known from the literature. A wide spectrum of additives were used, including curcumin and its derivatives [12], essential oils [2,5,8,28-36], esculine [37], butylated hydroxytoluene and α-tocopherol [38], lignin [9,39], liquid smoke [40], tannin [11,42], carvacrol [41], vanillin [43], riboflavin [44], gallic acid [45], aloe vera gel [46,47], tea polyphenols and green tea extracts loaded into chitosan nanoparticles [15,47-50], grapefruit seed extract [51], tomato pulp [52], tomato pomace oil extract [79] and other plant extracts [80]. The control films, devoid of melanin showed radical-scavenging activity to some extent, which may be ascribed to the gelatin, particularly to the peptide fraction with its content of particular amino acids such as glycine and proline [48]. The addition of melanin into gelatin films caused a significant increase in their antioxidant activity ($p<0.05$). In our study, modified gelatin films have shown good radical scavenging activity that increased with increasing melanin content. This observation is comparable with the results of other authors who also observed that radical scavenging activity is dependent on antioxidant activity concentration [1,11,36,46].

Sensitive components of food such as lipids, flavours, vitamins and pigments may undergo photodegradation reactions. The spectrum and the intensity of the light source, the conditions of light exposure and the degree of packaging material light transmittance are factors that can significantly affect food quality. Thus, packaging plays a pivotal role in the prevention of the photodegradation of food components during storage [1,8]. The design of the packaging for a specific food product involves not only the choice of appropriate packaging material, but also the addition of the right additives or stabilizers to the packaging in order to provide a more efficient UV-Vis light barrier, and thus a significant improvement in the protection of food quality after storage. The absorption and transmission of light by polymers is particularly important in the food packaging industry where the packaged goods are light sensitive. Transparency of a film to some extent is determined by the miscibility of the various components in the film forming solution and hence transparency values can provide information about the regularity of the microstructure of the blends [52]. Thus, films with high transparency values are less prone to damage by UV light owing to limited light penetration into the films [8]. Another issue in fresh food packaging is the effect of irradiation in the package, since ultraviolet light irradiation is a common method used for lowering

microbial population in foods. A food packaging film is required to protect food from the effects of light, especially UV radiation. Therefore the gelatin film enriched with melanin may improve light barrier against UV light, thereby protecting and prolonging the shelf life of the food. The transmission of UV and visible light at a wavelength range of 200-1100 nm of the films were studied. Decreases in light transmission of films modified with melanin were observed at all wavelengths, compared with control film. The result indicated that melanin was able to impede the light transmission through the film. Fig. 1 shows the spectroscopic scans of the films at wavelengths between 200 and 1100 nm. All gelatin films with the melanin addition films showed a pronounced increase in the absorbance level within the UV region than compared to the gelatin films. Although the addition of melanin gelatin led to the films losing their colourless appearance, they still remained transparent. Núñez-Flores *et al.* [39], observed that gelatin films blended with lignin were not transparent. It is well known that the film light transmission depends on many factors such as thickness, the presence of a dispersed phase within the matrix with a particle size bigger than the wavelength of the visible light, as well as the presence of interactions between film components [11]. There are many reports of the addition of some chemical compounds resulted in the improvement of UV-Vis barrier properties in modified films, such as: coconut husk extract [73], catechin [75], lignin [9,39], ferulic, gallic and tannic acids [3,45], tea polyphenol-loaded chitosan nanoparticles [48], vegetable carbon black [23], metal nanoparticles [76] or essential oils [2,8]. There are several proposed mechanisms of influencing the light barrier properties of films, some compounds are able to absorb or reflect the light [73], and some cause light scattering within the polymer matrix, such as essential oil droplets [2,8]. Also, Schiff's base reaction between amine groups and the carbonyl groups of gelatin and additives may occur which can lead to increased barrier property against UV light as well [26]. The chromophoric nature of melanin is known to be well capable of protecting against UV radiation [57-61]. In addition, melanin has been also reported to act as a UV absorber in PLA/melanin composite films or as an additive to coatings for packaging materials [1,63,64].

Films with various amounts of melanin are of a similar pattern, which indicates that there were no major changes in the functional groups of the gelatin films as demonstrated in Figure 2. Addition of melanin triggered noticeable changes in intensity of Amide I band at 1633.38 cm^{-1}, Amide II band at 1538.39 cm^{-1}, and Amide III band at 1237.36 cm^{-1} than compared with pure gelatin film. The amide-I vibration mode is primarily a C=O stretching vibration coupled with the CN stretch, CCN deformation and in plane NH bending modes. The spectral differences between different film samples in amide-I region were largely attributed to the different conformation and orientation of polypeptide chains, affected by the incorporation of melanin. The amide-II vibration modes are attributed to an out-of-plane combination of the NH in plane bend and the CN stretching vibration with smaller contributions from the CO in plane bend and the CC and NC stretching vibrations. The amide-III represents the combination peaks between C-N stretching vibrations and NH deformation from amide linkages as well as absorptions arising from wagging vibrations from CH_2 groups from the glycine backbone and proline side-chains of gelatin molecules. In addition, absorption bands at the wavenumbers of 1033.28 cm^{-1} were found. Those bands most likely arose from asymmetric stretching vibrations of -OH groups of glycerol (plasticizer) coupled to the -CH_2 of the amino acid residues of the gelatin molecules. Moreover, amide-A band, arising from the stretching vibration of NH group appeared at wave numbers of 3294.26 cm^{-1}. According to Ahmad *et al.* [6] when the NH group of a peptide is

involved in a hydrogen bond, the position shifted to lower frequencies. Signal intensity lowering in the amide-A region of the modified films in comparison to the control film suggest that gelatin peptide NH groups and melanin functional groups are involved in hydrogen bonds. The amide-B bands were observed at wavenumber 3074.88 cm^{-1} corresponding to an asymmetric stretch vibration of =C-H, as well as $-NH_3^+$ of peptide fragments of gelatin molecules. Modified gelatin films showed a lower wavenumber at amide-B region, compared to the control film, suggesting an interaction of $-NH_3^+$ group between peptide chains. In addition, the hydrocarbon chains of melanin give asymmetric and symmetric CH_2 vibrations at 2937.05 cm^{-1} and 2877.50 cm^{-1}, respectively. The most pronounced changes in the films were in the range of 1633–650 cm^{-1} indicating intrusion caused by melanin in the hydrogen bonding between water and imide residues. Initially, the hydrophobic groups of polyphenol interact with the hydrophobic region of the protein via hydrophobic interaction followed by hydrogen bonding between the phenolic hydroxyl groups of polyphenols and the polar group of the protein. Based upon the above mechanism and FT-IR data, it is tempting to suggest that the hydroxyl and carboxyl group of melanin interact with the amino acids of the gelatin via hydrogen bonding and hydrophobic interaction. Therefore, the incorporation of melanin altered the molecular organisation and intermolecular interaction in the film matrix.

Results of the Raman spectroscopy analysis showed noticeable differences in the obtained spectra. With higher melanin content peaks were observed with greater insensitivity. Similar observations have been made in previous study [1]. The peaks can be interrelated as originating from the in-plane stretching of the aromatic rings and the linear stretching of the C–C bonds within the rings, along with some contributions from the C–H vibrations in the methyl and methylene groups in the melanin molecules [81]. A peak at 2000 cm^{-1} is similar to those obtained by Galvan *et al.* from eumelanin and may be caused by the stretching of three of the six C–C bonds within the melanin aromatic rings [82]. It was noted, that on all modified films, Raman spectra peaks at 395 cm^{-1} are present, which are thought to correspond to peaks obtained from pheomelanin and eumelanin and are caused by an out-of-plane deformation of the phenyl rings. Peaks at 2010 cm^{-1} are also similar to peaks seen in pheomelanin and are probably due to overtone or combination bands [81,82].

The optical properties of films are an important attribute which influences its appearance, marketability, and suitability for various applications. Clear edible films are typically desirable with higher applicability and acceptability in food packaging systems [6,73]. Generally, a clear film is preferable as the appearance of the contents is be displayed clearly [46]. Film colour can be affected by the type, nature and concentration of the incorporated additive [46,52]. It is commonly found that the addition of natural extracts alters the original colour of protein-based films to a certain extent and the magnitude of such is determined by the type and concentration of polyphenols which are believed to confer yellow-brown coloration [2,11]. The results are in agreement with Cao *et al.* who reported that gelatin film incorporated with phenolic compounds (tannic acid and ferulic acid) at alkaline pH showed changes in colour [3]. There are several reports that some additives may cause changes in gelatin films, to yellow-brown coloration or influence the their lightness such as vanillin [43], catechin [75], lignin [39], seaweed extract [83], gallic acid and silver nanoparticles [84], curcumin [12], some essential oils [2], tannin [11], riboflavin [44], metal nanoparticles [85,86], ferulic and caffeic acids [87], amino acids (histidine and lysine) [50], coconut husk extract [73]. Melanins are known for their dark-brown colouration [57-59], and

there some reports, that their addition into a polymer matrix may influence the colour values [1]. In general, melanins are dark because they do not re-radiate the absorbed visible or invisible light, but transform the energy into rotational and vibrational activity within the molecule and then dissipate it as heat. This phenomenon protects melanised tissues against light-induced damage. In general interaction between natural phenolic compounds and proteins in the presence of O_2 and alkaline conditions leads to the oxidation of the phenolic structure and the formation of a quinon compound. In fact quinon is a dimmer compound, which reacts with amino or polypeptide sulfhydryl chain to a form covalent bond of C-N or C-S. Polyphenol compounds are able to create cross-link bounds between individual protein molecules. Zhang *et al.* [88] found a colour change in bovine gelatin-based film containing caffeic acid from pale yellow to dark brown. These results were found to be in accord with our findings. In addition, all the films were dried at room temperature (25°C), thus eliminating the occurrence of the Maillard reaction which may cause the browning of gelatin [46]. Our results indicate that the increasing addition of melanin influences the colour values than where compared to pure gelatin film, leading to a reduction in lightness (L), as well as an increase in the redness (a), yellowness (b), chroma (C^*ab) and hue angle (h^*ab) values. ΔE values ranged from 4.67 to 32.39. $\Delta E > 1$ is considered perceptible to the human eye, so all melanin concentrations caused noticeable colour changes. The yellowness index (YI) increased with increasing melanin amount, while the whitening index (WI) decreased when the melanin content was increased. The yellowness index or a change in the degree of yellowness is a number calculated from spectrophotometric data that describes the change in colour of a test sample from clear or white to yellow. The opacity of gelatin films decreased with the addition of melanin (from 9.08 ± 0.02 of sample "0" to 6.71 ± 0.06 of sample "3"). This was probably due to the colour of the melanin powder. The changes of film colour and opacity as a consequence of the addition of melanin had been reported with PLA films [1]. The difference in opacity among the film samples was perceptible to the human eye and was statistically significant ($p<0.05$). Gelatin/melanin films in all melanin concentrations still had good transparency, even at high melanin content. This result suggested high gelatin/melanin films transparency, meaning that the packaging film could be transparent, which an important requirement for consumers and would have a clear influence on customer choice.

The water contact angle of the material is associated with its hydrophilicity. In general, the smaller the water contact angle, the higher the hydrophilicity. Gelatin is a kind of hydrophilic material owing to the functional groups in the molecule, such as amino, carboxyl, and hydroxyl. The hydrophilic property has restricted its application in many aspects. Based on this fact, it is necessary to carry out a hydrophobic modification of gelatin [4,89] In our work the contact angle of pure gelatin film was approximately 53.3°. The contact angle of non-modified gelatin films observed by other authors was 52.4° [86], 76.2° [90], 77.8° [89], 89.5° [91], 97.3° [40]. This discrepancy may be a result of the gelatin type and glycerol content. The addition of melanin into gelatin significantly ($p<0.05$) affected the surface properties of the polymer, increased the contact angle from 53.3° to 72.9°. Shankar *et al.* [86] modified gelatin films with ZnO nanoparticles which increased the water contact angle from 52.4° to 63°. This results are comparable to results of Nafchi *et al.*, who also observed increased hydrophobocity of ZnO amended gelatin films [76]. Yue *et al.* [90] noted that addition of polydimethylsiloxane or glycidol increased the gelatin films contact angle to 112.8°, Wang *et al.* using liquid smoke increased the contact angle of modified films to 111.2° [40], while Wang *et al.* [91] using cellulose nanofibres and palmitic acid achieved

123.7°. The results of this study are quite opposite to the results of our previous study, where melanin particles incorporation into poly(lactic acid) films did not significantly ($p>0.05$) affected their contact angle [1].

One of the most important properties of bio-based films for the application of packaging is to minimize the moisture transfer from the environment to the packed goods. Water vapor permeability (WVP) is one of the most important properties in food packaging due to the noticeable role water has in deteriorative reactions and microbial growth. For this purpose, the WVP of packaging materials should be as low as possible [1]. However, gelatin films have poor water barrier vapor property, thereby limiting their use as potential packaging. This is due to its hydrophilicity in nature. To tackle this problem, the incorporation of hydrophobic substances such as lipids, fatty acids, waxes and essential oils has been implemented to improve water barrier property [2,5,35]. A possible means to minimize the problem of the moisture content in gelatin films is also their association with some synthetic polymers through blending, such as poly(vinyl alcohol) (PVA) [72]. Polymer blending is a technique widely applied in polymer science to obtain materials with improving properties. On the other hand, some additives may exacerbate the water vapor barrier properties such as ZnO nanoparticles, which is probably due to the discontinuous phase formed between nanoparticles and the polymer matrix, making the nanocomposite film more porous, resulting in an increase in the WVP of the composite films [86]. The pivotal role in WVP of films plays the presence of plasticizer in polymer matrix. Generally, plasticizer e.g., glycerol located between adjacent chains of gelatin molecules decrease the intermolecular forces, thus increasing the free volume of the system and favouring the mobility of polypeptide chains in the film matrix. The increased mobility results in greater free volume and segmental motions, which facilitates the migration of water vapor molecules through the film. The water vapor transfer process in the films also depends on the hydrophilic/hydrophobic ratio of the film constituents [6].

Oxygen is an essential factor for the oxidation of food. The lower oxygen transmission rate of film could better prevent the oxidation of food, gelatin films are known for their oxygen barrier properties, due to their amino acid composition [23,87]. Polyphenolic components can interact with proteins (especially gelatin protein, rich in proline), resulting in the formation of protein–polyphenol complexes and forming hydrogen and covalent bonds with the polar groups of polypeptide gelatin chain [4]. It is speculated that these protein–polyphenol complexes could be responsible for OTR changes, while Ding *et al.* [23] and also Nassiri and Nafchi [25] suggested that more compact structures than the pure gelatin films, are difficult for oxygen to permeate.

Modified gelatin films did not show antimicrobial activity against *E. faecalis*, *P. aeruginosa* and *P. putida*. This data are opposite to the results obtained in a previous study on PLA/melanin modified films which were active against the above-mentioned bacteria species [1]. It is tempting to suggest that another film preparation mechanism (melanin was incorporated into the PLA matrix as particles, whereas in this study melanin was dissolved in alkaline conditions and reacted with melanin) could influence the antimicrobial activity of melanin and resulting modified films. The increased number of bacterial cells incubated with non-modified and modified films in comparison to control samples devoid of any films may result from the utilisation of gelatin as a nutrient source by bacteria. The solution could be the addition of some other antimicrobial compounds. These additives can be obtained from different sources, including plants, animals, bacteria, algae, fungi and by-products generated

during fruit and vegetable processing [4]. Some authors observed the antimicrobial activity of gelatin films modified with essential oils [2,5,8,28-36], chitin nanoparticles [26], lyzosyme [92], tomato pulp [52], vanillin [43], tannin [11] and metal nanoparticles, which are known from their excellent antimicrobial activity [4,25,26,76,85,86,93,94].

References

[1] Łopusiewicz Ł., Jędra F., Mizielińska M. 2018. New Poly(lactic acid) Active Packaging Composite Films Incorporated with Fungal Melanin. *Polymers* 10(4): 386. DOI: 10.3390/polym10040386

[2] Tongnuanchan P., Benjakul S., Prodpran T. 2012. Properties and antioxidant activity of fish skin gelatin film incorporated with citrus essential oils. *Food Chemistry* 134: 1571-1579. DOI: 10.1016/j.foodchem.2012.03.094

[3] Cao N., Fu Y., He J. 2007. Mechanical properties of gelatine films cross-linked, respectively, by ferulic acid and tannin acid. Food Hydrocolloids 21: 575-584. DOI: 10.1016/j.foodhyd.2006.07.001

[4] Ramos M., Valdés A., Beltrán A., Garrigós M.C. 2016. Gelatin-Based Films and Coatings for Food Packaging Applications. *Coatings* 6(41): 1-20. DOI: 10.3390/coatings6040041

[5] Tongnuanchan P., Benjakul S., Prodpran T., Pisuchpen S., Osako K. 2016. Mechanical, thermal and heat sealing properties of fish skin gelatin film containing palm oil and basil essential oil with different surfactants. *Food Hydrocolloids* 56: 93-107. DOI: 10.1016/j.foodhyd.2015.12.005

[6] Ahmad M., Benjakul S., Prodpran T., Agustini T.W. 2012. Physico-mechanical and antimicrobial properties of gelatin film from the skin of unicorn leatherjacket incorporated with essential oils. *Food Hydrocolloids* 28: 189-199. DOI: 10.1016/j.foodhyd.2011.12.003

[7] Sunday E.A., Joseph J.O. 2017. Production of regenerated cellulose polymeric films from plantain pseudostem. *World News of Natural Sciences* 7: 26-29.

[8] Wu X., Liu Y., Wang W., Han Y., Liu A. 2016. Improved mechanical and thermal properties of gelatin films using a nano inorganic filler. *Journal of Food Process Engineering* 40(3). DOI: 10.1111/jfpe.12469

[9] Aadil K.R., Barapatre A., Jha H. 2016. Synthesis and characterization of *Acacia* lignin-gelatin film for its possible application in food packaging. *Bioresources and Bioprocessing* 3: 27. DOI: 10.1186/s40643-016-0103-y

[10] Etxabide A., Leceta I., Cabezudo S., Guerrero P., de la Caba K. 2016. Sustainable Fish Gelatin Films: from Food Processing Waste to Compost. *ACS Sustainable Chemistry & Engineering* 4(9): 4626-4634. DOI: 10.1021/acssuschemeng.6b00750

[11] Peña-Rodriguez C., Martucci J.F., Neira L.M., Arbelaiz A., Eceiza A., Ruseckaite R.A. 2015. Functional properties and in vitro antioxidant and antibacterial effectiveness of

pigskin gelatin films incorporated with hydrolysable chestnut tannin. *Food Science and Technology International* 21(3): 221-231. DOI: 10.1177/1082013214525429

[12] Etxabide A., Coma V., Guerrero P., Gardrat C., de la Caba K. 2017. Effects of cross-linking in surface properties and antioxidant activity of gelatin films incorporated with a curcumin derivative. *Food Hydrocolloids* 66: 168-175. DOI: 10.1016/j.foodhyd.2016.11.036

[13] Bigi A., Panzavolta S., Rubini K. 2004. Relationship between triple-helix content and mechanical properties of gelatin films. *Biomaterials* 25: 5675-5680. DOI: 10.1016/j.biomaterials.2004.01.033

[14] Bigi A., Cojazzi G., Panzavolta S., Roveri N., Rubini K. 2002. Stabilization of gelatin films by crosslinking with genipin. *Biomaterials* 23: 4827-4832. DOI: 10.1016/S0142-9612(02)00235-1

[15] Giménez B., de Lacey L., Pérez-Santín E., López-Caballero M.E., Montero P. 2013. Release of active compounds from agar and agar-gelatin films with green tea extract. *Food Hydrocolloids* 30: 264-271. DOI: 10.1016/j.foodhyd.2012.05.014

[16] Prasertsung I., Mongkolnavin R., Kanokpanont S., Damrongsakkul S. 2010. The effects of pulsed inductively coupled plasma (PICP) on physical properties and biocompatibility of crosslinked gelatin films. *International Journal of Biological Macromolecules* 46: 72–78. DOI: 10.1016/j.ijbiomac.2009.11.001

[17] Schejtman S.D.G., Toselli R., Strumia M.C., Martinelli M. 2015. Gelatin films dendronized selectively on one side: enhancing antimicrobial properties and water repellence. *Polymer Bulletin* 72: 3043-3062. DOI: 10.1007/s00289-015-1452-y

[18] Yi J.B., Kim Y.T., Bae H.J., Whiteside W.S., Park H.J. 2006. Influence of Transglutaminase-Induced Cross-Linking on Properties of Fish Gelatin Films. *Journal of Food Science* 71(9): 376-383. DOI: 10.1111/j.1750-3841.2006.00191.x

[19] Bigi A., Panzavolta S., Roveri N. 1998. Hydroxyapatite-gelatin films: a structural and mechanical characterization. *Biomaterials* 19: 739-744. DOI: 10.1016/S0142-9612(97)00194-4

[20] Pranoto Y., Lee C.M., Park H.Y. 2007. Characterizations of fish gelatin films added with gellan and κ-carrageenan. *LWT- Food Science and Technology* 40(5): 766-774. DOI: 10.1016/j.lwt.2006.04.005

[21] Boanini E., Rubini K., Panzavolta S., Bigi A. 2010. Chemico-physical characterization of gelatin films modified with oxidized alginate. *Acta Biomaterialica* 6: 383-388. DOI: 10.1016/j.actabio.2009.06.015

[22] Soo P.Y., Sarbon N.M. 2018. Preparation and characterization of edible chicken skin gelatin film incorporated with rice flour. *Food Packaging and Shelf Life* 15: 1-8. DOI: 10.1016/j.fpsl.2017.12.009

[23] Ding J., Wu X., Qi X., Guo H., Liu A., Wang W. 2018. Impact of nano/micron vegetable carbon black on mechanical, barrier and anti-photooxidation properties of fish gelatin film. *Journal of the Science of Food and Agriculture* 98(7): 2632-2641. DOI: 10.1002/jsfa.8756

[24] Ortiz-Zarama M.A., Jiménez-Aparicio A., Perea-Flores M.J., Solorza-Feria J. 2014. Barrier, mechanical and morpho-structural properties of gelatin films with carbon nanotubes addition. *Journal of Food Engineering* 120: 223–232. DOI: 10.1016/j.jfoodeng.2013.08.004

[25] Nassiri R., Nafchi A.M. 2013. Antimicrobial and Barrier Properties of Bovine Gelatin Films Reinforced by Nano TiO_2. *Journal of Chemical Health Risks* 3(3): 12-28.

[26] Sahraee S., Ghanbarzadeh B., Milani J.M., Hamishehkar H. 2017. Development of Gelatin Bionanocomposite Films Containing Chitin and ZnO Nanoparticles. *Food and Bioprocess Technology* 10(8): 1441-1453. DOI: 10.1007/s11947-017-1907-2

[27] Nor Amalini A., Norziah M.H., Khan I., Haafiz M.K.M. 2018. Exploring the properties of modified fish gelatin films incorporated with different fatty acid sucrose esters. *Food Packaging and Shelf Life* 15: 105–112. DOI: 10.1016/j.fpsl.2017.12.003

[28] Ahmad M., Benjakul S., Sumpavapol P., Nirmal B.P. 2012. Quality changes of sea bass slices wrapped with gelatin film incorporated with lemongrass essential oil. *International Journal of Food Microbiology* 155: 171-178. DOI: 10.1016/j.ijfoodmicro.2012.01.027

[29] Alparslan Y., Yapıcı, Metin C., Baygar T., Günlü A., Baygar T. 2016. Quality assessment of shrimps preserved with orange leaf essential oil incorporated gelatin. *LWT – Food Science and Technology* 72: 457-466. DOI: 10.1016/j.lwt.2016.04.066

[30] Alparslan Y. 2018. Antimicrobial and antioxidant capacity of biodegradable gelatin film forming solutions incorporated with essential oils. Food Measure 12: 317-322. DOI: 10.1007/s11694-017-9643-x

[31] Wu J., Liu H., Ge S., Wang S., Qin Z., Chen L. Zheng Q., Liu Q., Zhang Q. 2015. The preparation, characterization, antimicrobial stability and in vitro release evaluation of fish gelatin films incorporated with cinnamon essential oil nanoliposomes. *Food Hydrocolloids* 43: 427-435. DOI: 10.1016/j.foodhyd.2014.06.017

[32] Tongnuanchan P., Benjakul S., Prodpran T. 2014. Comparative studies on properties and antioxidative activity of fish skin gelatin films incorporated with essential oils from various sources. *International Aquatic Research* 6: 62. DOI: 10.1007/s40071-014-0062-x

[33] Maryam Adilah Z.A., Nur Hanani Z.A. 2016. Active packaging of fish gelatin films with *Morinda citrifolia* oil. *Food Bioscience* 16: 66–71. DOI: 10.1016/j.fbio.2016.10.002

[34] Martucci J.F., Gende L.B., Neira L.M., Ruseckaite R.A. 2015. Oregano and lavender essential oils as antioxidant and antimicrobial additives of biogenic gelatin films. *Industrial Crops and Products* 71: 205–213. DOI: 10.1016/j.indcrop.2015.03.079

[35] Lee K-Y., Lee J-H., Yang H-J., Song K.B. 2016. Production and characterisation of skate skin gelatin films incorporated with thyme essential oil and their application in chicken tenderloin packaging. *International Journal of Food Science and Technology* 51: 1465–1472. DOI: 10.1111/ijfs.13119

[36] Kavoosi G., Rahmatollahi A., Dadfar S.M.M., Purfard A.M. 2014. Effects of essential oil on the water binding capacity, physicomechanical properties, antioxidant and antibacterial activity of gelatin films. *LWT - Food Science and Technology* 57(2): 556-561. DOI: 10.1016/j.lwt.2014.02.008

[37] Liang C., Jia M., Tian D., Tang Y., Ju W., Ding S., Tian L., Ren X., Wang X. 2017. Edible sturgeon skin gelatine films: Tensile strength and UV light-barrier as enhanced by blending with esculine. *Journal of Functional Foods* 37: 219–228. DOI: 10.1016/j.jff.2017.07.051

[38] Jongjareonrak A., Benjakul S., Visessanguan W., Tanaka M. 2008. Antioxidative activity and properties of fish skin gelatin films incorporated with BHT and a-tocopherol. *Food Hydrocolloids* 22: 449-458. DOI: 10.1016/j.foodhyd.2007.01.002

[39] Núñez-Flores R., Giménez B., Fernández-Martín F., López-Caballero M.E., Montero M.P., Gómez-Guillén M.C. 2013. Physical and functional characterization of active fish gelatin films incorporated with lignin. *Food Hydrocolloids* 30: 163-172. DOI: 10.1016/j.foodhyd.2012.05.017

[40] Wang W., Li C., Zhang H., Ni Y. 2016. Using Liquid Smoke to Improve Mechanical and Water Resistance Properties of Gelatin Films. *Journal of Food Science* 81(5): 1151-1157. DOI: 10.1111/1750-3841.13282

[41] Kavoosi G., Dadfar S.M.M., Purfard A.M., Mehrabi R. 2013. Antioxidant and Antibacterial Properties of Gelatin Films Incorporated with Carvacrol. *Journal of Food Safety* 33: 423-432. DOI: 10.1111/jfs.12071

[42] Tammineni N., Rasco B., Powers J., Nindo C., Ünlu G. 2014. Bovine and fish gelatin coatings incorporating tannins: effect on physical properties and oxidative stability of salmon fillets. *Journal of Food Chemistry and Nutrition* 2: 93-102.

[43] Lee K-Y., Lee J-H., Yang H-J., Song K.B. 2016. Characterization of a starfish gelatin film containing vanillin and its application in the packaging of crab stick. *Food Science and Biotechnology* 25(4):1023-1028. DOI: 10.1007/s10068-016-0165-9

[44] Wang K., Wang W., Wu X., Xiao J., Liu Y., Liu A. 2016. Effect of photochemical UV/riboflavin-mediated cross-links on different properties of fish gelatin films. *Journal of Food Process Engineering* 40: 1-10. DOI: 10.1111/jfpe.12536

[45] Limpisophon K., Schleining G. 2017. Use of Gallic Acid to Enhance the Antioxidant and Mechanical Properties of Active Fish Gelatin Film. *Journal of Food Science* 82(1): 80-89. DOI: 10.1111/1750-3841.13578

[46] Sui Chin S., Han Lyn F., Nur Hanani Z.A. 2017. Effect of *Aloe vera* (*Aloe barbadensis* Miller) gel on the physical and functional properties of fish gelatin films as active packaging. *Food Packaging and Shelf Life* 12: 128-134. DOI: 10.1016/j.fpsl.2017.04.008

[47] Radi M., Firouzi E. Akhavan H., Amiri S. 2017. Effect of Gelatin-Based Edible Coatings Incorporated with *Aloe vera* and Black and Green Tea Extracts on the Shelf Life of Fresh-Cut Oranges. *Journal of Food Quality* 9764650: 1-10. DOI: 10.1155/2017/9764650

[48] Bao S., Xu S., Wang Z. 2009. Antioxidant activity and properties of gelatin films incorporated with tea polyphenol-loaded chitosan nanoparticles. *Journal of the Science of Food and Agriculture* 89: 2692-2700. DOI: 10.1002/jsfa.3775

[49] Giménez B., Moreno S., López-Caballero M.E., Montero P., Gómez-Guillén M.C. 2013. Antioxidant properties of green tea extract incorporated to fish gelatin films after simulated gastrointestinal enzymatic digestion. *LWT- Food Science and Technology* 53: 445-451. DOI: 10.1016/j.lwt.2013.03.020

[50] Lin J., Wang Y., Pan D., Sun Y., Ou C., Cao J. 2018. Physico-mechanical properties of gelatin films modified with Lysine, Arginine and Histidine. *International Journal of Biological Macromolecules* 108: 947-952. DOI: 10.1016/j.ijbiomac.2017.11.015

[51] Lim G-O., Jang S.A., Kim J-Y., Kim H-J., Song K.B. 2010. Use of a Gelatin Film Containing Grapefruit Seed Extract in the packaging of Strawberries. *Korean Journal of Food Preservation* 17(2): 196-201.

[52] Kannat S.R., Jethwa T., Sawant K., Chawla S.P. 2017. PVA-Gelatin Films Incorporated With Tomato Pulp: A Potential Primary Food Packaging Film. *International Journal of Current Microbiology and Applied Sciences* 6(10): 1-14. DOI: 10.20546/ijcmas.2017.610.xx

[53] Łopusiewicz Ł., Mizielińska M. 2017. Antifungal activity of PLA foils covered with ethylocelulose containing essential oils. *World News of Natural Sciences* 12: 27-32.

[54] Sun X., Wu B., Zhou L., Liu Z., Dong Y., Yang A. 2017. Isolation and characterization of melanin pigment from yesso scallop *Patinopecten yessoensis*. *Journal of Ocean University China* 16(2): 279-284. DOI: 10.1007/s11802-017-3162-6

[55] Łopusiewicz Ł. 2018. Antioxidant, antibacterial properties and the light barrier assessment of of raw and purified melanins isolated from *Citrullus lanatus* (watermelon) seeds. *Herba Polonica* 64(2).

[56] Kurian N.K., Nair H.P., Bhat S.G. 2014. Melanin producing *Pseudomonas stutzeri* BTCZ10 from marine sediment at 96 m depth (Sagar Sampada cruise #305). *International Journal of Current Biotechnology* 2(5): 6-11.

[57] Łopusiewicz Ł. 2018. *Scleroderma citrinum* melanin: isolation, purification, spectroscopic studies with characterization of antioxidant, antibacterial and light barrier properties. *World Scientific News* 94(2): 114-129.

[58] Łopusiewicz Ł. 2018. Isolation, characterisation and biological activity of melanin from *Exidia nigricans*. *World Scientific News* 91: 111–129.

[59] Łopusiewicz Ł. 2018. The isolation, purification and analysis of the melanin pigment extracted from *Armillaria mellea* rhizomorphs. *World Scientific News* 100: 135-153.

[60] Solano F. Melanins: skin pigments and much more – types, structural models, biological functions, and formation routes. *New Journal of Science* 2014.

[61] Łopusiewicz Ł., Lisiecki S. 2016. „Czarne złoto" – melaniny w życiu człowieka [In Polish]. *Kosmos* 65(4): 621-629.

[62] Łopusiewicz Ł., Lisiecki S., Mizielińska M. 2017. Aktywność przeciwutleniające folii PE i PLA modyfikowanych powłokami zawierającymi melaniny grzybowe [In Polish]. *Opakowanie* 3: 81-85.

[63] Łopusiewicz Ł., Lisiecki S., Mizielińska M. 2017. Właściwości przeciwutleniające i optyczne folii BOPP i PET modyfikowanych powłokami zawierającymi melaniny grzybowe [In Polish]. *Opakowanie* 7: 48-55.

[64] Dong, W., Wang Y., Huang C., Xiang S., Ma P., Ni Z.. Chen M. 2014. Enhanced thermal stability of poly(vinyl alcohol) in presence of melanin. *Journal of Thermal Analysis and Calorimetry* 115: 1661–1668. DOI: 10.1007/s10973-013-3419-2

[65] Shanmuganathan K., Cho J.H., Iyer P., Baranowitz S., Ellison C.J. 2011. Thermooxidative Stabilization of Polymers Using Natural and Synthetic Melanins. *Macromolecules* 44: 9499–9507. DOI: 10.1021/ma202170n

[66] Kiran G.S., Jackson S.A., Priyadharsini S., Dobson A.D.W., Selvin J. 2017. Synthesis of Nm-PHB (nanomelanin-polyhydroxybutyrate) nanocomposite film and its protective effect against biofilm-forming multi drug resistant *Staphylococcus aureus*. *Scientific Reports* 7: 9167. DOI: 10.1038/s41598-017-08816-y

[67] Cao N., Yang X., Fu Y. 2009. Effects of various plasticizers on mechanical and water vapour barrier properties of gelatin films. *Food Hydrocolloids* 23:729-735. DOI: 10.1016/j.foodhyd.2008.07.017

[68] Thomazine M., Carvalho R.S., Sobral P.J.A. 2005. Physical Properties of Gelatin Films Plasticized by Blends of Glycerol and Sorbitol. *Journal of Food Science* 70(3): 172-176. DOI: 10.1111/j.1365-2621.2005.tb07132.x

[69] Bandeira S.F., De Souza Gomes da Silva R., de Moura J.M., de Almeida Pinto L.A. 2015. Modified gelatin films from croaker skins: effects of pH, and addition of glycerol and chitosan. *Journal of Food Process Engineering* 38: 613-620. DOI: 10.1111/jfpe.12191

[70] Król Ż., Malik M., Marycz K., Jarmoluk A. 2016. Characteristic of Gelatine, Carrageenan and Sodium Alginate Hydrosols Treated by Direct Electric Current. *Polymers* 8: 275. DOI: 10.3390/polym8080275

[71] Karnnet S., Potiyaray P., Pimpan V. 2005. Preparation and properties of biodegradable stearic acid-modified gelatin films. *Polymer Degradation and Stability* 90: 106-110. DOI: 10.1016/j.polymdegradstab.2005.02.016

[72] Bergo P., Moraes I.C.F., Sobral P.J.A. 2012. Effects of Different Moisture Contents on Physical Properties of PVA-Gelatin Films. *Food Biophysics* 7: 354-361. DOI: 10.1007/s11483-012-9273-0

[73] Nagarajan M., Prodpran T., Benjakul S., Songtipya P. 2017. Properties and Characteristics of Multi-Layered Films from Tilapia Skin Gelatin and Poly(Lactic Acid). *Food Biophysics* 12: 222. DOI: 10.1007/s11483-017-9478-3

[74] Javadian N., Mirzai H., Nafchi A.M. 2014. The Effects of Ribose on Mechanical and Physicochemical Properties of Cold Water Fish Gelatin Films. *Journal of Chemical Health Risks* 4(2): 39-45.

[75] Rawdkuen S., Suthiluk P., Kamhangwong D., Benjakul S. 2012. Mechanical, physico-chemical, and antimicrobial properties of gelatin-based film incorporated with catechin-lysozyme. *Chemistry Central Journal* 6: 131. DOI: 10.1186/1752-153X-6-131

[76] Nafchi A.M., Moradpour M., Saeidi M., Alias A.K. 2014. Effects of nanorod-rich ZnO on rheological, sorption isotherm, and physicochemical properties of bovine gelatin films. *LWT - Food Science and Technology* 58: 142-149. DOI: 10.1016/j.lwt.2014.03.007

[77] Peña C., de la Caba K., Eceiza A., Ruseckaite R., Mondragon I. 2010. Enhancing water repellence and mechanical properties of gelatin films by tannin addition. *Bioresource Technology* 101: 6836–6842. DOI: 10.1016/j.biortech.2010.03.112

[78] De Carvalho R.A., Grosso C.R.F. 2006. Properties of chemically modified gelatin films. *Brazilian Journal of Chemical Engineering* 23(1): 45-53. DOI: 10.1590/S0104-66322006000100006

[79] Jirukkakul N., Sodtipinta J. 2017. Effects of the Tomato Pomace Oil Extract on the Physical and Antioxidant Properties of Gelatin Films. *International Journal of Food* Engineering 3(1): 1-5. DOI: 10.18178/ijfe.3.1.1-5

[80] Lee K-Y., Yang H-J., Song K.B. 2016. Application of a puffer fish skin gelatin film containing Moringa oleifera Lam. leaf extract to the packaging of Gouda cheese. *Journal of Food Science and Technology* 53(11): 3876-3883. DOI: 10.1007/s13197-016-2367-9

[81] Huang Z., Lui, H., Chen M.X.K., Alajlan A., McLean D.I., Zeng, H. 2004. Raman spectroscopy of in vivo cutaneous melanin. *Journal of Biomedical Optics* 9: 1198–1205. DOI: 10.1117/1.1805553

[82] Galván I., Jorge A., Ito K., Tabuchi K., Solano F., Wakamatsu K. 2013. Raman spectroscopy as non-invasive technique for quantification of melanins in feathers and hairs. *Pigment Cell & Melanoma Research* 26(6): 917-923. DOI: 10.1111/pcmr.12140

[83] Rattaya S., Benjakul S., Prodpran T. 2009. Properties of fish skin gelatin film incorporated with seaweed extract. *Journal of Food Engineering* 95: 151-157. DOI: 10.1016/j.jfoodeng.2009.04.022

[84] Pilaquinga F., Escobar V., Cueva G., Meneses L. 2018. Preparation of a degradable hydrocolloid film with silver nanoparticles synthetized with aqeous garlic extract (*Allium sativum*). *Southern Brazilian Journal of Chemistry* 15(29): 266-275.

[85] Jamróz E., Kopel P., Juszczak L., Kawecka A., Bytesnikova Z., Milosavljević V., Kucharek M., Makarewicz M., Adam V. 2018. Development and characterisation of furcellaran-gelatin films containing SeNPs and AgNPs that have antimicrobial activity. *Food Hydrocolloids* 83: 9-16. DOI: 10.1016/j.foodhyd.2018.04.028

[86] Shankar S., Teng X., Li G., Rhim J-W. 2015. Preparation, characterization, and antimicrobial activity of gelatin/ZnO nanocomposite films. *Food Hydrocolloids* 45: 264-271. DOI: 10.1016/j.foodhyd.2014.12.001

[87] Araghi M., Moslehi Z., Nafchi A.M., Mostahsan A., Salamat N., Garmakhany A.D. 2015. Cold water fish gelatin modification by a natural phenolic cross-linker (ferulic acid and caffeic acid). *Food Science & Nutrition* 3(5): 370-375. DOI: 10.1002/fsn3.230

[88] Zhang X., Do M.D., Casey P., Sulistio A., Qiao G.G., Lundin L., Lillford P., Kosaraju S. 2010. Chemical Cross-Linking Gelatin with Natural Phenolic Compounds as Studied by High-Resolution NMR Spectroscopy. *Biomacromolecules* 11(4): 1125-1132. DOI: 10.1021/bm1001284

[89] Shi C., Zhuang C., Ciu Y., Tao F. 2017. Preparation and characterization of gelatin film modified by cellulose active ester. *Polymer Bulletin* 74: 3505-3525. DOI: 10.1007/s00289-017-1907-4

[90] Yue L., Li J., Zhu W-Y., Xu J., Li T-D. 2017. Fabrication, andcharacterization of polydimethylsiloxane/ glycidol-grafted gelatin film. *Journal of Coatings Technology and Research* 14: 225-231. DOI: 10.1007/s11998-016-9846-9

[91] Wang W., Liu Y., Jia H., Liu Y., Zhang H., He Z., Ni Y. 2016. Effects of Cellulose Nanofibers Filling and Palmitic Acid Emulsions Coating on the Physical Properties of Fish Gelatin Films. *Food Biophysics* 12: 23-32. DOI: 10.1007/s11483-016-9459-y

[92] Ma W., Tang C-H., Yin S-W., Yang X-Q., Qi J-R. 2013. Genipin-crosslinked gelatin films as controlled releasing carriers of lysozyme. *Food Research International* 51: 321–324. DOI: 10.1016/j.foodres.2012.12.039

[93] Rosi H., Kalyanasundaram S. 2018. Synthesis, characterization, structural and optical properties of titanium-dioxide nanoparticles using *Glycosmis cochinchinensis* Leaf extract and its photocatalytic evaluation and antimicrobial properties. *World News of Natural Sciences* 17: 1-15.

[94] Shivakumar Sings P., Vidyasagar G.M. 2018. Biosynthesis of antibacterial silver nano-particles from Aspergillus terreus. *World News of Natural Sciences* 16: 117-124.

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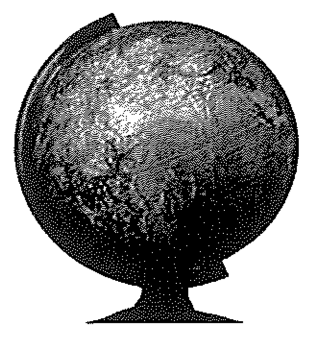

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The application of melanin modified gelatin coatings for packaging and the oxidative stability of pork lard

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ABSTRACT

The influence of gelatin coatings modified with fungal melanin on pork lard oxidative stability was studied. The lard was coated with gelatin coatings containing 0.1%; 0.5%; 1% of fungal melanin and control gelatin coating devoid of melanin. The peroxide values (POV), iodine values (IV) and acid values (AV) were studied after 7, 14 and 21 days storage in controlled conditions. Lard covered with modified coatings had lowered oxidative rancidity. Hence, modified coatings containing fungal melanin can be use effectively for the prevention of lard oxidation.

Keywords: melanin, packaging, antioxidant, lard, gelatin, lipid oxidation

1. INTRODUCTION

In the last few decades, there has been a marked increase in the use of natural polymer-based film materials and coatings in packaging for the food industry, which protect food from external contamination, retarding its deterioration by extending shelf life and maintaining quality and safety [1]. In addition to consumer requirements and in order to substitute petroleum-based plastic packaging, a wide variety of biopolymers that come from agro-food industrial wastes and renewable low cost natural resources have emerged [1,2].

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Packaging is widely used for the protection of food quality, thereby ensuring hygiene and extending the shelf life of perishable items, especially those susceptible to oxidative and microbiological deterioration [3]. Gelatin based edible films and coatings have already been proposed to protect, maintain or extend the shelf-life of food products [1,4,5]. Factors that should be considered when designing this type of system include the chemical nature of food, the controlled release mechanism, food organoleptic characteristic and additive toxicity, storage and distribution, physical and the mechanical properties of packaging and regulations to be applied in this framework [1].

Nowadays, research in the field of active packaging is also focused on the development of novel food packaging materials with antioxidant agents from natural sources, such as plant and spices extracts rather than synthetic antioxidants such as butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA), since synthetic antioxidants are suspected of raising some safety concerns and have been restricted in their use as food additives [4]. Antioxidant active packaging, an innovative concept, is defined as a packaging incorporated with certain antioxidants, such as natural antioxidants, in order to provide a sustained release of antioxidant during storage.

The antioxidant active packaging can retard lipid oxidation, which is one of main food deteriorations to extend the shelf-life of food products [6]. In this context, some studies have reported that natural antioxidants show sufficient capacity to control lipid oxidation inside the food packaging because the oxidative processes can cause the degradation of proteins, pigments and fats, limiting food shelf-life [1,7]. A great number of reports of gelatin modifications by antioxidants are known from literature. A wide spectrum of additives were used including: curcumin and its derivatives [8], essential oils [9-17], esculine [4], butylated hydroxytoluene and α-tocopherol [7], lignin [18,19], liquid smoke [20], tannin [21,22], carvacol [23], vanillin [24], riboflavin [25], gallic acid [6], aloe vera gel [26,27], tea polyphenols and green tea extracts loaded into chitosan nanoparticles [27], tomato pulp and tomato pomace oil extract [28,29], as well as other plant extracts [30].

In a previous study we developed modified gelatin/melanin films with antioxidant activity and improved the oxygen barrier properties [31]. The aim of this study was to investigate the influence of modified gelatin/melanin coatings based on a film forming formulation on the rancidity stability of coated pork lard.

2. MATERIAL AND METHODS

2. 1. Lard coating

Gelatin coatings were prepared based on formulation developed in the previous study [31]. Firstly, ammonia water was added to the distilled water and pH was adjusted to 10 (to enable a solubilisation of melanin). Next, fungal melanin was added to alkaline solutions to obtain a 0.1% (sample „1"), a 0.5% (sample „2") and a 1% (sample „3") (on gelatin dry basis), and the solutions were placed in a stirrer (120 rpm), at 50 °C for 24 h. The solution devoid of melanin addition served as a control coating solution (sample „0"). When the melanin was dissolved, the solutions were filtered through Whatman filter paper under vacuum. 8 g of gelatin was added to 100 mL of filtered alkaline solutions and kept for 2 h at 60 °C under continuous stirring (120 rpm) to obtain a homogenous solution. Then, 10 % w/w (based on gelatin dry mass) of glycerol was added (to be used as a plasticizer).

The solutions were allowed to stand until their temperature reached 40 °C, then the lard samples (2 cm × 2 cm × 2 cm) were immersed in the coating solution, immediately removed and inserted under an air fan to solidify the coatings. The procedure was repeated twice for each lard sample. The samples were conditioned in a chamber at 25 °C and 50% RH. All measurements were conducted at the beginning of experiment (time 0) and after 7, 14 and 21 days.

2. 2. Peroxide values (POV) measurements

The POV values of lard were measured according to Bao *et al.* based on the IUPAC method [32]. The sample (1–2 g) was mixed with 25 mL of chloroform/acetic acid (2:3 v/v) followed by 1 mL of saturated potassium iodide solution. The reaction mixture was allowed to stand for 5 min in the dark. Distilled water (75 mL) was added to the mixture, which was then titrated with 0.01 mol/L sodium thiosulfate using 1 mL of 10 g/L starch solution as an indicator. POV was calculated using the following Equation:

$$\text{POV (meq } O_2\,\text{kg}^{-1}) = (a - b) \times N \times 100/w \quad (1)$$

where: a and b are the volumes (mL) of sodium thiosulfate used for the sample and blank (distilled water) titrations respectively, N is the concentration of sodium thiosulfate (mol/L) and w is the sample weight (g).

2. 3. Iodine values (IV) measurements

The IV values of lard were measured according to PN-EN ISO 3961:2013-10 [33]. The sample (1-2 g) was mixed with 20 mL of cyclohexane: acetic acid (1:1 v/v) followed by 25 mL of Wijs solution. The reaction mixture was allowed to stand for 1 hour in the dark. After an incubation period, 20 mL of 10% KI and 150 mL of distilled water were added to the mixture, which was then titrated with 0.1 mol/L sodium thiosulfate to a light brown-yellow colour. Then 1 mL of 1 mL of 10 g/L starch solution was added and the mixture was continuously titrated until it became colourless. The iodine value was calculated using the following Equation:

$$\text{IV} = 12.69 \times N \times (a - b)/w \quad (2)$$

Where a and b are the volumes of sodium thiosulfate used for the sample and blank (distilled water) titrations, respectively, N is the concentration of sodium thiosulfate (mol/L), w is the sample weight (g) and 12.69 is a calculation coefficient.

2. 4. Acid values (AV) measurements

The acid value (AV) values of lard were measured according to PN-EN ISO 660:2010 [34]. The sample (1-2) g was mixed with 50 mL of ethanol: diethyl eter (1:1 v/v). The mixture was titrated with 0.1 mol/L KOH solution in the presence of phenolphthalein, as an indicator. The acid value was calculated using the following Equation:

$$\text{AV} = 56.1 \times (a - b) \times N/w \quad (3)$$

where: a and b are the volumes of potassium hydroxide used for the sample and blank (distilled water) titrations, respectively, N is the concentration of potassium hydroxide (mol/L), w is the sample weight (g) and 56.1 is a calculation coefficient.

2. 5 Statistical analysis

All determinations were carried out in triplicate as a minimum. Statistical significance was determined using an analysis of variance (ANOVA) followed by a Duncans's test. The values were considered as significantly different when $p < 0.05$. All analyses were performed with Statistica version 10 (StatSoft Polska, Kraków, Poland).

3. RESULTS AND DISCUSSION

Food products are very susceptible to rancidity caused by the oxidation of lipids that contain unsaturated fatty acids that can be attacked by oxygen free radicals. Antioxidants are added to foods to intercept and react with these free radicals at a faster rate than the lipid substrate. Nevertheless, the current incorporation of antioxidants throughout the entire food matrix in one large initial dose is not an efficient process due to the oxidation occurring at the surface and high initial doses of antioxidant having a pro-oxidant effect [35]. Therefore, one emerging technology is the use of antioxidant active packaging, where the antioxidant is incorporated into a packaging material with the purpose of being delivered to the food surface during commercialization, at an appropriate rate. Most of the active packaging developments base their work on the mass transportation properties of plastic materials (sorption, migration, and permeation), and the release of the active agents depends on several factors, such as the type of polymer and type of food. However, the presence of synthetic antioxidants in food is questionable, owing to the potential risks. This has been encouraged by strong consumer demand, as synthetic compounds are frequently perceived as undesirable or harmful. Natural antioxidants are preferred to artificial substances, especially by consumers [4,7]. Moreover, the use of active antioxidant packaging that incorporates natural antioxidants presents important advantages. The addition of a natural compound to the packaging may reduce the need to use synthetic antioxidants in the plastic, reducing the risk of potential toxicity by migration [1].

Animal fats have long been recognized as a raw material for food and industrial applications [36]. Lard is a form of pig fat which in a saturated or unsaturated form derived from its adipose tissue and may be used in its raw form as frying medium or after modification of its physical properties, as shortening for baking applications and is often used in food production as an emulsion, shortening, or as a substitute to butter, margarine or cooking oils. Usually, it is extracted from the back skin, muscle, surrounding digestive organs, surrounding the kidneys of pig. Scientifically, lard is known as triglyceride, it is mainly consists of fats or fatty acid. It contains considerable proportions of palmitic, stearic, oleic and linoleic acids. There are also small amounts of palmitoleic, traces of linoleic, arachidonic and myristic acids [37]. Lard is a common choice due to its cheap market price and easy availability [38-40]. The application of antioxidants and antioxidant-gelatin composite films for lard are known from literature. Mihaylova and Schalow used quercetin-containing flavonoid extract obtained from *Sophora japonica* flower for the antoxidant stabilization of lard [41]. Antonietta Paleari *et al.* covered lard with antioxidant spices and

aromatic herbs [42]. Yeo *et al.* applied free radical scavengers such as α-tocopherol, BHT, sesamol, tert-butylhydroquinone in thermally-oxidized lard [43]. Dziedzic *et al.* used polyhydroxydihydrochalcones as antioxidants for lard [44]. Jongjareonrak et al. applied modified fish skin gelatin films incorporated with BHT and α-tocopherol on plastic cups containing lard [7].

Recent studies have focused on techniques to develop active gelatin-based packaging films and coatings, including antimicrobial, antioxidant and other agents which can enhance the biological features of food [1]. Liu *et al.* developed gelatin-based films packaged with sunflower oils with different free/encapsulated tea polyphenols in chitosan nanoparticles [45]. Their results showed a reduction in the oxidation of sunflower oil obtaining lower peroxide (POV) and thiobarbituric acid reactive substance (TBARS) values for oils packed in the films. In addition, an improvement in antioxidant activity when using and optimum ratio of free and encapsulated additives was demonstrated over a long period of storage (6 weeks) as well as the preservation of the functional properties of the developed films. Gómez-Estaca *et al.* [46] observed that pigskin gelatin film with oregano and rosemary extracts delayed the lipid oxidation of cold smoked sardine coated with the films in storage at 5 °C.

Kakei *et al.* noted that chitosan-gelatin incorporated with ethanolic red grape seed extract and *Ziziphora clinopodioides* essential oil improved the lipid stability of minced trout fillet [47]. Chottanom *et al.* [48] noted that gelatin-starch pouches modified with plant extract decreased the hydrolytic rancidity rate of meat products. Lee *et al.* [5] applied a fish skin gelatin film containing *Moringa oleifera* leaf extract to the packaging of Gouda cheese and noted lower POV and TBARS values in covered samples after a period of storage. Alparslan *et al.* [13] observed that a gelatin film enriched with laurel essential oil was suitable for the preservation of rainbow trout fillet, retarded fish lipid oxidation and the ability of laurel essential oil to preserve the film depended on its ratio. Also Alparslan *et al.* [12] used gelatin coatings enriched with orange leaf essential oil for the preservation of shrimps, and noted that coatings effectively preserved lipid stability. On the other hand, Antoniewski *et al.* [49] found that a gelatin coating had no effect on lipid oxidation in salmon fillet stored at 4 °C for 14 days. Ahmad *et al.* [3] used modified gelatin films with lemongrass essential oil to wrap sea bass slices. During storage, a significant increase in TBARS was observed in control samples than compared to the samples wrapped with non-modified and modified films. This result suggested that the lipid oxidation in sea bass slices could be delayed when a lemongrass essential oil film was applied, probably due to the antioxidant activity of the lemongrass essential oil, as well as the low oxygen permeability characteristics of the modified film. Those results are in line with our results in our previous study, which showed that modified melanin/gelatin films have a significantly lower oxygen transmission rate in comparison to the non-modified films [31].

According to Ahmad *et al.* [3] the antioxidant activities of the essential oils have been attributed to assorted mechanisms, including the prevention of radical chain initiation, the binding of transition metal ion catalysts, the decomposition of peroxides and the interaction with the free radicals. Melanins are also known from their antioxidant activity as well as metal ions chelating ability [50-54]. Also there are several reports that packaging materials and coatings containing melanins have antioxidant activity [35,55,56].

The mechanism of action of these natural antioxidants in contact with food are related to lipid oxidation reactions. In addition, they are focused on phenolic groups present in the melanin structure. Hydrogen atoms from phenol hydroxyl groups could react with peroxyl

radicals produced at the early stages of the oxidation mechanism to yield stable phenoxyl radicals and, consequently result in the termination of the lipid peroxidation chain reactions. However, understanding the antioxidant activity mechanism of melanins in films is a hard task as this activity depends on the electronic and steric effects on their ring substituents, the strength of hydrogen-bonding interactions between the phenolic groups and the solvent, and the interactions with the film matrix and the packaged food [1].

POV was used to monitor the oxidation of lard packaged with gelatin coatings, both with and without the addition of incorporated melanin. Peroxide value, an oxidative rancidity parameter is measured from hyroperoxide content. The hyperoxide species generally occurs in the initial phase of oxidative rancidity and then reacts with additional lipid molecules to form other reactive chemical species [48]. As can be seen in Table 1, the POV of lard without film coating (uncoated) increased continuously and rapidly within a short time under controlled incubation. However, the POV of lard covered with films coatings increased slowly, even in the case of non-modified coatings. The differences between all the samples were statistically significant ($p<0.05$). This phenomenon can be attributed to the effect of the coatings in retarding oxygen of the lard. During the entire incubation period the POV of lard coated with coatings containing melanin was lower than that of lard coated with coatings devoid of melanin. It is tempting to suggest that it was due the antioxidant activity of melanin.

Table 1. The influence of pure gelatin and gelatin/melanin coatings on lard peroxide values (POV) [meq O_2 kg^{-1}]

	Uncovered	Control	0.1% melanin	0.5% melanin	1% melanin
0 day	1.45±0.11	1.45±0.11	1.45±0.11	1.45±0.11	1.45±0.11
7 day	28.44±0.13	7.93±0.20	5.67±0.09	1.83±0.04	1.88±0.06
14 day	73.92±0.25	25.22±0.33	7.22±0.18	2.44±0.07	2.33±0.15
21 day	111.12±1.45	30.11±0.55	9.91±0.22	2.77±0.09	2.49±0.05

The acid value (AV) is a number that expresses the quantity of potassium hydroxide, in milligrams, required to neutralize the free acids present in 1 g of the substance. The acid value may be overestimated if other acid components are present in the system, *e.g.* amino acids or acid phosphates. The acid value is often a good measure in the breakdown of the triacylglycrols into free fatty acids, which has an adverse effect on the quality of many lipids. Acid value is the measure of hydrolytic rancidity. In general, it gives an indication about edibility of the fat. As can be seen in Table 2, the AV of lard without film coating (uncoated) increased continuously and rapidly within a short time under controlled incubation. However, the AV of lard covered with films coatings increased slowly, even in the case of non-modified coatings, indicating that gelating coatings and melanin/gelatin coatings influenced the amount of free fatty acids in lard samples. The differences between all the samples were statistically significant ($p<0.05$).

Table 2. The influence of pure gelatin and gelatin/melanin coatings on lard acid values (AV) [mg KOH/g]

	Uncovered	Control	0.1% melanin	0.5% melanin	1% melanin
0 day	1.31±0.11	1.31±0.11	1.31±0.11	1.31±0.11	1.31±0.11
7 day	1.77±0.14	1.68±0.09	1.45±0.33	1.37±0.06	1.35±0.04
14 day	2.84±0.25	1.99±0.15	1.78±0.13	1.45±0.03	1.44±0.09
21 day	3.15±0.07	2.52±0.08	2.22±0.05	1.55±0.04	1.52±0.12

Iodine number indicates the degree of unsaturation i.e. the number of double bonds present at the length of the chain. Iodine value is low in animal fats and high in vegetable oils. The higher the iodine value, the lower the melting point. As can be seen in Table 3, the IV of lard without film coating (uncoated) decreased continuously under controlled incubation. However, the IV of lard covered with films coatings decreased slowly, even in the case of non-modified coatings, indicating that gelating coatings and melanin/gelatin coatings influenced the amount of unsaturated bonds in lard samples. The differences between all the samples were statistically significant ($p<0.05$).

Table 3. The influence of pure gelatin and gelatin/melanin coatings on lard iodine values (IV) [g/100 g]

	Uncovered	Control	0.1% melanin	0.5% melanin	1% melanin
0 day	58.11±1.11	58.11±1.11	58.11±1.11	58.11±1.11	58.11±1.11
7 day	52.34±1.98	57.13±3.15	56.98±1.67	56.33±2.34	57.45±2.17
14 day	45.22±1.45	52.84±3.19	55.12±3.12	55.23±3.78	56.11±1.56
21 day	43.23±2.45	50.13±2.63	51.34±2.88	52.18±2.52	53.66±1.34

The preventive effect on lard stabilization was possibly also caused by the increased hydrophobicity of the coatings matrix in the presence of melanin. In a previous study, the contact angle of films increased from 53.3° (unmodified film) 72.9° (1% of melanin in film) [31]. Similar mechanism has been proposed by Jongjareonrak *et al.* [7], who used hydrophobic BHT and α-tocopherol for the modification of fish skin gelatin films used for lard packaging. Gelatin films might function as a barrier to oxygen permeability on the lard surface. Therefore, only a small amount of oxygen could penetrate into the lard, leading to a lower oxidation rate. On the other hand, Jonjareonrak *et al.* [7] concluded that the addition of (BHT) and *α*-tocopherol had a negligible antioxidant effect on lard. It is tempting to suggest

that when they covered the cup containing the lard with films incorporated with BHT or α-tocopherol only changed the permeability of oxygen, and the antioxidants could not directly affect the oxidation of lard because of the separation between them and the lard, which was speculated also by Bao *et al.* [32] Their research found a direct connection between product and antioxidant coating, thus possibly maximising the effect of melanin against lard oxidation in our study.

4. CONCLUSION

Oxidation of lard was effectively retarded when covering with gelatin coatings containing fungal melanin. Hence, modified gelatin coatings can be applied to preserve of lipid stability.

References

[1] Ramos M., Valdés A., Beltrán A., Garrigós M.C. 2016. Gelatin-Based Films and Coatings for Food Packaging Applications. *Coatings* 6(41): 1-20. DOI: 10.3390/coatings6040041

[2] Tongnuanchan P., Benjakul S., Prodpran T., Pisuchpen S., Osako K. 2016. Mechanical, thermal and heat sealing properties of fish skin gelatin film containing palm oil and basil essential oil with different surfactants. *Food Hydrocolloids* 56: 93-107. DOI: 10.1016/j.foodhyd.2015.12.005

[3] Ahmad M., Benjakul S., Sumpavapol P., Nirmal B.P. 2012. Quality changes of sea bass slices wrapped with gelatin film incorporated with lemongrass essential oil. *International Journal of Food Microbiology* 155: 171-178. DOI: 10.1016/j.ijfoodmicro.2012.01.027

[4] Liang C., Jia M., Tian D., Tang Y., Ju W., Ding S., Tian L., Ren X., Wang X. 2017. Edible sturgeon skin gelatin films: Tensile strength and UV light-barrier as enhanced by blending with esculine. *Journal of Functional Foods* 37: 219–228. DOI: 10.1016/j.jff.2017.07.051

[5] Lee K-Y., Lee J-H., Yang H-J., Song K.B. 2016. Production and characterisation of skate skin gelatin films incorporated with thyme essential oil and their application in chicken tenderloin packaging. *International Journal of Food Science and Technology* 51: 1465–1472. DOI: 10.1111/ijfs.13119

[6] Limpisophon K., Schleining G. 2017. Use of Gallic Acid to Enhance the Antioxidant and Mechanical Properties of Active Fish Gelatin Film. *Journal of Food Science* 82(1): 80-89. DOI: 10.1111/1750-3841.13578

[7] Jongjareonrak A., Benjakul S., Visessanguan W., Tanaka M. 2008. Antioxidative activity and properties of fish skin gelatin films incorporated with BHT and a-tocopherol. *Food Hydrocolloids* 22: 449-458. DOI: 10.1016/j.foodhyd.2007.01.002

[8] Etxabide A., Coma V., Guerrero P., Gardrat C., de la Caba K. 2017. Effects of cross-linking in surface properties and antioxidant activity of gelatin films incorporated with a curcumin derivative. *Food Hydrocolloids* 66: 168-175. DOI: 10.1016/j.foodhyd.2016.11.036

[9] Kavoosi G., Rahmatollahi A., Dadfar S.M.M., Purfard A.M. 2014. Effects of essential oil on the water binding capacity, physicomechanical properties, antioxidant and antibacterial activity of gelatin films. *LWT - Food Science and Technology* 57(2): 556-561. DOI: 10.1016/j.lwt.2014.02.008

[10] [Alparslan Y., Baygar T., Baygar T., Hasanhocaoglu H., Metin C. 2014. Effects of Gelatin-Based Edible Films Enriched with Laurel Essential Oil on the Quality of Rainbow Trout (*Oncorhynchus mykiss*) Fillets During Refrigerated Storage. *Food Technology and Biotechnology* 52(3): 325-333.

[11] Martucci J.F., Gende L.B., Neira L.M., Ruseckaite R.A. 2015. Oregano and lavender essential oils as antioxidant and antimicrobial additives of biogenic gelatin films. *Industrial Crops and Products* 71: 205–213. DOI: 10.1016/j.indcrop.2015.03.079

[12] Alparslan Y., Yapıcı, Metin C., Baygar T., Günlü A., Baygar T. 2016. Quality assessment of shrimps preserved with orange leaf essential oil incorporated gelatin. *LWT – Food Science and Technology* 72: 457-466. DOI: 10.1016/j.lwt.2016.04.066

[13] Alparslan Y. 2018. Antimicrobial and antioxidant capacity of biodegradable gelatin film forming solutions incorporated with essential oils. *Food Measure* 12: 317-322. DOI: 10.1007/s11694-017-9643-x

[14] Wu J., Liu H., Ge S., Wang S., Qin Z., Chen L. Zheng Q., Liu Q., Zhang Q. 2015. The preparation, characterization, antimicrobial stability and in vitro release evaluation of fish gelatin films incorporated with cinnamon essential oil nanoliposomes. *Food Hydrocolloids* 43: 427-435. DOI: 10.1016/j.foodhyd.2014.06.017

[15] Tongnuanchan P., Benjakul S., Prodpran T. 2014. Comparative studies on properties and antioxidative activity of fish skin gelatin films incorporated with essential oils from various sources. *International Aquatic Research* 6: 62. DOI: 10.1007/s40071-014-0062-x

[16] Maryam Adilah Z.A., Nur Hanani Z.A. 2016. Active packaging of fish gelatin films with *Morinda citrifolia* oil. *Food Bioscience* 16: 66–71. DOI: 10.1016/j.fbio.2016.10.002

[17] Tongnuanchan P., Benjakul S., Prodpran T. 2012. Properties and antioxidant activity of fish skin gelatin film incorporated with citrus essential oils. *Food Chemistry* 134: 1571-1579. DOI: 10.1016/j.foodchem.2012.03.094

[18] Aadil K.R., Barapatre A., Jha H. 2016. Synthesis and characterization of *Acacia* lignin-gelatin film for its possible application in food packaging. *Bioresources and Bioprocessing* 3: 27. DOI: 10.1186/s40643-016-0103-y

[19] Núñez-Flores R., Giménez B., Fernández-Martín F., López-Caballero M.E., Montero M.P., Gómez-Guillén M.C. 2013. Physical and functional characterization of active fish

gelatin films incorporated with lignin. *Food Hydrocolloids* 30: 163-172. DOI: 10.1016/j.foodhyd.2012.05.017

[20] Wang W., Li C., Zhang H., Ni Y. 2016. Using Liquid Smoke to Improve Mechanical and Water Resistance Properties of Gelatin Films. *Journal of Food Science* 81(5): 1151-1157. DOI: 10.1111/1750-3841.13282

[21] Peña-Rodriguez C., Martucci J.F., Neira L.M., Arbelaiz A., Eceiza A., Ruseckaite R.A. 2015. Functional properties and in vitro antioxidant and antibacterial effectiveness of pigskin gelatin films incorporated with hydrolysable chestnut tannin. *Food Science and Technology International* 21(3): 221-231. DOI: 10.1177/1082013214525429

[22] Tammineni N., Rasco B., Powers J., Nindo C., Ünlu G. 2014. Bovine and fish gelatin coatings incorporating tannins: effect on physical properties and oxidative stability of salmon fillets. *Journal of Food Chemistry and Nutrition* 2: 93-102.

[23] Kavoosi G., Dadfar S.M.M., Purfard A.M., Mehrabi R. 2013. Antioxidant and Antibacterial Properties of Gelatin Films Incorporated with Carvacrol. *Journal of Food Safety* 33: 423-432. DOI: 10.1111/jfs.12071

[24] Lee K-Y., Lee J-H., Yang H-J., Song K.B. 2016. Characterization of a starfish gelatin film containing vanillin and its application in the packaging of crab stick. *Food Science and Biotechnology* 25(4):1023-1028. DOI: 10.1007/s10068-016-0165-9

[25] Wang K., Wang W., Wu X., Xiao J., Liu Y., Liu A. 2016. Effect of photochemical UV/riboflavin-mediated cross-links on different properties of fish gelatin films. *Journal of Food Process Engineering* 40: 1-10. DOI: 10.1111/jfpe.12536

[26] Sui Chin S., Han Lyn F., Nur Hanani Z.A. 2017. Effect of *Aloe vera* (*Aloe barbadensis* Miller) gel on the physical and functional properties of fish gelatin films as active packaging. Food Packaging and Shelf Life 12: 128-134. DOI: 10.1016/j.fpsl.2017.04.008

[27] Radi M., Firouzi E. Akhavan H., Amiri S. 2017. Effect of Gelatin-Based Edible Coatings Incorporated with *Aloe vera* and Black and Green Tea Extracts on the Shelf Life of Fresh-Cut Oranges. *Journal of Food Quality* 9764650: 1-10. DOI: 10.1155/2017/9764650

[28] Kannat S.R., Jethwa T., Sawant K., Chawla S.P. 2017. PVA-Gelatin Films Incorporated With Tomato Pulp: A Potential Primary Food Packaging Film. *International Journal of Current Microbiology and Applied Sciences* 6(10): 1-14. DOI: 10.20546/ijcmas.2017.610.xx

[29] Jirukkakul N., Sodtipinta J. 2017. Effects of the Tomato Pomace Oil Extract on the Physical and Antioxidant Properties of Gelatin Films. *International Journal of Food Engineering* 3(1): 1-5. DOI: 10.18178/ijfe.3.1.1-5

[30] Lee K-Y., Yang H-J., Song K.B. 2016. Application of a puffer fish skin gelatin film containing Moringa oleifera Lam. leaf extract to the packaging of Gouda cheese. *Journal of Food Science and Technology* 53(11): 3876-3883. DOI: 10.1007/s13197-016-2367-9

[31] Łopusiewicz Ł., Jędra F., Bartkowiak A. 2018. New Active Packaging Films Made from Gelatin Modified with Fungal Melanin. *World Scientific News* 101: 1-30.

[32] Bao S., Xu S., Wang Z. 2009. Antioxidant activity and properties of gelatin films incorporated with tea polyphenol-loaded chitosan nanoparticles. *Journal of the Science of Food and Agriculture* 89(15): 2692-2700. DOI: 10.1002/jsfa.3775

[33] Polish Norm PN-EN ISO 3961:2013-10

[34] Polish Norm PN-EN ISO 660:2010

[35] Łopusiewicz Ł., Jędra F., Mizielińska M. 2018. New Poly(lactic acid) Active Packaging Composite Films Incorporated with Fungal Melanin. *Polymers* 10(4): 386. DOI: 10.3390/polym10040386

[36] Chizoo E., Sunday U.C., Chimankpam E.M., Nwoye O.V., Uwaoma O.A. 2017. Extraction of Nigerian Beef Tallow by Wet Rendering Process and its Characterization. *World News of Natural Sciences* 15: 129-138.

[37] Sharma H., Giriprasad R., Goswami M. 2013. Animal FAT-Processing and Its Quality Control. *Food Processing & Technology* 4: 252. DOI: 0.4172/2157-7110.1000252

[38] Latief M., Khorsidtalab A., Saputra I., Akmeliawati R., Nurashikin A., Jaswir I., Witjaksono G. 2017. Rapid lard identification with portable electronic nose. *IOP Conf. Series: Materials Science and Engineering* 260: 012043. DOI: 10.1088/1757-899X/260/1/012043

[39] Yanty N.A.M. Marikkar J.M.N., Che Man Y.B., Long K. 2011. Composition and Thermal Analysis of Lard Stearin and Lard Olein. *Journal of Oleo Science* 60(7): 333-338. DOI: 10.5650/jos.60.333

[40] Khamil K.N., Yin C.K. 2016. An Analysis of lard by using Dielectric Sensing Method. *Journal of Telecommunication* 8(9): 131-137.

[41] Mihaylova D., Shalow S. 2013. Antioxidant and Stabilization Activity of a Quercitin-Containing Flavonoid Extract Obtained from Bulgarian *Sophora japonica* L. *Brazilian Arichives of Biology and Technology* 56(3): 431-438. DOI: 10.1590/S1516-89132013000030001

[42] Antonietta Paleari M., Maria Moretti V., Bersani C., Beretta G., Mentasti T. 2004. Characterisation of a lard cured with spices and aromatic herbs. *Meat Science* 67(4): 549-557. DOI: 10.1016/j.meatsci.2003.12.002

[43] Yeo J.D., Jeong M.K., Park C.U. Lee J. 2010. Comparing antioxidant effectiveness of natural and synthetic free radical scavengers in thermally-oxidized lard using DPPH method. *Journal of Food Science* 75(3):258-262. DOI: 10.1111/j.1750-3841.2010.01534.x

[44] Dziedzic S.Z., Hudson B.J.F., Barnes G. 1985. Polyhydroxydihydrochalcones as antioxidants for lard. *Journal of Agricultural and Food Chemistry* 33(2): 244-246. DOI: 10.1021/jf00062a022

[45] Liu F., Antiniou J., Li Y., Yi J., Yokoyama W., Ma J., Zhong F. 2015. Preparation of gelatin films incorporated with tea polyphenol nanoparticles for enhancing controlled-

release antioxidant properties. *Journal of Agricultural and Food Chemistry* 63(15): 3987-3995. DOI: 10.1021/acs.jafc.5b00003

[46] Gómez-Estaca J., Montero P., Giménez B., Gómez-Guillén M.C. 2007. Effect of functional edible films and high pressure processing on microbial and oxidative spoilage In cold-smoked sardine (*Sardina pilchardus*). *Food Chemistry* 105: 511-520. DOI: 10.1016/j.foodchem.2007.04.006

[47] Kakaei S., Shahbazi Y. 2016. Effect of chitosan-gelatin film incorporated with ethanolic red grape seed extract *and Ziziphora clinopodioides* essential oil on survival of *Listeria monocytogenes* and chemical, microbial and sensory properties of minced trout fillet. *LWT - Food Science and Technology* 72: 432-438. DOI: 10.1016/j.lwt.2016.05.021

[48] Chottanom P., Srisa-ard M., Seehangkot, Rungram N. 2014. Certain Properties of Gelatin-Starch Film Incorporated With Plant Extract (*Kaempferia parviflora* Wall. Ex Baker) and its Application to Meat Product. *American Journal of Food Technology* 9(7): 330-339. DOI: 10.3923/ajft.2014.330.339

[49] Antoniewski M.N., Barringer S.A., Knipe C.L., Zerby H.N. 2007. Effect of a gelatin coating on the shelf life of fresh meat. *Journal of Food Science* 72(6): 382-387. DOI: 10.1111/j.1750-3841.2007.00430.x

[50] Łopusiewicz Ł. 2018. Isolation, characterisation and biological activity of melanin from *Exidia nigricans*. *World Scientific News* 91, 111–129.

[51] Łopusiewicz Ł. 2018. *Scleroderma citrinum* melanin: isolation, purification, spectroscopic studies with characterization of antioxidant, antibacterial and light barrier properties. *World Scientific News* 94(2), 114-129.

[52] Łopusiewicz Ł. 2018. The isolation, purification and analysis of the melanin pigment extracted from *Armillaria mellea* rhizomorphs. *World Scientific News* 100: 135-153.

[53] Łopusiewicz Ł. 2018. Antioxidant, antibacterial properties and the light barrier assessment of of raw and purified melanins isolated from *Citrullus lanatus* (watermelon) seeds. *Herba Polonica* 64(2).

[54] Łopusiewicz Ł., Lisiecki S. 2016. „Czarne złoto" – melaniny w życiu człowieka [In Polish]. *Kosmos* 65(4): 621-629.

[55] Łopusiewicz Ł., Lisiecki S., Mizielińska M. 2017. Aktywność przeciwutleniające folii PE i PLA modyfikowanych powłokami zawierającymi melaniny grzybowe [In Polish]. *Opakowanie* 3: 81-85.

[56] Łopusiewicz Ł., Lisiecki S., Mizielińska M. 2017. Właściwości przeciwutleniające i optyczne folii BOPP i PET modyfikowanych powłokami zawierającymi melaniny grzybowe [In Polish]. *Opakowanie* 7: 48-55.

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Arkusz przewodni

Łukasz

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Strona początkowa

9. Przebieg rozwoju naukowego i dorobek naukowy doktoranta:

10.2014- obecnie Zachodniopomorski Uniwersytet Technologiczny w Szczecinie
Wydział Nauk o Żywności i Rybactwa
Dyscyplina Technologia żywności i żywienia, studia stacjonarne III stopnia

02.2013-07.2014 Zachodniopomorski Uniwersytet Technologiczny w Szczecinie
Wydział Biotechnologii i Hodowli Zwierząt
Kierunek Biotechnologia, studia stacjonarne II stopnia
specjalność Biotechnologia w Produkcji Zwierzęcej i Ochronie Środowiska

10.2010-04.2013 Zachodniopomorski Uniwersytet Technologiczny w Szczecinie
Wydział Biotechnologii i Hodowli Zwierząt
Kierunek Bioinformatyka, studia stacjonarne I stopnia

09.2011-05.2012 Mendel University in Brno (Czechy) - Studia w ramach programu Eramus

10.2009-02.2013 Zachodniopomorski Uniwersytet Technologiczny w Szczecinie
Wydział Biotechnologii i Hodowli Zwierząt
Kierunek Biotechnologia, studia stacjonarne I stopnia

2006-2009 I Liceum Ogólnokształcące im. Adama Mickiewicza w Stargardzie
klasa o profilu matematyczno-fizycznym

Staże i praktyki zawodowe, członkowstwo w organizacjach:

03-2017 – obecnie Członek zwyczajny Polskiego Towarzystwa Mikrobiologów

03.2013-07.2013 Praktyka w ramach programu Erasmus, Department of Agrochemistry, Soil Science, Microbiology and Plant Nutrition, Mendel University w Brnie, Czechy (badania wpływu grzybów mykoryzowych na aktywność enzymatyczną gleby)

08.2012 2 tygodniowa praktyka w Katedrze Immunologii, Mikrobiologii i
Chemii Fizjologicznej,
Zachodniopomorski Uniwersytet Technologiczny w Szczecinie

08.2012 2 tygodniowa praktyka w Ogrodzie Dendrologicznym w Przelewicach

07.2011	4 tygodniowa praktyka w Ogrodzie Dendrologicznym w Przelewicach
2010-2013	Członek Studenckiego Koła Naukowego Enzymologów

Dorobek naukowy:

Sumaryczny IF = 10.918

Indeks Hirscha wg. Scopus = 2

Indeks Hirscha wg. Google Scholar = 4

Liczba cytowań wg. Scopus = 10

Liczba cytowań wg. Google Scholar = 56

Autor:

1. Łopusiewicz Ł, Mizielińska M. 2016. Aktywność antymikrobiologiczna folii PLA powlekanych ekstraktami z Macrolepiota konradii. *Opakowanie* 4: 57-60.
2. Łopusiewicz Ł., Lisiecki S. 2016. „Czarne złoto" – melaniny w życiu człowieka. *Kosmos* 65, 4(313): 621-629.
3. Łopusiewicz Ł., Lisiecki S., Mizielińska M. 2017. Aktywność przeciwutleniająca folii PE i PLA modyfikowanych powłokami zawierającymi melaniny grzybowe. *Opakowanie* 3: 81-85.
4. Łopusiewicz Ł., Lisiecki S., Mizielińska M. 2017. Właściwości przeciwutleniające i optyczne folii BOPP i PET modyfikowanych powłokami zawierającymi melaniny grzybowe. *Opakowanie* 7: 48-55.
5. Łopusiewicz Ł, Mizielińska M.. 2017. Antifungal activity of PLA foils covered with ethylocelulose containing essential oils. *World News of Natural Sciences* 12, EISSN 2543-5426.
6. Łopusiewicz Ł. 2018. Isolation, characterisation and biological activity of melanin from *Exidia nigricans*. *World Scientific News* 91: 111-129.
7. Łopusiewicz Ł. 2018. *Scleroderma citrinum* melanin: isolation, purification, spectroscopic studies with characterization of antioxidant, antibacterial and light barrier properties. *World Scientific News* 94(2): 115-130.
8. Łopusiewicz Ł., Jędra F., Mizielińska M. 2018. New Poly(lactic acid) active packaging composite films incorporated with fungal melanin. *Polymers* 10: 386, IF=3,364, ISSN 2073-4360.
9. Łopusiewicz Ł. 2018. Isolation, purification and analysis of the melanin pigment extracted from *Armillaria mellea* rhizomorphs. *World Scientific News* 100: 135-153.
10. Łopusiewicz Ł., Jędra F., Bartkowiak A. New Active Packaging Films Made from Gelatin Modified with Fungal Melanin. *World Scientific News* 2018, 101: 1-30.

11. Łopusiewicz Ł., Jędra F., Bartkowiak A. 2018. The application of melanin modified gelatin coatings for packaging and oxidative stability of lard. *World Scientific News* 101: 108-119.
12. Łopusiewicz Ł. 2018. Antioxidant, antibacterial properties and the light barrier assessment of raw and purified melanins isolated from *Citrullus lanatus* (watermelon) seeds. *Herba Polonica* 64(2).
13. Łopusiewicz Ł., Lisiecki S. 2018. Melaniny w diecie człowieka. *Kosmos* – praca zaakceptowana do druku, ukazanie się nr 3/2018.
14. Łopusiewicz Ł. 2018. Waste from the harvesting of button mushroom (*Agaricus bisporus*) as a source of natural melanin. *Folia Pomeranae Universitatis Technologiae Stetinensis seria Agricultura, Alimentaria, Piscaria et Zootechnica* – praca zaakceptowana do druku, ukazanie się nr 47 (III kwartał 2018 r.).

Współautor:

1. Mizielińska M., Łopusiewicz Ł., Mężyńska M., Bartkowiak A. 2017. The influence of accelerated UV-A and Q-SUN irradiation on the antimicrobial properties of coatings containing ZnO nanoparticles. *Molecules* 22(9), IF = 2,861.
2. Mizielińska M., Salachna P., Ordon M., Łopusiewicz Ł. 2017. Antimicrobial activity of water and acetone extracts of some *Eucomis* taxa. *Asian Pacific Journal of Tropical Medicine* 10(8), IF = 0,925.
3. Mizielińska M., Kowalska U., Salachna P., Łopusiewicz Ł., Jarosz M. 2018. The Influence of Accelerated UV-A and Q-SUN Irradiation on the Antibacterial Properties of Hydrophobic Coatings Containing *Eucomis comosa* Extract. *Polymers*, 10(4): 421, IF=3,364.
4. Mizielińska M., Łopusiewicz Ł. 2018. Encapsulation and evaluation of probiotic bacteria survival in simulated gastrointestinal conditions. *Romanian Biotechnological Letters Journal* – praca zaakceptowana do druku, IF= 0,404.
5. Mazurkiewicz-Zapałowicz K., Golianek A., Łopusiewicz Ł. 2016. Microscopic fungi on *Nymphaeaceae* plants of the Lake Płociczno in Drawa National Park (NW Poland). *Acta Mycologica* 51(1):
6. Mizielińska M., Jankowska P., Bień A., Łopusiewicz Ł., Bartkowiak A. 2016. Ocena zdolności wybranych szczepów grzybów z gromady Basidiomycota do syntezy lipaz oraz esteraz. *Żywność. Nauka. Technologia. Jakość* 3(106): 78-90.
7. Mizielińska M., Kowalska U., Łopusiewicz Ł. 2017. The influence of PU foams modification on the efficiency of *Citrobacter freundii* cells immobilization. *World Scientific News* 77(2): 211-225.
8. Mizielińska M., Kowalska U., Łopusiewicz Ł. 2017. Evaluation of hydrocarbons adsorbtion and biodegradation by *Pseudomonas aeruginosa* cells entrapped into silica-alginate beads. *World Scientific News* 80: 256-267.
9. Mazurkiewicz-Zapałowicz K., Rybińska A., Łopusiewicz Ł. 2017. Microscopic fungi on *Schoenoplectus lacustris* in lakes Płociczno and Płociowe in Drawa National Park (northwest Poland). *Acta Mycologica* 52(2).
10. Mizielińska M., Łopusiewicz Ł., Soból M. 2018. Polihydroksyalkanolany – obiecujące polimery biodegradowalne. *Kosmos* – praca zaakceptowana do druku, ukaże się w nr 2/2018, ISSN 0023-4249.

Wystąpienia ustne:

1. Lopusiewicz Ł. Phytotoxic activity of extract from fungus *Macrolepiota konradii*. 60th Anniversary Conference The Contribution of the natural sciences to the development of politics of sustainable development of agriculture. Wystąpienie w języku angielskim.
2. Łopusiewicz Ł. Analiza właściwości antymikrobiologicznych grzyba *Macrolepiota konradii*. Konferencja Badania i Rozwój Młodych Naukowców w Polsce 2015. Wystąpienie w języku polskim.
3. Łopusiewicz Ł. Analiza aktywności lipolitycznej wybranych gatunków grzybów. I Konferencja Naukowa Enzymos Enzymy w nauce i przemyśle 2015. Wystąpienie w języku polskim.
4. Łopusiewicz Ł. Ocena możliwości zastosowania grzybni pozyskanej z hodowli płynnej do wytworzenia materiału opakowaniowego o strukturze spienionej. BioOrg 2015 I Wielkopolskie Seminarium Chemii Bioorganicznej, Organicznej i Biomateriałów Poznań 2015. Wystąpienie w języku polskim.
5. Łopusiewicz Ł. Ocena możliwości zastosowania ekstraktu z grzyba *Macrolepiota konradii* jako naturalnego bioherbicydu. Badania i Rozwój Młodych Naukowców w Polsce 2015. Wystąpienie w języku polskim

Postery:

Autor:

1. Łopusiewicz Ł., Mizielińska M., Łabuda M., Bańcarz G., Lisiecki S., Bartkowiak A. Modification on PLA foil surface by ethylcellulose and antimicrobial extract from fungus *Macrolepiota konradii*. 7th International Conference of PhD Students of Szczecin Univeristy 17.10.2014.

2. Łopusiewicz Ł., Lisiecki S. Comparision of coffee beand and grounds as additives for *Macrolepiota konradii* biomass production. 4th European Young Engineers Conference 2015.

3. Łopusiewicz Ł. Natural Sources of Melanin. Ekologia Człowieka II Międzynarodowa Konferencja Szczecin 2016

4. Łopusiewicz Ł., Golianek A. Analiza aktywności enzymatycznej *Fusarium sulphureum*. I Konferencja Naukowa Enzymos Enzymy w nauce i przemyśle 2015.

5. Łopusiewicz Ł. Analiza aktywności enzymatycznej grzyba *Isaria fumosorosea*. Wpływ Młodych Naukowców Na Osiągnięcia Polskiej Nauki 2015

6. Łopusiewicz Ł. Wykorzystanie grzyba *Macrolepiota konradii* do produkcji spienionego materiału opakowaniowego. VIII Międzydyscyplinarna Konferencja Doktorantów Uniwersytetu Szczecińskiego.

7. Łopusiewicz Ł. Odpad z produkcji pieczarki dwuzarodnikowej (*Agaricus bisporus*) jako źródło naturalnej melaniny. Konferencja Badania i Rozwój Młodych Naukowców w Polsce 2016.

Współautor:

1. Golianek A., Łopusiewicz Ł. Ocena zdolności do produkcji esteraz przez wybrane gatunki grzybów gromady Basidiomycota. I Konferencja Naukowa Enzymos, Enzymy w nauce i przemyśle 2015
2. Kołodziejczyk L., Mazurkiewicz-Zapałowicz K., Twarużek M., Grajewski J., Golianek A., Łopusiewicz Ł., Dzika E., Pilarczyk B. Grzyby z rodzaju *Fusarium* a rozwój zarodkowy *Ascaris suum*. Ekologia Człowieka II Międzynarodowa Konferencja Szczecin.
3. Kołodziejczyk L., Mazurkiewicz-Zapałowicz K., Tkaczuk C., Twarużek M., Grajewski J., Golianek A., Łopusiewicz Ł., Dzika E., Pilarczyk B. Enthomopathogenic fungi vs. *Ascaris summ* embryogenesis. XXIV Zjazd Polskiego Towarzystwa Parazytologicznego.
4. Kołodziejczyk L., Mazurkiewicz-Zapałowicz K., Twarużek M., Grajewski J., Golianek A., Łopusiewicz Ł., Dzika E., Pilarczyk B. Wpływ herbicydów ChwastoxTrio 540 SL i Roudap Ultra 170 SL na rozwój zarodkowych *Ascaris suum*. Nauki Przyrodnicze we współczesnym świecie, Szczecin 2.06.2017
5. Kołodziejczyk L., Mazurkiewicz-Zapałowicz K., Twarużek M., Grajewski J., Golianek A., Łopusiewicz Ł., Dzika E. Wpływ herbicydów Afalon, Command i Mistral na rozwój zarodkowy Ascaris suum. Biotechnologia - problemy i wyzwania. Ogólnopolska Konferencja Naukowa, Szczecin, 8 czerwca 2018 r.

Publikacje książkowe:

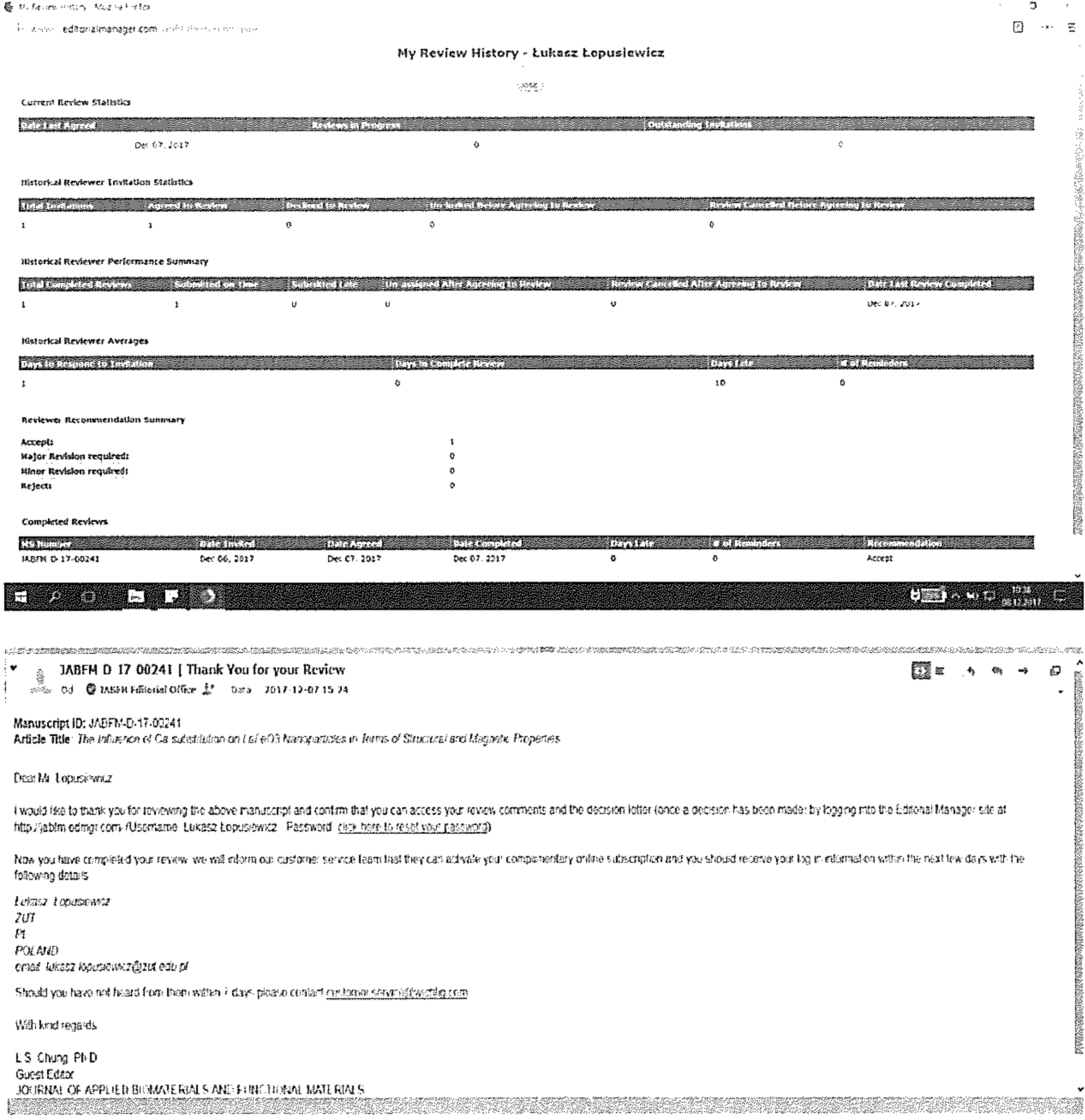
1. Łopusiewicz Ł., Lisiecki S. Rola i znaczenie grzybów w środowisku naturalnym. Badania i Rozwój Młodych naukowców w Polsce – Nauki przyrodnicze część II ISBN 978-83-942083-1-8.
2. Łopusiewicz Ł., Lisiecki S. Analiza właściwości bakteriostatycznych i fungistatycznych grzyba *Macrolepiota konradii*. Badania i Rozwój Młodych naukowców w Polsce – Nauki przyrodnicze część II ISBN 978-83-942083-1-8.
3. Łopusiewicz Ł. Ocena możliwości zastosowania ekstraktu z grzyba *Macrolepiota konradii* jako naturalnego bioherbicydu. Badania i Rozwój Młodych Naukowców w Polsce – Nauki Przyrodnicze część IV, Monografie (6) 2016, ISBN 978-83-65362-19-3.
4. Łopusiewicz Ł. Grzyby wyższe jako źródło substancji aktywnych. Badania i Rozwój Młodych Naukowców w Polsce – Nauki Przyrodnicze część IV, Monografie (6) 2016, ISBN 978-83-65362-19-3.
5. Łopusiewicz Ł. Analiza determinantów katalitycznego optimum pH wybranych alfa-amylaz pochodzących z bakterii z rodzaju *Bacillus*. Monografia Przegląd wybranych prac z zakresu enzymologii Lublin 2016 Wydawnictwo Tygiel, ISBN 978-83-65272-34-8.

6. Łopusiewicz Ł. Analiza właściwości lipolitycznych wybranych gatunków grzybów z typu Basidiomycota. Badania i Rozwój Młodych Naukowców w Polsce - Nauki Przyrodnicze część I. Monografie (6) 2016. ISBN 978-83-65677-79-2.

Wykonane recenzje:

1. Recenzja publikacji *The Influence of Ca-substitution on LaFeO3 Nanoparticles in Terms of Structural and Magnetic Properties* - Manuscript ID: JABFM-D-17-00241. Journal of Applied Biomaterials & Functional Materials (Zał.1)

Zał. 1



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MS Number	Date Invited	Date Agreed	Date Completed	Days Late	# of Reminders	Recommendation
JABFM-D-17-00241	Dec 06, 2017	Dec 07, 2017	Dec 07, 2017	0	0	Accept

JABFM D 17 00241 | Thank You for your Review

Od: JABFM Editorial Office Data: 2017-12-07 15:24

Manuscript ID: JABFM-D-17-00241
Article Title: *The Influence of Ca substitution on LaFeO3 Nanoparticles in Terms of Structural and Magnetic Properties*

Dear Mr. Lopusiewicz

I would like to thank you for reviewing the above manuscript and confirm that you can access your review comments and the decision letter (once a decision has been made) by logging into the Editorial Manager site at http://jabfm.edmgr.com/ (Username: Lukasz Lopusiewicz Password: click here to reset your password)

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With kind regards

L.S. Chung, PhD
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JOURNAL OF APPLIED BIOMATERIALS AND FUNCTIONAL MATERIALS