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Badania nad enzymatyczną modyfikacją związków fenolowych i ich wykorzystaniem jako dodatków funkcjonalnych do olejów roślinnych

Research on the enzymatic modification of phenolic compounds and their use as functional additives to vegetable oils

Rozprawa doktorska

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STRESZCZENIE

Badania nad enzymatyczną modyfikacją związków fenolowych i ich wykorzystaniem jako dodatków funkcjonalnych do olejów roślinnych.

Celem rozprawy było opracowanie immobilizowanego biokatalizatora opartego na odpadach spożywczych oraz jego zastosowanie w lipofilizacji kwasów fenolowych, w szczególności kwasu chlorogenowego (CGA), dominującego w ekstraktach z fusów kawowych, wytłoków aronii i jabłek. Postawiono hipotezę, że odpady te mogą stanowić zrównoważone nośniki do immobilizacji lipaz, umożliwiając syntezę bioaktywnych estrów o ulepszonych właściwościach przeciwutleniających i potencjalnie przeciwdrobnoustrojowych, a także poprawiających stabilność olejów roślinnych.

Wykazano, że spośród lipaz unieruchamianych na trzech testowanych nośnikach najwyższą aktywność syntetyczną (1036,0 U/g) osiągnęła lipaza z *A. oryzae* immobilizowana na natywnych fusach kawowych (NSCG), co przypisano ich porowatej strukturze, wysokiej zawartości hemicelulozy oraz obecności lipidów sprzyjających aktywacji międzyfazowej enzymu. Biokatalizator NSCG skutecznie katalizował estryfikację CGA alkoholami o różnej długości łańcucha węglowego. Zaobserwowano zależność wydajności tej reakcji od długości łańcucha węglowego, w przeciwieństwie do enzymu komercyjnego Novozym 435. Najwyższą wydajność biotransformacji CGA uzyskano w przypadku dodekanolu (34,06%).

Ester butylowy CGA charakteryzował się najwyższą aktywnością antyoksydacyjną, porównywalną z wolnym CGA i BHT. Estry średniołańcuchowe (C6, C8) wykazały selektywną aktywność przeciwdrobnoustrojową wobec bakterii Gram-dodatnich. Dodatek estrów krótkołańcuchowych (C4, C6) do oleju rzepakowego znacząco poprawiał jego stabilność oksydacyjną, dzięki lepszej rozpuszczalności w matrycy lipidowej. W przypadku lipofilizacji ekstraktów butanolem najwyższą wydajność reakcji otrzymywania estru butylowego kwasu chlorogenowego (~90%) odnotowano dla ekstraktów z wytłoków jabłkowych i aroniowych. Mimo wysokiej wydajności zaobserwowano obniżenie aktywności antyoksydacyjnej w stosunku do ekstraktów niemodyfikowanych oraz brak wpływu na stabilność oksydacyjną oleju.

Przeprowadzone badania dowodzą, że odpady spożywcze mogą pełnić podwójną rolę, jako nośniki biokatalizatorów i źródło związków fenolowych. Biokatalizator oparty na fusach kawowych umożliwił efektywną syntezę bioaktywnych estrów kwasu chlorogenowego.

ABSTRACT

Research on the enzymatic modification of phenolic compounds and their use as functional additives to vegetable oils.

The aim of the dissertation was to develop an immobilized biocatalyst based on food waste and to apply it to the lipophilization of phenolic acids, in particular chlorogenic acid (CGA), which predominates in extracts from spent coffee grounds, chokeberry pomace, and apple pomace. It was hypothesized that these wastes could serve as sustainable carriers for lipase immobilization, enabling the synthesis of bioactive esters with enhanced antioxidant and potentially antimicrobial properties, as well as improving the oxidative stability of vegetable oils.

It was demonstrated that, among the lipases immobilized on the three tested carriers, *A. oryzae* lipase immobilized on native spent coffee grounds (NSCG) exhibited the highest synthetic activity (1036.0 U/g). This was attributed to the porous structure of the carrier, its high hemicellulose content, and the presence of lipids that favor interfacial activation of the enzyme. The NSCG biocatalyst effectively catalyzed the esterification of CGA with alcohols of varying carbon-chain lengths. In contrast to the commercial biocatalyst Novozym 435, a clear dependence of reaction yield on alcohol chain length was observed. The highest CGA biotransformation yield was obtained with dodecanol (34.06%).

The butyl ester of CGA exhibited the highest antioxidant activity, comparable to that of free CGA and butylated hydroxytoluene (BHT). Medium-chain esters (C6, C8) showed selective antimicrobial activity against Gram-positive bacteria. The addition of short-chain esters (C4, C6) to rapeseed oil significantly improved its oxidative stability due to their better solubility in the lipid matrix. In the lipophilization of extracts with butanol, the highest yield of butyl chlorogenate formation (~90%) was recorded for extracts from apple and chokeberry pomace. Despite the high conversion, a reduction in antioxidant activity was observed compared to unmodified extracts, with no effect on oil oxidative stability.

The conducted studies demonstrate that food waste can play a dual role, serving both as biocatalyst carriers and as sources of phenolic compounds. A biocatalyst based on spent coffee grounds enabled the efficient synthesis of bioactive chlorogenic acid esters.

WYKAZ SKRÓTÓW I OZNACZEŃ

Symbol	Znaczenie
ADF	włókno detergentowe kwaśne (ang. *Acid Detergent Fibre*)
ADL	lignina detergentowa kwaśna (ang. *Acid Detergent Lignin*)
APE	ekstrakt z wytłoków jabłkowych (ang. *Apple Pomace Extract*)
AP	wytłoki jabłkowe (ang. *Apple Pomace*)
NAP	lipaza z *A.oryzae* (Novozym 51032) immobilizowana na wytłokach jabłkowych
BHT	butylohydroksytoluen
CALB	lipaza B z *C. antarctica*
CGA	kwas chlorogenowy
ChoP	wytłoki aroniowe (ang. *Chokeberry Pomace*)
NChoP	lipaza z *A.oryzae* (Novozym 51032) immobilizowana na wytłokach aroniowych
CUPRAC	zdolność antyoksydacyjna oparta na redukcji jonów miedzi (II) (ang. *Cupric Reducing Antioxidant Capacity*)
DM	sucha masa (ang. *dry mass*)
DPPH	rodnik 1,1-difenylo-2-pikrylohydrazylu
ESI – MS	spektrometria mas z jonizacją elektrorozpylającą
FTIR	spektroskopia w podczerwieni z transformacją Fouriera
IC_{50}	stężenie substancji powodujące 50% redukcję rodnika DPPH
NDF	włókno detergentowe obojętne (ang. *Neutral Detergent Fibre*)
NSCG	lipaza z *A.oryzae* (Novozym 51032) immobilizowana na fusach kawowych
PDSC	wysokociśnieniowa, różnicowa kalorymetria skaningowa
ROS	reaktywne formy tlenu (ang. *Reactive Oxygen Species*)
RSM	metoda powierzchni odpowiedzi (ang. *Response Surface Methodology*)
SCG	fusy kawowe (ang. *Spent Coffee Grounds*)
SCGE	ekstrakt z fusów kawowych (ang. *Spent Coffee Grounds extract*)

WYKAZ PUBLIKACJI STANOWIĄCYCH PRACĘ DOKTORSKĄ

[P1] Jasińska, K., Fabiszewska, A., Białecka-Florjańczyk, E., Zieniuk, B. (2022). Mini-Review on the enzymatic lipophilization of phenolics present in plant extracts with the special emphasis on anthocyanins. Antioxidants, 11(8), 1528. https://doi.org/10.3390/antiox11081528

IF: 7,0; $MEiN_{2022}$: 100 pkt.

[P2] Jasińska, K., Zieniuk, B., Jankiewicz, U., Fabiszewska, A. (2023). Bio-Based materials versus synthetic polymers as a support in lipase immobilization: impact on versatile enzyme activity. Catalysts, 13(2), 395. https://doi.org/10.3390/catal13020395

IF: 3,8; $MEiN_{2023}$: 100 pkt.

[P3] Jasińska, K., Zieniuk, B., Piasek, A.M., Wysocki, Ł., Sobiepanek, A., Fabiszewska, A. (2024). Obtaining a biodegradable biocatalyst – study on lipase immobilization on spent coffee grounds as potential carriers. Biocatalysis and Agricultural Biotechnology, 59, 103255. https://doi.org/10.1016/j.bcab.2024.103255

IF: 3,8; $MEiN_{2024}$: 70 pkt.

[P4] Jasińska, K., Nowosad, M., Perzyna, A., Bielacki, A., Dziwiński, S., Zieniuk, B., Fabiszewska, A. (2024). Sustainable Lipase Immobilization: Chokeberry and Apple Waste as Carriers. Biomolecules, 14(12), 1564. https://doi.org/10.3390/biom14121564

IF: 4,8; $MEiN_{2024}$: 100 pkt

[P5] Jasińska K., Zieniuk B., Bryła M., Padewska D., Brzezińska R., Kruszewski B., Nowak D., Fabiszewska A. (2025). The Double Life of Plant-Based Food Waste: A Source of Phenolic Acids and a Carrier for Immobilization of Lipases Capable of Their Lipophilization. International Journal of Molecular Sciences, 26(23), 11400. https://doi.org/10.3390/ijms262311400

IF: 4,9; $MEiN_{2024}$: 140 pkt

Łączna wartość IF: 24,3; liczba punktów MEiN: 510 pkt.

WSTĘP

Powszechnie wiadomo, że związki fenolowe stanowią dużą i zróżnicowaną grupę metabolitów wtórnych występujących głównie w roślinach, a także w grzybach. W ostatnich latach wzrosło zainteresowanie tymi związkami ze względu na ich cechy funkcjonalne, takie jak właściwości przeciwutleniające, przeciwzapalne, przeciwdrobnoustrojowe i przeciwnowotworowe, co przekłada się na ich znaczenie biologiczne, żywieniowe i farmakologiczne. Cechą, która utrudnia ich zastosowanie, jest niska rozpuszczalność w tłuszczach i olejach. Tę właściwość można jednak poprawić w wyniku dołączenia do cząsteczki grup lipofilowych (np. reszt acylowych z kwasów tłuszczowych lub łańcuchów alkilowych z alkoholi tłuszczowych), co w praktyce oznacza estryfikację związków fenolowych. Pochodne otrzymane w rezultacie lipofilizacji cechuje wyższa rozpuszczalność w lipidach. Ponadto, w wielu przypadkach modyfikacja poprawia również ich aktywność biologiczną. Do przeprowadzenia powyższych reakcji, zgodnie z podejściem zielonej chemii, można wykorzystać enzymy, a dokładnie lipazy, należące do klasy hydrolaz.

W celu otrzymania stabilnego preparatu enzymatycznego stosuje się technikę immobilizacji, która polega na unieruchomieniu cząsteczek białek enzymatycznych na odpowiednim nośniku. Proces immobilizacji umożliwia ochronę enzymu przed czynnikami stresowymi pochodzącymi ze środowiska, a także łatwe oddzielenie i oczyszczanie produktów z mieszanin reakcyjnych oraz wydajne odzyskiwanie biokatalizatorów. Nowy trend stanowi poszukiwanie bardziej przyjaznych dla środowiska matryc w celu stworzenia w pełni biodegradowalnego biokatalizatora, który będzie nową alternatywą dla syntetycznych nośników.

1. PRZEGLĄD PIŚMIENNICTWA

1.1. Wybrane odpady spożywcze jako cenny surowiec do ponownego zagospodarowania

Marnowanie żywności to poważny problem o wymiarze globalnym, który niesie za sobą skutki środowiskowe, społeczne i ekonomiczne. Według Food Waste Index Report (2021), w 2019 roku na świecie wygenerowano około 931 milionów ton odpadów żywnościowych. Z tej ilości 61% pochodziło z gospodarstw domowych, 26% z sektora gastronomicznego, a 13% z handlu detalicznego. Oznacza to, że nawet 17% całkowitej globalnej produkcji żywności może zostać zmarnowane. Jak wskazują Poore i Nemecek (2018), niemal 24% emisji gazów cieplarnianych związanych z produkcją żywności wynika ze strat w łańcuchu dostaw lub marnotrawstwa na poziomie konsumentów. Spośród tych emisji, 15% przypisuje się niewłaściwym praktykom w łańcuchu dostaw, takim jak nieodpowiednie warunki przechowywania, obsługi, chłodzenia czy transportu, natomiast 9% związanych jest z wyrzucaniem żywności przez sprzedawców detalicznych i konsumentów. W rezultacie marnowanie żywności odpowiada za około 6% globalnych emisji gazów cieplarnianych.

Głównymi grupami żywności, które przyczyniają się do marnotrawstwa lub utraty składników odżywczych, są zboża i rośliny strączkowe, owoce i warzywa, mięso i produkty pochodzenia zwierzęcego, korzenie, bulwy i rośliny oleiste. Spośród tych grup żywności, korzenie, bulwy i rośliny oleiste, stanowiące prawie 26% oraz owoce i warzywa, stanowiące 22%, są dwiema największymi grupami pod względem strat żywności (Capanoglu i in. 2022). Odpady powstałe z tych grup obejmują m.in. skórki, łodygi, nasiona, łupiny, otręby, zarodki, odpady sortownicze, wytłoki i miazgę. Szczególnym wyzwaniem dla przemysłu przetwórstwa owoców i warzyw jest zagospodarowanie surowców, które nie są bezpośrednio wykorzystywane w procesach technologicznych. Szacuje się, że wytłoki stanowią od 10 do 35% masy przetwarzanego surowca (Tarko i in. 2012).

Problem ten ma szczególne znaczenie w Polsce, jednym z czołowych producentów owoców w Europie, takich jak np. jabłka, gruszki i aronia, które są surowcem do produkcji soków, dżemów czy koncentratów. Według danych Głównego Urzędu Statystycznego, w sezonie 2018/2019 aż 3,15 miliona ton jabłek przeznaczono na produkcję soku zagęszczonego (Płocharski i in. 2019). Wytłoki jabłkowe są jednym z najpowszechniej generowanych rodzajów odpadów spożywczych, ich światowa

produkcja wynosi około 4 miliony ton rocznie (Gołębiewska i in. 2022). W przypadku aronii, według danych Agencji Rynku Rolnego, powierzchnia jej upraw w Polsce wzrosła z 5 000 ha do 8 000 ha w latach 2004–2013, a zbiory zwiększyły się z 38 000 do 58 000 ton (Platonova i in. 2021).

Wzrost zainteresowania gospodarką o obiegu zamkniętym oraz praktykami zrównoważonego rozwoju stymuluje badania nad możliwością ponownego wykorzystania lignocelulozowych odpadów owocowo-warzywnych, w tym właśnie jabłkowych i aroniowych, w zastosowaniach technologicznych. Ze względu na odpowiednią porowatość, ładunek powierzchniowy oraz chemiczną obojętność, odpady te stanowią atrakcyjne nośniki w procesach unieruchamiania enzymów (Bilal i in. 2019; Nájera-Martínez i in. 2022).

Innym odpadem o podobnych właściwościach są fusy po kawie, których globalna roczna produkcja wynosi około 6 milionów ton i stanowi aż do 60% masy przetworzonych ziaren kawy. Wraz ze wzrostem światowego spożycia kawy, ilość tych odpadów również systematycznie rośnie. Fusy po kawie to lignocelulozowy odpad o niskim koszcie, łatwej dostępności i odnawialnym charakterze, spełniający wiele wymagań fizykochemicznych stawianych nośnikom enzymów. Ich struktura charakteryzuje się odpowiednią wytrzymałością, obojętnością chemiczną i stabilnością. Co więcej, zawartość związków organicznych, takich jak kwasy tłuszczowe, lignina, celuloza, hemiceluloza oraz inne polisacharydy, czyni z nich doskonałą matrycę do immobilizacji enzymów (Campos-Vega i in. 2015; Mak i in. 2023; Atabani i in. 2022; Srinivasan i in. 2023; Colantoni i in. 2021).

Poza rolą technologiczną, odpady spożywcze, w tym wytłoki owocowe i fusy po kawie, stanowią także bogate źródło związków bioaktywnych, w tym związków fenolowych, błonnika pokarmowego, białek i węglowodanów. Ze względu na właściwości przeciwutleniające, przeciwzapalne i przeciwdrobnoustrojowe związki fenolowe znajdują zastosowanie w żywności funkcjonalnej, suplementach diety, a także w formulacjach farmaceutycznych i kosmetycznych (Arun i in. 2020).

1.2. Lipazy jako cenne biokatalizatory

Lipazy (EC 3.1.1.3), klasyfikowane jako hydrolazy, to enzymy lipolityczne, których naturalną funkcją jest katalizowanie reakcji hydrolizy triacylogliceroli do diacylogliceroli, monoacylogliceroli, glicerolu i wolnych kwasów tłuszczowych. Wykazują aktywność zależną od pH, zazwyczaj stabilną w neutralnym pH lub w zakresie

od 4,0 do 8,0. Mogą być pozyskiwane z różnych źródeł: zwierzęcych, roślinnych oraz mikrobiologicznych. Ostatnie z nich budzą coraz większe zainteresowanie z uwagi na niższy koszt produkcji oraz większą dostępność w porównaniu do lipaz pochodzenia zwierzęcego i roślinnego. Charakteryzują się wysoką stabilnością katalityczną i enancjoselektywnością, a także szeroką specyficznością substratową i tolerancją na czynniki środowiskowe, takie jak pH, temperatura czy rozpuszczalniki organiczne (Coelho i Orlandelli 2020; Chandra i in. 2020). Lipazy w centrach aktywnych posiadają triadę katalityczną aminokwasów: serynę (Ser), histydynę (His) oraz kwas asparaginowy (Asp) lub glutaminowy (Glu). Ich cechą charakterystyczną jest obecność „wieczka" (ang. *lid*), które jest amfipatyczną strukturą zbudowaną z jednej lub kilku α-helis. Ma ona kluczowe znaczenie funkcjonalne, ponieważ odpowiada za odsłonięcie hydrofobowego centrum katalitycznego w obecności substratu. W jednorodnym środowisku wodnym lipazy zwykle przyjmują konformację zamkniętą i wykazują niską aktywność; natomiast na granicy faz woda–olej lub w środowisku hydrofobowym przechodzą w konformację otwartą i ulegają aktywacji międzyfazowej (ang. *interfacial activation*) (Filho i in. 2019; Albayati i in. 2020).

Podstawową funkcją lipaz jest hydroliza wiązań estrowych na granicy faz hydrofilowej i hydrofobowej. W odpowiednich warunkach, szczególnie przy ograniczonej obecności wody, mogą one również katalizować reakcje syntezy, takie jak estryfikacja i transestryfikacja (de Araujo i in. 2017; Chandra i in. 2020). Enzymy te znajdują szerokie zastosowanie w różnych dziedzinach, głównie ze względu na ich selektywność chemiczną, regio- i stereospecyficzność. Odgrywają znaczącą rolę w przetwarzaniu szerokiej gamy olejów roślinnych. Proces ten, bez udziału enzymów, wymaga wysokich temperatur i ciśnień, co czyni go energochłonnym. Zastosowanie lipaz pochodzenia mikrobiologicznego, m.in. z hodowli takich mikroorganizmów jak: *Thermomyces lanuginosus*, *Aspergillus niger*, *Pseudomonas aeruginosa*, pozwala prowadzić reakcję w łagodniejszych warunkach (40–60 °C) oraz uzyskiwać produkty o lepszych właściwościach użytkowych i wysokiej czystości. Enzymy lipolityczne umożliwiają także modyfikację struktury tłuszczów roślinnych, co jest istotne w produkcji preparatów do żywienia niemowląt oraz w produkcji tłuszczów o zmniejszonej kaloryczności. Lipazy wykorzystuje się do wytwarzania substytutów masła kakaowego o zbliżonych właściwościach smakowych i użytkowych do naturalnego produktu, do produkcji bioemulgatorów oraz estrów, które są istotnymi składnikami aromatów (Mehta i in. 2021; Chandra i in. 2020, Rodrigues i in. 2019; Mokhtar i in. 2020). Są cennymi

biokatalizatorami w produkcji biodiesla, katalizują transestryfikacje olejów roślinnych krótkołańcuchowymi alkoholami alifatycznymi (Narwal i in. 2013; Quayson i in. 2020). W przemyśle kosmetycznym i farmaceutycznym wykorzystuje się je m.in. do wytwarzania filtrów UV, składników preparatów myjących, olejków zapachowych oraz do syntezy enancjomerycznie czystych leków. Przykładem jest lipaza B z *Candida antarctica* (CALB), używana do produkcji inihibitora katepsyny K (Mouad i in. 2016; Ugo i in. 2017; Brault i in. 2014; Manoel i in. 2016). Swoje zastosowanie lipazy odnajdują też w przemyśle środków czyszczących. Detergenty wytwarzane przy udziale lipaz preferowane są do usuwania cząsteczek lipidów z zabrudzonych powierzchni, co przedłuża żywotność tkanin i jest efektywne w temperaturze otoczenia. Są wszechstronnymi narzędziami w syntezie organicznej i bioremediacji (Fernandez-Lafuente i in. 2010; Mhetras i in. 2021; Albayati i in. 2020).

1.3. Proces immobilizacji lipaz

Wykorzystanie wolnych enzymów w przemyśle wiąże się jednak z pewnymi ograniczeniami, takimi jak niska stabilność w warunkach syntezy. Aby zminimalizować te problemy, stosuje się proces immobilizacji enzymów. Technika ta poprawia ich stabilność w szerokim zakresie pH i temperatur oraz umożliwia ich łatwe odzyskiwanie i ponowne wykorzystanie z mieszaniny reakcyjnej (Xie i in. 2022). Immobilizację dzieli się na dwa typy ze względu na rodzaj oddziaływań pomiędzy nośnikiem a białkiem enzymatycznym. Oddziaływania fizyczne obejmują wiązania wodorowe, siły van der Waalsa oraz oddziaływania hydrofobowe, natomiast oddziaływania chemiczne polegają na tworzeniu wiązań kowalencyjnych. Do podstawowych metod immobilizacji zalicza się adsorpcję fizyczną, pułapkowanie, enkapsulację, wiązanie kowalencyjne oraz sieciowanie. Na przestrzeni lat przebadano wiele różnych nośników, w tym przede wszystkim polimery syntetyczne, takie jak żywice akrylowe, metakrylowe i styrenowe (Hanefeld i in. 2009), nanomateriały (Shuai i in. 2017), np. nanocząstki magnetyczne i nieorganiczne (Liu i in. 2011), nanocząstki złota (Du i in. 2013), tlenek grafenu (Kumar i Pal 2021; Nematian i in. 2020), czy nanocząstki krzemionki. Wśród innych badanych nośników znajdują się również materiały węglowe oraz tlenki metali nieorganicznych (np. tlenki glinu, tytanu, cyrkonu) (Ismail i Baek, 2020), jak i biopolimery, m.in. polisacharydy (celuloza, skrobia, dekstran, agaroza, chitozan) oraz białka (albumina, żelatyna) (Cantone i in. 2013). Jednym z najbardziej znanych preparatów enzymatycznych jest Novozym 435, czyli lipaza B z *C. antarctica* (CALB),

unieruchomiona na makroporowatej żywicy polimerowej Lewatit VP OC 1600, kopolimerze metakrylanu z diwinylobenzenem. Biokatalizator ten cechuje się wyjątkową skutecznością w syntezie organicznej i procesach biokatalitycznych (Coelho i Orlandelli 2020; Filho i in. 2019).

Ponadto tendencje badawcze coraz częściej koncentrują się na rozwoju bardziej ekologicznych i ekonomicznie korzystnych rozwiązań (Thangaraj i Solomon, 2019). W tym kontekście rosnące zainteresowanie wzbudzają odpady lignocelulozowe, które stanowią obiecujące nośniki dla lipaz i innych enzymów. Wzrost liczby publikacji naukowych oraz analiza danych statystycznych jednoznacznie wskazują na stale rozwijający się trend badań w tym obszarze. Ze względu na rosnącą populację ludności oraz intensyfikację działalności rolniczej i leśnej, każdego roku generowane są miliardy ton biomasy roślinnej. Biomasa ta składa się głównie z drewna, roślin zielnych oraz odpadów rolniczych. Jej podstawowe składniki chemiczne to: celuloza (16–50%), hemiceluloza (10–38%), lignina (7–36%) i popiół (0,4–15%), a także niewielka ilość takich składników, jak pektyny, żywice, woski i sole mineralne (Mokhena i in. 2021; Trache i in. 2020).

Do tej pory podjęto próby adsorpcji lipaz na odpadach z przemysłu rolno-spożywczego, takich jak kolby kukurydzy, trzcina cukrowa, łuski ryżowe, fusy kawowe (de S. Lira 2021, Girelli i in. 2023), włókno kokosowe, młóto browarniane (Najera-Martinez 2022) oraz skorupki jaj (Budzaki i in. 2022). W niektórych przypadkach zastosowanie tych surowców nie tylko okazało się skuteczne, ale również prowadziło do uzyskania preparatów, cechujących się wyższą aktywnością niż w przypadku tradycyjnych nośników. Główne zalety odpadów żywnościowych jako nośników do immobilizacji enzymów to ich wysoka porowatość i duża powierzchnia właściwa, a także obecność różnorodnych grup chemicznych, które pozytywnie wpływają na adsorpcję enzymów (Girelli i in. 2020).

1.4. Kwasy fenolowe i enzymatyczne otrzymywanie ich pochodnych

Znaczna liczba związków fenolowych i ich pochodnych występuje w naturze, szczególnie w świecie roślin. Polifenole, będące wtórnymi metabolitami roślinnymi, obejmują takie klasy związków jak kwasy fenolowe, flawonoidy, stilbeny oraz lignany. Stanowią one istotny składnik codziennej diety człowieka i pełnią kluczową rolę w systemie obronnym roślin, wykazując przy tym szereg korzystnych właściwości biologicznych, w tym aktywność przeciwutleniającą. Kwasy fenolowe charakteryzują się

obecnością grupy karboksylowej oraz co najmniej jednej grupy hydroksylowej bezpośrednio przyłączonej do pierścienia aromatycznego (fenolowej). Są klasyfikowane jako pochodne kwasu hydroksybenzoesowego np. kwas *p*-hydroksybenzoesowy, protokatechowy, wanilinowy, galusowy i kwasu hydroksycynamonowego np. kwas *p*-kumarowy, ferulowy, kawowy, sinapowy, chlorogenowy, neochlorogenowy, rozmarynowy) (Korzeniowska 2017; Vermerris i Nicholson 2008). Są naturalnymi, hydrofilowymi przeciwutleniaczami, które powszechnie występują w owocach, warzywach, przyprawach oraz ziołach aromatycznych. Ze względu na swoje właściwości antyoksydacyjne, chelatujące, a także działanie przeciwzapalne, antyalergiczne i przeciwdrobnoustrojowe, są one szczególnie interesujące z punktu widzenia zdrowia publicznego oraz zastosowania w żywności funkcjonalnej i nutraceutykach (Proestos i in. 2006; Szajdek i Borowska 2004).

Naturalne kwasy fenolowe są powszechnie stosowane jako przeciwutleniacze w przemyśle spożywczym, kosmetycznym i farmaceutycznym, ale ich niska rozpuszczalność w środowiskach apolarnych np. olejach znacząco ogranicza zakres zastosowań w produktach tłuszczowych oraz układach emulsyjnych. Aby zwiększyć ich biodostępność i skuteczność w tego typu matrycach, możliwe jest przeprowadzenie ich lipofilizacji, czyli modyfikacji cząsteczki w celu nadania jej amfifilowego charakteru. Taka modyfikacja zmienia równowagę hydrofilowo-lipofilową, umożliwiając otrzymywanie nowych, funkcjonalnych związków bioaktywnych o ulepszonych właściwościach w porównaniu do tradycyjnych hydrofilowych związków fenolowych. Dzięki temu związki te mogą lokalizować się na granicach faz (olej–woda lub olej–powietrze), gdzie zachodzą reakcje utleniania lipidów, skuteczniej chroniąc przed ich degradacją oksydacyjną (Durand i in. 2017; Ramadan, 2021, Laguerre i in. 2013).

Istnieją dwa główne sposoby przeprowadzania lipofilizacji: chemiczny i enzymatyczny. Metoda chemiczna jest bardziej złożona ze względu na wrażliwość kwasów fenolowych na temperaturę i ich podatność na utlenianie w środowisku zasadowym. Proces ten jest często mało selektywny, wymaga wielu etapów oczyszczania, usuwania katalizatorów i rozpuszczalników oraz generuje znaczne ilości odpadów. Z kolei metoda enzymatyczna jest preferowana, ponieważ zachodzi w łagodniejszych warunkach (niska temperatura, neutralne pH), przy jednoczesnym ograniczeniu reakcji ubocznych i wysokiej selektywności substratowej, co czyni ją bardziej przyjazną dla środowiska i skutkuje mniejszą ilością produktów ubocznych (Mardani i in. 2024; Aleman i in. 2015; Figueroa-Espinoza i in. 2013). Do

przeprowadzenia takiej modyfikacji najczęściej wykorzystywane są lipazy, działające w środowiskach o niskiej zawartości wody. Substratami mogą być np. kwas ferulowy i różne alkohole tłuszczowe, które w obecności enzymu ulegają estryfikacji, tworząc nowe bioaktywne związki o zwiększonej rozpuszczalności w olejach (Campos-Vega i in. 2015; Du i in. 2013). Poza lipazami, w tego typu reakcjach wykorzystywane są także inne enzymy: esterazy, tannazy oraz kutynazy (Shuai i in. 2017; Pasangulapati i in. 2012).

W ostatnich latach enzymatyczna lipofilizacja kwasów fenolowych zyskała na znaczeniu jako metoda zwiększania ich hydrofobowości i funkcjonalności przeciwutleniającej. Guyot i in. (1997, 2000) wykorzystali lipazę B z *C. antarctica*, do estryfikacji różnych kwasów fenolowych (np. cynamonowego, kawowego, ferulowego) z alkoholami o różnej długości łańcucha (C4–C18), osiągając nawet 97% wydajności dla kwasu cynamonowego z butanolem w systemie bezrozpuszczalnikowym. Z kolei Buisman i in. (1998) odnotowali wydajność do 85% w estryfikacji kwasu cynamonowego z 1-butanolem w *n*-pentanie, podczas gdy dla pochodnych kwasu benzoesowego uzyskano znacznie niższe wartości (<2%). Badania Stamatis i in. (1999, 2001) wykazały, że lipaza z *R. miehei* może być skuteczniejsza od lipazy z *C. antarctica* w syntezie estrów kwasu ferulowego, osiągając do 30% wydajności w bezrozpuszczalnikowych warunkach z 1-oktanolem. Compton i in. (2000) uzyskali jedynie 14% wydajności dla ferulanu oktylu, co wskazuje na znaczenie warunków reakcji i typu alkoholu. Giuliani i in. (2001) z powodzeniem zastosowali feruloesterazę z *A. niger* w mikroemulsji do syntezy ferulanu pentylu, osiągając 60% wydajności w 8 godzin. Kolejne badania (Topakas i in. 2003) potwierdziły znaczenie struktury substratu: feruloesteraza z *Fusarium oxysporum* wykazała wysoką aktywność wobec kwasów z grupą hydroksylową w pozycji *para*, osiągając do 75% wydajności. Natomiast Yu i in. (2004) syntetyzowali estry kwasu galusowego z alkoholami (C1–C18) przy użyciu mikrokapsułkowanej tanazy z *A. niger*, uzyskując najwyższą wydajność (44,3%) dla galusanu propylu. Zebrane dane dowodzą, że efektywność enzymatycznej lipofilizacji kwasów fenolowych jest silnie zależna od struktury substratów, typu enzymu, długości łańcucha węglowego alkoholu oraz warunków reakcji, co stanowi kluczowy element optymalizacji procesów otrzymywania bioaktywnych, lipofilowych estrów fenolowych.

1.5. Enzymatyczna lipofilizacja związków fenolowych w ekstraktach roślinnych

Poprawa rozpuszczalności związków fenolowych w środowiskach organicznych jest najczęściej realizowana poprzez modyfikację pojedynczych związków (Ramadan

2022; de Araujo i in. 2017). Jednakże wykazano, że ekstrakty roślinne zazwyczaj charakteryzują się lepszymi właściwościami przeciwutleniającymi niż większość czystych związków fenolowych, co może sugerować istnienie synergistycznych interakcji pomiędzy obecnymi w nich przeciwutleniaczami (Szajdek i Borowska 2004).

Szczególny nacisk w badaniach kładziony jest na antocyjany, które choć są wysoko aktywne, są również często niestabilne i wrażliwe na zmiany pH, światło i ciepło. Lipofilizacja antocyjanów, najczęściej występujących w postaci glikozydów, odbywa się poprzez estryfikację grupy hydroksylowej w cząsteczce cukru kwasami tłuszczowymi, co ma na celu poprawę ich stabilności oraz rozpuszczalności w tłuszczach. Zastosowanie lipofilizowanych ekstraktów roślinnych wykazuje obiecujące rezultaty. Pochodne te są zazwyczaj bardziej lipofilowe niż związki wyjściowe i umożliwiają poprawę stabilności olejów jadalnych (np. podczas smażenia), zwiększenie stabilności barwy podczas obróbki termicznej oraz obniżenie zawartości akrylamidu w produktach spożywczych (Jasińska i in. 2022, **[P1]**). Owoce stanowią bogate źródło związków fenolowych. Do tej pory wykorzystano antocyjany z mącznicy alpejskiej (*Arctostaphylos alpina*), takie jak cyjanidyno-3-*O*-galaktozyd, acylowany kwasem laurynowym. Modyfikacja ta poprawiła termostabilność i lipofilowość estru, mierzoną współczynnikiem podziału oktanol/woda (logP). Badania na czarnej porzeczce (*Ribes nigrum* L.) wykazały, że enzymatyczna acylacja glukozydów i rutynozydów cyjanidyny i delfinidyny kwasem laurynowym doprowadziła do powstania monoacylowanych pochodnych. Estryfikacja ta zwiększyła lipofilowość i termostabilność antocyjanów, a także poprawiła hamowanie peroksydacji lipidów w środowisku lipofilowym. Ekstrakty z kanadyjskiej jabłoni (ang. *Canadian crabapple*), zawierające głównie florydzynę, zostały zmodyfikowane za pomocą kwasu oktadekanowego. Otrzymany ester wykazał lepsze działanie antyoksydacyjne w oleju rzepakowym podczas smażenia chipsów ziemniaczanych, choć nie miał istotnego wpływu na jego stabilność podczas przechowywania. Ponadto, proantocyjanidyny z nasion winogron estryfikowano kwasem laurynowym, uzyskując pochodne o wyższej lipofilowości i najwyższej aktywności zmiatającej rodniki DPPH w porównaniu do związków wyjściowych oraz syntetycznych antyoksydantów BHT i TBHQ (Jasińska i in. 2022, **[P1]**).

Lipofilizacje przeprowadzano w ekstraktach, nie tylko z owoców, ale także z kwiatów oraz liści. Badania na płatkach róży wykazały, że enzymatyczna estryfikacja antocyjanów (głównie cyjanidyno-3,5-*O*-diglukozyd) kwasem laurynowym prowadzi do

powstania monoestrów laurylowych. Pochodne te wykazały lepszą stabilność barwy podczas obróbki cieplnej w ekstrudatach ryżowych i kremach do biszkoptów, co podkreśla ich potencjał jako naturalnych barwników. Delfinidyno-3-*O*-sambubiozyd z kwiatów hibiskusa (*Hibiscus sabdariffa*) został acylowany kwasem oktanowym. Otrzymany produkt miał poprawioną lipofilowość i wykazywał stabilizację zasady chinoidalnej, która odpowiada za niebieski kolor w umiarkowanie zasadowym pH, co pozwala na jego zastosowanie jako stabilnego pigmentu. W przypadku ekstraktów z liści modyfikacja była stosowana głównie w celu poprawy rozpuszczalności flawonoidów w matrycach tłuszczowych. Ekstrakt z liści bambusa, bogaty we flawonoidy *C*-glikozydowe, takie jak izoorientyna i izowiteksyna, poddano enzymatycznej acylacji kwasem laurynowym. Zastosowanie tych acylowanych flawonoidów wykazało kluczową zdolność do hamowania tworzenia akrylamidu w smażonych chipsach ziemniaczanych, sugerując ich interakcję z reaktywnymi związkami karbonylowymi powstającymi w reakcjach Maillarda (Jasińska i in. 2022, **[P1]**). Ze względu na ograniczoną lipofilowość ekstraktu z jarzębu pospolitego (*Sorbus aucuparia* L.), którego dominującym składnikiem jest kwas chlorogenowy (ok. 80% całkowitej zawartości fenoli), Aladedunye i in. (2015) wykorzystali enzymatyczną estryfikację kwasu chlorogenowego alkoholem oktadekanowym, otrzymując oktadecylowy ester kwasu chlorogenowego. Produkt ten wykazał zwiększoną skuteczność antyoksydacyjną w warunkach smażenia, znacząco hamując tworzenie związków polarnych oraz di- i polimerycznych triacylogliceroli w porównaniu z ekstraktem niemodyfikowanym i próbą kontrolną. Wyniki te potwierdzają, że enzymatyczna lipofilizacja może być efektywną strategią poprawy funkcjonalności fenoli w matrycach lipidowych i sprzyjać wykorzystaniu surowców roślinnych jako źródła przeciwutleniaczy o podwyższonej aktywności. Zastosowanie pełnych ekstraktów roślinnych, zawierających różne rodzaje związków fenolowych, oraz analiza ich synergistycznego działania wciąż stanowi nowe i złożone podejście badawcze. Oczywiste jest, że ekstrakty z poszczególnych rodzajów owoców różnią się zawartością przeciwutleniaczy, a ich aktywność przeciwutleniająca zależy od typu i ilości poszczególnych związków fenolowych. Metoda lipofilizacji ekstraktów roślinnych powinna być dostosowana do składu danego ekstraktu (Osmulescu i in. 2017).

2. CEL I HIPOTEZY BADAWCZE

Celem rozprawy było opracowanie immobilizowanego biokatalizatora opartego na odpadach spożywczych oraz jego zastosowanie w lipofilizacji kwasów fenolowych, w szczególności kwasu chlorogenowego (CGA), dominującego w ekstraktach z fusów kawowych, wytłoków aronii i jabłek. Otrzymane estry i lipofilizowane ekstrakty oceniono jako dodatki poprawiające stabilność oksydacyjną oleju roślinnego.

Cel pracy realizowany był w oparciu o następujące hipotezy badawcze:

H1: Odpady spożywcze z produkcji napojów (np. wytłoki jabłkowe i aroniowe oraz fusy kawowe) mogą stanowić nośnik do immobilizacji enzymów lipolitycznych, umożliwiając otrzymanie biokatalizatorów o wysokiej aktywności syntetycznej.

H2: Lipazy immobilizowane na nośnikach pochodzących z odpadów spożywczych skutecznie katalizują estryfikację dominującego w uzyskanych ekstraktach kwasu fenolowego.

H3: Estry dominującego kwasu fenolowego otrzymane w lipofilizacji zachowują lub przewyższają właściwości przeciwutleniające i/lub przeciwdrobnoustrojowe w porównaniu do związku wyjściowego, a ich dodatek do oleju roślinnego istotnie zwiększa jego stabilność oksydacyjną.

H4: Lipofilizacja ekstraktów roślinnych za pomocą opracowanego biokatalizatora skutkuje otrzymaniem produktu o zwiększonej lipofilowości, przy zachowaniu lub poprawie właściwości przeciwutleniających i/lub przeciwdrobnoustrojowych w porównaniu do ekstraktu niezmodyfikowanego.

3. MATERIAŁY I METODY BADAWCZE

3.1. Materiały do badań

Główny materiał do badań stanowiły:

- lipazy pochodzenia mikrobiologicznego:
  - **[P2, P3, P5]** płynna nieimmobilizowana lipaza z *Aspergillus oryzae* (Novozym 51032, Novozymes, Bagsvaerd, Dania)
  - **[P3, P4]** płynna nieimmobilizowana lipaza z *Rhizomucor miehei* (Palatase 20000L, Novozymes, Bagsvaerd, Dania)
  - **[P3]** płynna nieimmobilizowana lipaza z *Thermomyces lanuginosus* (Lipozyme TL, Novozymes, Bagsvaerd, Dania)
  - **[P2]** płyn po hodowli szczepu drożdży *Yarrowia lipolytica* KKP 379 pochodzącego z Kolekcji Kultur Drobnoustrojów Przemysłowych, należącej do Instytutu Biotechnologii Przemysłu Rolno-Spożywczego im. Prof. Wacława Dąbrowskiego – Państwowego Instytutu Badawczego w Warszawie (Polska).
  - **[P2, P3, P5]** immobilizowana lipaza B z *Candida antarctica* (Novozym 435, Sigma-Aldrich, Poznań, Polska) – jako próba referencyjna

- nośniki do procesu immobilizacji:
  - **[P2]** chitozan (Glentham Life Sciences, Corsham, UK)
  - **[P2]** Lewatit VP OC 1600 (Lanxess, Kolonia, Niemcy) – makroporowaty adsorbent zbudowany z kopolimeru metakrylanu i diwinylobenzenu
  - **[P2, P3, P4, P5]** odpady spożywcze: wytłoki z aronii i z jabłek (Greenherb, Wysoka, Polska), fusy kawowe (lokalna kawiarnia Costa Coffee oraz EcoBean (Warszawa, Polska)).

- odczynniki chemiczne, stanowiące substraty oraz rozpuszczalnik do optymalizacji reakcji syntezy
  - **[P5]** kwas chlorogenowy, 1-butanol, 1-heksanol, 1-oktanol, 1-dekanol, 1-dodekanol, eter *tert*-butylowo-metylowy (Sigma-Aldrich, Poznań, Polska)

3.2. Hodowla drożdży, liofilizacja i oczyszczanie lipaz

Do hodowli drożdży **[P2]** zastosowano podłoże YPO (2% peptonu, 2% oliwy z oliwek, 1% ekstraktu drożdżowego) o pH 5,0, z dodatkiem 0,1% Tween 80 jako emulgatora. Hodowle eksperymentalne inokulowane poprzez dodanie 0,1% (v/v) 24-godzinnej hodowli szczepu drożdży *Y. lipolytica* KKP 379 w podłożu YPG (2% peptonu, 2% glukozy, 1% ekstraktu drożdżowego, pH = 5,0) do 200 cm^3 sterylnego podłoża w kolbach okrągłych płaskodennych. Następnie kolby inkubowano przez 48 godzin na wytrząsarce posuwisto-zwrotnej, przy prędkości obrotowej 140 rpm (IKA KS 4000 IC Control, Niemcy). Hodowlę drożdży odwirowano, a płyn pohodowlany z lipazami zewnątrzkomórkowymi oddzielono od biomasy i przelano na szalki Petriego. Próbki zamrożono w zamrażarce Irinox (Corbanese, Włochy) w temperaturze −40 °C, a następnie liofilizowano w aparacie Christ Gamma 1-16 (Osterode am Harz, Niemcy). Uzyskany materiał przechowywano na półkach w temperaturze 0 °C. Liofilizowany płyn pohodowlany oczyszczono z wykorzystaniem chromatografii jonowymiennej (elucja za pomocą liniowego gradientu z 0,7 M NaCl i 15 mM buforu Tris-HCl, pH = 6,8) oraz chromatografii żelowej z użyciem sita molekularnego (bufor fosforanowy 50 mM, pH = 7,0). Otrzymane aktywne frakcje enzymów lipolitycznych zagęszczono w wirówce (4 000× g, 10 min) z zastosowaniem membrany VIVASPIN Centrifugal Concentrator 10 000 MWCO PES (Sartorius, Göttingen, Niemcy).

3.3. Wstępna obróbka fusów z kawy

Badania przeprowadzono na surowcach pobranych na różnych etapach ciągłego procesu technologicznego firmy Ecobean, opisanego w zgłoszeniu patentowym nr WIPO ST 10/C PL447416 **[P3]**. Poniżej opisano wstępną obróbkę każdej próbki:

SCG1 – fusy z kawy zostały zebrane z warszawskiej kawiarni. Po transporcie SCG zostały wysuszone do wilgotności około 5%.

SCG2 – SCG1 ekstrahowano heksanem w reaktorze pod ciśnieniem przez 30 minut, a następnie filtrowano w suszarce filtracyjnej w celu oddzielenia fazy stałej od heksanu i oleju kawowego.

SCG3 – SCG2 ekstrahowano 40 – 50% etanolem, w celu uzyskania kolejnej frakcji. Ekstrakcję przeprowadzono w reaktorze przez 2 godziny w stosunku SCG: rozpuszczalnik (1:10). Koniecznym etapem była filtracja i suszenie analogiczne do przygotowania SCG2.

SCG4 – Odpady SCG3 poddano hydrolizie w reaktorze z 2,5% wodnym roztworem kwasu siarkowego w stosunku 1:9, przez 3 godziny w temperaturze 100 °C. SCG4 oddzielono od cieczy i wysuszono.

3.4. Immobilizacja lipaz

3.4.1. Immobilizacja na nośniku Lewatit VP OC 1600

Immobilizację na nośniku Lewatit VP OC 1600 przeprowadzono zgodnie z metodologią opisaną przez Barrera-Rivera i Martínez-Richa (2017), z drobnymi modyfikacjami **[P2]**. W pierwszym etapie makroporowaty nośnik aktywowano etanolem w stosunku 1:10 (nośnik:etanol) przez 5 godzin, następnie przemywano go wodą destylowaną i suszono próżniowo w temperaturze pokojowej przez 24 godziny. Następnie do 1 g nośnika dodano 15 cm^3 płynu po hodowli *Y. lipolytica* lub roztworu lipazy (1 cm^3 oczyszczonej lipazy uzyskanej z płynu po hodowli *Y. lipolytica* lub 1 cm^3 nieimmobilizowanej lipazy Novozym 51032 w 14 cm^3 wody). Roztwory lipaz wraz z nośnikiem inkubowano na wytrząsarce orbitalnej w temperaturze 4 °C przez 14 godzin. Po inkubacji, unieruchomione lipazy na nośniku przemywano wodą destylowaną i suszono próżniowo w temperaturze pokojowej przez 24 godziny.

3.4.2. Immobilizacja na chitozanie

Na podstawie metodologii opisanej przez Pereira i in. (2003), z drobnymi modyfikacjami, przeprowadzono immobilizację lipaz na chitozanie poprzez adsorpcję fizyczną **[P2].** Przed immobilizacją, chitozan (2 g) zawieszono w 30 cm^3 heksanu i mieszano przez 1 godzinę. Heksan usunięto poprzez filtrację próżniową, a chitozan przemyto wodą destylowaną. Do immobilizacji użyto 20 cm^3 płynu po hodowli drożdży *Y. lipolytica* oraz 1 cm^3 nieimmobilizowanej lipazy Novozym 51032 (rozcieńczonej w 14 cm^3 wody destylowanej). Roztwór lipaz mieszano z chitozanem w kolbie Erlenmeyera i inkubowano przez 3 godziny w temperaturze pokojowej, a następnie przez kolejne 18 godzin w warunkach statycznych w temperaturze 4 °C. Na zakończenie, unieruchomione preparaty filtrowano próżniowo, przemyto wodą destylowaną i wysuszono w temperaturze pokojowej.

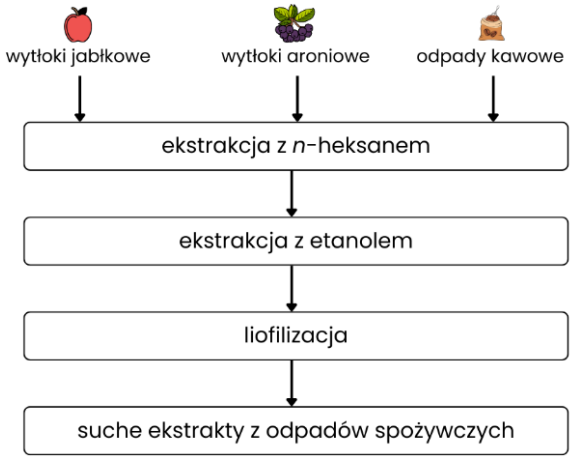
3.4.3. Immobilizacja na odpadach spożywczych

Procedura immobilizacji na odpadach spożywczych (wytłokach z aronii i jabłek oraz fusach kawowych) opierała się na metodologii przedstawionej przez Buntić i in. (2018), z niewielkimi modyfikacjami **[P2, P3, P4, P5]**. Roztwór nieimmobilizowanej, komercyjnej lipazy (1 cm^3 ciekłej lipazy i 14 cm^3 wody destylowanej), dodawano do kolby zawierającej 1 g nośnika, a następnie mieszano przez 2 godziny. Immobilizowaną lipazę filtrowano pod próżnią, przemyto wodą destylowaną i wysuszono w temperaturze pokojowej.

3.5. Charakterystyka odpadów spożywczych

3.5.1. Obróbka wstępna i przygotowanie ekstraktów

10 g każdego odpadu spożywczego (wytłoków z aronii i jabłek oraz fusów po kawie – **[P5]**) odważono, owinięto w bibułę filtracyjną i umieszczono w aparacie Soxhleta. Ekstrakcję prowadzono zgodnie ze schematem (Rys.1), najpierw o obecności 150 cm^3 hekasanu, a następnie 150 cm^3 etanolu, do uzyskania 10 pełnych cykli przelania rozpuszczalnika prze aparat Soxhleta. Po tym czasie pozostały rozpuszczalnik został odparowany pod zmniejszonym ciśnieniem z wykorzystaniem wyparki rotacyjnej. Z frakcji po ekstrakcji heksanem określono zawartość lipidów w odpadach spożywczych. Procentową ilość wyekstrahowanego tłuszczu obliczono na podstawie masy początkowej próbki i masy wyekstrahowanych lipidów. Uzyskane ekstrakty nałożono na płytki i zamrożono w temperaturze -42°C w zamrażarce Irinox (Corbanese, Włochy) przez 1 godzinę, a następnie przeniesiono na 24 godziny do liofilizacji w aparacie Christ Gamma 1-16 LSC (Osterode am Harz, Niemcy). Zastosowane warunki to: temperatura półek 10°C oraz ciśnienie 63 Pa. Ciśnienie bezpieczeństwa wynosiło 103 Pa, chroniąc materiał przed temperaturami powyżej -20°C. Suche ekstrakty przechowywano w temperaturze pokojowej.



Rys. 1. Schemat procesu wstępnej obróbki odpadów spożywczych.

3.5.2. Analiza włókna

Frakcję włókna oznaczano za pomocą systemu FibertecMC 8000 (Foss Analytics, Warszawa, Polska) **[P3, P4]**. Zawartość włókna surowego oznaczono metodą PN-ISO 5498 (1996). Zawartość włókna detergentowego kwaśnego (ADF) oraz ligniny detergentowej kwaśnej (ADL) określono zgodnie z normą PN-EN ISO 13906 (2009). Natomiast oznaczenie zawartości włókna detergentowego obojętnego (NDF), po trawieniu z użyciem amylazy, przeprowadzono zgodnie z normą PN-EN ISO 16472 (2007). Zawartość celulozy obliczono jako różnicę między ADF a ADL, natomiast zawartość hemicelulozy – jako różnicę między NDF a ADF.

3.5.3. Analiza pierwiastków

Całkowitą zawartość pierwiastków: C, N i S oznaczono metodą spalania na sucho (Vario MacroCube, Elementar, Niemcy) **[P5]**.

3.6. Charakterystyka biokatalizatorów

3.6.1. Aktywność hydrolityczna

Pomiar aktywności hydrolitycznej przeprowadzono metodą spektrofotometryczną, opartą na hydrolizie laurynianu *p*-nitrofenylu **[P2, P3, P4, P5]**. Reakcję prowadzono w probówkach typu Eppendorf. Do badania użyto 100 µl wolnej (ciekłej) lipazy lub 25 mg unieruchomionego biokatalizatora zawieszonego w 100 µl wody destylowanej, które mieszano, w temperaturze 37 °C, z 25 µl 0,3 mmol laurynianu *p*-nitrofenylu rozpuszczonym w 2 cm^3 heptanu. Po 15 minutach inkubacji mierzono absorbancję przy długości fali 410 nm za pomocą spektrofotometru UV–Vis. Jednostkę aktywności

enzymatycznej lipazy (1 U) zdefiniowano jako ilość enzymu uwalniającą 1 µmol *p*-nitrofenolu na minutę w warunkach oznaczenia.

3.6.2. Aktywność syntetyczna

Aktywność syntetyczną immobilizowanej lipazy oceniano za pomocą metody kolorymetrycznej opracowanej przez Zheng i in. (2014), które została zmodyfikowana na potrzeby pracy badawczej **[P2, P3, P4, P5]**. Pomiar oparto na reakcji transestryfikacji pomiędzy octanem winylu a 1-butanolem. MBTH (hydrazon 3-metylobenzo-2-tiazolinonu) reaguje z uwalnianym aldehydem octowym, tworząc odpowiednią aldazynę, która następnie przekształcana jest w niebiesko zabarwiony związek TAPMC (tetraazapentametylenowa cyjanina). Reakcję prowadzono w probówkach typu Eppendorf, w roztworze zawierającym 100 mM octanu winylu i 100 mM 1-butanolu w 1 cm^3 heksanu. W celu zainicjowania transestryfikacji dodawano 5 µl wolnej (ciekłej) lipazy lub 5 mg immobilizowanej lipazy. Inkubację prowadzono przez 5 minut w temperaturze 30 °C z mieszaniem. Próba kontrolna nie zawierała dodatku enzymu. Po zakończeniu reakcji przygotowywano rozcieńczone próbki (200-krotnie lub zgodnie z wymaganiami) do pomiaru spektrofotometrycznego. Analizę rozpoczynano od dodania 1 cm^3 0,1% (m/v) roztworu MBTH do każdej próbki i inkubacji przez 10 minut w temperaturze 30 °C. Następnie dodawano 0,4 cm^3 1% (m/v) roztworu $NH_4Fe(SO_4)_2$ x12H_2O (w 0,1 M HCl) i inkubowano przez kolejne 30 minut w tej samej temperaturze. Pomiar kolorymetryczny przeprowadzano przy długości fali 595 nm przy użyciu spektrofotometru. Krzywą wzorcową opracowano na podstawie różnych stężeń aldehydu octowego. Jednostkę aktywności syntetycznej lipazy (1 U) zdefiniowano jako ilość enzymu przekształcającą 0,1 mmol octanu winylu w aldehyd octowy na minutę w warunkach oznaczenia.

3.6.3. Specyficzność substratowa

Do określenia specyficzności działania lipaz wobec różnych substratów zastosowano metodę spektrofotometryczną opartą na reakcji hydrolizy laurynianu *p*-nitrofenylu (podrozdział 3.6.1) **[P3, P4].** W badaniu wykorzystano następujące estry *p*-nitrofenylowe: maślan (C4:0), kaprylan (C8:0), laurynian (C12:0), palmitynian (C16:0), stearynian (C18:0) oraz oleinian (C18:1).

3.6.4. Zawartość białka

Stężenie białka w wolnej lipazie oraz w filtratach po procesie immobilizacji oznaczano spektrofotometrycznie metodą Lowry'ego (Toczko i Grzelińska 1997) **[P2, P3, P4]**. Metoda ta opiera się na reakcji wiązań peptydowych i aromatycznych aminokwasów z odczynnikiem fenolowym Folina–Ciocalteu. Do pomiaru używano 1 cm^3 każdego badanego roztworu. Przygotowano również 50-krotne rozcieńczenia próbek. Reakcję prowadzono w probówkach, do których dodawano 5 cm^3 odczynnika miedziowego (2% Na_2CO_3 w 0,1 M NaOH, 1% $CuSO_4$ oraz 2% winianu sodowo-potasowego w stosunku objętościowym 100:1:1). Po 10 minutach dodawano 0,5 cm^3 odczynnika fenolowego Folina–Ciocalteu. Inkubację prowadzono przez 30 minut, po czym mierzono absorbancję przy długości fali 750 nm za pomocą spektrofotometru Rayleigh UV-1601 (BRAIC, Pekin, Chiny). Do obliczeń zawartości białka wykorzystano krzywą kalibracyjną sporządzoną z użyciem albuminy jako wzorca. Ilość białka zaadsorbowanego na nośniku obliczono na podstawie różnicy pomiędzy zawartością białka w wolnej lipazie, a jego ilością w roztworze po immobilizacji.

3.6.5. Specyficzna aktywność hydrolityczna i syntetyczna

Na podstawie uzyskanych wyników dotyczących aktywności hydrolitycznej i syntetycznej oraz zawartości białka, obliczono aktywności właściwe wolnej lipazy oraz immobilizowanych biokatalizatorów zgodnie z poniższymi równaniami **[P3, P4]:**

1. Wolna, nieimmobilizowana lipaza

$$Aktywność\ właściwa\ \left[\frac{U}{mg}\right] = \frac{aktywność\ hydrolityczna\ lub\ syntetyczna\ [\frac{U}{ml}]}{zawartość\ białka\ w\ wolnej\ lipazie\ [\frac{mg\ białka}{ml}]}$$

2. Immobilizowana lipaza

$$Aktywność\ właściwa\ \left[\frac{U}{mg}\right] = \frac{aktywność\ hydrolityczna\ lub\ syntetyczna\ [\frac{U}{mg}]}{ilość\ białka\ unieruchomionego\ na\ nośniku\ [\frac{mg\ białka}{mg\ nośnika}]}$$

3.6.6. Profil aktywności w różnym pH i temperaturze

Do określenia wpływu różnych wartości pH środowiska reakcji oraz temperatury na immobilizowane preparaty enzymatyczne, zastosowano metodę oceny aktywności hydrolitycznej (podrozdział 3.6.1) **[P3, P4]**. W pierwszym wariancie reakcje prowadzono w buforze fosforanowym (10 mM) o pH 5, 6, 7 oraz 8, a temperatura reakcji dla każdego pomiaru była stała i wynosiła 37 °C. W drugim wariancie, reakcje

prowadzono w temperaturach od 30 °C do 70 °C, przy zachowaniu niezmienionych, pozostałych parametrów.

3.6.7. Spektroskopia FT-IR

Widma FT-IR wszystkich preparatów rejestrowano za pomocą spektrometru Nicolet iS5 ATR firmy Thermo Scientific, wyposażonego w diamentowy kryształowy moduł do pomiarów ATR iD7 (Thermo Fisher Scientific, Waltham, USA). Pomiary wykonywano w zakresie liczby falowej od 4000 do 600 cm^{-1} **[P3].**

3.6.8. Możliwość ponownego użycia

Możliwość ponownego wykorzystania biokatalizatorów oceniono za pomocą metody pomiaru aktywności hydrolitycznej opisanej w podrozdziale 3.6.1 **[P3, P4]**. Reakcję hydrolizy laurynianu *p*-nitrofenylu przeprowadzano w temperaturze 37 °C i pH 7 przez 15 minut. Następnie mierzono absorbancję za pomocą spektrofotometru. Immobilizowany enzym oddzielano od mieszaniny reakcyjnej, dwukrotnie przemywano heksanem oraz buforem fosforanowym (pH 7), po czym ponownie używano do katalizowania tej samej reakcji. Biokatalizatory stosowano w pięciu kolejnych cyklach.

3.6.9. Skaningowa Mikroskopia Elektronowa (SEM).

Natywne nośniki oraz preparaty enzymatyczne po immobilizacji poddano analizie struktury powierzchni za pomocą skaningowego mikroskopu elektronowego (HITACHI TM 3000, Ramsey, New Jersey, USA) **[P2, P3, P4].** Próbki zostały wysuszone w warunkach próżni i pokryte warstwą złota przy użyciu napylarki Cressington Sputter Coater 108 auto (Cressington Scientific Instruments, Watford, Wielka Brytania), a następnie obserwowane mikrofotografie wykonano przy powiększeniu ×400.

3.7. Optymalizacja reakcji syntezy z wykorzystaniem modelu Boxa-Behnkena

Modelowa reakcja lipofilizacji kwasu chlorogenowego z 1-butanolem została przeprowadzona z użyciem biokatalizatora – lipazy z *A. oryzae* immobilizowanej na fusach po kawie (NSCG) **[P5]**. W celu optymalizacji warunków reakcji zaprojektowano eksperyment trójczynnikowy o trzech poziomach każdego z analizowanych czynników, wykorzystując plan Boxa-Behnkena (Tabela 1). Analizowanymi czynnikami były temperatura (35, 45 i 55 °C), stężenie enzymu (20%, 35% i 50% względem sumy masy substratów) oraz molowy stosunek substratów (alkoholu do kwasu chlorogenowego – 2:1, 5:1 oraz 8:1). Jako rozpuszczalnik zastosowano eter *tert*-butylowo-metylowy (15

cm^3). Optymalizowano również czas trwania reakcji (3, 5, 7 i 9 dni). W kolejnych reakcjach lipofilizacja kwasu chlorogenowego była przeprowadzana z alkoholami o różnej długości łańcucha węglowego (C6, C8, C10, C12) przez 7 dni. Optymalne parametry stanowią część zgłoszenia patentowego „Sposób otrzymywania estrów kwasu chlorogenowego i alkoholi, na drodze biokatalizy", WIPO ST 10/C PL451973 (Jasińska K., Fabiszewska A., Zieniuk B.). Eksperymenty prowadzono z użyciem dwóch biokatalizatorów: lipazy z *A. oryzae* immobilizowanej na fusach kawowych oraz Novozym 435 jako wzorca referencyjnego. Skład mieszaniny reakcyjnej badano za pomocą techniki HPLC. Wydajność reakcji obliczano na podstawie pola pod krzywą piku substratu i produktu na chromatogramie.

Tabela 1. Poziomy kodowane i wartość dekodowana planu Boxa-Behnkena

Czynnik	Nazwa	Jednostka	Minimum (-1)	Średnia (0)	Maximum (+1)
1	Temperatura	°C	35	45	55
2	Molowy stosunek substratów	Alkohol : kwas chlorogenowy	2:1	5:1	8:1
3	Stężenie enzymu względem sumy masy substratów	%	20	35	50

3.8. Analiza chromatograficzna

3.8.1. Chromatografia kolumnowa – oczyszczanie produktu reakcji

Po zakończeniu reakcji estryfikacji (podrozdział 3.7) mieszaninę przefiltrowano w celu oddzielenia biokatalizatora. Następnie rozpuszczalnik odparowano pod obniżonym ciśnieniem, a powstały osad poddano oczyszczaniu metodą chromatografii kolumnowej. Jako fazę stacjonarną zastosowano żel krzemionkowy (wielkość cząstek: 0,040–0,063 mm; rozmiar oczek: 230–400), natomiast fazą ruchomą była mieszanina chloroformu i metanolu w stosunku objętościowym 1:1. Obecność poszukiwanych związków monitorowano za pomocą cienkowarstwowej chromatografii (TLC) na płytkach z żelem krzemionkowym. Frakcje zawierające pożądany produkt połączono, a rozpuszczalnik usunięto przez odparowanie. Estry wykrystalizowano z heptanu, aby uzyskać związek oczyszczony **[P5]**.

3.8.2. Wysokosprawna chromatografia cieczowa - określenie wydajności reakcji

Proces przygotowania estrów po reakcji polegał na odparowaniu rozpuszczalnika pod strumieniem azotu, rozcieńczeniu otrzymanego osadu metanolem oraz przeniesieniu do kolb miarowych o objętości 25 cm^3. Przygotowane roztwory estrów po reakcji przefiltrowano przez filtr strzykawkowy PTFE o średnicy porów 0,45 μm do fiolek chromatograficznych.

Analiza chromatograficzna estrów kwasu chlorogenowego została przeprowadzona zgodnie ze zmodyfikowaną procedurą analityczną opisaną przez Głowacką i in. (2019). Analiza HPLC odbyła się przy użyciu układu wysokosprawnej chromatografii cieczowej Shimadzu (Kioto, Japonia), wyposażonego w odgazowywacz DGU-20A SR, pompę LC-20AD, autosampler SIL-20A HT, termostat kolumny CTO-10AS VP oraz detektor diodowy SPD-M20A. Separacja próbek została przeprowadzona na kolumnie analitycznej Supelco SUPELCOSIL LC-18-S o wielkości cząstek 5 μm (25 cm × 4,6 mm). Jako fazy ruchome zastosowano wodny 0,1% roztwór kwasu mrówkowego (faza A) oraz metanol (faza B) w trybie elucji gradientowej, przy przepływie 0,8 cm^3/min. Profil gradientu był następujący: 0 min – 80% fazy B, 12–15 min – 50% fazy B, 28–45 min – 80% fazy B. Detekcję prowadzono przy długości fali 325 nm. Wydajność reakcji wyznaczano na podstawie pól pod krzywymi odpowiednich pików analitów **[P5]**.

3.8.3. Chromatografia cieczowa sprzężona ze spektrometrią mas - analiza zawartości kwasów fenolowych w wybranych ekstraktach

Porównanie zawartości wybranych kwasów fenolowych oraz ich pochodnych w przygotowanych ekstraktach przed i po procesie lipofilizacji określono za pomocą chromatografii cieczowej sprzężonej ze spektrometrią mas (LC-MS) **[P5]**, w Zakładzie Bezpieczeństwa i Analizy Chemicznej Żywności, Instytutu Biotechnologii Przemysłu Rolno-Spożywczego im. Prof. Wacława Dąbrowskiego w Warszawie.

Każdy liofilizowany ekstrakt dokładnie odważono (10 mg) i rozpuszczono w 1 cm^3 metanolu. Zawiesiny poddano sonikacji w kąpieli ultradźwiękowej przez 30 minut w temperaturze pokojowej. Następnie próbki przefiltrowano przez strzykawkowe filtry o porowatości 0,22 μm (membrana PTFE). Na podstawie tych roztworów macierzystych przygotowano trzy poziomy stężenia analitów: 10, 1 oraz 0,1 mg/cm^3. W tym celu odpowiednie objętości roztworów macierzystych odparowano do sucha pod strumieniem

azotu w temperaturze pokojowej, a pozostałości rozpuszczono w 30% metanolu (v/v), aby uzyskać pożądane stężenia.

Do określenia zawartości kwasów fenolowych w ekstraktach pochodnych pobierano próbki z mieszaniny reakcyjnej. Odpowiednie objętości (50 lub 200 µL) pobierano z fiolek i odparowywano do sucha pod strumieniem azotu. Pozostałości rozpuszczano w roztworze 30% metanolu (v/v) i dopełniano do objętości 200 lub 250 µL, w zależności od początkowej objętości ekstraktu. Przygotowane próbki następnie analizowano za pomocą chromatografii cieczowej sprzężonej ze spektrometrią mas (LC-MS).

3.9. Identyfikacja związków

3.9.1. Analiza struktury estrów

Potwierdzenie struktury oczyszczonych estrów przeprowadzono za pomocą spektroskopii magnetycznego rezonansu jądrowego - ^{13}C NMR na Wydziale Chemii Uniwersytetu Warszawskiego, w Warszawie. Widma rejestrowano w spektrometrze Bruker AVANCE 500 MHz (Bruker, Billerica, MA, USA) z zastosowaniem DMSO-d6 jako rozpuszczalnika. Przesunięcia chemiczne (δ) podano w częściach na milion (ppm) względem tetrametylosilanu (TMS) jako standardu wewnętrznego **[P5]**.

Ester butylowy kwasu chlorogenowego (C4)

^{13}C NMR (126 MHz, DMSO-d6) δ 173.19, 165.43, 148.61, 145.70, 145.17, 125.34, 121.34, 115.85, 114.55, 113.81, 73.12, 71.09, 69.40, 66.91, 64.12, 37.23, 35.09, 30.03, 18.54, 13.56.

Ester heksylowy kwasu chlorogenowego (C6)

^{13}C NMR (126 MHz, DMSO-d6) δ 173.16, 165.35, 148.54, 145.66, 145.15, 125.32, 121.27, 115.79, 114.55, 113.75, 73.01, 71.07, 69.27, 66.75, 64.37, 37.22, 34.97, 30.80, 27.88, 24.90, 21.97, 13.78.

Ester oktylowy kwasu chlorogenowego (C8)

^{13}C NMR (126 MHz, DMSO-d6) δ 173.16, 165.35, 148.53, 145.65, 145.15, 125.31, 121.26, 115.77, 114.53, 113.73, 73.01, 71.07, 69.28, 66.77, 64.36, 37.22, 34.99, 31.11, 28.57, 27.92, 25.24, 22.05, 13.94.

Ester decylowy kwasu chlorogenowego (C10)

^{13}C NMR (126 MHz, DMSO-d6) δ 173.16, 165.36, 148.57, 145.67, 145.16, 125.29, 121.27, 115.77, 114.51, 113.71, 73.04, 71.06, 69.29, 66.80, 64.37, 37.22, 35.03, 31.31, 29.12, 29.00, 28.97, 28.92, 28.85, 28.73, 22.10, 13.97.

Ester dodecylowy kwasu chlorogenowego (C12)

^{13}C NMR (126 MHz, DMSO-d6) δ 173.15, 165.35, 148.54, 145.65, 145.14, 125.30, 121.25, 115.76, 114.51, 113.73, 73.04, 71.05, 69.31, 66.80, 64.35, 37.21, 35.03, 31.30, 29.02, 28.99, 28.91, 28.89, 28.72, 28.60, 27.92, 25.24, 22.11, 13.97.

3.9.2. Analiza za pomocą spektrometrii mas

Widma wysokorozdzielczej spektrometrii masowej z jonizacją elektrorozpylającą (ESI-MS) butylowego estru chlorogenowego zostały pozyskane za pomocą ultra-wydajnego chromatografu cieczowego ACQUITY UPLC I-Class (Waters, Milford, MA, USA) sprzężonego ze spektrometrem masowym Synapt G2-S HDMS (Waters, Milford, MA, USA), wyposażonym w źródło jonów ESI oraz analizator mas typu q-TOF. Zarejestrowane dane przetworzono za pomocą pakietu oprogramowania MassLynx V4.1 (Waters, Milford, MA, USA). Widma ESI-MS rejestrowano w trybie jonów dodatnich i ujemnych w zakresie stosunku masy do ładunku (m/z) od 50 do 3000. Analiza została przeprowadzona w Pracowni Spektrometrii Mas, Instytutu Chemii Organicznej PAN w Warszawie **[P5]**.

3.10. Reakcja lipofilizacji

Na podstawie optymalizacji syntezy pochodnych kwasu chlorogenowego opisanej w podrozdziale 3.7 wybrano parametry do przeprowadzenia lipofilizacji ekstraktów z fusów po kawie, wytłoków z aronii i jabłek **[P5]**. Reakcje prowadzono w kolbach stożkowych w temperaturze 55 °C, z ciągłym mieszaniem (150 obr./min). Do mieszaniny reakcyjnej dodano 1-butanol oraz wysuszone ekstrakty z odpadów spożywczych w stosunku 8:1 (m/m) oraz 15 cm^3 eteru *tert*-butylowo-metylowego. Reakcję katalizowano lipazą z *A. oryzae* immobilizowaną na fusach kawowych oraz lipazą Novozym 435 jako biokatalizatorem referencyjnym, przez 7 dni. Biokatalizatory usunięto z mieszaniny reakcyjnej przez filtrację. Ekstrakty, po odparowaniu pozostałego rozpuszczalnika, poddano analizie technikami chromatograficznymi wg metodyki opisanej w rozdziale 3.8.3.

3.11. Charakterystyka estrów kwasu fenolowego oraz ekstraktów z odpadów spożywczych

3.11.1 Całkowita zawartość związków fenolowych

Całkowitą zawartość związków fenolowych w ekstraktach (1 mg/cm^3 w metanolu) przed procesem lipofilizacji określono metodą Folina–Ciocalteu **[P5]**. Do szklanych probówek dodano 0,18 cm^3 ekstraktu, który rozcieńczono 4,92 cm^3 wody destylowanej, a następnie dodano 0,3 cm^3 odczynnika Folina–Ciocalteu i wymieszano. Po 3 minutach pH roztworu zmieniono poprzez dodanie 0,6 cm^3 nasyconego roztworu węglanu sodu. Inkubację prowadzono w ciemności, w temperaturze 25 °C, przez godzinę. Absorbancję roztworów

zmierzono spektrofotometrem Rayleigh UV-1601 (BRAIC, Pekin, Chiny) przy długości fali 750 nm, z użyciem próbki kontrolnej jako punktu odniesienia. Zawartość polifenoli wyrażono jako równoważniki kwasu chlorogenowego (mg równoważników CGA na g ekstraktu). Analizę przeprowadzono w trzech powtórzeniach.

3.11.2. Analiza właściwości przeciwutleniających

3.11.2.1. Metoda z rodnikiem DPPH

Test DPPH został przeprowadzony zgodnie z protokołem opisanym przez Zanetti i in. (2017), z niewielkimi modyfikacjami, w celu oceny właściwości antyoksydacyjnych otrzymanych związków i ekstraktów **[P5]**. Do przeprowadzenia doświadczenia przygotowano 1 mM roztwór wyizolowanych estrów kwasu chlorogenowego oraz 1 mg/cm^3 otrzymanych ekstraktów w metanolu. Do probówek z rozcieńczeniami próbek dodano 2,7 cm^3 roztworu DPPH (4 mg DPPH w 100 cm^3 metanolu). Aktywność antyoksydacyjną badanych związków zmierzono za pomocą spektrofotometru Rayleigh UV-1601 (BRAIC, Pekin, Chiny) przy długości fali 517 nm. Na podstawie wyników czterech różnych rozcieńczeń badanych roztworów obliczono wartość IC_{50}, czyli stężenie potrzebne do redukcji 50% rodnika DPPH$^{\cdot}$. Jako odniesienie użyto butylohydroksytoluen (BHT).

3.11.2.2. Metoda CUPRAC

Test CUPRAC został zastosowany jako uzupełniająca metoda oceny aktywności antyoksydacyjnej badanych substancji. Analiza została przeprowadzona zgodnie z metodą opisaną przez Özyürek i in. (2011) **[P5]**. W tej metodzie spektrofotometrycznie mierzono absorbancję kompleksu utworzonego przez neokuproinę (2,9-dimetylo-1,10-fenantrolinę) i jon Cu(I) przy długości fali 450 nm. Na podstawie pomiarów absorbancji badanych estrów chlorogenowych i ekstraktów w porównaniu do Troloxu, który służył jako wzorzec, obliczono ekwiwalentne zdolności antyoksydacyjne Troloxu (TEAC). Jako odniesienie użyto butylohydroksytoluen (BHT).

3.11.3. Analiza właściwości przeciwdrobnoustrojowych

Aktywność przeciwdrobnoustrojową oceniano metodą dyfuzyjno-krążkową **[P5].** Estry i ekstrakty rozpuszczono najpierw w etanolu, uzyskując stężenie 50 mg/cm^3. Następnie na jałowe dyski o średnicy 6 mm naniesiono po 10 µL każdego związku lub ekstraktów. Zawiesiny bakterii dostosowano do standardu 0,5 McFarlanda i równomiernie rozprowadzone na płytkach z agarowym podłożem Mueller–Hinton (30% wywar z

wołowiny, 1,75% hydrolizat kazeiny, 0,15% skrobia, 1,7% agar). Następnie dyski nasączone wybranymi estrami i ekstraktami umieszczono na powierzchni podłoża z inokulum. Płytki inkubowano w temperaturze 37 °C przez 16–18 godzin. Po inkubacji zmierzono średnice stref zahamowania wzrostu wokół dysków, aby ocenić skuteczność antybakteryjną testowanych związków i ekstraktów. Do analizy użyto następujących szczepów bakterii, zakupionych w Polskiej Kolekcji Mikroorganizmów (PCM) Instytutu Immunologii i Terapii Doświadczalnej Polskiej Akademii Nauk (Wrocław, Polska): *Bacillus cereus* PCM 482, *Bacillus subtilis* PCM 486, *Enterobacter cloacae* PCM 2848, *Enterococcus faecalis* PCM 2909, *Escherichia coli* PCM 2057, *Listeria monocytogenes* PCM 2191, *Serratia marcescens* PCM 549 oraz *Staphylococcus aureus* PCM 2054.

3.11.4. Analiza stabilności oksydacyjnej olejów roślinnych

Maksymalny czas utleniania (τ_{max}) dla olejów rzepakowych bez dodatków (próbka kontrolna) oraz z dodatkami został określony za pomocą analizy wysokociśnieniowej, różnicowej kalorymetrii skaningowej (PDSC) **[P5].** Do 30 g oleju rzepakowego w probówkach typu Falcon dodano etanolowe ekstrakty (3 mg związku/ekstraktu w 30 µl etanolu), co odpowiadało stężeniu 0,01% związku/ekstraktu w oleju. Pomiar przeprowadzono zaraz po dodaniu wszystkich reagentów oraz po 2 miesiącach przechowywania. Próbka oleju o masie 3–4 mg umieszczona została w otwartej aluminiowej szalce, obok której znajdowała się pusta szalka referencyjna, w komorze ciśnieniowej aparatu DSC Q20 (TA Instruments, New Castle, DE, USA). Wartość τ_{max} rejestrowano w stałej temperaturze 120 °C oraz przy początkowym ciśnieniu 1400 kPa, w środowisku czystego tlenu. Maksymalny czas utleniania τ_{max}, wyrażony w minutach, został wyznaczony poprzez analizę przepływu ciepła w czasie przy użyciu oprogramowania TA Software (wersja 4.5A).

3.11.5. Analiza lipofilowości estrów wybranego kwasu fenolowego

Aby określić lipofilowość otrzymanych estrów kwasu chlorogenowego, współczynnik podziału oktanol/woda (logP) oraz rozpuszczalność w wodzie (logS) zostały obliczone za pomocą oprogramowania MOE (Molecular Operating Environment, Chemical Computing Group, Kanada) **[P5]**.

3.12. Analiza statystyczna

Wyniki zostały poddane analizie statystycznej za pomocą oprogramowania STATISTICA 13.3 (TIBCO Software Inc., Palo Alto, CA, USA). Zastosowano

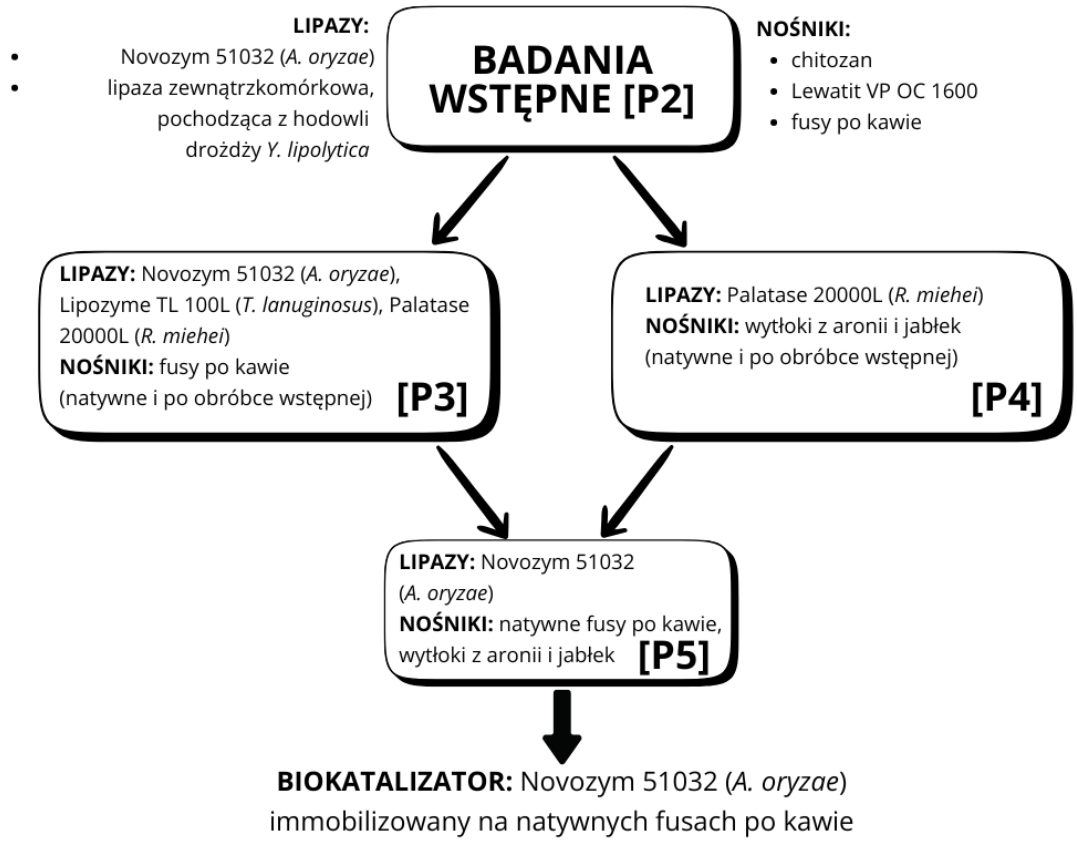
następujące metody: test Shapiro-Wilka do oceny hipotezy statystycznej dotyczącej normalności rozkładu danych eksperymentalnych, testy Levene'a i Brown-Forsythe'a do oceny hipotezy jednorodności wariancji, analizę wariancji (ANOVA), test post-hoc Tukeya oraz plan eksperymentu Boxa-Behnkena do optymalizacji reakcji syntezy. Za istotne statystycznie uznano wyniki przy poziomie istotności $p \leq 0,05$.

4. OMÓWIENIE I DYSKUSJA WYNIKÓW

4.11. Charakterystyka opracowanych biokatalizatorów, otrzymanych w wyniku immobilizacji enzymów lipolitycznych pochodzenia mikrobiologicznego na różnych nośnikach

Weryfikacja hipotezy **H1:** Odpady spożywcze z produkcji napojów (np. wytłoki jabłkowe i aroniowe oraz fusy kawowe) mogą stanowić nośnik do immobilizacji enzymów lipolitycznych, umożliwiając otrzymanie biokatalizatorów o wysokiej aktywności syntetycznej.

W ramach badań wstępnych przeprowadzono analizę możliwości wykorzystania wybranych lipaz pochodzenia mikrobiologicznego w procesach immobilizacji na nośnikach pochodzenia naturalnego oraz syntetycznego, której celem była selekcja najbardziej efektywnych układów enzym–nośnik do dalszych etapów pracy (Rys. 2).



Rys. 2. Schemat prac badawczych dotyczących otrzymania i charakterystyki immobilizowanych preparatów enzymatycznych, pochodzenia mikrobiologicznego, które opisano w publikacjach **[P2, P3, P4, P5]**.

W tym celu porównano kilka komercyjnych preparatów enzymatycznych, obejmujących:

- lipazę B z drożdży *C. antarctica* (CALB), immobilizowaną na hydrofobowym nośniku Lewatit VP OC 1600 (Novozym 435), powszechnie stosowaną w przemyśle jako biokatalizator, a w przypadku niniejszej pracy jako odnośnik referencyjny,
- lipazę z pleśni *A. oryzae* (Novozym 51032),
- lipazę z pleśni *T. lanuginosus* (Lipozyme TL 100L),
-lipazę z pleśni *R. miehei* (Palatase 20000L).

Jako nośniki do immobilizacji wykorzystano zarówno syntetyczny Lewatit VP OC 1600, jak i alternatywne, biodegradowalne matryce takie jak odpady pochodzenia spożywczego, obejmujące zużyte fusy kawowe (SCG), wytłoki z jabłek (AP), wytłoki z aronii (ChoP) oraz naturalny biopolimer - chitozan.

Uzyskane wyniki jednoznacznie wykazały, że aktywność katalityczna badanych preparatów zależała nie tylko od rodzaju enzymu, ale przede wszystkim od specyfiki zastosowanego nośnika. Lipaza Novozym 51032 immobilizowana na nośniku Lewatit VP OC 1600 charakteryzowała się najwyższą aktywnością hydrolityczną (0,0059 U/mg), przewyższającą nawet preparat referencyjny, przy jednocześnie wysokiej zdolności adsorpcji białka na powierzchni nośnika **[P2].** Z kolei enzymy immobilizowane na nośnikach lignocelulozowych (fusy po kawie) oraz chitozanie wykazały zdecydowanie wyższy potencjał w zakresie aktywności syntetycznej, co jest szczególnie istotne w procesach estryfikacji i transestryfikacji. Dla przykładu, lipaza Novozym 51032 immobilizowana na chitozanie oraz fusach kawowych osiągnęła aktywność syntetyczną odpowiednio 0,23 i 0,40 U/mg, podczas gdy analogiczna wartość dla tego samego enzymu immobilizowanego na nośniku Lewatit VP OC 1600 wynosiła jedynie 0,17 U/mg **[P2]**. Podobne zjawisko zaobserwowano w przypadku lipazy z *R. miehei*, której aktywność syntetyczna na natywnych wytłokach jabłkowych osiągnęła wartość 461 U/g, a na natywnych wytłokach aroniowych 370 U/g, co jednoznacznie wskazuje na ich przydatność jako taniego, biodegradowalnego nośnika **[P4]**. Co więcej, immobilizacja lipaz Lipozyme TL i Palatase 20000L na fusach kawowych prowadziła do uzyskania biokatalizatorów o wyższej aktywności hydrolitycznej niż w przypadku zastosowania nośnika Lewatit VP OC 1600 **[P3]**.

Analiza porównawcza pozwoliła stwierdzić, że na efektywność immobilizacji decydujący wpływ miała jakość interakcji między enzymem, a nośnikiem, a nie sama ilość zaadsorbowanego białka. Lewatit VP OC 1600, mimo zdolności do wiązania dużych ilości enzymu, prawdopodobnie sprzyjał powstawaniu agregatów ograniczających dostęp

do centrum aktywnego. Porowate materiały lignocelulozowe, takie jak fusy kawowe czy wytłoki owocowe, mogły sprzyjać zachowaniu otwartej konformacji lipazy, co mogło skutkować wzrostem aktywności syntetycznej. Wpływ miała również obróbka wstępna nośników – oczyszczanie poprawiało stabilność hydrolityczną biokatalizatora, lecz jednocześnie prowadziło do obniżenia jego aktywności syntetycznej. Fakt ten wskazuje na korzystny udział naturalnych komponentów roślinnych w stabilizacji enzymów. Potwierdzono to m.in. w przypadku fusów kawowych, gdzie usunięcie hemicelulozy w procesie oczyszczania skutkowało istotnym spadkiem aktywności lipolitycznej. Dodatkowo zaobserwowano, że biokatalizatory immobilizowane na oczyszczonych wytłokach aroniowych wykazały większą aktywność podczas ponownego użycia, utrzymując 5–10% początkowej aktywności hydrolitycznej po pięciu cyklach, co stanowiło lepszy wynik niż dla układów opartych na fusach kawowych.

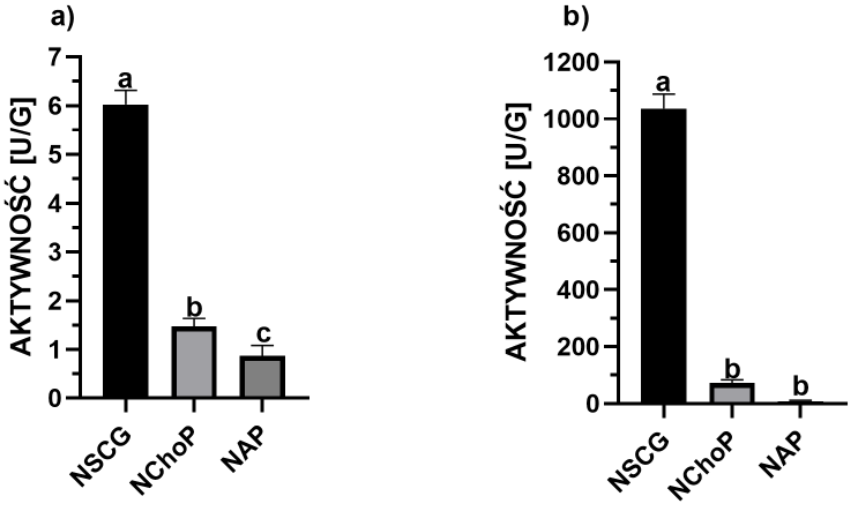
W toku badań przesiewowych skoncentrowano się na dwóch głównych kierunkach. W pierwszym [**P3**] prowadzono badania nad immobilizacją lipaz Novozym 51032, Lipozyme TL oraz Palatase na fusach kawowych w formie natywnej i po wstępnej ich obróbce, natomiast w drugim [**P4**] skoncentrowano się na immobilizacji lipazy Palatase na wytłokach z aronii i jabłek.

Na podstawie wyników aktywności i stabilności biokatalizatorów ostatecznie wytypowano układy z udziałem lipazy Novozym 51032 immobilizowanej na natywnych fusach kawowych, a także na wytłokach aroniowych i jabłkowych. Wybór ten uzasadniono wysoką aktywnością katalityczną, stabilnością oraz potencjałem wykorzystania tanich, powszechnie dostępnych materiałów odpadowych zgodnych z zasadami gospodarki o obiegu zamkniętym, co w dalszej perspektywie otwiera drogę do ich praktycznego zastosowania w syntezie związków o wysokiej wartości dodanej.

Ostatnim krokiem badań na tym etapie było opracowanie trzech rodzajów biokatalizatorów, uzyskanych poprzez adsorpcję lipazy mikrobiologicznej pochodzącej z pleśni *A. oryzae* na fusach po kawie, wytłokach z aronii i jabłek **[P5]**. Do weryfikacji efektywności procesu immobilizacji przeprowadzono analizę elementarną zarówno otrzymanych biokatalizatorów, jak i surowców zastosowanych jako nośniki. Ocenie poddano zawartość węgla, azotu oraz siarki w badanych próbkach. W przypadku fusów kawowych (SCG) stwierdzono stosunkowo wysoką zawartość węgla (48,41%), niską zawartość azotu (2,23%) oraz jedynie 0,12% siarki. Zbliżony układ wartości odnotowano

dla wytłoków z aronii (ChoP), które zawierały 50,31% węgla, 1,87% azotu oraz 0,09% siarki. Odmienny profil charakteryzował natomiast wytłoki jabłkowe (AP), dla których zawartość pierwiastków była niższa i wynosiła odpowiednio: 44,62% węgla, 0,99% azotu i 0,07% siarki. Otrzymane dane pozostają w zgodzie z wartościami raportowanymi w literaturze dla fusów kawowych (Girelli i in., 2023), wytłoków jabłkowych (Guerrero i in., 2014) oraz wytłoków jagodowych (Osman i in., 2020). Istotnym spostrzeżeniem jest zaobserwowany we wszystkich przypadkach wzrost udziału procentowego azotu w immobilizowanych preparatach enzymatycznych w stosunku do zawartości tego pierwiastka w materiale odpadowym, co wskazuje na skuteczną adsorpcję białek na powierzchni nośnika.

Spośród analizowanych układów, najwyższą aktywność katalityczną wykazywał biokatalizator immobilizowany na fusach kawowych, osiągając aktywność hydrolityczną na poziomie 6,0 U/g oraz aktywność syntetyczną wynoszącą 1036,0 U/g, co istotnie przewyższało wartości uzyskane dla pozostałych nośników (Rys. 3). Wysoka aktywność katalityczna otrzymanych immobilizowanych preparatów enzymatycznych może wynikać z szeregu czynników determinujących efektywność procesu.



Rys. 3. Aktywność hydrolityczna (a) i syntetyczna (b) lipazy z *A. oryzae* immobilizowanej na fusach z kawy (NSCG), wytłokach z aronii (NChoP) i wytłokach z jabłek (NAP). Średnie oznaczone tą samą literą (a-c) nie różniły się istotnie ($\alpha = 0,05$).

Adsorpcja, będąca jedną z najprostszych i najczęściej stosowanych metod immobilizacji enzymów, opiera się na oddziaływaniach fizycznych o niewielkiej sile, takich jak siły van

der Waalsa, interakcje hydrofobowe czy wiązania wodorowe. Z tego względu kluczowe znaczenie dla efektywności immobilizacji oraz stabilności enzymu ma struktura chemiczna i funkcjonalność powierzchni nośnika. Obecność określonych grup funkcyjnych, takich jak hydroksylowe, karboksylowe czy fenylowe, na powierzchni nośnika sprzyja powstawaniu oddziaływań niekowalencyjnych z enzymem, co umożliwia tworzenie stabilnych układów biokatalitycznych (Mokhtar i in., 2020; Alcañiz-Monge i in., 2022; Girelli i Chiappini, 2023).

W kontekście badanych materiałów odpadowych pochodzenia rolno-spożywczego wcześniejsze doniesienia wskazują, iż istotnym czynnikiem mogącym wpływać na zdolność adsorpcyjną nośnika jest skład włókna surowego, w szczególności zawartość hemicelulozy. Natywne fusy kawowe (SCG), w porównaniu z wytłokami jabłkowymi i aroniowymi, charakteryzują się wyraźnie wyższą zawartością hemicelulozy w suchej masie (Tabela 2). Podwyższona zawartość tego polisacharydu może zwiększać gęstość dostępnych miejsc wiązania na powierzchni, co z kolei sprzyja interakcjom enzymu z nośnikiem oraz poprawia stabilność immobilizowanego układu.

Tabela 2. Zawartość celulozy, hemicelulozy i ligniny w odpadach: wytłokach jabłkowych i aroniowych oraz fusach po kawie. Skróty: ADF – włókno detergentowe kwasowe, ADL – lignina detergentowa kwasowa, NDF – włókno detergentowe neutralne, DM – sucha masa.

ODPAD SPOŻYWCZY	JEDNOSTKA	CELULOZA (ADF-ADL)	HEMICELULOZA (NDF – ADF)	LIGNINA (ADL)	
Natywne fusy kawowe	%DM	21,06 ± 0,41	23,69 ± 0,97	16,87 ± 0,48	**[P3]**
Natywne wytłoki aroniowe		18,87 ± 0,32	3,53 ± 1,30	32,76 ± 0,08	**[P4]**
Natywne wytłoki jabłkowe		20,99 ± 0,07	5,87 ± 0,97	9,46 ± 0,20	**[P4]**

Kolejnym istotnym aspektem jest struktura surowca, stosowanego jako nośnik. Natywne fusy kawowe charakteryzowały się strukturą bardziej porowatą w porównaniu z pozostałymi badanymi nośnikami, co przekłada się na zwiększoną powierzchnię właściwą. Zwiększona dostępność powierzchni mogła sprzyjać skuteczniejszej adsorpcji cząsteczek lipazy na powierzchni nośnika. Dodatkowo, obecność lipidów w materiałach surowych może wspomagać stabilizację lipaz poprzez oddziaływania hydrofobowe, odzwierciedlające ich naturalne środowisko katalizy. Analiza zawartości tłuszczu w

badanych materiałach odpadowych wykazała, że fusy kawowe cechowały się najwyższą jego zawartością (11% m/m) w porównaniu do wytłoków aroniowych (0,3% m/m) oraz jabłkowych (0,2% m/m).

Lipazy zbudowane są z łańcuchów polipeptydowych zawierających zarówno regiony hydrofilowe, jak i hydrofobowe, a ich centra aktywne są zwykle zasłonięte przez hydrofobowe „wieczko" w konformacji zamkniętej (Rodrigues i in., 2019). W kontakcie z hydrofobową powierzchnią nośników, „wieczko" może ulec konformacyjnej zmianie, odsłaniając centrum aktywne w procesie określanym mianem aktywacji międzyfazowej. Zmiana ta zwiększa wydajność katalityczną enzymu oraz ułatwia jego adsorpcję na powierzchni hydrofobowej, stabilizując otwartą, monomeryczną formę enzymu bez konieczności stosowania dodatkowych etapów aktywacji (Manoel i in., 2015).

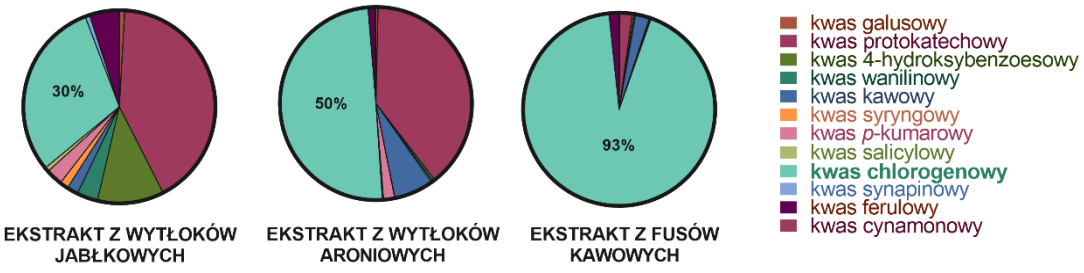
Stopień hydrofobowości oraz właściwości fizykochemiczne nośnika istotnie wpływają zarówno na efektywność wiązania lipazy, jak i na aktywność enzymatyczną enzymu (Silveira i in., 2017). Podczas gdy niektóre lipazy preferują nośniki o umiarkowanej hydrofobowości, w literaturze powszechnie wskazuje się, iż bardziej hydrofobowe powierzchnie umożliwiają uzyskanie wyższych wydajności immobilizacji w zależności od ich powinowactwa do poszczególnych nośników (Brabcová i in., 2013). W związku z tym wyższa zawartość lipidów w fusach kawowych może sprzyjać stabilizacji lipazy poprzez oddziaływania hydrofobowe, co pozostaje w zgodzie z opisanymi mechanizmami aktywacji międzyfazowej i immobilizacji enzymów.

Na podstawie uzyskanych wyników potwierdzono hipotezę **H1,** że odpady spożywcze takie jak wytłoki jabłkowe i aroniowe, a także fusy kawowe mogą stanowić nośnik do immobilizacji enzymów lipolitycznych, umożliwiając otrzymanie biokatalizatorów o wysokiej aktywności syntetycznej. Natomiast, do dalszych badań, jako biokatalizator, wybrano lipazę z *A. oryzae* immobilizowaną na natywnych fusach kawowych, która była najbardziej aktywna syntetycznie oraz stabilna w szerokim zakresie pH oraz temperatur.

4.12. Przygotowanie ekstraktów z odpadów spożywczych oraz ich analiza pod kątem składu kwasów fenolowych

Liofilizowane ekstrakty uzyskane z fusów kawowych, wytłoków aroniowych oraz wytłoków jabłkowych poddano analizie zawartości wybranych kwasów fenolowych **[P5]**. Ocenie poddano następujące związki: kwas galusowy, protokatechowy, 4-hydroksybenzoesowy, wanilinowy, kawowy, syryngowy, *p*-kumarynowy, salicylowy,

chlorogenowy, synapinowy, ferulowy oraz cynamonowy (Rys. 4). Spośród analizowanych próbek, ekstrakt pochodzący z fusów kawowych wykazał największą zawartość kwasu chlorogenowego, który stanowił około 93% całkowitej zawartości zidentyfikowanych kwasów fenolowych. Pozostałe 7% tworzyły przede wszystkim kwas protokatechowy, kawowy i ferulowy. W ekstrakcie z wytłoków aroniowych kwas chlorogenowy również był dominującym kwasem fenolowym, stanowiąc około 50% całkowitej zawartości oznaczonych kwasów fenolowych. Kolejnymi pod względem udziału były kwasy protokatechowy oraz kawowy. Najbardziej zróżnicowany profil kwasów fenolowych stwierdzono w ekstrakcie z wytłoków jabłkowych. W tym przypadku dominującym związkiem był kwas protokatechowy (42%), natomiast kwas chlorogenowy stanowił około 30% całkowitej zawartości oznaczanych związków, a trzecim kwas 4-hydroksybenzoesowy. Na podstawie uzyskanych wyników do dalszych badań wybrano kwas chlorogenowy jako substrat modelowy, ze względu na jego obecność we wszystkich trzech ekstraktach oraz wysoką zawartość w każdym z nich. Wybór tego kwasu w przypadków fusów kawowych umożliwiałby także otrzymanie jednorodnego produktu.



Rys. 4. Procentowy udział wybranych kwasów fenolowych w liofilizowanych ekstraktach z wytłoków jabłkowych, wytłoków z aronii i fusów z kawy (wyrażony jako % wszystkich zidentyfikowanych kwasów fenolowych).

4.13. Synteza estrów kwasu chlorogenowego.

Weryfikacja hipotezy **H2:** Lipazy immobilizowane na nośnikach pochodzących z odpadów spożywczych skutecznie katalizują estryfikację dominującego w uzyskanych ekstraktach kwasu fenolowego.

Kwas chlorogenowy jest szeroko rozpowszechnionym związkiem pochodzenia roślinnego, występującym w znacznych ilościach w ziarnach kawy, owocach pestkowych i jagodowych oraz warzywach kapustnych. Znany jest z szeregu korzystnych efektów

biologicznych, obejmujących silne właściwości antyoksydacyjne, ochronę bariery jelitowej i wątrobowej oraz potwierdzoną skuteczność w profilaktyce i terapii otyłości oraz cukrzycy typu II. Ze względu na polarny charakter kwas chlorogenowy cechuje się ograniczoną rozpuszczalnością w matrycach lipidowych, co stanowi czynnik ograniczający jego zastosowanie w przemyśle spożywczym, farmaceutycznym i kosmetycznym. Jego hydrofobowość można zwiększyć poprzez chemiczną bądź enzymatyczną lipofilizację, polegającą na estryfikacji grupy karboksylowej alkoholem tłuszczowym. Szczególne znaczenie przypisuje się podejściu enzymatycznemu, ponieważ zastosowanie biokatalizatorów umożliwia prowadzenie reakcji w łagodniejszych warunkach, zapewnia większą selektywność oraz ogranicza powstawanie produktów ubocznych. Co więcej, procesy enzymatyczne są przyjazne środowisku, wymagają mniejszych nakładów energetycznych i generują mniej odpadów (López-Giraldo i in., 2007; Wang i in., 2021; Gil i Wianowska, 2017).

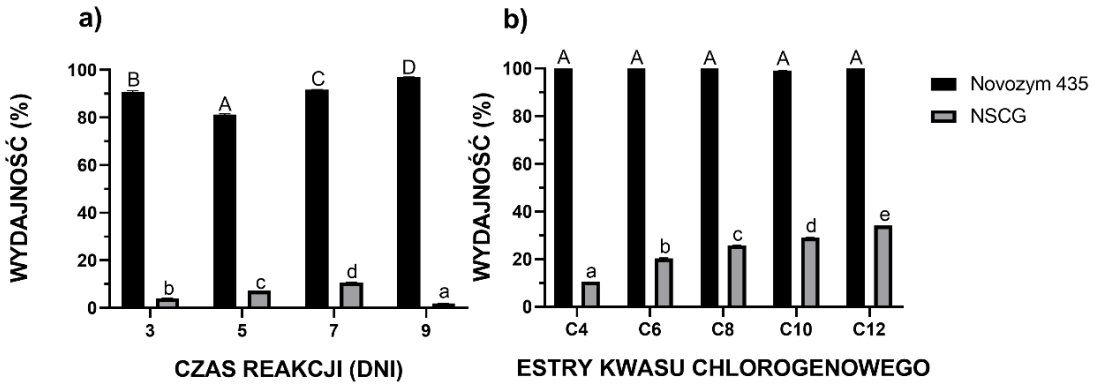
W niniejszej pracy wykorzystano metodę powierzchni odpowiedzi (RSM) w celu optymalizacji enzymatycznej syntezy estru butylowego kwasu chlorogenowego. Biokatalizatorem referencyjnym był Novozym 435, natomiast badanym układem – lipaza z *A. oryzae* immobilizowana na natywnych fusach kawowych (NSCG). Cały proces optymalizacji wybranej syntezy został dokładnie opisany w publikacji **[P5]**. Łącznie przeprowadzono 15 doświadczeń, w których analizowano wpływ temperatury (35–55 °C), molowego stosunku substratów (alkohol: kwas chlorogenowy – 2:1, 5:1, 8:1) oraz stężenia enzymu względem masy substratów (20–50%). Oceniany model odpowiedzi, uzyskany za pomocą planu Boxa-Behnkena wykazał wysoką zgodność z danymi eksperymentalnymi – współczynnik determinacji (R^2) wyniósł 0,999, a R^2 skorygowane 0,999 dla reakcji katalizowanej przez NSCG, natomiast dla Novozym 435 wartości te wynosiły odpowiednio 0,988 i 0,913.

Analiza wykresu Pareto dla syntezy estru butylowego kwasu chlorogenowego z udziałem biokatalizatora NSCG wykazała, że najistotniejszym czynnikiem wpływającym na wydajność reakcji był liniowy efekt molowego stosunku substratów (butanol: kwas chlorogenowy) oraz stężenie enzymu względem całkowitej ilości substratów. Trzecim pod względem znaczenia czynnikiem był efekt kwadratowy temperatury. Odpowiednie trójwymiarowe wykresy odpowiedzi potwierdziły, że zwiększanie stosunku alkoholu do kwasu prowadziło do wyższych wydajności reakcji. Ponadto, zarówno zwiększone

stężenie enzymu, jak i podwyższona temperatura wywierały pozytywny wpływ na efektywność estryfikacji.

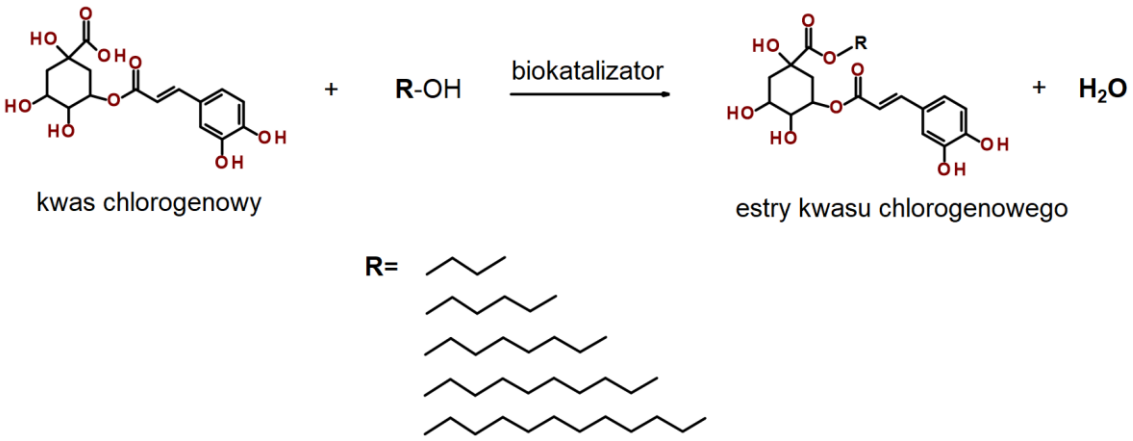
W przypadku reakcji katalizowanej enzymem referencyjnym Novozym 435 analiza wykresu Pareto wykazała, iż jedynie liniowy efekt stosunku substratów istotnie wpływał na wydajność procesu – wyższy udział alkoholu korelował z lepszą wydajnością reakcji. Pozostałe czynniki, takie jak stężenie enzymu i temperatura, nie wykazały istotności statystycznej. Porównanie obu układów wskazuje na istotną różnicę pomiędzy nimi. Immobilizowana lipaza z *A. oryzae* na nośniku odpadowym (NSCG) charakteryzowała się większą wrażliwością na badane warunki reakcji, podczas gdy enzym komercyjny immobilizowany na syntetycznym nośniku (Novozym 435) wykazywał większą stabilność. Na podstawie trójczynnikowej analizy Boxa–Behnkena wyznaczono optymalne warunki reakcji, które okazały się zgodne dla obu układów. Maksymalne wydajności osiągnięto w temperaturze 55 °C i przy najwyższym badanym molowym stosunku substratów (8:1, butanol: kwas chlorogenowy). Z ekonomicznego punktu widzenia do dalszych doświadczeń wybrano stężenie enzymu 35% (m/m względem masy substratów), co pozwoliło zoptymalizować proces poprzez redukcję ilości użytego biokatalizatora bez obniżania wydajności reakcji.

W kolejnym kroku badano wpływ czasu reakcji w ustalonych warunkach optymalnych (Rys. 5a). Dla biokatalizatora NSCG maksymalną wydajność (10,61%) uzyskano po 7 dniach, podczas gdy Novozym 435 osiągnął najwyższą wydajność (97%) po 9 dniach. Co istotne, komercyjny biokatalizator już na wczesnym etapie procesu wykazywał wysoką aktywność katalityczną, osiągając wydajność 90,86% po zaledwie 3 dniach, co podkreśla jego efektywność w katalizie tej reakcji estryfikacji.



Rys. 5. Wydajność reakcji otrzymania estru butylowego kwasu chlorogenowego w zależności od czasu reakcji (a) oraz wydajność reakcji otrzymania estrów kwasu chlorogenowego z alkoholami o różnej długości łańcucha węglowego (b). Wartości oznaczone tą samą małą literą (a-e) lub wielką literą (A-D) nie różniły się istotnie (α = 0,05).

Zoptymalizowane warunki reakcji zastosowano do lipofilizacji kwasu chlorogenowego z użyciem szeregu alkoholi tłuszczowych o zróżnicowanej długości łańcucha węglowego (C6, C8, C10, C12). Wyniki zaprezentowano na Rysunku 5b, a reakcje prowadzono zgodnie ze schematem przedstawionym na Rysunku 6. W przypadku biokatalizatora NSCG zaobserwowano, iż wydajność reakcji rosła proporcjonalnie do długości łańcucha alkoholu. Najwyższą wydajność (34,06%) uzyskano w reakcji z dodekanolem (C12), natomiast najniższą (10,62%) w reakcji z butanolem (C4). Z kolei komercyjny biokatalizator Novozym 435 zapewniał wysokie wydajności we wszystkich analizowanych przypadkach, co wskazuje, iż wcześniej zoptymalizowane warunki były szeroko skuteczne w syntezie różnych estrów kwasu chlorogenowego z jego udziałem.



Rys. 6. Estryfikacja kwasu chlorogenowego za pomocą lipazy z alkoholem butylowym (C4), heksylowym (C6), oktylowym (C8), decylowym (C10) i dodecylowym (C12).

Choć biokatalizator immobilizowany na odpadach kawowych (NSCG) charakteryzował się relatywnie niskimi wydajnościami całkowitymi, niniejsze badanie stanowi pierwsze doniesienie dotyczące jego zastosowania w enzymatycznej lipofilizacji kwasu chlorogenowego. Uzyskane wyniki potwierdzają, że biodegradowalne nośniki pochodzące z odpadów przemysłu spożywczego mogą stanowić obiecującą, przyjazną środowisku alternatywę wobec konwencjonalnych materiałów stosowanych w procesach immobilizacji. Co więcej, optymalizacja parametrów reakcji metodą Boxa–Behnkena (temperatura, molowy stosunek substratów, stężenie enzymu) wykazała, iż wydajność estryfikacji kwasu chlorogenowego katalizowanej przez biokatalizator NSCG jest istotnie zależna od tych czynników. Zjawisko to pozostaje zgodne z ogólnymi trendami obserwowanymi w reakcjach estryfikacji enzymatycznej, w których podwyższanie temperatury i zwiększanie ilości enzymu prowadzi zwykle do poprawy efektywności procesu do momentu, w którym mogą wystąpić zjawiska dezaktywacji termicznej lub nasycenia enzymu substratem. Z kolei biokatalizator komercyjny Novozym 435 cechował się wysokimi wydajnościami, które pozostawały w dużej mierze niezależne od zmienności badanych parametrów reakcji, co podkreśla jego stabilność operacyjną i tolerancję substratową. NSCG okazał się bardziej wrażliwym biokatalizatorem na zmienne warunki reakcji, co wymaga w przyszłości starannego doboru parametrów w planowaniu doświadczeń oraz podczas jego praktycznego stosowania.

Enzymatyczna modyfikacja kwasu chlorogenowego z wykorzystaniem lipaz była szeroko badana w poprzednich pracach, jednak dotychczas koncentrowano się przede wszystkim na biokatalizatorach komercyjnych. López-Giraldo i in. (2007) opisali

dwustopniową syntezę estrów kwasu chlorogenowego, obejmującą wstępną chemiczną estryfikację prowadzącą do powstania metylowego estru, a następnie enzymatyczną transestryfikację z udziałem alkoholi tłuszczowych (C4–C16) katalizowaną lipazą B z *Candida antarctica* (CALB). Reakcje prowadzone w temperaturze 55°C przez 96 godzin, przy stężeniu enzymu 2,5–10% (m/m), pozwoliły uzyskać wydajność w zakresie 61–93%, zależnie od długości łańcucha alkoholu. Podobne podejście zastosowali Guyot i in. (2000), wykorzystując ten sam enzym w reakcji bezpośredniej estryfikacji z 1-oktanolem, 1-dodekanolem i 1-heksadekanolem przy niższych stężeniach enzymu (1,2–1,5% m/m) i dłuższym czasie prowadzenia reakcji (30 dni), osiągając wydajności 40–75%.

W badaniach porównawczych Lorentz i in. (2010) przeprowadzili syntezę *O*-palmitoilowych pochodnych kwasu chlorogenowego z wykorzystaniem panelu komercyjnych lipaz, w tym Novozym 435, Lipozyme RM-IM, TL-IM, lipaza A (*A. niger*), lipaza M (*M. javanicus*), lipaza DF (*R. oryzae*), lipaza AY (*C. rugosa*), lipaza G (*P. camembertii*) oraz lipaza PS (*P. cepacia*). W warunkach standardowych (60 °C, 1000 rpm) wydajności mieściły się w przedziale 14–60% po 7 dniach, przy czym decydującym czynnikiem okazał się stosunek molowy substratów (kwas palmitynowy: kwas chlorogenowy w zakresie 10:1–80:1). Z kolei Wang i in. (2021) zastosowali Lipozyme RM do acylacji kwasu chlorogenowego winylowymi estrami (C2–C12), prowadząc reakcje w temperaturze 55°C i przy szybkości mieszania 400 rpm, uzyskując pięć odmiennych pochodnych 4-*O*-acylowanych w ciągu 7 dni. Zhu i in. (2020) skupili się na optymalizacji szeregu parametrów reakcji, obejmujących rodzaj rozpuszczalnika, stężenie i formę enzymu, stosunek substratów oraz czas reakcji, i zidentyfikowali warunki optymalne (55 °C, stosunek molowy substratów 1:10, 400 rpm, 7 dni), umożliwiające wydajną syntezę pochodnych kwasu chlorogenowego. W tym przypadku, w przeciwieństwie do badań w ramach tej pracy doktorskiej, acylowana była grupa OH w kwasie chinowym, będącym częścią kwasu chlorogenowego.

Pomimo szerokiego zastosowania komercyjnych immobilizowanych lipaz w badaniach opisanych w literaturze, nadal istnieje istotna luka badawcza dotycząca wykorzystania biokatalizatorów o charakterze zrównoważonym środowiskowo. Do tej pory nie raportowano zastosowania enzymów immobilizowanych na biodegradowalnych nośnikach pochodzących z odpadów, takich jak fusy kawowe, w procesach lipofilizacji kwasu chlorogenowego. Prezentowana praca wypełnia tę lukę, dostarczając danych porównawczych dotyczących wydajności katalitycznej nowego biokatalizatora NSCG

względem układu komercyjnego, potwierdza hipotezę **H2,** że lipazy immobilizowane na nośnikach pochodzących z odpadów spożywczych mogą skutecznie katalizować estryfikację dominującego w uzyskanych ekstraktach kwasu fenolowego (w tym przypadku kwasu chlorogenowego) oraz wnosi nowe spojrzenie na rozwój bardziej przyjaznych środowisku i ekonomicznie korzystnych alternatyw dla modyfikacji związków fenolowych.

4.14. Analiza właściwości otrzymanych estrów kwasu chlorogenowego.

Weryfikacja hipotezy **H3:** Estry dominującego kwasu fenolowego otrzymane w lipofilizacji zachowują lub przewyższają właściwości przeciwutleniającei/lub przeciwdrobnoustrojowe w porównaniu do związku wyjściowego, a ich dodatek do oleju roślinnego istotnie zwiększa jego stabilność oksydacyjną.

Otrzymane estry kwasu chlorogenowego (C4–C12), poddano ocenie pod kątem właściwości funkcjonalnych, obejmujących aktywność przeciwutleniającą, działanie przeciwdrobnoustrojowe oraz zdolność stabilizacji oksydacyjnej oleju roślinnego. Analizie poddano także kwas chlorogenowy oraz syntetyczny antyoksydant BHT jako kontrola odniesienia. Szczegółowe wyniki umieszczono w publikacji **[P5]**.

4.14.1. Aktywność przeciwutleniająca estrów kwasu chlorogenowego

Spośród wszystkich otrzymanych pochodnych, najwyższą aktywność przeciwutleniającą wykazał ester butylowy kwasu chlorogenowego (IC_{50} = 0,34 mM; TEAC = 3,26). Jego skuteczność była porównywalna zarówno z kwasem chlorogenowym (IC_{50} = 0,26 mM; TEAC = 3,49), jak i BHT (IC_{50} = 0,35 mM; TEAC = 3,14), przy jednoczesnym wzroście lipofilowości, co potwierdza wartość logP. Zaobserwowano ponadto, iż wraz ze wzrostem długości łańcucha węglowego alkoholu użytego w reakcji estryfikacji, aktywność antyoksydacyjna estrów zmniejszała się, niezależnie od zastosowanej metody analitycznej (CUPRAC, DPPH), pomimo zwiększonej lipofilowości cząsteczki.

Zjawisko to odpowiada dobrze znanemu w literaturze „efektowi odcięcia" (ang. *cut-off effect*), polegającemu na nieliniowej zależności pomiędzy aktywnością przeciwutleniającą a hydrofobowością związku. Wzrost lipofilowości nie zawsze prowadzi do proporcjonalnej poprawy zdolności antyoksydacyjnych związku, po osiągnięciu optymalnej długości łańcucha następuje wyraźny spadek aktywności przeciwutleniającej (Shahidi i Zhong, 2011; Laguerre i in., 2010, 2011, 2013). W badaniach własnych maksymalną aktywność odnotowano dla estru butylowego,

natomiast dane literaturowe wskazują zarówno na optimum przy krótszych łańcuchach (C4, C8) (López-Giraldo i in., 2009), jak i przy dłuższych (C12) np. w modelach komórkowych z nadprodukcją ROS oraz w emulsjach olej-woda (Laguerre i in., 2009, 2011, 2013). Rozbieżności te mogą wynikać z odmiennych systemów badawczych (roztwory homogeniczne, emulsje, modele komórkowe), metod oceny antyoksydacyjnej lub warunków eksperymentalnych.

W literaturze wskazuje się kilka mechanizmów mogących tłumaczyć efekt odcięcia. Najczęściej przywoływana jest hipoteza podziału fazowego (ang. *partitioning hypothesis*), zgodnie z którą estry o optymalnej długości łańcucha węglowego (C4, C8, C12) lokują się najefektywniej na granicy faz olej–woda bądź w obrębie błon komórkowych – tam, gdzie inicjowane jest peroksydacja lipidów i reakcje rodnikowe (Laguerre i in., 2011; Pappalardo i in., 2023). Przy dłuższych łańcuchach węglowych dochodzi do nadmiernego zanurzenia cząsteczki w fazie lipidowej, co ogranicza dostęp grup hydroksylowych do reaktywnych form tlenu (Sørensen i in., 2012). Kolejnym czynnikiem są efekty steryczne – masywne łańcuchy alkilowe mogą utrudniać dostęp wolnych rodników do miejsc reaktywnych (López-Giraldo i in., 2009). Zjawisko to może także wpływać na ograniczenie zdolności do tworzenia dimerów, co dodatkowo zmniejsza aktywność antyoksydacyjną związku. Trzecim potencjalnym mechanizmem jest zdolność do samorzutnej organizacji cząsteczek w środowisku wodnym. Dodecylowy ester kwasu chlorogenowego wykazywał zdolność do tworzenia struktur supramolekularnych w roztworach wodnych, co sprzyjało jego interakcji z błonami komórkowymi i wyższemu potencjałowi antyoksydacyjnemu (Laguerre i in., 2011).

4.14.2. Właściwości przeciwdrobnoustrojowe estrów kwasu chlorogenowego

Otrzymane pochodne kwasu chlorogenowego oceniono również pod kątem aktywności przeciwdrobnoustrojowej wobec ośmiu szczepów bakteryjnych, reprezentujących bakterie Gram-dodatnie i Gram-ujemne. Badania wykazały, że ester butylowy kwasu chlorogenowego, mimo wysokiej aktywności przeciwutleniającej, nie wykazywał żadnych właściwości przeciwdrobnoustrojowych, podobnie jak kwas chlorogenowy oraz BHT. Natomiast estry o średniej długości łańcucha (heksylowy i oktylowy) wykazały wyraźne działanie hamujące wzrost bakterii Gram-dodatnich (m.in. *B. cereus*, *B. subtilis*, *E. faecalis*, *S. aureus*). Estry o dłuższych łańcuchach węglowych (C10, C12) działały słabiej i wykazywały ograniczoną aktywność selektywną. Żaden z badanych związków nie wykazywał aktywności wobec bakterii Gram-ujemnych.

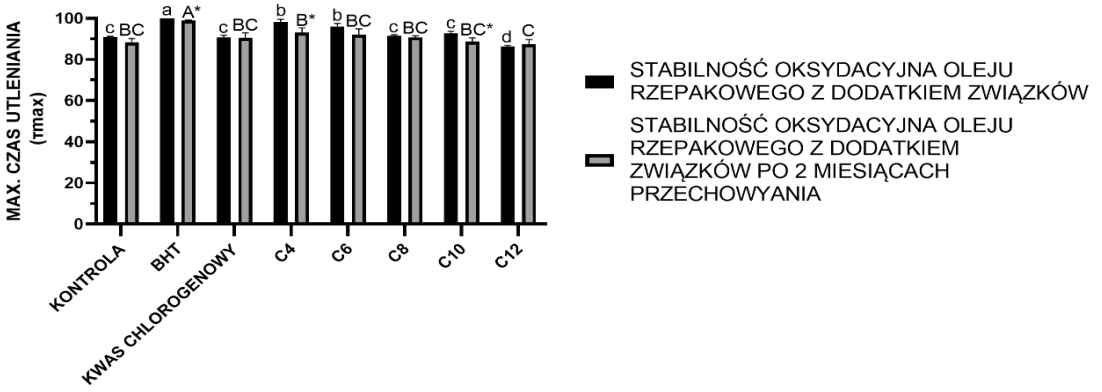
Otrzymane wyniki częściowo pokrywają się z doniesieniami literaturowymi. Suárez-Quiroz i in. (2013) stwierdzili, że dodecylowy ester kwasu chlorogenowgo, pozyskany z zielonej kawy, wykazuje aktywność wobec wybranych bakterii Gram-dodatnich (*B. cereus, Clostridium sporogenes, Listeria innocua*), lecz nie działa na Gram-ujemne szczepy (*E. coli, Pseudomonas fluorescens, Salmonella enterica*). Co interesujące, kwas chlorogenowy wykazywał szersze spektrum działania, obejmujące również bakterie Gram-ujemne, choć komórki *S. enterica* pozostawały oporne na jego działanie. Z kolei Ma i in. (2007) wykazali, że modyfikacje kwasu chlorogenowego poprzez wprowadzenie lipofilowych łańcuchów zawierających reszty aminokwasowe mogą znacząco zwiększać aktywność przeciwdrobnoustrojową zwłaszcza wobec opornych szczepów drożdży *Candida krusei,* przy jednoczesnym ograniczeniu toksyczności wobec organizmów morskich.

Podsumowując, aktywność przeciwdrobnoustrojowa estrów kwasu chlorogenowego ma wyraźnie charakter zależny od długości i charakteru łańcucha alkilowego. Największy potencjał wykazują pochodne o średniej długości (C6, C8), aktywne wobec bakterii Gram-dodatnich. Wyniki te sugerują możliwość zastosowania estrów kwasu chlorogenowego jako naturalnych środków przeciwdrobnoustrojowych, jednak ich selektywność względem bakterii Gram-ujemnych wskazuje na potrzebę dalszej modyfikacji strukturalnej w celu poszerzenia spektrum działania.

4.14.3. Stabilność oksydacyjna oleju rzepakowego z dodatkiem estrów kwasu chlorogenowego

Wyniki stabilności oksydacyjnej oleju rzepakowego z dodatkiem kwasu chlorogenowego, jego estrów oraz BHT, w momencie dodania oraz po dwóch miesiącach przechowywania przedstawiono na Rysunku 7. Najwyższą stabilność oksydacyjną (τ_{max} = 101,8 min) wykazywał olej z dodatkiem BHT, co było zgodne z oczekiwaniami wobec skuteczności tego syntetycznego przeciwutleniacza. Porównywalne wartości uzyskano dla estrów z krótkim łańcuchem węglowym – butylowego (C4, τ_{max} = 98,3 min) oraz heksylowego (C6, τ_{max} = 95,9 min). Natomiast olej zawierający natywny kwas chlorogenowy charakteryzował się niższą odpornością na utlenianie (τ_{max} = 90,7 min), która dodatkowo spadła po przechowywaniu. Wyniki te korelują z aktywnością przeciwutleniającą badanych związków, gdzie estry butylowy i heksylowy wykazywały najwyższą zdolność zmiatania wolnych rodników. Zwiększona lipofilowość tych pochodnych w stosunku do natywnego kwasu chlorogenowego mogła sprzyjać lepszemu

rozpuszczaniu się w matrycy lipidowej, co przekładało się na wyższą skuteczność antyoksydacyjną. Po dwóch miesiącach przechowywania odnotowano niewielki, lecz statystycznie istotne zmniejszenie stabilności oleju w przypadku dodatku niektórych estrów, w tym butylowego (C4, τ_{max} = 93,2 min) oraz decylowego (C10, τ_{max} = 88,7 min). Obniżenie stabilności oleju wskazuje na ograniczoną trwałość ochronnego działania estrów w dłuższym okresie, pomimo ich wyjściowej wysokiej aktywności.



Rys. 7 Stabilność oksydacyjna oleju rzepakowego z dodatkiem kwasu chlorogenowego, jego estrów z łańcuchami alkilowymi o różnej długości (C4-C12) oraz BHT (użytego jako antyoksydant referencyjny, mierzona w momencie dodania oraz po dwóch miesiącach przechowywania. Wartości oznaczone tą samą małą literą (a–c) lub wielką literą (A–C) nie różnią się znacząco (α = 0,05). Symbol * oznacza istotną statystycznie różnicę między początkowym stanem stabilności oksydacyjnej oleju z dodatkami, a stanem po 2 miesiącach przechowywania.

Wyniki badań potwierdzają hipotezę **H3**, że lipofilizacja jest skuteczną strategią, dzięki której wybrane pochodne kwasu chlorogenowego zachowują lub przewyższają pożądane właściwości w stosunku do wyjściowej niemodyfikowanej cząsteczki kwasu, zwłaszcza w kontekście ich zastosowania w matrycach lipidowych i zwiększania stabilności oksydacyjnej olejów roślinnych.

4.15. Lipofilizacja ekstraktów z odpadów spożywczych za pomocą wybranego biokatalizatora oraz analiza ich właściwości przeciwutleniających i przeciwdrobnoustrojowych oraz wpływu na stabilność oksydacyjną oleju roślinnego.

Weryfikacja hipotezy **H4:** Lipofilizacja ekstraktów roślinnych za pomocą opracowanego biokatalizatora skutkuje otrzymaniem produktu o zwiększonej lipofilowości, przy

zachowaniu lub poprawie właściwości przeciwutleniających i/lub przeciwdrobnoustrojowych w porównaniu do ekstraktu niezmodyfikowanego.

Na podstawie uzyskanych wyników optymalizacji syntezy estru butylowego kwasu chlorogenowego zaplanowano reakcje lipofilizacji z wykorzystaniem liofilizowanych ekstraktów pochodzących z odpadów przemysłu spożywczego. Ze względu na to, że kwas chlorogenowy został zidentyfikowany we wszystkich trzech ekstraktach, reakcje prowadzono przy parametrach uznanych za optymalne w układzie modelowym. Do estryfikacji zastosowano 1-butanol w stosunku masowym 1:8 (alkohol: ekstrakt). W badaniach wykorzystano zarówno biokatalizator biodegradowalny NSCG, jak i komercyjny Novozym 435 jako punkt odniesienia. Wyniki uzyskane w procesie lipofilizacji ekstraktów zostały umieszczone w publikacji **[P5]**.

Obecność głównego produktu reakcji: estru butylowego kwasu chlorogenowego potwierdzono w analizach LC-MS przeprowadzonych dla wszystkich badanych próbek po 7 dniach inkubacji. Najwyższą wydajność reakcji (Tabela 3) odnotowano dla ekstraktów z wytłoków jabłkowych i aronii, która osiągnęła ok. 90%. Co istotne, biokatalizator oparty na fusach kawowych (NSCG) katalizował reakcję w sposób porównywalny do enzymu referencyjnego. Niższe wyniki uzyskano natomiast dla ekstraktu z fusów kawowych, gdzie wydajność reakcji upochodnienia kwasu chlorogenowego wyniosła 47,7% w przypadku Novozymu 435 oraz 15,2% dla NSCG. Można przypuszczać, iż z uwagi na dominację kwasu chlorogenowego w ekstraktach z fusów kawowych, konieczny jest dłuższy czas reakcji, aby umożliwić całkowite przereagowanie substratu. W przypadku ekstraktu z wytłoków jabłkowych i aroniowych udział kwasu chlorogenowego był znacznie niższy, co mogło ułatwiać efektywniejszą estryfikację.

Tabela 3. Wydajność reakcji otrzymywania estru butylowego kwasu chlorogenowego, z mieszaniny reakcyjnej po lipofilizacji ekstraktów z odpadów spożywczych, katalizowanej przez Novozym 435 i NSCG.

WYDAJNOŚĆ REAKCJI OTRZYMYWANIA ESTRU BUTYLOWEGO KWASU CHLOROGENOWEGO W REAKCJI LIPOFILIZACJI EKSTRAKTÓW Z ODPADÓW SPOŻYWCZYCH [%]					
Ekstrakt z wytłoków aroniowych		Ekstrakt z wytłoków jabłkowych		Ekstrakt z fusów kawowych	
NOVOZYM 435	NSCG	NOVOZYM 435	NSCG	NOVOZYM 435	NSCG
90.9%	92.0%	91.5%	87.6%	47.7%	15.2%

Niniejsze badania stanowią pierwsze doniesienie dotyczące zastosowania lipazy mikrobiologicznej immobilizowanej na fusach kawowych do upochodnienia kwasu chlorogenowego zawartego w ekstraktach odpadowych. Do tej pory biokatalizator tego typu stosowany był wyłącznie w procesach hydrolizy tłuszczów mlecznych. Girelli i in. (2023) wykorzystali lipazę z drożdży *C. rugosa* immobilizowaną na fusach kawowych do hydrolizy tłuszczu mlecznego, uzyskując niemal 60% konwersję po 18 godzinach reakcji i utrzymując tę wartość w trakcie trzech kolejnych cykli użycia. Z kolei K. de S. Lira i in. (2021) podjęli próbę immobilizacji lipazy z *T. lanuginosus* (TLL) na fusach kawowych, jednak biokatalizator ten nie wykazywał żadnej aktywności katalitycznej.

Uzyskane wyniki wskazują, że lipazy mogą być wysoce efektywne w transformacjach wybranych związków w ekstraktach odpadowych, choć skuteczność katalizy zależy od składu chemicznego poszczególnych matryc. W zależności od rodzaju ekstraktu, lepsze rezultaty osiągano z użyciem biokatalizatora NSCG albo Novozymu 435. Podkreśla to konieczność starannego doboru immobilizowanego enzymu do określonego procesu. Ostateczna ilość otrzymanego produktu była ściśle uzależniona zarówno od początkowego stężenia kwasu chlorogenowego, jak i obecności innych związków fenolowych, które mogą blokować centrum aktywne enzymu. Dodatkowo na przebieg reakcji mogą wpływać inne składniki ekstraktów, takie jak antocyjany, taniny, kofeina, cukry czy pektyny, które wykazują zdolność wiązania lub hamowania enzymów (Antonic i in., 2020; Saracila i in., 2024; Ballesteros i in., 2014; Girelli i in., 2020).

4.15.1. Aktywność przeciwutleniająca i przeciwdrobnoustrojowa lipofilizowanych ekstraktów z odpadów spożywczych

Głównym celem tego etapu było zweryfikowanie, czy modyfikacja poprzez lipofilizację z użyciem 1-butanolu może poprawić właściwości ekstraktów, szczególnie w kontekście opracowania dodatków spożywczych o zwiększonej lipofilowości.

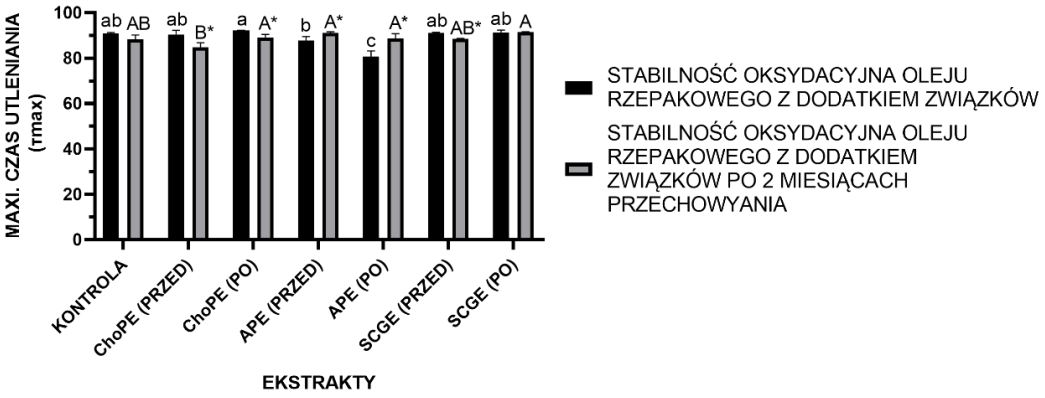
Najwyższą całkowitą zawartość związków fenolowych odnotowano w ekstrakcie z fusów kawowych, co potwierdziła analiza LC-MS. Na drugim miejscu znalazł się ekstrakt z wytłoków z aronii, natomiast ekstrakt z wytłoków jabłkowych wykazywał najniższe całkowite stężenie związków fenolowych. Podobną tendencję zaobserwowano w przypadku aktywności przeciwutleniającej, ekstrakty bogatsze w związki fenolowe charakteryzowały się większym potencjałem antyoksydacyjnym.

Istotnym spostrzeżeniem jest wpływ lipofilizacji na aktywność przeciwutleniającą. W przypadku ekstraktów z wytłoków z aronii oraz fusów kawowych zaobserwowano istotne obniżenie aktywności antyoksydacyjnej po lipofilizacji, co sugeruje, że proces ten może negatywnie wpływać na ogólną skuteczność antyoksydacyjną. Obniżenie to można tłumaczyć modyfikacją lub degradacją związków bioaktywnych w trakcie reakcji lipofilizacji. Odmienna sytuacja wystąpiła w przypadku ekstraktu z wytłoków jabłkowych, gdzie zmiany w aktywności antyoksydacyjnej były minimalne. Choć wytłoki jabłkowe uznawane są za stosunkowo bogate źródło związków polifenolowych, ponad 50% z nich stanowią procyjanidyny zaliczane do flawan-3-oli. Pozostałe związki to glikozydy kwercetyny, kwasy fenolowe oraz chalkony, takie jak florydzyna i floretyna (Bhushan i in. 2008; Tarko i in. 2012). W niniejszym badaniu analiza LC-MS wykazała, że liofilizowany ekstrakt z wytłoków jabłkowych zawierał najniższą całkowitą zawartość kwasów fenolowych (0,422 mg/g) w porównaniu do wytłoków aronii (10,442 mg/g) oraz fusów kawowych (15,723 mg/g). W związku z tym, efekty lipofilizacji mogły być bardziej wyraźne w przypadku dwóch ostatnich surowców, ze względu na wyższą zawartość kwasów fenolowych, a tym samym podatność na modyfikacje chemiczne.

Lipofilizacja całych ekstraktów niesie ze sobą ryzyko zmiany synergistycznych interakcji pomiędzy składnikami fenolowymi, co może prowadzić zarówno do obniżenia, jak i potencjalnego zwiększenia ich właściwości przeciwutleniających. W niniejszej pracy analizowano obecność jednego estru kwasu chlorogenowego w upochodnionych ekstraktach, natomiast potencjał innych pochodnych kwasów fenolowych powstałych w trakcie procesu pozostaje nieznany, a ich identyfikacja i ocena wymagają dalszych badań. Warto również podkreślić, że żaden z badanych ekstraktów z odpadów spożywczych – zarówno w formie natywnej, jak i po lipofilizacji nie wykazał aktywności przeciwdrobnoustrojowej wobec żadnego z testowanych szczepów bakterii.

4.15.2. Stabilność oksydacyjna oleju rzepakowego z dodatkiem ekstraktów z odpadów spożywczych

Zarówno natywne, jak i lipofilizowane ekstrakty z fusów kawowych (SCGE, τ_{max} = 91,0 min) oraz wytłoków aroniowych (ChoPE, τ_{max} = 90,4 min), nie poprawiały stabilności oksydacyjnej oleju w porównaniu do kontroli (Rys. 8). Odwrotny efekt odnotowano w przypadku ekstraktu z wytłoków jabłkowych (APE), który obniżał odporność oleju na utlenianie (τ_{max} = 87,7 min przed lipofilizacją i 80,7 min po lipofilizacji).



Rys. 8. Stabilność oksydacyjna oleju rzepakowego z dodatkiem ekstraktów z odpadów spożywczych: wytłoków aroniowych (ChoPE), wytłoków jabłkowych (APE) i fusów kawowych (SCGE) przed i po lipofilizacji, mierzona w momencie dodania ekstraktów i po dwóch miesiącach przechowywania. Wartości oznaczone tą samą małą literą (a–c) lub wielką literą (A–C) nie różnią się znacząco ($\alpha = 0{,}05$). Symbol * oznacza istotną statystycznie różnicę między początkowym stanem stabilności oksydacyjnej oleju z dodatkami, a stanem po 2 miesiącach przechowywania.

Po dwóch miesiącach przechowywania oleje ze wszystkimi badanymi ekstraktami wykazywały podobny poziom stabilności oksydacyjnej, zbliżony do prób kontrolnych, co wskazuje, że choć dodatek ekstraktów nie pogarszał znacząco parametrów jakościowych, nie zapewnił również istotnej ochrony przed utlenianiem w dłuższej perspektywie. Szczególnie słabe działanie ekstraktu jabłkowego sugeruje, że skład fenolowy tego surowca, charakteryzujący się stosunkowo niskim udziałem kwasów fenolowych, może ograniczać jego potencjał antyoksydacyjny w lipidowym środowisku oleju.

Niezadowalające wyniki w zakresie poprawy stabilności oksydacyjnej przy zastosowaniu ekstraktów odpadowych nie potwierdzają hipotezy **H4** i mogą wynikać z niedostatecznej rozpuszczalności fenolowych składników w fazie lipidowej. Proces lipofilizacji prowadzony z wykorzystaniem butanolu mógł okazać się niewystarczający dla uzyskania odpowiedniego stopnia solubilizacji w matrycy oleju. Wskazuje to na potrzebę zastosowania alkoholi o dłuższych łańcuchach alkilowych, co potwierdzają wcześniejsze prace Aladedunye i współpracowników (2014, 2015). W badaniach tych wprowadzenie łańcucha oktadecylowego do cząsteczki kwasu chlorogenowego bądź flawonoidów (m.in. florydzyny) poprawiało zdolność przeciwutleniającą w warunkach smażenia w

głębokim tłuszczu, choć nie przekładało się na istotne zwiększenie stabilności podczas przechowywania oleju. Co istotne, fenolowe pochodne lipofilowe wykazywały wyższą retencję w smażonym produkcie, poprawiając jego wartość odżywczą i potencjalnie wydłużając okres przydatności do spożycia.

5. WNIOSKI

1. Odpady przetwórstwa roślinnego z przemysłu napojowego stanowią jednocześnie źródło kwasów fenolowych oraz nośnik do immobilizacji lipaz.
2. Lipaza z pleśni *A. oryzae* została skutecznie unieruchomiona na trzech biodegradowalnych nośnikach: fusach kawowych (NSCG), wytłokach aroniowych (NChoP) i jabłkowych (NAP). Najwyższą aktywność uzyskano dla biokatalizatora NSCG, prawdopodobnie dzięki większej zawartości hemicelulozy, bardziej porowatej struktury i wyższej zawartości frakcji lipidowej (~11%), sprzyjających aktywacji międzyfazowej i stabilizacji enzymu poprzez oddziaływania hydrofobowe.
3. Obecny we wszystkich trzech ekstraktach (z wytłoków aroniowych i jabłkowych oraz fusów kawowych) kwas chlorogenowy poddano enzymatycznej lipofilizacji dla poprawy kompatybilności z matrycami lipidowymi. Dla NSCG wydajność lipofilizacji rosła wraz z długością łańcucha węglowego alkoholu (C4-C12), podczas gdy komercyjny biokatalizator Novozym 435 wykazywał aktywność katalityczną zbliżoną dla wszystkich cząsteczek substratu niezależnie od długości łańcucha węglowego alkoholi.
4. Wśród otrzymanych estrów kwasu chlorogenowego najwyższą aktywność antyoksydacyjną wykazał ester butylowy (C4), porównywalną ze swoim prekursorem i BHT, przy jednoczesnym wzroście lipofilowości. Zaobserwowano efekt „cut-off", gdzie wydłużenie łańcucha alkilowego obniżało aktywność antyoksydacyjną estrów kwasu chlorogenowego.
5. W zakresie aktywności przeciwdrobnoustrojowej najsilniejsze działanie wykazały estry średniołańcuchowe (C6, C8) kwasu chlorogenowego, szczególnie wobec bakterii Gram-dodatnich. Pozostałe badane substancje nie wykazywały istotnej aktywności przeciwdrobnoustrojowej.
6. Lipofilizacja ekstraktów z wytłoków aroniowych oraz fusów kawowych spowodowała istotny spadek ich aktywności antyoksydacyjnej, a ponadto żaden z ekstraktów, natywny ani lipofilizowany, nie wykazał aktywności przeciwdrobnoustrojowej w zastosowanych warunkach oznaczenia.
7. Dodatek krótkołańcuchowych (C4, C6) estrów kwasu chlorogenowego zapewnił istotną poprawę stabilności oksydacyjnej oleju rzepakowego, co przypisuje się lepszej rozpuszczalności tych estrów w fazie lipidowej względem wolnego kwasu

chlorogenowego. Same ekstrakty, mimo wysokiej zawartości kwasów fenolowych, nie poprawiły istotnie stabilności oksydacyjnej oleju, co wskazuje m.in. na potrzebę poprawy ich rozpuszczalności w środowisku lipidowym.

Podsumowując, odpady przemysłu spożywczego stanowią obiecujące, przyjazne środowisku nośniki do immobilizacji enzymów, umożliwiając otrzymywanie pochodnych kwasów fenolowych. Chociaż wydajność syntezy i funkcjonalność otrzymanych pochodnych zależą od składu ekstraktu oraz długości łańcucha alkilowego, niniejsze badanie otwiera nowe perspektywy wykorzystania odpadów spożywczych jako surowca do opracowywania innowacyjnych dodatków do żywności o zwiększonej lipofilowości oraz potencjalnych właściwościach antyoksydacyjnych i przeciwdrobnoustrojowych. Dalsze badania powinny objąć szczegółową identyfikację produktów lipofilizacji, optymalizację warunków reakcji, jak również weryfikację bezpieczeństwa stosowania w żywności przy praktycznych poziomach dodatku.

6. SPIS LITERATURY

Abreu Silveira, E., Moreno-Perez, S., Basso, A., Serban, S., Pestana Mamede, R., Tardioli, P. W., Sanchez Farinas, C., Rocha-Martin, J., Fernandez-Lorente, G., Guisan, J. M. (2017). Modulation of the regioselectivity of Thermomyces lanuginosus lipase via biocatalyst engineering for the Ethanolysis of oil in fully anhydrous medium. BMC Biotechnology, 17(1), 88. https://doi.org/10.1186/s12896-017-0407-9

Agricultural Food Products-Determination of Crude Fibre Content-General Method. Polish Committee for Standardization. (1996). PN ISO, 5498.

Aladedunye, F., Matthäus, B. (2014). Phenolic extracts from Sorbus aucuparia (L.) and Malus baccata (L.) berries: antioxidant activity and performance in rapeseed oil during frying and storage. Food Chemistry, 159, 273–281. https://doi.org/10.1016/j.foodchem.2014.02.139

Aladedunye, F., Niehaus, K., Bednarz, H., Thiyam-Hollander, U., Fehling, E., Matthäus, B. (2015). Enzymatic lipophilization of phenolic extract from rowanberry (Sorbus aucuparia) and evaluation of antioxidative activity in edible oil. Lebensmittel-Wissenschaft und Technologie [Food Science and Technology], 60(1), 56–62. https://doi.org/10.1016/j.lwt.2014.08.008

Albayati, S. H., Masomian, M., Ishak, S. N. H., Mohamad Ali, M. S. B., Thean, A. L., Mohd Shariff, F. B., Muhd Noor, N. D. B., Raja Abd Rahman, R. N. Z. (2020). Main structural targets for engineering lipase substrate specificity. Catalysts (Basel, Switzerland), 10(7), 747. https://doi.org/10.3390/catal10070747

Alcañiz-Monge, J., Román-Martínez, M. D. C., Lillo-Ródenas, M. Á. (2022). Chemical activation of lignocellulosic precursors and residues: What else to consider? Molecules (Basel, Switzerland), 27(5), 1630. https://doi.org/10.3390/molecules27051630

Alemán, M., Bou, R., Guardiola, F., Durand, E., Villeneuve, P., Jacobsen, C., Sørensen, A.-D. M. (2015). Antioxidative effect of lipophilized caffeic acid in fish oil enriched mayonnaise and milk. Food Chemistry, 167, 236–244. https://doi.org/10.1016/j.foodchem.2014.06.083

Animal Feeding Stuffs-Determination of Amylase-Treated Neutral Detergent Fibre Content (ANDF). (2007).

Antonic, B., Jancikova, S., Dordevic, D., Tremlova, B. (2020). Apple pomace as food fortification ingredient: A systematic review and meta-analysis. Journal of Food Science, 85(10), 2977–2985. https://doi.org/10.1111/1750-3841.15449

Arun, K. B., Madhavan, A., Sindhu, R., Binod, P., Pandey, A., Reshmy, Sirohi, R. (2020). Remodeling agro-industrial and food wastes into value-added bioactives and biopolymers. Industrial Crops and Products, 154(112621), 112621. https://doi.org/10.1016/j.indcrop.2020.112621

Atabani, A. E., Ali, I., Naqvi, S. R., Badruddin, I. A., Aslam, M., Mahmoud, E., Almomani, F., Juchelková, D., Atelge, M. R., Khan, T. M. Y. (2022). A state-of-the-art review on spent coffee ground (SCG) pyrolysis for future biorefinery. Chemosphere, 286(Pt 2), 131730. https://doi.org/10.1016/j.chemosphere.2021.131730

Ballesteros, L. F., Teixeira, J. A., Mussatto, S. I. (2014). Chemical, functional, and structural properties of spent coffee grounds and coffee silverskin. Food and Bioprocess Technology, 7(12), 3493–3503. https://doi.org/10.1007/s11947-014-1349-z

Barrera-Rivera, K. A., Martínez-Richa, A. (2017). Yarrowia lipolytica extracellular lipase Lip2 as biocatalyst for the ring-opening polymerization of ε-caprolactone. Molecules (Basel, Switzerland), 22(11). https://doi.org/10.3390/molecules22111917

Bhushan, S., Kalia, K., Sharma, M., Singh, B., Ahuja, P. S. (2008). Processing of apple pomace for bioactive molecules. Critical Reviews in Biotechnology, 28(4), 285–296. https://doi.org/10.1080/07388550802368895

Bilal, M., Fernandes, C. D., Mehmood, T., Nadeem, F., Tabassam, Q., Ferreira, L. F. R. (2021). Immobilized lipases-based nano-biocatalytic systems - A versatile platform with incredible biotechnological potential. International Journal of Biological Macromolecules, 175, 108–122. https://doi.org/10.1016/j.ijbiomac.2021.02.010

Brabcová, J., Demianová, Z., Vondrášek, J., Jágr, M., Zarevúcka, M., Palomo, J. M. (2013). Highly selective purification of three lipases from Geotrichum candidum 4013 and their characterization and biotechnological applications. Journal of Molecular Catalysis. B, Enzymatic, 98, 62–72. https://doi.org/10.1016/j.molcatb.2013.09.012

Brault, G., Shareck, F., Hurtubise, Y., Lépine, F., Doucet, N. (2014). Short-chain flavor ester synthesis in organic media by an E. coli whole-cell biocatalyst expressing a newly characterized heterologous lipase. PloS One, 9(3), e91872. https://doi.org/10.1371/journal.pone.0091872

Budžaki, S., Velić, N., Ostojčić, M., Stjepanović, M., Rajs, B. B., Šereš, Z., Maravić, N., Stanojev, J., Hessel, V., Strelec, I. (2022). Waste management in the agri-food industry: The conversion of eggshells, spent coffee grounds, and brown onion skins into carriers for lipase immobilization. Foods (Basel, Switzerland), 11(3), 409. https://doi.org/10.3390/foods11030409

Buisman, G. J. H., van Helteren, C. T. W., Kramer, G. F. H., Veldsink, J. W., Derksen, J. T. P., Cuperus, F. P. (1998). Enzymatic esterifications of functionalized phenols for the synthesis of lipophilic antioxidants. Biotechnology Letters, 20(2), 131–136. https://doi.org/10.1023/a:1005368222340

Buntić, A., Pavlović, M., Antonović, D., Pavlović, V., Vrućinić, D., Šiler-Marinković, S., Dimitrijević-Branković, S. (2018). Customizing the spent coffee for Trichoderma reesei cellulase immobilization by modification with activating agents. International Journal of Biological Macromolecules, 107, 1856–1863. https://doi.org/10.1016/j.ijbiomac.2017.10.060

Campos-Vega, R., Loarca-Piña, G., Vergara-Castañeda, H. A., Oomah, B. D. (2015). Spent coffee grounds: A review on current research and future prospects. Trends in Food Science & Technology, 45(1), 24–36. https://doi.org/10.1016/j.tifs.2015.04.012

Cantone, S., Ferrario, V., Corici, L., Ebert, C., Fattor, D., Spizzo, P., Gardossi, L. (2013). Efficient immobilisation of industrial biocatalysts: criteria and constraints for the

selection of organic polymeric carriers and immobilisation methods. Chemical Society Reviews, 42(15), 6262–6276. https://doi.org/10.1039/c3cs35464d

Capanoglu, E., Nemli, E., Tomas-Barberan, F. (2022). Novel approaches in the valorization of agricultural wastes and their applications. Journal of Agricultural and Food Chemistry, 70(23), 6787–6804. https://doi.org/10.1021/acs.jafc.1c07104

Chandra, P., Enespa, Singh, R., Arora, P. K. (2020). Microbial lipases and their industrial applications: a comprehensive review. Microbial Cell Factories, 19(1), 169. https://doi.org/10.1186/s12934-020-01428-8

Coelho, A. L. S., Orlandelli, R. C. (2021). Immobilized microbial lipases in the food industry: a systematic literature review. Critical Reviews in Food Science and Nutrition, 61(10), 1689–1703. https://doi.org/10.1080/10408398.2020.1764489

Colantoni, A., Paris, E., Bianchini, L., Ferri, S., Marcantonio, V., Carnevale, M., Palma, A., Civitarese, V., Gallucci, F. (2021). Spent coffee ground characterization, pelletization test and emissions assessment in the combustion process. Scientific Reports, 11(1), 5119. https://doi.org/10.1038/s41598-021-84772-y

Compton, D. L., Laszlo, J. A., Berhow, M. A. (2000). Lipase-catalyzed synthesis of ferulate esters. Journal of the American Oil Chemists' Society, 77(5), 513–519. https://doi.org/10.1007/s11746-000-0082-9

de Araújo, M. E. M. B., Franco, Y. E. M., Messias, M. C. F., Longato, G. B., Pamphile, J. A., Carvalho, P. de O. (2017). Biocatalytic synthesis of flavonoid esters by lipases and their biological benefits. Planta Medica, 83(1–02), 7–22. https://doi.org/10.1055/s-0042-118883

Du, X., Liu, X., Li, Y., Wu, C., Wang, X., Xu, P. (2013). Efficient biocatalyst by encapsulating lipase into nanoporous gold. Nanoscale Research Letters, 8(1), 180. https://doi.org/10.1186/1556-276X-8-180

Durand, E., Lecomte, J., Villeneuve, P. (2017). The Biological and Antimicrobial activities of Phenolipids. Lipid Technology, 29(7–8), 67–70. https://doi.org/10.1002/lite.201700019

Environment, U. N. (2021). UNEP Food Waste Index.

Fernandez-Lafuente, R. (2010). Lipase from Thermomyces lanuginosus: Uses and prospects as an industrial biocatalyst. Journal of Molecular Catalysis. B, Enzymatic, 62(3–4), 197–212. https://doi.org/10.1016/j.molcatb.2009.11.010

Figueroa-Espinoza, M. C., Laguerre, M., Villeneuve, P., Lecomte, J. (2013). From phenolics to phenolipids: Optimizing antioxidants in lipid dispersions. Lipid Technology, 25(6), 131–134. https://doi.org/10.1002/lite.201300277

Filho, D. G., Silva, A. G., Guidini, C. Z. (2019). Lipases: sources, immobilization methods, and industrial applications. Applied Microbiology and Biotechnology, 103(18), 7399–7423. https://doi.org/10.1007/s00253-019-10027-6

Gil, M., Wianowska, D. (2017). Chlorogenic acids-their properties, occurrence and analysis. Annales Universitatis Mariae Curie-Skłodowska, sectio AA-Chemia 72.

Girelli, A. M., Astolfi, M. L., Scuto, F. R. (2020). Agro-industrial wastes as potential carriers for enzyme immobilization: A review. Chemosphere, 244(125368), 125368. https://doi.org/10.1016/j.chemosphere.2019.125368

Girelli, A. M., Chiappini, V. (2023). Renewable, sustainable, and natural lignocellulosic carriers for lipase immobilization: A review. Journal of Biotechnology, 365, 29–47. https://doi.org/10.1016/j.jbiotec.2023.02.003

Girelli, A. M., Chiappini, V., Amadoro, P. (2023). Immobilization of lipase on spent coffee grounds by physical and covalent methods: A comparison study. Biochemical Engineering Journal, 192(108827), 108827. https://doi.org/10.1016/j.bej.2023.108827

Giuliani, S., Piana, C., Setti, L., Hochkoeppler, A., Pifferi, P. G., Williamson, G., Faulds, C. B. (2001). Synthesis of pentylferulate by a feruloyl esterase from Aspergillus niger using water-in-oil microemulsions. Biotechnol. Lett, 23, 325–330.

Głowacka, R., Górska, A., Wirkowska-Wojdyła, M., Wołosiak, R., Majewska, E., Derewiaka, D. (2019). The influence of brewing method on bioactive compounds residues in spent coffee grounds of different roasting degree and geographical origin. International Journal of Food Science & Technology, 54(11), 3008–3014. https://doi.org/10.1111/ijfs.14213

Gołębiewska, E., Kalinowska, M., Yildiz, G. (2022). Sustainable Use of Apple Pomace (AP) in Different Industrial Sectors. Materials (Basel) 15(5), 1788. https://doi.org/10.3390/ma15051788

Guerrero, M. R. B., Marques da Silva Paula, M., Zaragoza, M. M., Gutiérrez, J. S., Velderrain, V. G., Ortiz, A. L., Collins-Martínez, V. (2014). Thermogravimetric study on the pyrolysis kinetics of apple pomace as waste biomass. International Journal of Hydrogen Energy, 39(29), 16619–16627.https://doi.org/10.1016/j.ijhydene.2014.06.012

Guyot, B., Bosquette, B., Pina, M., Graille, J. (1997). Esterification of phenolic acids from green coffee with an immobilized lipase from Candida antarctica in solvent-free medium. Biotechnology Letters, 19(6), 529–532. https://doi.org/10.1023/a:1018381102466

Guyot, Bernard, Gueule, D., Pina, M., Graille, J., Farines, V., Farines, M. (2000). Enzymatic synthesis of fatty esters in 5-caffeoyl quinic acid. European Journal of Lipid Science and Technology: EJLST, 102(2), 93–95. https://doi.org/10.1002/(sici)1438-9312(200002)102:2<93::aid-ejlt93>3.0.co;2-b

Hanefeld, U., Gardossi, L., Magner, E. (2009). Understanding enzyme immobilisation. Chemical Society Reviews, 38(2), 453–468. https://doi.org/10.1039/b711564b

Ismail, A. R., Baek, K.-H. (2020). Lipase immobilization with support materials, preparation techniques, and applications: Present and future aspects. International Journal of Biological Macromolecules, 163, 1624–1639. https://doi.org/10.1016/j.ijbiomac.2020.09.021

Iso, P.-E. (2009). Animal Feeding Stuffs-Determination of Acid Detergent Fibre (ADF) and Acid Detergent Lignin (ADL) Contents. Polish Committee for Standardization.

K de S Lira, R., T Zardini, R., C C de Carvalho, M., Wojcieszak, R., G F Leite, S., Itabaiana, I., Jr. (2021). Agroindustrial wastes as a support for the immobilization of lipase from Thermomyces lanuginosus: Synthesis of hexyl laurate. Biomolecules, 11(3), 445. https://doi.org/10.3390/biom11030445

Korzeniowska, K. (2017). Możliwości zastosowania ekstraktów roślinnych zawierających związki fenolowe w rolnictwie ekologicznym. Przemysł Chemiczny, 1(7), 100–104. https://doi.org/10.15199/62.2017.7.15

Kumar, R., Pal, P. (2021). Lipase immobilized graphene oxide biocatalyst assisted enzymatic transesterification of Pongamia pinnata (Karanja) oil and downstream enrichment of biodiesel by solar-driven direct contact membrane distillation followed by ultrafiltration. Fuel Processing Technology, 211(106577), 106577. https://doi.org/10.1016/j.fuproc.2020.106577

Laguerre, M., Bayrasy, C., Lecomte, J., Chabi, B., Decker, E. A., Wrutniak-Cabello, C., Cabello, G., Villeneuve, P. (2013). How to boost antioxidants by lipophilization? Biochimie, 95(1), 20–26. https://doi.org/10.1016/j.biochi.2012.07.018

Laguerre, M., Giraldo, L. J. L., Lecomte, J., Figueroa-Espinoza, M.-C., Baréa, B., Weiss, J., Decker, E. A., Villeneuve, P. (2009). Chain length affects antioxidant properties of chlorogenate esters in emulsion: the cutoff theory behind the polar paradox. Journal of Agricultural and Food Chemistry, 57(23), 11335–11342. https://doi.org/10.1021/jf9026266

Laguerre, M., López Giraldo, L. J., Lecomte, J., Figueroa-Espinoza, M.-C., Baréa, B., Weiss, J., Decker, E. A., Villeneuve, P. (2010). Relationship between hydrophobicity and antioxidant ability of "phenolipids" in emulsion: a parabolic effect of the chain length of rosmarinate esters. Journal of Agricultural and Food Chemistry, 58(5), 2869–2876. https://doi.org/10.1021/jf904119v

Laguerre, M., Wrutniak-Cabello, C., Chabi, B., López Giraldo, L. J., Lecomte, J., Villeneuve, P., Cabello, G. (2011). Does hydrophobicity always enhance antioxidant drugs? A cut-off effect of the chain length of functionalized chlorogenate esters on ROS-overexpressing fibroblasts: Cut-off effect of phenolipid antioxidants. The Journal of Pharmacy and Pharmacology, 63(4), 531–540. https://doi.org/10.1111/j.2042-7158.2010.01216.x

Liu, X., Lei, L., Li, Y., Zhu, H., Cui, Y., Hu, H. (2011). Preparation of carriers based on magnetic nanoparticles grafted polymer and immobilization for lipase. Biochemical Engineering Journal, 56(3), 142–149. https://doi.org/10.1016/j.bej.2011.05.013

López Giraldo, L. J., Laguerre, M., Lecomte, J., Figueroa-Espinoza, M.-C., Barouh, N., Baréa, B., Villeneuve, P. (2007). Lipase-catalyzed synthesis of chlorogenate fatty esters in solvent-free medium. Enzyme and Microbial Technology, 41(6–7), 721–726. https://doi.org/10.1016/j.enzmictec.2007.06.004

López-Giraldo, L. J., Laguerre, M., Lecomte, J., Figueroa-Espinoza, M.-C., Baréa, B., Weiss, J., Decker, E. A., Villeneuve, P. (2009). Kinetic and stoichiometry of the reaction of chlorogenic acid and its alkyl esters against the DPPH radical. Journal of Agricultural and Food Chemistry, 57(3), 863–870. https://doi.org/10.1021/jf803148z

Lorentz, C., Dulac, A., Pencreac'h, G., Ergan, F., Richomme, P., Soultani-Vigneron, S. (2010). Lipase-catalyzed synthesis of two new antioxidants: 4-O- and 3-O-palmitoyl chlorogenic acids. Biotechnology Letters, 32(12), 1955–1960. https://doi.org/10.1007/s10529-010-0386-6

Ma, C.-M., Kully, M., Khan, J. K., Hattori, M., Daneshtalab, M. (2007). Synthesis of chlorogenic acid derivatives with promising antifungal activity. Bioorganic & Medicinal Chemistry, 15(21), 6830–6833. https://doi.org/10.1016/j.bmc.2007.07.038

Mak, S. L., Wu, M. Y. T., Chak, W. Y., Kwong, W. K., Tang, W. F., Li, C. H., Lee, C. C., Li, C. Y. (2023). A review of the feasibility of producing polylactic acid (PLA) polymers using spent coffee ground. Sustainability, 15(18), 13498. https://doi.org/10.3390/su151813498

Manoel, E. A., Dos Santos, J. C. S., Freire, D. M. G., Rueda, N., Fernandez-Lafuente, R. (2015). Immobilization of lipases on hydrophobic supports involves the open form of the enzyme. Enzyme and Microbial Technology, 71, 53–57. https://doi.org/10.1016/j.enzmictec.2015.02.001

Manoel, E. A., Robert, J. M., Pinto, M. C. C., Machado, A. C. O., Besteti, M. D., Coelho, M. A. Z., Simas, A. B. C., Fernandez-Lafuente, R., Pinto, J. C., Freire, D. M. G. (2016). Evaluation of the performance of differently immobilized recombinant lipase B from Candida antarctica preparations for the synthesis of pharmacological derivatives in organic media. RSC Advances, 6(5), 4043–4052. https://doi.org/10.1039/c5ra22508f

Mardani, M., Badakné, K., Farmani, J., Shahidi, F. (2024). Enzymatic lipophilization of bioactive compounds with high antioxidant activity: a review. Critical Reviews in Food Science and Nutrition, 64(15), 4977–4994. https://doi.org/10.1080/10408398.2022.2147268

Mehta, A., Guleria, S., Sharma, R., Gupta, R. (2021). The lipases and their applications with emphasis on food industry. In Microbial Biotechnology in Food and Health (pp. 143–164). Elsevier.

Mhetras, N., Mapare, V., Gokhale, D. (2021). Cold active lipases: Biocatalytic tools for greener technology. Applied Biochemistry and Biotechnology, 193(7), 2245–2266. https://doi.org/10.1007/s12010-021-03516-w

Mokhena, T. C., Sadiku, E. R., Mochane, M. J., Ray, S. S., John, M. J., Mtibe, A. (2021). Mechanical properties of cellulose nanofibril papers and their bionanocomposites: A review. Carbohydrate Polymers, 273(118507), 118507. https://doi.org/10.1016/j.carbpol.2021.118507

Mokhtar, N. F., Abd. Rahman, R. N. Z. R., Muhd Noor, N. D., Mohd Shariff, F., Mohamad Ali, M. S. (2020). The immobilization of lipases on porous support by adsorption and hydrophobic interaction method. Catalysts (Basel, Switzerland), 10(7), 744. https://doi.org/10.3390/catal10070744

Mouad, A. M., Taupin, D., Lehr, L., Yvergrauy, F., Porto, A. L. M. (2016). Aminolysis of linoleic and salicylic acid derivatives with Candida antartica lipase B: a solvent free

process to obtain amphiphilic amides for cosmetic application. Journal of Molecular Catalysis B: Enzymatic, 126, 64-e68.

Nájera-Martínez, E. F., Melchor-Martínez, E. M., Sosa-Hernández, J. E., Levin, L. N., Parra-Saldívar, R., Iqbal, H. M. N. (2022). Lignocellulosic residues as supports for enzyme immobilization, and biocatalysts with potential applications. International Journal of Biological Macromolecules, 208, 748–759. https://doi.org/10.1016/j.ijbiomac.2022.03.180

Narwal, S. K., Gupta, R. (2013). Biodiesel production by transesterification using immobilized lipase. Biotechnology Letters, 35(4), 479–490. https://doi.org/10.1007/s10529-012-1116-z

Nematian, T., Shakeri, A., Salehi, Z., Saboury, A. A. (2020). Lipase immobilized on functionalized superparamagnetic few-layer graphene oxide as an efficient nanobiocatalyst for biodiesel production from Chlorella vulgaris bio-oil. Biotechnology for Biofuels, 13(1), 57. https://doi.org/10.1186/s13068-020-01688-x

Osman, A. I., Young, T. J., Farrell, C., Harrison, J., Al-Muhtaseb, A. H., Rooney, D. W. (2020). Physicochemical characterization and kinetic modeling concerning combustion of waste berry pomace. ACS Sustainable Chemistry & Engineering, 8(47), 17573–17586. https://doi.org/10.1021/acssuschemeng.0c07390

Osmulescu, S., Trandafir, I., Nour, V. (2017). Phenolic acids and flavonoids profiles of extracts from edible wild fruits and their antioxidant properties. Int. J. Food Prop, 20, 3124–3134.

Özyürek, M., Güçlü, K., Apak, R. (2011). The main and modified CUPRAC methods of antioxidant measurement. Trends in Analytical Chemistry: TRAC, 30(4), 652–664. https://doi.org/10.1016/j.trac.2010.11.016

Pappalardo, V., Ravasio, N., Falletta, E., De Rosa, M. C., Zaccheria, F. (2023). A green lipophilization reaction of a natural antioxidant. Antioxidants (Basel, Switzerland), 12(2), 218. https://doi.org/10.3390/antiox12020218

Pasangulapati, V., Ramachandriya, K. D., Kumar, A., Wilkins, M. R., Jones, C. L., Huhnke, R. L. (2012). Effects of cellulose, hemicellulose and lignin on thermochemical conversion characteristics of the selected biomass. Bioresource Technology, 114, 663–669. https://doi.org/10.1016/j.biortech.2012.03.036

Pereira, E. B., Zanin, G. M., Castro, H. F. (2003). Immobilization and catalytic properties of lipase on chitosan for hydrolysis and esterification reactions. Brazilian Journal of Chemical Engineering, 20(4), 343–355. https://doi.org/10.1590/s0104-66322003000400002

Platonova, E. Y., Shaposhnikov, M. V., Lee, H.-Y., Lee, J.-H., Min, K.-J., Moskalev, A. (2021). Black chokeberry (Aronia melanocarpa) extracts in terms of geroprotector criteria. Trends in Food Science & Technology, 114, 570–584. https://doi.org/10.1016/j.tifs.2021.06.020

Płocharski, W., Mieszczakowska-Frąc, M., Rutkowski, K., Konopacka, D. (n.d.). Tradycyjne i innowacyjne kierunki zagospodarowania jabłek w Polsce. Inhort.Pl.

Retrieved September 17, 2025, from https://www.inhort.pl/files/program_wieloletni/PW_2015_2020_IO/spr_2019/Broszura_Plocharski_2019_Tradycyjne_zad.3.5.pdf

Poore, J., Nemecek, T. (2018). Reducing food's environmental impacts through producers and consumers. Science (New York, N.Y.), 360(6392), 987–992. https://doi.org/10.1126/science.aaq0216

Proestos, C., Sereli, D., Komaitis, M. (2006). Determination of phenolic compounds in aromatic plants by RP-HPLC and GC-MS. Food Chemistry, 95(1), 44–52. https://doi.org/10.1016/j.foodchem.2004.12.016

Quayson, E., Amoah, J., Rachmadona, N., Morita, K., Darkwah, L., Hama, S., Yoshida, A., Kondo, A., Ogino, C. (2020). Valorization of palm biomass waste into carbon matrices for the immobilization of recombinant Fusarium heterosporum lipase towards palm biodiesel synthesis. Biomass & Bioenergy, 142(105768), 105768. https://doi.org/10.1016/j.biombioe.2020.105768

Ramadan, M. F. (2022). Pheno-phospholipids and lipo-phenolics: Novel structured antioxidants. Springer Nature.

Rodrigues, R. C., Virgen-Ortíz, J. J., Dos Santos, J. C. S., Berenguer-Murcia, Á., Alcantara, A. R., Barbosa, O., Ortiz, C., Fernandez-Lafuente, R. (2019). Immobilization of lipases on hydrophobic supports: immobilization mechanism, advantages, problems, and solutions. Biotechnology Advances, 37(5), 746–770. https://doi.org/10.1016/j.biotechadv.2019.04.003

Saracila, M., Untea, A. E., Oancea, A. G., Varzaru, I., Vlaicu, P. A. (2024). Comparative analysis of black chokeberry (Aronia melanocarpa L.) fruit, leaves, and pomace for their phytochemical composition, antioxidant potential, and polyphenol bioaccessibility. Foods (Basel, Switzerland), 13(12), 1856. https://doi.org/10.3390/foods13121856

Shahidi, F., Zhong, Y. (2011). Revisiting the polar paradox theory: a critical overview. Journal of Agricultural and Food Chemistry, 59(8), 3499–3504. https://doi.org/10.1021/jf104750m

Shuai, W., Das, R. K., Naghdi, M., Brar, S. K., Verma, M. (2017). A review on the important aspects of lipase immobilization on nanomaterials: Lipase Immobilization on Nanomaterials. Biotechnology and Applied Biochemistry, 64(4), 496–508. https://doi.org/10.1002/bab.1515

Sørensen, A.-D. M., Nielsen, N. S., Yang, Z., Xu, X., Jacobsen, C. (2012). Lipophilization of dihydrocaffeic acid affects its antioxidative properties in fish-oil-enriched emulsions. European Journal of Lipid Science and Technology: EJLST, 114(2), 134–145. https://doi.org/10.1002/ejlt.201100002

Srinivasan, G. R., Mahajan, A., Seth, R., Mahajan, R. (2023). Spent coffee ground briquettes: A critical review. In Advances in Environmental Engineering and Green Technologies (pp. 244–270). IGI Global.

Stamatis, H., Sereti, V., Kolisis, F. N. (1999). Studies on the enzymatic synthesis of lipophilic derivatives of natural antioxidants. Journal of the American Oil Chemists' Society, 76(12), 1505. https://doi.org/10.1007/s11746-999-0193-1

Stamatis, H., Sereti, V., Kolisis, F. N. (2001). Enzymatic synthesis of hydrophilic and hydrophobic derivatives of natural phenolic acids in organic media. Journal of Molecular Catalysis. B, Enzymatic, 11(4–6), 323–328. https://doi.org/10.1016/s1381-1177(00)00016-3

Suárez-Quiroz, M. L., Taillefer, W., López Méndez, E. M., González-Ríos, O., Villeneuve, P., Figueroa-Espinoza, M. C. (2013). Antibacterial activity and antifungal and anti-mycotoxigenic activities against Aspergillus flavus and A. ochraceus of green coffee chlorogenic acids and dodecyl chlorogenates: Antimicrobial chlorogenic acids and chlorogenates. Journal of Food Safety, 33(3), 360–368. https://doi.org/10.1111/jfs.12060

Szajdek, A., Borowska, J. (2004). Właściwości przeciwutleniające żywności pochodzenia roślinnego. Żywność Nauka Technologia Jakość, 11(4).

Tarko, T., Duda-Chodak, A., Bebak, A. (2012). Aktywność biologiczna wybranych wytłoków owocowych oraz warzywnych. Żywność Nauka Technologia Jakość.

Thangaraj, B., Solomon, P. R. (2019). Immobilization of lipases – A review. Part I: Enzyme immobilization. ChemBioEng Reviews, 6(5), 157–166. https://doi.org/10.1002/cben.201900016

Toczko, M., Grzelińska, A. (Eds.). (1997). Materiały do ćwiczeń z biochemii. Wydaw. SGGW. Warsaw, Poland, 2001. (n.d.).

Topakas, E., Stamatis, H., Biely, P., Kekos, D., Macris, B. J., Christakopoulos, P. (2003). Purification and characterization of a feruloyl esterase from Fusarium oxysporum catalyzing esterification of phenolic acids in ternary water–organic solvent mixtures. Journal of Biotechnology, 102(1), 33–44. https://doi.org/10.1016/s0168-1656(02)00363-2

Trache, D., Tarchoun, A. F., Derradji, M., Hamidon, T. S., Masruchin, N., Brosse, N., Hussin, M. H. (2020). Nanocellulose: From fundamentals to advanced applications. Frontiers in Chemistry, 8, 392. https://doi.org/10.3389/fchem.2020.00392

Ugo, A. K., Amara, A. V., Igwe, C. N., Kenechuwku, U. (2017). Microbial Lipases: A Prospect for Biotechnological Industrial Catalysis for Green Products: A Review. Fermentation Technology, 6.

Vermerris, W., Nicholson, R. (2006). Phenolic Compound Biochemistry. Springer.

Wang, S., Li, Y., Meng, X., Chen, S., Huang, D., Xia, Y., Zhu, S. (2021). Antioxidant activities of chlorogenic acid derivatives with different acyl donor chain lengths and their stabilities during in vitro simulated gastrointestinal digestion. Food Chemistry, 357(129904), 129904. https://doi.org/10.1016/j.foodchem.2021.129904

Xie, J., Zhang, Y., Simpson, B. (2022). Food enzymes immobilization: novel carriers, techniques and applications. Current Opinion in Food Science, 43, 27–35. https://doi.org/10.1016/j.cofs.2021.09.004

Yu, X., Li, Y., Wu, D. (2004). Enzymatic synthesis of gallic acid esters using microencapsulated tannase: effect of organic solvents and enzyme specificity. Journal of Molecular Catalysis. B, Enzymatic, 30(2), 69–73. https://doi.org/10.1016/j.molcatb.2004.03.009

Zanetti, M., Carniel, T. K., Valério, A., Oliveira, J. V. de, Oliveira, D. de, Araújo, P. H. H. de, Riella, H. G., Fiori, M. A. (2017). Synthesis of geranyl cinnamate by lipase-catalyzed reaction and its evaluation as an antimicrobial agent: Lipase-catalyzed synthesis of geranyl cinnamate. Journal of Chemical Technology and Biotechnology (Oxford, Oxfordshire: 1986), 92(1), 115–121. https://doi.org/10.1002/jctb.4998

Zheng, J., Fu, X., Ying, X., Zhang, Y., Wang, Z. (2014). A sensitive colorimetric high-throughput screening method for lipase synthetic activity assay. Analytical Biochemistry, 452, 13–15. https://doi.org/10.1016/j.ab.2014.02.001

Zhu, S., Wang, S., Chen, S., Xia, Y., Li, Y. (2020). Lipase-catalyzed highly regioselective synthesis of acylated chlorogenic acid. Food Bioscience, 37(100706), 100706. https://doi.org/10.1016/j.fbio.2020.100706

7. DOROBEK NAUKOWY

PRACE BADAWCZE STANOWIĄCE ROZPRAWĘ DOKTORSKĄ:

1. **Jasińska Karina**, Fabiszewska Agata, Białecka-Florjańczyk Ewa, Zieniuk Bartłomiej: Mini-Review on the Enzymatic Lipophilization of Phenolics Present in Plant Extracts with the Special Emphasis on Anthocyanins, Antioxidants, 2022, vol. 11, nr 8, s.1-16, Numer artykułu:1528. DOI:10.3390/antiox11081528
2. **Jasińska Karina**, Zieniuk Bartłomiej, Jankiewicz Urszula, Fabiszewska Agata: Bio-Based Materials versus Synthetic Polymers as a Support in Lipase Immobilization: Impact on Versatile Enzyme Activity, Catalysts, 2023, vol. 13, nr 2, s.1-14, Numer artykułu:395. DOI:10.3390/catal13020395
3. **Jasińska Karina**, Zieniuk Bartłomiej, Piasek Adrianna Maria, Wysocki Łukasz, Sobiepanek Anna, Fabiszewska Agata: Obtaining a biodegradable biocatalyst – study on lipase immobilization on spent coffee grounds as potential carriers, Biocatalysis and Agricultural Biotechnology, 2024, nr 59, s.1-14, Numer artykułu:103255. DOI:10.1016/j.bcab.2024.103255
4. **Jasińska Karina**, Nowosad Maksym, Perzyna Aleksander, Bielacki Andrzej, Dziwiński Stanisław, Zieniuk Bartłomiej, Fabiszewska Agata: Sustainable Lipase Immobilization: Chokeberry and Apple Waste as Carriers, Biomolecules, 2024, vol. 14, nr 12, s.1-14, Numer artykułu:1564.DOI:10.3390/biom14121564
5. **Jasińska Karina**, Zieniuk Bartłomiej, Bryła Marcin, Padewska Daria, Brzezińska Rita, Kruszewski Bartosz, Nowak Dorota, Fabiszewska Agata: The Double Life of Plant-Based Food Waste: A Source of Phenolic Acids and a Carrier for Immobilization of Lipases Capable of Their Lipophilization. International Journal of Molecular Sciences, 2025, 26(23), 11400. DOI:10.3390/ijms262311400

POZOSTAŁE PRACE (CHRONOLOGICZNIE):

6. Zieniuk Bartłomiej, Ononamadu Chimaobi James, **Jasińska Karina**, Wierzchowska Katarzyna, Fabiszewska Agata: Lipase-Catalyzed Synthesis, Antioxidant Activity, Antimicrobial Properties and Molecular Docking Studies of Butyl Dihydrocaffeate, Molecules, 2022, vol. 27, nr 15, s.1-18, Numer artykułu:5024.DOI:10.3390/molecules27155024
7. Zieniuk Bartłomiej, **Jasińska Karina**, Wierzchowska Katarzyna, Fabiszewska Agata: Enzymatic Synthesis of Flavours and Fragrances, Antioxidants and Antimicrobials on the Example of Benzyl Alcohol and Its Selected Derivatives, Biology and Life Sciences Forum, 2022, vol. 18, nr 2, s.1-6. DOI:10.3390/Foods2022-13066
8. Wierzchowska Katarzyna, Musiałek Aleksandra, Zieniuk Bartłomiej, **Jasińska Karina**, Nowak Dorota, Fabiszewska Agata: Is There Any Possibility to Use Ultrasounds, High Pressure Homogenization or Pulsed Electric Field in Single Cell Oil Release from Oleaginous Yeast Cells, Biology and Life Sciences Forum, 2022, vol. 2, s.1-6. DOI:10.3390/Foods2022-12959

9. **Jasińska Karina**, Zieniuk Bartłomiej, Fabiszewska Agata, Wierzchowska Katarzyna: Investigating Culture Media for Obtaining Lipolytic Biocatalysts Based on Rhizopus oryzae Fungi, Biology and Life Sciences Forum, 2022, vol. 2, s.1-5. DOI:10.3390/Foods2022-12965
10. Fabiszewska Agata, Pakulska Anna, Zieniuk Bartłomiej, Wierzchowska Katarzyna, **Jasińska Karina**, Małajowicz Jolanta, Nowak Dorota: Unconventional Extraction Methods of Oleaginous Yeast Cell Pretreatment and Disruption, Applied Sciences-Basel, 2023, vol. 13, nr 24, s.1-15, Numer artykułu:13135. DOI:10.3390/app132413135
11. Zieniuk Bartłomiej, **Jasińska Karina**, Wierzchowska Katarzyna, Şuheda Uğur, Fabiszewska Agata: Yarrowia lipolytica Yeast: A Treasure Trove of Enzymes for Biocatalytic Applications—A Review, Fermentation, 2024, vol. 10, nr 5, s.1-30, Numer artykułu:263. DOI:10.3390/fermentation10050263
12. Zieniuk Bartłomiej, Małajowicz Jolanta, **Jasińska Karina**, Wierzchowska Katarzyna, Şuheda Uğur, Fabiszewska Agata: Agri-Food and Food Waste Lignocellulosic Materials for Lipase Immobilization as a Sustainable Source of Enzyme Support—A Comparative Study, Foods, 2024, vol. 13, nr 23, s.1-18, Numer artykułu:3759. DOI:10.3390/foods13233759
13. Fabiszewska Agata, Wierzchowska Katarzyna, Dębkowska Ilona, Śliczniak Weronika, Ziółkowska Magdalena, **Jasińska Karina**, Kobus Joanna, Nowak Dorota, Zieniuk Bartłomiej: Plant-Based Alternatives to Mold-Ripened Cheeses as an Innovation among Dairy Analogues, Foods, 2024, vol. 13, nr 14, s.1-19, Numer artykułu:2305. DOI:10.3390/foods13142305
14. Fabiszewska Agata, Zieniuk Bartłomiej, **Jasińska Karina**, Nowa Dorota, Sasal Katarzyna, Kobus Joanna, Jankiewicz Urszula: Extracellular Lipases of Yarrowia lipolytica Yeast in Media Containing Plant Oils—Studies Supported by the Design of Experiment Methodology, Applied Sciences-Basel, 2024, vol. 14, nr 23, s.1-18, Numer artykułu:11449. DOI:10.3390/app142311449
15. Małajowicz Jolanta, Wierzchowska Katarzyna, **Jasińska Karina**, Fabiszewska Agata. Turning mushy lipids into fruity notes: Unlocking lactone biosynthesis potential in fat industry lipid waste. Foods (Basel, Switzerland), 2025, 14(24), 4326. https://doi.org/10.3390/foods14244326

PATENTY:

- Zgłoszenie patentowe: WIPO ST 10/C PL451973 „Sposób otrzymywania estrów kwasu chlorogenowego i alkoholi na drodze biokatalizy".
- Zgłoszenie patentowe: WIPO ST 10/C PL448784 „Sposób wytwarzania roślinnej alternatywy sera z porostem pleśni"

STAŻE NAUKOWE ORAZ WYJAZDY ZAGRANICZNE

1. 3-miesięczny staż naukowy, Program Badawczy Informatyki Biomedycznej, Universitat Pompeu Fabra (UPF), Wydział Medycyny i Nauk Przyrodniczych, Barcelona, Hiszpania (01.04 – 30.06.2025)

2. Uczestnictwo w Szkole Letniej – BIP Erasmus „Nutrition and nutrition education", University of Ljubljana, Słowenia (09 – 13.09.2024)
3. Uczestnictwo w Szkole Letniej - XIII International Summer School (BIP) Erasmus „Decentralized water and waste management systems: appropriate technologies and solutions for resilience and sustainability. Rural and isolated communities in developing countries"; University of Brescia, Włochy (24.06. – 02.07.2024)
4. Staż naukowy – Politechnika Warszawska, Wydział Chemiczny, Katedra Biotechnologii Środków Leczniczych i Kosmetyków, 00-662 Warsaw (Listopad - Grudzień 2022)
5. Uczestnictwo w 6-miesięcznym międzynarodowym programie - FOEBE project - FOSTERING ENTREPRENEURSHIP FOR THE BIOECONOMY (Erasmus +), zakończonym tygodniowym wyjazdem do Bolonii, Włochy (intensive study) (Styczeń-Czerwiec 2022)

KONFERENCJE I PREZENTACJE

1. XXIII European Conference on Food Chemistry, Bratislava, Slovakia, 11-13.06.2025 – ustna prezentacja w j.angielskim – "*Lipophilization of chlorogenic acid by biodegradable biocatalysts immobilized on spent coffee grounds"*.
2. Natural Science Baltic Conference – International Conference Online, 10-11.05.2025 – ustna prezentacja w j.angielskim - *"Sustainable esterification of chlorogenic acid using lipase immobilized on spent coffee grounds: Characterization of a potential food additive"* .
3. XIth International Session of Young Scientific Staff "Food in the face of the challenges of the modern world", Gdańsk, Poland, 16-17.05.2024 – ustna prezentacja w j.angielskim - *"Sustainable Lipase Immobilization: Chokeberry and Apple Waste as Carriers"*.
4. 12 European Young Engineers Conference, Warsaw, Poland, 15-17.04.2024 – poster w j. angielskim - *"Development of chokeberry and apple pomace as matrices for immobilization of microbial lipases"* i ustna prezentacja w j.angielskim - *"The upcycling of spent coffee grounds and their application in the immobilization of microbial lipases"*.
5. Przeprowadzenie warsztatów „Jak popularyzować naukę" dla studentów i doktorantów w Szkole Głównej Gospodarstwa Wiejskiego w Warszawie, Polska, 23.01.2024 r. (w ramach projektu „Popularyzacja nie boli 2.0)
6. XXVII Sesja Naukowa Sekcji Młodej Kadry Naukowej „Rozwój Nauk o Żywności. Zrównoważona przyszłość"/ Xth International Session of Young Scientific Staff "Food Science Development. Sustainable future. Prezentacja pt: Odpady kawowe jako potencjalny nośnik do immobilizacji lipaz. 11-12.05.2023 Warszawa
7. The 3rd International Electronic Conference on Foods: Food, Microbiome, and Health, 01 – 15.10.2022 – proceedings paper – "*Investigating culture media for obtaining lipolytic biocatalysts based on Rhizopus oryzae fungi"*.
8. XXVI Sesja Naukowa Sekcji Młodej Kadry Naukowej Żywność dzisiaj – lokalna czy globalna? Tradycyjna czy innowacyjna? / IXth International Session of Young

Scientific Staff Nowadays food-local or global? Traditional or innovative? Prezentacja pt: Immobilizacja lipolitycznych preparatów enzymatycznych pochodzących z drożdży Yarrowia lipolytica. 19-20.05.2022, Poznań.

9. IV. Konferencja Doktorantów „Cztery Żywioły – współczesne problemy w naukach o życiu" – online. Prezentacja pt.": Naturalne nośniki stosowane w immobilizacji lipaz pochodzenia mikrobiologicznego. 14.12.2021.

NAGRODY I WYRÓŻNIENIA

1. II miejsce w konkursie „Popularyzacja nie boli" organizowanym przez Krajową Reprezentację Doktorantów oraz Ministerstwo Edukacji i Nauki za stworzenie e-booka popularnonaukowego dla studentów i doktorantów „Akademickie selfCare - jak zadbać o zdrowie w trakcie studiów"(2022)
2. Szkoła Główna Gospodarstwa Wiejskiego w Warszawie, Zespołowa Nagroda Naukowa II stopnia w 2022 r. i Zespołowa Nagroda Naukowa III stopnia w 2023 r. za działalność badawczą, Zespołowa Nagroda Naukowa III stopnia w 2024 za działalność badawczą, Warszawa, Polska

INNE OSIĄGNIĘCIA:

1. Kierownik projektu Preludium 24 pt. „Odpady spożywcze jako bioplatforma: nośniki do immobilizacji lipaz i źródło polifenoli w procesie enzymatycznej lipofilizacji związków bioaktywnych" (2026-2029)
2. Członek zespołu projektowego drugiej edycji "Popularyzacja nie boli", prowadząca warsztaty z zakresu popularyzacji nauki. Projekt tworzony przez KRD i finansowany ze środków Ministra Edukacji i Nauki w ramach Programu „Społeczna Odpowiedzialność Nauki II" (2024).
3. Członek Komitetu Organizacyjnego Sesji Naukowej Sekcji Młodej Kadry Naukowej „Rozwój Nauk o Żywności. Zrównoważona przyszłość"/ Xth International Session of Young Scientific Staff "Food Science Development. Sustainable future" 11-12 maja 2023.
4. Pomoc w organizacji Dni SGGW oraz Dni Otwartych Wydziału Technologii Żywności 2022 oraz Dni Otwartych SGGW 2023.
5. Członek Polskiego Towarzystwa Technologów Żywności (od 2021)
6. Członek Zespołu roboczego ds. mediów społecznościowych Wydziału Technologii Żywności SGGW (2023-2024)
7. Członek Zespołu Promocji i Projektów przy Radzie Doktorantów SGGW (2023-2024)
8. Członek Komisji ds. popularyzacji nauki Krajowej Reprezentacji Doktorantów (od 2022)
9. Opiekun projektu badawczego studentów należących do Koła Naukowego Biotechnologów KNBiotech, sekcji biokatalizy enzymatycznej, który otrzymał I i III nagrodę podczas 50 Przeglądu Dorobku Kół Naukowych SGGW (2023).

8. PUBLIKACJE STANOWIĄCE ROZPRAWĘ DOKTORSKĄ WRAZ Z OŚWIADCZENIAMI WSPÓŁAUTORÓW

antioxidants

MDPI

Review

Mini-Review on the Enzymatic Lipophilization of Phenolics Present in Plant Extracts with the Special Emphasis on Anthocyanins

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Abstract: Different plant extracts have the potential to be important sources of phenolic compounds. Their antibacterial, antifungal and antioxidant properties are of interest to researchers due to various possibilities for use in the pharmacy, cosmetic and food industries. Unfortunately, the direct application of phenolics in food is limited because of their hydrophilic nature and low solubility. The review is devoted to the recent advances in the methods of lipophilization of phenolic extracts along with the use of enzymes. The concept of extract modification instead of single compound modification is based on the expected synergistic effect of many phenolic compounds. The main focus is on the phenolic compounds found in fruits, flowers and leaves of different common and underutilized as well as medicinal, folk-medicinal or endemic plants. The compiled papers point to the great interest in the modification of anthocyanins, highly active but often unstable phenolics. Some examples of other flavonoids are also outlined. The possible applications of the lipophilized plant extracts are presented for improving the stability of edible oils, decreasing the content of acrylamide, exhibiting higher color stability in thermal processing and increasing the nutritional value.

Keywords: plant extracts; lipophilization; phenolic compounds; lipase; anthocyanins; esterification

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1. Introduction

Plant extracts have been used since ancient times. People were using phytonutrients and biologically active compounds long before they were described and their structures were discovered. Nowadays, it is assumed that approximately 20% of known plants have been applied in pharmaceutical studies and have positively affected human health [1]. Many plant origin substances have well-described antifungal, inflammatory, antibacterial and antitumor properties. It is well known that many plants are abundant in polyphenolic compounds; therefore, plant extracts are also a good source of these secondary plant metabolites. For example, vegetables, fruits, nuts, seeds, stems, flowers and roots are plentiful in polyphenols. To date, there are more than 8000 polyphenolic structures known, and for many years, research on their functional and therapeutic properties has been carried out [2]. The diversity in the quantity and specific structures of phenolics is observed between plants. They are a large and diverse group, including flavonoids, e.g., flavonols, flavanols, anthocyanins and nonflavonoids, e.g., phenolic acids (hydroxycinnamic and hydroxybenzoic acids), lignans and stilbenes [3]. Phenolic compounds are of general interest because of their ability to be very effective in free radical scavenging in foods. They can easily donate hydrogen or electrons to convert free radicals into more stable and safe compounds [4]. Food technologists are trying to fortify groceries with phenolics which can serve as antioxidants, but their use in lipophilic matrices is limited due to their hydrophilic nature and low solubility. It is necessary to derive them into the more amphiphilic form

which let them keep their original functional properties. The improvement can be made by a modification defined as lipophilization. This term encompasses chemically or enzymatically esterifying the appropriate group of a phenolic compound with a fatty alcohol or a fatty acid [4,5]. In the case of phenolic acids, this will be esterification with aliphatic alcohols. Flavonoids, which are usually present in nature in the form of glycosides, can be lipophilized by esterification of the primary (predominantly) alcohol group in the sugar molecule with fatty acids. It should be noted that this reaction does not concern phenolic groups and therefore should not adversely affect the antioxidant potential of the esterified compounds (Figure 1). The esterification of phenolic compounds can be performed by chemical or enzymatic methods. The latter is especially recommended because the use of biocatalysts allows for applying milder conditions, assures selective specificity and minimizes side reactions and formation of by-products. Enzymatic reactions are more environmentally friendly because of the reduced amount of energy consumption and waste material production. Sometimes these two methods are applied alongside in chemoenzymatic lipophilization, which can be carried out in two ways: under solvent or solvent-free conditions [6].

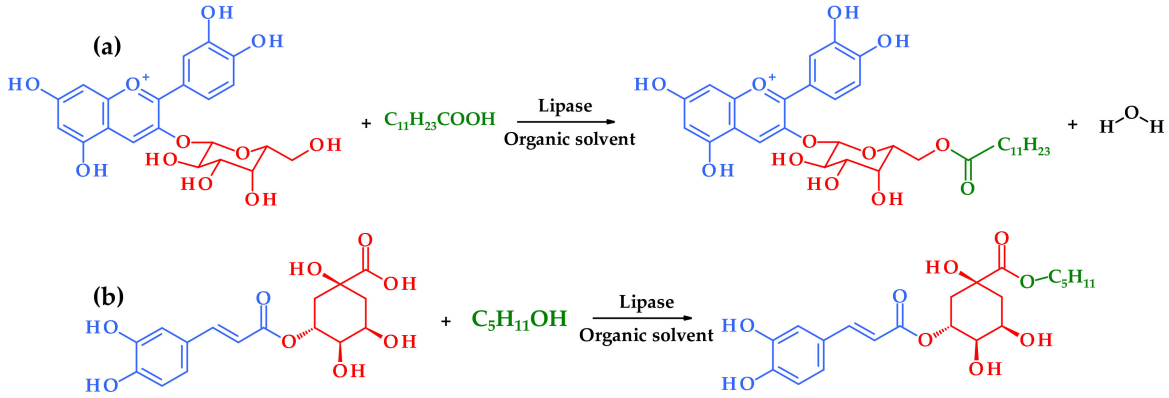

Figure 1. Enzymatic synthesis of (**a**) cyanidin-3-*O*-(6″-dodecanoyl)galactoside, and (**b**) pentyl chlorogenate. The phenolic moiety is highlighted in blue, sugar moiety and quinic acid are in red, and lipophilic molecules (fatty acid/alcohol) are marked in green.

Improving the solubility of phenolics in organic media is performed often as a single compound modification [5,7]. However, plant extracts have been found to generally show better antioxidant properties than most pure phenols, which may suggest a synergistic interaction of antioxidants with each other [8].

Using whole plant extracts, which contain different kinds of phenolic compounds, and verifying their synergistic effect is still a novel approach. It is obvious that extracts from the individual types of fruits differ in the content of various antioxidants and their antioxidant activity is related to the content of the type of individual phenolic compounds. Therefore, the method of lipophilization depends on the composition of the extract [9].

This review aims to summarize the current knowledge concerning enzymatic lipophilization of plant extracts. Recent advances in the methods of enzymatic modification of phenolic extracts and investigated enzymes as well as exerted biological activities, including nutritional and pharmaceutical applications, are discussed. In the paper, we present results for enzymatic modifications of phenolic compounds occurring in multi-compound mixtures (whole-plant extracts or extracts from parts of plants) as well as purified mono-component phenolic extracts. The main group of phenolic derivatives via enzymatic lipophilization are anthocyanins and other flavonoids.

2. Lipophilization of Extracts from Fruits

Extracts of fruits such as grapes, raspberries, cherries, blackberries, blueberries, apples or rowan are characterized by a wide spectrum of antioxidant properties and contain many

beneficial substances, especially polyphenols, which are one of the essential groups of compounds in secondary metabolism.

Rowan (*Sorbus aucuparia* L.) is certainly an interesting source of phenolic compounds. The ripe fruits of rowan are spherical with orange to red color and tart-bitter in taste. The major constituent of rowan phenolic extract is chlorogenic acid, which is amounted to approximately 80% of phenolics. In smaller quantities, there are neochlorogenic and feruloylquinic acids present, which are isomers and derivatives of chlorogenic acid, respectively [10].

Due to the poor lipophilic character of rowan phenolic extract, Aladedunye et al. [10] changed its lipid solubility by lipase-catalyzed esterification of the carboxyl group of the phenolic acid with aliphatic alcohol-octadecanol. In the enzymatic reaction octadecyl chlorogenate (Figure 2a) was obtained and subsequently was evaluated as an antioxidant agent in frying and storage tests.

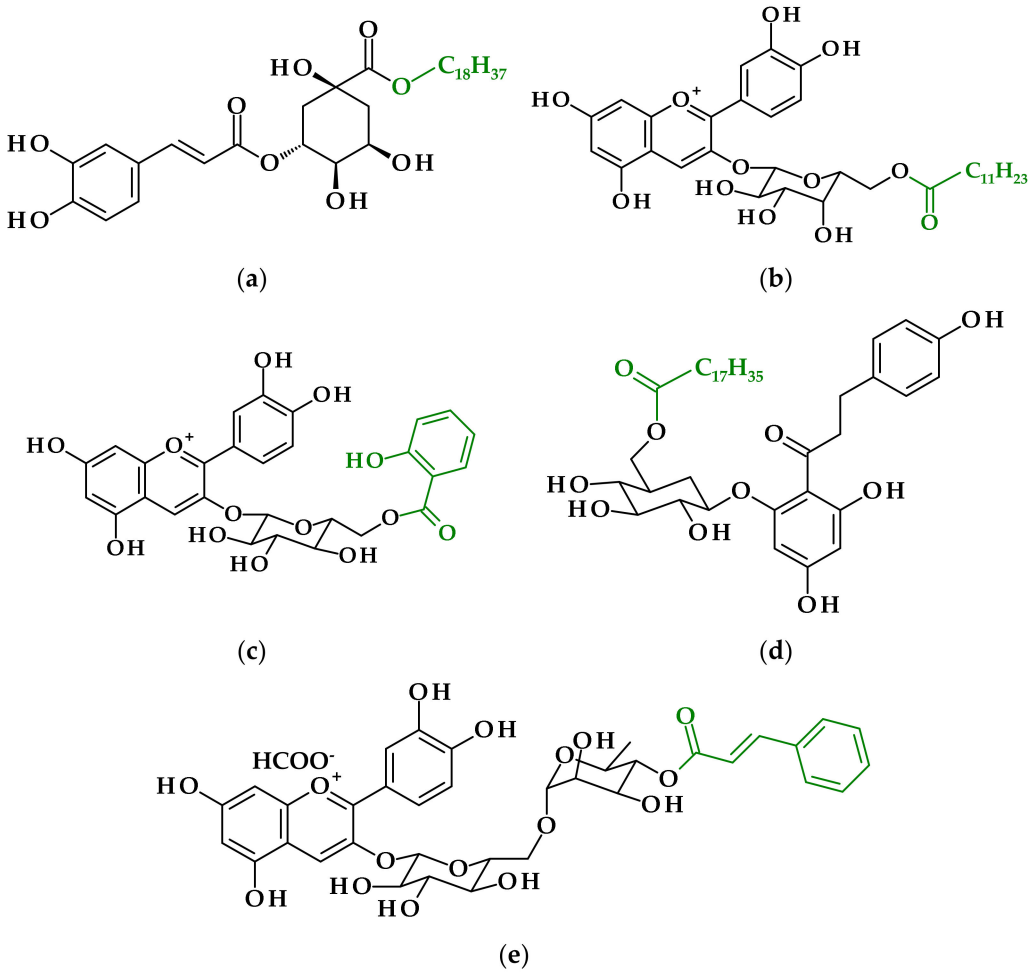

Figure 2. Phenolic derivatives from lipophilized extracts of fruits: (**a**) octadecyl chlorogenate derivative of chlorogenic acid-depside of caffeic acid and quinic acid [10]; (**b**) cyanidin-3-*O*-(6′′-dodecanoyl)galactoside derivative of anthocyanins (glycosides of anthocyanidins) [11]; (**c**) cyanidin-3-(6-salicyloyl)glucoside–derivative of anthocyanins (glycosides of anthocyanidins) [12]; (**d**) phloridzyl octadecenoate-derivative of dihydrochalcone glucoside [13]; (**e**) cyanidin-3-*O*-(4′′′-cinnamoyl)rutinoside derivative of anthocyanins (glycosides of anthocyanidins) [14].

The presence of chlorogenic acid in rowan phenolic extract, and more specifically a catechol ring, resulted in an antioxidant activity and increasing the oxidative stability of oils. In the storage test, both purified natural extract (PNE) and its lipophilized form (LE) inhibited the primary oxidation, which was observed by lower peroxide values compared with the control (oil without addition of any compounds). Interestingly, there were no significant differences between PNE and LE. On the contrary, the incorporation of the

octadecyl chain to the structure of chlorogenic acid impacted on oil protection during the deep frying of potato chips. Additionally, in comparison with the control and non-modified rowan extract formation of polar components and di- or polymeric triacylglycerols was remarkably inhibited. The abovementioned paper suggests the feasibility of developing functional antioxidants with the use of wild/ornamental edible fruits [10].

Fruits can stand as a good source of anthocyanins, which are glycosides of anthocyanidins that are based on the flavylium cation with hydroxyl and methoxyl groups substituted for its hydrogen atoms. The vast majority of anthocyanins are derived from six aglycones: cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin [11]. Anthocyanins are the main water-soluble class of flavonoids and occur in vegetables such as red onion, red cabbage and sweet potatoes, as well as in fruits, e.g., strawberries, raspberries and cranberries [15]. They are also present in flowers and can absorb visible light. Those natural pigments are responsible for flower or other plants' color with the range from red to blue, which result in using them as described by the authors of [16]. The unique properties of described compounds are attributed to their structures. They have shown diverse biological activity such as antioxidant, antimicrobial and anti-inflammatory properties; moreover, they have played a role in cardiovascular protection and obesity prevention, and are capable of decreasing serum triglyceride [11,14,15]. Nonetheless, the use of anthocyanins is limited because their instability and hydrophilic properties restrict their application in lipidic media. Mentioned compounds are sensitive to pH values, light, heat or ion concentration. The physiological activity is also hindered due to the poor solubility in fat, that they cannot pass through the lipid bilayer [17]. Anthocyanin stability can be improved, for example, through esterification that can be achieved by chemical or enzymatic methods. Definitely, the latter is performed in mild conditions and with high selectivity, without hazardous catalysts or appearing as by-products [14,16].

In the research by Yang et al. [11], anthocyanins isolated from alpine bearberry (*Arctostaphylos alpina*) were enzymatically acylated through hydroxyl group in sugar moiety with lauric, myristic, palmitic and stearic acids, respectively, with the use of *C. antarctica* lipase B (CALB) as a catalyst. A saturated fatty acid with 12 carbons proved to be the best acyl donor to synthesize ester of cyanidin-3-*O*-galactoside (Figure 2b) and the conversion rate amounted to 73% when *tert*-butanol was a solvent, the temperature was 60 °C for 72 h and the molar ratio of anthocyanin to lauric acid was 1:10.

Interestingly, the introduction of lauric acid into the cyanidin-3-*O*-galactoside structure improved thermo-stability as well as lipophilicity by determining the octanol/water partition coefficient (logP). Comparison of antioxidant activity of cyanidin-3-*O*-galactoside and its lauric ester assessed by DPPH· and FRAP assays revealed that acylation only slightly affected the antioxidant capacity, and activity of lauric ester measured in the hydrophilic system was largely retained [11].

Blackcurrant (*Ribes nigrum* L.) is plentiful in anthocyanins, which are natural colorants and bioactive ingredients. Using them as fruit extracts has some barriers to being successfully applied in food, cosmetic and pharmaceutical industries because of the hydrophilic nature of phenolic compounds. Cruz's group [18] tried to improve the lipophilicity and enlarge the application of blackcurrant anthocyanins by enzymatic acylation using *Candida antarctica* lipase B. They extracted anthocyanins from waste blackcurrant (*Ribes nigrum* L.) fruit skins which contained rutinosides and glucosides of cyanidin and delphinidin and used them as a substrate in reaction with octanoic acid. The research showed that cyanidin and delphinidin glucosides were acylated, whereas the rutinosides were not. This was probably caused by the absence of a primary hydroxyl group in the disaccharide moiety. The lipophilization method was proposed as a new technique to separate anthocyanins with different glycosylation patterns in mixtures otherwise difficult to separate [18]. Furthermore, Yang et al. [19] enzymatically acylated anthocyanin-rich fractions isolated from blackcurrant (*Ribes nigrum* L.) with lauric acid. They successfully monoacylated delphinidin-3-*O*-glucoside, delphinidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-rutinoside showing that there is a possibility to acylate rutinose moieties of

anthocyanins. In the case of rutinosides, esterification occurred at 4-OH of the rhamnose unit. According to the authors, the discrepancy with the Cruz results comes from using different reaction conditions and acylating agents. Conducted reactions enhanced lipophilicity and thermostability, as well as improved the inhibition of lipid peroxidation in a lipophilic environment. Yang et al. [19] noticed also that more hydrophilic anthocyanin rutinosides after esterification were more lipophilic than glucosides of the same anthocyanidins [19].

Raspberry (*Rubus* L.) belongs to the same group of fruit as blackcurrant so it is very rich in anthocyanins. Therefore, Teng et al. [12] acylated anthocyanins present in an extract from raspberry with methyl salicylate via lipase-catalyzed reaction under reduced pressure and achieved a conversion rate of 84.26%. The product of the reaction was cyanidin-3-(6-salicyloyl)glucoside, and the few analyses of it were obtained (Figure 2c). The researchers found that enzymatic acylation could be a helpful way for developing the processing stability of anthocyanins and also to maintain their powerful antioxidant capacity [12].

Stevenson et al. [20] chose a blueberry extract and apple extract. They used immobilized *Candida antarctica* lipase B to acylate flavonoid glycosides from fruit with carboxylic acids. Depending on the kind of acyl donors (palmitic, cinnamic and phenylpropionic acids) the conversions ranged from ~25% to ~95%. The enzymatic lipophilization of tested extracts, which were a good source of flavonoids, presented strong selectivity for acylation of glucosides with a primary aliphatic hydroxyl group on the sugar moiety, such as phloridzin and anthocyanidin glucosides and galactosides. For compounds lacking a primary aliphatic hydroxyl group, such as flavonoid aglycones, chlorogenic acid or anthocyanidin arabinosides, no acylation was noticed. They observed that acylation, especially with palmitic acid, enhanced lipophilicity and solubility in the reaction medium [20].

Aladedunye and Matthäus [13] also tried to take advantage of apple extract. They used it for the study of *Canadian crabapple* and reported that the major polyphenolic component of the extract was phloridzin, which includes seven hydroxyl functional groups, but only the primary aliphatic group in glucose moiety was active. Enzymatic acylation was carried out with octadecanoic acid by *Candida antarctica* lipase B. The phloridzyl octadecanoate (Figure 2d) was a product of the reaction and after lipophilization was added to rapeseed oil to assess the antioxidant activity of the modified extract during storage and frying. Results showed that attachment of the octadecyl tail enhanced the antioxidant performance of phloridzin in rapeseed oil during the frying of potato chips but had no relevant impact on the oxidative stability of the oil under storage conditions [13].

The significant potential of grape seed was discovered by Chen and Yu [21]. Proanthocyanidins, which are major polyphenol components in grape seed, were modified structurally to improve their lipophilicity using esterification catalyzed by immobilized lipase Lipozyme TL IM with lauric acid. The outcome showed that the conversion rate was 84.1%. The presence of GSP (grape seed proanthocyanidin) derivatives was confirmed by HPLC-MS-MS. It was found that GSP derivatives had higher lipophilicity than GSP and gained the highest DPPH radical scavenging activity compared to GSP, BHT (butylated hydroxytoluene) and TBHQ (*tert*-butylhydroquinone). The researchers also proved that GSP derivatives were nontoxic so this indicated its potential addition to food as an oil-soluble antioxidant. It is worth underlining that the authors used Lipozyme TL IM as a biocatalyst instead of the commonly used CALB. Moreover, it was unusual that there were esterified hydroxyl groups present in the phenolic ring and despite the lower number of hydroxyl groups in the modified compound, the antioxidant effectiveness was higher than GSP [21].

The work of Fernandez-Aulis et al. [14] showed a study on the preparation of a series of acylated anthocyanins from five purified extracts from underutilized plants. Among the series of reactions carried out, several of them clearly differ from the above-cited papers. Cyanidin glycoside from trueno fruits (*Ligustrum japonicum*) was subjected to transesterification with vinyl cinnamate and cyanidin-3-*O*-(4‴-cinnamoyl)rutinoside (Figure 2e) and was synthesized at a 45.5% conversion yield [14]. The usage of vinyl esters in the enzymatic reactions is a well-known way to increase the conversion yield due to

the shifting of the equilibrium state towards the formation of the product because of the tautomerization of vinyl alcohol to acetaldehyde [22].

The fruits mentioned above are well known because of their attractive biofunctional properties and high content of flavonoids and other polyphenols. However, other examples that are not as popular as the berry fruit group, but share the same features, can be found on the planet. In the first instance, jaboticaba (*Myrciaria cauliflora*) can be provided, which easily grows in tropical regions of the planet, especially in Brazil, but also in Argentina, Bolivia, Paraguay and Peru. De Castro et al. [23] extracted polyphenols from the skin of jaboticaba fruits and then performed enzymatic acylation using palmitic acid as an acyl donor and lipase B from *Candida antarctica* (CALB) as a biocatalyst. The researchers analyzed antioxidant properties and content of the fraction of phenolic compounds. They detected the presence of ellagic acid, quercetin, rutin, delphinidin-3-glucoside and cyanidin-3-glucoside and confirmed that the activity of the jaboticaba peel extracts were at least as high as those reported in the literature for several more known fruits. Due to enzymatic modification, it was possible to obtain two anthocyanin derivatives, namely, delphinidin-3-*O*-(6′′-palmitoyl)glucoside and cyanidin-3-*O*-(6′′-palmitoyl)glucoside [23]. The second example can be jambolan (*Syzygium cumini*), a tropical, edible fruit with a deep purple peel when ripe, native to the Indian subcontinent. It has an attractive color due to the presence of anthocyanins (mainly delphinidin-3,5-diglucoside and petunidin-3,5-diglucoside [24], especially in its peel part and is a novel source of natural colorants for the food system. For this reason, Sari et al. [25] tried to acylate anthocyanins of jambolan fruit. They enzymatically synthesized derivatives of phenolic compounds by lipase-catalyzed transesterification reaction with cinnamic acid. The results showed that acylation with chosen acid gave a change of color from red to purplish-red in the beverage model system, pH = 3 and increased thermal and light stability of modified anthocyanins. Unfortunately, enzymatic lipophilization partially declined antioxidant activity [25]. The data on the use of fruit extracts in the synthesis of lipophilic antioxidants are summarized in Table 1.

Table 1. Plant extracts from fruits used in enzymatic modifications of phenolic compounds.

The Origin of the Plant Extract	Main Components of the Extract	Used Enzyme	Reaction Conditions	The Obtained Ester(s)	Research Highlights	Reference
Rowan (*Sorbus aucuparia* L.)	Chlorogenic acid	*Candida antarctica* lipase B	55 °C, 120 h, 2-methyl-2-butanol as a solvent, molar ratio of 1:20 (acid:alcohol)	Octadecyl chlorogenate	(a) A 43% decrease in peroxide value of rapeseed oil after fortifying with octadecyl chlorogenate. (b) Inhibition of triacylglycerols polymerization and polar compound formation during potato chip frying due to the addition of lipophilized extract.	[10]
Alpine bearberry (*Arctostaphylos alpina*)	Cyanidin-3-*O*-galactoside	*Candida antarctica* lipase B	60 °C, 72 h, *tert*-butanol as a solvent and the molar ratio of anthocyanin to lauric acid was 1:10	Cyanidin-3-*O*-(6′′-dodecanoyl)galactoside	(a) Improved thermostability of the obtained ester. (b) Improved lipophilicity (higher logP value).	[11]
Blackcurrant (*Ribes nigrum* L.)	Delphinidin-3-*O*-rutinoside, Cyanidin-3-*O*-rutinoside, Delphinidin-3-*O*-glucoside, Cyanidin-3-*O*-glucoside	*Candida antarctica* lipase B	60 °C, 9 h, acetonitrile:DMSO 10:1 (*v*/*v*), 20 g/L of enzyme, molecular sieves (100 g/L), a anthocyanins:octanoic acid ratio of 1:100	Cyanidin-3-*O*-(6′′-octanoyl)glucoside, Delphinidin-3-*O*-(6′′-octanoyl)glucoside	(a) Lipophilization as a new anthocyanin separation technique with different glycosidic moieties.	[18]
Blackcurrant (*Ribes nigrum* L.)	Delphinidin-3-*O*-rutinoside, Cyanidin-3-*O*-rutinoside, Delphinidin-3-*O*-glucoside, Cyanidin-3-*O*-glucoside	*Candida antarctica* lipase B	60 °C, 72 h, *tert*-butanol as a solvent and the molar ratio of anthocyanin to lauric acid was 1:10, 10 g/L of enzyme, molecular sieves (100 g/L)	Cyanidin-3-*O*-(6′′-dodecanoyl)glucoside, Delphinidin-3-*O*-(6′′-dodecanoyl)glucoside, Cyanidin-3-*O*-(6′′-dodecanoyl)rutinoside, Delphinidin-3-*O*-(6′′-dodecanoyl)rutinoside,	(a) Improved thermostability due to the enzymatic acylation. (b) Acylation significantly improved inbitition of lipid peroxidation in the β-carotene bleaching method.	[19]

Table 1. *Cont.*

The Origin of the Plant Extract	Main Components of the Extract	Used Enzyme	Reaction Conditions	The Obtained Ester(s)	Research Highlights	Reference
Trueno (*Ligustrum japonicum*) fruits	Cyanidin-3-*O*-rutinoside	*Candida antarctica* lipase B	60 °C, 48 h, 300 rpm, 20 g/L of enzyme, *tert*-butanol as a solvent, a cyanidin:vinyl cinnamate ratio of 1:250, molecular sieves (100 g/L)	Cyanidin-3-*O*-(4‴-cinnamoyl)rutinoside	(a) Optimized synthesis methodology with 45.5% conversion yield.	[14]
Raspberry (*Rubus* L.)	Cyanidin-3-*O*-glucoside	Novozym 435 (*Candida antarctica* lipase B)	40 °C, 24 h, pyridine (10 mL), 0.9 KPa of pressure (rotary evaporator), 10 mg of purified anthocyanins, 500 mg of methyl salicylate, 200 mg of Novozym 435	Cyanidin-3-(6-salicyloyl)glucoside	(a) Acylation improved thermostability and stability in the light and oxidation environments.	[12]
Penglai apple polyphenolic extract	Phloridzin	Novozym 435 (*Candida antarctica* lipase B)	60 °C, 168 h, 20 mg of fruit extract, acyl donor substrate (2 molar equivalents), 100 mg of enzyme, 100 mg of molecular sieves, *tert*-butanol as a solvent (1 mL, containing 0.2% of BHT)	Phloridzyl palmitate, Phloridzyl 4-hydroxyphenylpropionate, Phloridzyl 2-hydroxyphenylpropionate, Phloridzyl 3,4-dihydroxyphenylpropionate, phloridzyl cinnamate, Phloridzyl 3-phenylpropionate	(a) Acylation improved the solubility of the obtained derivatives in the reaction solvent.	[20]
Blueberry extract	Chlorogenic acid, Quercetin-3-glycosides, Delphinidin, cyanidin, petunidin and malvidin glycosides	Novozym 435 (*Candida antarctica* lipase B)	60 °C, 168 h, 20 mg of fruit extract, acyl donor substrate (2 molar equivalents), 100 mg of enzyme, 100 mg of molecular sieves, *tert*-butanol as a solvent (1 mL, containing 0.2% of BHT)	3-phenylpropionate esters of quercetin, isoquercetin, and delphinidin, cyanidin, petunidin and malvidin glycosides	(a) Novozym 435 showed selectivity for a primary aliphatic hydroxyl group of the sugar moiety in the acylation of different groups of polyphenols.	[20]
Canadian crabapple	Phloridzin	*Candida antarctica* lipase B	55 °C, 120 h, 250 rpm, 2-methyl-2-butanol (10 mL) as a solvent, 1000 mg of enzyme, molecular sieves (1000 mg), phenolic extract (750 mg), octadecanoic acid (acyl donor, 1500 mg)	Phloridzyl octadecanoate	(a) Improved stability of the rapeseed oil during potato chip frying (inhibition of polymerization of triacylglycerols and polar components formation). (b) Higher amount of tocopherols presented in deep fried potato chips in the oil with the addition of phloridzyl octadecanoate.	[13]
Grape seeds (GSP)	Epicatechin, Procyanidin B1, and other 9 phenolic compounds	Lipozyme TL IM (immobilized lipase from *Thermomyces lanuginosus*)	45 °C, 22 h, a ratio of lauric acid:grape seeds extract of 1:1, enzyme (2%), ethanol as a solvent	Lauroyl epicatechin, Tri-lauroyl epicatechin gallate, Lauroyl catechin	(a) GSP derivatives had the highest DPPH· scavenging activity compared to GSP, BHT (butylated hydroxytoluene) and TBHQ (*tert*-butylhydroquinone)	[21]
Skin of jaboticaba (*Myrciaria cauliflora*) fruits	Delphinidin-3-*O*-glucoside and Cyanidin-3-*O*-glucoside	Novozym 435 (*Candida antarctica* lipase B)	50 °C, 48 h, 600 rpm, 200 mbar, 20 g/L of enzyme, 20 mg of jaboticaba extract, palmitic acid as an acyl donor (2 molar equivalents), 2-methyl-2-butanol as a solvent (5 mL)	Delphinidin-3-*O*-(6″-palmitoyl)glucoside, Cyanidin-3-*O*-(6″-palmitoyl)glucoside	(a) Acylation increased the hydrophobicity of anthocyanins.	[23]
Jambolan (*Syzygium cumini*) fruits	Anthocyanins	*Candida antarctica* lipase B	40 °C, 48 h, acetone with 10% of DMSO as solvents, vinyl cinnamate as an acyl donor	Cinnamate esters of anthocyanins	(a) Higher thermal and light stability in the acylated anthocyanins compared to the native anthocyanins.	[25]

3. Enzyme-Assisted Derivatization of Phenolics Present in Flower Extracts

Flower extracts are also a natural source of bioactive compounds providing, for example, antioxidant or anti-inflammatory properties, but focus on them has been as natural dyes, widely used to produce natural cosmetics and foods. Plant pigments are classified into four main categories: chlorophylls, anthocyanins, carotenoids and betalains.

Among them, anthocyanins are of particular interest and colored anthocyanin pigments have been traditionally used as natural food colorants. Unfortunately, as mentioned in the case of fruit anthocyanins, the color and stability of these pigments are influenced by pH, light, temperature and structure. In acidic conditions, anthocyanins appear red but turn blue when the pH increases. Therefore, both solubility and resistance to changes under acid pH are important [26,27].

The possibility of increasing the lipophilicity of anthocyanins derived from flower extracts was studied by Marquez-Rodriguez et al. [16]. Delphinidin 3-*O*-sambubioside was extracted and purified from *Hibiscus sabdariffa* flowers, and then was subjected to enzymatic acylation with octanoic acid. Due to the hydrolysis occurring in the disaccharide residue, the reaction conditions had to be optimized. Two solvents, the ratio of substrates, enzyme concentrations and three different counter-ions, were evaluated. The highest conversion yield (15%) with no observed hydrolysis was achieved when 2-methyl-2-butanol was a solvent, and delphinidin 3-*O*-sambubioside was applied with formate as counter-ions.

The physicochemical characterization of the new synthesized lipophilic pigment, such as partition coefficient and color properties, were investigated. Acylation of delphinidin 3-*O*-sambubioside improved the lipophilicity of the obtained compound, as well as improved the stability by hydration resistance and presented a stabilization of the quinoidal base, one of the equilibrium forms of flavylium cation, responsible for blue color at a neutral or moderate alkaline pH. The presented results show the possibility of synthesizing and the potential use of natural-based lipophilic pigments in different industries [16].

Marathe et al. [15] observed that flower petal waste is generated in tremendous quantities in places of worship, and such waste is usually disposed of in wastelands. Rose petals occurred more often. Roses are definitely a good source of anthocyanins, and due to their low stability and sensitivity to different conditions, the authors proposed carrying out esterification reactions as an idea to partially reduce the formation of organic waste and improve the physicochemical properties and biological activity of flavonoid compounds present in rose petals.

Similar to other authors, lauric acid was used as an acyl donor, and molar conversion of 63% of anthocyanins to laurate esters was reached when the temperature of 40 °C, acetonitrile, a molar ratio of 1:100 (cyanidin to a fatty acid), *C. antarctica* lipase, and molecular sieves were used. Isolated anthocyanins and their lauric acid esters were compared in the $DPPH^{\bullet}$, $ABTS^{\bullet+}$ and FRAP assays to assess their antioxidant activity. Three mentioned tests revealed that antioxidant activity of acylated anthocyanins was significantly reduced in comparison with anthocyanins that were not subjected to esterification, but it can be linked to the higher lipophilic character of the obtained esters as well as partial degradation of anthocyanins during the reaction. The use of anthocyanin lauric acid esters was studied as a colorant in cupcakes and in filling cream to sandwich biscuits as well as a pre-extrusion colorant in puffed rice extrudates. Likewise, to their precursors, the color of laurate esters of anthocyanins was pH-dependent, which was confirmed at varying pH using a colorimeter and in the color of cupcakes. Better thermo-stability of biscuit cream filling was also observed when the products of esterification were used compared to native anthocyanins and synthetic colorants. Similar observations were provided by the authors in extrusion experiments, and the laurate esters exhibited higher color stability in thermal processing. Enzymatic esterification of anthocyanins from rose petals and confirmation of their better thermal stability and other properties may in the near future affect their consideration as natural-based colorants in the food industry [15].

Fernandez-Aulis et al. [14] acquired cyanidin glycosides from bottlebrush (*Callistemon citrinus*) flowers from trees growing in the streets of Mexico City, which are also used in traditional medicine. It may be that the content of isolated anthocyanins was not very high, namely 6.15 ± 0.39 g/g DW for bottlebrush flower, but cyanidin-3-*O*-(6″-cinnamoyl)glucoside-5-*O*-glucoside was obtained (Figure 3a). In the enzymatic reactions, vinyl cinnamate and cinnamic, dihydrocinnamic, dihydroferulic and dihydrosinapic acids were used as acyl donors, and some new acylated derivatives were obtained with improved

antioxidant activity and thermostability, in comparison to the cyanidin-3-glucoside [14]. Enzyme-assisted derivatizations of phenolics present in flower extracts are summarized in Table 2.

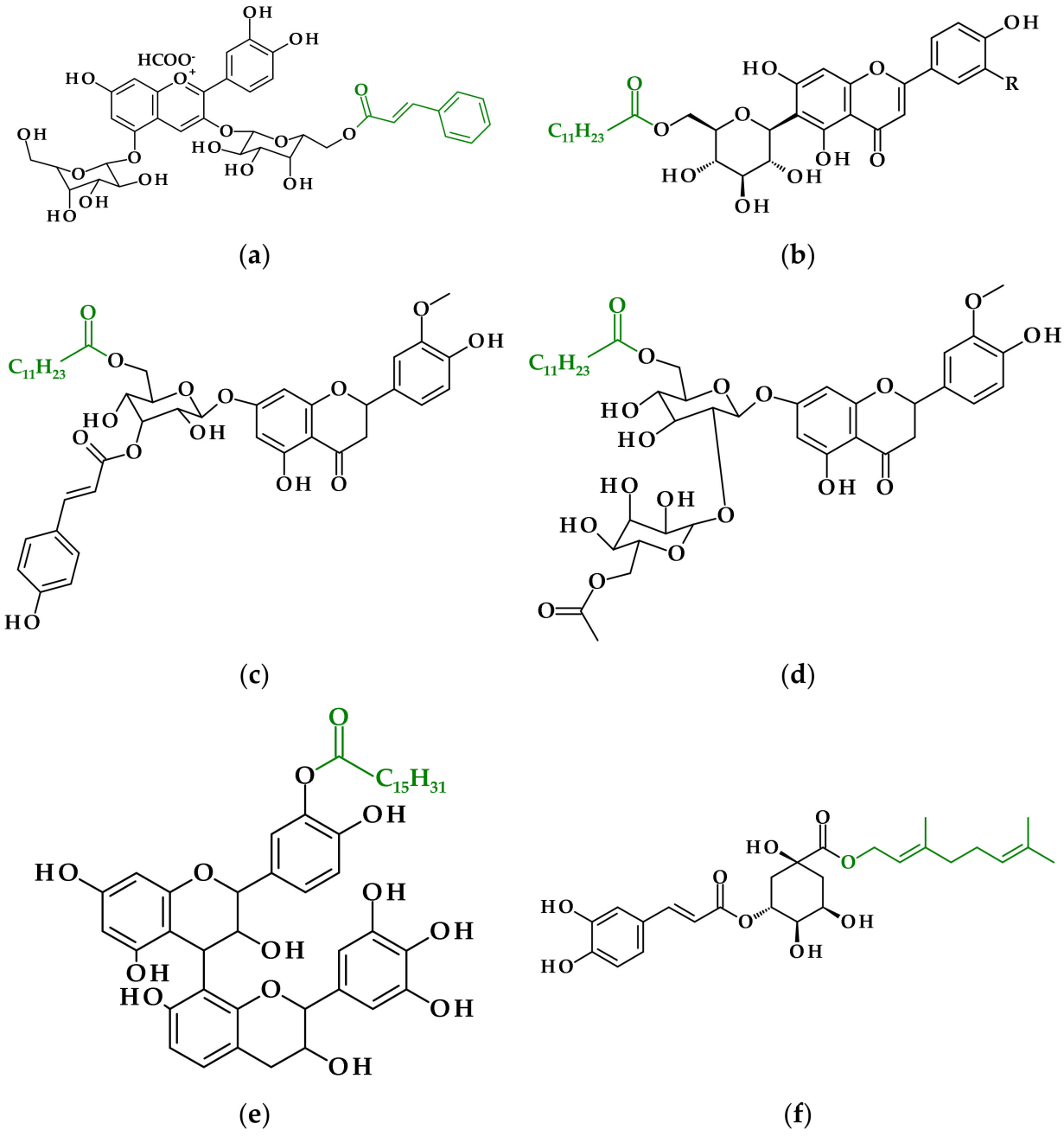

Figure 3. Phenolic derivatives from lipophilized plant extracts other than fruits extracts: (**a**) cyanidin-3-*O*-(6″-cinnamoyl)glucoside-5-*O*-glucoside-derivative of anthocyanins (glycosides of anthocyanidins) [14]; (**b**) isoorientin-6″-laurate/isovitexin-6″-laurate-derivative of flavonoid C-glycosides [28], isoorientin: R = OH, isovitexin: R = H; (**c**) laurate ester of chrysoeriol-7-*O*-β-D-(3″-*E*-*p*-coumaroyl)-glucopyranoside-derivative of monosaccharidic flavone [29]; (**d**) laurate ester of chrysoeriol-7-[6‴-*O*-acetyl-β-D-allosyl-(1→2)-β-D-glucopyranoside]–derivative of disaccharidic flavone [29]; (**e**) procyanidin and prorobinetinidin heteroduplex esterified with palmitic acid–derivative of proanthocyanidin [17]; (**f**) geranyl chlorogenate–derivative of chlorogenic acid [30].

Table 2. Plant extracts from flowers and leaves used in enzymatic modifications of phenolic compounds.

The Origin of the Plant Extract	Main Components of the Extract	Used Enzyme	Reaction Conditions	The Obtained Ester(s)	Research Highlights	Reference
Hibiscus sabdariffa flowers	Delphinidin 3-*O*-sambubioside	*Candida antarctica* lipase B	60 °C, 48 h, 20 g/L of enzyme, 2-methyl-2-butanol as a solvent, molecular sieves (100 g/L), a anthocyanin:octanoic acid ratio of 1:250	Octanoic acid ester of delphinidin-3-*O*-sambubioside	(a) Stable quinoidal base with blue color at a wide range of pH.	[16]
Bottlebrush (*Callistemon citrinus*) flowers	Cyanidin-3,5-*O*-diglucoside	*Candida antarctica* lipase B	60 °C, 48 h, 300 rpm, 20 g/L of enzyme, *tert*-butanol as a solvent, a cyanidin:vinyl cinnamate ratio of 1:250, molecular sieves (100 g/L)	Cyanidin-3-*O*-(6′′-cinnamoyl) glucoside-5-*O*-glucoside	(a) Optimized synthesis methodology with 85.7% conversion yield.	[14]
Rose petals	Cyanidin-3,5-*O*-diglucoside	Fermase CALB™10000 (lipase B from *Candida antarctica* immobilized on polyacrylate beads)	40 °C, 24 h, acetonitrile as a solvent, 20 mg/mL of enzyme, a cyanidin:lauric acid molar ratio of 1:100, molecular sieves (100 mg/mL)	Lauryl monoesters of cyanidin-3,5-*O*-diglucoside	(a) Enhanced color stability in thermal processing of rice extrudates. (b) The use of esterified anthocyanins as a colorant in cupcakes and as a filling in sandwich biscuit cream.	[15]
Bamboo leaves	Isoorientin and Isovitexin	*Candida antarctica* lipase B	65 °C, 48 h, molecular sieves (100 mg/mL), 10 g/L of enzyme, 2-methyl-2-butanol as a solvent, the acyl donor/flavonoid molar ratio of 5:1 or 10:1	Isoorientin-6′′-laurate, Isovitexin-6′′-laurate	(a) The lipophilicity was improved due to the acylation, and simultaneously, reduction in the antioxidant activity was observed.	[28]
Bamboo leaves	Isoorientin	Novozym 435 (*Candida antarctica* lipase B)	60 °C, 48 h, 210 rpm, molecular sieves (140 mg/mL), 12 g/L of enzyme, 2-methyl-2-butanol as a solvent, the acyl donor/flavonoid molar ratio reached of 12:1	Isoorientin-6′′-palmitate	(a) High yield of the acylation (90%). (b) Acylation improved lipophilicity, but the antioxidant activity of the obtained derivative was lower.	[31]
Bamboo leaves	Orientin, Isoorientin, Vitexin, and Isovitexin	Novozym 435 (*Candida antarctica* lipase B)	65 °C, 48 h, 2-methyl-2-butanol as a solvent (100 mL), bamboo leaves extract (2.5 g–4.5 mM of falvonoids), 4.51 g of lauric acid (22.5 mM), a ratio of acid:extract of 5:1, 10 g of enzyme, 100 mg/mL of molecular sieves	Orientin-6′′-laurate, Vitexin-6′′-laurate, Isoorientin-6′′-laurate, Isovitexin-6′′-laurate	(a) Acylated antioxidants from bamboo leaves in the concentration of 0.05% and 0.1% inhibited the formation of acrylamide during potato chip frying.	[32]

4. Lipophilization of Extracts from Plant Leaves

Enzymatic acylation was applied to improve solubility in lipidic matrices or lipophilic media of flavonoids present in the bamboo-leaf extract. Ma et al. [28] focused on the enzymatic acylation of two flavonoids from bamboo-leaf extract-isoorientin and isovitexin, with three fatty acids as acyl donors (lauric C12, myristic C14 and palmitic C16 acids). The acylation in the presence of CALB occurred at the primary hydroxyl group of glucose moiety and only monoesters were detected. The highest conversion yields exceeding 75% were achieved when lauric acid was used in *tert*-amyl-alcohol as the reaction medium. The partition coefficients of acylated derivatives, which are attributable to their lipophilicity, increased with elongation of the acyl chain length [28]. Palmitoyl isoorientin ester was also synthesized via lipase-catalyzed esterification, whereby the product was isolated in a high purity (>95%) and conversion yield of 90% in a system containing dried *tert*-amyl alcohol. Several factors were investigated in depth, with the effect on the performance of the acylation reaction. The optimal conditions seemed to be a lipase amount of 12 g/L, a temperature of 60 °C and molecular sieves of 4 Å, which amounted to 166 g/L [31].

Ma's group continued their work on bamboo-leaf flavonoid lipophilization in order to enhance glycosylated flavonoids' inhibitory activity toward the formation of acrylamide in a lipidic food system. In China, the antioxidant of bamboo leaves as a natural food additive was approved in 2007; it has also been reported that it can effectively reduce acrylamide formation in model food systems. The antioxidant extract from bamboo-leaves, which consist of 80% (*w*/*w*) flavonoids (two pairs of position isomers-13% orientin, 39% isoorientin, 5% vitexin and 22% isovitexin), was enzymatically acylated with lauric acid (Figure 3b). Results showed that 0.05% and 0.1% solution of acylated antioxidant extract significantly reduced the content of acrylamide in potato crisps by 44.5%, and 46.9%, respectively and this extract was more efficient for inhibiting acrylamide formation than the non-modified. According to the authors, bamboo-leaf flavonoid esters scavenged reactive carbonyls formed via Maillard reactions responsible for acrylamide formation in foods [32].

Moreover, it should be pointed out that Ma et al. [32] did not show significant concentration- and anti-concentration-dependent relationships in different ranges of treatments [32]. This phenomenon was described as an example of the "polar paradox" which had been first reported in 1989. The lipophilic antioxidants tend to be more effective in the emulsion or liposome system and less effective in bulk dry oils. The reverse is generally true for the more polar antioxidants (or amphiphiles with higher hydrophile–lipophile balance). Thus, in the members of a homologous series, a reciprocal relationship has been shown. The lower (less alkylated) members tend to be more active in dry oils and the higher members more active in emulsions [33]. Leaves of polyphenolic extracts used for their lipophilization are summarized in Table 2.

5. Acylation of Other Plant Derived Matrices Rich in Phenolic Compounds

In the flowering plant family, *Lamiaceae*, which includes widely used culinary and medicinal herbs, Stachys is one of the largest genera. Two Greek endemic plants, *Stachys swainsonii* ssp. argolica (Boiss.) Phitos and Damboldt and *St. swainsonii* ssp. Swainsonii, were used by Mellou et al. [29] for the preparation of lipophilic antioxidant agents. Flavonoid glycosides, namely, chrysoeriol-7-*O*-β-D-(3′′-*E*-*p*-coumaroyl)-glucopyranoside and chrysoeriol-7-[6′′′-*O*-acetyl-β-D-allosyl-(1→2)-β-D-glucopyranoside] (Figure 3c,d), were isolated from the purified methanol extracts of the aerial parts of two mentioned Greek plants and were acylated with vinyl laurate as acylation agent with immobilized *Candida antarctica* lipase B as a biocatalyst [29].

Based on the model reaction, acylation of naringin, the influence of the molar ratio of the flavonoid to acyl donor, its nature (in the form of lauric acid or its vinyl ester) and the reaction solvent (acetone or *tert*-butanol), were evaluated. Vinyl laurate, acetone and 10-fold excess of acyl donor proved to be the right choice to achieve a high conversion yield. Moreover, the used lipase showed regiospecificity for the primary alcohol of the glucose residue of tested flavonoid glycosides. Purified flavonoid esters and their precursors

were compared in their ability to impact on the resistance of isolated LDL (low-density lipoprotein) and total serum to copper-induced oxidation. In line with the predictions, the use of the more lipophilic compounds caused higher prolongation of LDL and serum resistance to oxidation. Despite that, LDL oxidation is a complex process and has not yet been fully understood; the provided research definitely exhibited the antioxidant capacity of esterified flavonoids [29].

Proanthocyanidins, which are oligomeric flavonoids, have also become the subject of research on the possibility of lipophilization of polyphenols. A dimeric compound (PA) used in the study of Xiao et al. [17] was isolated from the bark of *Acacia mearnsii* and was a heteroduplex composed of procyanidin and prorobinetinidin linked by a single C4–C8 bond. The reaction conditions for the acylation of PA with palmitic acid (Figure 3e) were optimized by choosing temperature, reaction time, amount of enzyme, the molar ratio of substrates and solvent together with the initial water content.

A conversion of 97% was achieved with the following conditions: 10-fold excess of palmitic acid, *tert*-amyl alcohol with 5% of water at 60 °C for 12 h with 30 g/L of CALB. As in the earlier quoted papers, the incorporation of fatty acid into a hydrophilic molecule resulted in a significant improvement in lipophilicity. The partition coefficient of mono-acylated PA was about 2.4 times higher in comparison with the non-esterified precursor. Furthermore, the antioxidant activity of the obtained compound, its precursor and vitamin E (as a control) were compared in the DPPH radical method, where half-maximal inhibitory concentrations (IC_{50}) were determined. Surprisingly, IC_{50} values for PA and its acylated derivative were 8.20 μmol/L and 5.20 μmol/L, respectively. The authors suggested that the provided research may be a way to obtain lipophilic antioxidants for possible application in the food and cosmetic industries, due to the abundance of procyanidin-prorobinetinidin heterodimers in *A. mearnsii* bark and the ease of their enzymatic acylation [17].

Coffee pulp is rich in chlorogenic acid (5-cafeoylquinic acid, 5-CGA), which has been found to be its major constituent (31–42%) [30]. The functionalization of chlorogenic acid has been proposed by enzymatic esterification of the carboxylic acid moiety of quinic acid with fatty alcohol in order to lipophilize the molecule [34]. An alternative and more efficient chemo-enzymatic strategy has also been proposed, where 5-CGA was first chemically esterified with methanol and then transesterified with fatty alcohols [35]. The lipase-catalyzed esterification of 5-CGA present in coffee pulp with 1-heptanol/pentanol/geraniol (Figure 3f) in supercritical carbon dioxide/t-butanol has been optimized, reaching an 85/82/77% conversion yield [30].

The main purpose of anthocyanins acylation is to improve their antioxidant activity and thermostability. It has been shown that acylation with aromatic acids has a particular impact on the stability of the obtained compounds due to the intramolecular co-pigmentation caused by π-π interactions [14]. In this study, the novel acylated cyanidin glycoside derivatives were obtained through the enzymatic synthesis in *tert*-butanol. Cyanidin-3-glucoside, cyanidin-3-rutinoside, and cyanidin-3,5-diglucoside were successfully extracted from underutilized plant sources including purple corn (*Zea mays*), tiliapo (*Sideroxylon palmeri*) and plum (*Prunus domestica*) husks. Acylated anthocyanins were synthesized, but only cyanidin-3-(6′′-dihydroferuloyl)glucoside and cyanidin-3-(6′′-dihydrosinapoyl)glucoside exhibited better thermostability than cyanidin-3-glucoside, and its antioxidant activity was improved with dihydrosinapoyl and cinnamoyl residues [14]. Similarly, Yan et al. [36] acylated anthocyanin from black rice with aromatic acid methyl ester of benzoic acid as acyl donors and CALB presence. Cyanidin 3-(6′′-benzoyl)-glucoside, cyanidin 3-(6′′-salicyloyl)-glucoside and cyanidin 3-(6′′-cinnamoyl)-glucoside were successfully synthesized and their thermostability and light-resistivity were improved [36].

6. Other Unconventional Uses of Polyphenols Lipophilization

Both examples relate to the use of acylation reactions to simultaneously purify and increase the durability of camellia seed oil.

Tea is one of the most popular beverages consumed around the globe and prepared from the leaves of *Camellia sinensis.* The beneficial effect of tea on human health is primarily due to its high antioxidant activity, which results mainly from the presence of tannins and catechins (epicatechin, epigallocatechin), as well as their derivatives (epicatechin gallate and epigallocatechin gallate) [37–39]. Seeds are another product in addition to the leaves of the tea plant; they are the source of vegetable oil, an important plant oil due to its high content of unsaturated fatty acids, especially essential linoleic acid. However, crude camellia seed oil contains some free fatty acids, which must be removed to obtain an oil of acceptable quality. In crude camellia seed oil, the main free fatty acids were found to be oleic acid (C18:1), palmitic acid (C16:0), stearic acid (C18:0) and linoleic acid (C18:2). Chen et al. [39] reduced the free fatty acid content by lipophilization of epicatechin with these free fatty acids catalyzed by *Candida antarctica* lipase B (Novozym 435). Epicatechin fatty acid esters were thus formed and the presence of these compounds enhanced the oxidative stability of the oil at the same time [39].

Similarly, Luo et al. [40] used anthocyanins found in blueberry extract to add to crude camellia seed oil and lipophilized with free fatty acids in the oil through an enzymatic process catalyzed by *Candida antarctica* lipase B. Lipophilized anthocyanin derivatives were synthesized, which improved the oxidative stability of the oil under high-temperature treatment and observed that content of free fatty acids of the crude camellia seed oil was decreased [40]. The extracts mentioned in both Sections 5 and 6 are collated in Table 3.

Table 3. Other plant extracts used in enzymatic modifications of phenolic compounds.

The Origin of the Plant Extract	Main Components of the Extract	Used Enzyme	Reaction Conditions	The Obtained Ester(s)	Research Highlights	Reference
Aerials parts of two Greek endemic plants, i.e., *Stachys swainsonii* ssp. argolica (Boiss.) Phitos and Damboldt and *St. swainsonii* ssp. swainsonii	Chrysoeriol-7-*O*-β-D-(3′′-*E*-*p*-coumaroyl) glucopyranoside and Chrysoeriol-7-[6′′′-*O*-acetyl-β-D-allosyl-(1→2)-β-D-glucopyranoside]	Novozym 435 (*Candida antarctica* lipase B)	50 °C, 96 h, 240 rpm, acetone as a solvent (10 mL), flavonoids–0.2 mmol, vinyl laurate as an acyl donor (2 mmol, ratio 1:10), 100 mg of enzyme	Laurate ester of chrysoeriol-7-*O*-β-D-(3′′-*E*-*p*-coumaroyl)-glucopyranoside and laurate ester of chrysoeriol-7-[6′′′-*O*-acetyl-β-D-allosyl-(1→2)-β-D-glucopyranoside]	(a) The obtained laurate esters caused higher prolongation of LDL and serum resistance to copper-induced oxidation.	[29]
Purple corn (*Zea mays*) or tiliapo (*Sideroxylon palmeri*) husks	Cyanidin-3-*O*-glucoside	*Candida antarctica* lipase B	60 °C, 48 h, 300 rpm, 20 g/L of enzyme, *tert*-butanol as a solvent, a cyanidin:vinyl cinnamate ratio of 1:250, molecular sieves (100 g/L)	Cyanidin-3-*O*-(6′′-cinnamoyl) glucoside, Cyanidin-3-*O*-(6′′-dihydro cinnamoyl)glucoside, Cyanidin-3-*O*-(6′′-dihydro feruloyl)glucoside, Cyanidin-3-*O*-(6′′-dihydro sinapoyl)glucoside	(a) Cyanidin-3-*O*-(6′′-dihydrosinapoyl) glucoside had the highest antioxidant activity in DPPH• assay.	[14]
Plum (*Prunus domestica*) husk	Cyanidin-3-*O*-rutinoside	*Candida antarctica* lipase B	60 °C, 48 h, 300 rpm, 20 g/L of enzyme, *tert*-butanol as a solvent, a cyanidin:vinyl cinnamate ratio of 1:250, molecular sieves (100 g/L)	Cyanidin-3-*O*-(4′′′-cinnamoyl)rutinoside	(a) Optimized synthesis methodology with a 45.5% conversion yield.	[14]
Black rice (*Oryza sativa* L. subsp. *Japonica*)	Cyanidin-3-*O*-glucoside	Novozym 435 (*Candida antarctica* lipase B)	40 °C, 48 h, 30 rpm, 900 mbar (vacuum pump), 0.5 g of black rice anthocyanins, 10 mL of acyl donor, pyridine (5 mL) as a solvent, 1 g of enzyme	Cyanidin-3-*O*-(6′′-benzoate) glucoside, Cyanidin-3-*O*-(6′′-salicylate) glucoside, Cyanidin-3-*O*-(6′′-cinnamate) glucoside	(a) Enzymatic acylation improved the half-life times of anthocyanins in the light treatments (dark, UV and fluorescent). (b) Improved thermostability due to the enzymatic acylation was observed.	[36]
Acacia mearnsii bark	Heteroduplex composed of procyanidin and prorobinetinidin linked by a single C4–C8 bond (PA dimers)	Novozym 435 (*Candida antarctica* lipase B)	60 °C, 12 h, 30 g/L of enzyme, 2-methyl-2-butanol (with 5% of water) as a solvent, a ratio of 10:1 (palmitic acid:PA dimers)	Procyanidin and prorobinetinidin heteroduplex esterified with palmitic acid	(a) The antioxidant activities of PA dimers and obtained palmitate ester were much higher than the activity of vitamin E.	[17]

Table 3. *Cont.*

The Origin of the Plant Extract	Main Components of the Extract	Used Enzyme	Reaction Conditions	The Obtained Ester(s)	Research Highlights	Reference
Coffee pulp	Chlorogenic acid	Novozym 435 (*Candida antarctica* lipase B)	Cell volume of 50 mL, 150 mbar of supercritical carbon dioxide (sCO_2), 55 °C, 25 h, *tert*-butanol as a solvent (10% *v*/*v*), 10% (*v*/*v*) of acyl donor (1-heptanol or 1-pentanol or geraniol), 20 mg/mL of enzyme, 20 mg/mL of molecular sieves	Heptyl chlorogenate	(a) The supercritical carbon dioxide proved to be useful in the lipophilization of chlorogenic acid from the coffee pulp.	[30]
Tea leaves	Epicatechin	Novozym 435 (*Candida antarctica* lipase B)	50 °C, 36 h, 14 mg of epicatechin (dissolved in polyoxyethylene stearate, 1:1 (m/m)), 10 g of crude camellia seed oil, 10 mg of enzyme, 2 g of molecular sieves	Epicatechin palmitate, Epicatechin oleate	(a) Reduced content of the free fatty acids as a result of the reaction of blueberry anthocyanins with components of the crude camellia seed oil. (b) The oxidative stability of the oil was improved.	[39]
Blueberry anthocyanin extract	Malvidin-3-*O*-galactoside, Cyanidin-3-*O*-galactoside, Delphinidin-3-*O*-galactoside, Malvidin-3-*O*-glucoside, and Cyanidin-3-*O*-arabinoside	Novozym 435 (*Candida antarctica* lipase B)	50 °C, 36 h, 200 rpm, 36 mg of the blueberry anthocyanin solution (dissolved in polyoxyethylene stearate, 1:2 (m/m)), 10 g of crude camellia seed oil, 10 mg of enzyme, 2 g of molecular sieves	Cyanidin-3-*O*-(6″-oleoyl) galactoside, Cyanidin-3-*O*-(6″-palmitoyl) galactoside	(a) Reduced content of the free fatty acids as a result of the reaction of blueberry anthocyanins with components of the crude camellia seed oil. (b) The oxidative stability of the oil was improved.	[40]

7. Conclusions

Different plants have the potential to be important sources of phenolic compounds which can be used as additives in food products. It should be outlined that the majority of studies focused on anthocyanins and other flavonoids. Presented studies pointed out that modified derivatives of polyphenols were more lipophilic than the native compounds and exhibited good antioxidant properties, and often higher thermal and light stability. Some acylated phenolics improved the stability of edible oils. This fact caused a positive impact on products that were fried in modified oils by increasing their nutrient values or decreasing the content of hazardous acrylamide. In the future, lipophilic compounds can be used as a possible alternative for synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and *tert*-butylhydroquinone (TBHQ). Still, toxicological and functional research is needed to confirm that the derivatives of phenolics are safe for human health. Moreover, the outstanding issue remains, the impact of the lipophilization reaction on antioxidant properties of many esterified phenolics compared to non-modified compounds, which could be double. The reason for those observations is still unknown, and it should be explained in the future with the use of computational studies and in-depth experiments.

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References

1. Altemimi, A.; Lakhssassi, N.; Baharlouei, A.; Watson, D.G.; Lightfoot, D.A. Phytochemicals: Extraction, Isolation and Identification of Bioactive Compounds from Plant Extracts. *Plants* **2017**, *6*, 42. [CrossRef] [PubMed]
2. Barberi, R.; Coppo, E.; Marchese, A.; Daglia, M.; Sabarzo-Sanchez, E.; Nabavi, S.F.; Nabavi, S.M. Phytochemicals for human disease: An update on plant-derived compounds antibacterial activity. *Microbiol. Res.* **2017**, *196*, 44–68. [CrossRef] [PubMed]

3. Strumiłło, J.; Gerszon, J.; Rodacka, A. Charakterystyka związków fenolowych pochodzenia naturalnego ze szczególnym uwzględnieniem ich roli w prewencji chorób neurodegeneracyjnych. In *Bory Tucholskie i Inne Obszary Leśne. Ochrona i Monitoring*; Gwoździński, K., Ed.; Wydawnictwo Uniwersytetu Łódzkiego: Łódź, Poland, 2015; pp. 231–245.
4. Figueroa-Espinoza, M.C.; Bourlieu, C.; Durand, E.; Lecomte, J.; Villeneuve, P. Lipophilized Antioxidants. In *Encyclopedia of Food Chemistry*; Melton, L., Shahidi, F., Varelis, P., Eds.; Academic Press: Cambridge, MA, USA, 2019; Volume 2, pp. 193–201. [CrossRef]
5. Ramadan, M.F. *Pheno-Phospholipids and Lipo-Phenolics. Novel Structured Antioxidants*; Springer: Cham, Switzerland, 2021. [CrossRef]
6. Farooq, S.; Abdullah; Zhang, H.; Weiss, J. A comprehensive review on polarity, partitioning, and interactions of phenolic antioxidants at oil-water interface of food emulsions. *Compr. Rev. Food Sci. Food Saf.* **2021**, *20*, 4250–4277. [CrossRef] [PubMed]
7. de Araújo, M.E.M.B.; Franco, Y.E.M.; Messias, M.C.F.; Longato, G.B.; Pamphile, J.A.; Carvalho, P.D.O. Biocatalytic Synthesis of Flavonoid Esters by Lipase and Their Biological Benefits. *Planta Med.* **2017**, *83*, 7–22. [CrossRef]
8. Szajdek, A.; Borowska, J. Właściwości przeciwutleniające żywności pochodzenia roślinnego. *Żywn. Nauka Technol. Jakość* **2004**, *11*, 5–28.
9. Cosmulescu, S.; Trandafir, I.; Nour, V. Phenolic acids and flavonoids profiles of extracts from edible wild fruits and their antioxidant properties. *Int. J. Food Prop.* **2017**, *20*, 3124–3134. [CrossRef]
10. Aladedunye, F.; Niehaus, K.; Bednarz, H.; Thiyam-Hollander, U.; Fehling, E.; Matthaus, B. Enzymatic lipophilization of phenolic extract from rowanberry (*Sorbus aucuparia*) and evaluation of antioxidative activity in edible oil. *LWT* **2015**, *60*, 52–62. [CrossRef]
11. Yang, W.; Kortesniemi, M.; Yang, B.; Zheng, J. Enzymatic Acylation of Anthocyanins Isolated from Alpine Bearberry (*Arctostaphylos alpina*) and Lipophilic Properties, Thermostability, and Antioxidant Capacity of the Derivatives. *J. Agric. Food Chem.* **2018**, *66*, 2909–2916. [CrossRef]
12. Marathe, S.J.; Shah, N.N.; Bajaj, S.R.; Singhal, R.S. Esterification of anthocyanins isolated from floral waste: Characterization of the esters and their application in various food systems. *Food Biosci.* **2021**, *40*, 100852. [CrossRef]
13. Marquez-Rodriguez, A.S.; Guimaraes, M.; Mateus, N.; de Freitas, V.; Ballinas-Casarrubias, M.L.; Fuentes-Montero, M.E.; Salas, E.; Cruz, L. Disaccharide anthocyanin delphinidin 3-*O*-sambubioside from *Hibiscus sabdariffa* L.: *Candida antarctica* lipase B-catalyzed fatty acid acylation and study of its color properties. *Food Chem.* **2021**, *344*, 128603. [CrossRef]
14. Fernandez-Aulis, F.; Torres, A.; Sanchez-Mendoza, E.; Cruz, L.; Navarro-Ocana, A. New acylated cyanidin glycosides extracted from underutilized potential sources: Enzymatic synthesis, antioxidant activity and thermostability. *Food Chem.* **2020**, *309*, 125796. [CrossRef] [PubMed]
15. Xiao, D.; Jin, X.; Song, Y.; Zhang, Y.; Li, X.; Wang, F. Enzymatic Acylation of Proanthocyanidin Dimers from *Acacia Mearnsii* Bark: Effect on Lipophilic and Antioxidant Properties. *J. Bioresour. Bioprod.* **2021**, *6*, 359–366. [CrossRef]
16. Cruz, L.; Benhoud, M.; Rayner, C.M.; Mateus, N.; de Freitas, V.; Blackburn, R.S. Selective enzymatic lipohilization of anthocyanin glucosides from blackcurrant (*Ribes nigrum L.)* skin extract and characterization of estrified anthocyanins. *Food Chem.* **2018**, *266*, 415–419. [CrossRef]
17. Yang, W.; Kortesniemi, M.; Ma, X.; Zheng, J.; Yang, B. Enzymatic acylation of blaccurrant (*Ribes nigrum*) anthocyanins and evaluation of lipohilic properties and antioxidant capacity of derivatives. *Food Chem.* **2019**, *281*, 189–196. [CrossRef] [PubMed]
18. Teng, H.; Mi, Y.; Cao, H.; Chen, L. Enzymatic acylation of raspberry anthocyanin: Evaluations on its stability and oxidative stress prevention. *Food Chem.* **2022**, *372*, 130766. [CrossRef] [PubMed]
19. Stevenson, D.E.; Wibisono, R.; Jensen, D.J.; Stanley, R.A.; Cooney, J.M. Direct acylation of flavonoid glycosides with phenolic acids catalysed by *Candida antarcitca* lipase B (Novozym 435). *Enzyme Microb. Technol.* **2006**, *39*, 1236–1241. [CrossRef]
20. Aladedunye, F.; Matthäus, B. Effective lipophilic antioxidant enzymatically derived from Canadian crabapple. *Eur. J. Lipid Sci. Technol.* **2016**, *118*, 919–927. [CrossRef]
21. Chen, M.; Yu, S.-J. Lipophilized Grape Seed Proanthocyanidin Derivatives as Novel Antioxidants. *J. Agric. Food Chem.* **2017**, *65*, 1598–1605. [CrossRef]
22. Do Nascimento, M.A.; Vargas, J.P.C.; Rodrigues, J.G.A.; Leao, R.A.C.; de Moura, P.H.B.; Leal, I.C.R.; Bassut, J.; de Souza, R.O.M.A.; Wojcieszak, R.; Itabaiana, I., Jr. Lipase-catalyzed acylation of levoglucosan in continuous flow: Antibacterial and biosurfactant studies. *RSC Adv.* **2022**, *12*, 3027–3035. [CrossRef]
23. de Castro, V.C.; da Silva, P.H.A.; de Oliveira, E.B.; Desobry, S.; Humeau, C. Extraction, identification and enzymatic synthesis of acylated derivatives of anthocyanins from jaboticaba (*Myrciaria cauliflowa*) fruits. *Int. J. Food Sci. Technol.* **2014**, *49*, 196–204. [CrossRef]
24. Chang, S.K.; Alasalvar, C.; Shahidi, F. Superfruits: Phytochemicals, antioxidant efficacies, and health effects—A comprehensive review. *Cri. Rev. Food Sci. Nutr.* **2018**, *59*, 1580–1604. [CrossRef] [PubMed]
25. Sari, P.; Setiawan, A.; Siswoyo, T.A. Stability and antioxidant activity of acylated jambolan (*Syzygium cumini*) anthocyanins synthesized by lipase-catalyzed transesterification. *Int. Food Res. J.* **2015**, *22*, 671–676.
26. Pires, T.C.S.P.; Dias, M.I.; Barros, L.; Calhelha, R.C.; Alves, M.J.; Oliveira, M.B.P.P.; Santos-Buelga, C.; Ferreira, I.C.F.R.; SantosBuelga, C.; Ferreira, I.C.F.R. Edible flowers as sources of phenolic compounds with bioactive potential. *Food Res. Int.* **2018**, *105*, 580–588. [CrossRef]
27. Pires, E.d.O., Jr.; Di Gioia, F.; Rouphael, Y.; Ferreira, I.C.F.R.; Caleja, C.; Barros, L.; Petropoulos, S.A. The Compositional Aspects of Edible Flowers as an Emerging Horticultural Product. *Molecules* **2021**, *26*, 6940. [CrossRef]
28. Ma, X.; Yan, R.; Yu, S.; Lu, Y.; Li, Z.; Lu, H.H. Enzymatic acylation of isoorientin and isovitexin from bamboo-leaf extracts with fatty acids and antiradical activity of the acylated derivatives. *J. Agric. Food Chem.* **2012**, *60*, 10844–10849. [CrossRef] [PubMed]

29. Xu, J.; Qian, J.; Li, S. Enzymatic acylation of isoorientin isolated from antioxidant of bamboo leaves with palmitic acid and antiradical activity of the acylated derivatives. *Eur. Food Res. Technol.* **2014**, *239*, 661–667. [CrossRef]
30. Ma, X.; Wang, E.; Lu, Y.; Wang, Y.; Ou, S.; Yan, R. Acylation of antioxidant of bamboo leaves with fatty acids by lipase and the acylated derivatives' efficiency in the inhibition of acrylamide formation in fried potato crisps. *PLoS ONE* **2015**, *10*, e0130680. [CrossRef]
31. Porter, W.L.; Black, E.D.; Drolet, A.M. Use of polyamide oxidative fluorescence test on lipid emulsions—contrast in relative effectiveness of antioxidants in bulk versus dispersed systems. *J. Agric. Food Chem.* **1989**, *373*, 615–624. [CrossRef]
32. Mellou, F.; Lazari, D.; Skaltsa, H.; Tselepis, A.D.; Kolisis, F.N.; Stamatis, H. Biocatalytic preparation of acylated derivatives of flavonoid glycosides enhances their antioxidant and antimicrobial activity. *J. Biotechnol.* **2005**, *116*, 295–304. [CrossRef]
33. Hernandez, C.E.; Chen, H.-H.; Chang, C.I.; Huang, T.-C. Direct lipase-catalyzed lipophilization of chlorogenic acid from coffee pulp in supercritical carbon dioxide. *Ind. Crops Prod.* **2009**, *30*, 359–365. [CrossRef]
34. Figueroa-Espinoza, M.C.; Villeneuve, P. Phenolic acids enzymatic lipophilization. *J. Agric. Food Chem.* **2005**, *53*, 2779–2787. [CrossRef]
35. Lopez Giraldo, L.J.; Laguerre, M.; Lecomte, J.; Figueroa-Espinoza, M.C.; Barouh, N.; Barea, B.; Villeneuve, P. Lipase-catalyzed synthesis of chlorogenate fatty esters in solvent-free medium. *Enzyme Microb. Technol.* **2007**, *41*, 721–726. [CrossRef]
36. Yan, Z.; Li, C.; Zhang, L.; Liu, Q.; Ou, S.; Zeng, X. Enzymatic acylation of anthocyanin isolated from black rice with methyl aromatic acid ester as donor: Stability of the acylated derivatives. *J. Agric. Food Chem.* **2016**, *64*, 1137–1143. [CrossRef] [PubMed]
37. Pinto, G.; Illiano, A.; Carpentieri, A.; Spinelli, M.; Melchiorre, C.; Fontanarosa, C.; di Serio, M.; Amoresano, A. Quantification of polyphenols and metals in Chinese tea infusions by mass spectrometry. *Foods* **2020**, *9*, 835. [CrossRef] [PubMed]
38. Klepacka, J.; Tońska, E.; Rafałowski, R.; Czarnowska-Kujawska, M.; Opara, B. Tea as a Source of Biologically Active Compounds in the Human Diet. *Molecules* **2021**, *26*, 1487. [CrossRef] [PubMed]
39. Chen, S.-S.; Luo, S.-Z.; Zheng, Z.; Zhao, Y.-Y.; Pang, M.; Jiang, S.-T. Enzymatic lipophilization of epicatechin with free fatty acids and its effect on antioxidative capacity in crude camellia seed oil. *J. Sci. Food Agric.* **2017**, *97*, 868–874. [CrossRef]
40. Luo, S.-Z.; Chen, S.-S.; Pan, L.-H.; Qin, X.-S.; Zheng, Z.; Zhao, Y.-Y.; Pang, M.; Jiang, S.-T. Antioxidative capacity of crude camellia seed oil: Impact of lipohilization products of blueberry anthocyanin. *Int. J. Food Prop.* **2017**, *20*, 1627–1636. [CrossRef]

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Bio-Based Materials versus Synthetic Polymers as a Support in Lipase Immobilization: Impact on Versatile Enzyme Activity

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Abstract: To improve enzyme stability, the immobilization process is often applied. The choice of a support on which the enzymes are adsorbed plays a major role in enhancing biocatalysts' properties. In this study, bio-based (i.e., chitosan, coffee grounds) and synthetic (i.e., Lewatit VP OC 1600) supports were used in the immobilization of lipases of various microbial origins (yeast (*Yarrowia lipolytica*) and mold (*Aspergillus oryzae*)). The results confirmed that the enzyme proteins had been adsorbed on the surface of the selected carriers, but not all of them revealed comparably high catalytic activity. Immobilized CALB (Novozym 435) was used as a commercial reference biocatalyst. The best hydrolytic activity (higher than that of CALB) was observed for Novozym 51032 (lipase solution of *A. oryzae*) immobilized on Lewatit VP OC 1600. In terms of synthetic activity, there were only slight differences between the applied carriers for *A. oryzae* lipase, and the highest measures were obtained for coffee grounds. All of the biocatalysts had significantly lower activity in the synthesis reactions than the reference catalyst.

Keywords: immobilization; lipase; synthetic activity; Lewatit; chitosan; coffee grounds

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1. Introduction

Some of the most widely industrially applied enzymes are lipases (triacylglycerol hydrolases, EC 3.1.1.3). These enzymes can be sourced from microorganisms, animals, and plants. Microbial lipases are of great interest due to their high stability in catalytic performance and broad tolerance of environmental factors (e.g., pH, temperature, and organic solvents). They can catalyze various reactions, such as hydrolysis, esterification, and transesterification, in both hydrophilic and hydrophobic media. A particular advantage of these biocatalysts is their specificity (stereo- and enantioselectivity), which is key to both the classification of lipases and their application. These special features allow lipases to be used in almost every field of biotechnology, including food chemistry, biodiesel production, and synthesis of biopolymers and pharmaceuticals [1–5].

Most lipase preparations are solutions with various degrees of concentration and purification, or preparations in solid form. In many studies, researchers apply commercial lipases such as CALB (immobilized lipase B from *Candida antarctica*), Lipozyme RMIM (from *Rhizomucor miehei*), and Lipozyme TLIM (from *Thermomyces lanuginosus)* [6].

To improve the stability of enzymes, the immobilization technique is often used. There are two main ways in which biocatalysts can be linked to special supports: the first is based on physical techniques such as adsorption, entrapment, and encapsulation, while the second includes chemical bonding processes such as covalent bonding and crosslinking [1]. Due to its reversibility and simplicity, the most favored method is physical adsorption. Its advantage is that there are no significant changes in the enzymes' native conformation and

it does not cause a loss of catalytic activity. In this single-step process, biocatalysts can be adsorbed at both porous and non-porous supports [7].

Carriers for enzyme immobilization are classified into four groups: inorganic materials, polymers, MOFs (metal–organic frameworks), and DNA origami. The most popular inorganic matrices are mica, silica, zeolites, activated carbon and, for polymers, agarose, chitin, chitosan, cellulose, lignin, starch, or gelatin (natural polymers), and polyethylene glycol, polyacrylamide, polyaniline, nylon, and epoxy-activated polymers (synthetic polymers) [2,8–12].

In recent years, research efforts have been focused on boosting the catalytic reaction to achieve more ecological and economical processes. The search for more biodegradable, biocompatible, and non-toxic supports is still necessary. The present study examined various supports for enzyme immobilization, including the synthetic, commercial matrix Lewatit VP OC 1600 as a reference, the natural biopolymer chitosan, and spent coffee grounds. The aim of this study was to compare the activity of lipase preparations immobilized on different supports—namely, lipases obtained in a wild-type strain of *Yarrowia lipolytica* yeast culture, and commercial lipase solution Novozym 51032 from *Aspergillus oryzae*. The investigations focused on the usability of spent coffee grounds in lipase immobilization in comparison to other carriers. This paper focuses on the examination of the hydrolytic and synthetic activity of the obtained preparations.

2. Results and Discussion

2.1. Morphology of Native Matrices and Immobilized Preparations

Lewatit VP OC 1600 is a synthetic matrix based on a macroporous resin of poly(methyl methacrylate) that is widely used for the physical immobilization of *Candida antarctica* lipase B (CALB) and commercially available as Novozym® 435 [13,14]. In this study, the synthetic support was applied for the immobilization of a crude enzyme solution of *Y. lipolytica* extracellular lipases and the liquid lipase Novozym 51032. The carrier was in the form of beads, as also shown in Figure 1. It was noticeable that the native Lewatit had a regular and smooth surface in comparison with the immobilized preparations (Figure 1A,B). Scanning electron microphotographs confirmed that both used enzymes were adsorbed on the surface of the support. Significant differences were observed. The carrier with lipase Novozym 51032 had a greater number of visibly adsorbed enzymes on the surface, which merged in some places to form agglomerates (Figure 1E,F). Otherwise, it looked for support with crude lipase solution from *Y. lipolytica* (Figure 1C,D). It was noticeable that the shape of both immobilized enzymes varied. Presumably, these differences resulted from the purification of the adsorbed lipases. The supernatant was separated from the yeast biomass by centrifugation and, despite the extracellular lipases, could contain some salts, sugars, other proteins or products of the yeast metabolism, and culture medium ingredients [15]. Novozym 51032, as a commercial biocatalyst, included only purified lipase proteins.

Chitosan is a natural polysaccharide that can be sourced from the walls of the shells of shellfish (mainly from shrimps, crabs, or lobsters). This derivative of chitin is characterized by biocompatibility, environmental friendliness, non-toxicity, and high stability [7,16]. In this research, the abovementioned biopolymer was used as a matrix for the immobilization of Novozym 51032. The form of the support was a powder. In Figure 2, the structure of the native chitosan and immobilized enzyme on the carrier can be observed. In comparison with commercial supports such as Lewatit, the visibility of lipases on the surface of the chitosan is scarce. Due to the more complex structure of this biopolymer, enzymes could adsorb on different elements, which might be less noticeable, or the presence of the protein could depend on the type of lipases used. Foresti et al. [17] used chitosan to carry out immobilization of different microbial lipases, such as *C. rugosa* AY lipase, *Pseudomonas fluorescens* AK lipase, and native lipase B from *C. antarctica*. Scanning electron microphotographs showed that agglomerates of *C. antarctica* B were clearly seen on the supports, while with *P. fluorescens* lipase proteins were barely visible.

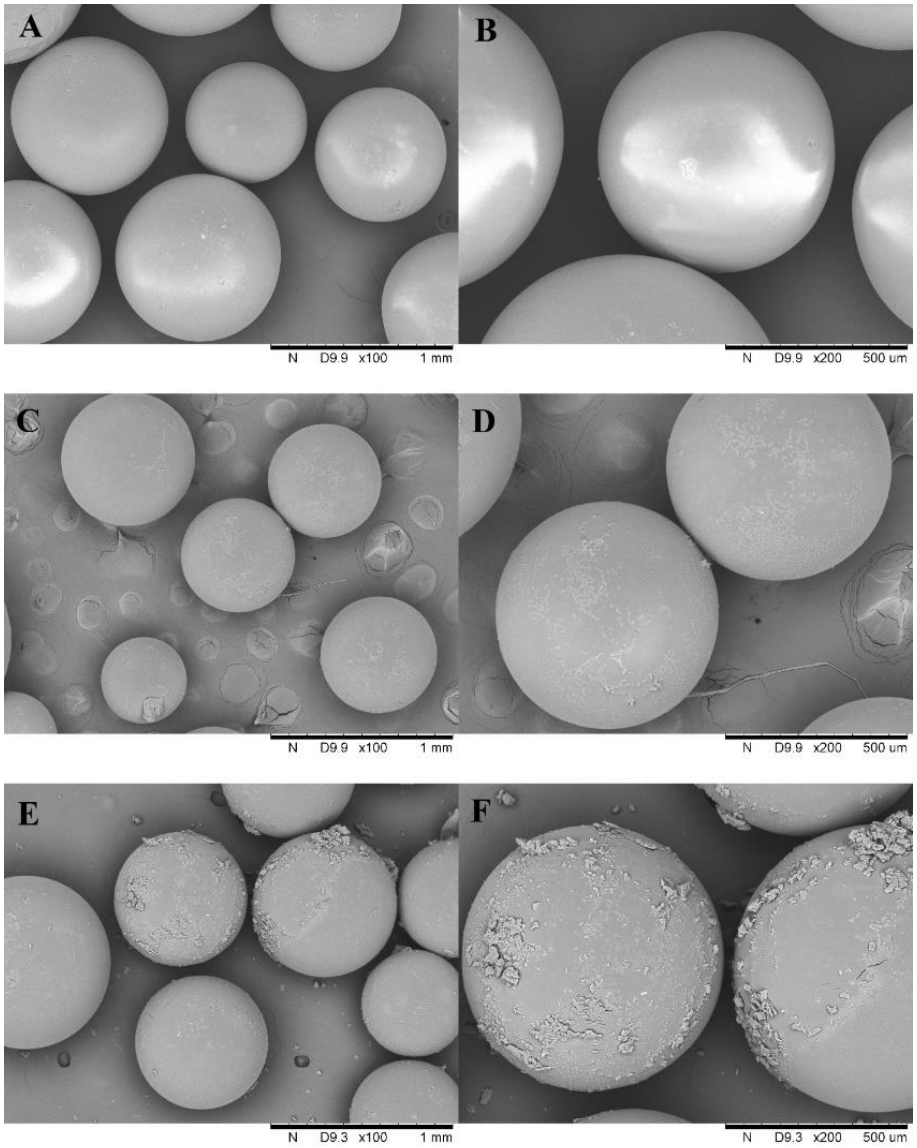

Figure 1. Scanning electron microphotographs of (**A**,**B**) beads of Lewatit VP OC 1600 (×100, ×200), (**C**,**D**) crude solution of *Y. lipolytica* enzymes immobilized on Lewatit VP OC 1600 (×100, ×200), and (**E**,**F**) liquid lipase Novozym 51032 immobilized on Lewatit VP OC 1600 (×100, ×200).

Spent coffee grounds (SCGs) are lignocellulosic wastes obtained from the preparation of coffee. Due to the popularity of this beverage, the amount of residues around the world is continually growing. For this reason, the interest in reusing their waste is increasing, and spent coffee grounds have become a new source with potential application as a support for the immobilization of enzymes. This kind of biomaterial is characterized by biodegradability, non-toxicity, and wide availability and consists of organic compounds such as fatty acids, cellulose, hemicellulose, lignin, protein, minerals, and total sugars [18–21]. Food waste also has various interesting properties, such as high porosity—which can be observed for coffee grounds in the presence of different chemical groups—and high surface area. It is worth highlighting that using the material can have a positive effect in reducing waste disposal problems [19]. Analyzing scanning electron microphotographs (Figure 3), it was found that the immobilization process was successful and the lipase Novozym 51032 was adsorbed on the coffee grounds support. Some of the enzyme molecules settled on the surface of the carrier, while others were adsorbed inside the pores, in the channels. The structure of the coffee grounds was porous, with visible flat leaf shapes, similar to that shown by Osorio-Arias et al. [22] and Ballesteros et al. [23].

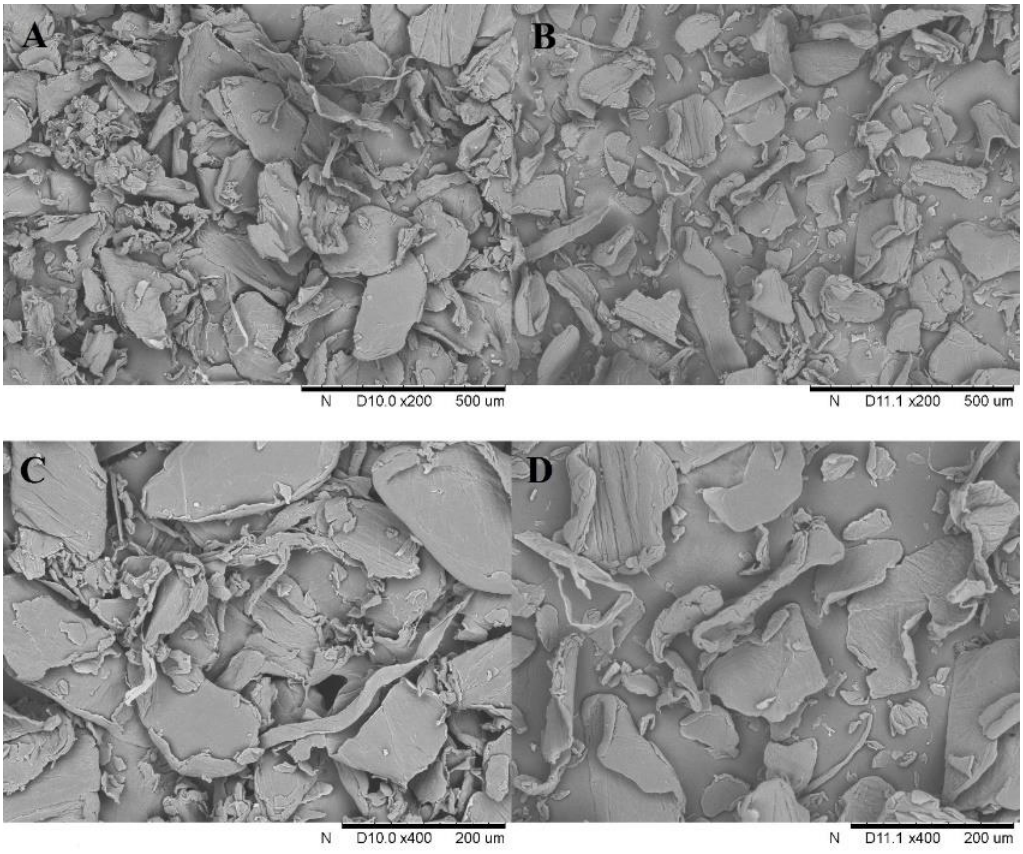

Figure 2. Scanning electron microphotographs of (**A**,**C**) liquid lipase Novozym 51032 immobilized on chitosan powder (×200, ×400) and (**B**,**D**) chitosan powder (×200, ×400).

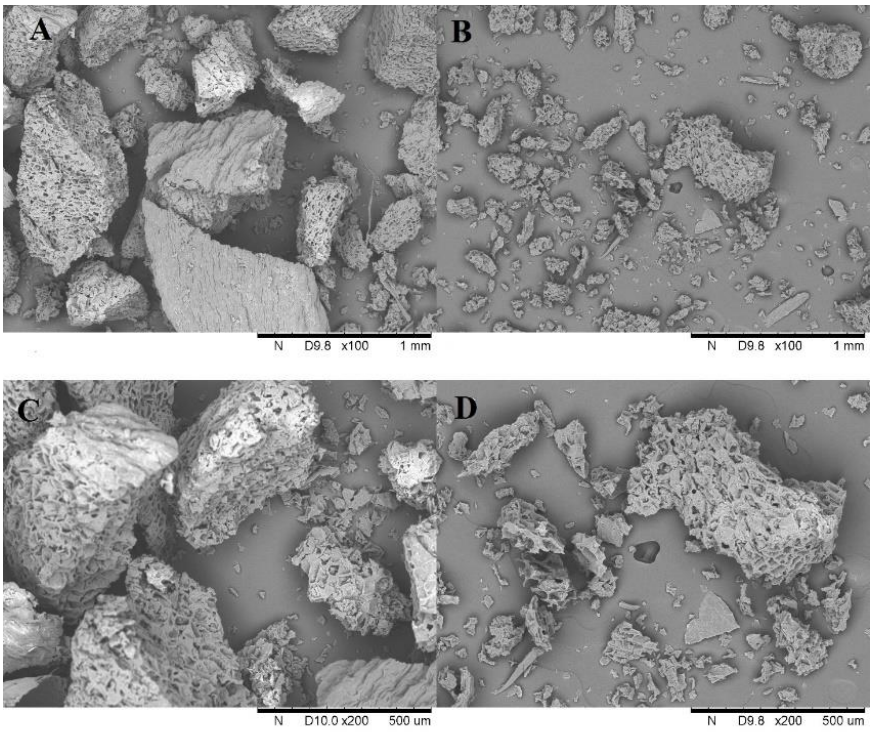

Figure 3. Scanning electron microphotographs of (**A**,**C**) liquid lipase Novozym 51032 immobilized on coffee grounds (×100, ×200) and (**B**,**D**) coffee grounds (×100, ×200).

2.2. Influence of the Lipase Source and Type of Support Used in Immobilization on the Hydrolytic and Synthetic Activity of the Enzyme Preparation

Immobilized biocatalysts were employed in the hydrolysis of *p*-nitrophenyl laurate and the transesterification between vinyl acetate and *n*-butanol. The obtained results are shown in Figures 4 and 5. The highest hydrolytic activity (0.0059 U/mg) was obtained for the preparation with immobilized lipase Novozym 51032 from *Aspergillus* on Lewatit VP OC 1600 (Figure 4). The results were greater than for the reference CALB when commercially immobilized on the same support. On the other hand, in Figure 5, it can be observed that the immobilized CALB had the best outcomes for synthetic activity. These differences confirm that the selection of the perfect support for immobilization had to be specific to the used enzymes. The commercial CALB supplied by Novozym is widely known in the industry, and the support on which it was immobilized was relatively hydrophobic [24]. This combination resulted in receiving a biocatalyst with great potential in many types of reactions, as also verified by the experiments in this study. Some laboratories have tried to repeat the success of Novozym 435 and immobilized other lipases on Lewatit VP OC 1600—for example, from *Rhizopus arrhizus* [25], *R. oryzae* and *Carica papaya* [26], or *Penicillium* sp. [27]—but the conclusion was often that the achievement of combining Lewatit VP OC 1600 with CALB cannot be extrapolated to other lipases. This paper showed that in some cases, e.g., catalyzing hydrolysis reactions, our own laboratory-made biocatalysts could be better than commercially available enzymes.

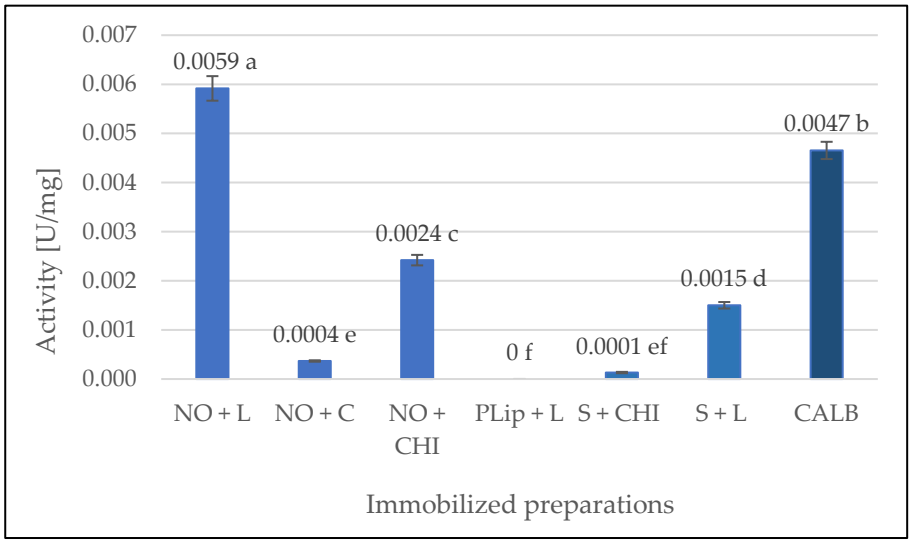

Figure 4. The hydrolytic activity of immobilized preparations: S + CHI: crude enzyme solution of *Y. lipolytica* immobilized on chitosan powder; S + L: crude enzyme solution of *Y. lipolytica* immobilized on Lewatit VP OC 1600; PLip + L: purified lipase from the supernatant of *Y. lipolytica* immobilized on Lewatit VP OC 1600; NO + L: liquid lipase Novozym 51032 immobilized on Lewatit VP OC 1600; NO + C: liquid lipase Novozym 51032 immobilized on coffee grounds; NO + CHI: liquid lipase Novozym 51032 immobilized on chitosan powder; CALB: immobilized lipase B from *Candida antarctica* as a reference. Means with the same capital letter (a, b, c, d, e, f) did not differ significantly ($\alpha = 0.05$).

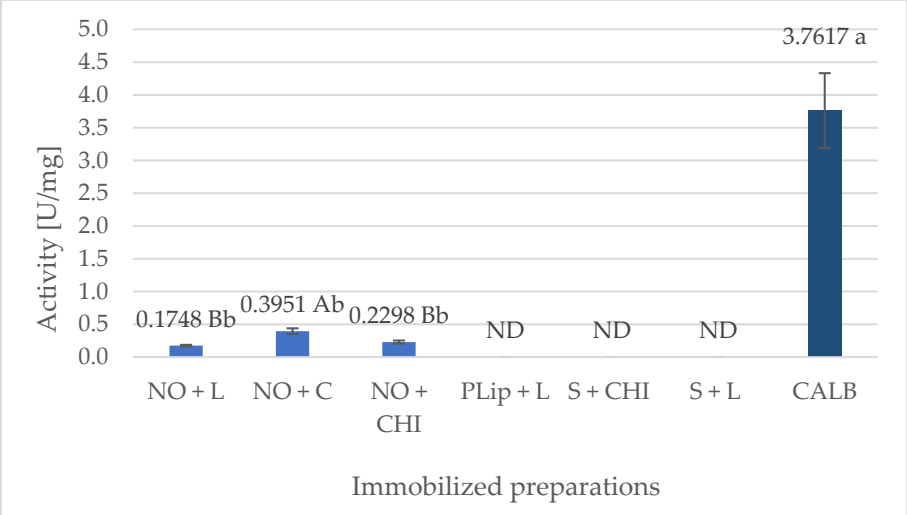

Figure 5. The synthetic activity of immobilized preparations: S + CHI: crude enzyme solution of *Y. lipolytica* immobilized chitosan powder; S + L: crude enzyme solution of *Y. lipolytica* immobilized on Lewatit VP OC 1600; PLip + L: purified lipase from *Y. lipolytica* immobilized on Lewatit VP OC 1600; NO + L: liquid lipase Novozym 51032 immobilized on Lewatit VP OC 1600; NO + C: liquid lipase Novozym 51032 immobilized on coffee grounds; NO + CHI: liquid lipase Novozym 51032 immobilized on chitosan powder; CALB: immobilized lipase B from *C. antarctica* as a reference; ND: not detected. Means with the same capital letter A or B in the case of liquid lipase Novozym 51032 immobilized on different supports (and a or b in the case of all biocatalysts) did not differ significantly ($\alpha = 0.05$).

The next carriers used in the experiments with lipase Novozym 51032 were chitosan and coffee grounds. Higher results for hydrolytic activity were achieved for samples with chitosan (0.0024 U/mg) than with coffee grounds (0.0004 U/mg), but for synthetic activity the outcomes were the opposite—a slightly higher score was obtained for coffee grounds (0.3951 U/mg). Chitosan had reactive functional groups such as amino- or hydroxyl groups, which can affect the immobilization process. Sometimes, modified functional groups in the carrier may influence the non-specific binding of the enzyme to the support, which can cause the loss of catalytic activity. There are some instances when blocking the remaining reactive groups in the carrier is necessary [7]. Due to this fact, chitosan as a support can behave in different ways in hydrolysis or synthesis because of the possibility of reacting with substrates or products of the reaction. In comparison with the literature, plenty of studies have tried to immobilize different types of lipase by physical adsorption on chitosan powder—for instance, from *A. niger* [28], C. *rugosa* [29], or *Burkholderia cepacia* [30]. Sanchez et al. [29] found that *Burkholderia cepacia* lipase immobilized on chitosan showed high activity in the *sn*-2 esterification of 1,3-dicaprin with palmitic acid. On the other hand, Kaja et al. [30] found that lipase from *C. rugosa* immobilized on chitosan had low activity values because of its lower porosity than other supports, e.g., Sephadex G-25 or Celite-545. Foresti et al. [17] obtained promising results in terms of hydrolytic and synthetic activity for chitosan-immobilized lipases from *C. rugosa*, *P. fluorescens*, and *C. antarctica* B.

Few studies are currently being conducted on coffee grounds as a carrier in the immobilization process. Lira et al. [31] made an attempt to immobilize lipase from *Thermomyces lanuginosus* (Lipozyme TL100L-Novozymes®) on spent coffee grounds. The obtained biocatalyst showed high hydrolytic activity (1715 U/g) but, surprisingly, did not present any esterification activity. Girelli et al. [20] prepared two types of biocatalyst—*Candida rugose* lipase immobilized on spent coffee grounds—by using physical adsorption and covalent methods. They obtained a preparation that could possibly be used in the hydrolysis of milk fat. In the present study, the results of enzyme activity showed that lipase Novozym 51032 immobilized on SCG can be a biocatalyst suitable for both hydrolysis and synthesis

reactions, but improvements in the values of catalytic activity are needed in future. The generally low catalytic activity of the enzyme linked to this porous support may have been due to the limitations on diffusion induced by the interaction between the biomass and the enzyme, or because of contaminants that may be present in the waste and not removed during the pretreatment [31,32].

In this study, we attempted to immobilize crude enzyme solutions of *Y. lipolytica* extracellular lipases on Lewatit VP OC 1600 and chitosan. These two supports were chosen based on the best results that were obtained for commercial lipase. Despite the fact that the crude solution of yeast lipase was a non-purified extract, it was possible to acquire an active preparation on synthetic supports that had slightly lower hydrolytic activity (0.0015 U/mg) than immobilized lipase Novozym 51032 on chitosan. Unfortunately, immobilization on chitosan was no longer as effective. It was also undertaken to carry out a laboratory experiment with self-purified lipase from lyophilized crude enzyme solution of *Y. lipolytica* extracellular lipases. However, the obtained results did not meet our expectations, and these preparations had the lowest hydrolytic activity. The process of self-purification of lipase has proven to be inefficient and needs to be refined in the future. For the measurement of synthetic activity, the method was not sensitive enough for such low activities, so the level of activity was not detected.

For preparations with detected synthetic activity, the percentage of conversion of vinyl acetate into acetaldehyde was determined, as presented in Figure 6. The highest result was obtained for commercial immobilized CALB, which Zheng et al. [33] also confirmed in their study. The best of our own laboratory-made biocatalysts was Novozym 51032 immobilized on coffee grounds. The significantly lower conversion enabled the biocatalyst to be adsorbed on chitosan.

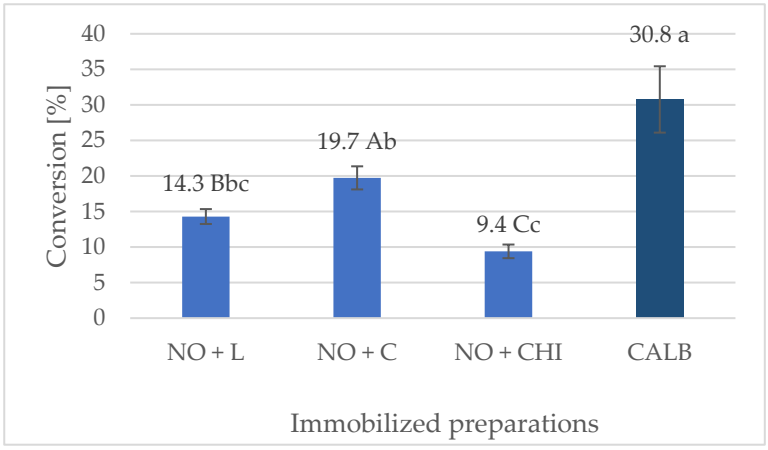

Figure 6. Conversion of vinyl acetate into acetaldehyde based on the colorimetric assay method with the immobilized preparations: NO + L: liquid lipase Novozym 51032 immobilized on Lewatit VP OC 1600; NO + C: liquid lipase Novozym 51032 immobilized on coffee grounds; NO + CHI: liquid lipase Novozym 51032 immobilized on chitosan powder; CALB: immobilized lipase B from *C. antarctica* as a reference. Means with the same capital letter A, B, or C in the case of liquid lipase Novozym 51032 immobilized on different supports, and a, b, or c in the case of all biocatalysts, did not differ significantly ($\alpha = 0.05$).

2.3. Protein Content and Specific Activity of Selected Immobilized Preparations in Comparison to the Native Forms of the Enzymes

The protein content and the specific hydrolytic and synthetic activity were determined for free lipase and for preparations with lipase Novozym 51032 immobilized on different supports (i.e., Lewatit VP OC 1600, chitosan, coffee grounds). Results were presented in Table 1. For each prepared biocatalyst, it was confirmed that the lipase had been well adsorbed, and the percentage of protein immobilization for the samples was 93.68%, 92.75%, and 88.26%, respectively. The scanning electron microphotographs presented earlier in

Section 2.1 also confirmed the presence of enzymes on the surface of the carriers. In comparison to free lipase, all of the biocatalysts had a higher catalytic activity, proving that physical adsorption is a proper method of immobilization for improving the hydrolytic and synthetic properties of enzymes. Yielding more active and selective biocatalysts—especially when used to catalyze complex reactions such as regioselective hydrolysis or synthesis—can be possible if physical adsorption takes place on hydrophobic supports, because it results in the lipase being mainly in its "open" form [7]. The physiological role of lipase is to catalyze the hydrolysis reaction, and this study showed that the best specific hydrolytic activity was that of the biocatalyst immobilized on the synthetic support Lewatit VP OC 1600 (0.314 U/mg protein), while for specific synthetic activity better results were obtained for lipase immobilized on biomaterials. There was only a slight difference between enzymes immobilized on chitosan (24.61 U/mg protein) and coffee grounds (22.23 U/mg protein). This may have been because the enzymes were located inside the pores of the support, protecting them against medium alterations and enabling transesterification reactions to take place under hydrophobic medium conditions [1]. The low specific synthetic activity of the biocatalyst immobilized on Lewatit VP OC 1600 may have been caused by lipase's formation of dimers or aggregates, as shown in Figure 1E,F, which may have negatively affected the immobilization [7].

Table 1. The protein content, specific hydrolytic activity, and specific synthetic activity of various free and immobilized lipases: NO + L: liquid lipase Novozym 51032 immobilized on Lewatit VP OC 1600; NO + C: liquid lipase Novozym 51032 immobilized on coffee grounds; NO + CHI: liquid lipase Novozym 51032 immobilized on chitosan powder.

	Protein Content (mg/mL)			Specific Hydrolytic Activity (U/mg Protein)		Specific Synthetic Activity (U/mg Protein)	
Preparations	**Free Lipase**	**Filtrate after Immobilization Process**	**% Protein Immobilization**	**Free Lipase**	**Immobilized Preparations**	**Free Lipase**	**Immobilized Preparations**
NO + L		1.27 ± 0.80	93.68		0.314 ± 0.013		9.27 ± 0.68
NO + CHI	20.13 ± 2.84	1.46 ± 0.69	92.75	0.022 ± 0.002	0.259 ± 0.011	5.20 ± 0.14	24.61 ± 2.51
NO + C		2.36 ± 0.71	88.26		0.021 ± 0.001		22.23 ± 2.48

3. Materials and Methods

3.1. Materials and Biocatalysts

In the present study, as a support for lipase immobilization, the following materials were used: chitosan (Glentham Life Sciences, Corsham, UK), Lewatit VP OC 1600 (Lanxess, Cologne, Germany), and coffee grounds (household waste). Chemical reagents were purchased from Sigma-Aldrich (Poznań, Poland) and Avantor Performance Materials Poland S.A. (Gliwice, Poland). Immobilized lipase B from *Candida antarctica* (CALB) (Sigma-Aldrich, Poznań, Poland), liquid lipase Novozym 51032 from *Aspergillus oryzae* (Novozymes, Basgvaerd, Denmark), and supernatant from *Yarrowia lipolytica* were used as biocatalysts. The supports Lewatit VP OC 1600 and commercial lipase Novozym 51032 were kindly gifted by the companies Novozymes and Lanxess, respectively. The yeast strain *Yarrowia lipolytica* KKP 379 was purchased from the Collection of Industrial Microorganisms at the Prof. Wacław Dąbrowski Institute of Agricultural and Food Biotechnology State Research Institute in Warsaw, Poland. Furthermore, culture media and their components were acquired from BTL Sp. z o.o. (Łódź, Poland).

3.2. Culture Media and Yeast Cultivation

For yeast cultivation, YPO medium (2% peptone, 2% olive oil, 1% yeast extract) pH 5.0 was used, with 0.1% Tween 80 as an emulsifier. Inoculation was conducted by adding 0.1% (*v/v*) of a 24-h *Yarrowia lipolytica* KKP 379 inoculum in YPD medium (2% peptone, 2% glucose, 1% yeast extract) to 200 mL of sterile medium in flat-bottomed flasks, which

were then cultured on a rotary shaker (140 rpm) for 48 h. The obtained yeast culture was centrifuged, and the crude enzyme solution of *Y. lipolytica* extracellular lipases was separated from biomass.

3.3. Freeze-Drying and Purification of Yeast Lipase

The obtained supernatant of *Y. lipolytica* KKP 379 was divided and poured into Petri dishes and then lyophilized. The samples were frozen in an Irinox freezer (Corbanese, Italy) at −40 °C and then freeze-dried in the Christ Gamma 1-16 apparatus (Osterode am Harz, Germany). The materials were stored on shelves at a temperature of 0 °C. The lyophilized supernatant was purified by using ion-exchange chromatography (elution with a linear gradient with 0.7 M NaCl + 15 mM Tris-HCl, pH = 6.8, TRIS buffer) and molecular sieves (50 mM phosphate buffer, pH = 7.0). The obtained active fractions were concentrated in a centrifuge (4.000× *g*, 10 min) on a VIVASPIN Centrifugal Concentrator Membrane 10.000 MWCO PES (Sartorius, Göttingen, Germany).

3.4. Lipase Immobilization

3.4.1. Immobilization on Lewatit VP OC 1600

Immobilization on Lewatit was carried out according to the methodology described by Barrera-Rivera and Martínez-Richa [34], with slight modifications. Firstly, the beads were activated with ethanol at a ratio of 1:10 (beads:ethanol) for 5 h, and then they were filtered with distilled water and, finally, dried under vacuum at room temperature for 24 h. After that, 1 g of beads was added to 15 mL of different lipase solutions. For immobilization, 15 mL of supernatant from *Yarrowia lipolytica*, 1 mL of purified lipase from supernatant from *Yarrowia lipolytica*, and 1 mL of lipase Novozym 51032 were used. The lipase solutions and beads were shaken in a rotatory shaker at 4 °C for 14 h. After incubation, the immobilized beads were filtered off with distilled water and dried under vacuum at room temperature for 24 h.

3.4.2. Immobilization on Chitosan

Based on the methodology described by Pereira et al. [35], with slight changes, lipase was immobilized on chitosan by physical adsorption. Before immobilization, the chitosan (2 g) was soaked in 30 mL of hexane and agitated for 1 h. After that, the hexane was removed by filtration under a vacuum, and the chitosan was washed with distilled water. The volume of lipase solution used was 20 mL of supernatant from *Yarrowia lipolytica* and 1 mL of lipase Novozym 51032 (14 mL of distilled water). The lipase solution and chitosan were mixed together for 3 h at room temperature in an Erlenmeyer flask, and then for an additional period of 18 h they were moved under static conditions at 4 °C. At the end, the immobilized preparations were filtered under vacuum, washed with distilled water, and dried at room temperature.

3.4.3. Immobilization on Coffee Grounds

Firstly, it was necessary to purify the coffee grounds before beginning the immobilization. Briefly, spent coffee grounds were subjected to extraction processes with the Soxhlet apparatus, and a 1:15 (*w*/*v*) ratio of coffee grounds to solvent was used. First of all, water was used as a solvent, and the extraction lasted about 4 h. Subsequently, *n*-hexane and then ethanol were used, where the solvents overflowed the Soxhlet chamber 12 times. The multistep extractions allowed for the removal of proteins, polyphenols, terpenes, and oils [36]. The immobilization procedure on coffee grounds was based on the methodology presented by Buntic et al. [18], with slight modifications. Lipase solution (1 mL of Novozym 51032 and 14 mL of distilled water) was agitated with 1 g of the prepared supports for 2 h. After that, the immobilized lipase was filtered under a vacuum, washed with distilled water, and dried at room temperature.

3.5. Lipase Activity Assay

3.5.1. Hydrolytic Activity

Measurement of hydrolytic activity was carried out via a spectrophotometric method based on the hydrolysis of *p*-nitrophenyl laurate. The reaction was carried out in Eppendorf test tubes. The 100 µL of free liquid lipase or 25 mg of immobilized biocatalyst in 100 µL of distilled water was stirred at 37 °C with 25 µL of 0.3 mmol *p*-nitrophenyl laurate dissolved in 2 mL of heptane. After 15 min of incubation, absorbance was measured at 410 nm with a UV–Vis spectrophotometer. The unit of lipase enzymatic activity was 1 U, i.e., the amount of enzyme that released 1 µmol *p*-nitrophenol per minute under the assay conditions.

3.5.2. Synthetic Activity

The synthetic activity of the immobilized lipase was checked using the colorimetric method developed by Zheng et al. [33], with some modifications. The measurement was based on transesterification between vinyl acetate and *n*-butanol. MBTH (3-methyl-2-benzothialinone) reacts with released acetaldehyde to produce the appropriate aldazine, which is converted to a blue-colored TAPMC (tetraaza-pentamethincyanine). The reaction was conducted in an Eppendorf tube and included 100 mM vinyl acetate and 100 mM *n*-butanol in 1 mL of hexane. To induce transesterification, 5 µL of free liquid lipase or 5 mg of immobilized lipase was added. Incubation was carried out for 5 min, at 30 °C, with agitation. The control experiment was conducted without the addition of an enzyme. After that, diluted samples (200 times, or in accordance with the requirements) for spectrophotometric measurement were prepared in test tubes. The assay began by adding 1 mL of 0.1% (*m*/*v*) MBTH solution to each sample and mixing for 10 min at 30 °C. Then, 0.4 mL of 1% (*m*/*v*) $H_4FeNO_4S_2 \cdot 12H_2O$ solution (in 0.1 M HCl) was added and agitated for another 30 min at 30 °C. The analytical wavelength was determined based on the UV–Vis spectrum for derivatives of acetaldehyde (Figure 7). Colorimetric measurements were carried out at 595 nm in a spectrophotometer. The standard curve was prepared by using different concentrations of acetaldehyde (Figure 8). The unit of lipase synthetic activity was 1 U, i.e., the amount of enzyme that converted 0.1 mmol vinyl acetate into acetaldehyde per minute under the assay conditions.

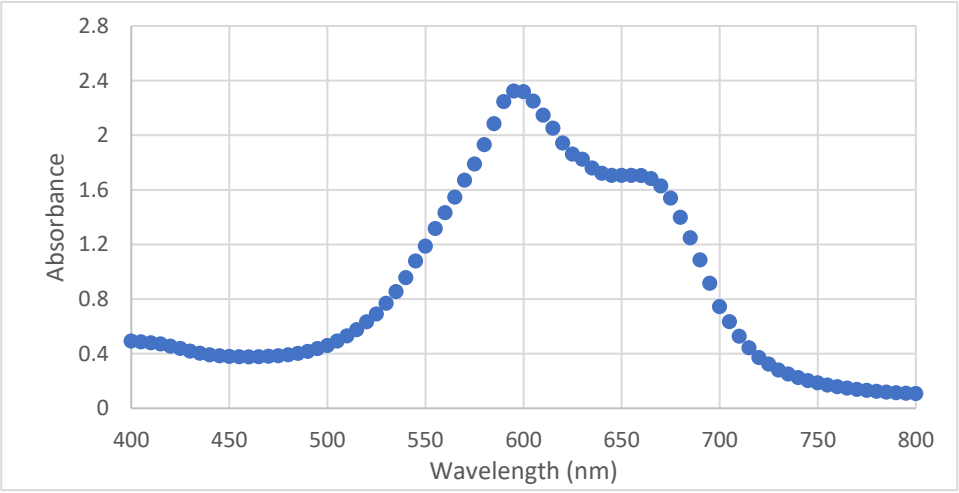

Figure 7. UV–Vis spectrum for derivatives of acetaldehyde.

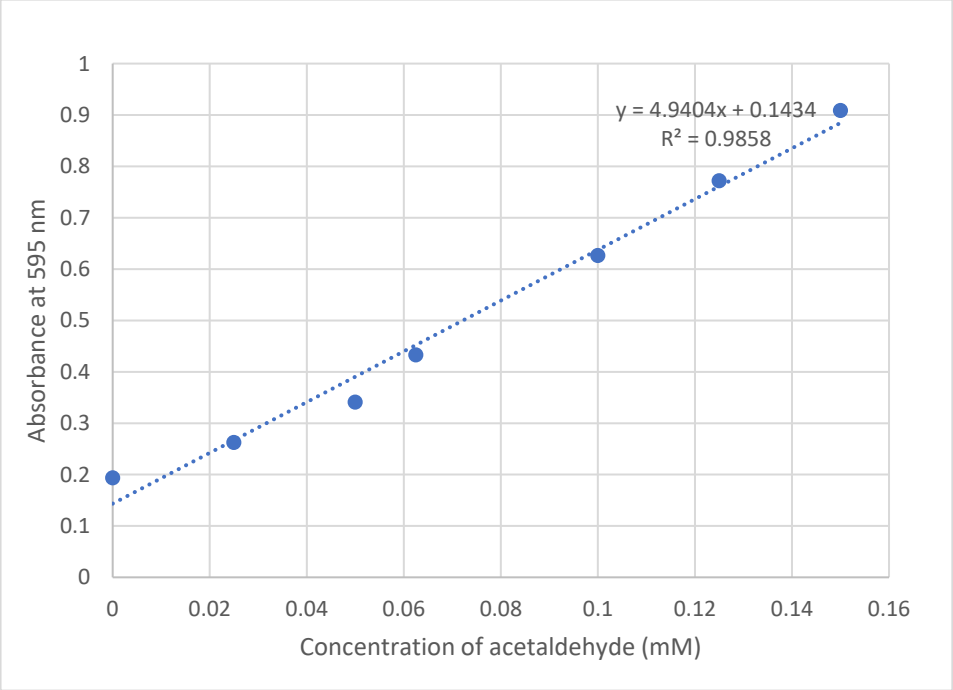

Figure 8. Standard curve of acetaldehyde by MBTH derivatization against absorbance at 595 nm.

3.6. Protein Content

The protein concentration in free lipase and in filtrates after immobilization was indicated spectrophotometrically using Lowry's method [37]. This process is based on the reaction between peptide bonds and aromatic amino acids with Folin–Ciocâlteu phenol reagent. For measurement, 1 mL of each tested solution was used. Fiftyfold dilutions of the samples were also prepared. The reaction was carried out in probes, to which 5 mL of copper reagent (2% Na_2CO_3 in 0.1 M NaOH, 1% $CuSO_4$, and 2% potassium sodium tartrate at a ratio of 100:1:1) was added. After 10 min, 0.5 mL of Folin–Ciocâlteu phenol reagent was added. The incubation lasted 30 min, and then the measurements were taken at 750 nm with a Rayleigh UV-1601 spectrophotometer (BRAIC, Beijing, China). A calibrated curve obtained with albumin as a standard was used to calculate the protein content. The amount of adsorbed protein on the particles was estimated based on differences between free lipase and the enzyme solution after immobilization.

3.7. Scanning Electron Microscopy (SEM)

The surface and morphology of the supports and immobilized preparations were studied using an electron microscope (HITACHI TM 3000, Ramsey, New Jersey, USA). Before observation, the samples were dried under a vacuum and coated with a layer of gold (Cressington Sputter Coater 108 auto, Cressington Scientific Instruments, Watford, UK). Microphotographs were taken at a magnification of 100, 200, and 400x.

3.8. Statistical Analysis

The obtained results of the conducted studies were analyzed by statistical methods, using the STATISTICA 13 program (StatSoft, Krakow, Poland). The Shapiro–Wilk test was used to verify the statistical hypothesis of normality of the distribution of the experimental data, while Levene's test and the Brown-Forsythe test were used to check the hypothesis of homogeneity of variance. The significance of the grouping variables was assessed by conducting an analysis of variance (ANOVA). Homogeneous groups for the experimental data that met the assumption of normal distribution were separated using Tukey's test. Values of $p \leq 0.05$ were considered to be statistically significant.

4. Conclusions

In this study, we investigated the process of immobilization of various microbial lipases on different supports. The obtained results proved the diverse influence of the used carriers (i.e., chitosan, coffee grounds, and synthetic Lewatit VP OC 1600) on the catalytic activity of the immobilized biocatalysts. The most promising results were achieved for coffee grounds and chitosan, which have potential in enzyme catalysis due to their biological origins, biodegradability, and susceptibility to chemical and/or physical modifications. Reusing food waste can provide more sustainable supports for enzyme immobilization. To the best of the authors' knowledge, this is one of the first papers to take into account immobilization on spent coffee grounds and chitosan liquid lipase Novozym 51032 from *Aspergillus niger*, opening new prospects for future researches. Moreover, it was found that, depending on the enzyme's biocatalytic properties, it is necessary to choose a specific support for the immobilization. Lipases are versatile catalysts with many contrasting activities, e.g., hydrolysis, esterification, transesterification, and aminolysis. It is crucial to indicate the types of reactions in which the prepared biocatalyst will be used, because of the differences in hydrolytic and synthetic activity observed in this study.

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References

1. Mulinari, J.; Oliveira, V.; Hotza, D. Lipase immobilization on ceramic supports: An overview on techniques and materials. *Biotechnol. Adv.* **2020**, *42*, 107581. [CrossRef]
2. Ismail, A.R.; Baek, K.H. Lipase immobilization with support materials, preparation techniques and applications: Present and future aspects. *Int. J. Biol. Macromol.* **2020**, *163*, 1624–1639. [CrossRef] [PubMed]
3. Sun, J.; Chen, Y.; Sheng, J.; Sun, M. Immobilization of *Yarrowia lipolytica* Lipase on Macroporous Resin Using Different Methods: Characterization of the Biocatalysts in Hydrolysis Reaction. *BioMed Res. Int.* **2015**, *2015*. [CrossRef] [PubMed]
4. Coelho, A.L.S.; Orlandelli, R.C. Immobilized microbial lipases in the food industry: A systematic literature review. *Crit. Rev. Food Sci. Nutr.* **2021**, *61*, 1689–1703. [CrossRef] [PubMed]
5. Zieniuk, B.; Białecka-Florjańczyk, E.; Wierzchowska, K.; Fabiszewska, A. Recent advances in the enzymatic synthesis of lipophilic antioxidant and antimicrobial compounds. *World J. Microbiol. Biotechnol.* **2022**, *38*, 11. [CrossRef]
6. Tanasković, S.J.; Jokić, B.; Grbavčić, S.; Drvenica, I.; Prlainović, N.; Luković, N.; Knežević-Jugović, Z. Immobilization of *Candida antarctica* lipase B on kaolin and its application in the synthesis of lipophilic antioxidants. *Appl. Clay Sci.* **2017**, *135*, 103–111. [CrossRef]
7. Alnoch, R.C.; Santos, L.A.; Almeida, J.M.; Krieger, N.; Mateo, C. Recent Trends in Biomaterials for Immobilization of Lipases for Application in Non-Conventional Media. *Catalysts* **2020**, *10*, 697. [CrossRef]
8. Thangaraj, B.; Solomon, P.R. Immobilization of Lipases-A Review. Part I: Enzyme Immobilization. *ChemBioEng Rev.* **2019**, *6*, 157–166. [CrossRef]
9. Bilal, M.; Fernandes, C.D.; Mehmood, T.; Nadeem, F.; Tabassam, Q.; Ferreira, L.F.R. Immobilized lipases-based nano-biocatalytic systems-A versatile platform with incredible biotechnological potential. *Int. J. Biol. Macromol.* **2021**, *175*, 108–122. [CrossRef]
10. Stanisz, M.; Bachosz, K.; Siwińska-Ciesielczyk, K.; Klapiszewski, Ł.; Zdarta, J.; Jesionowski, T. Tailoring Lignin-Based Spherical Particles as a Support for Lipase Immobilization. *Catalysts* **2022**, *12*, 1031. [CrossRef]
11. Tomaino, T.; Capecchi, E.; Piccinino, D.; Saladino, R. Lignin Nanoparticles Support Lipase-Tyrosinase Enzymatic Cascade in the Synthesis of Lipophilic Hydroxytyrosol Ester Derivatives. *ChemCatChem* **2022**, *14*, e202200380. [CrossRef]
12. Sipponen, M.H.; Farooq, M.; Kovisto, J.; Pellis, A.; Seitsonen, J.; Österberg, M. Spatially confined lignin nanospheres for biocatalytic ester synthesis in aqueous media. *Nat. Commun.* **2018**, *9*, 2300. [CrossRef] [PubMed]

13. Basso, A.; Froment, L.; Hesseler, M.; Serban, S. New highly robust divinyl benzene/acrylate polymer for immobilization of lipase CALB. *Eur. J. Lipid Sci. Technol.* **2013**, *115*, 468–472. [CrossRef]
14. Tambe, A.; Vyasarayani, R.; Datla, A.; Ponrathnam, S.; Demnerova, K. Macroporous Poly(Vinyl Acetate-Co-Divinyl Benzene) Copolymer Beads as Adsorptive Support for the Direct Immobilization of *Candida Antarctica* Lipase B. *Enzym. Eng.* **2015**, *4*, 1000130.
15. Brígida, A.I.S.; Amaral, P.F.F.; Coelho, M.A.Z.; Gonçalves, L.R.B. Lipase from *Yarrowia lipolytica*: Production, characterization and application as an industrial biocatalyst. *J. Mol. Catal. B: Enzym.* **2014**, *101*, 148–158. [CrossRef]
16. Rafiee, F.; Rezaee, M. Different strategies for the lipase immobilization on the chitosan based supports and their applications. *Int. J. Biol. Macromol.* **2021**, *179*, 170–195. [CrossRef]
17. Foresti, M.L.; Ferreira, M.L. Chitosan-immobilized lipases for the catalysis of fatty acid esterifications. *Enzym. Microb. Technol.* **2007**, *40*, 769–777. [CrossRef]
18. Buntić, A.; Pavlović, M.; Antonović, D.; Pavlović, V.; Vrućinić, D.; Šiler-Marinković, S.; Dimitrijević-Branković, S. Customizing the spent coffee for *Trichoderma reesei* cellulase immobilization by modification with activating agents. *Int. J. Biol. Macromol.* **2018**, *107*, 1856–1863. [CrossRef]
19. Girelli, A.M.; Astolfi, M.L.; Scuto, F.R. Agro-industrial wastes as potential carriers for enzyme immobilization: A review. *Chemosphere* **2019**, *244*, 125368. [CrossRef]
20. Girelli, A.M.; Chiappini, V.; Amadoro, P. Immobilization of lipase on spent coffee grounds by physical and covalent methods: A comparison study. *Biochem. Eng. J.* **2023**, *192*, 108827. [CrossRef]
21. Campos-Vega, R.; Loarca-Piña, G.; Vergara-Castañeda, H.A.; Oomah, B.D. Spent coffee grounds: A review on current research and future prospects. *Trends in Food Science & Technology* **2015**, *45*, 24–36.
22. Osorio-Arias, J.; Delgado-Arias, S.; Duarte-Correa, Y.; Largo-Ávila, E.; Montaño, D.; Simpson, R.; Vega-Castro, O. New powder material obtained from spent coffee ground and whey protein; Thermal and morphological analysis. *Mater. Chem. Phys.* **2020**, *240*, 122171. [CrossRef]
23. Ballesteros, L.F.; Teixeira, J.A.; Mussatto, S.I. Chemical, Functional, and Structural Properties of Spend Coffee Grounds and Coffee Silverskin. *Food Bioprocess Technol.* **2014**, *7*, 3493–3503. [CrossRef]
24. Ortiz, C.; Ferreira, M.L.; Barbosa, O.; Santos, J.C.S.; Rodrigues, R.C.; Berenguer-Murcia, A.; Briand, L.E.; Fernandez-Lafuente, R. Novozym 435: The "perfect" lipase immobilized biocatalyst? *Catal. Sci. Technol.* **2019**, *9*, 2380–2420. [CrossRef]
25. Tahir, M.N.; Adnan, A.; Strömberg, E.; Mischnick, P. Stability of lipase immobilized on O-pentynyl dextran. *Bioprocess Biosyst. Eng.* **2012**, *35*, 535–544. [CrossRef] [PubMed]
26. Rodrigues, J.; Canet, A.; Rivera, I.; Osório, N.M.; Sandoval, G.; Valero, F. Biodiesel production from crude Jatropha oil catalyzed by non-commercial immobilized heterologous *Rhizopus oryzae* and *Carica papaya* lipases. *Bioresour. Technol.* **2016**, *213*, 88–95. [CrossRef]
27. Turati, D.; Morais Junior, W.; Terrasan, C.; Moreno-Perez, S.; Pessela, B.; Fernandez-Lorente, G. Immobilization of Lipase from *Penicillium* sp. Section Gracilenta (CBMAI 1583) on Different Hydrophobic Supports: Modulation of Functional Properties. *Molecules* **2017**, *22*, 339. [CrossRef]
28. Dumore, N.S.; Mukhopadhyay, M. Removal of oil and grease using immobilized triacylglycerin lipase. *Int. Biodeterior. Biodegrad.* **2012**, *68*, 65–70. [CrossRef]
29. Sanchez, D.A.; Tonetto, G.M.; Ferreira, M.L. Screening of Lipases with Unusual High Activity in the *sn*-2 Esterification of 1,3-Dicaprin under Mild Operating Conditions. *J. Agric. Food Chem.* **2017**, *65*, 5010–5017. [CrossRef]
30. Kaja, B.S.; Lumor, S.; Besong, S.; Taylor, B.; Ozbay, G. Investigating Enzyme Activity of Immobilized *Candida rugosa* Lipase. *J. Food Qual.* **2018**.
31. Lira, R.K.; Zardini, R.T.; de Carvalho, M.C.C.; Wojcieszak, R.; Leite, S.G.F.; Itabaiana, I., Jr. Agroindustrial Wastes as a Support for the Immobilization of Lipase from *Thermomyces lanuginosus*: Synthesis of Hexyl Laurate. *Biomolecules* **2021**, *11*, 445. [CrossRef] [PubMed]
32. Rodrigues, R.C.; Virgen-Ortiz, J.J.; dos Santos, J.C.S.; Berenguer-Murcia, A.; Alcantara, A.R.; Barbosa, O.; Ortiz, C.; Fernandez-Lafuente, R. Immobilization of lipases on hydrophobic supports: Immobilization mechanism, advantages, problems and solutions. *Biotechnol. Adv.* **2019**, *37*, 746–770. [CrossRef]
33. Zheng, J.; Fu, X.; Ying, X.; Zhang, Y.; Wang, Z. A sensitive colorimetric high-throughput screening method for lipase synthetic activity assay. *Anal. Biochem.* **2014**, *452*, 13–15. [CrossRef]
34. Barrera-Rivera, K.A.; Martinez-Richa, A. *Yarrowia lipolytica* Extracellular Lipase Lip2 as Biocatalyst for the Ring-Opening Polymerization of ε-Caprolactione. *Molecules* **2017**, *22*, 1917. [CrossRef] [PubMed]
35. Pereira, E.B.; Zanin, G.M.; Castro, H.F. Immobilization and catalytic properties of lipase on chitosan for hydrolysis and esterification reactions. *Braz. J. Chem. Eng.* **2003**, *20*, 343–355. [CrossRef]

36. Budžaki, S.; Velić, N.; Ostojcić, M.; Stjepanovć, M.; Bilić Rajs, B.; Šereš, Z.; Maravć, N.; Stanojev, J.; Hessel, V.; Strelec, I. Waste Management in the Agri-Food Industry: The Conversion of Eggshells, Spent Coffee Grounds, and Brown Onion Skins into Carriers for Lipase Immobilization. *Foods* **2022**, *11*, 409. [CrossRef]
37. Toczko, M.; Grzelińska, A. *Materiały do ćwiczeń z Biochemii*; Wydaw. SGGW: Warsaw, Poland, 2001.

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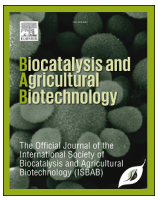

Obtaining a biodegradable biocatalyst – study on lipase immobilization on spent coffee grounds as potential carriers

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ABSTRACT

Coffee waste can be reused as matrices for enzyme immobilization, as it contains various organic compounds able to adsorb catalytic proteins. In this study, spent coffee grounds were used as a support in the immobilization process in their native form and after being pretreated with hexane, ethanol, and sulphuric acid solution, respectively. Microbial lipases from *Aspergillus oryzae*, *Thermomyces lanuginosus*, and *Rhizomucor miehei* were adsorbed on the carrier, and as a reference, the abovementioned lipases were also immobilized on a synthetic matrix - Lewatit VP OC 1600. The research investigated the impact of the purification step on the immobilization process and the full characteristics of the obtained biocatalysts. The hydrolytic and synthetic activities of the immobilized enzyme preparations were tested, as well as substrate specificity, recovery, and temperature and pH activity profiles. SEM and FTIR analyses were also performed. The results showed that the chemical composition of coffee waste influenced the activity of the obtained biocatalysts, and the lack of hemicellulose caused a reduction in lipolytic activity. The study reveals that spent coffee grounds can be a potential support for enzyme immobilization, and lipases adsorbed on them have improved properties such as hydrolytic or synthetic activity, and stability in pH.

1. Introduction

The still-growing amount of agro-industrial waste constitutes an enormous challenge in its management. The Food Waste Index Report 2021 states that around 1.3 billion tons of produced food is lost and only some of it is being recycled into fertilizer (Food Waste Index Report, 2021). Reusing food waste is profitable from a bio-economic standpoint because of the greenhouse gas emission reduction, lowering processing costs, and carbon footprint issues. Agricultural and food materials have great potential due to the plentifulness of bioactive compounds and high availability. The presence of different chemical groups (hydroxyl, amino, carboxyl, and phosphate groups), their surface, and porosity make such materials a possible carrier for enzyme immobilization (Girelli et al., 2020; Bilal and Iqbal, 2019; Nájera-Martínez et al., 2022).

Due to environmental aspects, developing new biocatalysts can be beneficial, because of bio-based carriers on which enzymes can be adsorbed and desirable properties compared to chemical catalysts such as higher substrate and reaction specificity, and lower en-

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ergy requirements. In addition, the immobilization of enzymes enables their separation from the reaction mixture after ending the process, and thus their recovery and reusing. It also improves their stability and allows them to be used in extreme pH or temperature conditions (Franssen et al., 2013; Budžaki et al., 2022). Moreover, the chemical stability of immobilized enzymes and therefore their resistance to the substances, both substrates and products or by-products present in the reaction mixture, are also widely discussed in the literature (Ismail and Baek, 2020).

The numerous industries that use enzymes are dominated by three groups of such proteins: carbohydrases, proteases, and lipases. Despite the latter accounting for less than 10% market share, they are irreplaceable in the food, animal feed, detergent, and pharmaceutical sectors (Guerrand, 2017; Mokhtar et al., 2020; Ittrat et al., 2014). The Future Market Insights study shows that in the next ten years, i.e. by 2033, the value of the lipase market will increase to US$ 1631.2 million, and a CAGR (compound annual growth rate) of 10.3% during the projected period is expected. The physiological role of the lipases (EC 3.1.1.3) is to catalyze the hydrolysis of triglycerides to diacylglycerols, monoacylglycerols, free fatty acids, and glycerol and these are enzymes that commonly occur in nature, e.g., in seeds and vegetative organs of plants, microorganisms, human and animals. Apart from hydrolysis occurring in an aqueous environment, a special advantage of lipases is their synthetic activity in organic solvents. Within such conditions, the opposite phenomenon of hydrolysis is observed, where Zaks and Klibanov (1985) described that lipases took part in esterification, as well as amidation, thioesterification, and transesterification - interesterification, acidolysis, and alcoholysis.

Recently, a trend has emerged in the use of lignocellulosic materials, often regarded as waste, for enzyme immobilization, including lipases. The examples of carriers given in the following sentences can be used as the so-called low-cost immobilization supports. So far, by using an adsorption method several materials have been applied as carriers, including bamboo (K de S Lira et al., 2021; G. Palma et al., 2021), cashew apple bagasse (Serpa et al., 2021), coconut waste (Oliveira-Ribeiro et al., 2019), corn cob (Ittrat et al., 2014; K de S Lira et al., 2021; Costa-Silva et al., 2016), rice husk (Ittrat et al., 2014; Costa-Silva et al., 2016; Srisaipet et al., 2005; Santos et al., 2021) and sugarcane bagasse (K de S Lira et al., 2021; Costa-Silva et al., 2016; Mittersteiner et al., 2018).

Spent coffee grounds (SCG) are generated in tremendous amounts with an annual generation of 6 million tons worldwide and representing up to 60% of the processed coffee beans. Global coffee consumption is still growing, hence annual coffee production is progressive continuously. That is why, the amount of generated SCG waste will increase. This lignocellulosic waste is a very affordable and renewable feedstock that possesses specific characteristics required for excellent enzyme support, including physical strength, inertness, and stability due to its composition. The abundance of organic compounds like fatty acids, lignin, cellulose, hemicellulose, and other polysaccharides makes spent coffee grounds valuable matrices for enzyme immobilization. (Campos-Vega et al., 2015; Mak et al., 2023; Atabani et al., 2022; Srinivasan et al., 2023; Colantoni et al., 2021). Despite its well-known characteristics, it is rarely used as an enzyme support. The literature only reports its use for immobilization of lipase (Girelli et al., 2023; K de S Lira et al., 2021), β-glucosidase (Chen et al., 2013), and cellulase (Buntić et al., 2018). It also emphasizes the importance of selecting an appropriate enzyme immobilization protocol, as various factors can influence the activities and properties of biocatalysts. Therefore, the area of research within the reuse of SCG as a matrix for immobilization process is still undiscovered and represents great potential. This article is another one that wants to expand the knowledge on this topic.

The main aim of this research was to obtain an active low-cost biocatalyst immobilized on lignocellulosic food waste. For the first time, three lipases of microbial origin from *Aspergillus oryzae*, *Thermomyces lanuginosus*, and *Rhizomucor miehei* were immobilized by the adsorption method on spent coffee grounds. The establishment of the study was to verify how different pretreatments of coffee waste and their chemical composition impact the immobilization process and how this translates into enzyme activity. Moreover, this research answers the question of which SCG ingredients are crucial in the adsorption process between the enzyme and the carrier. The study contained a full characterization of obtained biocatalysts, including the most important properties such as the ability to catalyze both hydrolysis and synthesis reactions. Moreover, the protein content on the carrier's surface, the stability in different conditions such as pH and temperature, and the possibility of recovery were tested. Additionally, the specificity of lipases against various substrates was carried out. In this study, the above-mentioned microbial-derived lipases were immobilized also on a synthetic carrier - macroporous acrylic resin to compare the properties with biocatalysts adsorbed on biodegradable support.

2. Materials and methods

2.1. Materials and biocatalysts

In the present research the following liquid lipases, which were kindly gifted from Novozymes (Basgvaerd, Denmark), were applied as biocatalysts: Lipozyme TL (from *Thermomyces lanuginosus*), Novozym 51032 (from *Aspergillus oryzae*) and Palatase 20000L (from *Rhizomucor miehei*). The supports for the immobilization of enzymes were four types of spent coffee grounds with varying degrees of purification which were received from EcoBean (Warsaw, Poland). As a reference for lignocellulosic support, the synthetic carrier – Lewatit VP OC 1600 was used (Lanxess, Cologne, Germany). The results of the lipase activity assay were compared with commercial *Candida antarctica* lipase B (CALB). All chemical reagents and solvents were purchased from Sigma-Aldrich (Poznań, Poland).

2.2. Pretreatment of spent coffee grounds

The research conducted raw materials taken at various stages of the company's continuous technological process described in patent application no. WIPO ST 10/C PL447416. The following pretreatment of each sample is described below:

SCG1 – spent coffee grounds were collected from Warsaw cafe. After transportation, SCG were dried to a moisture content of approximately 5%.

SCG2 – SCG1 were boiled with hexane in a reactor under pressure for 30 min, and filtrated in a filter drier to separate the solid phase from hexane and coffee oil.
SCG3 – To obtain SCG3 extraction 40–50% ethanol solution was required. Extraction was carried out in the reactor for 2 h at a ratio SCG: solvent (1:10). The necessary step was filtration and drying analogous to the preparation of SCG2.
SCG4 – SCG4 are the most purified grounds. SCG3 waste was hydrolyzed in a reactor with 2,5% aqueous sulphuric acid solution at radio 1:9, for - 3 h and temperature 100 °C. SCG4 was separated from the liquid and dried grounds.

2.3. Determination of fiber of spent coffee grounds

The fiber fraction was determined in the Fibertec^{MC} 8000 system (Foss Analytics, Warsaw, Poland). The percentage of crude fiber was carried out by the method PN-ISO 5498 (1996). The content of acid detergent fiber (ADF) and acid detergent lignin (ADL) was determined according to PN-EN ISO 13906 (2009). Meanwhile, determining neutral detergent fiber (NDF) content after amylase treatment was carried out according to PN-EN ISO 16472 (2007). Cellulose concentration was calculated as the difference between ADF and ADL, and hemicellulose concentration as the difference between NDF and ADF.

2.4. Lipase immobilization procedures

2.4.1. Immobilization on Lewatit VP OC 1600

Three liquid lipases were immobilized by adsorption on Lewatit according to the methodology described by Jasińska et al. (2023) in preliminary studies. Before the process, synthetic beads were activated in ethanol. For immobilization, 1 g of support and 15 mL of lipase solution were used. Each reaction contained 1 mL of lipase (Lipozyme TL, Novozym 51032, Palatase, 20000L) in the solution. All components were shaken in flasks in a rotatory shaker for 14 h, at 4 °C. Then, immobilized beads were separated from the mixture by filtration, washed with distilled water, and dried under vacuum for 24 h, at room temperature. Three repetitions of each preparation were performed.

2.4.2. Lipase immobilization on spent coffee grounds

Lipases were immobilized by adsorption on different samples of spent coffee grounds (SCG) delivered by EcoBean. There were four fractions: the native one without any pretreatment (SCG1), spent coffee grounds after hexane treatment (SCG2), spent coffee grounds after ethanol treatment (SCG3), and spent coffee grounds after sulphuric acid treatment (SCG4). The immobilization procedure was based on the methodology included in the study by Jasińska et al. (2023). Lipase solutions – 1 mL of liquid lipase and 14 mL of distilled water were added to a flask with 1 g of support and then agitated for 2 h. After this time, the obtained enzyme preparations were filtered, washed with distilled water, and dried at room temperature.

2.5. Lipase activity assay

2.5.1. Hydrolytic activity

The measurements were based on the spectrophotometric method. The reaction – hydrolysis of *p*-nitrophenyl laurate was carried out at 37 °C. 100 μl of free liquid lipase or 25 mg of immobilized enzyme preparation in 100 μl of distilled water and 25 μl *p*-nitrophenyl laurate solution (0.3 mmol dissolved in 2 ml of heptane) were added to Eppendorf test tubes and stirred for 15 min. After this time, the absorbance was measured immediately at 410 nm by a UV–Vis spectrophotometer. The unit of lipase enzymatic activity was 1U, i.e., the amount of enzyme that released 1 μmol of *p*-nitrophenol per minute under the assay conditions.

2.5.2. Synthetic activity

The synthetic activity of free and immobilized lipases was checked based on the methodology described in Jasińska et al. (2023) study. The transesterification reaction was carried out in an Eppendorf tube and contained 100 mM vinyl acetate and 100 mM *n*-butanol in 1 mL of *n*-hexane. To the mixture, 10 μL of free liquid lipase or 5 mg of immobilized lipase were added. After 5 min of incubation, diluted samples were prepared in test tubes. To each one 1 mL of 0.1% (*m/v*) MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate) solution was added and agitated for 10 min at 30 °C. After that time 0.4 mL of 1% (*m/v*) $H_4FeNO_4S_2 \cdot 12H_2O$ solution (in 0.1M HCl) was added and mixed for 30 min at 30 °C. Then, the content of released acetaldehyde converted to a blue-colored tetraaza-pentamethincyanine (TAPMC) was measured by spectrophotometric assay at 595 nm.

2.5.3. Substrate specificity

A spectrophotometric method based on hydrolysis reaction (section 2.5.1.) was used to determine how specifically lipases act against different substrates. The following substrates were used for the study: *p*-nitrophenyl butyrate (C4:0), *p*-nitrophenyl caprylate (C8:0), *p*-nitrophenyl laurate (C12:0), *p*-nitrophenyl palmitate (C16:0), *p*-nitrophenyl stearate (C18:0), and *p*-nitrophenyl oleate (C18:1).

2.5.4. Recovery

The study aimed to evaluate the reusability of particular biocatalysts using the hydrolytic activity measurement method outlined in section 2.5.1. The hydrolysis reaction of *p*-nitrophenyl laurate was conducted at a temperature of 37 °C and at a pH of 7 for 15 min. Afterward, the absorbance was measured by a spectrophotometer. The immobilized enzyme was separated from the reaction mixture, washed twice with hexane and phosphate buffer (pH 7), and then reused to catalyze the same reaction. The biocatalysts were used for five cycles.

2.5.5. Profile of pH activity

To determine the influence of different pH on immobilized enzyme preparations, the method of assessing the hydrolytic activity with hydrolysis of *p*-nitrophenyl laurate (section 2.5.1.) was used. The reaction was carried out in phosphate buffer (10 mM) at pH 5, 6, 7, and 8 instead of water. The temperature for each reaction was constant and reached 37 ˚C.

2.5.6. Profile of temperature activity

To assess the impact of temperature on the biocatalysts, measurements of hydrolytic activity were conducted while keeping all conditions constant except for the temperature range. The reaction was carried out at temperatures ranging from 30 ˚C to 70 ˚C and the hydrolytic activity was monitored as described in section 2.5.1.

2.6. Protein content

The analysis of protein content in free lipase and filtrates after the immobilization process was provided by Lowry's method fully described in Jasińska et al. (2023) work. The samples were diluted 50 times. 1 mL of each solution and 5 mL of copper reagent were incubated for 10 min. After that time the Folin–Ciocâlteu phenol reagent was added. After 30 min of incubation, spectrophotometric measurements were carried out at 750 nm in a Rayleigh UV-1601 spectrophotometer (BRAIC, Bejing, China). The percentage of adsorbed proteins on a carrier was calculated based on the difference between the protein content of free lipase solutions and in filtrates after immobilization.

2.7. Hydrolytic and synthetic specific activity

Based on the obtained results of hydrolytic and synthetic activity and protein content, the specific activities of immobilized biocatalysts and free lipase were calculated according to the equations:

1. Liquid, free lipase

$$Specific\ activity\ \left[\frac{U}{mg}\right] = \frac{hydrolytic\ or\ synthetic\ activity\ \left[\frac{U}{ml}\right]}{protein\ content\ in\ liquid, free\ lipase\ \left[\frac{mg\ protein}{ml}\right]}$$

2. Immobilized lipase

$$Specific\ activity\ \left[\frac{U}{mg}\right] = \frac{hydrolytic\ or\ synthetic\ activity\ \left[\frac{U}{mg}\right]}{pr\ otein\ immobilized\ on\ support\ \left[\frac{mg\ protein}{mg\ support}\right]}$$

2.8. Scanning electron microscopy (SEM)

The native supports and immobilized enzyme preparations were analyzed in terms of the surface structure by an electron microscope (HITACHI TM 3000, Ramsey, New Jersey, USA). The samples were dried under vacuum and coated with layers of gold (Cessington Sputter Coater 108 auto, Cressington Scientific Instruments, Watford, UK) and then observed. Microphotographs were taken at a magnification of 400x.

2.9. FTIR spectroscopy

The FTIR spectra of all preparations were recorded on a Nicolet iS5 ATR Thermo Scientific spectrometer equipped with a diamond crystal iD7 ATR sampling component (Thermo Fisher Scientific, Waltham, USA). The measurement was taken in the range 4000–600 cm^{-1}.

2.10. Statistical analysis

The obtained results were statistically analyzed using the STATISTICA 13.3 software (StatSoft, Krakow, Poland). The following methods were used: Shapiro-Wilk test to verify the statistical hypothesis of normality of the distribution of the experimental data, Levene's and Brown-Forsythe tests for checking the hypothesis of homogeneity of variance, analysis of variance (ANOVA), and post-hoc Tukey's test. Values of $p \leq 0.05$ were considered to be statistically significant. For cluster analysis, the obtained data for the prepared biocatalysts (n = 15), i.e., adsorbed protein content, hydrolytic activity, synthetic activity, specific hydrolytic activity, and specific synthetic activity were standardized. Ward's method was applied and the distances between clusters were estimated with the Euclidean distance.

3. Results and discussion

3.1. Characterization of the support material – spent coffee grounds

3.1.1. Chemical composition of SCG – cellulose, hemicellulose and lignin

Spent coffee grounds are a waste product obtained after the coffee brewing process and in the production of instant coffee. They are abundant in sugars polymerized into cellulose and hemicellulose structures, which constitute about half of the dry material (Table 1). The content of these two organic compounds in native spent coffee grounds (SCG1) was comparable to others reported in the liter-

Table 1
Cellulose, hemicellulose, and lignin content of SCG1 – native spent coffee grounds, SCG2 – spent coffee grounds after hexane treatment, SCG3 – spent coffee grounds after ethanol treatment, and SCG4 – spent coffee grounds after sulphuric acid treatment. CF – crude fiber, NDF – neutral detergent fiber, CEL – cellulose, HEM – hemicellulose, ADF – acid detergent fiber, ADL – acid detergent lignin, DM – dry mass.

PARAMETER	UNIT	SCG1	SCG2	SCG3	SCG4
CF	%DM	28.09 ± 0.56	30.57 ± 0.40	33.53 ± 0.04	23.37 ± 0.04
NDF	%DM	61.61 ± 0.90	67.38 ± 1.11	74.90 ± 0.98	48.24 ± 0.04
ADF	%DM	37.92 ± 0.07	40.86 ± 0.03	44.69 ± 0.03	47.81 ± 0.19
CELLULOSE (ADF-ADL)	%DM	21.06 ± 0.41	23.12 ± 0.57	25.11 ± 0.07	28.90 ± 0.05
HEMICELLULOSE (NDF-ADF)	%DM	23.69 ± 0.97	26.52 ± 1.09	30.21 ± 1.00	0.43 ± 0.22
TOTAL (CEL AND HEM)	%DM	44.75 ± 1.38	49.63 ± 0.52	55.31 ± 0.94	29.33 ± 0.17
LIGNIN (ADL)	%DM	16.87 ± 0.48	17.75 ± 0.60	19.59 ± 0.04	18.91 ± 0.14

ature for SCG (Ballesteros et al., 2014; Mussatto et al., 2011). The total amount of cellulose and hemicellulose changed depending on the stage of waste pretreatment. With the removal of fats and then polyphenols the content was increasing (SCG2 and SCG3), but for the samples that were purified the most (SCG4), the results showed that they consisted mainly of cellulose and traces of hemicellulose. Moreover, the fraction SCG4 included the lowest amount of NDF–neutral detergent fiber in comparison to other samples. It confirmed that the pretreatment with acids (such as sulphuric acid) impacts the hemicellulose and breaks glycosidic bonds (Nájera-Martínez et al., 2022), resulting in its removal from the fraction.

Lignin is a natural polymer that is also present in spent coffee grounds. Its content was similar in each sample (SCG1 – 16.87%, SCG2 – 17.75%, SCG3 – 19.59%, and SCG4 – 18.91%), but the values were slightly lower than shown in other studies - Ballesteros et al. (2014) – 23.90%, Karmee (2018) – 25%, respectively. These minor differences could be attributed to the diversity of spent coffee ground sources.

3.1.2. SCG morphology by SEM

The morphological structures of SCGs (Fig. 1) were analyzed using a Scanning Electron Microscope. The modification of the carrier affected its porosity. The structure of native coffee waste (SCG1) was finer and more compact with itself. After pretreatment, in the microphotographs of SCG2 (Fig. 1B), SCG3 (Fig. 1C), and SCG4 (Fig. 1D) the irregular shape and numerous macropores. In accordance with the literature (Buntić et al., 2018; Girelli et al., 2023) the removal of different functional groups from coffee waste support probably affected the structure of the material and caused uprising channels with various sizes.

3.1.3. SCG analysis by FTIR spectroscopy

The FTIR technique was employed to investigate the functional groups of the native and modified spent coffee grounds. The received FTIR spectra showed similar attitudes but with different peak heights (Fig. 2). The obtained results revealed a broad peak between 3600 and 3200 cm^{-1} specific to O–H bond vibration, and two visible sharp peaks at 2920 and 2851 cm^{-1} which are characteristic of C–H bond in aliphatic chains and confirmed the presence of methyl and methylene groups, as well as, the peak at 1741 cm^{-1} connected to C=O bonds, peak at 1647 cm^{-1} related to C=C bonds (aromatic) and two peaks at 1374 and 1025 cm^{-1} specific to C–O bonds (Ballesteros et al., 2014; Buntić et al., 2018; Cruz-Lopes et al., 2017; Bejenari et al., 2021). The main presence of cellulose and lignin in this coffee material likely caused the intensification of peaks that are characteristic of the aforementioned compounds. Especially, higher results were noticed at 1025 cm^{-1} which might be specific for cellulose C–O–C pyranose ring vibration, at 1519 cm^{-1} which can be connected to lignin aromatic rings C = C bonds (Girelli et al., 2023; Cruz-Lopes et al., 2017) and at 2920 cm^{-1} which probably corresponds to the stretching vibration of C–H band in CH_2, CH_3, and OCH_3 groups of lignin structures (Liu et al., 2014).

3.2. Characterization of immobilized biocatalysts

3.2.1. Lipase activity assay

The physiological role of lipases is to hydrolyze triglycerides into fatty acids and glycerol. However, they can be also applied to catalyze esterification, transesterification, and interesterification, which are widely used in biotechnology (Ismail and Baek, 2020). The activity of three different lipases (Lipozyme TL - L, Novozym 51032 - N, and Palatase, 20000L - P) immobilized on biodegradable support – spent coffee grounds and synthetic carrier – Lewatit VP OC 1600 was compared with commercial lipase B from *Candida antarctica* (Figs. 3 and 4). All of the received biocatalysts were capable of catalyzing hydrolysis of *p*-nitrophenyl laurate but it was noticed that pretreatment of spent coffee grounds influences their activity. The lowest values were reached for the enzyme preparations which were immobilized on the most purified coffee waste, respectively NSCG4 – 0.0018 U/mg, LSCG4 – 0.0002 U/mg, and PSCG4 – 0.0014 U/mg (Fig. 3). This may indicate that absence of hemicellulose in the support and mainly the presence of cellulose as well as lignin had an impact on the decrease of hydrolytic activity of the analyzed lipases from *Th. lanuginosus*, *A. oryzae* and *R.miehei.* Some authors found that lignin may limit the interaction between the support and the enzyme. Moreover, they also reported that there are differences in the fundamental mechanism of the enzyme's adsorption onto lignin from various lignocellulosic biomass, due to the diverse composition and structural features of the compound (Buntić et al., 2018; Baig, 2020; Rodríguez-Restrepo and Orrego, 2020). For all of the investigated lipases (Lipozyme TL, Novozym 51032, and Palatase, 20000L) there were only slight differences in the hydrolytic activity of enzymes adsorbed between native (SCG1) and defatted (SCG2) or defatted and free of polyphenols (SCG3) forms of the carrier. Removal of the selected functional groups on the carrier did not highly affect the effectiveness of catalyzing the hydrolysis reaction by the adsorbed enzyme.

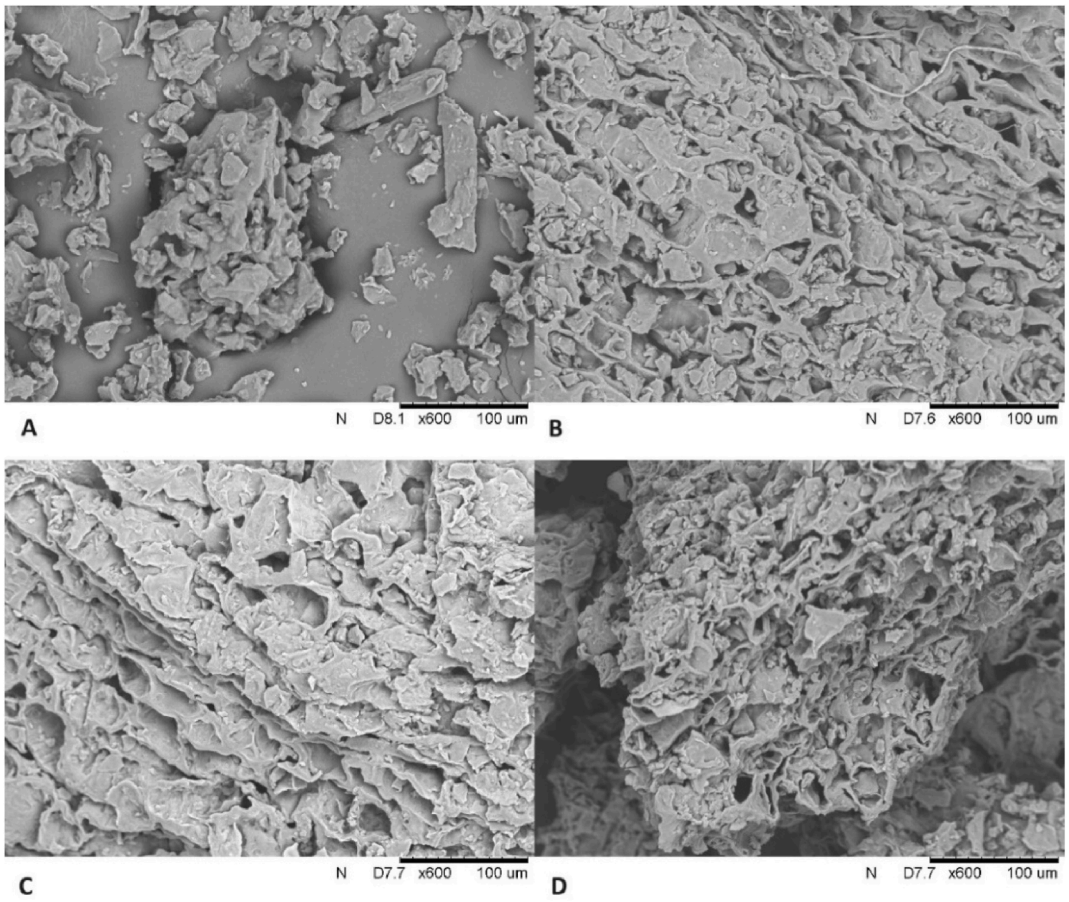

Fig. 1. Scanning electron microphotographs (x600) of A – native spent coffee grounds (SCG1), B – spent coffee grounds after hexane treatment (SCG2), C – spent coffee grounds after ethanol treatment (SCG3), D – – spent coffee grounds after sulphuric acid treatment (SCG4).

The study assumed also a comparison between biodegradable biocatalysts, those immobilized on synthetic carriers, and commercial CALB. In the case of immobilization of lipase Lipozyme TL and Palatase it succeeded in obtaining more active biocatalysts adsorbed on spent coffee grounds (SCG1, SCG2, SCG3) than on Lewatit VP OC 1600 – macroporous acrylic resin, the same on which lipase B from *Candida antarctica* is immobilized (Ortiz et al., 2019). A similar tendency was observed with the hydrolytic activity of the commercial enzyme preparation. Still, for lipase, Novozym 51032 immobilized on the native spent coffee grounds the activity was almost equal for the biocatalysts with Lewatit as support.

The results of the synthetic activity of designed biocatalysts are shown in Fig. 4. Immobilized lipases were tested for their ability to catalyze the synthesis reaction of vinyl acetate and *n*-butanol with *n*-hexane as a solvent. The best activity (3.76 U/mg) was presented by commercially available immobilized lipase – CALB. Under study microbial lipases did not receive such successful results and reached respectively NL – 0.17 U/mg, LL – 0.05 U/mg, and PL – 0.21 U/mg. These three biocatalysts were immobilized on the same synthetic support as well as CALB but gained much lower activity. The combination of Lewatit VP OC 1600 with CALB cannot be extrapolated to other lipases. Despite the general preference of lipases to be immobilized on hydrophobic support, it was shown that not all of them revealed the same affinity for a given support (Rodríguez-Restrepo and Orrego, 2020). Among the biocatalysts adsorbed on spent coffee grounds, the leading one was lipase Novozym 51032 immobilized on SCG1 – native form. Along with the degree of purification, the synthetic activity of this enzyme decreased. For the preparation of lipase Lipozyme TL and Palatase, the activity on SCG1, SCG2, and SCG3 was similar. Biocatalysts immobilized on the most pretreated carrier did not show the activity, which confirmed that the lack of hemicellulose and increased participation of lignin in support negatively influence the synthetic activity of all studied lipases.

One of the advantages of the immobilization process is improving the catalytic properties of enzymes, which was also tested in this research. In Figs. 5–7, it was shown, how the physical adsorption of three microbial lipases onto different supports influences lipolytic activity in comparison with the non-immobilized enzymes. The results of the hydrolytic and synthetic activities were calculated based on concentrations of proteins adsorbed on the carrier and presented as a specific activity. For lipase – Novozym 51032 all immobilized enzyme preparations had significantly higher specific hydrolytic activity than the free lipase. Two biocatalysts – NSCG1 and NSCG2 reached also better results in specific synthetic activity compared to the non-immobilized lipase. A similar tendency was observed for other lipases. The immobilization process improved hydrolytic activity for all biocatalysts of lipase Palatase and most of li-

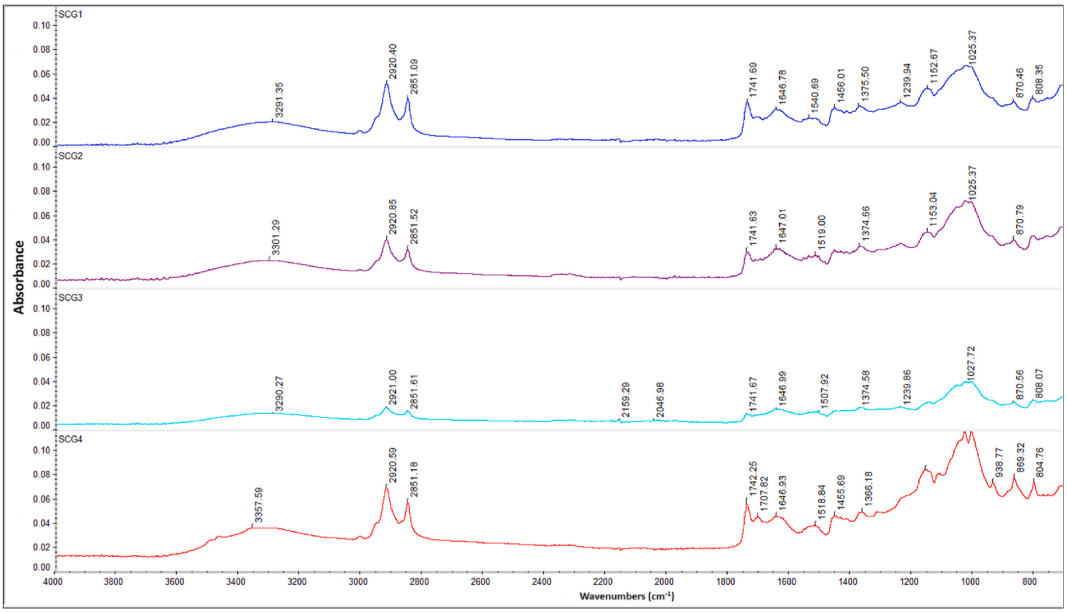

Fig. 2. FTIR spectra of SCG1 – native spent coffee grounds, SCG2 – spent coffee grounds after hexane treatment, SCG3 – spent coffee grounds after ethanol treatment, and SCG4 – spent coffee grounds after sulphuric acid treatment.

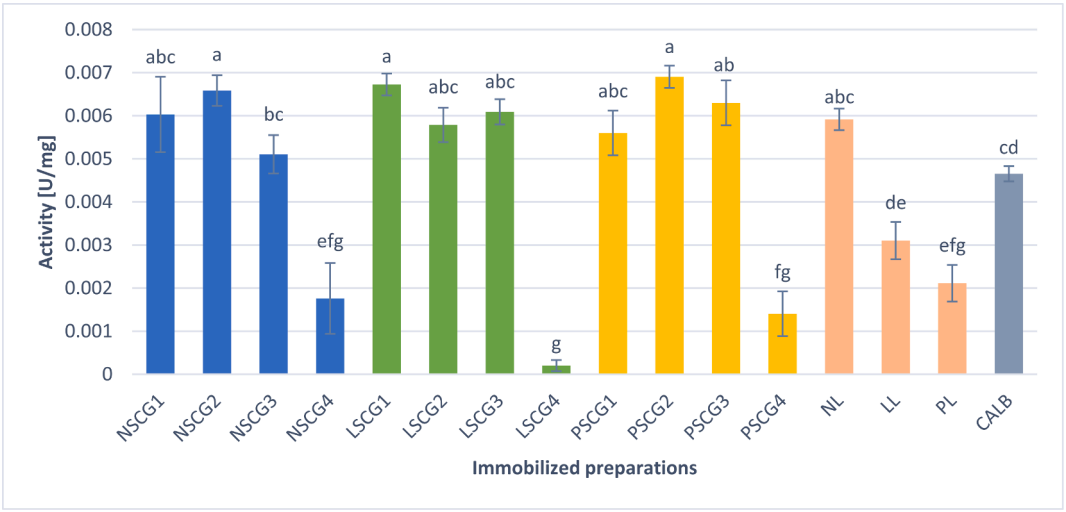

Fig. 3. The hydrolytic activity of immobilized lipases (N – Novozym 51032, L – Lipozyme TL 100L. P – Palatase, 20000L) on spent coffee grounds (SCG1, SCG2, SCG3, SCG4) and on L – Lewatit VP OC 1600. As reference CALB – lipase B of *Candida antarctica*. Means with the same letter (a–g) did not differ significantly ($\alpha = 0.05$).

pase Lipozyme TL. The ability to catalyze synthesis reaction was higher for preparations LSCG2, LSCG3, PSCG1, PSCG2, and PSCG3 in comparison with the free lipase. This proved that the immobilized lipases can boost their lipolytic activity and it showed how it is important to select a proper support for this process as not all matrices gave the same effect.

In Figs. 5–7 the percentage of protein content on support was also presented. Biocatalysts immobilized on spent coffee grounds reached content of adsorbed proteins between 68% and 80% for lipase Novozym 51032, 83%–92% for lipase Lipozyme TL, and 55%–90% for Palatase, which were lower than for synthetic carrier respectively. Despite this observation, the specific activity – hydrolytic and synthetic of biocatalysts immobilized on SCG1, SCG2, and SCG3 was significant. It suggests that spent coffee grounds may be a better support for those lipases and the quality of how proteins are immobilized on matrices is more important than the quantity of molecules. Probably in this configuration adsorbed lipases on spent coffee grounds still had an open active center. What is more, it was noticeable for microbial lipases immobilized on Lewatit VP OC 1600 that the high results of adsorbed proteins did not correlate with the high activity of biocatalysts. Despite the ability of lipases to adsorb on hydrophobic surfaces and the high content of proteins immobilized on macroporous acrylic polymer resin, both hydrolytic and synthetic specific activities were low. The general rule is that a more hydrophobic carrier can immobilize more lipases than a less hydrophobic one. However, lipases tend to form li-

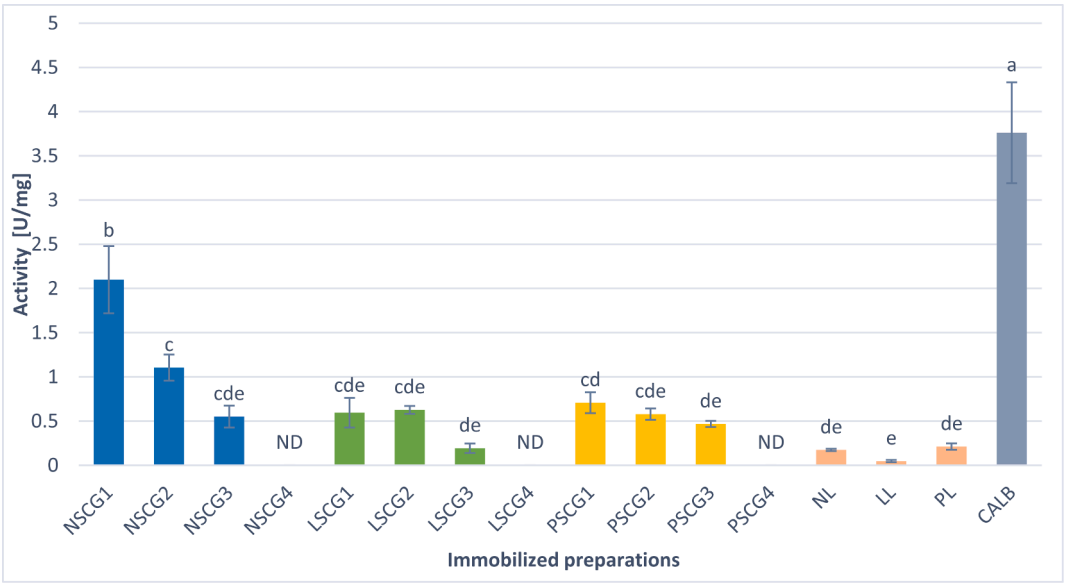

Fig. 4. The synthetic activity of immobilized lipases (N – Novozym 51032, L – Lipozyme TL 100L. P – Palatase, 20000L) on spent coffee grounds (SCG1, SCG2, SCG3, SCG4) and on L – Lewatit VP OC 1600. As reference CALB – lipase B of *Candida antarctica.* Means with the same letter (a–g) did not differ significantly (α = 0.05). ND – not detected.

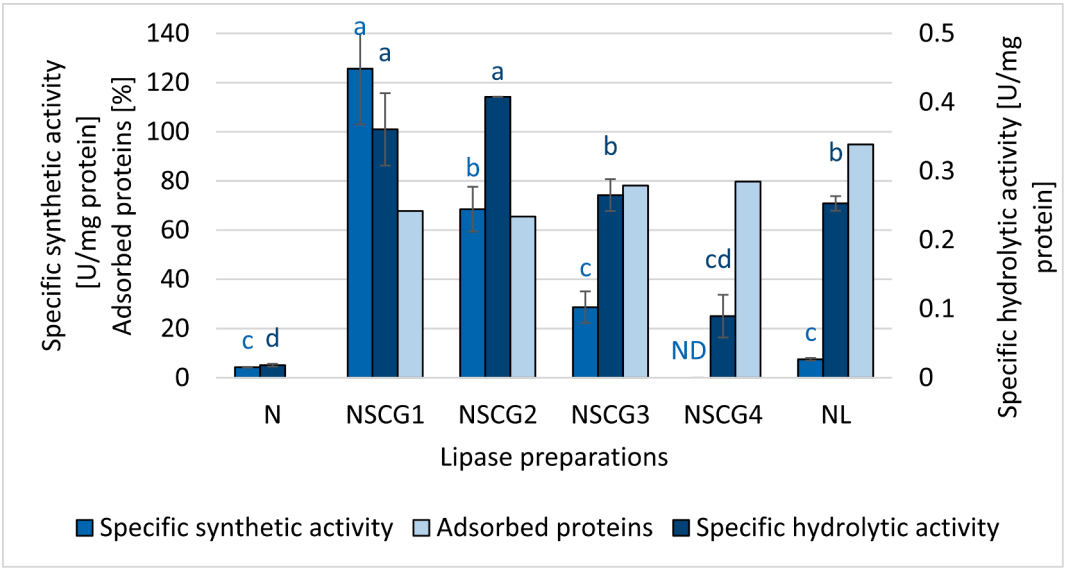

Fig. 5. Specific hydrolytic and synthetic activities of free (N) and immobilized lipase Novozym 51032 on different carriers (NSCG1, NSCG2, NCSG3, NCG4, NL) and level of adsorbed proteins on support after immobilization process. ND – not detected. The means compared within one parameter (specific hydrolytic or synthetic activity), marked with different lowercase letters, are statistically different (α = 0.05).

pase-lipase dimers containing the open form of each of the lipase molecules. It may cause problems with handling enzymes because the features of monomers and dimers can be very different. Dimer formation can result in the creation of enzyme aggregation on the support's surface, which can affect the open form and hinder lipase immobilization by interfacial activation on the hydrophobic carrier (Rodríguez-Restrepo and Orrego, 2020).

3.2.2. Profile of pH activity of tested biocatalysts

The pH influence on the enzyme activity has been monitored. The concentration of hydrogen ions may impact the ability of lipases to catalyze different reactions, thus the hydrolytic activity in various pH, from 5 to 8, was studied (Fig. 8). The majority of immobilized enzyme preparations were stable in a whole range of pH and the activity did not differ significantly. It suggested that lipases adsorbed on the investigated SCG supports have a more stable configuration and can be easily used as biocatalysts in reactions that require varied pH conditions. Similar properties were examined by Girelli et al. (2023). The researchers tested the pH stability in the range 6–8 of the immobilized form of lipase *Candida rugosa* on spent coffee grounds. They gained the maximum activity at pH 7, but

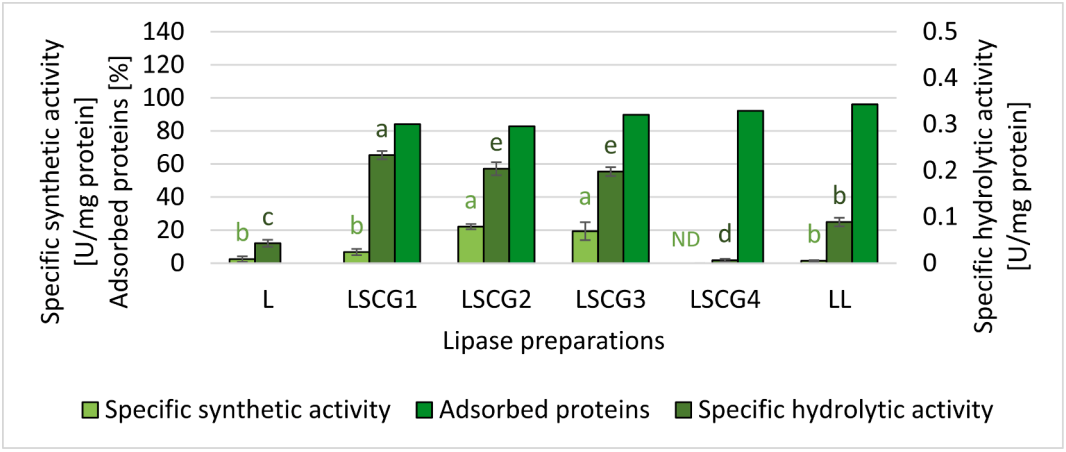

Fig. 6. Specific hydrolytic and synthetic activities of free (L) and immobilized lipase Lipozyme TL on different carriers (LSCG1, LSCG2, LCSG3, LCG4, LL) and level of adsorbed proteins on support after immobilization process. ND – not detected. The means compared within one parameter (specific hydrolytic or synthetic activity), marked with different lowercase letters, are statistically different (α = 0.05).

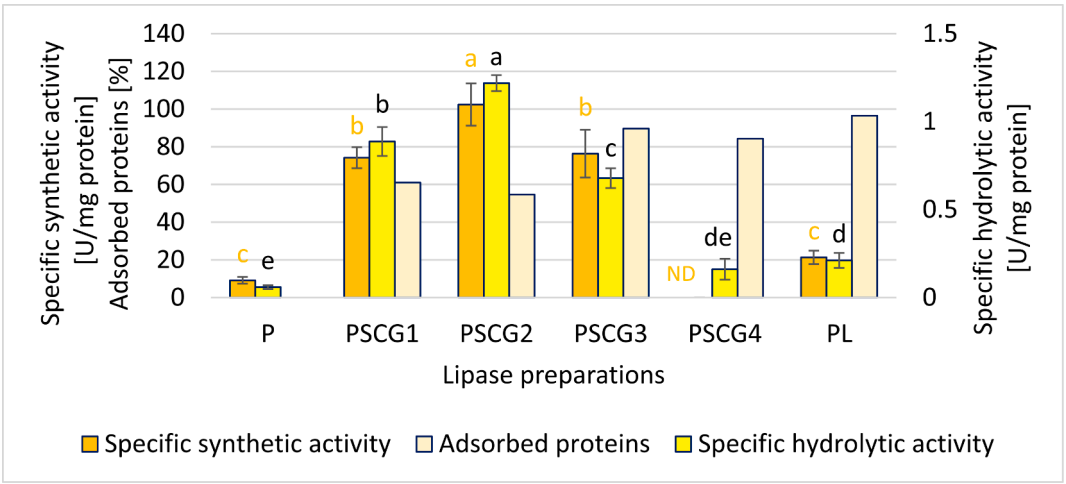

Fig. 7. Specific hydrolytic and synthetic activities of free (P) and immobilized lipase Palatase 20000L on different carriers (PSCG1, PSCG2, PCSG3, PCG4, PL) and level of adsorbed proteins on support after immobilization process. ND – not detected. The means compared within one parameter (specific hydrolytic or synthetic activity), marked with different lowercase letters, are statistically different (α = 0.05).

the results in the whole spectrum were higher than for the free lipase, which also highlights the advantages of the immobilization process.

3.2.3. Selection of the best immobilized biocatalyst

To select the best biocatalysts a cluster analysis was performed (Fig. 9). The following data were tested: adsorbed protein content, hydrolytic and synthetic activities, and specific hydrolytic and synthetic activities. The received results were divided into proper groups. The first cluster including the immobilized enzyme preparations such as NSCG3, LSCG1, LSCG2, LSCG3, and NL have reached the highest value of the adsorbed protein content. The second with biocatalysts – NSG1 and NSG2 have shown the best synthetic and specific hydrolytic activities. Within the third cluster with PSCG1, PSCG2, and PSCG3 the highest specific synthetic activity, even though they had the lowest adsorbed protein content on the support was noted. The least active hydrolytically and synthetically preparations, i.e., NSCG4, LSCG4, PSCG4, LL, and PL were found in the fourth cluster. For further tests, one biocatalyst of each microbial lipase – NSCG1 (Novozym 51032), PSCG1 (Palatase, 20000L), and LSG1 (Lipozyme TL 100L) immobilized on native spent coffee grounds were chosen. The selection was mainly based on the activity of immobilized enzyme preparation. The level of waste pretreatment was also an important criterion. Immobilized preparations on a native carrier were chosen, which is economically advantageous. The aim for the further part of the study was to focus on the analysis of lipases adsorbed on the spent coffee grounds as an alternative to synthetic carriers and to test their other features, like substrate specificity, temperature activity profile and the ability to reuse the biocatalysts in reaction.

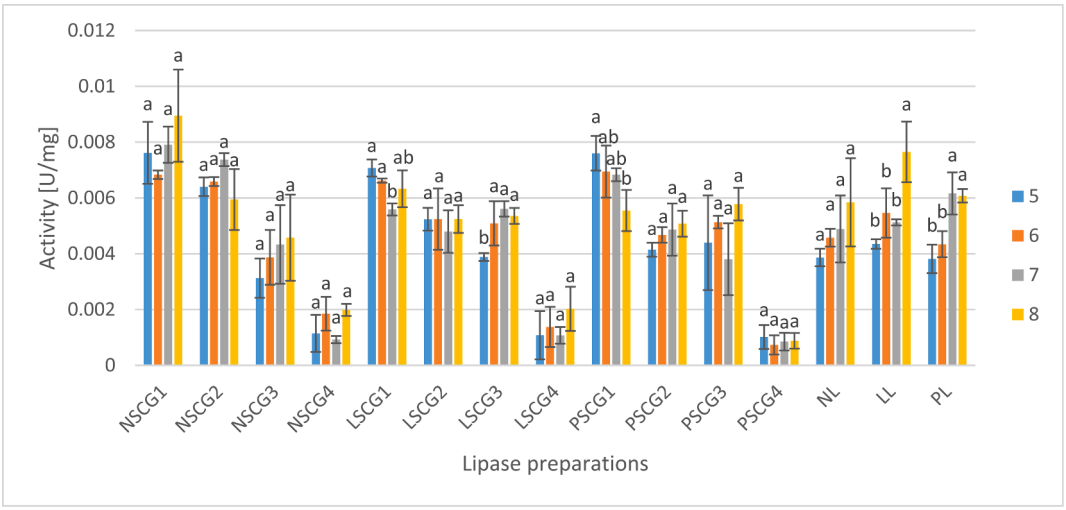

Fig. 8. The influence of pH (from 5 to 8) on hydrolytic activity of obtained biocatalyst. The means compared within one enzyme preparation in the tested pH range, marked with different lowercase letters, are statistically different (α = 0.05).

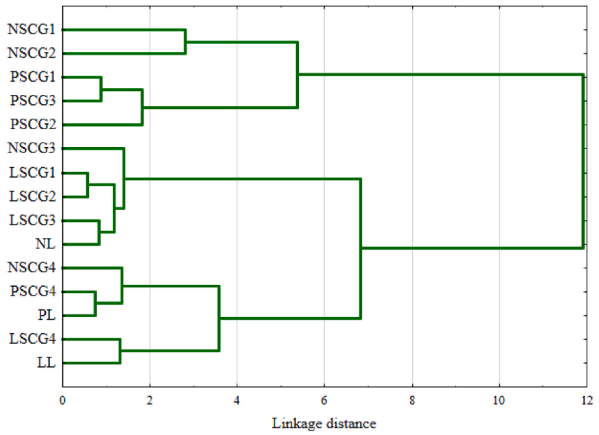

Fig. 9. Clustering tree diagram for the obtained biocatalysts according to Ward's method for Euclidean distances.

3.2.4. Substrate specificity of immobilized lipases

Substrate specificity was determined for three biocatalysts – LSCG1, PSCG1 and NSCG1 (Fig. 10). Six esters of *p*-nitrophenyl and fatty acids of varying carbon chain lengths were used in this experiment, in the presence of which the hydrolytic activity of microbial lipases was measured. From the obtained results, it can be observed that the highest lipolytic activities were achieved against the substrate *p*-nitrophenyl butyrate (C4:0) for LSCG1 (0.0079 U/mg); *p*-nitrophenyl butyrate (C4:0), *p*-nitrophenyl caprylate (C8:0) and *p*-nitrophenyl laurate (C12:0) for PSCG1 (0.0074 U/mg, 0.0071 U/mg, and 0.0076 U/mg); *p*-nitrophenyl butyrate (C4:0) and *p*-nitrophenyl caprylate (C8:0) for NSCG1 (0.0103 U/mg and 0.0102 U/mg). Lipases belong to the class of hydrolases and what distinguishes them from other enzymes, such as esterases is that they are mainly active toward water-insoluble substrates like triglycerides (Lopes et al., 2011). They can catalyze both hydrolysis of butyrate and long-chain esters which was confirmed in this study. A similar observation was described by Song et al. (2008), where they tested nine microbial lipases against 15 substrates with different carbon chain lengths and found that several lipases presented strong selectivity for short-chain esters, some for medium-chain or long-chain fatty acid esters. It pointed out that analysis of substrate specificities of microbial lipases is important for predicting the catalytic activity of enzymes and is helpful for researchers to select proper lipases for catalyzing characteristic reactions.

3.2.5. Temperature activity profile of immobilized biocatalysts

The study investigated the hydrolytic activity of immobilized lipases – LSCG1, PSCG1, and NSCG1 with respect to temperature, to understand the protective effect of the matrix on the immobilized enzyme in relation to thermal denaturation (Fig. 11). The temperature range of 30–70 °C was considered while measuring the enzyme activity. The study found that lipase Lipozyme TL showed the highest activity at 50 °C, while lipase Palatase 20000L had the highest activity at 60 °C, and lipase Novozym 51032 performed best at 70 °C. The results demonstrate that spent coffee grounds used as a support for immobilization can protect lipases at high temperatures, with only slight differences observed across the temperature range. Additionally, the study found that the origin of the lipase

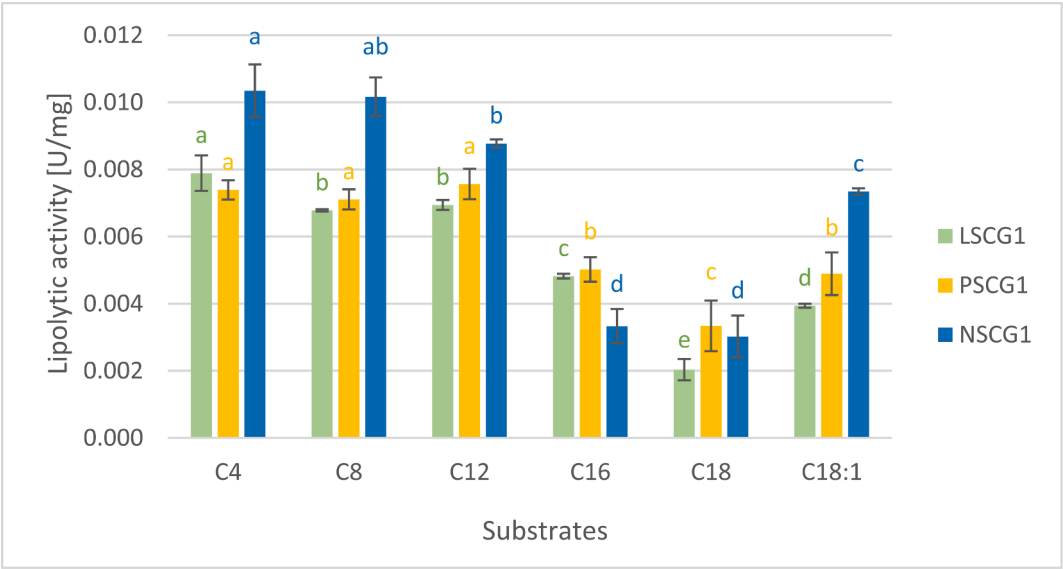

Fig. 10. Comparison of lipolytic activity of biocatalysts: LSCG1, PSCG1 and NSCG1 for the following substrates: *p*-nitrophenyl butyrate (C4:0), *p*-nitrophenyl caprylate (C8:0), *p*-nitrophenyl laurate (C12:0), *p*-nitrophenyl palmitate (C16:0), *p*-nitrophenyl stearate (C18:0), and *p*-nitrophenyl oleate (C18:1). The means compared within one enzyme preparation, marked with different lowercase letters, are statistically different (α = 0.05).

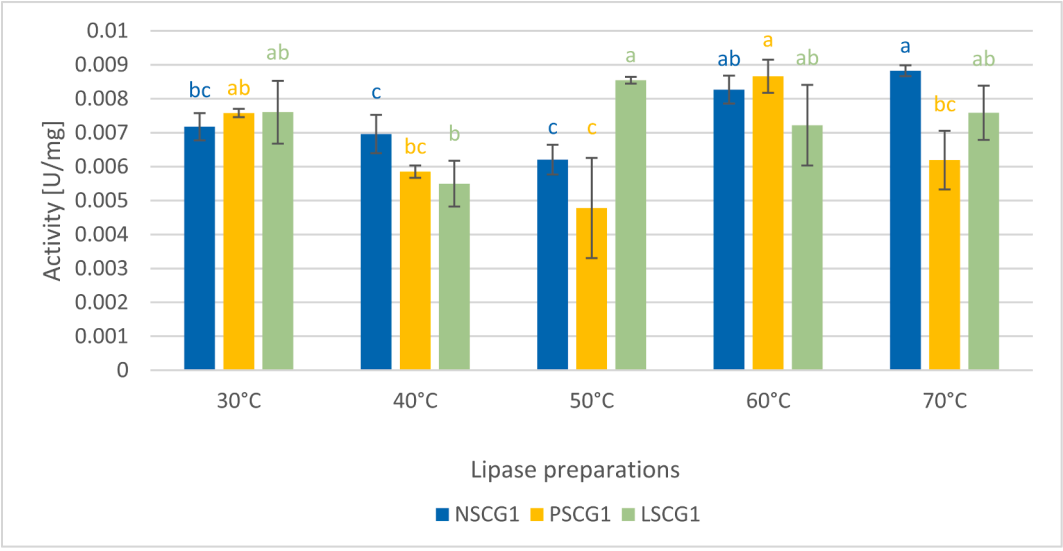

Fig. 11. Comparison of hydrolytic activity of biocatalysts: LSCG1, PSCG1 and NSCG1 at different temperatures – 30 °C, 40 °C, 50 °C, 60 °C and 70 °C). The means compared within one enzyme preparation, marked with different lowercase letters, are statistically different (α = 0.05).

may have an influence on its maximum activity. Similar results were obtained by Girelli et al. (2023), who immobilized lipase type VII from *Candida rugosa* (CRL) on SCG and for the immobilized enzyme, the maximum activity was obtained at 60 °C.

3.2.6. The recovery of immobilized biocatalysts

Another advantage of the immobilization of enzymes is the possible recovery of biocatalysts. Adsorption is a reversible method based on weak non-specific forces like van der Waals, hydrophobic interaction, hydrogen bonds, and ionic bonding. This simple procedure allows to reuse of the biocatalyst in multiple process cycles (Nájera-Martínez et al., 2022; Ittrat et al., 2014). In this study, microbial lipases immobilized on spent coffee grounds were tested in 5 cycles (Fig. 12). It is noticeable that in the second cycle, the relative activity was more than half lower, and after four cycles the activity dropped almost to zero. Despite the decreasing tendency of all biocatalyst activities, it was successful that immobilized enzyme preparations were still capable of catalyzing the hydrolysis reaction till 3rd cycle. The electrostatic and hydrophobic interactions can be influenced by several immobilization factors such as pH, quantity and quality of reagents, type of enzyme, surface area, chemical structure of the support, and ionic strength. Despite the potential of lignocellulosic waste as a support due to the presence of specific functional groups on their surface which can establish a bond with li-

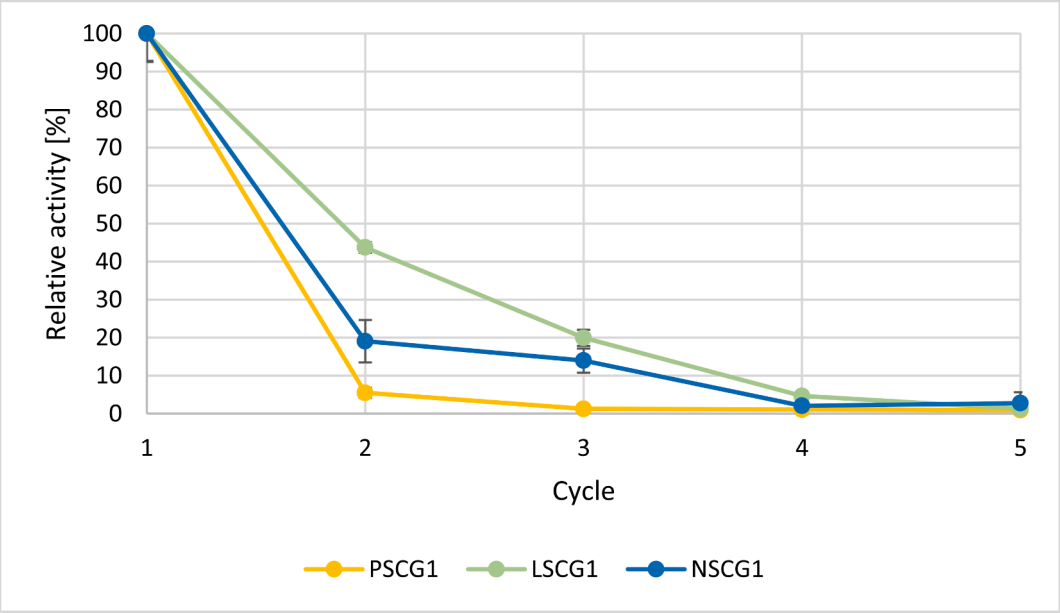

Fig. 12. Analyzing the recovery of immobilized lipases – PSCG1, LSCG1 and NSCG1. The highest hydrolytic activities of all biocatalysts were defined as 100%.

pase and their high porosity, lipases may be leached out from support (Ismail and Baek, 2020; Girelli and Chiappini, 2023). This may cause a loss of activity through the next process cycles.

4. Conclusion

In this research lipase from *Th. lanuginosus* (Lipozyme TL), *A. oryzae* (Novozym 51032), and *R. miehei* (Palatase, 20000L) were immobilized on various supports derived from spent coffee grounds and synthetic macroporous acrylic resin (Lewatit VP OC 1600) used as a reference to investigate the possibility of receiving efficient biocatalysts. The SCG were pretreated differently to optimize the process of immobilization. It was verified that the chemical composition of coffee waste has an influence on the activity of obtained biocatalysts and the lack of hemicellulose caused the reduction of lipolytic activity. As part of the conducted study, it was possible to gain immobilized enzyme preparations on biodegradable support which were hydrolytically and synthetically active and stable in different pH conditions. One of the received biocatalysts (lipase Novozym 51032) adsorbed on spent coffee grounds, primarily native and defatted form, revealed especially beneficial results compared to the commercial reference enzyme immobilized on the synthetic carrier (CALB). Although the preparations contained different protein content on the surface of the carrier, the higher amount of enzyme adsorbed on the carrier did not directly translate into their activity.

The study has confirmed that spent coffee grounds can be a potential support for enzyme immobilization. Reusing lignocellulosic waste can be a proper direction in the industry to ensure a circular economy and contribute to the reduction of the already existing waste. Lipases adsorbed on coffee waste have improved their properties such as hydrolytic or synthetic activities and stability in pH. Each microbial enzyme had various specificities and reached different results, which indicates the importance of selecting a properly prepared carrier. More detailed studies are still needed to characterize the biocatalysts adsorbed on spent coffee grounds. It would also be crucial to optimize the immobilization process, which will allow obtaining an enzyme preparation that can be reused in reactions multiple times and will not decrease its activity.

CRediT authorship contribution statement

Karina Jasińska: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Conceptualization. **Bartłomiej Zieniuk:** Writing – review & editing, Methodology, Conceptualization. **Adrianna Maria Piasek:** Writing – review & editing, Resources. **Łukasz Wysocki:** Writing – review & editing, Resources. **Anna Sobiepanek:** Writing – review & editing. **Agata Fabiszewska:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Wysocki has patent #WIPO ST 10/C PL447416 pending to EcoBean. Piasek has patent #WIPO ST 10/C PL447416 pending to EcoBean. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The samples of spent coffee grounds with different types of pretreatment were kindly received from EcoBean (Warsaw, Poland). As a reference, the synthetic carrier – Lewatit VP OC 1600 was gifted from Lanxess (Cologne, Germany). The enzymes were kindly gifted from Novozyme (Bagsvaerd, Denmark).

References

Atabani, A.E., Ali, I., Naqvi, S.R., Badruddin, I.A., Aslam, M., Mahmoud, E., Almomani, F., Juchelková, D., Atelge, M.R., Khan, T.M.Y., 2022. A state-of-the-art review on spent coffee ground (SCG) pyrolysis for future biorefinery. Chemosphere 286, 131730. https://doi.org/10.1016/j.chemosphere.2021.131730.

Baig, K.S., 2020. Interaction of enzymes with lignocellulosic materials: causes, mechanism and influencing factors. Bioresour. Bioprocess. 7. https://doi.org/10.1186/s40643-020-00310-0.

Ballesteros, L.F., Teixeira, J.A., Mussatto, S.I., 2014. Chemical, functional, and structural properties of spent coffee grounds and coffee silverskin. Food Bioprocess Technol. 7, 3493–3503. https://doi.org/10.1007/s11947-014-1349-z.

Bejenari, V., Marcu, A., Ipate, A.-M., Rusu, D., Tudorachi, N., Anghel, I., Şofran, I.-E., Lisa, G., 2021. Physicochemical characterization and energy recovery of spent coffee grounds. J. Mater. Res. Technol. 15, 4437–4451. https://doi.org/10.1016/j.jmrt.2021.10.064.

Bilal, M., Iqbal, H.M.N., 2019. Sustainable bioconversion of food waste into high-value products by immobilized enzymes to meet bio-economy challenges and opportunities - a review. Food Res. Int. 123, 226–240. https://doi.org/10.1016/j.foodres.2019.04.066.

Budžaki, S., Velić, N., Ostojčić, M., Stjepanović, M., Rajs, B.B., Šereš, Z., Maravić, N., Stanojev, J., Hessel, V., Strelec, I., 2022. Waste management in the agri-food industry: the conversion of eggshells, spent coffee grounds, and brown onion skins into carriers for lipase immobilization. Foods 11, 409. https://doi.org/10.3390/foods11030409.

Buntić, A., Pavlović, M., Antonović, D., Pavlović, V., Vrućinić, D., Šiler-Marinković, S., Dimitrijević-Branković, S., 2018. Customizing the spent coffee for Trichoderma reesei cellulase immobilization by modification with activating agents. Int. J. Biol. Macromol. 107, 1856–1863. https://doi.org/10.1016/j.ijbiomac.2017.10.060.

Campos-Vega, R., Loarca-Piña, G., Vergara-Castañeda, H.A., Oomah, B.D., 2015. Spent coffee grounds: a review on current research and future prospects. Trends Food Sci. Technol. 45, 24–36. https://doi.org/10.1016/j.tifs.2015.04.012.

Chen, K.-I., Lo, Y.-C., Liu, C.-W., Yu, R.-C., Chou, C.-C., Cheng, K.-C., 2013. Enrichment of two isoflavone aglycones in black soymilk by using spent coffee grounds as an immobiliser for β-glucosidase. Food Chem. 139, 79–85. https://doi.org/10.1016/j.foodchem.2013.01.093.

Colantoni, A., Paris, E., Bianchini, L., Ferri, S., Marcantonio, V., Carnevale, M., Palma, A., Civitarese, V., Gallucci, F., 2021. Spent coffee ground characterization, pelletization test and emissions assessment in the combustion process. Sci. Rep. 11, 5119. https://doi.org/10.1038/s41598-021-84772-y.

Costa-Silva, T.A., Carvalho, A.K.F., Souza, C.R.F., De Castro, H.F., Said, S., Oliveira, W.P., 2016. Enzymatic synthesis of biodiesel using immobilized lipase on a non-commercial support. Energy Fuels 30, 4820–4824. https://doi.org/10.1021/acs.energyfuels.6b00208.

Cruz-Lopes, L., Domingos, I., Ferreira, J., Esteves, B., 2017. A new way of using spent coffee ground. J. Int. Scientific Pub.: Agricult. Food 5, 85–93.

Environment, U.N., 2021. UNEP Food Waste Index Report 2021. UNEP - UN Environment Programme. [WWW Document]. https://www.unep.org/resources/report/unep-food-waste-index-report-2021. 3.27.24.

Franssen, M.C.R., Steunenberg, P., Scott, E.L., Zuilhof, H., Sanders, J.P.M., 2013. Immobilised enzymes in biorenewables production. Chem. Soc. Rev. 42, 6491–6533. https://doi.org/10.1039/c3cs00004d.

Palma, B.G., Leão, R.A.C., de Souza, R.O.M.A., Pandoli, O.G., 2021. Immobilization of lipases on lignocellulosic bamboo powder for biocatalytic transformations in batch and continuous flow. Catal. Today 381, 280–287. https://doi.org/10.1016/j.cattod.2020.04.041.

Girelli, A.M., Chiappini, V., 2023. Renewable, sustainable, and natural lignocellulosic carriers for lipase immobilization: a review. J. Biotechnol. 365, 29–47. https://doi.org/10.1016/j.jbiotec.2023.02.003.

Girelli, A.M., Astolfi, M.L., Scuto, F.R., 2020. Agro-industrial wastes as potential carriers for enzyme immobilization: a review. Chemosphere 244, 125368. https://doi.org/10.1016/j.chemosphere.2019.125368.

Girelli, A.M., Chiappini, V., Amadoro, P., 2023. Immobilization of lipase on spent coffee grounds by physical and covalent methods: a comparison study. Biochem. Eng. J. 192, 108827. https://doi.org/10.1016/j.bej.2023.108827.

Guerrand, D., 2017. Lipases industrial applications: focus on food and agroindustries. OCL 24, D403. https://doi.org/10.1051/ocl/2017031.

Ismail, A.R., Baek, K.-H., 2020. Lipase immobilization with support materials, preparation techniques, and applications: present and future aspects. Int. J. Biol. Macromol. 163, 1624–1639. https://doi.org/10.1016/j.ijbiomac.2020.09.021.

Ittrat, P., Chacho, T., Pholprayoon, J., Suttiwarayanon, N., Charoenpanich, J., 2014. Application of agriculture waste as a support for lipase immobilization. Biocatal. Agric. Biotechnol. 3, 77–82. https://doi.org/10.1016/j.bcab.2014.02.002.

Jasińska, K., Zieniuk, B., Jankiewicz, U., Fabiszewska, A., 2023. Bio-based materials versus synthetic polymers as a support in lipase immobilization: impact on versatile enzyme activity. Catalysts 13, 395. https://doi.org/10.3390/catal13020395.

K de S Lira, R., T Zardini, R., C C de Carvalho, M., Wojcieszak, R., G F Leite, S., Itabaiana, Jr, I., 2021. Agroindustrial wastes as a support for the immobilization of lipase from Thermomyces lanuginosus: synthesis of hexyl laurate. Biomolecules 11, 445. https://doi.org/10.3390/biom11030445.

Karmee, S.K., 2018. A spent coffee grounds based biorefinery for the production of biofuels, biopolymers, antioxidants and biocomposites. Waste Manag. 72, 240–254. https://doi.org/10.1016/j.wasman.2017.10.042.

Liu, Y., Hu, T., Wu, Z., Zeng, G., Huang, D., Shen, Y., He, X., Lai, M., He, Y., 2014. Study on biodegradation process of lignin by FTIR and DSC. Environ. Sci. Pollut. Res. Int. 21, 14004–14013. https://doi.org/10.1007/s11356-014-3342-5.

Lopes, D.B., Fraga, L.P., Fleuri, L.F., Macedo, G.A., 2011. Lipase and esterase: to what extent can this classification be applied accurately? Food Sci. Technol. 31, 603–613. https://doi.org/10.1590/s0101-20612011000300009.

Mak, M.L.Y.T., Wu, W.Y., Chak, W.K., Kwong, W.F., Tang, C.H., Li, C.C., Lee, C.Y., 2023. A review of the feasibility of producing polylactic acid (PLA) polymers using spent coffee ground. Sustainability 15.

Mittersteiner, M., Linshalm, B.L., Vieira, A.P.F., Brondani, P.B., Scharf, D.R., De, P.C., 2018. Convenient enzymatic resolution of (R, S)-2-methylbutyric acid catalyzed by immobilized lipases. Chirality 30, 106–111.

Mokhtar, N.F., Abd Rahman, R.N.Z.R., Muhd Noor, N.D., Mohd Shariff, F., Mohamad Ali, M.S., 2020. The immobilization of lipases on porous support by adsorption and hydrophobic interaction method. Catalysts 10, 744. https://doi.org/10.3390/catal10070744.

Mussatto, S.I., Carneiro, L.M., Silva, J.P.A., Roberto, I.C., Teixeira, J.A., 2011. A study on chemical constituents and sugars extraction from spent coffee grounds. Carbohydr. Polym. 83, 368–374. https://doi.org/10.1016/j.carbpol.2010.07.063.

Nájera-Martínez, E.F., Melchor-Martínez, E.M., Sosa-Hernández, J.E., Levin, L.N., Parra-Saldívar, R., Iqbal, H.M.N., 2022. Lignocellulosic residues as supports for enzyme immobilization, and biocatalysts with potential applications. Int. J. Biol. Macromol. 208, 748–759. https://doi.org/10.1016/j.ijbiomac.2022.03.180.

Oliveira-Ribeiro, L.M., Meili, L., Silva-Belo-Gois, G.N., Rosas-Garcia-Almeida, R.M., da Silva-Duarte, J.L., 2019. Immobilization of lipase in biochar obtained from Manihot esculenta Crantz. Rev. Ion Investig. Optim. Nuevos Procesos Ing. 32, 7–13. https://doi.org/10.18273/revion.v32n2-2019001.

Ortiz, C., Ferreira, M.L., Barbosa, O., dos Santos, J.C.S., Rodrigues, R.C., Berenguer-Murcia, Á., Briand, L.E., Fernandez-Lafuente, R., 2019. Novozym 435: the "perfect" lipase immobilized biocatalyst? Catal. Sci. Technol. 9, 2380–2420. https://doi.org/10.1039/c9cy00415g.

Rodríguez-Restrepo, Y.A., Orrego, C.E., 2020. Immobilization of enzymes and cells on lignocellulosic materials. Environ. Chem. Lett. 18, 787–806. https://doi.org/

10.1007/s10311-020-00988-w.
Santos, L.F.S., Silva, M.R.L., Ferreira, E.E.A., Gama, R.S., Carvalho, A.K.F., Barboza, J.C.S., Luiz, J.H.H., Mendes, A.A., Hirata, D.B., 2021. Decyl oleate production by enzymatic esterification using Geotrichum candidum lipase immobilized on a support prepared from rice husk. Biocatal. Agric. Biotechnol. 36, 102142. https://doi.org/10.1016/j.bcab.2021.102142.
Serpa, J. de F., Matias, G.A.B., Fechine, P.B.A., da Costa, V.M., Freire, R.M., Denardin, J.C., Gonçalves, L.R.B., de Macedo, A.C., Rocha, M.V.P., 2021. New nanocomposite made of cashew apple bagasse lignin and Fe3O4 for immobilizing of lipase B from Candida Antarctica aiming at esterification. J. Chem. Technol. Biotechnol. 96, 2472–2487. https://doi.org/10.1002/jctb.6770.
Song, X., Qi, X., Hao, B., Qu, Y., 2008. Studies of substrate specificities of lipases from different sources. Eur. J. Lipid Sci. Technol. 110, 1095–1101. https://doi.org/10.1002/ejlt.200800073.
Srinivasan, G.R., Mahajan, A., Seth, R., Mahajan, R., 2023. Spent coffee ground briquettes: a critical review. Opportunities and Challenges in Climate-Friendly Clean Water and Energy Technologies 244–270.
Srisaipet, A., Jeyashoke, N., Krisnangkura, K., 2005. Resolution of R/S-α-Hydroxy fatty acids by immobilized lipase on rice hull ash. KMUTT Research and Development Journal 28, 429–443.
Zaks, A., Klibanov, A.M., 1985. Enzyme-catalyzed processes in organic solvents. Proc. Natl. Acad. Sci. U. S. A 82, 3192–3196. https://doi.org/10.1073/pnas.82.10.3192.

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Sustainable Lipase Immobilization: Chokeberry and Apple Waste as Carriers

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Abstract: In the modern world, the principles of the bioeconomy are becoming increasingly important. Recycling and reusability play a crucial role in sustainable development. Green chemistry is based on enzymes, but immobilized biocatalysts are still often designed with synthetic polymers. Insoluble carriers for immobilized biocatalysts, particularly those derived from agro-industrial waste such as mesoporous lignocellulosic materials, offer a promising alternative. By using waste materials as support for enzymes, we can reduce the environmental impact of waste disposal and contribute to the development of efficient bioprocessing technologies. The current study aimed to assess the possibility of using apple and chokeberry pomace as carriers for the immobilization of Palatase 20000L (lipase from *Rhizomucor miehei*). The analysis of lignocellulosic materials revealed that chokeberry pomace has a higher neutral detergent fiber (NDF) and lignin contents than apple pomace. Moreover, Scanning Electron Microscopy (SEM) observations indicated similar compact structures in both pomaces. The lipase activity assays demonstrated that immobilization of lipase from *R. miehei* onto apple and chokeberry pomace improves their properties, especially the synthetic activity. The findings highlight the potential of utilizing fruit pomaces not only as a source of bioactive compounds but also in enhancing enzyme stability for industrial applications.

Keywords: lipase; immobilization; apple pomace; chokeberry pomace

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1. Introduction

Climate change and environmental degradation pose a serious threat to East European countries and the world as a whole. Waste processing and landfilling are contributing factors. The global food supply chain is one of the most complex and interconnected systems worldwide and one of the least sustainable [1]. The European Union, as part of the European Green Deal, aims to achieve zero emissions and maintain clean soils and waters to mitigate climate change. Therefore, it is crucial to explore new green and cost-effective solutions. One approach that aligns with these objectives is upcycling, which involves repurposing discarded materials to increase their value [2]. In fruit and vegetable processing, a significant problem is the disposal of products that are not used in the technological process. Pomace accounts for 10 to 35% of processed raw materials [3]. This problem is particularly relevant for countries such as Poland, one of the leading producers of fruits such as apples, pears, and chokeberries, which are used to produce juices, jams, or concentrates. According to the Central Statistical Office in Poland, in the 2018/2019 season, only 3150 thousand tons of apples were used to produce concentrated juice [4].

In numerous countries, apple pomace (AP) is one of the most frequently produced types of agri-food waste, with about 4 million tons being produced each year worldwide [5]. According to Poland's Agricultural Market Agency, the area of chokeberry fields in Poland increased from 5000 hectares to 8000 hectares between 2004 and 2013, with the yield rising from 38,000 to 58,000 metric tons [6].

The growing interest in sustainable practices has led to increased efforts in finding effective ways to repurpose lignocellulosic materials, including apple and chokeberry waste, from juice and jam manufacturing. Waste materials from the fruit and vegetable industry with adequate porosity, surface charge, and inertness to the product can serve as effective carriers in the immobilization process [7,8]. Over the years, scientists have tested many waste products as suitable carriers, i.e., spent grain after beer production, spent coffee grounds, cashew apple and sugarcane bagasse, and coconut waste, some of which have translated into providing better yields of the enzyme used [9–13].

Many types of enzymes are used in biotechnology and industry, such as hydrolases, lipases, and peptidases. Lipases, also known as triacylglycerol acylhydrolases (EC 3.1.1.3.), are a group of enzymes that can act on insoluble substrates emulsified in water and catalyze the hydrolysis of long-chain fatty acids in triacylglycerides. Lipases differ from esterases, which act on water-soluble substrates like simple esters with short-chain fatty acids. Furthermore, they are the third largest family of digestive enzymes, following proteases and carbohydrates, and the main group of biocatalysts in the biotechnology industry. In addition to their hydrolytic activity on triacylglycerides, lipases catalyze reverse reactions in non-aqueous media, such as esterification and transesterification [14].

The lipase from *Rhizomucor miehei* (RML) is a commercially available enzyme in free and immobilized forms [15]. An important issue from an industrial perspective is the insufficient activity and stability of free enzymes [16]. Researchers have discovered that immobilization is an effective solution to these challenges. The functions and properties of the immobilized enzyme depend on the type of protein used, as well as the immobilization technique and the material used as a carrier [17]. An ideal immobilization method should not only provide high activity and stability for the enzyme but also maintain a good balance between performance and price, which is particularly important in large-scale production [18]. The method of enzyme immobilization can be based on chemical reactions or physical adsorption. Adsorption relies on the physical interactions between the carrier and enzyme, such as van der Waals forces, ionic interactions, and hydrogen bonding. These interactions are relatively weak and, importantly, typically do not alter the native structure of the enzyme. This helps to maintain the enzyme's active sites and allows it to retain its activity [19].

In the current study, the main goal was to answer whether chokeberry and apple pomace could be effective carriers for enzyme immobilization. The purpose of the work was also to see if this opens up new avenues for the use of food waste in the field of biotechnology with simultaneous recycling and finding new methods of environmental protection and whether this will allow a change in the approach to enzyme immobilization, combining new ecological outputs and saving finances spent on immobilization. So far, several studies have examined food waste as carriers for immobilizing lipolytic enzymes, such as eggshells, spent coffee grounds, or brown onion skins [20]. Therefore, to the authors' knowledge, fruit pomaces have never been utilized in the immobilization process.

2. Materials and Methods

2.1. Materials and Biocatalyst

The present study used apple and chokeberry pomaces (Greenherb Company, Wysoka, Poland) as a support for lipase immobilization. Liquid lipase Palatase 20000L (from *Rhizomucor miehei*), kindly gifted from Novozymes (Basgvaerd, Denmark), was used as a biocatalyst. All chemical reagents and solvents were purchased from Sigma-Aldrich (Poznań, Poland).

2.2. Pretreatment of Apple and Chokeberry Pomace

Three different types of matrices were used for lipase immobilization. The first was a native carrier without any pretreatment, the second involved pretreatment through hexane extraction of pomace, and the third used a hexane-ethanol extraction pretreatment of pomace. The obtained apple and chokeberry pomaces were purified of polyphenols, lipids, and other polar and non-polar substances using a Soxhlet apparatus, with a pomace-to-solvent ratio of 1:15 (w/v). The solvents employed for this process were n-hexane and ethanol, and the extraction was performed until each solvent had cycled through the system 12 times.

2.3. Determination of Fiber of Apple and Chokeberry Pomace

The fiber content was analyzed using the FibertecMC 8000 system (Foss Analytics, Warsaw, Poland). The percentage of crude fiber was measured using the PN-ISO 5498 [21] method. The content of acid detergent fiber (ADF) and acid detergent lignin (ADL) was assessed according to the PN-EN ISO 13906 standards [22]. The neutral detergent fiber (NDF) content, following amylase treatment, was determined based on PN-EN ISO 16472 standards. [23]. The concentration of cellulose was calculated as the difference between ADF and ADL, while the concentration of hemicellulose was calculated as the difference between NDF and ADF.

2.4. Lipase Immobilization Procedure

Based on the methodology described by Jasinska et al. [24], lipase was immobilized by the adsorption method onto native and pretreated apple and chokeberry pomace. Then, 1 mL of lipase (Palatase 20000L) was dissolved in 14 mL of distilled water, added to 1 g of support in a flask, and stirred for two hours. After this time, the biocatalyst was filtered using a vacuum, washed with distilled water, and dried under room conditions. After immobilizing the lipase onto the carrier, the material was stored in vials at room temperature and used for further analyses.

2.5. Lipase Activity Assay

2.5.1. Hydrolytic Activity

The measurements were conducted using a spectrophotometric technique based on the hydrolysis of *p*-nitrophenyl laurate. The reaction was executed in Eppendorf test tubes. A total of 100 µL of free liquid lipase or 25 mg of immobilized lipase in 100 µL of distilled water was mixed at 37 °C with 25 µL of 0.3 mmol *p*-nitrophenyl laurate dissolved in 2 mL of heptane. After an incubation period of 15 min, the absorbance was promptly measured at 410 nm using a UV–Vis spectrophotometer. The enzymatic activity of lipase was defined as 1 U, meaning the quantity of enzyme that produced 1 µmol of *p*-nitrophenol per minute under the specified assay conditions.

2.5.2. Synthetic Activity

The colorimetric method outlined by Jasińska et al. [24] was used to assess the synthetic activity of free and immobilized lipase. The transesterification process occurred in Eppendorf test tubes filled with 100 mM vinyl acetate, 100 mM n-butanol, and 1 mL of n-hexane. Subsequently, 10 µL of free liquid lipase or 5 mg of immobilized lipase was introduced. After incubating for 5 min, diluted samples were prepared in test tubes. To each of these samples, 1 mL of a 0.1% (m/v) MBTH (3-methyl-2-benzothiazolinone hydrazone) solution was added and stirred for 10 min at 30 °C. Then, 0.4 mL of a 1% (m/v) $H_4FeNO_4S_2 \times 12H_2O$ solution was incorporated and mixed for 30 min at 30 °C. Ultimately, the analysis of released acetaldehyde, which was converted into a blue-colored tetraazapentamethincyanine (TAPMC), was performed using spectrophotometric measurement at a wavelength of 595 nm.

2.6. Substrate Specificity

A spectrophotometric method (described in Section 2.5.1) was used to test the enzyme's substrate specificity. Four esters were used as substrates: *p*-nitrophenyl butyrate (C4:0), *p*-nitrophenyl laurate (C12:0), *p*-nitrophenyl palmitate (C16:0), and *p*-nitrophenyl oleate (C18:1).

2.7. Immobilized Lipases Recovery

An evaluation of the biocatalyst's ability to be reused was performed using a spectrophotometric technique focused on the hydrolytic activity of the samples (as described in Section 2.5.1). A single sample was utilized to hydrolyze *p*-nitrophenyl laurate in five separate instances. Following each cycle, the biocatalyst was extracted from the reaction mixture and rinsed with n-hexane and subsequently phosphate buffer (pH = 7). The prepared biocatalyst was then employed for the subsequent hydrolysis cycle.

2.8. Profile of pH Activity

To assess the stability of the enzyme used in the study under different pH conditions, spectrophotometric measurements of the *p*-nitrophenyl laurate hydrolysis reaction in of various solutions were conducted. Four phosphate buffers (10 mM) were prepared at pH levels 5.8, 6, 7, and 8. Solutions of pH 5 (buffer pH was lowered with hydrochloric acid), 6, 7, and 8 were used for analysis.

2.9. Protein Content

The concentration of protein in free liquid lipase and the filtrates following immobilization was determined using Lowry's method [25], which involves a reaction between peptide bonds and aromatic amino acids with the Folin–Ciocâlteu phenol reagent. In this study, each examined solution comprised 1 mL, and samples were diluted fiftyfold. The reaction was conducted in test tubes by adding 5 mL of copper reagent (composed of 2% Na_2CO_3 in 0.1 M NaOH, 1% $CuSO_4$, and 2% potassium sodium tartrate in a 100:1:1 ratio). After 10 min, 0.5 mL of the Folin–Ciocâlteu phenol reagent was incorporated. The mixture was incubated for 30 min, after which measurements were performed at 750 nm using a Rayleigh UV-1601 spectrophotometer (BRAIC, Beijing, China). A calibration curve created with albumin as a standard was employed to determine the protein content.

2.10. Hydrolytic and Synthetic Specific Activity

Based on the obtained results of hydrolytic and synthetic activity and protein content, the specific activities of immobilized biocatalysts and free lipase were calculated using the provided equations:

1. Liquid, free lipase:

$$Specific\ activity\left[\frac{U}{\mathrm{mg}}\right] = \frac{hydrolytic\ or\ synthetic\ activity\left[\frac{U}{\mathrm{mL}}\right]}{protein\ content\ in\ liquid,\ free\ lipase\left[\frac{\mathrm{mg}\ protein}{\mathrm{mL}}\right]} \quad (1)$$

2. Immobilized lipase:

$$Specific\ activity\left[\frac{U}{\mathrm{mg}}\right] = \frac{hydrolytic\ or\ synthetic\ activity\left[\frac{U}{\mathrm{mg}}\right]}{protein\ immobilized\ on\ support\left[\frac{\mathrm{mg}\ protein}{\mathrm{mg}\ support}\right]} \quad (2)$$

2.11. Scanning Electron Microscopy (SEM)

The native supports and immobilized enzyme preparations were examined for their surface structure using an electron microscope (HITACHI TM 3000, Ramsey, NJ, USA). The samples were dried in a vacuum, coated with gold layers (Cressington Sputter Coater

108 auto, Cressington Scientific Instruments, Watford, UK), and subsequently observed. Microphotographs were captured at magnifications of 200× and 600×.

2.12. Statistical Analysis

The outcomes were examined using the STATISTICA 13.3 software (StatSoft, Kraków, Poland). The analysis involved the following methods: the Shapiro–Wilk test to assess the normality of the data, Levene's and Brown-Forsythe tests to evaluate the equality of variances, analysis of variance (ANOVA), and the post hoc Tukey's test. A p-value of ≤ 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Characterization of Support for Immobilization

After the juice extraction and cider production process, approximately a quarter of the fresh fruit weight is left as apple pomace. This pomace comprises residual flesh, peels, seeds, and stems from various apple cultivars. Despite being naturally considered waste, these residual materials are rich in carbohydrates, proteins, vitamins, and minerals. They also serve as a valuable source of natural antioxidants and pectins [26,27]. Chokeberry pomace is also distinguished by its composition. The residue from juice production contains significant amounts of dietary fiber, polyphenols, anthocyanins, and pectins [28].

Quantifying the amount of lignocellulosic material in carrier samples is essential due to its potential impact on immobilization. The neutral detergent fiber (NDF) was used to measure all components of plant cell walls, such as cellulose, hemicellulose, and lignin. Based on the obtained results (Table 1) for native apple and chokeberry pomace, the differences between those two sources were noticeable. The NDF value for native chokeberry residuals (55.16%) was higher than for apple waste matrices (36.32%). Chokeberry pomace had lignin, cellulose, and hemicellulose contents of 32.76%, 18.87%, and 3.53%, respectively, which were lower in comparison to data presented by Nawirska and Kwaśniewska [29], whereas cellulose (34.6%) and hemicellulose (33.5%) occurred in the most significant amounts while lignin content (24.1%) was lower. In apple pomace, lignin, cellulose, and hemicellulose contents were obtained at 9.46%, 20.99%, and 5.87%. At the same time, other researchers found lignin, cellulose, and hemicellulose contents of 18.92%, 11.56%, and 10.0% [27], or 8.87%, 16.44%, and 4.09% observed by Wang and Thomas [30], as well as 20.4%, 43.6%, and 24.4% [29], while in the study of Gullón et al. [31], these values were in the ranges 13.8–17.1%, 20.2–26.4%, and 20.0–29.9%, respectively, when nine samples of apple pomace were compared. The fruit pomace lignocellulosic content analysis yielded varying results compared to prior studies, suggesting the heterogeneous nature and diversity of apple and chokeberry byproducts.

Table 1. Cellulose, hemicellulose, and lignin content of supports—native apple and chokeberry pomaces.

Parameter	Unit	Native Apple Pomace	Native Chokeberry Pomace
CF—crude fiber	%DM	21.72 ± 0.21	24.45 ± 1.12
NDF		36.32 ± 0.06	55.16 ± 4.80
ADF		30.45 ± 0.13	51.63 ± 0.40
Cellulose (ADF-ADL)		20.99 ± 0.07	18.87 ± 0.32
Hemicellulose (NDF-ADF)		5.87 ± 0.97	3.53 ± 1.30
Total (CEL and HEM)		26.86 ± 0.13	22.40 ± 4.73
Lignin (ADL)		9.46 ± 0.20	32.76 ± 0.08

Abbreviations: CF—crude fiber, NDF—neutral detergent fiber, CEL—cellulose, HEM—hemicellulose, ADF—acid detergent fiber, ADL—acid detergent lignin, DM—dry mass.

The morphological structures of native carriers were analyzed using a Scanning Electron Microscope (Figure 1). It was observed that the surface of both waste matrices is similar and forms a compact structure, flattened with some visible gaps in between. Both carriers were not highly porous and did not contain loose spaces in the structure. In the case of apple pomace, fibers were more visible and arranged in layers. This structure

increases the likelihood of the enzyme adsorbing onto the surface of the carrier rather than entering the matrix.

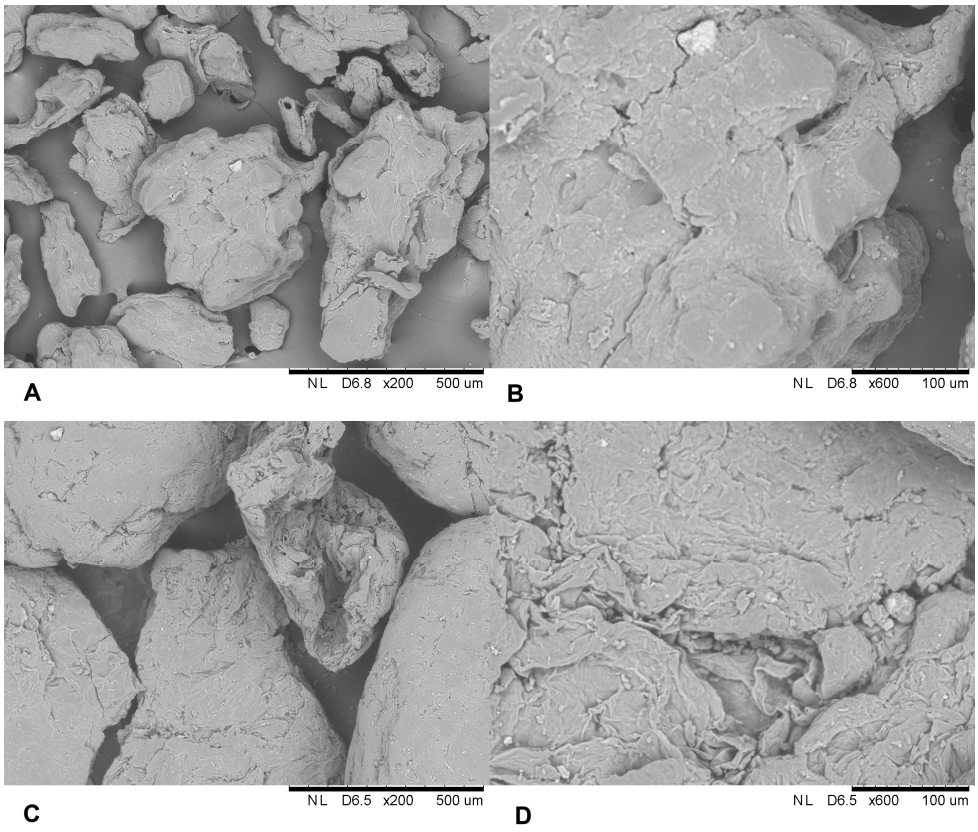

Figure 1. Scanning electron microphotographs (×200 and ×600) of (**A**,**B**)—native chokeberry pomace and (**C**,**D**)—native apple pomace.

3.2. Evaluation of Lipase Activity

RML (Lipase from *R. miehei*) is a type of lipase initially applied and primarily manufactured to modify oils and fats. Its use in the industry is attributed mainly to the enzyme's high stability in anhydrous systems, which provides advantages compared to other lipases. These advantages are further supported by its high esterification activity in anhydrous environments. Consequently, RML is often the preferred lipase for esterification reactions or any process involving esterification in the initial stages (such as acidolysis and interesterification) [15].

The primary function of lipases is to break down oils and fats through hydrolysis. Triglycerides are molecules that are not very soluble in water. However, lipases can carry out this function, which sets them apart from standard esterases. Lipases are considered interfacial enzymes [32]. The current research analyzed the ability of lipases to catalyze hydrolysis and transesterification reactions under appropriate conditions. The lipolytic activities of lipase immobilized onto chokeberry and apple pomace after different pretreatment methods (Figures 2 and 3) were compared. Additionally, the effect of storage on both activities was investigated.

The highest hydrolytic activity was obtained for biocatalysts immobilized onto native chokeberry pomace (12.07 U/g), but after 6 months of storage, it sharply decreased (2.15 U/g). However, after storage, lipases immobilized onto chokeberry pomace pretreated with ethanol and hexane did not significantly change their activity. The hydrolytic activity of enzymes immobilized onto native apple pomace and carriers after solvent pretreatments showed no statistically significant differences between preparations and after storage. It was also noticed that the immobilization process onto apple pomace, regardless of pretreatment of the carrier, positively affects the stability of lipase hydrolytic activity over time. The

same conclusion can be made for biocatalysts immobilized onto chokeberry pomace after hexane and ethanol pretreatment. This indicates that although the highest hydrolytic activity was achieved by the enzyme preparation immobilized onto native chokeberry pomace, unfortunately, this result did not persist after storage. It may seem that the compounds in the native waste had an inhibitory effect on the enzyme activity during storage, which was no longer observed for the purified pomace [33].

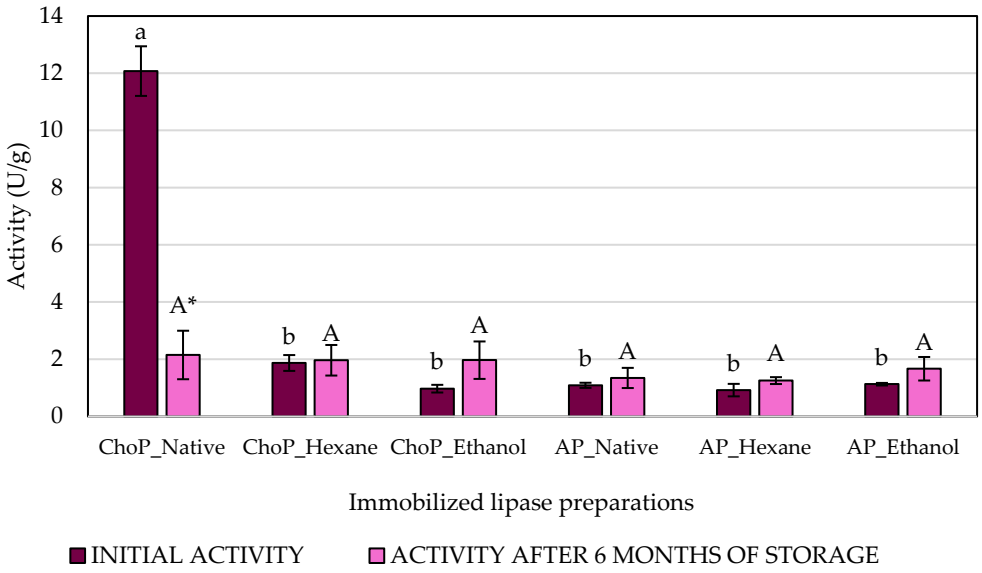

Figure 2. The hydrolytic activities of immobilized lipase onto chokeberry pomace—ChoP (native, after hexane and ethanol treatment) and apple pomace—AP (native, after hexane and ethanol treatment, measured initially (dark magenta bars) and after 6 months of storage (violet bars). Means with the same letter a or b for initial activity and A for activity after 6 months of storage did not differ significantly ($\alpha = 0.05$). Means with * within one immobilized lipase preparation differ significantly ($\alpha = 0.05$).

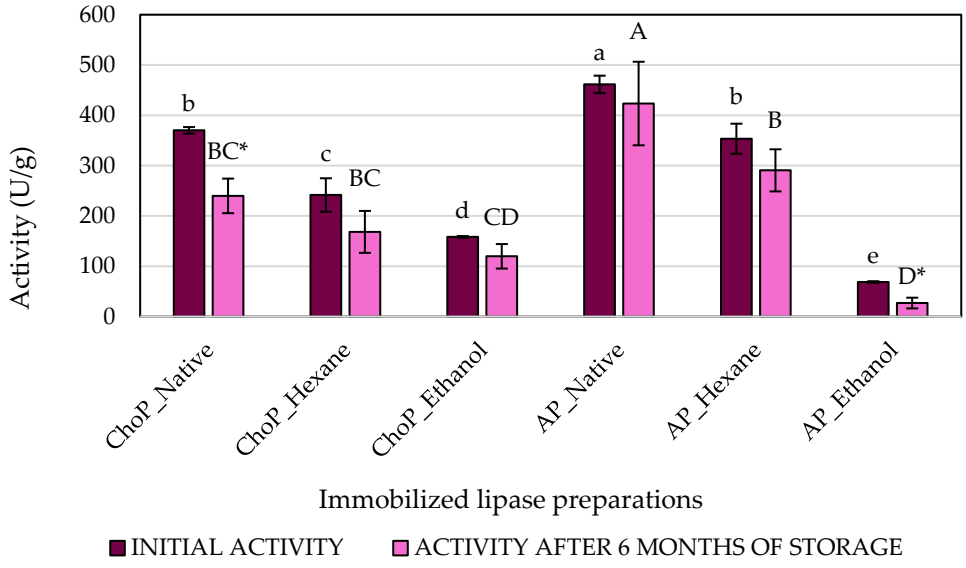

Figure 3. The synthetic activities of immobilized lipase onto chokeberry pomace—ChoP (native, after hexane and ethanol treatment) and apple pomace—AP (native, after hexane and ethanol treatment, measured initially (dark magenta bars) and after 6 months of storage (violet bars). Means with the same letter (a–e) or (A–D) did not differ significantly. Means with * within one immobilized lipase preparation differ significantly ($\alpha = 0.05$).

However, different observations were made regarding the synthetic activity of the biocatalysts. Enzyme preparations immobilized onto native apple and chokeberry pomace received the highest synthetic activities, i.e., 461 and 370 U/g, respectively. It was also observed that synthetic activity decreased with the degree of fruit waste pretreatment. Comparing the two carriers with each other, the biocatalyst immobilized onto native apple pomace had higher synthetic activity than that immobilized onto native chokeberry pomace. After 6 months of storage, lipase preparations immobilized onto chokeberry pomace after hexane and ethanol modification and onto native apple pomace after hexane pretreatment were still equally active. The only notable differences were in the biocatalyst adsorbed on native chokeberry pomace and apple pomace following ethanol pretreatment. The results indicate that the immobilization process generally ensures the stability of most biocatalysts after storage, except for the two variants mentioned earlier.

In another study, where researchers were using spent coffee grounds as carriers for the immobilization process of lipases, there was a conclusion that lipolytic enzyme preparations demonstrated the highest synthetic activity immobilized onto native spent coffee grounds, while the purification process using hexane or ethanol resulted in lower enzyme activity. The research indicated minimal variations in the hydrolytic activity of enzymes that were adsorbed on native compared to defatted forms or between defatted forms and those free of polyphenols [34].

The process of immobilization enhances the catalytic features of enzymes, a concept that was also explored in this study. Figures 4 and 5 illustrate the effect of physically adsorbed microbial lipase on various supports regarding lipolytic activity when compared to non-immobilized enzymes. The results for both hydrolytic and synthetic activities were determined based on the protein concentrations adsorbed onto the carrier and presented as a specific activity. The free enzyme showed the lowest specific hydrolytic—0.075 U/g protein and synthetic—9.999 U/g protein activities compared to immobilized biocatalysts. The enzyme immobilized onto untreated chokeberry pomace showed the highest specific hydrolytic activity (2.538 U/g protein). As the degree of purification of the pomace increased successively with hexane and ethanol, the activity of chokeberry pomace decreased. In contrast, apple pomace remained at a constant, small level, a level that is not significantly different from free lipase. Unprocessed apple pomace showed lower specific hydrolytic activity compared to chokeberry waste. This may be due to differences in the chemical composition and surface structure of the two types of carriers. Studies showed that chemical treatment of the carriers can alter their surface properties, affecting the enzyme activity. Hexane extraction can remove some lipids from the surface of the carriers, changing their properties and affecting enzyme–carrier interactions. In the case of chokeberry waste, hexane extraction lowered the specific activity, but it was still higher than for the free enzyme. Ethanol extraction, especially conducted in the Soxhlet apparatus, can remove various organic compounds (e.g., phenolics, sugars), which can change the surface properties of the carrier more dramatically, explaining the much lower enzyme activity after ethanol extraction [35]. The study by Girelli et al. [10] also showed better results than free lipase, demonstrating the importance of the immobilization process.

In the specific synthetic activity, the highest result was achieved for enzyme preparation immobilized onto native apple pomace (115 U/g protein) and was slightly lower in chokeberry pomace (77 U/g protein). Purification of the pomace led to a decrease in activity, as in the previous results. Perhaps the chemical surface area of the pomace influences the catalytic activity [36]. In the case of unprocessed carriers, natural components (e.g., lipids, polyphenols, sugars) can stabilize the enzyme structure, which is particularly important for enzymatic processes [37,38].

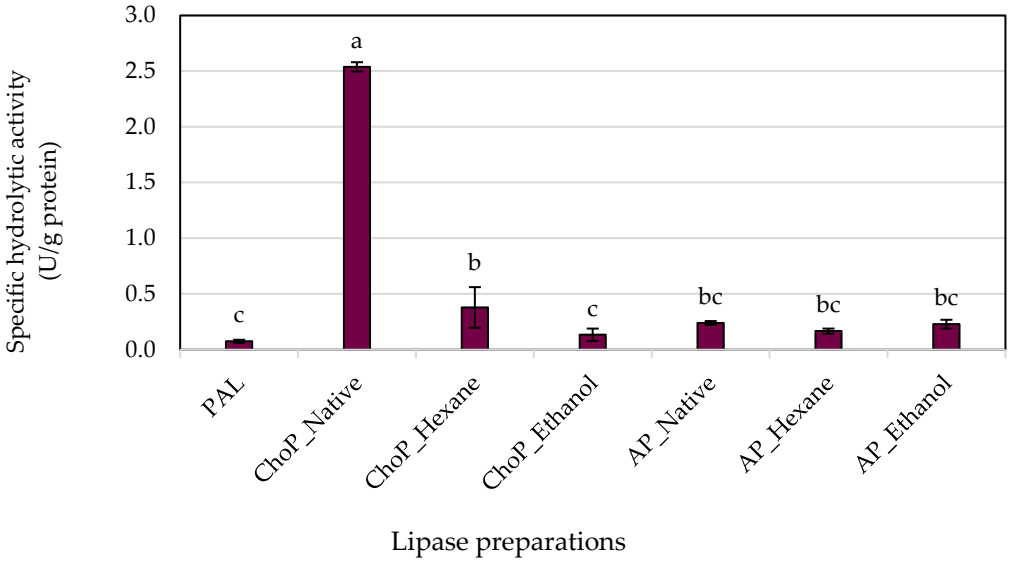

Figure 4. The specific hydrolytic activities of Palatase 20000L in the free form (PAL) and immobilized onto different carrier forms. Means with the same letter (a–c) did not differ significantly ($\alpha = 0.05$). Abbreviations: ChoP—chokeberry pomace, AP—apple pomace.

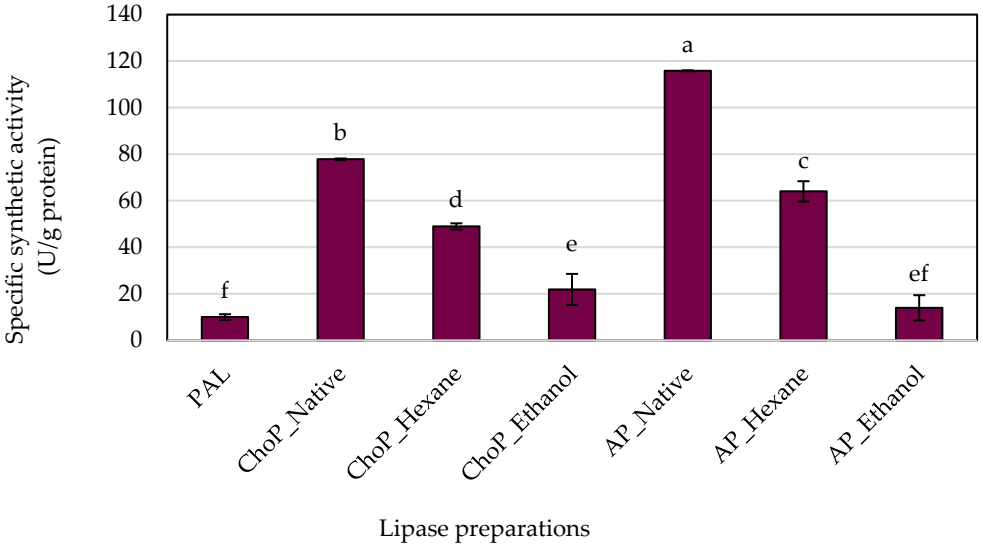

Figure 5. The specific synthetic activities of Palatase 20000L in the free form (PAL) and immobilized onto different carrier forms. Means with the same letter (a–f) did not differ significantly ($\alpha = 0.05$). Abbreviations: ChoP—chokeberry pomace, AP—apple pomace.

3.3. Substrate Specificity of Immobilized Lipases

Substrate specificity was analyzed for all the obtained biocatalysts (Figure 6). The four following esters were used in this experiment: *p*-nitrophenyl butyrate (C4:0), *p*-nitrophenyl laurate (C12:0), *p*-nitrophenyl palmitate (C16:0), and *p*-nitrophenyl oleate (C18:1). The results showed that the highest lipolytic activities were achieved against the substrate *p*-nitrophenyl butyrate (C4:0), where it was up to 7 U/g for lipase immobilized onto apple carrier purified with ethanol. The obtained biocatalysts also catalyzed reactions of other substrates (*p*-nitrophenyl laurate, palmitate, and oleate) but with lower activities. Similar observations were made by Druteika et al. [39], where a parental *Geobacillus* lipase GD-95 had the highest lipolytic activity with *p*-NP butyrate (C4:0) as a substrate. The lipase from *R. miehei* (RML) is known as a *sn*-1,3-specific lipase, whose natural function is the hydrolysis of triglycerides. Therefore, it is possible for them to catalyze hydrolysis reactions of both short-chain and long-chain fatty acid esters [15]. Our results confirmed

that lipases immobilized onto apple and chokeberry pomaces are able to catalyze the hydrolysis reactions of *p*-nitrophenyl esters specifically.

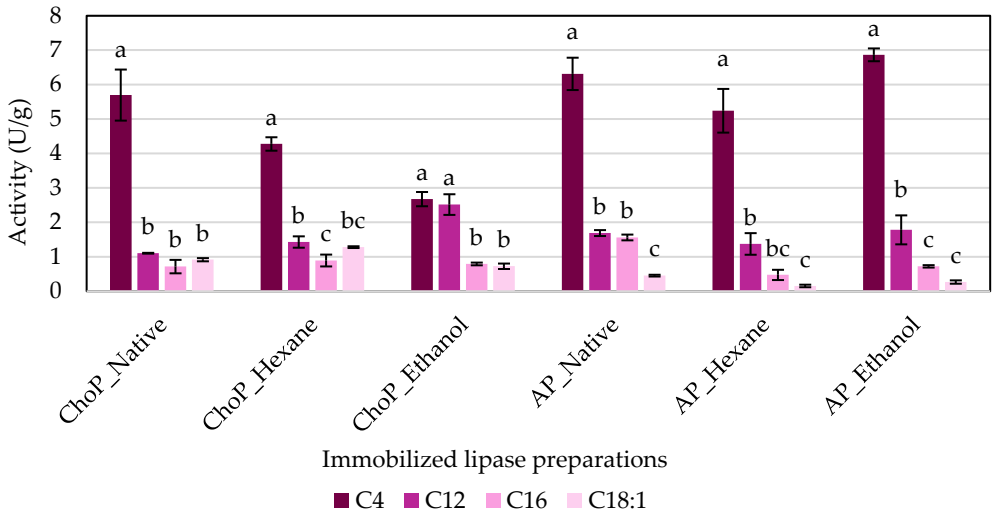

Figure 6. Comparison of hydrolytic activity of biocatalysts for the following substrates: *p*-nitrophenyl butyrate (C4:0), *p*-nitrophenyl laurate (C12:0), *p*-nitrophenyl palmitate (C16:0), and *p*-nitrophenyl oleate (C18:1). The means compared within one enzyme preparation, marked with different lowercase letters, are statistically different ($\alpha = 0.05$). Abbreviations: ChoP—chokeberry pomace, AP—apple pomace.

3.4. Profile of pH Activity of Immobilized Lipase

The impact of pH on the activity of lipase was also evaluated. Hydrogen ion concentration can affect the ability of lipases to catalyze various reactions, so hydrolytic activity was studied at pH in the range of 5 to 8 (Figure 7). Most of the biocatalysts immobilized onto chokeberry pomace were stable over the entire pH range, and their activities did not vary significantly. The exception is hexane-purified chokeberry preparations. In this case, the highest activity—4.4 U/g—was obtained for pH 5, and it was significantly higher compared to pH 7, where an activity of 2.5 U/g was obtained. The result suggests that lipases adsorbed on chokeberries had a more stable configuration and could be easily used as biocatalysts in reactions requiring varying pH conditions. Similar properties were investigated by Girelli et al. [10] and Jasinska et al. [34]. The researchers conducted a study on used coffee grounds in identical pH ranges and observed no clear differences between the study groups. Significantly different results were obtained for preparations immobilized onto apple pomace. In these groups, each purification method indicated independent groups that were significantly different depending on the pH value. In each group, the highest activity was obtained for tests with pH 8, which indicates that these are the proper conditions for biocatalysts immobilized onto apple pomace.

Takó et al. [40] reported that isolated and purified *R. miehei* lipase NRRL 5282 had a optimum pH between 6.8 and 7.4. The pH stability tests revealed that the purified lipase demonstrated significant stability, retaining over 70% of its activity within a pH range of 7.0 to 8.0. This indicates its classification as an alkaline-tolerant enzyme. Fé et al. [41] also found that free lipase RML (Palatase) showed the highest activity at pH 7. However, Zhang et al. [42] observed that pH 8 is optimum for RML immobilized onto the surface of *Saccharomyces cerevisiae* using whole-cell biocatalyst techniques. With this information and the data shown in the present paper, perhaps chokeberry pomace used as a support maintains the protective effect of the material, ensuring lipase stability in a wider range of pH compared with free enzymes. In contrast, apple pomace leads to higher activity of lipases in the alkaline zone.

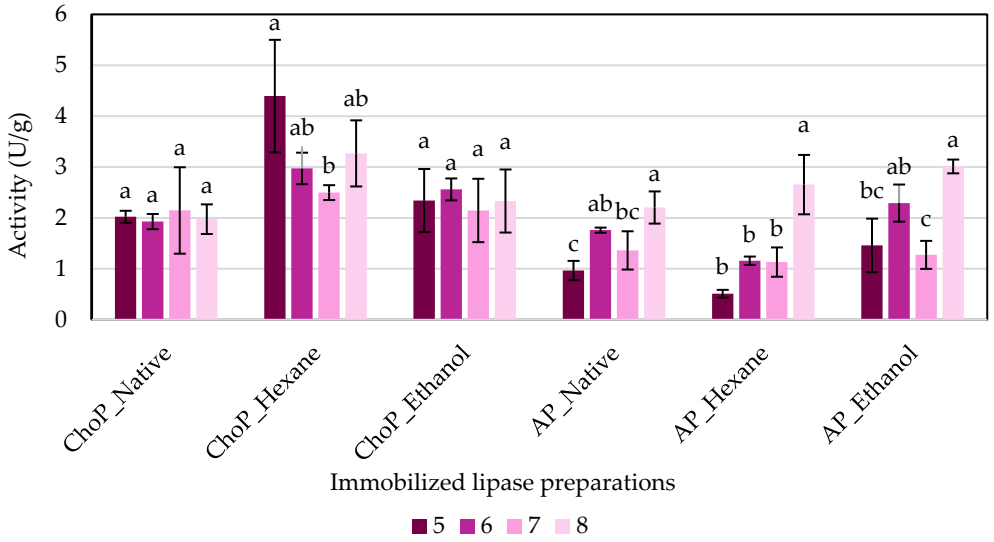

Figure 7. The effect of pH (ranging from 5 to 8) on the hydrolytic activity of the produced biocatalyst. The averages compared within a single enzyme preparation across the examined pH range, indicated by distinct lowercase letters, are significantly different ($\alpha = 0.05$). Abbreviations: ChoP—chokeberry pomace, AP—apple pomace.

3.5. The Recovery of Immobilized Biocatalysts

Another important advantage of enzyme immobilization from an industrial standpoint is the ability to recover biocatalysts. Adsorption is a reversible method based on weak bonds, including van der Waals forces, hydrogen bonds, or hydrophobic interactions [8,34,43]. In the current research, lipase from *R. miehei* immobilized onto chokeberry and apple pomace was tested in five cycles. After each reaction, biocatalysts were retrieved, washed, and recycled (Figures 8 and 9). Figure 8 describes the reusability of biocatalysts adsorbed on chokeberry pomace. It can be seen that the highest activity in each cycle, except for the third cycle, was for ethanol-treated chokeberry, while the lowest was for native chokeberry. The activity in the second cycle for hexane- and ethanol-treated chokeberries was lower by only 40 and 30%, respectively. Also, even in the fifth cycle, 5–10% activity was shown. These are promising observations considering that in the study presented by Karra-Châabouni et al. [44], where lipases were immobilized onto a cellulose carrier, *Rhizopus oryzae* lipase immobilized onto modified cellulose fibers yielded only 25% of its initial esterification activity after just three cycles. Activities of enzymes immobilized onto spent coffee grounds analyzed by Jasinska et al. [34] dropped by half as early as the second cycle, and after the fourth cycle, they were practically inactive. Similar observations to the abovementioned study were shown for apple pomaces, where the highest activity in the second cycle was observed for hexane-treated apple pomace, but it was less than 40%. Similarly, after the fourth cycle, the activities for all test groups ranged from 5–12%. The hydrophobic and electrostatic interactions can be affected by various immobilization factors, including pH levels, the amount and quality of reagents used, the type of enzyme, surface area, the chemical composition of the support, and ionic strength. Lignocellulosic waste shows promise as a support material due to the specific functional groups on its surface that can bond with lipases, as well as its considerable porosity. However, there is a risk of lipases leaching from the carrier, which may result in decreased activity during subsequent processing cycles [17,45].

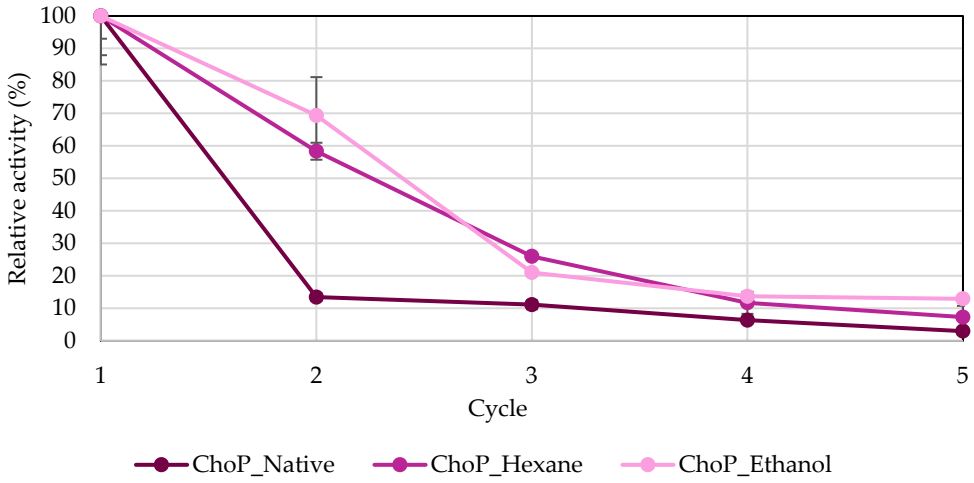

Figure 8. Recovery analysis of lipase immobilized onto chokeberry pomace. The highest hydrolytic activities of all biocatalysts were defined as 100%. Abbreviation: ChoP—chokeberry pomace.

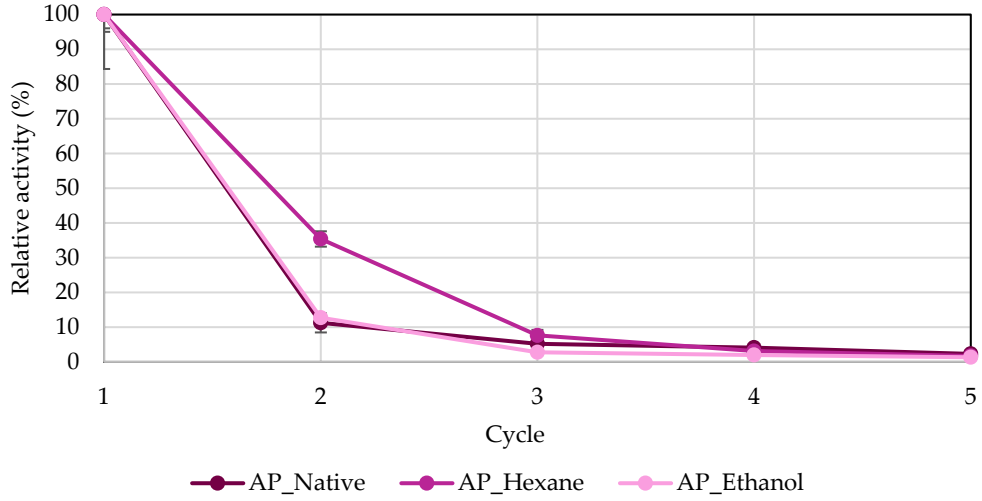

Figure 9. Recovery analysis of lipase immobilized onto apple pomace. The highest hydrolytic activities of all biocatalysts were defined as 100%. Abbreviation: AP—apple pomace.

4. Conclusions

This study examined reusing fruit waste as potential support for enzyme immobilization. The biocatalyst was microbial lipase from *R. miehei*, which was adsorbed on apple and chokeberry pomace, which are various and heterogeneous materials with different contents of cell walls. Their simple structure allowed lipase adsorption on the surface, resulting in the obtaining of lipolytic biocatalysts. The obtained immobilized enzyme preparations could catalyze the hydrolytic and synthetic reactions. It was observed that pretreating the fruit pomace before the immobilization process had a greater impact on the synthetic activity of biocatalysts than on hydrolytic activity, leading to a decrease in activity for samples with more purified pomaces (with removed lipids and polyphenols fractions).

The study showed how important is to choose the right carrier for lipase immobilization, as it can affect the properties of the biocatalyst. Although the lipase immobilized on native pomace had the highest synthetic activity, from an economic and environmental point of view, it is very beneficial since the waste obtained can be directly managed without purification. Further experiments showed that compounds contained in native pomace can potentially inhibit the activity of lipases. Moreover, higher activity in different pH ranges and the possibility of recovery in subsequent reaction cycles had biocatalysts immobilized on purified pomace (defatted and with removed polyphenols).

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References

1. Govindan, K. Sustainable consumption and production in the food supply chain: A conceptual framework. *Int. J. Prod. Econ.* **2018**, *195*, 419–431. [CrossRef]
2. Singh, S.; Marwah, R. *Politics of Climate Change: Crises, Conventions and Cooperation*; World Scientific Publishing Co.: Singapore, 2023.
3. Tarko, T.; Duda-Chodak, A.; Bebak, A. Aktywność biologiczna wybranych wytłoków owocowych oraz warzywnych. *Żywność Nauka Technologia Jakość* **2012**, *19*, 55–65.
4. Płocharski, W.; Mieszczakowska-Frąc, M.; Rutkowski, K.; Konopacka, D. Tradycyjne i Innowacyjne Kierunki Zagospodaro-wania Jabłek w Polsce. Available online: https://www.inhort.pl/files/program_wieloletni/PW_2015_2020_IO/spr_2019/Broszura_Plocharski_2019_Tradycyjne_zad.3.5.pdf (accessed on 7 October 2024).
5. Gołębiewska, E.; Kalinowska, M.; Yildiz, G. Sustainable Use of Apple Pomace (AP) in Different Industrial Sectors. *Materials* **2022**, *15*, 1788. [CrossRef]
6. Platonova, E.Y.; Shaposhnikov, M.V.; Lee, H.-Y.; Lee, J.-H.; Min, K.-J.; Moskalev, A. Black chokeberry (*Aronia melanocarpa*) extracts in terms of geroprotector criteria. *Trends Food Sci. Technol.* **2021**, *114*, 570–584. [CrossRef]
7. Bilal, M.; Iqbal, H.M. Sustainable bioconversion of food waste into high-value products by immobilized enzymes to meet bio-economy challenges and opportunities—A review. *Food Res. Int.* **2019**, *123*, 226–240. [CrossRef] [PubMed]
8. Nájera-Martínez, E.F.; Melchor-Martínez, E.M.; Sosa-Hernández, J.E.; Levin, L.N.; Parra-Saldívar, R.; Iqbal, H.M. Lignocellulosic residues as supports for enzyme immobilization, and biocatalysts with potential applications. *Int. J. Biol. Macromol.* **2022**, *208*, 748–759. [CrossRef] [PubMed]
9. Pospiskova, K.; Safarik, I. Low-cost, easy-to-prepare magnetic chitosan microparticles for enzymes immobilization. *Carbohydr. Polym.* **2013**, *96*, 545–548. [CrossRef]
10. Girelli, A.M.; Chiappini, V.; Amadoro, P. Immobilization of lipase on spent coffee grounds by physical and covalent methods: A comparison study. *Biochem. Eng. J.* **2023**, *192*, 108827. [CrossRef]
11. Lira, R.K.d.S.; Zardini, R.T.; de Carvalho, M.C.C.; Wojcieszak, R.; Leite, S.G.F.; Itabaiana, I. Agroindustrial Wastes as a Support for the Immobilization of Lipase from *Thermomyces lanuginosus*: Synthesis of Hexyl Laurate. *Biomolecules* **2021**, *11*, 445. [CrossRef]
12. Serpa, J.d.F.; AB Matias, G.; Fechine, P.B.A.; da Costa, V.M.; Freire, R.M.; Denardin, J.C.; Gonçalves, L.R.B.; de Macedo, A.C.; Rocha, M.V.P. New nanocomposite made of cashew apple bagasse lignin and Fe_3O_4 for immobilizing of lipase B from *Candida antarctica* aiming at esterification. *J. Chem. Technol. Biotechnol.* **2021**, *96*, 2472–2487. [CrossRef]
13. Oliveira-Ribeiro, L.M.; Meili, L.; Silva-Belo-Gois, G.N.; Rosas-Garcia-Almeida, R.M.; da Silva-Duarte, J.L. Immobilization of lipase in biochar obtained from Manihot esculenta Crantz. *Rev. ION* **2019**, *32*, 7–13. [CrossRef]
14. Demera, L.L.; Barahona, P.P.; Barriga, E.J.C. Production, Extraction and Characterization of Lipases from the Antarctic Yeast *Guehomyces pullulans*. *Am. J. Biochem. Biotechnol.* **2019**, *15*, 75–82. [CrossRef]
15. Rodrigues, R.C.; Fernandez-Lafuente, R. Lipase from Rhizomucor miehei as a biocatalyst in fats and oils modification. *J. Mol. Catal. B Enzym.* **2010**, *66*, 15–32. [CrossRef]
16. Agyei, D.; He, L. Evaluation of cross-linked enzyme aggregates of Lactobacillus cell-envelope proteinases, for protein degradation. *Food Bioprod. Process.* **2015**, *94*, 59–69. [CrossRef]
17. Ismail, A.R.; Baek, K.-H. Lipase immobilization with support materials, preparation techniques, and applications: Present and future aspects. *Int. J. Biol. Macromol.* **2020**, *163*, 1624–1639. [CrossRef]
18. Miladi, B.; El Marjou, A.; Boeuf, G.; Bouallagui, H.; Dufour, F.; Di Martino, P.; Elm'Selmi, A. Oriented immobilization of the tobacco etch virus protease for the cleavage of fusion proteins. *J. Biotechnol.* **2012**, *158*, 97–103. [CrossRef]
19. Jesionowski, T.; Zdarta, J.; Krajewska, B. Enzyme immobilization by adsorption: A review. *Adsorption* **2014**, *20*, 801–821. [CrossRef]

20. Budžaki, S.; Velić, N.; Ostojčić, M.; Stjepanović, M.; Rajs, B.B.; Šereš, Z.; Maravić, N.; Stanojev, J.; Hessel, V.; Strelec, I. Waste Management in the Agri-Food Industry: The Conversion of Eggshells, Spent Coffee Grounds, and Brown Onion Skins into Carriers for Lipase Immobilization. *Foods* **2022**, *11*, 409. [CrossRef] [PubMed]
21. *PN ISO 5498:1996*; Agricultural Food Products—Determination of Crude Fibre Content—General Method. Polish Committee for Standardization: Warsaw, Poland, 1996.
22. *PN-EN ISO 13906:2009*; Animal Feeding Stuffs—Determination of Acid Detergent Fibre (ADF) and Acid Detergent Lignin (ADL) Contents. Polish Committee for Standardization: Warsaw, Poland, 2009.
23. *PN-EN ISO 16472:2007*; Animal Feeding Stuffs—Determination of Amylase-Treated Neutral Detergent Fibre Content (ANDF). Polish Committee for Standardization: Warsaw, Poland, 2007.
24. Jasińska, K.; Zieniuk, B.; Jankiewicz, U.; Fabiszewska, A. Bio-Based Materials versus Synthetic Polymers as a Support in Lipase Immobilization: Impact on Versatile Enzyme Activity. *Catalysts* **2023**, *13*, 395. [CrossRef]
25. Toczko, M.; Grzelińska, A. *Materiały do Ćwiczeń z Biochemii*; Wydawnictwo SGGW: Warsaw, Poland, 2001.
26. Arraibi, A.A.; Liberal, Â.; Dias, M.I.; Alves, M.J.; Ferreira, I.C.F.R.; Barros, L.; Barreira, J.C.M. Chemical and Bioactive Characterization of Spanish and Belgian Apple Pomace for Its Potential Use as a Novel Dermocosmetic Formulation. *Foods* **2021**, *10*, 1949. [CrossRef]
27. Gowman, A.C.; Picard, M.C.; Rodriguez-Uribe, A.; Misra, M.; Khalil, H.; Thimmanagari, M.; Mohanty, A.K. Physicochemical analysis of apple and grape pomaces. *BioResources* **2019**, *14*, 3210–3230. [CrossRef]
28. Schmid, V.; Steck, J.; Mayer-Miebach, E.; Behsnilian, D.; Briviba, K.; Bunzel, M.; Karbstein, H.P.; Emin, M.A. Impact of defined thermomechanical treatment on the structure and content of dietary fiber and the stability and bioaccessibility of polyphenols of chokeberry (*Aronia melanocarpa*) pomace. *Food Res. Int.* **2020**, *134*, 109232. [CrossRef] [PubMed]
29. Nawirska, A.; Kwaśniewska, M. Dietary fibre fractions from fruit and vegetable processing waste. *Food Chem.* **2004**, *91*, 221–225. [CrossRef]
30. Wang, H.J.; Thomas, R.L. Direct Use of Apple Pomace in Bakery Products. *J. Food Sci.* **1989**, *54*, 618–620. [CrossRef]
31. Gullón, B. Evaluation of Apple Pomace as a Raw Material for Alternative Applications in Food Industries. *Food Technol. Biotechnol.* **2007**, *45*, 426–433.
32. Rodríguez-Restrepo, Y.A.; Orrego, C.E. Immobilization of enzymes and cells on lignocellulosic materials. *Environ. Chem. Lett.* **2020**, *18*, 787–806. [CrossRef]
33. Buchholz, T.; Melzig, M.F. Polyphenolic Compounds as Pancreatic Lipase Inhibitors. *Planta Medica* **2015**, *81*, 771–783. [CrossRef]
34. Jasińska, K.; Zieniuk, B.; Piasek, A.M.; Wysocki, Ł.; Sobiepanek, A.; Fabiszewska, A. Obtaining a biodegradable biocatalyst—Study on lipase immobilization on spent coffee grounds as potential carriers. *Biocatal. Agric. Biotechnol.* **2024**, *59*, 103255. [CrossRef]
35. Chella, N.; Shastri, N.R. Lipid Carriers: Role and Applications in Nano Drug Delivery. In *Particulate Technology for Delivery of Therapeutics*; Springer: Singapore, 2017; pp. 253–289. ISBN 9789811036460.
36. Zaharia, C.; Musteret, C.-P.; Afrasinei, M.-A. The Use of Coagulation–Flocculation for Industrial Colored Wastewater Treatment—(I) The Application of Hybrid Materials. *Appl. Sci.* **2024**, *14*, 2184. [CrossRef]
37. Jurendić, T.; Ščetar, M. *Aronia melanocarpa* Products and By-Products for Health and Nutrition: A Review. *Antioxidants* **2021**, *10*, 1052. [CrossRef]
38. Skinner, R.C.; Gigliotti, J.C.; Ku, K.-M.; Tou, J.C. A comprehensive analysis of the composition, health benefits, and safety of apple pomace. *Nutr. Rev.* **2018**, *76*, 893–909. [CrossRef] [PubMed]
39. Druteika, G.; Sadauskas, M.; Malunavicius, V.; Lastauskiene, E.; Statkeviciute, R.; Savickaite, A.; Gudiukaite, R. New engineered Geobacillus lipase GD-95RM for industry focusing on the cleaner production of fatty esters and household washing product formulations. *World J. Microbiol. Biotechnol.* **2020**, *36*, 41. [CrossRef] [PubMed]
40. Tako, M.; Kotogan, A.; Papp, T.; Kadaikunnan, S.; Alharbi, N.S.; Vagvolgyi, C. Purification and Properties of Extracellular Lipases with Transesterification Activity and 1,3-Regioselectivity from Rhizomucor miehei and Rhizopus oryzae. *J. Microbiol. Biotechnol.* **2017**, *27*, 277–288. [CrossRef]
41. Fé, L.X.; dos Santos, M.M.; Costa, C.S.; Pinto, M.C.; de Oliveira, R.A.; Cipolatti, E.P.; Pinto, J.C.; Langone, M.A.; Dellamora-Ortiz, G.M.; Manoel, E.A. Lipase from Rhizomucor miehei onto home-made hydrophobic polymers: Stable and efficient biocatalysts. *Process. Biochem.* **2024**, *142*, 136–148. [CrossRef]
42. Zhang, W.-G.; Han, S.-Y.; Wei, D.; Lin, Y.; Wang, X.-N. Functional Display of *Rhizomucor miehei* Lipase on Surface of *Saccha-romyces cerevisiae* with Higher Activity and Its Practical Properties. *J. Chem. Technol. Biotechnol.* **2008**, *83*, 329–335. [CrossRef]
43. Ittrat, P.; Chacho, T.; Pholprayoon, J.; Suttiwarayanon, N.; Charoenpanich, J. Application of agriculture waste as a support for lipase immobilization. *Biocatal. Agric. Biotechnol.* **2014**, *3*, 77–82. [CrossRef]
44. Karra-Châabouni, M.; Bouaziz, I.; Boufi, S.; Botelho do Rego, A.M.; Gargouri, Y. Physical immobilization of Rhizopus oryzae lipase onto cellulose substrate: Activity and stability studies. *Colloids Surf. B Biointerfaces* **2008**, *66*, 168–177. [CrossRef]
45. Girelli, A.M.; Chiappini, V. Renewable, sustainable, and natural lignocellulosic carriers for lipase immobilization: A review. *J. Biotechnol.* **2023**, *365*, 29–47. [CrossRef]

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The Double Life of Plant-Based Food Waste: A Source of Phenolic Acids and a Carrier for Immobilization of Lipases Capable of Their Lipophilization

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Abstract

Addressing global food waste challenges, this study investigated plant-based byproducts, spent coffee grounds, apple, and chokeberry pomaces, as sources of phenolic acids and biodegradable carriers for lipase immobilization. The goal was to enhance the lipophilicity and functionality of natural phenolics by enzymatic lipophilization. Microbial lipase from *A. oryzae* was immobilized on these materials, with native spent coffee grounds (NSCG) showing the highest activity (6.0 U/g hydrolytic; 1036 U/g synthetic). Chlorogenic acid (CGA), predominant in extracts, served as a model substrate. Using response-surface methodology, optimal conditions for butyl-CGA synthesis were determined. This is the first report of CGA lipophilization using food-waste-immobilized biocatalysts, where reaction yield for NSCG increased with alcohol chain length, peaking with dodecanol (34.06%). Among synthesized esters, butyl chlorogenate displayed the highest antioxidant activity, comparable to free CGA and BHT, and increased lipophilicity, though a "cut-off" effect appeared for longer chains. Medium-chain esters (C6, C8) showed selective antimicrobial activity against Gram-positive bacteria. While lipophilization of chokeberry pomace and spent coffee grounds extracts reduced antioxidant activity, short-chain esters (C4–C6) improved rapeseed oil stability. The findings highlight food waste as a sustainable source for developing biocatalysts and value-added bioactives with enhanced functional properties.

Keywords: food waste; lipase immobilization; chlorogenic acid; enzymatic lipophilization; bioactive compounds

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1. Introduction

Food waste is a significant global issue that affects environmental, social, and economic concerns. According to the Food Waste Index Report [1], approximately 931 million tons of food waste were generated in 2019. Of this total, 61% originated from households, 26% from

the food service sector, and 13% from retail. This suggests that approximately 17% of the total global food production may be wasted. A study conducted by Poore and Nemecek [2] revealed that nearly one-quarter (24%) of the emissions associated with food production result from losses in supply chains or waste generated by consumers. Specifically, 15% of these emissions are attributed to issues in the supply chain, including inadequate storage, improper handling methods, insufficient refrigeration, and spoilage during transportation and processing. The remaining 9% stems from food discarded by retailers and consumers. Consequently, food waste accounts for approximately 6% of the total global greenhouse gas emissions. The two main groups based on food loss are roots, tubers, and oil-bearing crops (26%) and fruits and vegetables (22%). These include peels, stems, seeds, shells, bran, germs, culls, pomace, pulp, and other residues from processing [3]. These byproducts represent significant sources of bioactive molecules with a wide range of applications. Food waste can be a source of polyphenols, dietary fiber, carbohydrates, and proteins. These molecules have potential for use in functional foods, nutraceuticals, pharmaceuticals, and beauty care products [4].

One of the groups of bioactive compounds of great interest is natural phenolics, which are known for their antioxidant, antimicrobial, anti-inflammatory, and anticarcinogenic properties [5]. However, their hydrophilic nature limits their biological functions. To enhance their effectiveness, a lipophilic group can be added to their structure, resulting in compounds known as lipo-phenolics. This modification changes the hydrophilic-lipophilic balance, enabling the development of new functionalized bioactive molecules with improved properties compared to traditional hydrophilic phenolics. In detail, lipophilization increases the solubility of phenolics in nonpolar environments and alters the positioning of these compounds within emulsions, thereby potentially enhancing their antioxidant capabilities. In emulsified systems, lipo-phenolics typically position themselves at the interface between the lipid and aqueous phases, providing better protection for fats and oils. As a result, lipo-phenolics have demonstrated remarkable antioxidant properties in both food and cosmetic applications [6–9]. There are two methods for carrying out lipophilization: chemical and enzymatic. The chemical method is more complex due to the thermal sensitivity and oxidation susceptibility of phenolic acids in alkaline media. It involves multiple purification steps and generates waste. In contrast, the enzymatic method is preferred, as it operates under milder conditions, making it an environmentally friendly process that produces fewer byproducts [10–12].

Lipases, known as triacylglycerol hydrolases (EC 3.1.1.3), are effective biocatalysts for modifying phenolic compounds. Their primary function is to catalyze the hydrolysis of ester bonds at the interface between hydrophilic and hydrophobic environments. Under specific conditions, particularly in the absence of water, lipases can also catalyze synthesis reactions, such as esterification and transesterification [13,14]. These enzymes find broad applications across various fields, including biotechnology, pharmacy, biodiesel production, bioremediation, detergents, and the food industry, primarily due to their chemo-, regio-, and stereoselectivity. Lipase can be obtained from various sources, including animal, plant, or microbial origins. The last one started to show promise due to its low manufacturing cost and greater availability compared to animal and plant lipases [15].

The use of free enzymes in industry presents several challenges, including low stability under extreme conditions. To address these issues, the immobilization process can be employed. This technique enhances the catalytic activity of enzymes, increases their stability across a broad range of pH levels and temperatures, and facilitates easy recovery and reuse from the reaction mixture [16]. Immobilization can be categorized into two types of interactions: physical and chemical. Physical interactions include hydrogen bonds, van der Waals forces, and hydrophobic interactions, while chemical interactions

involve covalent bonding. The primary methods of immobilization include physical adsorption, entrapment or encapsulation, covalent binding, and cross-linking. Various materials serve as carriers for enzyme immobilization. Natural polymers such as gelatin, cellulose, chitin, chitosan, and agarose, as well as nanostructured materials like metal–organic frameworks (MOFs), nanoparticles, carbon nanotubes, and graphene oxide, have already been utilized [17–19].

One of the most recognized enzyme preparations is Novozym 435, which is a lipase B from *Candida antarctica* (CALB) immobilized onto macroporous acrylic polymer resin—Lewatit VP OC 1600. This enzyme performs exceptionally well in organic synthesis and biocatalysis [15,20]. Nowadays, new, more biodegradable carriers for enzyme immobilization are being searched for, which would be as effective biocatalysts as the Lewatit adsorbed one. The increasing interest in sustainable practices has heightened efforts to develop effective strategies for repurposing lignocellulosic materials, including apple and chokeberry waste generated during the production of juice and jam. Waste materials from the fruit and vegetable industry, which possess appropriate porosity, surface charge, and chemical inertness, can serve as efficient carriers in enzyme immobilization processes. Over the years, various by-products have been evaluated as potential supports for immobilization, such as brewer's spent grain, spent coffee grounds, cashew apple waste, sugarcane bagasse, and coconut residues [21–28]. Several of these materials have shown improved enzyme yields. Notably, spent coffee grounds (SCG) have received significant attention, as their global annual generation reaches approximately 6 million tons, accounting for up to 60% of processed coffee beans. With the continuous rise in global coffee consumption, the volume of SCG waste is expected to increase further. SCGs are an inexpensive, renewable lignocellulosic feedstock with desirable properties for enzyme immobilization, such as mechanical strength, chemical inertness, and structural stability. Their composition, rich in organic compounds such as fatty acids, lignin, cellulose, hemicellulose, and other polysaccharides, makes them a valuable matrix for biocatalyst support [29–31]. Despite these well-documented characteristics, SCG has rarely been employed as an enzyme carrier in practice. Literature reports on their use have been limited to the immobilization of lipase [24,32], β-glucosidase [33], and cellulase [34].

This study implements a dual-valorization approach for beverage-processing residues: spent coffee grounds, apple pomace, and chokeberry pomace, by using them both as (i) biodegradable supports for immobilizing a microbial lipase and (ii) sources of phenolic acids. We initially developed a chlorogenic acid (CGA) model to optimize enzymatic lipophilization (adjusting temperature, enzyme loading, alcohol: CGA ratio, and reaction time), then applied the optimal conditions to non-purified, polyphenol-rich extracts. To our knowledge, this is the first demonstration of CGA lipophilization with a biocatalyst immobilized onto carriers derived from food waste. By modifying individual phenolics and then whole extracts, we aimed to develop lipophilized preparations with improved functional properties, particularly antioxidant activity, for potential use as clean-label food additives.

2. Results

2.1. Activity Evaluation of Immobilized Lipase

In the first stage of the study, three different biocatalysts were prepared by immobilizing microbial lipase from *A. oryzae* onto biodegradable carriers. Based on previous observations [35,36] demonstrating that lipases adsorbed onto native residues from beverage production displayed the highest catalytic activity, three untreated agri-food residues were selected as supports: chokeberry pomace, apple pomace, and spent coffee grounds. The lipase was adsorbed directly onto these native supports, yielding three dry-form bio-

catalysts. To confirm the success of the immobilization process, an elemental analysis was conducted on the biocatalysts obtained, as well as on the raw material used as a support. This analysis focused on the carbon, nitrogen, and sulfur content of the samples, as detailed in Table 1. The samples of spent coffee grounds (SCG) exhibited a relatively high carbon content of 48.41%, a low nitrogen content of 2.23%, and a negligible sulfur content of 0.12%. A similar pattern was observed in chokeberry pomace (ChoP), which contained 50.31% carbon, 1.87% nitrogen, and 0.09% sulfur. In contrast, apple pomace (AP) samples showed lower values: 44.62% carbon, 0.99% nitrogen, and 0.07% sulfur. The results for the elemental content align with existing literature for spent coffee grounds [37], apple pomace [38], and berry pomace [39]. Notably, in all immobilized enzyme preparations, an increase in nitrogen percentage was observed compared to the nitrogen content in the food waste alone. This observation suggests a positive adsorption of proteins onto the carrier.

Table 1. Carbon, nitrogen and sulfur content of three supports and three immobilized lipases. The * symbol indicates a significant difference between the results for each support and biocatalyst, which is immobilized on this carrier.

Source	Percentage Content (%)		
	C	N	S
Spent coffee grounds (SCG)	48.41 ± 0.01	2.23 ± 0.01	0.12 ± 0.00
Novozym 51032® immobilized on spent coffee grounds (NSCG)	50.07 ± 0.02 *	2.37 ± 0.00 *	0.12 ± 0.00
Chokeberry pomace (ChoP)	50.31 ± 0.01	1.87 ± 0.02	0.09 ± 0.00
Novozym 51032® immobilized on chokeberry pomace (NChoP)	50.49 ± 0.42	2.17 ± 0.01 *	0.10 ± 0.00 *
Apple pomace (AP)	44.62 ± 0.06	0.99 ± 0.02	0.07 ± 0.00
Novozym 51032® immobilised on apple pomace (NAP)	44.65 ± 0.09	1.26 ± 0.01 *	0.07 ± 0.00

The results with hydrolytic and synthetic activity are presented in Figure 1. Among them, the biocatalyst immobilized on spent coffee grounds showed the highest enzymatic activity, with a hydrolytic activity of 6.0 U/g and a synthetic activity of 1036.0 U/g. Significantly outperforming the other two formulations: lipase immobilized on apple and chokeberry pomace. Based on the obtained results, an immobilized biocatalyst onto native spent coffee grounds was selected for further research.

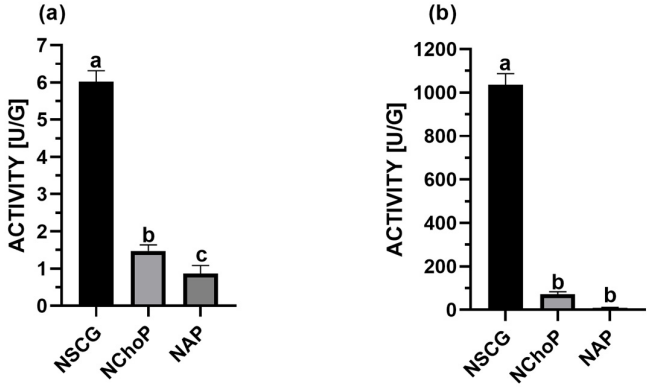

Figure 1. The hydrolytic (**a**) and synthetic (**b**) activities of immobilized lipase onto spent coffee grounds (NSCG), chokeberry pomace (NChoP), and apple pomace (NAP). Means with the same letter (a–c) did not differ significantly ($\alpha = 0.05$).

2.2. Content of Selected Phenolic Acids in Lyophilized Extracts

Lyophilized dry extracts obtained from spent coffee grounds, chokeberry pomace, and apple pomace were analyzed for their content of selected phenolic acids using LC-MS (Figure 2). The analysis included the following compounds: gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, vanillic acid, caffeic acid, syringic acid, *p*-coumaric acid, salicylic acid, chlorogenic acid, sinapic acid, ferulic acid, and cinnamic acid. Among the tested samples, the extract derived from spent coffee grounds exhibited the highest dominance of a single compound, with chlorogenic acid accounting for approximately 93% of the total identified phenolic acids. The remaining 7% consisted primarily of protocatechuic, caffeic, and ferulic acids. In the chokeberry pomace extract, chlorogenic acid also represented a significant portion, accounting for approximately 50% of the total phenolic acid content. Protocatechuic and caffeic acids were the next most abundant compounds. The extract from apple pomace displayed a more diverse phenolic profile. In this case, protocatechuic acid was the most prevalent (42%), while chlorogenic acid accounted for approximately 30% of the total, followed by 4-hydroxybenzoic acid as the third most abundant component. Based on the obtained results, chlorogenic acid was selected as a model substrate for further investigations, due to its presence in all three extracts and its relatively high proportion in each sample.

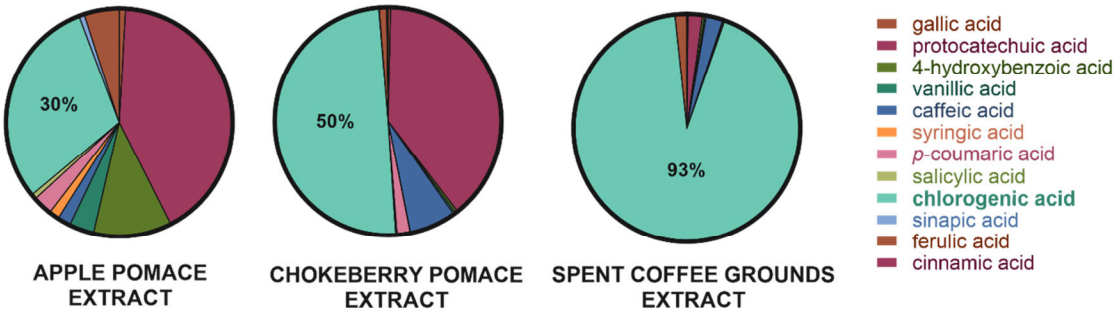

Figure 2. Percentage contribution of selected phenolic acids in freeze-dried extracts from apple pomace, chokeberry pomace, and spent coffee grounds (expressed as % of total identified phenolic acids).

2.3. Optimization of Synthesis Reaction

In the current study, a response surface methodology was utilized to optimize the enzymatic synthesis of butyl chlorogenate via the direct esterification of chlorogenic acid with 1-butanol using NSCG as a biocatalyst and Novozym 435 as a reference. A total of 15 runs (Table 2) were conducted to evaluate different temperatures (35–55 °C), substrate molar ratios (alcohol to chlorogenic acid—2:1, 5:1, and 8:1), and enzyme concentration (20–50%). The results, presented in the form of a Pareto chart and 3D response surface plots, are shown in Figures 3 and 4. The response model evaluated in this study had a coefficient of determination (R^2) equal to 0.999 and an adjusted R^2 = 0.999 for reaction catalyzed by NSCG and an R^2 of 0.988 and an adjusted R^2 = 0.913 for Novozym 435, with a 95% confidence level.

Table 2. Experimental matrix for the three-variable Box–Behnken design with the yield of the butyl chlorogenate synthesis catalyzed by lipase from *A. oryzae* immobilized onto spent coffee grounds (NSCG) and Novozym 435.

Exp. No.	Temperature (°C)	Substrate Molar Ratio (Alcohol: Chlorogenic Acid)	Enzyme Concentration Relative to the Total Mass of Substrates (%)	Yield (%) NSCG	Yield (%) Novozym 435
1	35	2:1	35	1.78	17.73
2	55	2:1	35	1.67	12.81
3	35	8:1	35	9.24	49.12
4	55	8:1	35	16.02	95.67
5	35	5:1	20	2.40	30.61
6	55	5:1	20	0.96	39.21
7	35	5:1	50	10.11	42.32
8	55	5:1	50	9.39	66.42
9	45	2:1	20	1.91	8.45
10	45	8:1	20	7.20	57.10
11	45	2:1	50	5.00	31.72
12	45	8:1	50	15.91	59.32
13	45	5:1	35	10.94	52.81
14	45	5:1	35	10.88	40.81
15	45	5:1	35	10.82	42.11

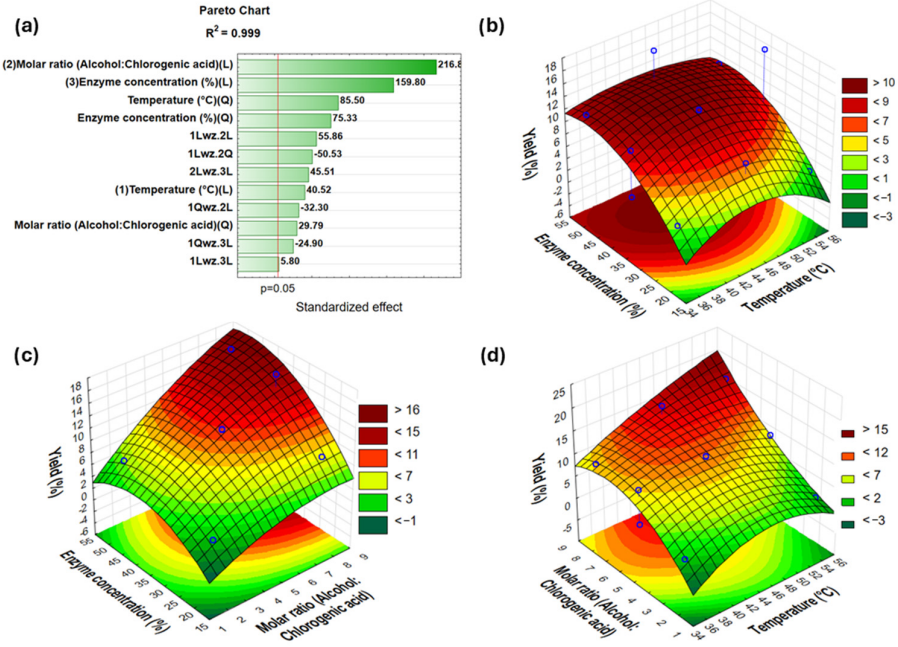

Figure 3. Pareto chart (**a**) and 3D response surface plots showing the effects of the interaction of enzyme concentration and temperature (**b**), enzyme concentration and substrate molar ratio (**c**), and substrate molar ratio and temperature (**d**) on the production yield of butyl chlorogenate catalyzed by NSCG. Explanations: 1—temperature; 2—substrate molar ratio (alcohol: chlorogenic acid); 3—enzyme concentration; L—linear; Q—quadratic; 1Qwz.2L—the interaction between a quadratic effect of temperature and a linear effect of substrate molar ratio in the statistical model, etc.

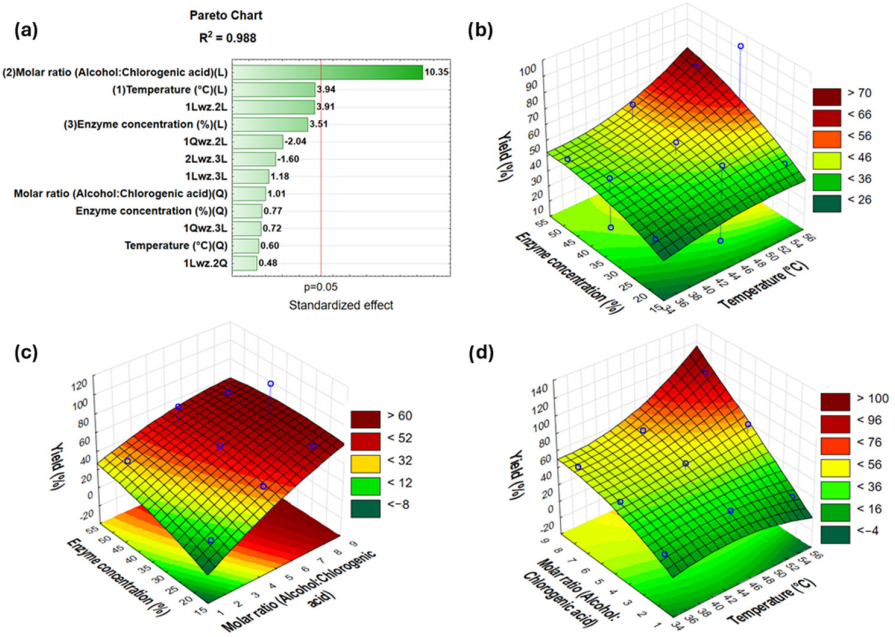

Figure 4. Novozym 435 Pareto chart (**a**) and 3D response surface plots showing the effects of the interaction of enzyme concentration and temperature (**b**), enzyme concentration and substrate molar ratio (**c**) and substrate molar ratio and temperature (**d**) on the production yield of butyl chlorogenate catalyzed by Novozym 435. Explanations: 1—temperature; 2—substrate molar ratio (alcohol: chlorogenic acid); 3—enzyme concentration; L—linear; Q—quadratic; 1Qwz.2L—the interaction between a quadratic effect of temperature and a linear effect of substrate molar ratio in the statistical model, etc.

The structure of the obtained butyl chlorogenate was confirmed by ESI-MS and NMR analysis. The fragmentation spectrum for the ester derivative of chlorogenic acid is shown in Figure 5.

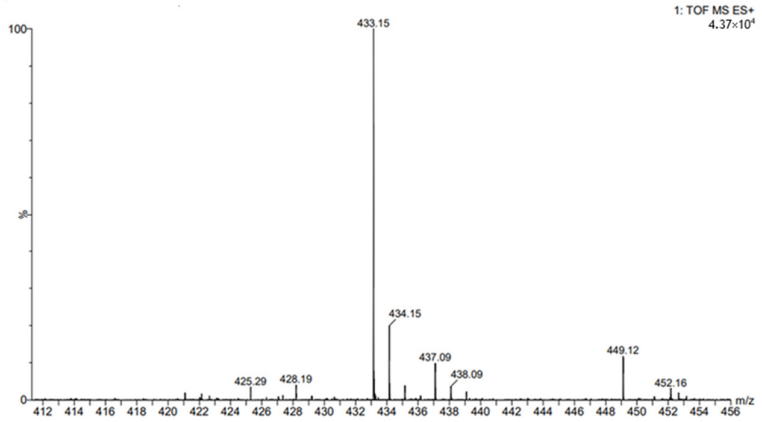

Figure 5. The ESI-MS spectrum (in the selected m/z range 412–456) for butyl chlorogenate.

Based on the Pareto chart for the synthesis of butyl chlorogenate using the NSCG biocatalyst, it was observed that the most significant factors influencing the reaction yield were the linear effect of the substrate molar ratio (1-butanol to chlorogenic acid) and the enzyme concentration relative to the total amount of substrates. The third most influential factor was the quadratic effect of temperature. The corresponding 3D response surface plots confirmed that increasing the alcohol-to-acid ratio led to higher reaction yields. Additionally, both elevated enzyme loading and higher temperatures had a positive impact on the overall efficiency of the esterification process. In contrast, the Pareto chart for the same reaction catalyzed by Novozym 435, used here as a commercial benchmark, showed

that only the linear effect of the substrate molar ratio significantly affected the reaction yield; higher alcohol content correlated with improved conversion. Other examined parameters, such as enzyme concentration and temperature, were not statistically significant. This comparison highlights a key distinction between the two biocatalysts: the immobilized lipase on the food waste-derived support (NSCG) exhibited sensitivity to a broader range of reaction conditions, whereas the commercial enzyme immobilized on a synthetic carrier (Novozym 435) demonstrated high robustness and versatility across varying parameters. Nevertheless, following a three-factor Box–Behnken analysis, optimal reaction conditions were established and found to be consistent for both systems. Maximum yields were achieved at 55 °C and at the highest substrate ratio tested, 8:1 (1-butanol: chlorogenic acid). From an economic standpoint, an enzyme concentration of 35% (*w*/*w* relative to total substrates) was selected for subsequent reactions to optimize the process while minimizing biocatalyst usage without compromising efficiency.

Further investigations were conducted to examine the influence of reaction time under the established optimal conditions (Figure 6a). For the NSCG biocatalyst, the maximum yield (10.61%) was achieved after 7 days of reaction, whereas Novozym 435 reached its highest yield (97%) after 9 days of reaction. Notably, the commercial immobilized lipase exhibited high catalytic performance early in the process, reaching 90.86% yield after just 3 days, highlighting its superior efficiency in catalyzing this specific esterification. The optimized reaction parameters described above were subsequently applied to lipophilization reactions of chlorogenic acid using a series of fatty alcohols with varying carbon chain lengths (C6, C8, C10, and C12), following the reaction scheme presented in Figure 7. It was observed that for the NSCG biocatalyst, the reaction yield increased proportionally with the length of the alcohol's carbon chain (Figure 6b). The highest yield (34.06%) was obtained in the reaction with dodecanol (C12), whereas the lowest (10.62%) was recorded with butanol (C4). In contrast, the commercial biocatalyst Novozym 435 showed consistently high yields across all tested alcohols, indicating that the previously optimized reaction conditions were broadly effective for the synthesis of various chlorogenic acid esters using this enzyme. Although the coffee waste-based biocatalyst (NSCG) demonstrated relatively low overall yields, this study represents the first report showcasing its applicability in the enzymatic lipophilization of chlorogenic acid. The results suggest that biodegradable supports derived from food industry residues may serve as a promising, environmentally friendly alternative to conventional immobilization materials in biocatalytic processes.

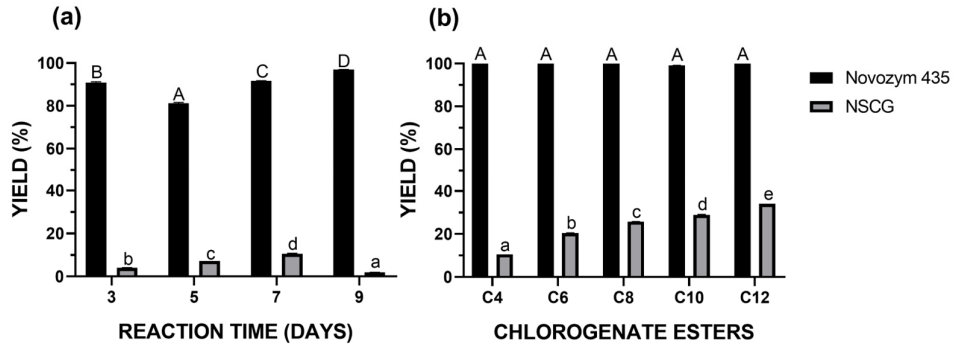

Figure 6. Yield of the reaction for obtaining chlorogenic acid butyl ester depending on the reaction time (**a**) and yield of the reaction for obtaining chlorogenic acid esters with alcohols of varying carbon chain lengths (**b**). The values with the same lowercase letter (a–e) or capital letter (A–D) did not differ significantly ($\alpha = 0.05$).

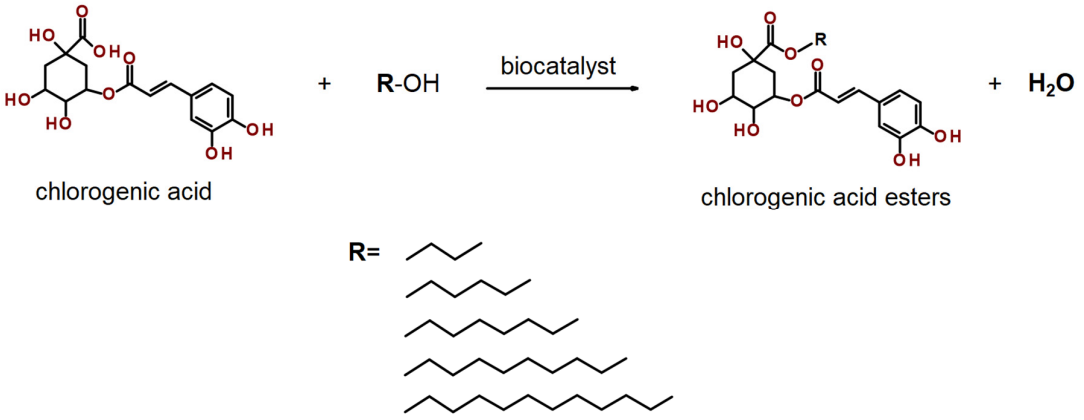

Figure 7. Lipase-catalyzed esterification of chlorogenic acid with butyl, hexyl, octyl, decyl, and dodecyl alcohol.

2.4. Extracts Lipophilization

The utilization of whole plant extracts containing diverse phenolic compounds, along with the assessment of their potential synergistic effects, remains a relatively innovative strategy. It is well recognized that extracts derived from different fruit types vary in their phenolic profiles, which directly influence their antioxidant capacities. Consequently, the lipophilization approach must be tailored to the specific composition of each extract [40]. Based on the optimization of butyl chlorogenate synthesis, lipophilization reactions were planned using the obtained lyophilized extracts from food waste. Since chlorogenic acid was identified in all three extracts, the reactions were conducted using the optimal parameters established in the model system. To derivatize chlorogenic acid, 1-butanol was used, with the extract added at a mass ratio of 1:8 (alcohol: extract). An attempt was also made to employ a biodegradable biocatalyst and Novozym 435 as a reference to carry out the lipophilization reaction. The presence of the main reaction product—butyl chlorogenate—was analyzed in these samples to confirm the successful derivatization of chlorogenic acid contained in the extracts. LC-MS analysis confirmed the presence of the chlorogenic acid ester in all reaction mixtures after 7 days of incubation. The conversion rate of butyl chlorogenate is presented in Table 3. The highest conversion rate of butyl chlorogenate was recorded for the lipophilized extract from apple and chokeberry pomace, which reached up to approx. 90%. The prepared NSCG biocatalyst catalyzed the derivatization of chlorogenic acid contained in these extracts in a manner comparable to Novozym 435. Lower results were obtained for the derivatized extract from spent coffee grounds. Here, a conversion rate of 47.7% was achieved with Novozym 435 and 15.2% with the NSCG biocatalyst. It is possible that because spent coffee grounds consist mainly of chlorogenic acid, as a representative of phenolic acids, more time is needed to hydrolyze the entire amount contained in the extract. In the case of the other pomace extracts, these amounts are significantly lower.

Table 3. Conversion rate of butyl chlorogenate in the reaction mixture after lipophilization of food waste extracts, catalyzed by Novozym 435 and NSCG.

Conversion Rate of Butyl Chlorogenate in Extracts After Lipophilization (%)					
Chokeberry Pomace Extract		Apple Pomace Extract		Spent Coffee Grounds Extract	
Novozym 435	NSCG	Novozym 435	NSCG	Novozym 435	NSCG
90.9%	92.0%	91.5%	87.6%	47.7%	15.2%

2.5. Properties of Obtained Esters of Chlorogenic Acid and Pre- and Postmodified Extracts

The derivatized extracts, along with individual chlorogenic acid esters (C4-C12), were analyzed for various functional properties, including antioxidant activity, antimicrobial effects, and the ability to enhance the oxidative stability of vegetable oil. The study aimed to determine whether modifying the extracts through 1-butanol-based lipophilization could improve their overall properties, particularly in the context of developing a food additive with enhanced lipophilicity. The results for the first group of compounds—chlorogenic acid, chlorogenic acid esters, and BHT (a synthetic antioxidant used as a reference) are presented in Table 4. Among all synthesized esters, butyl chlorogenate exhibited the highest antioxidant activity (IC_{50} = 0.34 mM; TEAC = 3.26). Its performance was comparable to that of free chlorogenic acid (IC_{50} = 0.26 mM; TEAC = 3.49 and BHT (IC_{50} = 0.35 mM; TEAC = 3.14, while also displaying increased lipophilicity, as indicated by the logP value—a partition coefficient between octanol and water that reflects a molecule's hydrophilic or hydrophobic character. It was also observed that as the carbon chain length of the alcohol used as a substrate in the esterification reaction increased, the antioxidant activity of the resulting esters decreased, regardless of the analytical method applied (CUPRAC or DPPH), despite the corresponding increase in molecular lipophilicity.

Table 4. Selected properties—antioxidant activity by DPPH and CUPRAC methods, and lipophilicity of chlorogenic acid and its esters, and BHT. The values with the same lowercase letter (a–d) within the method did not differ significantly (α = 0.05).

Compound	Antioxidant Properties		Lipophilicity	
	CUPRAC—TEAC	DPPH—IC_{50} (mM)	logP	logS
BHT	3.14 ± 0.22 ^{a}	0.35 ± 0.02 $^{a\ b}$	4.964	−4.909
Chlorogenic acid	3.49 ± 0.32 ^{a}	0.26 ± 0.00 ^{a}	0.415	−1.489
Butyl chlorogenate	3.26 ± 0.17 ^{a}	0.34 ± 0.00 $^{a\ b}$	2.076	−2.945
Hexyl chlorogenate	1.15 ± 0.15 ^{b}	0.40 ± 0.01 ^{b}	2.960	−3.976
Octyl chlorogenate	0.80 ± 0.05 $^{b\ c}$	0.58 ± 0.01 ^{c}	3.844	−5.007
Decyl chlorogenate	0.64 ± 0.03 ^{c}	0.57 ± 0.01 ^{c}	4.728	−6.037
Dodecyl chlorogenate	0.45 ± 0.02 ^{c}	0.91 ± 0.12 ^{d}	5.612	−7.067

The antioxidant properties and total content of phenolic compounds in selected food waste extracts before and after lipophilization are presented in Table 5. The highest total phenolic content was observed in the spent coffee grounds extract, as confirmed by LC-MS analysis. This was followed by the chokeberry pomace extract, while the apple pomace extract exhibited the lowest concentration of phenolic compounds. A similar trend was noted in antioxidant activity, with extracts richer in phenolic content demonstrating greater antioxidant potential. A noteworthy observation concerns the impact of lipophilization on antioxidant activity. In the case of chokeberry pomace and spent coffee grounds extracts, a significant decrease in antioxidant activity was observed after lipophilization, suggesting that the process may negatively affect the overall antioxidant efficacy. This reduction could be attributed to the modification or degradation of bioactive compounds during derivatization. In contrast, only minimal changes were detected in the antioxidant activity of the apple pomace extract. Although apple pomace is considered a relatively rich source of polyphenolic compounds, over 50% of these are procyanidins, classified as flavan-3-ols. The remaining compounds include quercetin glycosides, phenolic acids, and chalcones, such as phloridzin and phloretin [41,42]. In the present study, LC-MS analysis showed that the lyophilized apple pomace extract contained the lowest total phenolic acid content

(422 μg/g), compared to the chokeberry pomace (10,442 μg/g) and spent coffee grounds (15,723 μg/g) extracts.

Table 5. Antioxidant properties and total content of phenolic compounds in selected food waste extracts before and after lipophilization process. Means with the same lowercase letter (a–c) or capital letter (A–C) within the method did not differ significantly (α = 0.05). The * symbol indicates a significant difference between the results for a given extract before and after the lipophilization process. Abbreviation: n.a.—not analyzed.

Type of Extract	Lipophilization	Antioxidant Properties		Total Content of Phenolic Compounds
		CUPRAC—TEAC	DPPH—IC_{50} (mg/mL)	Average CGA Content (mg/mL)
Chokeberry pomace extract	BEFORE	2.00 ± 0.17^{b}	0.26 ± 0.02^{a}	0.26 ± 0.03^{b}
	AFTER	0.85 ± 0.07^{B} *	0.90 ± 0.02^{B} *	n.a.
Apple pomace extract	BEFORE	0.23 ± 0.01^{c}	4.55 ± 5.97^{b}	0.03 ± 0.01^{c}
	AFTER	0.23 ± 0.01^{C}	5.97 ± 0.02^{C} *	n.a.
Spent coffee grounds extract	BEFORE	2.50 ± 0.10^{a}	0.13 ± 0.09^{a}	0.36 ± 0.00^{a}
	AFTER	1.60 ± 0.07^{A} *	0.42 ± 0.04^{A} *	n.a.

The chlorogenic acid derivatives obtained in this study, along with extracts from food processing by-products, were also assessed for their antimicrobial properties. Eight bacterial strains, representing both Gram-positive and Gram-negative species, were tested. To each sterile disc, 10 μL of the selected compounds and extracts, prepared at a concentration of 50 μg/mL, were added. The results are summarized in Tables 6 and 7.

Table 6. Comparison of the antimicrobial activity of chlorogenic acid, its esters and BHT as a reference. The 6 mm indicates no growth inhibition. *—A diameter of an inhibition zone of 6 mm is considered as no antimicrobial activity.

Compound	Inhibition Zone Diameter (mm)							
	B. cereus PCM 482	*B. subtilis* PCM 486	*E. faecalis* PCM 2909	*L. monocytogenes* PCM 2191	*S. aureus* PCM 2054	*E. cloacae* PCM 2848	*E. coli* PCM 2057	*S. marcescens* PCM 549
BHT	6 *	6	6	6	6	6	6	6
Chlorogenic acid	6	6	6	6	6	6	6	6
Butyl chlorogenate	6	6	6	6	6	6	6	6
Hexyl chlorogenate	10	8	10	6	12	6	6	6
Octyl chlorogenate	10	8	6	6	10	6	6	6
Decyl chlorogenate	8	6	6	9	9	6	6	6
Dodecyl chlorogenate	7	6	6	7	6	6	6	6

Interestingly, while butyl chlorogenate exhibited antioxidant activity comparable to that of unmodified chlorogenic acid, it did not show any antimicrobial effects. Similarly, no bacterial growth inhibition was observed for BHT or chlorogenic acid. In contrast, hexyl and octyl chlorogenates demonstrated clear antimicrobial activity, particularly against Gram-positive bacteria such as *B. cereus*, *B. subtilis*, *E. faecalis*, and *S. aureus*, with the exception of *E. faecalis* in the case of octyl chlorogenate. Additionally, decyl and dodecyl chlorogenates produced smaller inhibition zones against *B. cereus*, *L. monocytogenes*, and *S. aureus*. Notably, all chlorogenic acid esters tested in this study showed selective activity

only against Gram-positive bacteria, with no detectable effects on Gram-negative strains. Among the derivatives, those with medium-length alkyl chains (C6 and C8) exhibited the strongest antimicrobial activity, whereas shorter (C4) and longer chains (C10 and C12) were significantly less effective. In contrast, none of the food waste extracts exhibited antimicrobial activity against any of the tested bacterial strains.

Table 7. Comparison of the antimicrobial activity of spent coffee grounds, chokeberry and apple pomace extracts before and after lipophilization. The 6 mm indicates no growth inhibition. *—A diameter of an inhibition zone of 6 mm is considered as no antimicrobial activity.

Type of Extract	Lipophilization	Inhibition Zone Diameter (mm)							
		B. cereus PCM 482	*B. subtilis* PCM 486	*E. faecalis* PCM 2909	*L. monocytogenes* PCM 2191	*S. aureus* PCM 2054	*E. cloacae* PCM 2848	*E. coli* PCM 2057	*S. marcescens* PCM 549
Chokeberry pomace extract	BEFORE	6 *	6	6	6	6	6	6	6
	AFTER	6	6	6	6	6	6	6	6
Apple pomace extract	BEFORE	6	6	6	6	6	6	6	6
	AFTER	6	6	6	6	6	6	6	6
Spent coffee grounds extract	BEFORE	6	6	6	6	6	6	6	6
	AFTER	6	6	6	6	6	6	6	6

The oxidative stability of rapeseed oil with the addition of chlorogenic acid, its alkyl esters (C4–C12), BHT (as a reference compound), and food waste extracts was evaluated both at the initial time point and after two months of storage. As shown in Figure 8, the highest initial maximum oxidation time of vegetable oil was observed with the addition of BHT (τ_{max} = 101.8 min), followed closely by the short-chain chlorogenates, particularly the butyl (C4—τ_{max} = 98.3 min.) and hexyl (C6—τ_{max} = 95.9 min) esters. In contrast, oil with native chlorogenic acid exhibited significantly lower stability (τ_{max} = 90.7 min), which further declined after storage. This correlates with the results obtained, as the butyl and hexyl esters of chlorogenic acid exhibited the strongest antioxidant activity among the tested derivatives. Their enhanced lipophilicity relative to native chlorogenic acid likely facilitated more effective dissolution within the lipid matrix, contributing to their improved performance. After two months of storage, a slight but statistically significant reduction in the stability of oil was observed for the addition of several esters, most notably in the case of the butyl (C4—τ_{max} = 93.2 min) and decyl (C10—τ_{max} = 88.7 min) derivatives.

At the initial stage of the study, oils supplemented with SCGE (maximum oxidation time = 91.0 min.) and ChoPE (maximum oxidation time = 90.4 min) extracts, both native and lipophilized, exhibited oxidative stability levels comparable to the control oil without additives, indicating no enhancement of oxidation resistance at the start of storage. In contrast, the addition of apple pomace extract (APE), before (τ_{max} = 87.7 min) and after (τ_{max} = 80.7 min) lipohilization, led to a decrease in oxidative stability. After two months of ambient storage, oils containing all tested extracts showed similar oxidative stability levels, suggesting that while the extracts did not negatively impact stability, they also failed to provide significant protection against oxidation during prolonged storage. Overall, no significant differences in initial oxidative stability were observed among the oils with various extracts, with APE demonstrating the least effect regardless of modification. The marked decline in stability over time across most samples highlights the challenge of maintaining antioxidant efficacy throughout storage.

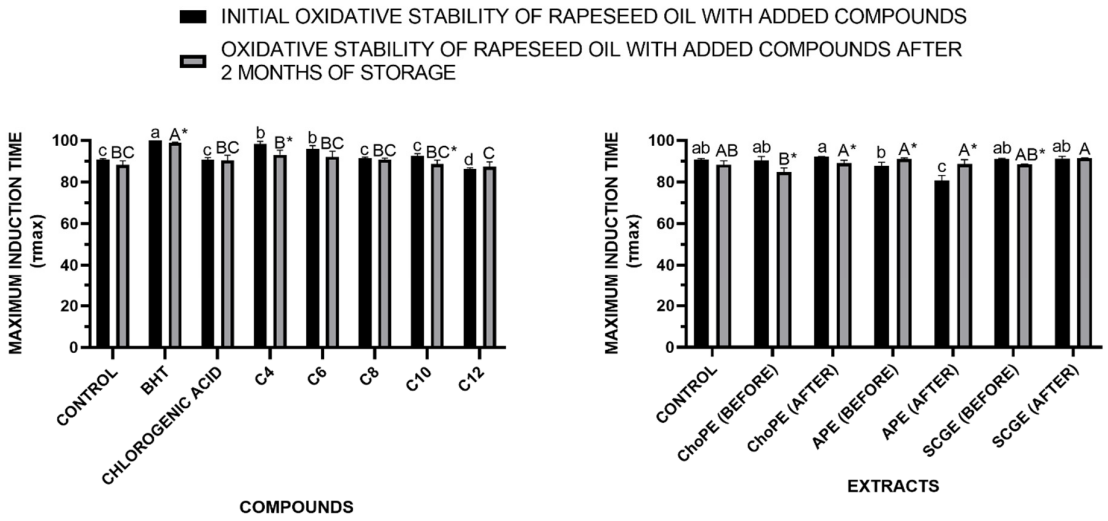

Figure 8. Oxidative stability of rapeseed oil with addition of chlorogenic acid (CGA), its esters with alkyl chains of different lengths (C4–C12), BHT (used as a reference antioxidant), and food waste extracts—chokeberry pomace extract (ChoPE), apple pomace extract (APE), and spent coffee grounds extract (SCGE), before and after lipophilization measured at the initial stage and after two months of storage. Values marked with the same lowercase (a–d) or uppercase (A–C) letters do not differ significantly ($\alpha = 0.05$). The * symbol indicates a significant difference between the initial stadium of oxidative stability and after 2 months of storage for a given compound or extract before and after the lipophilization process. Abbreviations: ChoPE—Chokeberry Pomace Extract, APE—Apple Pomace Extract and SCGE—Spent Coffee Grounds Extract.

3. Discussion

3.1. Activity of Biocatalysts Immobilized on Food Waste Materials

Lipases are enzymes that primarily catalyze the hydrolysis of triacylglycerols into diglycerides, monoglycerides, free fatty acids, and glycerol. Beyond their hydrolytic activity, lipases also exhibit broad catalytic versatility, participating in esterification, interesterification (including acidolysis, alcoholysis, and transesterification), and aminolysis reactions. These transformations can occur in both aqueous and organic media, provided that water content is carefully regulated [15]. Given their dual ability to catalyse both hydrolysis and synthesis reactions, depending on the reaction environment, this study aimed to assess the hydrolytic and synthetic activities of selected lipase-based biocatalysts.

The high catalytic activity of the obtained immobilized preparations may be attributed to several contributing factors. Adsorption, as one of the simplest and most commonly used methods for enzyme immobilization, relies on weak physical interactions such as Van der Waals forces, hydrophobic interactions, and hydrogen bonding. Consequently, the chemical structure and surface functionality of the support material play a critical role in determining immobilization efficiency and enzyme stability. The presence of specific functional groups, such as hydroxyl, carboxyl, or phenyl moieties, on the support surface can facilitate non-covalent interactions with the enzyme, thereby enabling the formation of stable biocatalyst systems [43–45]. In the context of the examined agro-industrial waste materials, previous studies have suggested that fiber composition, particularly hemicellulose content, may significantly influence the adsorption capacity of the support [45,46]. Native spent coffee grounds (SCG), in comparison to apple and chokeberry pomace, exhibit a markedly higher hemicellulose content in their dry matter (Table 8). This higher polysaccharide content could enhance the density of available binding sites on the surface, thereby improving enzyme-support interactions and contributing to the stability of the immobilized system.

Indeed, earlier research has demonstrated a positive correlation between hemicellulose content and the synthetic activity of biocatalysts, which is also relevant to the present study.

Table 8. Cellulose, hemicellulose, and lignin content of supports—native apple and chokeberry pomaces and spent coffee grounds. Abbreviations: ADF—acid detergent fiber, ADL—acid detergent lignin, NDF—neutral detergent fiber, DM—dry mass.

Food Waste	Unit	Cellulose (ADF-ADL)	Hemicellulose (NDF-ADF)	Lignin (ADL)	
Native Apple Pomace	%DM	20.99 ± 0.07	5.87 ± 0.97	9.46 ± 0.20	[35]
Native Chokeberry Pomace		18.87 ± 0.32	3.53 ± 1.30	32.76 ± 0.08	[35]
Native Spent Coffee Grounds		21.06 ± 0.41	23.69 ± 0.97	16.87 ± 0.48	[36]

Moreover, the physical morphology of the support material is another key factor. Native SCGs possess a more porous structure relative to the other tested carriers, resulting in an increased specific surface area. This enhanced surface availability likely facilitates more effective adsorption of lipase molecules onto the carrier. Finally, the presence of residual lipids in raw materials may contribute to the stabilization of lipases through hydrophobic interactions that resemble the enzyme's native environment. This is supported by the lipid content analysis of the tested waste materials, where spent coffee grounds exhibited the highest fat fraction (11%), compared to chokeberry pomace (0.3%) and apple pomace (0.2%). Lipases are characterized by polypeptide chains containing both hydrophobic and hydrophilic regions, with their active sites typically concealed by a hydrophobic lid in the closed conformation [18]. Upon contact with hydrophobic interfaces such as substrate droplets or hydrophobic supports, this lid undergoes a conformational change, exposing the active site in a process known as interfacial activation. This structural rearrangement enhances the enzyme's catalytic efficiency and facilitates its adsorption onto hydrophobic surfaces, stabilizing the open monomeric form of the enzyme without the need for additional activation steps [46]. Furthermore, the hydrophobicity and physicochemical properties of the immobilization support significantly influence both the efficiency of lipase binding and the resulting enzymatic activity [47]. While certain lipases exhibit preferences for supports with moderate hydrophobicity, it is generally observed that more hydrophobic surfaces enable greater immobilization yields and allow for the selective fractionation of lipase mixtures based on their affinity to different supports [48]. Therefore, the higher lipid content in spent coffee grounds likely promotes enhanced lipase stabilization through such hydrophobic interactions, which is consistent with established mechanisms of lipase interfacial activation and immobilization.

3.2. Enzymatic Modification of Chlorogenic Acid and Food Waste Extracts

Chlorogenic acid is a widely distributed plant-derived compound, found in significant amounts in coffee beans, stone fruits, berries, and cruciferous vegetables. It is known for a range of beneficial biological effects, including strong antioxidant properties, protection of the intestinal and hepatic barriers, and demonstrated efficacy in the prevention and treatment of obesity and type II diabetes. Due to its polar nature, chlorogenic acid has limited solubility in lipid matrices, which restricts its applicability in the food, pharmaceutical, and cosmetic industries. Its hydrophobicity can be increased through chemical or enzymatic lipophilization, which involves the esterification of the carboxyl group with a fatty alcohol. The enzymatic approach is particularly favoured, as the use of biocatalysts allows for milder reaction conditions, enhanced selectivity, and minimized formation of

by-products. Moreover, enzymatic reactions are environmentally friendly, requiring less energy and producing lower amounts of waste [40,49–51].

The optimization of the reaction parameters using the Box–Behnken design (temperature, substrate molar ratio, and enzyme concentration) revealed that the yield of chlorogenic acid esterification is especially sensitive to these factors when catalyzed by the NSCG biocatalyst. This aligns with common trends in enzymatic esterification, where increasing temperature and enzyme loading generally improve reaction efficiency, up to a point beyond which thermal deactivation or substrate saturation may occur. Conversely, the commercial immobilized biocatalyst Novozym 435 achieved high reaction yields that remained largely unaffected by variations in reaction conditions, highlighting its operational robustness and substrate tolerance. The NSCG biocatalyst was found to be more sensitive to different reaction conditions, necessitating more careful selection of parameters in experimental planning and enzyme use. In contrast, Novozym 435 performs reliably, making it a more suitable choice for large-scale and standardized production processes where stability and predictability are vital.

The enzymatic modification of chlorogenic acid using lipases has been extensively explored in prior studies, primarily using commercially available immobilized enzymes. López-Giraldo et al. [49] reported a two-step synthesis of chlorogenic acid esters: initial chemical esterification to produce methyl chlorogenate, followed by enzymatic transesterification with fatty alcohols (C4–C16) using *Candida antarctica* lipase B (CALB). The reactions, conducted at 55 °C for 96 h with varying enzyme loadings (2.5–10%, *w*/*w*), achieved conversion efficiencies ranging from 61% to 93%, depending on the alcohol chain length. Similarly, Guyot et al. [52] employed the same enzyme for direct esterification with octanol, dodecanol, and hexadecanol under milder enzyme concentrations (1.2–1.5%, *w*/*w*) and longer reaction times (30 days), achieving yields ranging from 40% to 75%. In a comparative study, Lorentz et al. [53] synthesized palmitoyl esters of chlorogenic acid using a panel of commercial lipases, including Novozym® 435, Lipozyme RM-IM, TL-IM, Lipase A (*A. niger*), Lipase M (*M. javanicus*), Lipase DF (*R. oryzae*), Lipase AY (*C. rugosa*), Lipase G (*P. camembertii*), and Lipase PS (*P. cepacia*). Under standardized conditions (60 °C, 1000 rpm), bioconversion efficiency varied from 14% to 60% after 7 days, depending primarily on the substrate molar ratio of palmitic acid to chlorogenic acid (ranging from 10:1 to 80:1). Further work by Wang et al. [50] applied Lipozyme RM to acylate chlorogenic acid with vinyl esters (C2–C12) under similar thermal (55 °C) and agitation conditions (400 rpm), yielding five distinct 4-O-acylated derivative, after 7 days. Zhu et al. [54] expanded also on this approach by systematically optimizing multiple reaction parameters—including solvent type, enzyme concentration and form, substrate ratio, and time—ultimately identifying optimal conditions (55 °C, 1:10 substrate molar ratio, 400 rpm, 7 days) for efficient vinyl ester synthesis.

Despite the widespread use of immobilized commercial lipases in these studies, a significant gap remains concerning the employment of environmentally sustainable biocatalysts. To date, no reports have investigated the enzymatic lipophilization of chlorogenic acid using enzymes immobilized on biodegradable, waste-derived supports such as spent coffee grounds. This study addresses this gap by assessing the catalytic performance of a novel NSCG biocatalyst and comparing it with a commercial reference, thereby offering new insights into developing greener, cost-effective alternatives for modifying phenolic compounds. It is the first study of its kind to use microbial lipase immobilized by adsorption on spent coffee grounds to derivatize chlorogenic acid contained in food waste extract. Until now, this type of biocatalyst has only been utilized for the hydrolysis of milk fat. The immobilized lipase from *Candida rugosa* onto spent coffee grounds was used to hydrolyze bovine milk to evaluate operational stability within a real sample. This solid biocatalyst

yielded nearly 60% conversion of milk fats into fatty acids after 18 h of reaction, maintaining this level across three reuses [37]. An attempt was also made to immobilize lipase from *Thermomyces lanuginosus* (TLL) onto spent coffee grounds, but during the research, it was found that it did not display any enzymatic activity and was not chosen to catalyze the synthesis of hexyl laurate [24].

This study shows that lipases are very effective in transforming specific food waste extracts. Depending on the composition of the extract, either NSCG or Novozym 435 performed better as a biocatalyst. This highlights the need to choose the right immobilized enzyme for specific reactions. The amount of product obtained can vary depending on the amount of CGA used initially and the presence of competing phenolic acids, which can block the enzyme's active site. Other substances, such as anthocyanins, tannins, caffeine, sugars, and pectins, can also bind to or inhibit enzymes, affecting the results [24,55–57].

3.3. Properties of Obtained Esters of Chlorogenic Acid and Pre- and Postmodified Extracts

3.3.1. Antioxidant Properties

When analyzing the antioxidant properties of chlorogenic acid (CGA) and its esters, a phenomenon known as "cut-off effect" or a nonlinear relationship between antioxidant activity and hydrophobicity is observed. It suggests that increasing the hydrophobicity of a polyphenolic compound does not always result in a proportional enhancement of its antioxidant capacity; on the contrary, after reaching an optimal chain length, a drastic decline in activity may occur [9,58–60]. Numerous studies have confirmed the nonlinear relationship between alkyl chain length and the antioxidant properties of CGA esters. One study, which included assays using the DPPH method, indicated that shorter-chain esters exhibited the highest antioxidant activity. López-Giraldo et al. [61] reported that C4 (butyl) and C8 (octyl) CGA esters exhibited significantly higher DPPH• scavenging capacity than the non-esterified 5-CQA. These esters also displayed higher reaction rate constants compared to 5-CQA. Similarly, Laguerre et al. [59], using a fibroblast model with overproduction of reactive oxygen species (ROS), observed optimal activity for the dodecyl ester (C12), with a notable 45% decrease in antioxidant capacity when the chain was extended to 16 carbon atoms, clearly illustrating the cut-off effect. Comparable results, with peak activity at C12, were also found in oil-in-water emulsions using the Conjugated Autoxidizable Triene (CAT) assay [9,62]. Conversely, a more recent study by Pappalardo et al. [63], also employing the DPPH method, reported a continuous increase in antioxidant activity with growing chain length, thus failing to confirm the classical cut-off pattern. However, it is important to note that this work referred to a general increase in activity, without a detailed analysis of a peak followed by a decline. In the context of ester synthesis, some studies [49,52] reported the highest esterification yields for C12 and C16 alkyl chain lengths. These discrepancies in optimal chain lengths (C4/C8 vs. C12) may arise from differences in the studied systems (emulsions, cellular environments, homogeneous solutions), the types of antioxidant assays, or experimental conditions.

Several mechanisms have been proposed to explain the cut-off effect. One of the most frequently cited is the partitioning hypothesis. According to this theory, esters with optimal chain lengths (e.g., butyl, octyl, dodecyl) are more effectively located at the oil–water interface in emulsions or within cellular membranes, where radical generation occurs [59,63]. In contrast, longer chains may embed too deeply in the lipid phase, thereby limiting the accessibility of hydroxyl groups to reactive species [63,64]. For example, dodecyl chlorogenate exhibited a greater partitioning capacity into the oil phase compared to longer esters (C16, C18, C20), which is crucial for optimal activity [59]. A second explanation involves steric effects: bulky alkyl chains may hinder interactions with free radicals by restricting access to reactive sites. López-Giraldo et al. [61] suggested that steric

hindrance may also limit dimer formation in longer esters (e.g., dodecyl) compared to shorter ones (e.g., butyl), thus affecting overall antioxidant potential. A third proposed mechanism relates to self-assembly behaviour. In cellular studies, the superior activity of dodecyl chlorogenate may be associated with its ability to self-organize in aqueous environments, forming structures that better interact with cell membranes [59].

Accordingly, the effects of lipophilization may have been more pronounced in the latter two by-products due to their higher content of phenolic acids, which are more susceptible to chemical modification. Lipophilization of whole extracts carries the inherent risk that the synergistic interactions among phenolic constituents may be altered, potentially diminishing or enhancing antioxidant properties. In this study, butyl chlorogenate exhibited the highest antioxidant activity among the derivatives tested. However, the antioxidant potential of other phenolic acid derivatives formed during the process remains unknown, and their identification and evaluation warrant further investigation.

3.3.2. Antimicrobial Properties

The obtained results are partially consistent with those of other studies. Suárez-Quiroz et al. [65] evaluated both native chlorogenic acid (CGA) and its dodecyl ester (DCGA) derived from green coffee for their antimicrobial potential. While DCGA was active against select Gram-positive bacteria (*B. cereus*, *Clostridium sporogenes*, *Listeria innocua*), it showed no activity against Gram-negative strains such as *E. coli*, *Pseudomonas fluorescens*, and *Salmonella enterica*. Interestingly, unmodified CGA exhibited a broader antimicrobial spectrum, including activity against both Gram-positive and Gram-negative bacteria such as *P. fluorescens* and *S. aureus*, although *S. enterica* was resistant to both forms. The authors suggested that lipophilization through esterification may enhance antimicrobial efficacy in certain contexts, although it has a narrower spectrum of activity in others.

Further support for the antimicrobial potential of CGA derivatives comes from Ma et al. [66], who synthesized chlorogenic acid analogues with lipophilic chains containing amino acid residues. Two of these compounds, featuring threonine moieties, showed significantly enhanced antifungal activity, particularly against drug-resistant *Candida krusei*, while also demonstrating reduced toxicity toward marine organisms.

These findings collectively support the notion that CGA and its derivatives possess notable, although structure-dependent, antimicrobial properties. In light of the current results, it appears that the antimicrobial efficacy of chlorogenic acid derivatives is strongly influenced by the length and nature of the alkyl chain introduced during the esterification process. Medium-chain esters such as hexyl and octyl chlorogenates offer the most promising activity, particularly against Gram-positive pathogens, and may serve as potential candidates for further development as natural antimicrobial agents. However, their selectivity and lack of effect on Gram-negative bacteria highlight the need for continued structural optimization to broaden their spectrum of activity.

3.3.3. Oxidative Stability in Rapeseed Oil

Despite the high antioxidant activity of the extracts themselves, particularly those derived from spent coffee grounds and chokeberry pomace, this effect was not reflected in improved oxidative stability of the oil. It is possible that derivatization of the food waste extracts with 1-butanol was insufficient to ensure adequate solubility in the oil matrix, and the use of alcohols with longer alkyl chains may be required in future studies. For instance, in the study by Aladedunye et al. [67], a polyphenolic extract from rowanberry (*Sorbus aucuparia*) was developed with the aim of enriching oil with the obtained compound. The incorporation of an octadecyl chain significantly enhanced the antioxidant performance of chlorogenic acid in rapeseed oil during deep-fat frying of French fries; however, it had no

considerable effect on the oxidative stability of the oil under storage conditions. Compared to the native compound, the lipophilized phenolic derivative showed better transfer into the fried product, potentially improving the functional properties, nutritional quality, and shelf life of the final food product. Similarly, in the study by Aladedunye and Matthäus [68], a native phenolic extract from Canadian crabapple, composed primarily of phloridzin, was enzymatically modified to obtain an octadecanoyl derivative. The introduction of an octadecyl chain significantly improved the antioxidant capacity of phloridzin in rapeseed oil during deep frying, although no significant effect was observed on oxidative stability during storage. The addition of the modified phenolic extract to frying oil substantially improved tocopherol retention in fried foods, thereby enhancing their nutritional value and potentially extending their shelf life during storage.

4. Materials and Methods

4.1. Materials

In the present research, the liquid lipase of microbial origin—Novozym 51032 from *Aspergillus oryzae* (current trade name Sustine 140)—was kindly provided by Novozymes (Bagsvaerd, Denmark) and used as a biocatalyst. The food waste materials, which were carriers for the enzyme immobilization process, were acquired from the coffee shops—spent coffee grounds and juices production—apple and chokeberry pomaces (Greenherb Company, Wysoka, Poland). The biocatalyst Novozym 435 was used as a reference. All chemical reagents and solvents were purchased from Sigma-Aldrich (Poznań, Poland).

4.2. Preparation of Food Waste

A short diagram of the extract pre-treatment process is shown in Figure 9. Ten grams of each food waste material was weighed, wrapped in filter paper, and placed in a Soxhlet apparatus. Initially, extraction was performed using 150 mL of *n*-hexane, followed by 150 mL of ethanol. For each step, the extract was poured for 10 cycles of solvent transfer. After that time, the residual solvent was evaporated. In the case of hexane extracts, the solvent was evaporated, and the lipid contents in the food waste were calculated. The amount of fat extracted was calculated by relating the weight of the extracted lipids to the volume of food waste used in the process. The obtained ethanol extracts were put on the plates and frozen at −42 °C in an Irinox freezer (Corbanese, Italy) for 1 h and then moved for 24 h to the lyophilization process in the Christ Gamma 1–16 LSC apparatus (Osterode am Harz, Germany). The applied conditions were: a temperature of 10 °C on the shelves and a pressure of 63 Pa. The safety pressure was 103 Pa, protecting the material from temperatures above −20 °C. The dry extracts were stored at room temperature.

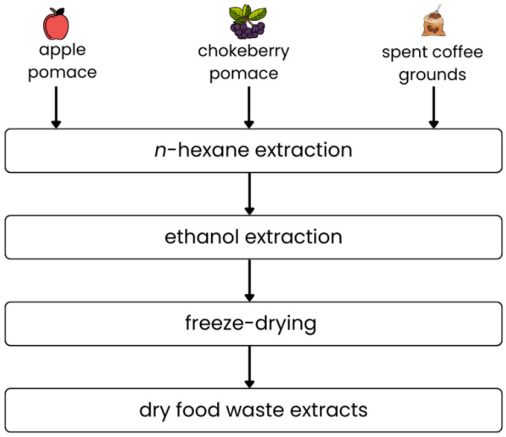

Figure 9. The scheme of the food waste material pretreatment process.

4.3. Lipase Immobilization Procedure

Liquid lipase from *A. oryzae* was immobilized based on the procedure described in the study by Jasińska et al. [69]. Briefly, 1 g of food waste (native spent coffee grounds—SCG; apple pomace—AP; or chokeberry pomace—ChoP) was added to a flask. Next, 1 mL of liquid lipase and 14 mL of distilled water were added to the flask. The lipase solution and the food waste carrier were agitated together for 2 h using a magnetic stirrer (140 rpm). After this period, the resulting biocatalysts were filtered, washed with distilled water, and dried at room temperature. The immobilization reactions were performed in triplicate for each type of food waste material.

4.4. Lipase Activity Assay—Hydrolytic and Synthetic Activities

To determine the hydrolytic and synthetic activities of the immobilized enzyme preparations, two methods were employed as detailed in the research by Jasińska et al. [69]. Both methods relied on spectrophotometric measurements. Based on the results of the lipolytic activities, a decision was made to select the most active biocatalyst for further testing.

The first method focused on the hydrolysis reaction of *p*-nitrophenyl laurate, which was conducted for 15 min at 37 °C. In this procedure, 25 mg of the immobilized enzyme preparation was combined with 100 µL of distilled water and 25 µL of *p*-nitrophenyl laurate solution (0.3 mmol dissolved in 2 mL of heptane) in Eppendorf test tubes. The mixture was stirred, and the absorbance was measured at 410 nm using a UV-Vis spectrophotometer. The unit of lipase enzymatic activity was defined as 1 U, which is the amount of enzyme that releases 1 µmol of *p*-nitrophenol per minute under the specified assay conditions.

The second method was based on the transesterification reaction, which occurred in an Eppendorf tube containing 100 mM vinyl acetate and 100 mM 1-butanol in 1 mL of *n*-hexane. To this mixture, 5 mg of immobilized lipase was added. After incubating for 5 min, diluted samples were prepared in test tubes. To each sample, 1 mL of a 0.1% (*m*/*v*) solution of MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride hydrate) was added and agitated for 10 min at 30 °C. Following this, 0.4 mL of a 1% (*m*/*v*) solution of $NH_4Fe(SO_4)_2$ $12H_2O$ (dissolved in 0.1M HCl) was added, and the mixture was mixed for 30 min at 30 °C. Spectrophotometric measurements were then carried out at 595 nm.

4.5. Elemental Compositions of Biocatalysts and Their Supports

Total C, N, and S contents of three biocatalysts and their supports were determined by dry combustion (Vario MacroCube, Elementar, Langenselbold, Germany).

4.6. Optimization of Synthesis Reaction—Box–Behnken Design

A model reaction was performed for the lipophilization of chlorogenic acid with 1-butanol (Figure 10) using biocatalyst—lipase from *A. oryzae* immobilized onto spent coffee grounds (NSCG). The following three-factor, three-level experiment was designed using Box–Behnken methodologies to optimize the reaction conditions (Table 9). The analyzed factors included temperature (35, 45, and 55 °C), enzyme concentration (20%, 35%, and 50%), and substrate molar ratio (alcohol to chlorogenic acid—2:1, 5:1, and 8:1). The tert-butyl methyl ether was used as a solvent (15 mL). The time duration of the reaction was also optimized (3, 5, 7, and 9 days). The subsequent reactions involved the lipophilization of chlorogenic acid with alcohols of varying carbon chain lengths (C6, C8, C10, C12), which were carried out over 7 days. The optimized parameters are a part of the patent application "Method for the preparation of chlorogenic acid esters and alcohols, by biocatalysis. WIPO ST 10/C PL451973". Experiments were conducted using two biocatalysts: NSCG and Novozym 435, which served as a reference. The reaction yield was calculated from the

area under the substrate and product peaks on the chromatogram obtained using the HPLC technique.

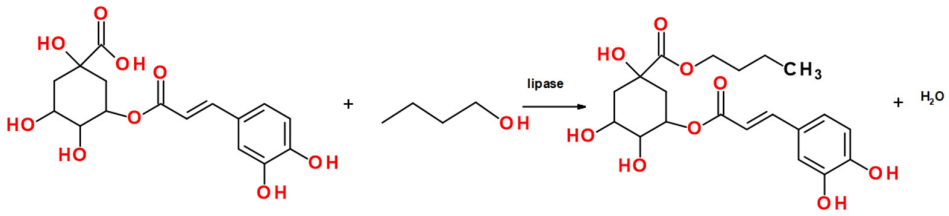

Figure 10. Reaction scheme for the synthesis of chlorogenic acid with 1-butanol.

Table 9. Coded levels and decoded value of the Box–Behnken design.

Factors	Name	Units	Low (−1)	Medium (0)	High (+1)
1	Temperature	°C	35	45	55
2	Substrate molar ratio	Alcohol:chlorogenic acid	2:1	5:1	8:1
3	Enzyme concentration	%	20	35	50

4.7. Chromatographic Determination of Esterified Post-Reaction Mixtures

The process for preparing esterified post-reaction mixtures involved evaporating the reaction solvent under nitrogen, diluting it with methanol, and then transferring the mixture to 25 mL volumetric flasks. Subsequently, the prepared solutions of esterified post-reaction mixtures were filtered using a PTFE syringe filter (0.45 µm) into chromatographic vials.

Chromatographic analysis of chlorogenic acid esters was performed using a modified analytical procedure reported by Głowacka et al. [70]. HPLC analysis was conducted by means of the high-performance liquid chromatography system from Shimadzu (Kyoto, Japan), which consisted of a DGU-20A SR degassing unit, an LC-20AD pump, a SIL-20A HT autosampler, a CTO-10AS VP column oven, and a SPD-M20A diode array detector. The obtained samples were separated on the Supelco 5-µm SUPELCOSIL LC-18-S analytical column (25 cm × 4.6 mm). The chromatographic separation of the obtained samples was performed in gradient elution mode using a 0.1% aqueous formic acid solution (phase A) and methanol (phase B) as mobile phases, at a flow rate of 0.8 mL/min. The composition of the mobile phase was as follows: 0 min, 80% B; 12–15 min, 50% B; 28–45 min, 80% B. Data were registered at a wavelength of 325 nm. In order to determine the degree of reaction, the areas under the peaks of the analytes of interest were utilized.

4.8. Column Chromatography and NMR Analysis

Upon completion of the reaction, the mixture was filtered to separate the biocatalyst. The solvent was then evaporated under reduced pressure, and the resulting residue was subjected to column chromatography for the purification of chlorogenic acid esters. Silica gel 60 (particle size: 0.040–0.063 mm; mesh size: 230–400) was used as the stationary phase, while a chloroform: methanol mixture (1:1, *v*/*v*) served as the mobile phase. The presence of target compounds was monitored by thin-layer chromatography (TLC) using silica gel plates.

Fractions containing the desired product were pooled, the solvent was removed by evaporation, and the esters were crystallized from heptane to obtain a purified compound. Structural confirmation of the purified esters was performed by carbon-13 nuclear magnetic resonance spectroscopy (^{13}C NMR). Spectra were recorded using a Bruker AVANCE

500 MHz spectrometer (Bruker, Billerica, MA, USA) with DMSO-d6 as the solvent. Chemical shifts (δ) are reported in parts per million (ppm) and referenced to tetramethylsilane (TMS) as the internal standard.

Butyl chlorogenate
^{13}C NMR (126 MHz, DMSO-d6) δ 173.19, 165.43, 148.61, 145.70, 145.17, 125.34, 121.34, 115.85, 114.55, 113.81, 73.12, 71.09, 69.40, 66.91, 64.12, 37.23, 35.09, 30.03, 18.54, 13.56.
Hexyl chlorogenate
^{13}C NMR (126 MHz, DMSO-d6) δ 173.16, 165.35, 148.54, 145.66, 145.15, 125.32, 121.27, 115.79, 114.55, 113.75, 73.01, 71.07, 69.27, 66.75, 64.37, 37.22, 34.97, 30.80, 27.88, 24.90, 21.97, 13.78.
Octyl chlorogenate
^{13}C NMR (126 MHz, DMSO-d6) δ 173.16, 165.35, 148.53, 145.65, 145.15, 125.31, 121.26, 115.77, 114.53, 113.73, 73.01, 71.07, 69.28, 66.77, 64.36, 37.22, 34.99, 31.11, 28.57, 27.92, 25.24, 22.05, 13.94.
Decyl chlorogenate
^{13}C NMR (126 MHz, DMSO-d6) δ 173.16, 165.36, 148.57, 145.67, 145.16, 125.29, 121.27, 115.77, 114.51, 113.71, 73.04, 71.06, 69.29, 66.80, 64.37, 37.22, 35.03, 31.31, 29.12, 29.00, 28.97, 28.92, 28.85, 28.73, 22.10, 13.97.
Dodecyl chlorogenate
^{13}C NMR (126 MHz, DMSO-d6) δ 173.15, 165.35, 148.54, 145.65, 145.14, 125.30, 121.25, 115.76, 114.51, 113.73, 73.04, 71.05, 69.31, 66.80, 64.35, 37.21, 35.03, 31.30, 29.02, 28.99, 28.91, 28.89, 28.72, 28.60, 27.92, 25.24, 22.11, 13.97.

4.9. ESI- MS Experiments

High-resolution ESI-MS spectra of butyl chlorogenate were acquired using an ultra-performance liquid chromatograph ACQUITY UPLC I-Class (Waters, Milford, MA, USA) coupled with a Synapt G2-S HDMS (Waters, Milford, MA, USA) mass spectrometer equipped with an electrospray ion source and q-TOF type mass analyser. The instrument was controlled, and the recorded data were processed using the MassLynx V4.1 software package (Waters, Milford, MA, USA). The electrospray ionization–mass spectrometry (ESI–MS) spectra were recorded in the positive and negative ion mode in the *m/z* range 50–3000.

4.10. Lipophilization Process

Based on the optimization of the synthesis of chlorogenic acid derivatives, parameters were selected to carry out the lipophilization of extracts. Reactions were maintained in conical flasks at 55 °C, stirring continuously (150 rpm). Into the reaction mixture, 1-butanol and dried extracts from food waste were added in a 8:1 (*w*/*w*) ratio, along with 15 mL of tert-butyl methyl ether. The reaction was catalyzed by immobilized onto spent coffee grounds lipase from *A. oryzae* and Novozym 435, as a reference, for 7 days. The biocatalysts were removed from the reaction mixture by filtration. The extracts, after evaporating the remaining solvent, were analyzed by chromatographic techniques.

4.11. LC-MS Assay of the Obtained Extracts

The comparison of the content of selected phenolic acids and their derivatives in prepared extracts before and after the lipophilization process was determined by Liquid Chromatography-Mass Spectrometry.

Each lyophilized extract was accurately weighed (10 mg) and dissolved in 1 mL of methanol. The suspensions were sonicated in an ultrasonic bath for 30 min at room temperature. Subsequently, the samples were filtered through 0.22 μm syringe filters (PTFE membrane). Based on these stock solutions, three concentration levels of analytes were

prepared: 10, 1, and 0.1 mg/mL. To achieve this, the stock solutions were evaporated to dryness under a stream of nitrogen at room temperature, and the residues were then dissolved in a 30% methanol solution (v/v) to obtain the desired concentrations. The contents of the compounds were expressed on a dry weight basis (per gram of dry extract).

To determine the content of phenolic acids in the derivatized extracts, aliquots were taken from the reaction mixture and processed to dryness under a stream of nitrogen. The residues were reconstituted in 30% methanol (v/v), and the final volume was adjusted to ensure comparability between samples. In most cases, this meant restoring the same concentration as in the original extract; in some instances, the volume was increased to introduce an additional dilution when required by the limited amount of material available. The prepared samples were then subjected to LC-MS analysis. The content of compounds was expressed per liter of derivatized extracts.

4.12. Total Polyphenol Content

The total polyphenolic content in extracts (1 mg/mL in methanol) before the lipophilization process was determined using the Folin–Ciocalteu method. 0.18 mL of extract was placed in glass tubes and diluted with 4.92 mL of distilled water. Afterwards, 0.3 mL of Folin–Ciocalteu reagent was added and mixed. After 3 min, the pH of the solution was adjusted by adding and mixing 0.6 mL of a supersaturated sodium carbonate solution. The incubation was carried out in the darkness at 25 °C for an hour. The absorbance of the solutions was measured using a Rayleigh UV-1601 spectrophotometer (BRAIC, Beijing, China) at a wavelength of 750 nm against a blank sample. The content of polyphenols was calculated as chlorogenic acid equivalents (mg CGA equivalents/ g extract). The analysis was conducted in triplicate.

4.13. Evaluation of Antioxidant Properties

4.13.1. The DPPH Assay

The DPPH assay was performed according to the protocol outlined by Zanetti et al. [71], with some minor adjustments made to assess the antioxidant properties of the obtained compounds and extracts. Briefly, 0.004% solutions of DPPH, 1 mM of isolated chlorogenic esters, and 1 mg/mL of the obtained extracts in methanol were prepared. The antioxidant activities of the tested compounds were measured using a Rayleigh UV-1601 spectrophotometer (BRAIC, Beijing, China) at 517 nm. Based on the results of four different dilutions of tested solutions, the IC_{50} parameters, i.e., the concentration required for a 50% reduction of the DPPH· radical, were calculated. Butylated hydroxytoluene (BHT) was used as a reference.

4.13.2. CUPRAC Method

The CUPRAC (cupric ion reducing antioxidant capacity) assay was utilized as a supplementary method to evaluate the antioxidant activities of the substances under investigation. This analysis was conducted following the approach outlined by Özyürek et al. [72]. In this method, the absorbance of the complex formed between neocuproine (2,9-dimethyl-1,10-phenanthroline) and the Cu(I) ion is assessed spectrophotometrically at 450 nm, with antioxidant compounds acting as electron donors. The Trolox Equivalent Antioxidant Capacities (TEAC) were calculated for the chlorogenic esters and extracts being tested based on the absorbance readings of these compounds compared to Trolox, which served as a standard reference. Butylated hydroxytoluene (BHT) was used as a reference.

4.14. Evaluation of Antimicrobial Properties

The antimicrobial activity was evaluated using the disc diffusion assay. Esters and extracts were first dissolved in ethanol to achieve a concentration of 50 mg/mL. Then, 10 µL

of each compound and extracts were applied onto sterile 6 mm diameter discs. Bacterial suspensions adjusted to a 0.5 McFarland standard (1.5×10^8 CFU/mL) were evenly spread onto Mueller–Hinton agar plates. The discs impregnated with the selected esters and extracts were then placed onto the inoculated agar surface. Plates were incubated at 37 °C for 16–18 h. After incubation, the diameters of the inhibition zones around the discs were measured to assess antibacterial efficacy. The following bacteria, purchased from the Polish Collection of Microorganisms (PCM) of the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wrocław, Poland), were used for analysis: *Bacillus cereus* PCM 482, *Bacillus subtilis* PCM 486, *Enterobacter cloacae* PCM 2848, *Enterococcus faecalis* PCM 2909, *Escherichia coli* PCM 2057, *Listeria monocytogenes* PCM 2191, *Serratia marcescens* PCM 549, and *Staphylococcus aureus* PCM 2054.

4.15. PDSC Measurements

The maximum oxidation time (τ_{max}) for rapeseed oils without (control sample) and with additives was determined using a pressure differential scanning calorimetry (PDSC) assay. Into 30 g of rapeseed oil in Falcon tubes, the ethanolic extracts were added (3 mg of compound/extract in 30 µL EtOH), resulting in a 0.01% concentration of the compound/extract in oil. The measurement was conducted immediately after the additives were applied and after a 2-month storage period had elapsed. An oil sample of 3–4 mg was placed in an open aluminum pan, with an empty reference pan next to it, within the pressure chamber of a DSC Q20 apparatus (TA Instruments, New Castle, DE, USA). The τ_{max} was recorded at a constant temperature of 120 °C and an initial pressure of 1400 kPa, all within a pure oxygen environment. The τ_{max}, measured in minutes, was derived by analyzing the heat flow over time with the help of TA Software (version 4.5A). All tested compounds were added at a concentration of 0.01% (m/m) in alignment with the maximum BHT addition to fats and oils designated for the production of heat-treated foods or frying oils and fats, in accordance with EU Regulation [73].

4.16. Lipophilicity Analysis

To determine the lipophilicity of the obtained chlorogenic acid esters, the octanol/water partition coefficient (logP) and aqueous solubility (logS) were calculated in MOE Software (MOE 2015.10, Molecular Operating Environment, Chemical Computing Group, Montreal, QC, Canada).

4.17. Statistical Analysis

The results were statistically analyzed using the STATISTICA 13.3 software (TIBCO Software Inc., Palo Alto, CA, USA). The following methods were applied: the Shapiro–Wilk test to assess the statistical hypothesis regarding the normality of the distribution of the experimental data, Levene's and Brown-Forsythe tests to evaluate the hypothesis of variance homogeneity, analysis of variance (ANOVA), post-hoc Tukey's test, and the Box–Behnken design for optimizing the synthesis reaction. A significance level of $p \leq 0.05$ was deemed statistically significant.

5. Conclusions

This study successfully demonstrates the dual potential of plant-based food processing by-products from beverage production as valuable sources of phenolic acids and sustainable supports for lipase immobilization. Microbial lipase from *A. oryzae* was successfully immobilized on three biodegradable carriers: spent coffee grounds (NSCG), chokeberry pomace (NChoP), and apple pomace (NAP). Among these, the biocatalyst immobilized on native spent coffee grounds (NSCG) exhibited the highest enzymatic activity, substantially surpassing the other preparations. This enhanced performance of NSCG may be attributed

to its higher hemicellulose content, more porous structure, and elevated lipid fraction (11%), which favour interfacial activation of lipase and stabilization of the enzyme through hydrophobic interactions. Chlorogenic acid (CGA), abundant in all extracts, was enzymatically lipophilized to enhance its compatibility with lipid matrices. The lipophilization yield increased with the length of the alcohol's carbon chain using the biocatalyst—NSCG, while the commercial enzyme showed consistent performance regardless of chain length. This is the first report of using a food waste-immobilized biocatalyst for chlorogenic acid lipophilization.

Characterization of the synthesized derivatives and modified extracts revealed diverse effects of structural modification. Butyl chlorogenate exhibited the highest antioxidant activity among all synthesized esters, comparable to that of free CGA and BHT, while also offering improved lipophilicity. A clear "cut-off effect" was observed, where longer alkyl chains led to decreased antioxidant activity, likely due to suboptimal orientation at the lipid–water interface or steric hindrance. Regarding antimicrobial activity, medium-chain chlorogenates (C6 and C8) showed the strongest antibacterial effects, particularly against Gram-positive bacteria. In contrast, free CGA, BHT, butyl chlorogenate (C4), and longer esters (C10 and C12) did not exhibit such activity. For the extracts, lipophilization of chokeberry pomace and spent coffee grounds extracts resulted in a significant decrease in antioxidant activity, suggesting that the process may have disrupted the stability of active components. Importantly, none of the tested extracts, either native or lipophilized, demonstrated antibacterial activity. In terms of oxidative stability of rapeseed oil, short-chain chlorogenates (C4 and C6) exhibited the highest initial stability, likely due to better solubility in the lipid matrix compared to free CGA. However, the extracts themselves, even those rich in polyphenols, did not significantly improve the oxidative stability of the oil. This indicates the need for further research to optimize their solubility in lipid environments, possibly through the use of alcohols with longer alkyl chains during the lipophilization process.

In conclusion, food processing by-products represent a promising, environmentally friendly, and sustainable material for enzyme immobilization in biocatalytic processes, enabling the production of valuable bioactive compounds. While the yield and functionality of the resulting derivatives depend on extract composition and alkyl chain length, this study opens new perspectives for utilizing food waste as a raw material for the development of innovative food additives with enhanced lipophilicity and potential antioxidant and antibacterial properties. Further investigations should focus on detailed identification of the derivatives formed during extract lipophilization and on optimizing reaction conditions to maximize desired properties for specific applications.

6. Patents

Karina Jasińska, Agata Fabiszewska and Bartłomiej Zieniuk have a patent application: "Method for the preparation of chlorogenic acid esters and alcohols, by biocatalysis." WIPO ST 10/C PL451973.

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Abbreviations

The following abbreviations are used in this manuscript:

AP	Apple pomace
NAP	Lipase from *A.oryzae* (Novozym 51032) immobilized on apple pomace
CGA	Chlorogenic acid
ChoP	Chokeberry pomace
NChoP	Lipase from *A.oryzae* (Novozym 51032) immobilized on chokeberry pomace
NSCG	Lipase from *A.oryzae* (Novozym 51032) immobilized on spent coffee grounds
SCG	Spent coffee grounds

References

1. UN Environment Programme. UNEP Food Waste Index Report 2021. Available online: https://www.unep.org/resources/report/unep-food-waste-index-report-2021 (accessed on 29 April 2025).
2. Poore, J.; Nemecek, T. Reducing Food's Environmental Impacts through Producers and Consumers. *Science* **2018**, *360*, 987–992; Erratum in *Science* **2019**, *363*, 6429. [CrossRef] [PubMed]
3. Capanoglu, E.; Nemli, E.; Tomas-Barberan, F. Novel Approaches in the Valorization of Agricultural Wastes and Their Applications. *J. Agric. Food Chem.* **2022**, *70*, 6787–6804. [CrossRef] [PubMed]
4. Arun, K.B.; Madhavan, A.; Sindhu, R.; Binod, P.; Pandey, A.; R, R.; Sirohi, R. Remodeling Agro-Industrial and Food Wastes into Value-Added Bioactives and Biopolymers. *Ind. Crops Prod.* **2020**, *154*, 112621. [CrossRef]
5. Vermerris, W.; Nicholson, R. *Phenolic Compound Biochemistry*, 2006th ed.; Springer: New York, NY, USA, 2008; ISBN 9781402051630.
6. Durand, E.; Bayrasy, C.; Laguerre, M.; Barouh, N.; Lecomte, J.; Durand, T.; Balas, L.; Wrutniak-Cabello, C.; Cabello, G.; Villeneuve, P. Regioselective Synthesis of Diacylglycerol Rosmarinates and Evaluation of Their Antioxidant Activity in Fibroblasts: Diacylglycerol Rosmarinates as Novel Antioxidants. *Eur. J. Lipid Sci. Technol.* **2015**, *117*, 1159–1170. [CrossRef]
7. Durand, E.; Lecomte, J.; Villeneuve, P. The Biological and Antimicrobial Activities of Phenolipids. *Lipid Technol.* **2017**, *29*, 67–70. [CrossRef]
8. Ramadan, M.F. *Pheno-Phospholipids and Lipo-Phenolics: Novel Structured Antioxidants*, 1st ed.; Springer Nature: Cham, Switzerland, 2022; ISBN 9783030674014.
9. Laguerre, M.; Bayrasy, C.; Lecomte, J.; Chabi, B.; Decker, E.A.; Wrutniak-Cabello, C.; Cabello, G.; Villeneuve, P. How to Boost Antioxidants by Lipophilization? *Biochimie* **2013**, *95*, 20–26. [CrossRef]
10. Mardani, M.; Badakné, K.; Farmani, J.; Shahidi, F. Enzymatic Lipophilization of Bioactive Compounds with High Antioxidant Activity: A Review. *Crit. Rev. Food Sci. Nutr.* **2024**, *64*, 4977–4994. [CrossRef]
11. Alemán, M.; Bou, R.; Guardiola, F.; Durand, E.; Villeneuve, P.; Jacobsen, C.; Sørensen, A.-D.M. Antioxidative Effect of Lipophilized Caffeic Acid in Fish Oil Enriched Mayonnaise and Milk. *Food Chem.* **2015**, *167*, 236–244. [CrossRef]
12. Figueroa-Espinoza, M.C.; Laguerre, M.; Villeneuve, P.; Lecomte, J. From Phenolics to Phenolipids: Optimizing Antioxidants in Lipid Dispersions. *Lipid Technol.* **2013**, *25*, 131–134. [CrossRef]
13. de Araújo, M.E.M.B.; Franco, Y.E.M.; Messias, M.C.F.; Longato, G.B.; Pamphile, J.A.; Carvalho, P.d.O. Biocatalytic Synthesis of Flavonoid Esters by Lipases and Their Biological Benefits. *Planta Med.* **2017**, *83*, 7–22. [CrossRef]
14. Chandra, P.; Enespa; Singh, R.; Arora, P.K. Microbial Lipases and Their Industrial Applications: A Comprehensive Review. *Microb. Cell Fact.* **2020**, *19*, 169. [CrossRef] [PubMed]
15. Coelho, A.L.S.; Orlandelli, R.C. Immobilized Microbial Lipases in the Food Industry: A Systematic Literature Review. *Crit. Rev. Food Sci. Nutr.* **2021**, *61*, 1689–1703. [CrossRef] [PubMed]
16. Xie, J.; Zhang, Y.; Simpson, B. Food Enzymes Immobilization: Novel Carriers, Techniques and Applications. *Curr. Opin. Food Sci.* **2022**, *43*, 27–35. [CrossRef]
17. Ismail, A.R.; Baek, K.-H. Lipase Immobilization with Support Materials, Preparation Techniques, and Applications: Present and Future Aspects. *Int. J. Biol. Macromol.* **2020**, *163*, 1624–1639. [CrossRef]

18. Rodrigues, R.C.; Virgen-Ortíz, J.J.; Dos Santos, J.C.S.; Berenguer-Murcia, Á.; Alcantara, A.R.; Barbosa, O.; Ortiz, C.; Fernandez-Lafuente, R. Immobilization of Lipases on Hydrophobic Supports: Immobilization Mechanism, Advantages, Problems, and Solutions. *Biotechnol. Adv.* **2019**, *37*, 746–770. [CrossRef]
19. Bilal, M.; Fernandes, C.D.; Mehmood, T.; Nadeem, F.; Tabassam, Q.; Ferreira, L.F.R. Immobilized Lipases-Based Nano-Biocatalytic Systems—A Versatile Platform with Incredible Biotechnological Potential. *Int. J. Biol. Macromol.* **2021**, *175*, 108–122. [CrossRef]
20. Filho, D.G.; Silva, A.G.; Guidini, C.Z. Lipases: Sources, Immobilization Methods, and Industrial Applications. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 7399–7423. [CrossRef]
21. Palma, B.G.; Leão, R.A.C.; M.A. de Souza, R.O.; G. Pandoli, O. Immobilization of Lipases on Lignocellulosic Bamboo Powder for Biocatalytic Transformations in Batch and Continuous Flow. *Catal. Today* **2021**, *381*, 280–287. [CrossRef]
22. de França Serpa, J.; Matias, G.A.B.; Fechine, P.B.A.; da Costa, V.M.; Freire, R.M.; Denardin, J.C.; Gonçalves, L.R.B.; de Macedo, A.C.; Rocha, M.V.P. New Nanocomposite Made of Cashew Apple Bagasse Lignin and Fe_3O_4 for Immobilizing of Lipase B from *Candida antarctica* Aiming at Esterification. *J. Chem. Technol. Biotechnol.* **2021**, *96*, 2472–2487. [CrossRef]
23. Oliveira-Ribeiro, L.M.; Meili, L.; Silva-Belo-Gois, G.N.; Rosas-Garcia-Almeida, R.M.; da Silva-Duarte, J.L. Immobilization of lipase in biochar obtained from Manihot esculenta Crantz. *Rev. Ion Investig. Optim. Nuevos Procesos Ing.* **2019**, *32*, 7–13. [CrossRef]
24. de S. Lira, R.K.; Zardini, R.T.; de Carvalho, M.C.C.; Wojcieszak, R.; Leite, S.G.F.; Itabaiana, I., Jr. Agroindustrial Wastes as a Support for the Immobilization of Lipase from *Thermomyces lanuginosus*: Synthesis of Hexyl Laurate. *Biomolecules* **2021**, *11*, 445. [CrossRef]
25. Ittrat, P.; Chacho, T.; Pholprayoon, J.; Suttiwarayanon, N.; Charoenpanich, J. Application of Agriculture Waste as a Support for Lipase Immobilization. *Biocatal. Agric. Biotechnol.* **2014**, *3*, 77–82. [CrossRef]
26. Costa-Silva, T.A.; Carvalho, A.K.F.; Souza, C.R.F.; De Castro, H.F.; Said, S.; Oliveira, W.P. Enzymatic Synthesis of Biodiesel Using Immobilized Lipase on a Non-Commercial Support. *Energy Fuels* **2016**, *30*, 4820–4824. [CrossRef]
27. Nájera-Martínez, E.F.; Melchor-Martínez, E.M.; Sosa-Hernández, J.E.; Levin, L.N.; Parra-Saldívar, R.; Iqbal, H.M.N. Lignocellulosic Residues as Supports for Enzyme Immobilization, and Biocatalysts with Potential Applications. *Int. J. Biol. Macromol.* **2022**, *208*, 748–759. [CrossRef] [PubMed]
28. Budžaki, S.; Velić, N.; Ostojčić, M.; Stjepanović, M.; Rajs, B.B.; Šereš, Z.; Maravić, N.; Stanojev, J.; Hessel, V.; Strelec, I. Waste Management in the Agri-Food Industry: The Conversion of Eggshells, Spent Coffee Grounds, and Brown Onion Skins into Carriers for Lipase Immobilization. *Foods* **2022**, *11*, 409. [CrossRef] [PubMed]
29. Campos-Vega, R.; Loarca-Piña, G.; Vergara-Castañeda, H.A.; Oomah, B.D. Spent Coffee Grounds: A Review on Current Research and Future Prospects. *Trends Food Sci. Technol.* **2015**, *45*, 24–36. [CrossRef]
30. Mak, S.L.; Wu, M.Y.T.; Chak, W.Y.; Kwong, W.K.; Tang, W.F.; Li, C.H.; Lee, C.C.; Li, C.Y. A Review of the Feasibility of Producing Polylactic Acid (PLA) Polymers Using Spent Coffee Ground. *Sustainability* **2023**, *15*, 13498. [CrossRef]
31. Atabani, A.E.; Ali, I.; Naqvi, S.R.; Badruddin, I.A.; Aslam, M.; Mahmoud, E.; Almomani, F.; Juchelková, D.; Atelge, M.R.; Khan, T.M.Y. A State-of-the-Art Review on Spent Coffee Ground (SCG) Pyrolysis for Future Biorefinery. *Chemosphere* **2022**, *286*, 131730. [CrossRef]
32. Girelli, A.M.; Astolfi, M.L.; Scuto, F.R. Agro-Industrial Wastes as Potential Carriers for Enzyme Immobilization: A Review. *Chemosphere* **2020**, *244*, 125368. [CrossRef]
33. Chen, K.-I.; Lo, Y.-C.; Liu, C.-W.; Yu, R.-C.; Chou, C.-C.; Cheng, K.-C. Enrichment of Two Isoflavone Aglycones in Black Soymilk by Using Spent Coffee Grounds as an Immobiliser for β-Glucosidase. *Food Chem.* **2013**, *139*, 79–85. [CrossRef]
34. Buntić, A.; Pavlović, M.; Antonović, D.; Pavlović, V.; Vrućinić, D.; Šiler-Marinković, S.; Dimitrijević-Branković, S. Customizing the Spent Coffee for Trichoderma Reesei Cellulase Immobilization by Modification with Activating Agents. *Int. J. Biol. Macromol.* **2018**, *107*, 1856–1863. [CrossRef] [PubMed]
35. Jasińska, K.; Nowosad, M.; Perzyna, A.; Bielacki, A.; Dziwiński, S.; Zieniuk, B.; Fabiszewska, A. Sustainable Lipase Immobilization: Chokeberry and Apple Waste as Carriers. *Biomolecules* **2024**, *14*, 1564. [CrossRef] [PubMed]
36. Jasińska, K.; Zieniuk, B.; Piasek, A.M.; Wysocki, Ł.; Sobiepanek, A.; Fabiszewska, A. Obtaining a Biodegradable Biocatalyst—Study on Lipase Immobilization on Spent Coffee Grounds as Potential Carriers. *Biocatal. Agric. Biotechnol.* **2024**, *59*, 103255. [CrossRef]
37. Girelli, A.M.; Chiappini, V.; Amadoro, P. Immobilization of Lipase on Spent Coffee Grounds by Physical and Covalent Methods: A Comparison Study. *Biochem. Eng. J.* **2023**, *192*, 108827. [CrossRef]
38. Guerrero, M.R.B.; Marques da Silva Paula, M.; Zaragoza, M.M.; Gutiérrez, J.S.; Velderrain, V.G.; Ortiz, A.L.; Collins-Martínez, V. Thermogravimetric Study on the Pyrolysis Kinetics of Apple Pomace as Waste Biomass. *Int. J. Hydrogen Energy* **2014**, *39*, 16619–16627. [CrossRef]
39. Osman, A.I.; Young, T.J.; Farrell, C.; Harrison, J.; Al-Muhtaseb, A.H.; Rooney, D.W. Physicochemical Characterization and Kinetic Modeling Concerning Combustion of Waste Berry Pomace. *ACS Sustain. Chem. Eng.* **2020**, *8*, 17573–17586. [CrossRef]
40. Jasińska, K.; Fabiszewska, A.; Białecka-Florjańczyk, E.; Zieniuk, B. Mini-Review on the Enzymatic Lipophilization of Phenolics Present in Plant Extracts with the Special Emphasis on Anthocyanins. *Antioxidants* **2022**, *11*, 1528. [CrossRef]

41. Bhushan, S.; Kalia, K.; Sharma, M.; Singh, B.; Ahuja, P.S. Processing of Apple Pomace for Bioactive Molecules. *Crit. Rev. Biotechnol.* **2008**, *28*, 285–296. [CrossRef]
42. Tarko, T.; Duda-Chodak, A.; Bebak, A. Aktywność Biologiczna Wybranych Wytłoków Owocowych Oraz Warzywnych. *Żywność Nauka Technologia Jakość* **2012**, *4*, 55–65.
43. Mokhtar, N.F.; Rahman, R.N.Z.R.A.; Muhd Noor, N.D.; Mohd Shariff, F.; Mohamad Ali, M.S. The Immobilization of Lipases on Porous Support by Adsorption and Hydrophobic Interaction Method. *Catalysts* **2020**, *10*, 744. [CrossRef]
44. Alcañiz-Monge, J.; Román-Martínez, M.D.C.; Lillo-Ródenas, M.Á. Chemical Activation of Lignocellulosic Precursors and Residues: What Else to Consider? *Molecules* **2022**, *27*, 1630. [CrossRef] [PubMed]
45. Girelli, A.M.; Chiappini, V. Renewable, Sustainable, and Natural Lignocellulosic Carriers for Lipase Immobilization: A Review. *J. Biotechnol.* **2023**, *365*, 29–47. [CrossRef] [PubMed]
46. Manoel, E.A.; Dos Santos, J.C.S.; Freire, D.M.G.; Rueda, N.; Fernandez-Lafuente, R. Immobilization of Lipases on Hydrophobic Supports Involves the Open Form of the Enzyme. *Enzyme Microb. Technol.* **2015**, *71*, 53–57. [CrossRef] [PubMed]
47. Abreu Silveira, E.; Moreno-Perez, S.; Basso, A.; Serban, S.; Pestana Mamede, R.; Tardioli, P.W.; Sanchez Farinas, C.; Rocha-Martin, J.; Fernandez-Lorente, G.; Guisan, J.M. Modulation of the Regioselectivity of *Thermomyces lanuginosus* Lipase via Biocatalyst Engineering for the Ethanolysis of Oil in Fully Anhydrous Medium. *BMC Biotechnol.* **2017**, *17*, 88. [CrossRef]
48. Brabcová, J.; Demianová, Z.; Vondrášek, J.; Jágr, M.; Zarevúcka, M.; Palomo, J.M. Highly Selective Purification of Three Lipases from Geotrichum Candidum 4013 and Their Characterization and Biotechnological Applications. *J. Mol. Catal. B Enzym.* **2013**, *98*, 62–72. [CrossRef]
49. López Giraldo, L.J.; Laguerre, M.; Lecomte, J.; Figueroa-Espinoza, M.-C.; Barouh, N.; Baréa, B.; Villeneuve, P. Lipase-Catalyzed Synthesis of Chlorogenate Fatty Esters in Solvent-Free Medium. *Enzyme Microb. Technol.* **2007**, *41*, 721–726. [CrossRef]
50. Wang, S.; Li, Y.; Meng, X.; Chen, S.; Huang, D.; Xia, Y.; Zhu, S. Antioxidant Activities of Chlorogenic Acid Derivatives with Different Acyl Donor Chain Lengths and Their Stabilities during in Vitro Simulated Gastrointestinal Digestion. *Food Chem.* **2021**, *357*, 129904. [CrossRef]
51. Gil, M.; Wianowska, D. Chlorogenic Acids-Their Properties, Occurrence and Analysis. *Ann. Univ. Mariae Curie-Skłodowska Sect. AA-Chem.* **2017**, *72*, 61. [CrossRef]
52. Guyot, B.; Gueule, D.; Pina, M.; Graille, J.; Farines, V.; Farines, M. Enzymatic Synthesis of Fatty Esters in 5-Caffeoyl Quinic Acid. *Eur. J. Lipid Sci. Technol.* **2000**, *102*, 93–95. [CrossRef]
53. Lorentz, C.; Dulac, A.; Pencreac'h, G.; Ergan, F.; Richomme, P.; Soultani-Vigneron, S. Lipase-Catalyzed Synthesis of Two New Antioxidants: 4-O- and 3-O-Palmitoyl Chlorogenic Acids. *Biotechnol. Lett.* **2010**, *32*, 1955–1960. [CrossRef]
54. Zhu, S.; Wang, S.; Chen, S.; Xia, Y.; Li, Y. Lipase-Catalyzed Highly Regioselective Synthesis of Acylated Chlorogenic Acid. *Food Biosci.* **2020**, *37*, 100706. [CrossRef]
55. Antonic, B.; Jancikova, S.; Dordevic, D.; Tremlova, B. Apple Pomace as Food Fortification Ingredient: A Systematic Review and Meta-Analysis. *J. Food Sci.* **2020**, *85*, 2977–2985. [CrossRef]
56. Saracila, M.; Untea, A.E.; Oancea, A.G.; Varzaru, I.; Vlaicu, P.A. Comparative Analysis of Black Chokeberry (*Aronia melanocarpa* L.) Fruit, Leaves, and Pomace for Their Phytochemical Composition, Antioxidant Potential, and Polyphenol Bioaccessibility. *Foods* **2024**, *13*, 1856. [CrossRef]
57. Ballesteros, L.F.; Teixeira, J.A.; Mussatto, S.I. Chemical, Functional, and Structural Properties of Spent Coffee Grounds and Coffee Silverskin. *Food Bioproc. Tech.* **2014**, *7*, 3493–3503. [CrossRef]
58. Shahidi, F.; Zhong, Y. Revisiting the Polar Paradox Theory: A Critical Overview. *J. Agric. Food Chem.* **2011**, *59*, 3499–3504. [CrossRef] [PubMed]
59. Laguerre, M.; Wrutniak-Cabello, C.; Chabi, B.; López Giraldo, L.J.; Lecomte, J.; Villeneuve, P.; Cabello, G. Does Hydrophobicity Always Enhance Antioxidant Drugs? A Cut-off Effect of the Chain Length of Functionalized Chlorogenate Esters on ROS-Overexpressing Fibroblasts: Cut-off Effect of Phenolipid Antioxidants. *J. Pharm. Pharmacol.* **2011**, *63*, 531–540. [CrossRef] [PubMed]
60. Laguerre, M.; López Giraldo, L.J.; Lecomte, J.; Figueroa-Espinoza, M.-C.; Baréa, B.; Weiss, J.; Decker, E.A.; Villeneuve, P. Relationship between Hydrophobicity and Antioxidant Ability of "Phenolipids" in Emulsion: A Parabolic Effect of the Chain Length of Rosmarinate Esters. *J. Agric. Food Chem.* **2010**, *58*, 2869–2876. [CrossRef]
61. López-Giraldo, L.J.; Laguerre, M.; Lecomte, J.; Figueroa-Espinoza, M.-C.; Baréa, B.; Weiss, J.; Decker, E.A.; Villeneuve, P. Kinetic and Stoichiometry of the Reaction of Chlorogenic Acid and Its Alkyl Esters against the DPPH Radical. *J. Agric. Food Chem.* **2009**, *57*, 863–870. [CrossRef]
62. Laguerre, M.; Giraldo, L.J.L.; Lecomte, J.; Figueroa-Espinoza, M.-C.; Baréa, B.; Weiss, J.; Decker, E.A.; Villeneuve, P. Chain Length Affects Antioxidant Properties of Chlorogenate Esters in Emulsion: The Cutoff Theory behind the Polar Paradox. *J. Agric. Food Chem.* **2009**, *57*, 11335–11342. [CrossRef]
63. Pappalardo, V.; Ravasio, N.; Falletta, E.; De Rosa, M.C.; Zaccheria, F. A Green Lipophilization Reaction of a Natural Antioxidant. *Antioxidants* **2023**, *12*, 218. [CrossRef]

64. Sørensen, A.-D.M.; Nielsen, N.S.; Yang, Z.; Xu, X.; Jacobsen, C. Lipophilization of Dihydrocaffeic Acid Affects Its Antioxidative Properties in Fish-oil-enriched Emulsions. *Eur. J. Lipid Sci. Technol.* **2012**, *114*, 134–145. [CrossRef]
65. Suárez-Quiroz, M.L.; Taillefer, W.; López Méndez, E.M.; González-Ríos, O.; Villeneuve, P.; Figueroa-Espinoza, M.C. Antibacterial Activity and Antifungal and Anti-mycotoxigenic Activities against Aspergillus Flavus and A. Ochraceus of Green Coffee Chlorogenic Acids and Dodecyl Chlorogenates: Antimicrobial Chlorogenic Acids and Chlorogenates. *J. Food Saf.* **2013**, *33*, 360–368. [CrossRef]
66. Ma, C.-M.; Kully, M.; Khan, J.K.; Hattori, M.; Daneshtalab, M. Synthesis of Chlorogenic Acid Derivatives with Promising Antifungal Activity. *Bioorg. Med. Chem.* **2007**, *15*, 6830–6833. [CrossRef] [PubMed]
67. Aladedunye, F.; Niehaus, K.; Bednarz, H.; Thiyam-Hollander, U.; Fehling, E.; Matthäus, B. Enzymatic Lipophilization of Phenolic Extract from Rowanberry (*Sorbus aucuparia*) and Evaluation of Antioxidative Activity in Edible Oil. Lebenson. *Wiss. Technol.* **2015**, *60*, 56–62. [CrossRef]
68. Aladedunye, F.; Matthäus, B. Phenolic Extracts from *Sorbus aucuparia* (L.) and *Malus baccata* (L.) Berries: Antioxidant Activity and Performance in Rapeseed Oil during Frying and Storage. *Food Chem.* **2014**, *159*, 273–281. [CrossRef]
69. Jasińska, K.; Zieniuk, B.; Jankiewicz, U.; Fabiszewska, A. Bio-Based Materials versus Synthetic Polymers as a Support in Lipase Immobilization: Impact on Versatile Enzyme Activity. *Catalysts* **2023**, *13*, 395. [CrossRef]
70. Głowacka, R.; Górska, A.; Wirkowska-Wojdyła, M.; Wołosiak, R.; Majewska, E.; Derewiaka, D. The Influence of Brewing Method on Bioactive Compounds Residues in Spent Coffee Grounds of Different Roasting Degree and Geographical Origin. *Int. J. Food Sci. Technol.* **2019**, *54*, 3008–3014. [CrossRef]
71. Zanetti, M.; Carniel, T.K.; Valério, A.; de Oliveira, J.V.; de Oliveira, D.; de Araújo, P.H.H.; Riella, H.G.; Fiori, M.A. Synthesis of Geranyl Cinnamate by Lipase-Catalyzed Reaction and Its Evaluation as an Antimicrobial Agent: Lipase-Catalyzed Synthesis of Geranyl Cinnamate. *J. Chem. Technol. Biotechnol.* **2017**, *92*, 115–121. [CrossRef]
72. Özyürek, M.; Güçlü, K.; Apak, R. The Main and Modified CUPRAC Methods of Antioxidant Measurement. *Trends Analyt. Chem.* **2011**, *30*, 652–664. [CrossRef]
73. Regulation-1333/2008-EN-Additives-EUR-Lex. Available online: https://eur-lex.europa.eu/eli/reg/2008/1333/oj/eng (accessed on 20 November 2025).

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Oświadczenie o współautorstwie

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Warszawa, 02.12.2025

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