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OCENA AKTYWNOŚCI ENANCJOSELEKTYWNEJ I LIPOLITYCZNEJ LIPAZ Z *BURKHOLDERIA SP.* ORAZ *ASPERGILLUS SP.* W FORMIE WOLNEJ ORAZ IMMOBILIZOWANEJ NA NOŚNIKACH POLIMEROWYCH

Rozprawa na stopień doktora nauk farmaceutycznych

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P1. Jacek Dulęba, Tomasz Siódmiak, Michał Piotr Marszałł, Amano Lipase PS from *Burkholderia cepacia* - Evaluation of the Effect of Substrates and Reaction Media on the Catalytic Activity, *Curr. Org Chem.*, 2020, 24 (7), 798-807.

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P2. Jacek Dulęba, Tomasz Siódmiak, Michał Piotr Marszałł, The influence of substrate systems on the enantioselective and lipolytic activity of immobilized Amano PS from *Burkholderia cepacia* lipase (APS-BCL), *Process Biochem.* 2022, 120, 126-137.

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P4. Tomasz Siódmiak*****, **Jacek Dulęba***, Natalia Kocot, Dorota Wątróbska-Świetlikowska, Michał Piotr Marszałł, The High 'Lipolytic Jump' of Immobilized Amano A Lipase from *Aspergillus niger* in Developed 'ESS Catalytic Triangles' Containing Natural Origin Substrates, *Catalysts,* 2022, 12, 853.

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SPIS TREŚCI

WYKAZ STOSOWANYCH SKRÓTÓW

A_{rec}	Odzysk aktywności enzymu (ang. *activity recovery*)
A_{rel}	Aktywność relatywna (ang. *relative activity*)
A_{ret}	Aktywność zachowana (ang. *activity retention*)
AA-ANL	Amano lipaza A z *Aspergillus niger* (ang. *Amano lipase A from Aspergillus niger*)
ALA	Kwas α-linolenowy (ang. *α-linolenic acid*)
AN	*Aspergillus niger*
ANL	Lipaza z *Aspergillus niger* (ang. *Aspergillus niger lipase*)
APS-BCL	Amano lipaza PS z *Burkholderia cepacia* (ang. *Amano lipase PS from Burkholderia cepacia*)
APS-BCL-D	Amano lipaza PS z *Burkholderia cepacia*, odmiana D (ang. *Amano lipase PS-D from Burkholderia cepacia*)
ARA	Kwas arachidonowy (ang. *arachidonic acid*)
ATL	Lipaza z *Aspergillus terrasus* (ang. *lipase from Aspergillus terrasus*)
BC	*Burkholderia cepacia*
BCL	Lipaza z *Burkholderia cepacia* (ang. *Burkholderia cepacia lipase*)
C	Konwersja (ang. *conversion*)
CALB	Lipaza B z *Candida antarctica* (ang. *lipase B from Candida antarctica*)
CML	Lipaza z *Candida methylica* (ang. *lipase from Candida methylica*)
CRL	Lipaza z *Candida rugosa* (ang. *lipase from Candida rugosa*)
DAG	Diacyloglicerol (ang. *diacylglycerol*)
DHA	Kwas dokozaheksaenowy (ang. *docosahexaenoic acid*)
E	Enancjoselektywność (ang. *enantiomeric ratio, enantioselectivity*)
ee_p	Nadmiar enancjomeryczny produktów (ang. *enantiomeric excess of products*)
ee_s	Nadmiar enancjomeryczny substratów (ang. *enantiomeric excess of substrates*)
EPA	Kwas eikozapentaenowy (ang. *eicosapentaenoic acid*)
EFAs	Niezbędne nienasycone kwasy tłuszczowe (ang. *essential fatty acids*)
FFAs	Wolne kwasy tłuszczowe (ang. *free fatty acids*)
HPLC	Wysokosprawna chromatografia cieczowa (ang. *High Performance Liquid Chromatography)*
I_e	Efektywność immobilizacji (ang. *immobilization efficiency*)
I_y	Wydajność immobilizacji (ang. *immobilization yield*)
IB-150A	Immobead 150A (ang. *Immobead 150A*)

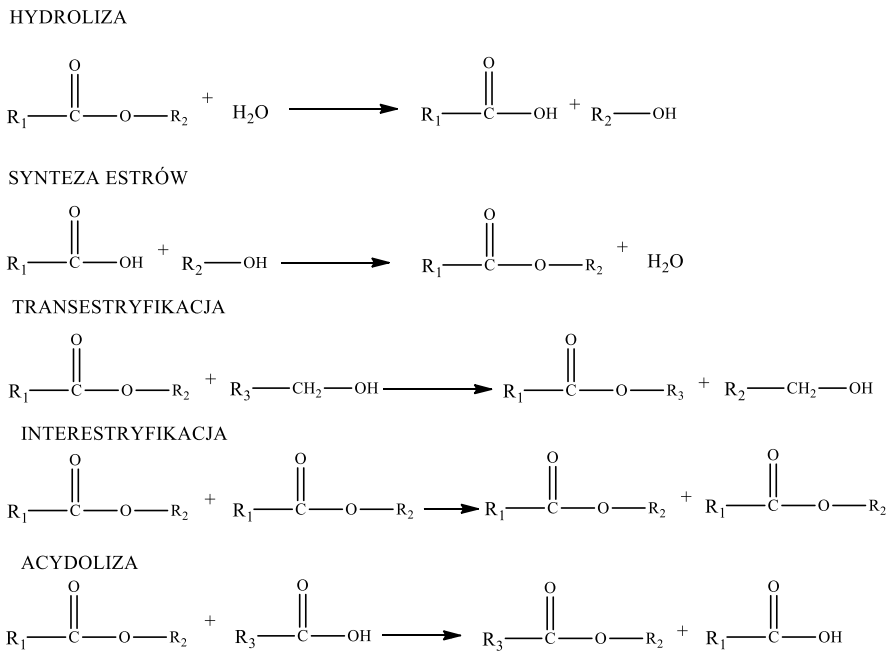
IB-150P	Immobead 150P (ang. *Immobead 150P*)
K_m	Stała kinetyczna Michaelis-Menten
L_L	Obciążenie lipazą (ang. *lipase loading*)
LA	Kwas α-linolowy (ang. *α-linoleic acid*)
MAG	Monoacyloglicerol (ang. *monoacylglycerol*)
MUFAs	Jednonienasycone kwasy tłuszczowe (ang. *monounsaturated fatty acids*)
OA	Kwas oleinowy (ang. *oleic acid*)
OTC	Lek dostępny bez recepty (ang. *over-the-counter drug*)
PC	*Pseudomonas cepacia*
PGE_2	Prostaglandyna E_2 (ang. *prostaglandin E_2*)
PGE_3	Prostaglandyna E_3 (ang. *prostaglandin E_3*)
PGI_2	Prostacyklina I_2 (ang. *prostacyclin I_2*)
PGI_3	Prostacyklina I_3 (ang. *prostacyclin I_3*)
PUFAs	Wielonienasycone kwasy tłuszczowe (ang. *polyunsaturated fatty acids*)
RS-PHE	(*R*,*S*)-1-fenyloetanol (ang. *(R,S)-1-phenylethanol*)
R-PHE	(*R*)-1-fenyloetanol (ang. *(R)-1-phenylethanol*)
R-PHE-ACE	octan (*R*)-1-fenyloetylu (ang. *(R)-1-phenylethyl acetate*)
S-PHE	(*S*)-1-fenyloetanol (ang. *(S)-1-phenylethanol*)
SFAs	Nasycone kwasy tłuszczowe (ang. *saturated fatty acids*)
TAG	Triacyloglicerol (ang. *triacylglycerol*)
TGs	Triglicerydy (ang. *triglycerides*)
TLL	Lipaza z *Thermomyces lanuginosus* (ang. *lipase from Thermomyces lanuginosus*)
UFAs	Nienasycone kwasy tłuszczowe (ang. *unsaturated fatty acids*)
V_{max}	Szybkość maksymalna reakcji (ang. *maximal velocity*)

1. Wstęp

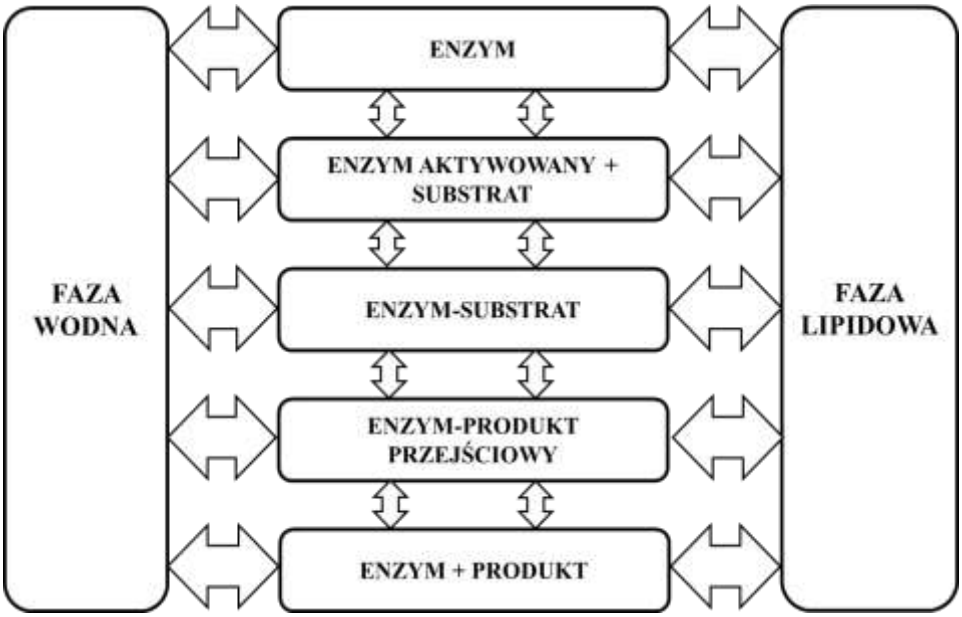
Biokataliza to proces oparty na zastosowaniu enzymów w katalizowaniu reakcji chemicznych. Za jej początek uznano badania naukowe Louisa Pasteura, który przeprowadzając fermentację cukrów do alkoholu, dowiódł, że proces ten jest związany z funkcjami życiowymi komórek drożdżowych [1]. Pojęcie „enzym" zostało zaproponowane po raz pierwszy w 1878 roku przez niemieckiego fizjologia, Wilhelma Kühnego [2]. Kontynuacja badań nad enzymami, prowadzonymi przez Eduarda Buchnera, polegała na katalizie bez udziału komórek żywych [3]. Stanowiło to początek studiów dotyczących izolacji enzymów w postaci krystalicznej takich jak np. trypsyna czy chymotrypsyna. Enzymy zastosowane w reakcjach chemicznych okazały się być użyteczne w szerokim spektrum reakcji o znaczeniu medycznym, farmaceutycznym, biotechnologicznym czy kosmetologicznym [4]. Głównym źródłem enzymów są mikroorganizmy oraz (w mniejszym stopniu) organizmy roślinne i zwierzęce. Udowodniono, że wykorzystanie białek katalitycznych znacząco zwiększa wydajność oraz selektywność reakcji (biokatalizator może zostać użyty nawet kilkukrotnie), obniża ich koszty oraz nie wymaga drastycznych warunków, przez co nie wywiera szkodliwego wpływu na otoczenie. Z tego powodu, biokataliza należy do tzw. zielonej chemii (ang. *green chemistry*), czyli koncepcji, zgodnie z którą reakcje prowadzi się w sposób najkorzystniejszy dla środowiska naturalnego, w celu ochrony przyrody oraz zapewnienia bezpieczeństwa klimatycznego [5, 6]. Jednakże, należy pamiętać, że enzymy pochodzenia naturalnego, mimo możliwości działania w szerokim spektrum warunków (temperatura, pH, czas przechowywania, siła jonowa, itp.) mogą ulec zniszczeniu w skrajnie ekstremalnym środowisku reakcji [7]. Enzymy wykazują specyficzność substratową i selektywność np. kinazy transportują grupy fosforanowe z wysokoenergetycznych związków na cząstki docelowe, proteazy hydrolizują wiązania peptydowe, transferazy przenoszą różne grupy chemiczne, amylazy rozkładają cukry złożone do cukrów prostych.

Szczególnym przykładem enzymów pozyskiwanych ze źródeł naturalnych są lipazy. Białka te należą do podklasy hydrolaz serynowych (EC 3.1.1.3) [8]. W organizmie są wytwarzane przez trzustkę. Następnie, pod wpływem żółci, ulegają aktywacji w dwunastnicy. Lipaza trzustkowa hydrolizuje pochodzące z pokarmu triacyloglicerole (TAG), do wolnych kwasów tłuszczowych (FFAs), diacylogliceroli (DAG) i monoacylogliceroli (MAG) [9]. Enzym ten jest niezbędny w prawidłowym utrzymaniu homeostazy. Inne lipazy, takie jak: językowa (wydzielana przez ślinianki), żołądkowa (wydzielana przez sok żołądkowy, działa w kwasowym pH), jelitowa (wspomaga działanie lipazy trzustkowej) odgrywają mniejszą rolę w procesie trawienia. Na **Rycinie 1** przedstawiono przykładowe typy reakcji chemicznych katalizowane przez lipazy. Jedną z cech lipaz,

istotną dla ich zastosowania w przemyśle farmaceutycznym jest aktywność katalityczna [10-14]. Ich znaczenie jest szczególne m.in. w reakcjach enancjoselektywnych m.in. kinetycznym rozdziale mieszaniny racemicznej związku chemicznego w celu uzyskania czystego optycznie enancjomeru [15, 16]. Lipazy działają na granicy faz, co umożliwia katalizowanie reakcji z udziałem związków zarówno hydrofilowych i hydrofobowych (**Rycina 2)** [17, 18]. Każda z lipaz posiada rdzeń zawierający 8 struktur β-fałdowych połączonych 6 fragmentami α-helisy oraz triadę katalityczną złożoną z trzech aminokwasów (seryna, histydyna, kwas glutaminowy lub asparaginowy), które tworzą tzw. miejsce aktywne (ang. *active site*) [19, 20]. Niektóre z tej grupy enzymów posiadają również tzw. wieczko (ang. *lid*), będące komponentem białkowym, zakrywającym centrum aktywne [21]. Ten element budowy umożliwia lipazie, poprzez przemieszczenie się wieczka, zmianę formy z zamkniętej na otwartą, co umożliwia większą dostępność dla substratów [17, 21-23]. Ponadto, lipazy pochodzenia bakteryjnego wykazują szczególnie wysoką stabilność termiczną oraz wysoką aktywność wobec reagentów hydrofobowych.



Rycina 1. *Przykładowe reakcje chemiczne katalizowane przez lipazy*



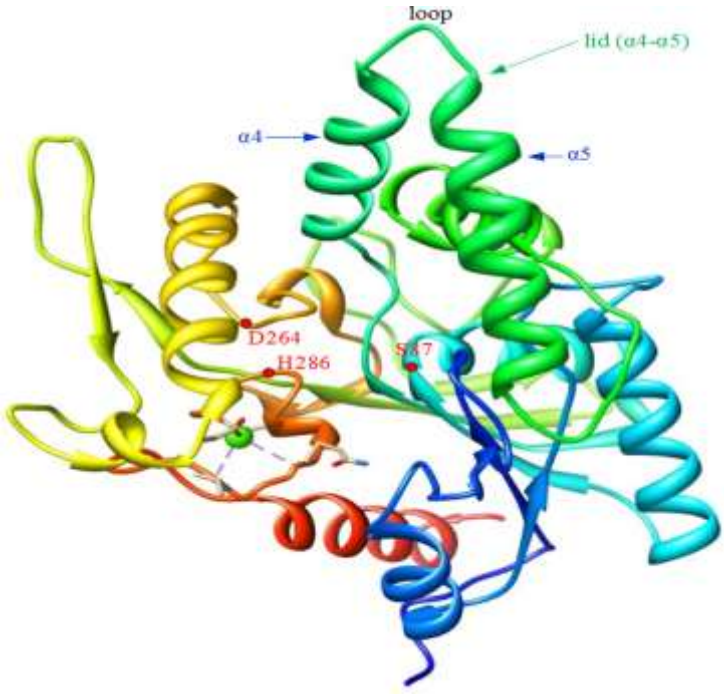
Rycina 2. *Uproszczony schemat działania lipazy na granicy faz wodnej i lipidowej*

W celu poprawy parametrów katalitycznych, lipazy poddaje się modyfikacjom. Często stosowaną techniką jest immobilizacja (ang. *immobilization*). Polega ona na unieruchomieniu lipazy w (lub na) materiale tzw. nośniku (ang. *support*) za pomocą różnych oddziaływań, zarówno chemicznych jak i fizycznych [24, 25]. Immobilizowany enzym powinien wykazywać zwiększoną aktywność w reakcjach katalitycznych. Jednakże, immobilizacja lipazy może również obniżyć jej aktywność, względem formy natywnej (wolnej) [12]. Liczne badania wykazały pozytywny wpływ immobilizacji na aktywność enzymatyczną lipaz [12, 26, 27]. Obecnie, stosuje się coraz bardziej zaawansowane techniki unieruchomienia, które, poprzez wzrost aktywności lipazy, mają znacząco zwiększyć wydajność przeprowadzanych reakcji [28-30].

Tematyka niniejszej pracy doktorskiej obejmuje wykorzystanie lipaz z *Burkholderia sp.* i *Aspergillus sp.* immobilizowanych na nośnikach polimerowych w katalizowaniu reakcji o znaczeniu farmaceutycznym. Przeprowadzone i opisane badania dotyczą oceny aktywności enancjoselektywnej lipaz w kinetycznym rozdziale (*R,S*)-1-fenyloetanolu oraz ocenie aktywności lipolitycznej poprzez hydrolizę enzymatyczną olejów pochodzenia naturalnego zawierających nienasycone kwasy tłuszczowe omega (ω).

2. Lipaza z *Burkholderia cepacia* jako przedstawiciel lipaz z *Burkholderia sp.*

Lipazy z *Burkholderia sp.* należą do najbardziej popularnych i powszechnie stosowanych enzymów w biokatalizie. Ze względu na swoje pochodzenie mikrobiologiczne, wykazują aktywność w szerokim spektrum reakcji katalitycznych oraz stabilność w różnych warunkach środowiskowych. Źródłem tych lipaz są różne gatunki pałeczek Gram-ujemnych (G(-)) bakterii *Burkholderia*, spośród których *Burkholderia cepacia* (BC) należy do najczęściej wykorzystywanych w badaniach naukowych. BC została opisana w 1950 roku, pod nazwą *Pseudomonas cepacia* (PC), przez Williama Burkholdera [31]. BC jest bakterią G(-), nieprzetrwalnikującą, powodującą częste zakażenia wewnątrzszpitalne. Ponadto, wykazuje oporność na antybiotyki aminoglikozydowe oraz z grupy penicylin. Cechuje się występowaniem w warunkach o dużej wilgotności, może wzrastać również w roztworach soli fizjologicznej, wodzie destylowanej, a nawet w środkach dezynfekujących. BCL jest pozyskiwana z mikroorganizmów za pomocą fermentacji a także metodą rekombinacji genetycznej. Lipaza ta, ze względu na swoją strukturę (**Rycina 3)**, przede wszystkim obecność wieczka, wykazuje wysoką zdolność do reagowania z szerokim spektrum substratów, odporność termiczną oraz tolerancję na wiele rozpuszczalników organicznych [32-34]. Z tego powodu, znajduje szerokie zastosowanie w katalizowaniu reakcji o znaczeniu farmaceutycznym.

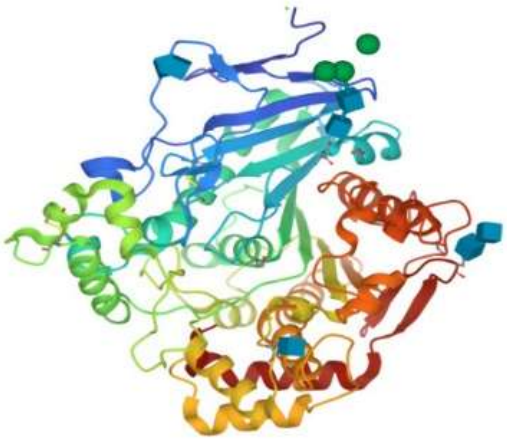


Rycina 3. *Struktura krystaliczna lipazy z Burkholderia cepacia (BCL)[34]. Skróty S87, D264 oraz H286 oznaczają triadę katalityczną wchodzącą w skład miejsca aktywnego enzymu. Miejsca α-4 oraz α-5 opisują lokalizację tzw. wieczka (ang. lid).*

3. Lipaza z *Aspergillus niger* jako przedstawiciel lipaz z *Aspergillus sp.*

Rodzina grzybów *Aspergillaceae* z rzędu kropidlakowców stanowi cenny materiał wyjściowy do pozyskiwania lipaz. Kropidlak czarny - *Aspergillus niger* – (AN) jest gatunkiem najczęściej wykorzystywanym w otrzymywaniu enzymu. Zwany potocznie, „czarną pleśnią", jest źródłem licznych toksyn takich jak aflatoksyny, które ze względu na swoją hepatotoksyczność i mutagenność, są traktowane jako substancje rakotwórcze. AN może być też przyczyną infekcji grzybiczej ucha (tzw. ucho pływaka). Wdychanie zarodników tego grzyba może być przyczyną tzw. aspergilozy – groźnego w skutkach grzybiczego zapalenia płuc. Z biotechnologicznego punktu widzenia, dzięki dużej zdolności do wydzielania białka oraz łatwość w izolowaniu biomasy, jest opisywany jako odpowiedni gatunek do produkcji lipaz. Ponadto, AN wykazuje optymalny wzrost na niskokosztowych podłożach mikrobiologicznych.

Lipaza z *Aspergillus niger* (ANL), ze względu na wyżej wymienione możliwości produkcyjne, należy do jednej z najczęściej stosowanych enzymów w biokatalizie (**Rycina 4)** [35, 36]. ANL wykazuje selektywność wobec kwasów karboksylowych o średniej długości łańcucha [37].



Rycina 4. *Struktura krystaliczna lipaz z Aspergillus niger (ANL) – wersja EstA [36]. Triada katalityczna składa się z sekwencji aminokwasów: Seryna*^{173}*, Kwas asparaginowy*^{228}*, Histydyna*^{285}

Najczęściej stosowanymi metodami otrzymywania ANL jest fermentacja oraz metody rekombinacji genetycznej. ANL znajduje szerokie zastosowanie w przemyśle, zwłaszcza farmaceutycznym, biotechnologicznym i spożywczym, ale także chemicznym, kosmetycznym i rolniczym. W badaniach farmaceutycznych, ANL jest wykorzystywana głównie w rozdziale kinetycznym mieszanin racemicznych substancji chemicznych lub prekursorów w celu otrzymywania chiralnie czystych enancjomerów. Niemniej jednak, ANL jest często używana również w hydrolizie triglicerydów kwasów tłuszczowych.

4. Aktywność enancjoselektywna lipaz w otrzymywaniu optycznie czystych enancjomerów leków

Leki chiralne należą do związków leczniczych stosowanych powszechnie w farmakoterapii. Otrzymuje się je w m.in. kinetycznym rozdziale mieszaniny racemicznej substancji leczniczych. Reakcja ta oparta jest na różnej szybkości reakcji enancjomerów z katalizatorem. Powstałe enancjomery najczęściej różnią się między sobą działaniem farmakodynamicznym, co zaobserwowano w przypadku enancjomerów leków z grupy niesteroidowych leków przeciwzapalnych (NLPZ) [38-41]. Enancjomer *S* ibuprofenu wykazuje *in vitro* 160 razy silniejsze działanie przeciwzapalne niż (*R*)-ibuprofen [39]. W przypadku flurbiprofenu, enancjomer *R*, w odróżnieniu od enancjomeru *S* wykazuje działanie antynocyceptywne [41].

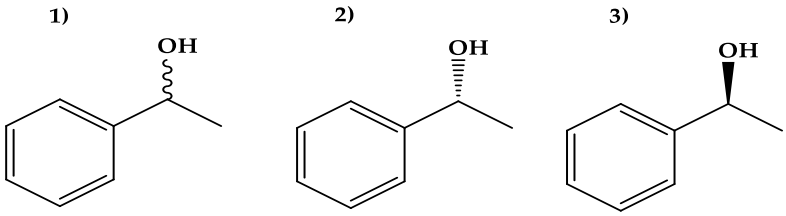
W reakcjach otrzymywania terapeutycznie czynnych enancjomerów leków często stosuje się biokatalizatory. Lipazy wykazujące aktywność enancjoselektywną, która oznacza szybsze reagowanie z jednym z enancjomerów – *R* lub *S*, należą do najczęściej stosowanych enzymów w katalizowaniu kinetycznego rozdziału związków chiralnych [38]. Lipaza z *Candida rugosa* (CRL) w kinetycznym rozdziale (*R*,*S*)-ibuprofenu, wykazuje preferencję w kierunku otrzymania (*S*)-ibuprofenu [38, 39]. Z drugiej strony, CALB katalizowała kinetyczny rozdział (*R*,*S*)-flurbiprofenu, w wyniku którego otrzymano (*R*)-enancjomer [12, 41-43]. W przypadku niektórych związków chiralnych, lipazy mogą wykazywać różną aktywność enancjoselektywną, np. w kinetycznym rozdziale (*R*,*S*)-1-fenyloetanolu – lipazy z *Burkholderia cepacia* (BCL), CALB oraz z *Aspergillus niger* (ANL) katalizują reakcję otrzymywania (*R*)-1-fenyloetanolu [44, 45], natomiast CRL wykazuje selektywność w kierunku (*S*)-1-fenyloetanolu [44]. Przykłady aktywności enancjoselektywnej w otrzymywaniu enancjomerów mieszanin racemicznych substancji leczniczych bądź ich prekursorów [46] przedstawiono w **Tabeli 1**.

Tabela 1. *Przykłady aktywności enancjoselektywnej lipaz w otrzymywaniu związków chemicznych o znaczeniu farmaceutycznym (lek, prekursory). ATL – Lipaza z Aspergillus terrasus, Novozyme-435 – CALB immobilizowana na makroporowatej żywicy akrylowej, TLL – lipaza z Thermomyces lanuginosus, APS-BCL-D – lipaza Amano z Burkholderia cepacia, odmiana D*

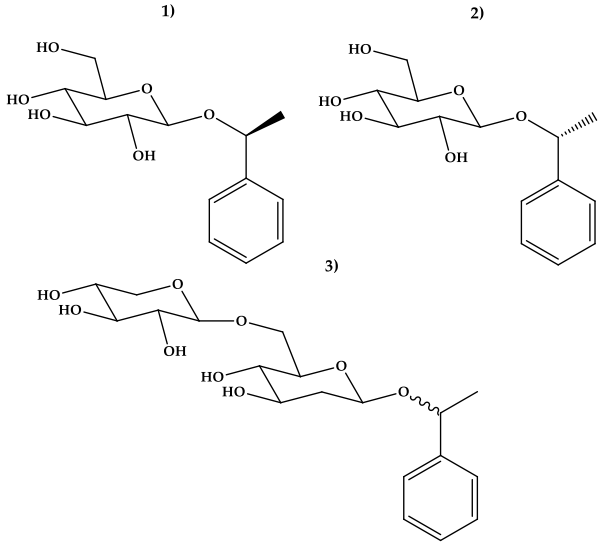
Lipaza	**Mieszanina racemiczna**	**Preferowany produkt**
CRL	Naproksen (ester metylowy)	(*S*)-naproksen
ATL	Ketopforen (ester winylowy)	(*R*)-ketoprofen
Novozyme 435	Ketorolak	Ester (*R*)-ketorolaku
CALB	Naftofuranodion	Ester (octan) (*R*)-naftofuranodionu
Novozyme 435	1-(2-furylo)-etanol	(*S*)-1-(2-furylo)-etanol
BCL	Kwas migdałowy	Ester metylowy kwasu (*R*) migdałowego
Novozyme 435	1-(1-naftylo)-etanol	(*S*)-1-(1-naftylo)-etanol
TLL	Indanol	(*S*)-indanol
APS-BCL-D	Hydroksylaktam	5(*S*)-alkohol

5. Kinetyczny rozdział (*R,S*)-1-fenyloetanolu

(*R,S*)-1-fenyloetanol (RS-PHE) jest związkiem chiralnym, posiadającym dwa enancjomery - (*R*)-1-fenyloetanol (R-PHE) i (*S*)-1-fenyloetanol (S-PHE) [47]. Według danych literaturowych, R-PHE znajdował zastosowanie jako prekursor w syntezie leków oraz był wykorzystany w produkcji soczewek kontaktowych oraz w jelitowej adsorpcji cholesterolu [48]. Ponadto, R-PHE był również testowany w przemyśle kosmetycznym jako łagodzący środek zapachowy. Warto wspomnieć, że poszczególne enancjomery – R-PHE oraz (*S*)-1-fenyloetanol (S-PHE) różnią się między sobą zapachami – R-PHE posiada aromat kwiatowy (podobny do wiciokrzewu), natomiast S-PHE cechuje się zapachem hiacyntu z domieszką truskawek [49, 50]. (*R,S*)-1-fenyloetanol w postaci octanu charakteryzuje się szerokim spektrum zapachu, od tropikalnego i mango, poprzez drewniany, do stęchłego. Oba enancjomery RS-PHE występują jako związki lotne w roślinach w postaci glikozydów (glukopiranozydy, primowerozydy), np. w kwiatach i liściach herbaty (*Camelia sinensis*). RS-PHE, R-PHE, S-PHE oraz ich glikozydy przedstawiono na **Rycinie 5** oraz **Rycinie 6.**

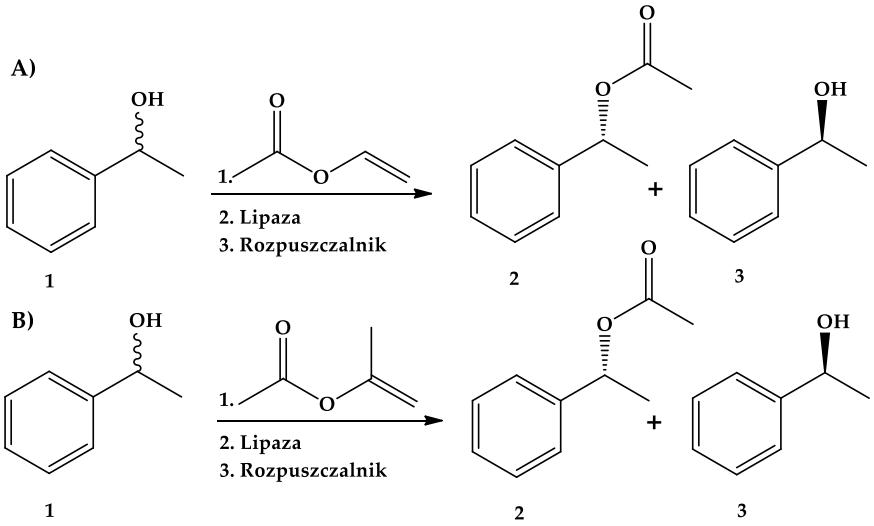


Rycina 5. *Struktury racemicznego RS-PHE (1) oraz enancjomerów: R-PHE (2) i S-PHE (3)*



Rycina 6. *Struktury glikozylowanego RS-PHE występujące w herbacie (Camellia sinensis): (R)-1-fenyloetylo-β-D-glukopiranozyd (1), (S)-1-fenyloetylo-β-D-glukopiranozyd (2), (R/S)-1-fenyloetylo-β-primowerozyd (3)*

Kinetyczny rozdział RS-PHE prowadzony jest najczęściej w obecności rozpuszczalnika o charakterze apolarnym. Reakcja ma charakter transestryfikacji poprzez przyłączenie grupy acylowej. Enancjomer R-PHE jest otrzymywany w postaci estru (**Rycina 7**). RS-PHE pełni rolę akceptora grupy acylowej. Jako donora grupy acylowej używa się często estru, zawierającego w swojej strukturze wiązanie podwójne. Octan winylu należy do powszechnie stosowanych donorów [44, 45, 51, 52], natomiast w ostatnich latach wykorzystuje się także inne związki, takie jak: octan izopropenylu, propionian winylu, maślan winylu.



Rycina 7. *Kinetyczny rozdział RS-PHE z zastosowaniem różnych donorów grupy acylowej: octan winylu (1), octan izopropenylu (2). 1 – RS-PHE, 2 – octan (R)-1-fenyloetylu (R-PHE-ACE), 3 – S-PHE.*

Ocena aktywności enancjoselektywnej w kinetycznym rozdziale opiera się na wyznaczeniu parametrów, które określają czystość enancjomeryczną reakcji. Czystość ta jest wyrażona jako nadmiar enancjomeryczny substratów (ang. *enantiomeric excess of substrates*, ee_s) oraz nadmiar enancjomeryczny produktów (ang. *enantiomeric excess of products*, ee_p). Obie wartości zwykle można wyrazić w procentach. Obliczane są za pomocą wzorów [41]:

$$[\%]ee_s = \frac{|R_s - S_s|}{|R_s + S_s|} \times 100$$

$$[\%]ee_p = \frac{|R_p - S_p|}{|R_p + S_p|} \times 100$$

gdzie R_s i R_p oznacza odpowiednio pola powierzchni pod pikiem *R*-enancjomeru substratu i *R*-enancjomeru produktu, a S_s i S_p odpowiednio pola powierzchni pod pikiem *S*-enancjomeru substratu i *S*-enancjomeru produktu

Kolejnymi parametrami, niezbędnym do określenia aktywności enancjoselektywnej, są konwersja (ang. *conversion, C*) oraz enancjoselektywność (ang. *enantiomeric ratio, enantioselectivity, E*). Konwersja oznacza stosunek procentowy nadmiaru enancjomerycznego substratu do sumy nadmiarów enancjomerycznych substratu i produktu, opisany poniższym wzorem:

$$[\%]C = \frac{ee_s}{ee_s + ee_p} \times 100$$

Natomiast enancjoselektywność, jako parametr wskazujący, która reakcja jest enancjoselektywna, przedstawiana jest wzorem:

$$E = \frac{\ln[(1 - C)(1 - ee_s)]}{\ln[(1 - C)(1 + ee_s)]}$$

Minimalna wartość *E* reakcji enancjoselektywnej wynosi 20 [12].

6. Aktywność lipolityczna lipaz w hydrolizie olejów zawierających nienasycone kwasy tłuszczowe

Nienasycone kwasy tłuszczowe (ang. *unsaturated fatty acids,* UFAs) są głównymi składnikami tłuszczów trawionych przez lipazy [53]. Kwasy te zawierają jedno lub więcej wiązań podwójnych: kwasy omega 3 (ω3, wiązanie podwójne przy trzecim od końca atomie węgla), omega 6 (ω6, wiązanie podwójne przy szóstym od końca atomie węgla) oraz omega 9 (ω9, wiązanie podwójne przy dziewiątym od końca atomie węgla). Kwasy ω3 i ω6 należą do wielonienasyconych kwasów tłuszczowych (ang. *polyunsaturated fatty acids*, PUFAs), natomiast ω9 do jednonienasyconych kwasów tłuszczowych (ang. *monounsaturated fatty acids*, MUFAs). Wszystkie grupy kwasów ω stanowią niezbędny składnik diety ludzkiej. Głównymi przedstawicielami kwasów ω3 są: kwas α-linolenowy (ALA), eikozapentaenowy (EPA) oraz dokozaheksaenowy (DHA); ω6 - kwas α-linolowy (LA); natomiast ω9 - kwas oleinowy (OA). Kwasy ω3 i ω6 należy dostarczyć z pożywieniem, ponieważ, ze względu na brak desaturaz wprowadzających wiązanie podwójne do cząsteczki kwasu w pozycje 3 i 6 grupy metylowej, organizm nie może produkować ich endogennie [54], dlatego należą do tzw. niezbędnych nienasyconych kwasów tłuszczowych (EFAs, ang. *essential fatty acids*) [55]. Z kolei, kwasy ω9, w przeciwieństwie do ω3 i ω6, z uwagi na możliwość syntezowania ich w ludzkim organizmie z kwasów nasyconych (SFAs, ang. *saturated fatty acids*), nie należą do EFAs.

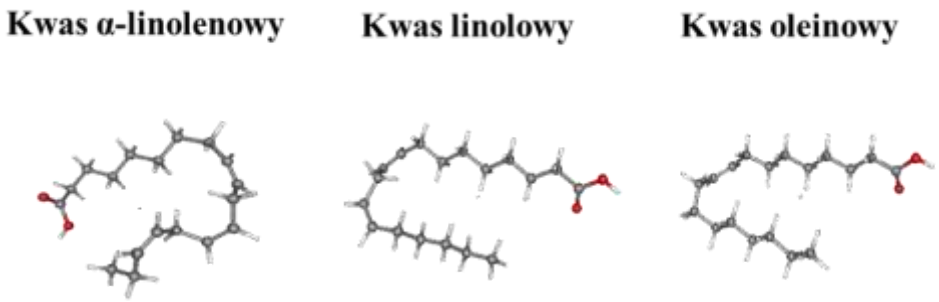
Kwasy ω3 należą do najważniejszych nienasyconych kwasów tłuszczowych [56, 57]. Metabolity kwasów ω3 i ω6 są substratami, które kompetycyjnie konkurują ze sobą o miejsce aktywne enzymu. Z tego powodu, wykazują wobec siebie działanie antagonistyczne. Kwas eikozapentaenowy (EPA), będący metabolitem kwasu ALA wytwarza eikozanoidy trienowe, podczas gdy kwas arachidonowy (ARA), jako metabolit kwasu α-linolowego, produkuje eikozanoidy dienowe [58]. Eikozanoidy trienowe, przede wszystkim prostaglandyna E_3 (PGE_3) i prostacyklina I_3 (PGI_3) wykazują działalnie przeciwzapalne, antyagregacyjne oraz wazodylatacyjne, w przeciwieństwie do prozapalnych i proagregacyjnych eikozanoidów dienowych, takich jak prostaglandyna E_2 (PGE_2) czy prostacyklina I_2 (PGI_2) [59]. Ponadto, eikozanoidy trienowe cechują się dużo niższą aktywnością biologiczną niż eikozanoidy dienowe. EPA, ze względu na swoje właściwości, stabilizuje naczynia krwionośne oraz zapobiega agregacji pływek krwi. Kwas dokozaheksaenowy (DHA) jest źródłem związków dokozatrienowych o silnym działaniu neuroprotekcyjnym np. neuroprotektyna D1. Z tego powodu, DHA odgrywa kluczową rolę w życiu płodowym, zwłaszcza podczas III trymestru ciąży. Zaleca się stosowanie DHA również w wieku dziecięcym, co może wpłynąć na lepsze zdolności poznawcze oraz optymalny proces wzrostu [56,

60]. Z uwagi na syntezę dokozatrienów w siatkówce oka, DHA pełni również rolę wspomagającą w procesie widzenia [61]. Kwasy ω6, za względu na prozapalną aktywność eikozanoidów dienowych, muszą być podawane w najniższych możliwych stężeniach. Z kolei, OA wchodzi w skład błon komórkowych, wpływając na zmniejszenie ich przepuszczalności. Wprowadzenie OA zamiast kwasów nasyconych do diety może zmniejszyć ryzyko miażdżycy i choroby wieńcowej. Badania kliniczne wykazały również poprawę profilu lipidowego u pacjentów z hipercholesterolemią. Dieta bogata w kwasy ω3 oraz ω6 powinna być stosowana z zachowaniem odpowiednich proporcji [62]. Stosunek ilościowy ω6 do ω3 nie powinien być większy niż 4-5:1. Rekomendacje dotyczące stosowania kwasów ω3 i ω6, przedstawiono w **Tabeli 2** [63]. Nadmiar poszczególnych kwasów, zwłaszcza ω6 i ω9, może zredukować korzystny efekt działania kwasów ω3 i prowadzić do otyłości, stłuszczenia wątroby oraz hipercholesterolemii [62]. W ostatnich dekadach, zwiększa się spożycie kwasów ω, zwłaszcza ω3 (wzrost średnio o 30% rocznie). Obecnie, na rynku farmaceutycznym zarejestrowana jest ogromna ilość preparatów zawierających nienasycone kwasy tłuszczowe. W dużej mierze są to suplementy diety zawierające kwasy ω3 i ω6. W ostatnich latach pojawiło się jednak kilka preparatów zarejestrowanych jako leki dostępne bez recepty (OTC) oraz środki specjalistyczne przeznaczenia żywieniowego.

Tabela 2. *Normy żywieniowe dotyczące spożywania kwasów ω3 i ω6 w różnych kategoriach wiekowych [63].*

Grupa wiekowa	**Ω3**	**Ω6**
Niemowlęta i małe dzieci		
7-11 miesięcy	ALA maksymalnie 0.5% diety DHA 100 mg/dobę	LA maksymalnie 4% diety
12-24 miesiące		
Dzieci i młodzież		
2-8 lat	ALA maksymalnie 0.5% diety DHA + EPA 250 mg/dobę	LA maksymalnie 4% diety
Osoby dorosłe		
Osoby dorosłe	ALA maksymalnie 0.5% diety DHA + EPA 250 mg/dobę	LA maksymalnie 4% diety
Kobiety w ciąży	ALA maksymalnie 0.5% diety DHA + EPA 250 mg/dobę + 100-200 mg DHA/dobę	
Kobiety karmiące piersią	ALA maksymalnie 0.5% diety DHA + EPA 250 mg/dobę + 100-200 mg DHA/dobę	

Lipazy, ze względu na ich aktywność lipolityczną, hydrolizują triglicerydy kwasów tłuszczowych do wolnych kwasów tłuszczowych, diacylogliceroli i monoacylogliceroli [9]. Szybkość reakcji enzymatycznych katalizowanych przez lipazy jest zależna m.in. od długości oraz struktury przestrzennej kwasów tłuszczowych ω, występujących w olejach w postaci triacylogliceroli. Większość lipaz wykazuje wysoką aktywność lipolityczną w stosunku do kwasów tłuszczowych o długości łańcucha węglowego C8-C18 substratów [64]. BCL jest aktywna wobec lipidów zawierających kwasy tłuszczowe niezależnie od ich długości [32], natomiast ANL cechuje się wysoką aktywnością w stosunku do kwasów o średniej długości łańcucha węglowego [37]. Budowa kwasów tłuszczowych ω tj. skręcalność łańcucha węglowego oraz ilość i położenie wiązań podwójnych (**Rycina 8**) mają znaczący wpływ na aktywność lipaz [65, 66]. Aknabi i wsp. [66] zauważyli, że lipaza A z *Candida antarctica* (CALA) wykazywała selektywność wobec kwasów tłuszczowych (nasyconych, jednonienasyconych i wielonienasyconych) wraz ze wzrostem liczby wiązań podwójnych w ich strukturach. Natomiast Ma i wsp. [67] zaobserwowali że aktywność lipazy była wyższa w hydrolizie kwasów ω6 niż ω3. Co więcej, zauważyli, że lipazy mogą wykazywać niższą aktywność wobec długołańcuchowych kwasów tłuszczowych ze względu na utrudnione wprowadzenie łańcucha acylowego do „tunelowej kieszeni" lipazy. Stabilizacja konformacji przestrzennej kwasu przez wiązania podwójne może ułatwić dostęp do miejsca aktywnego enzymu, zwiększając jego aktywność. Z drugiej strony, może to powodować zawadę steryczną, co wpływa negatywnie na katalizowanie reakcji. Casas-Godoy i wsp. [68] zauważyli, że zawada steryczna może być spowodowana obecnością podwójnego wiązania w pozycji 6 od grupy karboksylowej. Ponadto, aktywność lipazy jest uzależniona również od ilości kwasów tłuszczowych ω zawartych w danym oleju oraz stosunków ilościowych pomiędzy nimi. Te cechy mogą być przydatne przy ich wzbogacaniu, celem uzyskania wyższego stężenia kwasów w badanych olejach.

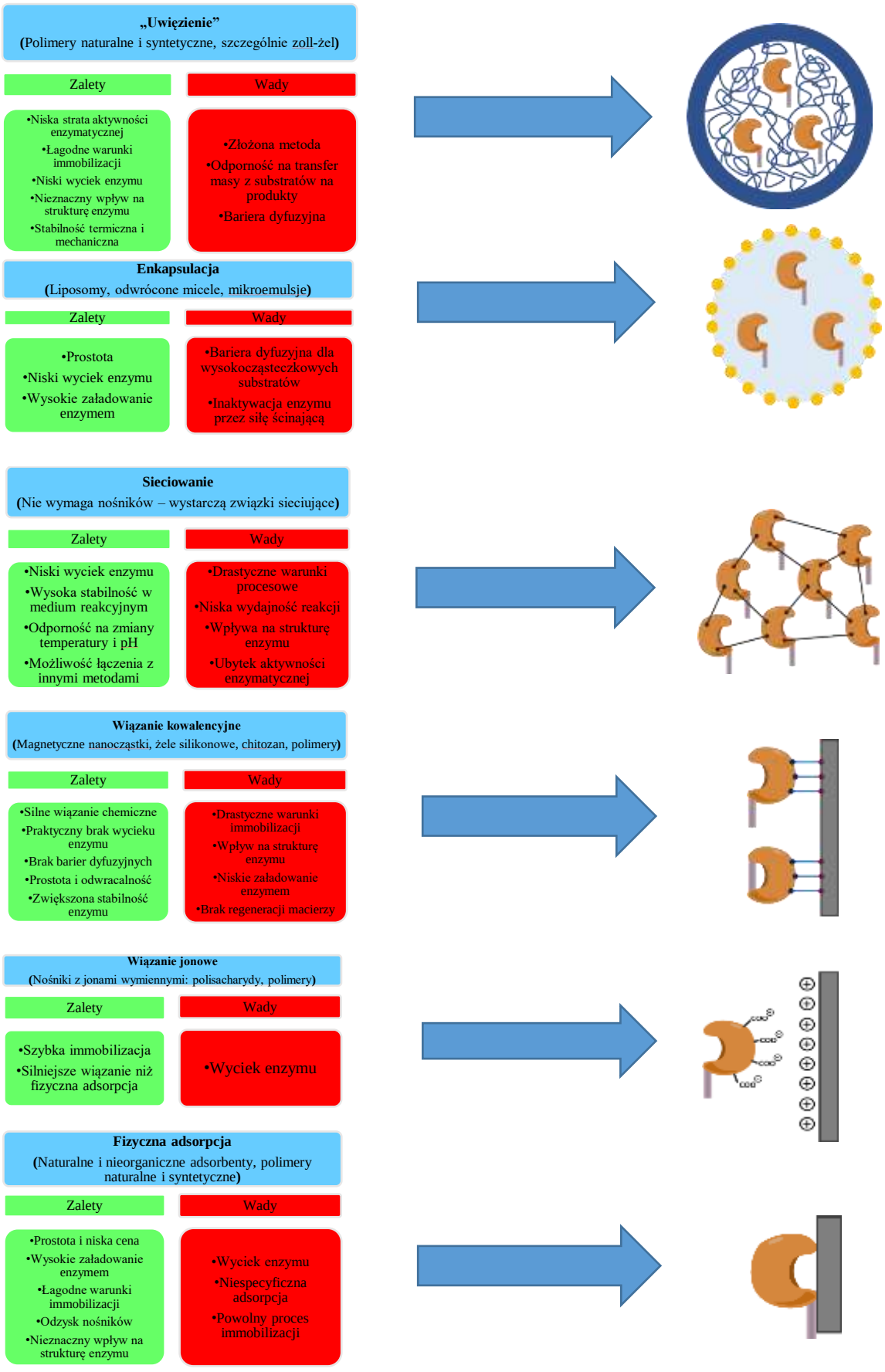


Rycina 8. *Struktury przestrzenne kwasów ω: ω3 – kwas α-linolenowy, ω6 – kwas linolowy, ω9 – kwas oleinowy [P1]*

7. Immobilizacja lipaz jako podstawowy proces w badaniach biokatalitycznych

Immobilizacja lipaz na nośnikach jest popularną techniką modyfikacji aktywności katalitycznej [25, 69, 70]. Proces ten wymaga optymalizacji. Unieruchomiona lipaza powinna wykazywać wysoką stabilność, w celu ograniczenia utraty aktywności podczas reakcji katalitycznej [71, 72]. Z drugiej strony, unieruchomienie i/lub usztywnienie lipazy utrudnia rotację struktury, co może mieć wpływ na obniżoną dostępność dla substratów [73]. Niektóre z metod wymagają bardziej drastycznych warunków procesowych, co niesie ze sobą ryzyko uszkodzenia struktury białkowej enzymu. Celem immobilizacji jest również wielokrotne wykorzystanie nośnika – stabilność immobilizowanej lipazy umożliwia prowadzenie reakcji w kilku cyklach [74]. W technikach immobilizacji ważna jest również prostota metody, niska ceny i przyjazność dla środowiska naturalnego [72]. Na **Rycinie 9** przedstawiono techniki immobilizacji, nośniki oraz wady i zalety niniejszych procesów. W immobilizacji, poza optymalizacją metody, niezwykle istotna jest dobór odpowiedniego materiału nośnikowego [25, 75, 76], uwzględniając ich parametry fizyczne i chemiczne. Poniżej przedstawiono cechy optymalnego nośnika [25, 77]:

1. Duża powierzchnia wewnętrzna pozwalająca na odpowiednie dopasowanie geometryczne do powierzchni enzymu
2. Grupa funkcyjna reagująca z enzymem powinna wykazać jak najmniejszą zawadę steryczną w celu odpowiedniego usztywnienia struktury oraz dostępności do centrum aktywnego enzymu
3. Grupa funkcyjna reagująca z enzymem powinna reagować z grupami obecnymi na powierzchni enzymu
4. Grupa funkcyjna reagująca z enzymem powinna utworzyć stabilny kompleks w celu dłuższego oddziaływania enzym-nośnik
5. Możliwość zablokowania lub zniszczenia grup reaktywnych na nośniku, które mogłyby utrudniać wiązanie z enzymem bez wpływu na jego strukturę



Rycina 9. *Podział technik immobilizacji ze względu na oddziaływania, rodzaj użytego nośnika oraz ich zalety i wady*

8. Nośniki polimerowe Immobead jako potencjalnie optymalny materiał do immobilizacji lipaz

W procesie immobilizacji znajdują zastosowanie nośniki polimerowe [78-80]. Ze względu na ich możliwości aplikacyjne w procesach technologicznych, firmy chemiczne i biotechnologiczne opracowały wiele zmodyfikowanych nośników polimerowych. Immobead (producent: ChiralVision, Niderlandy) są materiałami charakteryzującymi się różną matrycą np. poliakrylowa, polistyrenowa, polipropylenowa, silikonowa, styrenowa, metakrylanowa; charakterystyczną grupą (lub grupami) funkcyjną np. epoksydowa (polarna lub apolarna), kwas karboksylowy, ester kwasu karboksylowego, alkilowa, aromatyczna, hydroksylowa; rozmiarem cząstek, głównie w zakresie 150-1500 μm; zawartością wody (lub wilgotności). Różnorodna budowa nośników polimerowych Immobead umożliwia ich zastosowanie w różnych technikach immobilizacji np. fizyczna adsorpcja, wiązanie kowalencyjne, wiązanie jonowe (kationowe lub anionowe).

Parametry fizykochemiczne nośnika stanowią podstawę jego zastosowania w badaniach biokatalitycznych. Matte i wsp. [81] zaobserwowali, że różnorodność w budowie matrycy, w szczególności porowatość (wgłębienia, kanały i szczeliny) jest czynnikiem kluczowym w zastosowaniu do immobilizacji enzymów. Wynika to z faktu, że cząsteczki enzymu większe lub równe rozmiarom porów nośnika polimerowego cechują się niskim obciążeniem (ang. *loading*). Z tego powodu, ich rola jest ograniczona do adsorpcji na powierzchni nośnika, przez co oddziaływanie jest słabsze. Przyjmuje się, że średnica porów nośnika powinna być około 5-krotnie większa od średnicy białka enzymatycznego. Jednakże, wydajność immobilizacji jest niezależna od średnicy porów większych niż 100 nm. Spośród nośników Immobead, często stosowane są materiały o matrycy poliakrylowej, takie jak nośnik Immobead 150A (IB-150A). Jest to sieciowany kopolimer metakrylanowy zawierający grupy oksiranowe (informacje uzyskane z danych należących do ChiralVision) oraz epoksydowe grupy funkcyjne. Rozmiar cząstek wynosi 150-300 μm, z zawartością wilgotności poniżej 5%. W odróżnieniu do polarnego nośnika poliakrylowego Immobead 150P (IB-150P), IB-150A ma charakter apolarny. Liczne publikacje potwierdzają pozytywny wpływ nośników poliakrylowych IB-150 na aktywność immobilizowanych lipaz np. CALB, BCL czy lipazy z *Candida methylica* (CML) [12, 81].

9. Parametry opisujące aktywność lipolityczną lipaz

Aktywność lipolityczną lipaz w formie wolnej i immobilizowanej określa się poprzez wyznaczenie parametrów katalitycznych [74, 82]. Podstawową wielkością opisującą aktywność lipazy jest jednostka aktywności enzymatycznej U, która jest definiowana, jako ilość hydrolizowanego substratu (wyrażona w μmol) w ciągu 1 minuty reakcji. Stosunek procentowy aktywności lipolitycznej lipazy w formie immobilizowanej do lipazy w formie wolnej jest opisany, jako efektywność immobilizacji I_e (ang. *immobilization efficiency*) lub aktywność zachowana A_{ret} (ang. *activity retention*), wyznaczone za pomocą poniższego wzoru:

$$A_{ret} = I_e = \frac{U_I}{U_B}$$

gdzie U_I oznacza aktywność lipazy immobilizowanej na nośniku, a U_B aktywność lipazy w formie wolnej w ilości odpowiadającej ilości enzymu immobilizowanego na nośniku

Ważnymi parametrami w ocenie ilości białka enzymatycznego pozostałego na nośniku po przeprowadzeniu procesu immobilizacji są: obciążenie lipazą L_L (ang. *lipase loading*) oraz wydajność immobilizacji I_y (ang. *immobilization yield*) [29, 83]. L_L oznacza ilość immobilizowanej lipazy na gram nośnika, natomiast I_y wyznacza się ze wzoru [74, 84]:

$$I_y = \frac{LA_B}{LA_{10}}$$

gdzie LA_B oznacza ilość lipazy będąca różnicą pomiędzy ilością lipazy w supernatancie po przeprowadzeniu immobilizacji, do ilości wyjściowej lipazy, natomiast LA_{10} oznacza ilość wyjściową lipazy. Procentowy stosunek aktywności lipolitycznej lipazy immobilizowanej do aktywności formy wolnej w ilości wyjściowej określa się jako odzysk aktywności enzymu A_{rec} (ang. *activity recovery*), wyznaczony za pomocą wzoru:

$$A_{rec} = \frac{U_I}{U_B}$$

Wpływ różnych warunków środowiskowych np. temperatura, pH, siła jonowa, itp. są kolejnymi elementami badania aktywności lipaz. W tym celu wyznacza się tzw. aktywność relatywną A_{rel} (ang. *relative activity*), porównującą aktywność lipazy w badanych warunkach do aktywności maksymalnej (optymalne warunki) [26, 27].

10. Cel pracy

Celem niniejszej rozprawy doktorskiej była ocena aktywności enancjoselektywnej oraz lipolitycznej lipaz z *Burkholderia sp.* i *Aspergillus sp.* w formie wolnej oraz immobilizowanej na nośnikach polimerowych. Cel ten realizowano poprzez:

1. Przeprowadzenie badań aktywności lipolitycznej lipaz z *Burkholderia sp.* i *Aspergillus sp.* w formie wolnej

2. Badanie aktywności enancjoselektywnej lipaz z *Burkholderia sp.* i *Aspergillus sp.* w formie wolnej

3. Unieruchomienie lipaz Amano PS z *Burkholderia cepacia* (APS-BCL) i Amano A z *Aspergillus niger* (AA-ANL) na nośniku polimerowym IB-150A

4. Badanie aktywności enancjoselektywnej lipazy APS-BCL w formie immobilizowanej

a) przeprowadzenie kinetycznego rozdziału (*R,S*)-1-fenyloetanolu na drodze transestryfikacji katalizowanej immobilizowaną APS-BCL

b) badanie wpływu rozpuszczalników i donorów grupy acylowej na aktywność enancjoselektywną immobilizowanej APS-BCL

5. Badanie aktywności lipolitycznej lipazy APS-BCL w formie immobilizowanej

6. Badanie aktywności enancjoselektywnej lipazy AA-ANL w formie immobilizowanej

7. Badanie aktywności lipolitycznej lipazy AA-ANL w formie immobilizowanej

a) określenie wpływu substratów, nośników, temperatury, pH, cykli reakcyjnych na aktywność lipolityczną immobilizowanej AA-ANL

b) sprawdzenie wpływu przechowywania immobilizowanej lipazy AA-ANL na jej stabilność

Celem analitycznym pracy była optymalizacja rozdziału chromatograficznego (*R,S*)-1-fenyloetanolu i jego estrów z zastosowaniem wysokosprawnej chromatografii cieczowej i chiralnych faz stacjonarnych.

Do cyklu publikacji eksperymentalnych dodano pracę poglądową dotyczącą zastosowania lipazy z *Burkholderia cepacia w* reakcjach o znaczeniu farmaceutycznym.

11. Wyniki i dyskusja wyników

11. 1. Ocena aktywności enancjoselektywnej i lipolitycznej lipazy Amano PS z *Burkholderia cepacia* w formie wolnej – P1

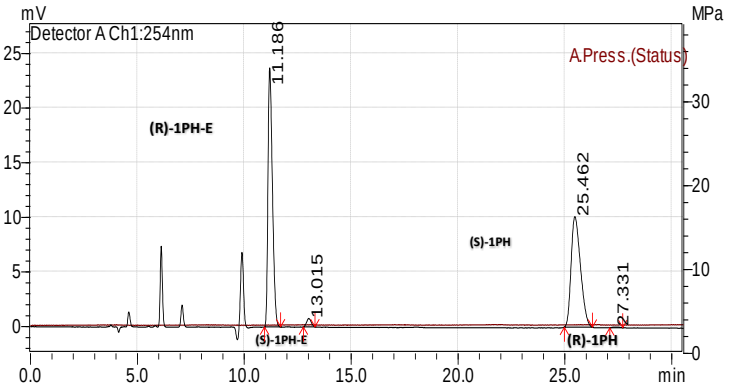
P1. **Jacek Dulęba,** Tomasz Siódmiak, Michał Piotr Marszałł, Amano Lipase PS from *Burkholderia cepacia* - Evaluation of the Effect of Substrates and Reaction Media on the Catalytic Activity, *Curr. Org Chem.*, 2020, 24 (7), 798-807.

Amano lipaza PS z *Burkholderia cepacia* (APS-BCL) należy do najczęściej stosowanych lipaz w biokatalizie. Jest wykorzystywana w reakcjach enancjoselektywnych np. w kinetycznym rozdziale związków chiralnych, między innymi leków lub ich prekursorów. APS-BCL bierze udział również w hydrolizie triglicerydów (TGs), do których należą m.in. wielonienasycone kwasy tłuszczowe (PUFAs), suplementowane w celu zmniejszenia ryzyka chorób sercowo-naczyniowych oraz poprawy pracy ośrodkowego układu nerwowego.

Badano aktywność enancjoselektywną APS-BCL w formie wolnej, przeprowadzając kinetyczny rozdział (*R*,*S*)-1-fenyloetanolu na drodze transestryfikacji. Otrzymano optycznie czysty octan (*R*)-1-fenyloetylu. Uzyskane parametry katalityczne (ee_p =94%, ee_s =99%, *C* =51%, *E* =170) wskazują na wysoką aktywność enancjoselektywną lipazy APS-BCL w formie wolnej **(Rycina 10)**.

Przeprowadzono badanie wpływu warunków reakcji, dobierając odpowiedni donor grupy acylowej oraz rozpuszczalnik. Wykazano, że octan izopropenylu może stanowić alternatywę dla octanu winylu jako donor grupy acylowej. Zaobserwowano pozytywny wpływ środowiska reakcji na aktywność enancjoselektywną APS-BCL. Nie wykazano natomiast liniowego wpływu wartości logP rozpuszczalników na aktywność APS-BCL.

Zbadano aktywność lipolityczną, prowadząc reakcję hydrolizy 13 olejów roślinnych o różnych zawartości kwasów ω3, ω6, ω9. Na podstawie otrzymanych wyników obliczono aktywność enzymatyczną U [μM/min] oraz aktywność relatywną [%]. Uzyskano wysoką aktywność lipolityczną lipazy APS-BCL. Ponadto, zauważono tendencję dotyczącą wpływu zawartości kwasów ω6/ω9 w substratach na aktywność enzymatyczną lipazy. Wzrost aktywności nastąpił w olejach, w których wartość ω6/ω9 była wyższa niż 2.3. Zatem, ustalono granicę odcięcia, powyżej której aktywność lipolityczna wyraźnie wzrastała. Zjawisko to mogło być spowodowane tzw. splątaniem łańcuchów węglowych oraz obecnością wiązań podwójnych w strukturach kwasów tłuszczowych ω [65, 67]. Otrzymane wyniki stanowiły podstawę do kolejnych badań, uwzględniających aktywność APS-BCL w formie immobilizowanej.



Rycina 10. *Chromatogram HPLC kinetycznego rozdziału (R,S)-1-fenyloetanolu katalizowanego przez APS-BCL w formie wolnej.*

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RESEARCH ARTICLE

Amano Lipase PS from *Burkholderia cepacia* - Evaluation of the Effect of Substrates and Reaction Media on the Catalytic Activity

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Abstract: Lipases in the native or immobilized form have commonly been used as catalysts in the chemical and pharmaceutical industry. One of the widely available enzyme catalysts on the market is lipase from *Burkholderia cepacia* (BCLs), previously called *Pseudomonas cepacia* (PCLs). This enzyme is applied, among others, in the stereoselective acylation of molecules to achieve chiral pure enantiomers of drugs or their building blocks. In this study, Amano lipase PS (APS-BCL), which is a commercial lipase from *Burkholderia cepacia* (BC) was tested. The lipolytic activity of APS-BCL by hydrolysis of vegetable oils and enantioselective activity of APS-BCL by the kinetic resolution of (*R,S*)-1-phenylethanol with using isopropenyl acetate as an acyl donor were evaluated. An effect of reaction media with different logP values (*t*-butyl methyl ether, dichloromethane, diisopropyl ether, toluene, cyclohexane, *n*-hexane, isooctane and *n*-heptane) on the enantioselective activity of lipase was also studied. The high value of the enantiomeric ratio (E =308.5) with the utilization of isopropenyl acetate was achieved. Whereas, the best reaction medium turned out to be diisopropyl ether, C =47.9%, ee_p =98%, ee_s =90%, after 24 h of incubation. Moreover, the influence of ω6/ω9 polyunsaturated fatty acids (PUFAs) ratio in commercial (peanut, camelina, rape, pumpkin seed, walnut, sesame, avocado, rice, corn, black cumin, hemp, safflower, grape seed) oils was investigated for the first time, the cut-off limit of ω6/ω9 ratio was proposed. The ratio equal to or higher than 2.3 allows achieving higher lipolytic activity.

Keywords: Lipase, Amano lipase PS from *Burkholderia cepacia*, (*R,S*)-1-phenylethanol, (*R*)-1-phenylethanol, isopropenyl acetate, ω-PUFAs, vegetable oil, the cut-off limit.

1. INTRODUCTION

Enzymatic processes are an important part of current trends in drug synthesis in the biotechnological and pharmaceutical industries. One of the ordinarily applied catalysts in enzymatic reactions are lipases (hydrolases of triacylglycerol, EC 3.1.1.3). They show an ability to act between the water and oil phase, and usually, undergo mechanism known as an "interfacial activation" [1-5]. Lipases are used in many reactions, *i.e.* ester synthesis, triglycerides hydrolysis, or transesterification, to obtain, among others, chiral pure drugs or compounds which are building blocks. Therefore, the meaning of lipases in pharmaceutical, biotechnological, chemical, cosmetic and food fields is still increasing [6-20].

Lipases from *Burkholderia cepacia* (BCLs), (earlier named *Pseudomonas cepacia* - PCLs) are one of the most useful enzymes applied in biocatalytic reactions. Sanchez *et al.* [20] described a catalytic triad of BCLs contained Ser87, His286 and Asp264, and dislocation of the α-5 helix as well as the change in the orientation of the α-9 helix during interfacial activation. Moreover, in literature, it is indicated that this enzyme is characterized by high hydrolytic activity and a lack of positional specificity [21-30].

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BCLs can be gained by various methods. Padilha *et al.* [6] used the PEG/phosphate aqueous two-phase system (ATPS) in achieving lipase from *Burkholderia cepacia* (BCL). The optimal pH conditions for the reaction were 6.0 and 8.0. Whereas, Lee *et al.* [18] tested a group of Good's buffer ionic liquids (GB-ILs), Good's buffer (GB) anions as well as salts K_3PO_4, K_2CO_3, and $(NH_4)_2SO_4$, and the polymers poly(ethylene glycol) (PEG), poly(propylene glycol) (PPG), and PEG-PPG copolymers at a purification step of lipase from *Burkholderia cepacia*.

One of the most popular BCLs is Amano lipase PS (APS-BCL), which is a commercial lipase from *Burkholderia cepacia* (BC). BCL is a widely applied enzyme, among others, in the stereoselective acylation of molecules. It should be noted that the catalytic reactions are often performed by the kinetic resolution of a racemic mixture of drugs or precursors, in order to obtain pure enantiomers. Some of them are used as building blocks, possessing in final form an antiviral, anti-inflammatory or antioxidant activity [6, 18-20, 22-33]. Above lipase is also applied in the synthesis or degradation of polymeric materials [20, 27]. Furthermore, using BCL for the synthesis of biodegradable diesel fuel has been studied. These reactions were based on the transesterification of conventional oils (soybean), unconventional oils (Babassu, Jatropha, Madhuca), or cooking oils, with alcohols [34-40].

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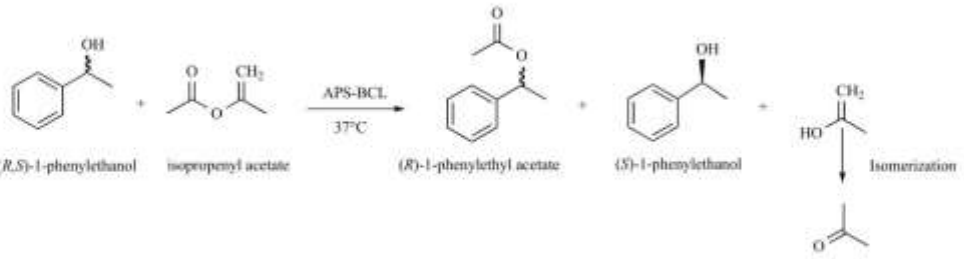


Scheme 1. The enantioselective transesterification of (*R,S*)-1-phenylethanol with isopropenyl acetate catalyzed by APS-BCL.

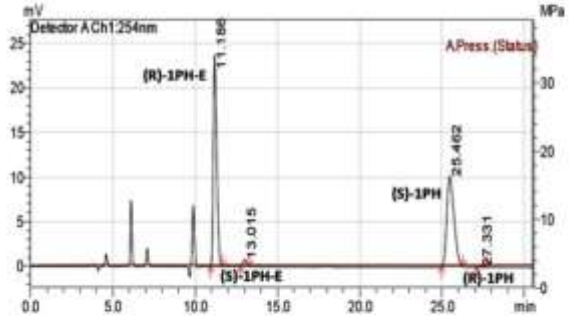


Fig. (1). HPLC chromatogram of (*R,S*)-1-phenylethanol and its esters: **(R)-1PH-E** – (*R*)-1-phenylethanol acetate; **(S)-1PH-E** – (*S*)-1-phenylethanol acetate; **(R)-1PH** – (*R*)-1-phenylethanol; **(S)-1PH** – (*S*)-1-phenylethanol; Chromatographic conditions: Lux Cellulose-3 (4.6 mm×250 mm×5 μm) column; mobile phase: *n*-heptane/2-propanol/ trifluoroacetic acid (98.7/1.3/0.15 v/v/v), flow rate=1 mL/min, t=15 °C, UV=254 nm. Reaction mixture: APS-BCL (8.0 mg), (*R,S*)-1-phenylethanol (9.98 μL), *n*-heptane (0.41 mL), isopropenyl acetate (28.25 μL). Incubation time: 48 h.

Lipolytic activity is undeniably one of the most important features of lipases. As a substrate, triglycerides (TG) of fatty acids contained in commercial animal and plant oils are mainly used. The vegetable oils are a source of unsaturated fatty acids like ω-3, ω-6, and ω-9 PUFAs (Polyunsaturated Fatty Acids). The favourable role of ω-acids in reducing the risk of heart diseases and normal working of the central nervous system is extensively described and confirmed in the recent studies [41-51]. During women's pregnancy, ω-3 PUFAs are responsible for developing brain structure and the sense of sight of a child. Hence, fish oil, which is the main source of PUFAs of animal origin, is widely applied in the diet of pregnant and breastfeeding women, in order to keep the optimal amount of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), belonging to ω-3 PUFAs. Creating PUFAs endogenously still remains the subject of studies [41]. Therefore, the only way to maintain a suitable concentration of PUFAs is exogenous delivery.

The evaluation of the lipolytic activity of lipases is often conducted using olive oil, one of the most popular vegetable oil, containing a high amount of ω-9 PUFAs (especially oleic acid) [42-45]. Whereas, determination of the enantioselective activity of lipase is usually based on the kinetic resolution of the racemic mixture. According to the literature [52-55], (*R,S*)-1-phenylethanol is widely applied model compound in order to obtain optically pure (*R*)-1-phenylethanol as a chiral building block in the synthesis of drugs. Furthermore, this alcohol is a useful reagent in cosmetics and biotechnological industries.

In the present work, the enantioselective activity of lipase APS-BCL by the kinetic resolution of (*R,S*)-1-phenylethanol in various media has been investigated. The optimal conditions for enzymatic transesterification using isopropenyl acetate as an acyl group donor were determined. Moreover, the effect of substrates (13 various commercial oils from vegetable sources with different amounts of ω-acids) on lipase lipolytic activity APS-BCL was tested. The ratio between ω-6/ω-9 PUFAs and their effect on the catalytic activity of lipase has been studied.

2. RESULTS AND DISCUSSION

2.1. Kinetic Resolution of (*R,S*)-1-Phenylethanol – Effect of Isopropenyl Acetate

The kinetic resolution of (*R,S*)-1-phenylethanol in *n*-heptane with isopropenyl acetate as an acyl donor, catalyzed by APS-BCL was performed (Scheme **1**). Moreover, the effect of the substrate on the reaction's enantioselectivity has been studied. Basing on the obtained results, 48 h was chosen as the optimal time of incubation (Fig. **1**). In the carried out reaction, the values of the enantiomeric excess of the substrate (ee_s) of 99%, the enantiomeric excess of the product (ee_p) of 94% and the enantioselectivity (*E*) of 170 were obtained. Analyzing the parameters of activity, it can be observed that lipase APS-BCL has a high ability to catalyze the kinetic resolution of (*R,S*)-1-phenylethanol in tested conditions. The results were compared with data in papers [53-55], depending on the used lipase, acyl donor and reaction time (Table **1**). It should be mentioned that according to our knowledge, kinetic resolution of (*R,S*)-1-phenylethanol catalyzed by APS-BCL with isopropenyl acetate as an acyl donor is not exhaustively described in the literature. Melais *et al.* [54] studied lipase-catalyzed transesterification of (*R,S*)-1-

Table 1. The results of transesterification of (*R,S*)-1-phenylethanol with isopropenyl acetate as an acyl donor depending on the used lipase, acyl donor and reaction time.

Lipase	Stereopreferention	Acyl donor	Time [h]	ee_p(%)	ee_s(%)	C(%)	*E*	References
APS-BCL[a]	*R*	Isopropenyl acetate	48	94	99	51	170	This study
CAL-B[b]	*R*	Isopropenyl acetate	24	99	99	50	>200	[54]
PCL[c]	*R*	Isopropenyl acetate	24	99	11.4	10.3	>200	[54]
CCL[d]	*S*	Isopropenyl acetate	24	50	8.9	15	3	[54]
APS-BCL[a]	*R*	Vinyl acetate	30	99	99	50	>200	[53]
PCL[c]	*R*	Vinyl acetate	24	98.5	44	31	148	[54]
RQ_3-BCL[e]	*R*	Vinyl acetate	20	99	99	50	>200	[55]

[a] – Amano lipase PS from *Burkholderia cepacia*
[b] – Lipase B from *Candida antarctica*
[c] – Lipase from *Pseudomonas cepacia*
[d] – Lipase from *Candida cylindracea*
[e] – Lipase RQ_3 from *Burkholderia cepacia*.

phenylethanol using various acyl donors and three different lipases – lipase B from *Candida antarctica* (CAL-B), lipase from *Pseudomonas cepacia* (PCL), and lipase from *Candida cylindracea* (CCL). The most beneficial results were achieved with the use of isopropenyl acetate and CAL-B - the enantioselectivity was above 200, at a conversion 50%. Whereas, the results received with the application of PCL were characterized by the lower values of the conversion. It should be emphasized that the values of catalytic parameters were compared with literature data at similar conversion values [53-55]. In the articles of citied authors, it seems that the extension time of incubation could have a positive effect on the conversion. In the case of acyl donor, the results of transesterification with vinyl acetate were slightly better in the reaction catalyzed by APS-BCL or RQ_3-BCL, than, when the APS-BCL with isopropenyl acetate were used (Table **1**). Regarding the time of reaction, transesterification performed using isopropenyl acetate required extended time of incubation when APS-BCL was applied. Comparing the reaction parameters for the same lipase APS-BCL (Table **1**), the transesterification time with the use of vinyl acetate was shorter, than when isopropenyl acetate was used. The vinyl acetate is the most commonly applicable donor in enzymatic reactions [53-64]. However, isopropenyl acetate can be considered as a suitable compound and alternative in delivery of the acyl group, due to high conversion and enantioselectivity.

2.2. Effect of Reaction Media with Various logP Values on the Enantioselective Activity of APS-BCL

The kinetic resolution of (*R,S*)-1-phenylethanol in 8 solvents, catalyzed by APS-BCL with different logP values has been studied. The chromatograms achieved in each medium have been shown (chromatogram with diisopropyl ether in Fig. (**2**) and the others in Supplementary material). The values of ee_p, ee_s, *C* and *E* have been calculated and presented in Table **2**. In the case of diisopropyl ether and *n*-hexane, the reaction in 24 h has been stopped, because of fully reacted one of the enantiomers of a substrate after 48 h. The values of conversion and enantioselectivity depending on the logP values of used solvent have been shown in Fig. (**3**), while the values of ee_p and ee_s depending on the logP value have been exhibited in Fig. (**4**). The obtained results showed a lack of relationship between increasing logP value of solvent and parameters describing the catalytic activity, expressed by *E*, *C*, ee_p, ee_s. Li *et al.* [53] have also studied the effect of solvent on the kinetic resolution of (*R,S*)-1-phenylethanol. The results presented in our paper confirmed the conclusions of the above-mentioned authors. The data demonstrate high values of conversion in all tested solvents (after 24 and 48 h), with the exception of dichloromethane (*C* =23.6%) (Table **2**). It is worthy to notice that this reaction medium (dichloromethane) was also applied in the kinetic resolution of racemic mixture other pharmaceuticals. Siódmiak *et al.* [65] determined the effect of a reaction medium in the esterification of (*R,S*)-flurbiprofen, catalyzed by Novozym 435. The conversion in dichloromethane after 96 h of incubation was 35.7%, the ee_p of 96.3%, the ee_s of 53.6%. In comparing with data achieved in this study (Table **2**), dichloromethane showed a similar profile of catalytic activity.

The highest values of enantiomeric excess of substrates were reached in *n*-heptane (ee_s =99%), cyclohexane (ee_s =92%), diisopropyl ether (ee_s =90%) and isooctane (ee_s =89%). Regarding the enantiomeric excess of products, in all solvents values ee_p were >94%, while the highest in *n*-hexane (ee_p=98%), diisopropyl ether (ee_p =98%) as well as in toluene (ee_p =98%) were observed. The highest values of conversion and enantioselectivity were achieved in a reaction carried out in diisopropyl ether (*C* =47.9%, *E* =308.5) after 24 h of incubation. Li *et al.* [52] investigated a kinetic resolution of (*R,S*)-1-phenylethanol catalyzed by a *Burkholderia cenocepacia* lipase (BCCL) with vinyl acetate as an acyl donor in various solvents. The reactions were conducted at 12 h. The *n*-heptane (ee_s =81.6%) *n*-decane (ee_s =81.1%) were the best solvents. Wang *et al.* [32] performed the kinetic resolution of (*R,S*)-1-phenylethanol (48 h) catalyzed by a BCL in encapsulated form, with vinyl acetate as the acyl donor in five solvents. The lowest values of conversion

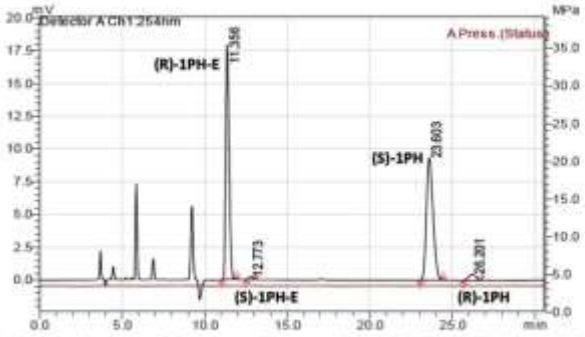


Fig. (2). HPLC chromatograms of (*R,S*)-1-phenylethanol and its esters: Diisopropyl ether; **(R)-1PH-E** – (*R*)-1-phenylethanol acetate; **(S)-1PH-E** – (*S*)-1-phenylethanol acetate; **(R)-1PH** – (*R*)-1-phenylethanol; **(S)-1PH** – (*S*)-1-phenylethanol; Chromatographic conditions: Lux Cellulose-3 (4.6 mm×250 mm×5 µm) column; mobile phase: *n*-heptane/2-propanol/ trifluoroacetic acid (98.7/1.3/0.15 v/v/v), flow rate =1 mL/min, t =15 °C, UV =254 nm. Reaction mixture: APS-BCL (8.0 mg), (*R,S*)-1-phenylethanol (9.98 µL), solvent (0.41 mL) isopropenyl acetate (28.25 µL). Incubation time: 24 h.

Table 2. The results of transesterification of (*R,S*)-1-phenylethanol catalyzed by lipase APS-BCL with isopropenyl acetate as an acyl donor depending on the used medium. The mixture was composed of 8 mg of APS-BCL, 9.98 µL (*R,S*)-1-phenylethanol, 0.41 mL of each solvent, 28.25 µL of isopropenyl acetate.

Solvent	LogP	ee_s(%)	ee_p(%)	*C*(%)	*E*
t-butylmethyl ether[a]	1.06	95	71	42.7	83.2
Dichloromethane[b]	1.25	97	30	23.6	88.4
Diisopropyl ether[b]	1.52	98	90	47.9	308.5
Toluene[a]	2.73	98	76	43.7	227.2
Cyclohexane[a]	3.44	96	92	48.9	162.8
n-Hexane[b]	3.60	98	67	40.6	202.4
Isooctane[a]	4.09	95	89	48.4	117.1
n-Heptane[a]	4.66	94	99	51.3	170.9

[a]Reaction was performed in 48 hours.
[b]Reaction was performed in 24 hours.

were reached in octane (*C* =18.7%). According to our knowledge, it could be caused by the process of encapsulation, which probably reduced the availability of lipase for the substrate.

In our study, the usefulness of all tested reaction media in catalytic reactions was documented. Additionally, the screening of solvents with different logP values showed high enantioselectivity of all performed reactions. The lack of a linear relationship between logP value and catalytic parameters was exposed. The optimal conditions of reaction were established what is an important aspect in obtaining the high purity of products. The data presented that diisopropyl ether can be a favourable reaction medium in conducting catalytic reactions.

2.3. Effect of Used Oils on the Lipolytic Activity of Lipase

The tested reaction mixture was composed of oil and arabic gum suspension. 13 different vegetable oils with various content of ω-3, ω-6 and ω-9 PUFAs have been studied. Lipolytic and relative activities were determined. The received results were shown in Table **3**, Figs. (**5** and **6**). It was observed that the application of oils with ω-6/ω-9 ratio equal to or higher than 2.3 allow obtaining the higher lipolytic activity than when oils were characterized by lower than 2.3 value of the ω-6/ω-9 ratio. Therefore, based on the results of lipolytic activity, the cut-off limit 2.3 of ω-6/ω-9 ratio has been proposed. As mentioned above, an interesting dependence during the performed experiments was observed. With an increasing ratio of ω-6 to ω-9 PUFAs in tested oils, lipase lipolytic activity also has been increasing. This trend does not have a linear relationship. When the hemp oil was used, the highest activity of lipase was achieved. Whereas, in safflower oil, the one with the highest value of the ω6/ω9 ratio, the enzymatic activity was slightly lower. In the tested group of vegetable oils, the obtained results were related to the ratio of ω-6/ω-9 PUFAs.

The variabilities in substrate (oil) availability to lipase can be caused by the molecular structure of fatty acids (Scheme **2**). The main fatty acids of ω-3, ω-6, ω-9 in the tested group of oils are, α-linolenic acid, linoleic acid and oleic acid, respectively. The differences in these oils structure can be observed. The intertangling of the carbon chain of oil results, among others, from the existence of double bonds C=C. α-Linolenic acid has three double bonds C=C, which can influence the worse accessibility of substrate to the active site of lipase, probably by steric hindrance. The linoleic acid (ω-6) has two double bonds C=C. The structure is less twisted, and

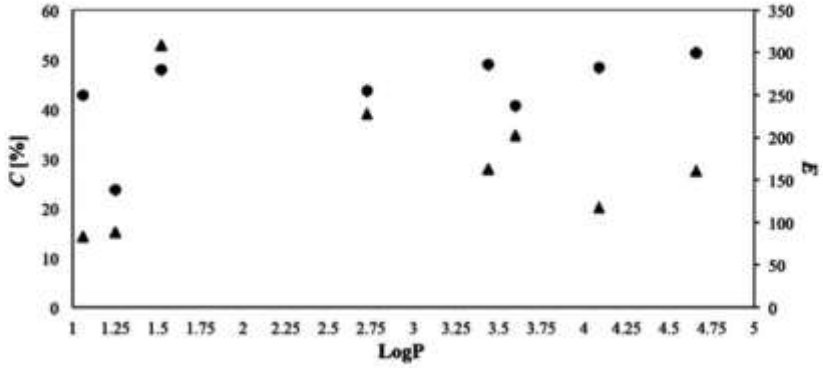


Fig. (3). Effect of logP value of solvent on the conversion (*C*) and enantioselectivity (*E*) of transesterification of (*R,S*)-1-phenylethanol catalyzed by lipase APS-BCL with isopropenyl acetate as the acyl donor. The filled circles mean conversion, while filled triangles mean enantioselectivity. The solvents were ordered on increasing logP value – *t*-butyl methyl ether (1.06), dichloromethane (1.25), diisopropyl ether (1.52), toluene (2.73), cyclohexane (3.44), hexane (3.60), isooctane (4.09), *n*-heptane (4.66). The time of reaction – 48 h; for diisopropyl ether and *n*-hexane – 24 h.

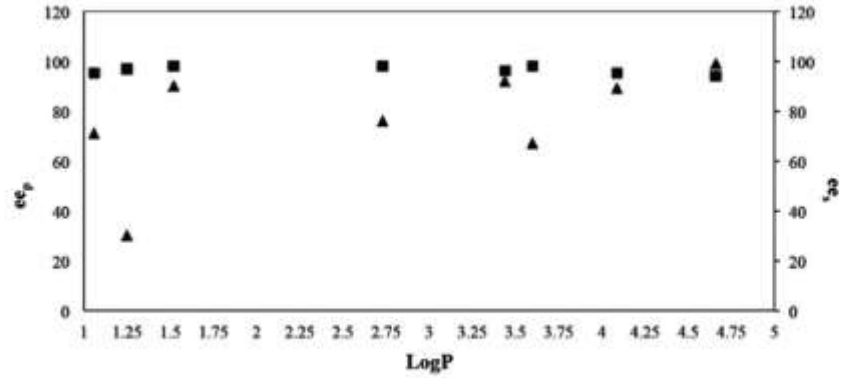


Fig. (4). Effect of logP value on enantiomeric excess of products (ee_p) and substrates (ee_s) of transesterification of (*R,S*)-1-phenylethanol catalyzed by lipase APS-BCL with isopropenyl acetate as the acyl donor. The filled squares mean ee_p, while filled triangles mean ee_s. The solvents were ordered on increasing logP value – *t*-butyl methyl ether (1.06), dichloromethane (1.25), diisopropyl ether (1.52), toluene (2.73), cyclohexane (3.44), *n*-hexane (3.60), isooctane (4.09), *n*-heptane (4.66). The time of reaction – 48 h; for diisopropyl ether and *n*-hexane – 24 h.

potentially promoting the catalytic activity of lipase. Gocen *et al.* [66] suggested that the presence of a double bond has an effect on geometrical isomerism of the fatty acid, and is associated with chain kinks. In another paper [67], it was demonstrated that the enzymatic activity of lipase with using of ω-6 PUFAs was higher than ω-3 PUFAs. It was shown that the length of the carbon chain and number, and position of double bonds have various effects on lipase activity. In the performed lipase-catalyzed experiment, the reaction yield decreased with an increasing amount of carbon atom in the chain. The longer carbon chain causes stronger steric hindrance, which could influence the interaction with the active site, and induces decrease of conversion. The cited authors stated that the presence and number of double bonds C=C may have a positive or negative effect on the lipase activity. The steric conformation of the chain can be stabilized by double bonds, promoting reaction with the active site of lipase, on the other hand, their presence can foster steric hindrance, consequently. Furthermore, the different locations of double bonds have an effect on steric hindrance and through it on the lipase activity. Casas-Godoy *et al.* [68] have also demonstrated that a high amount of double bonds enhanced steric hindrance to the active site of lipase. It was suggested that lipase had a higher activity on fatty acids when a first double bond is on carbon with an even number. The authors described also the hypothesis that when thermodynamic equilibrium is achieved, the enzymatic reaction is stopped. Aknabi *et al.* [69] used the lipase A from *Candida antarctica* (CAL-A) to concentrate DHA from fish and thraustochytrid oils. They confirmed that the presence of double bonds had an essential effect on lipase activity.

Analyzing published data concerning the structure of the main compounds of PUFAs, it can be assumed that linoleic acid (ω-6) is characterized by better accessibility for the active site of lipase than α-linolenic acid (ω-3) [67]. In our experiments, similar values of lipase activity have been observed, confirming the results from the literature. It should be emphasized that a significant number of published papers pertained to oils of animal origin, which contain large amounts of EPA and DHA. However, our study covers only

Table 3. The results of enzymatic activity depending on used oil in the reaction mixture, containing different amounts of ω-3, ω-6, ω-9 acids.

Oil	ω3[a]	ω6[a]	ω9[a]	U[μM/min][b]	ω6/ω9 Ratio[c]	Relative Activity [%][d]
Peanut	0	6.3	69	27.50	0.09	62
Camelina	29	17	0	31.00	0.00	70
Rape	7.5	16	56	39.00	0.29	88
Pumpkin seed	0	43	30	34.00	1.43	77
Walnut	10	17	17	27.17	1.00	61
Sesame	0	38	37	38.33	1.03	86
Avocado	0	9.6	51	34.17	0.19	77
Rice	0	32	44	34.17	0.73	77
Corn	0	49.9	28.5	32.17	1.75	73
Black cumin	0	52	22	42.67	2.36	96
Hemp	16	51	11	44.33	4.64	100
Safflower	0	69	13	42.17	5.31	95
Grape seed	0	62	18	42.17	3.44	95

[a]The amount of main PUFA (ω-3 – α-linolenic acid, ω-6 – linoleic acid, ω-9 – oleic acid) in 100 g of oil. Data were received from the Oleofarm company (Poland).
[b]The activity of lipase
[c]The quantitative ratio between ω-6 and ω-9 PUFAs.
[d]The percentage ratio between the enzymatic activity of the tested sample and the enzymatic activity of the sample which has the highest value of U[μM/min].

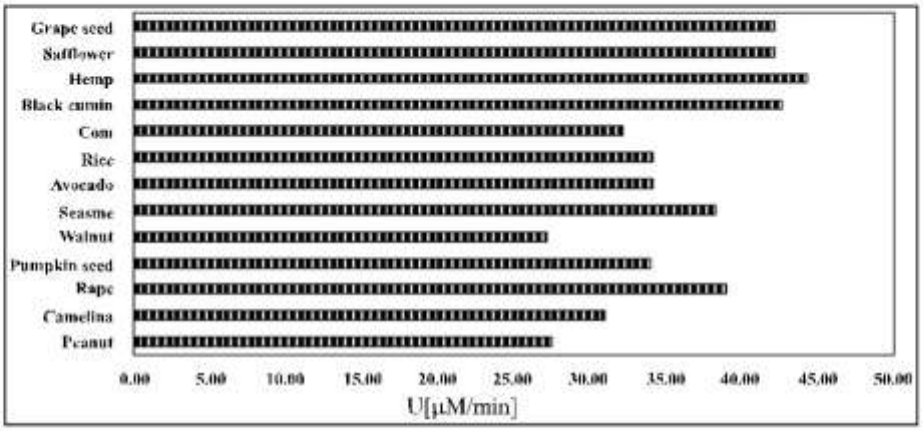


Fig. (5). The value of enzymatic activity (U) of APS-BCL applied in tested oils.

oils of vegetable origin. Regarding the main compound of ω-9 PUFAs group, oleic acid, has only one double bond C=C, and thus, the structure is less twisted than linoleic acid. The double bond in the oleic acid (ω-9) is more distant from the acyl group than in linoleic acid (ω-6), but despite this fact, no positive impact on the lipase activity, in our experiments, was observed. Comparing the results of enzyme activity in oils possessing a higher quantity of ω-9 PUFAs than ω-6 PUFAs (two double bonds), the lipolytic activity of the enzyme was lower, probably because of hindered access substrates to lipase.

As mentioned above, based on the molecular structure and existence of double bonds, the oleic acid should be less twisted than linoleic acid. The influence of intertangling of ω-9 acids on lipase activity is not exhaustively described in the literature. We assume that if in tested oils, the value of the ω-6/ω-9 ratio is equal to or higher than 2.3, the steric hindrance is lower and the combination of substrates with the active site is promoted. Below this value, the molecular conformation of substrates is less beneficial for lipase activity. It can be noted that the effect of ω-PUFAs ratio in oils on the lipase lipolytic activity is significant. As it was demonstrated, APS-BCL in native form shows the various level of activity depending on used substrate (oil). It is an important aspect of the stage of designing catalytic systems to achieve high enzymatic activity. The suggested cut-off limit require further investigation in the aim of development of useful and optimal parameter for modeling of enzymatic reactions. It is worth observing that the assessment of the

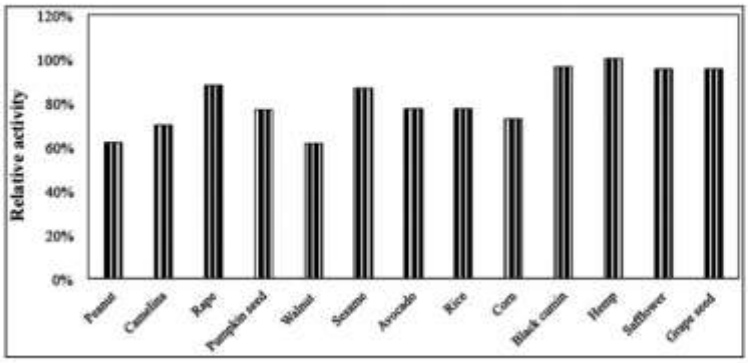
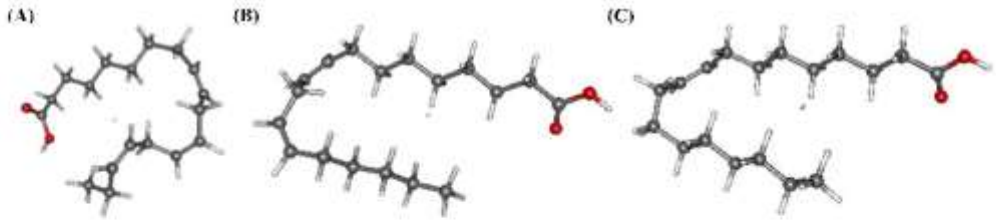


Fig. (6). The values of the relative activity of APS-BCL applied in studied oils.



Scheme 2. The 3D structures of the main compounds of ω-PUFAs: α-linolenic acid (**A**), linoleic acid (**B**) and oleic acid (**C**). The data was achieved from the PubChem website [70-72].

impact of the ratio of ω-3, ω-6, ω-9 acids in oils on the lipase activity is poorly discussed in the literature. It should also be realized that not only the quantity amount of individual PUFAs in oils has an effect on the lipase activity, but also the origin of tested oils might have a potential influence on enzyme activity.

3. EXPERIMENTAL

3.1. General Information

3.1.1. Chemicals

APS-BCL, (*R,S*)-1-phenylethanol (98%), *n*-heptane (≥99%) , *n*-hexane (≥97%), 2-propanol (99.9%), isopropenyl acetate (99%), isooctane (2,2,4-trimethylpentane, 99%), acetyl chloride (99%), trifluoroacetic acid (99%), toluene (99.9%), diisopropyl ether (98.5%) and *t*-butyl methyl ether (99.7%) were purchased from Sigma Aldrich. Cyclohexane (99.5%), dichloromethane (99.8%), sodium bicarbonate, sodium dihydrogen phosphate, and disodium hydrogen phosphate were obtained from POCH (Poland), oils from Oleofarm Company (Poland) and sodium chloride from Pharma Cosmetic (Poland).

3.1.2. Instrumentation and Conditions

The Shimadzu HPLC system (Japan) used was equipped with a pump, model LC-20AD; a UV-VIS detector, model SPD-20A; a degasser, model DGU-20A5; an autosampler, model SIL-20ACHT; and a column oven, model CTO-10AS$_{VP}$. A Lux Cellulose-3 (LC-3) (4.6 mm×250 mm) column with cellulose tris(4-methylbenzoate) as a chiral selector was applied. The column had 5 μm particle size. The most optimal chromatographic conditions for (R)- and (S)-1-phenylethanol and their esters were established with n-heptane/2-propanol/trifluoroacetic acid (98.7/1.3/0.15, v/v/v) mobile phase at a flow rate of 1 mL/min. The UV detection wavelength was set at 254 nm.

3.2. Methods

3.2.1. Chemical Acetylation of (R,S)-1-Phenylethanol

The acetylation of (*R,S*)-1-phenylethanol was performed according to Sikora *et al*. [73] with a few modifications. 8.4 μL (0.07 mM) (*R,S*)-1-phenylethanol and 8 μL (0.07 mM) of acetyl chloride were added to n-heptane (10 mL) and incubated at 30°C for 2 h. After incubation the mixture was washed by a solution (equal ratio) of sodium chloride (10 mL) and sodium bicarbonate (10 mL). The organic phase was collected and left for evaporation for a few hours. The same steps for pure enantiomers of (*R*)- and (*S*)-phenylethanol were performed. The retention times were compared with standards of the racemic mixture and pure enantiomers without acetylation. The results were used to choose the optimal conditions of the chromatographic method.

3.2.2. Kinetic Resolution of (R,S)-1-Phenylethanol

The kinetic resolution was carried out using a methodology described in the literature [53-55] with a few changes. 8 mg APS-BCL, 1 mM (*R,S*)-1-phenylethanol and 3.12 mM isopropenyl acetate were mixed with 0.41 mL *n*-heptane. The samples were collected after 48 h. Then, 5 μL of each sample were taken and 0.9 mL

of *n*-heptane was added. The mixture was filtrated after 10 minutes of stirring. 5 µL of the sample was injected into the HPLC column. All steps for other solvents with various logP values (incubation in 48 h) were repeated.

The enantiomeric excesses of the substrate (ee_s) and the product (ee_p) as well as the conversion (C), enantiomeric ratio (E) (also called enantioselectivity) were calculated [65, 74].

The E was determined as follows:

$$E = \frac{\ln[(1-C)(1-ee_s)]}{\ln[(1-C)(1+ee_s)]}$$

The ee_s and ee_p values were calculated as follows:

$$ee_s = \frac{|S-R|}{|S+R|} \times 100$$

$$ee_p = \frac{|S-R|}{|S+R|} \times 100$$

The C was determined:

$$C = \frac{ee_s}{ee_p + ee_s} \times 100$$

The results values (C, ee_p, ee_s) were expressed in a percentage.

3.2.3. Assay of Lipolytic Activity Depending on the Substrates

The lipolytic activity tests of lipase were carried out by titration of the reaction mixture, containing TG of fatty acids [75, 76]. The suspension of arabic gum (7%, w/v) was prepared by the suspense of arabic gum in distilled water and incubation for 1 h in a water bath to inactivate the enzymes, contained in arabic gum. After incubation, the suspension was cooled to room temperature and mixed, in an equal volume, with oil to obtain an emulsion. The assay mixture was composed of emulsion (5 mL), phosphate buffer (2 mL, 100 mM, pH 7.4) and free enzyme (1 mL, 10 mg/mL).

CONCLUSION

The performed studies have shown the usability in the application of isopropenyl acetate as an acyl donor in the kinetic resolution of (*R,S*)-1-phenylethanol catalyzed by Amano lipase PS from *Burkholderia cepacia* (APS-BCL). Furthermore, diisopropyl ether was chosen as an optimal reaction medium for biocatalytic reactions that were carried out. The lack of a linear relationship between the values of logP of tested solvents and parameters characterizing the catalytic activity was documented. The optimal conditions of the chromatographic method were established. The lipolytic activity of APS-BCL using 13 different oils from plant sources containing different amounts of ω-acids was determined. The effect of the ratio of polyunsaturated fatty acids (PUFAs) contained in examined oils on the lipolytic activity of lipase was proven. For the first time, the cut-off limit (value of 2.3) of the ratio of ω-6/ω-9 acids has been proposed. Above this value, are more favourable conditions for the activity of studied lipase. The showed dependence might be in the future potentially used as a preliminary parameter in designing of the enzymatic catalytic systems. However, this data requires further studies.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available from the corresponding author on reasonable request.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's Website along with the published article.

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11.2. Ocena aktywności enancjoselektywnej i lipolitycznej lipazy Amano PS z *Burkholderia cepacia* immobilizowanej na nośniku poliakrylowym IB-150A – P2

P2: **Jacek Dulęba,** Tomasz Siódmiak, Michał Piotr Marszałł, The influence of substrate systems on the enantioselective and lipolytic activity of immobilized Amano PS from *Burkholderia cepacia* lipase (APS-BCL), *Process Biochem.* 2022, 120, 126-137.

Lipazę APS-BCL poddano procesowi immobilizacji, celem zwiększenia jej aktywności. W opisanej pracy, APS-BCL immobilizowano na apolarnym nośniku poliakrylowym IB-150A. Zbadano aktywność enancjoselektywną immobilizowanej APS-BCL na drodze kinetycznego rozdziału (*R,S*)-1-fenyloetanolu w reakcji transestryfikacji. Ponadto, sprawdzono wpływ donorów grupy acylowej oraz rozpuszczalników na aktywność immobilizowanej APS-BCL. Wybrano *n*-heptan jako optymalne środowisko reakcji. W badaniach wstępnych zoptymalizowano czas inkubacji - 12 h. Reakcję prowadzono w obecności 2 donorów grupy acylowej: octanu izopropenylu i octanu winylu. Uzyskane wyniki potwierdziły wysoką aktywność enancjoselektywną APS-BCL w kinetycznym rozdziale (*R,S*)-1-fenyloetanolu. Zaobserwowano, że immobilizacja skróciła czas reakcji potrzebny do osiągnięcia wysokich parametrów katalitycznych. Podobnie jak w reakcji katalizowanej przez APS-BCL w formie wolnej, wskazano na octan izopropenylu jako alternatywę donora grupy acylowej oraz nie stwierdzono wpływu wzrostu parametru logP na aktywność enancjoselektywną enzymu.

Zbadano aktywność lipolityczną immobilizowanej APS-BCL w reakcji hydrolizy 13 olejów pochodzenia roślinnego zawierających różne ilości kwasów ω3/ω6/ω9. Badanie poprzedzono wyznaczeniem ilości immobilizowanego białka na nośniku metodą Bradforda [85]. Na podstawie uzyskanych danych, obliczono parametry katalityczne dotyczące wydajności immobilizacji oraz stosunku aktywności lipolitycznej immobilizowanej lipazy do aktywności enzymu w formie wolnej (I_y, A_{rec}, A_{ret}). Wyznaczono parametry kinetyczne reakcji, opierając się na krzywej Linweavera-Burka.

Otrzymane wyniki wskazywały na obniżenie aktywności immobilizowanej APS-BCL w porównaniu z aktywnością lipazy w formie wolnej. Do dalszych badań lipolitycznych, z uwagi na wyższą aktywność immobilizowanej APS-BCL, w porównaniu w formą wolną, wybrano olej arachidowy jako substrat. Potwierdzono granicę odcięcia aktywności APS-BCL w formie immobilizowanej – lipaza wykazywała wysoką aktywność w olejach o stosunku zawartości ω6/ω9 powyżej 2.3.

Wyznaczone parametry kinetyczne (wyższe K_m immobilizowanej APS-BCL w porównaniu z formą wolną) wskazują na zmniejszenie powinowactwa enzymu do substratu. Analizując wyniki uzyskane w przeprowadzonych badaniach jak i danych literaturowych [64, 66], stwierdzono, że na aktywność lipolityczną lipazy znacząco wpływają również właściwości chemiczne oraz fizyczne użytych substratów.

W badaniu aktywności enancjoselektywnej oraz lipolitycznej APS-BCL otrzymano wysokie parametry katalityczne immobilizowanej APS-BCL przy znacznie skróconym czasie reakcji, w porównaniu z lipazą w formie wolnej. Ponadto, wykazano pozytywny wpływ środowiska reakcji na aktywność enancjoselektywną immobilizowanej APS-BCL. Jednakże, w badaniach aktywności lipolitycznej zaobserwowano spadek aktywności lipazy w formie immobilizowanej.

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The influence of substrate systems on the enantioselective and lipolytic activity of immobilized Amano PS from *Burkholderia cepacia* lipase (APS-BCL)

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ABSTRACT

In this study, Amano lipase PS from *Burkholderia cepacia* (APS-BCL) was immobilized onto polyacrylic support IB-150A, which allowed to improve immobilized lipase activity in nonaqueous media compared with the free (native) form. The enantioselectivity of the immobilized APS-BCL (8 mg/mL) in the kinetic resolution of (*R,S*)−1-phenylethanol was evaluated. High values of catalytic parameters with isopropenyl acetate (E =775.4, C =49 % ± 0.4) and vinyl acetate (E =206.6, C = 50 % ± 0.3) as acyl donors were achieved after a reaction time of 12 h. The effects of 7 different non-aqueous solvents on catalyst enantioselectivity in the kinetic resolution of (*R,S*)−1−phenylethanol were also assessed. Diisopropyl ether and *n*-hexane were selected as the optimal reaction media. A novel lipolytic activity study of immobilized APS-BCL (7.9–9.3 mg/mL) with the application of the developed multicomponent unsaturated fatty acids (MC-UFAs) method using 13 vegetable oils in an aqueous medium has been proposed (pH =7, T =37 °C). The effect of the ratio of polyunsaturated fatty acids (PUFAs) to monounsaturated fatty acids (MUFAs) on lipase activity was demonstrated and a cut-off limit (ω6/ω9 =2.3) was determined. Hyperactivation of immobilized APS-BCL in peanut oil (A_{inc}=104.85 %, A_{int}=110.19 %) as the substrate was observed.

1. Introduction

Lipases (EC 3.1.1.3), thanks to the properties of catalyzing biochemical reactions, are among the classes of enzymes with the most widespread applications for biotechnological and pharmaceutical synthesis. Lipases exhibit their activity between hydrophilic and hydrophobic surfaces [1,2]. They also have an interesting feature known as 'interfacial activation'. The mechanism of action is based on the displacement of the amino acid lid (a helical flap) which leads to an opened conformational state of the lipase (which is more active and stable than the closed form [3]) with an exposed active site. This ability allows lipases, among others, to hydrolyze compounds containing carboxylic ester groups that are aggregated in water [2]. Moreover, in the presence of a hydrophobic surface, the enzyme becomes adsorbed on it, establishing a new structure called 'open', in which the active centre is fully exposed, rendering the lipase able to hydrolyze drops of oils (with the conformational equilibrium shifted towards the open form) [3,4]. The open form is expressed as a large hydrophobic pocket available to the reaction medium [4]. This conformation is crucial for lipase activity (e.g. enantioselective and lipolytic activity). The open form of one lipase molecule can also stabilize the open form of other lipase molecules (which leads to the formation of dimers with altered enzymatic properties). However, lid mobility does not have any significant effect on lipase selectivity or specificity, and these properties can be modified by genetic manipulation and/or physio-chemical modifications [3–5]. Therefore, lipases are investigated in a wide range of reactions of pharmaceutical, biotechnological, chemical, and agricultural significance, e.g. esterification, transesterification, hydrolysis, alcoholysis [6]. Their use is also associated with low costs and low toxicity, which makes them an important part of 'green chemistry'[6–9] and an alternative to chemical catalysts [10].

Amano lipase PS from *Burkholderia cepacia* (APS-BCL), also known as *Pseudomonas cepacia* (APS-PCL), belongs to an important class of extracellular lipases originating from *Burkholderia cepacia* (BC) and characterized by an α/β fold and a catalytic triad (Ser^{87}, Asp^{264}, His^{286}) at the active site [11]. This type of lipase is characterized by the lid as

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the mobile unit [12], however, without positional specificity [13]. The isoelectric point (pI) of this lipase is 5. The optimal conditions for this lipase are characterized by a neutral pH (7) and a temperature of 50 °C [14]. APS-BCL shows resistance to some metal ions (Na^{+}, Ca^{2+}, Li^{+}, Sr^{2+}) with significant sensitivity to the action of heavy metal ions (e.g. Zn^{2+}, Cu^{2+}) [14]. APS-BCL is a commercial lipase (lyophilized powder with the supporting and stabilizing materials) available in the free form, as Amano Lipase SD, and in immobilized forms, as Amano Lipase PS-C (immobilized on ceramic particles) and PS-D (immobilized on diatomite) [13]. The lipase can be purified by e.g. ultrafiltration, precipitation or affinity chromatography [14]. APS-BCL is commonly used in various reactions of medical importance. One notable example is kinetic resolution, based on the esterification or hydrolysis of one of the stereoisomers in a racemic mixture [15]. Thanks to its enantioselective activity, APS-BCL catalyzes the formation of chirally pure enantiomers of drugs or their building blocks in various reactions in non-aqueous media [15]. One of the common applications of kinetic resolution in the pharmaceutical industry is to obtain enantiomers of profens, i.e. (*R,S*)−ibuprofen or (*R,S*)-flurbiprofen, in reactions catalyzed by lipase B from *Candida antarctica* (CAL-B) and lipase from *Candida rugosa* (CRL) [15–24]. Of the other class of these enzymes, lipases from *Burkholderia cepacia* (BCLs), including APS-BCL, are widely tested in kinetic resolution [25–27]. The standard compound used in the reaction is a racemic mixture of (*R,S*)−1−phenylethanol. According to many research groups, kinetic resolution of (*R,S*)−1−phenylethanol is characterized as an enantioselectively efficient process [25–35]. As regards the course of the reaction, (*R,S*)−1−phenylethanol acts as an acyl acceptor, while the role of the donor is played by an ester of a carboxylic acid, most commonly unsaturated, e.g. vinyl acetate, isopropenyl acetate. As a result of this transesterification, a new ester and an alcohol are formed. The resulting chirally pure (*R*)−1−phenylethanol (as the ester form) is an important chiral building block which is used in the pharmaceutical industry [13, 25–39], e.g. for the inhibition of intestinal adsorption of cholesterol and as a component of ophthalmic preservatives [39,40].

Lipases are distinguished by activities which allow them to catalyze various reactions. As previously mentioned, thanks to the enantioselectivity of lipases it is possible to obtain optically pure organic compounds for use as drugs [41–43]. On the other hand, lipolytic activity is characterized by the hydrolysis of triacylglycerols (TAGs) to free fatty acid (FFAs) and glycerol. The main compounds that are broken down by hydrolysis are polyunsaturated fatty acids (PUFAs), specifically ω3- and ω6-PUFAs, and monounsaturated fatty acids (MUFAs), specifically ω9-MUFAs [44,45]. The carbon chains in PUFAs contain three (ω3-acids) or two (ω6-acids) double bonds, while MUFAs contain one (ω9-acids). PUFAs are used as ingredients of dietary supplements commercially available on the pharmaceutical market. PUFAs are considered as the compounds ancillary in the treatment of heart disease and improving the functioning of the nervous system [46–49]. Moreover, in order to optimize the most balanced system exerting beneficial effects on the human body, the ω6/ω3 ratio was employed [50,51]. It was suggested that a high ω6/ω3 PUFA ratio increased the risk of obesity due to fat deposition. It should also be noted that dietary excess of ω6-acids is deleterious to health [52]. In the case of a diet rich in MUFAs (ω9), due to decreasing the concentration of low-density lipoprotein (LDL-C) and increasing the concentration of high-density lipoprotein (HDL-C), reduction coronary risk and prevalence of cancer were noted [53,54].

Lipases from *Burkholderia cepacia* are studied in native or immobilized forms. Immobilization allows to modify the catalytic properties of these enzymes [55]. To carry out this process, supports are used. From the molecular point of view, immobilization is based on the intramolecular interaction between the protein part of the enzyme and the characteristic functional group in the support. Supports also affect the reagents (capture of substrates, facilitated diffusion, and/or reduced substrate/product inhibition) and the reaction media (dispersion). Hydrophobic supports interact with fatty acids (as the substrates), bringing them closer, so they are located near the lipase. As regards the properties of the lipase, the presence of hydrophobic supports (due to adsorption) renders the open form of the lipase (the lid exposing the active centre) more active, a feature that is then applied in immobilization, especially on hydrophobic materials [56]. Mateo et al. have described relevant supports characteristics (e.g. large internal surface, spacer arm, superficial density of reaction groups, minimal steric hindrance), and suitable reaction conditions (e.g. optimal reaction time, temperature, pH value, buffers, protein inhibitors of protectors) [55]. The support structure also requires enough mechanical strength, and the resistance to chemical attack and microbial decomposition [5]. The expected results include altered (increased or decreased) enzymatic activity, improved stability (three-dimensional structure of enzyme) and selectivity, and process control [57–61]. As a result of those, immobilized lipase is better adapted to reaction conditions and structurally different reagents. However, some interactions during immobilization can cause the steric hindrance, and as a result, a lower affinity of the enzyme for the substrate, which in turn affects enzyme activity. Furthermore, the presence of compounds that could denature the enzyme protein should be avoided. The following techniques of enzyme immobilization have been described in the literature [55,62–71]: as attachment by physical adsorption or covalent binding, entrapment, and cross-linking.

Physical adsorption is the simplest immobilization method and one of the most commonly used ones. Based on non-covalent binding, van der Waals forces, and electrostatic interactions, this technique is relatively inexpensive and can be used in mild conditions [68–71]. The amount and the activity of the adsorbed lipase are, however, dependent on the physicochemical properties of the supports (polarity, molarity, size, surface, porosity, and the range of the of hydrophilic and hydrophobic groups) [55]. The support materials used for this process are mainly polymers, e.g. polystyrene, polypropylene, polyacrylate, polyacrylonitrile, and polymethacrylate. In the mechanism of ionic binding, immobilization is based on the formation of a salt linkage between the enzyme and the support. Compared to physical adsorption, the intramolecular lipase-support interaction is stronger [55,65]. The supports used in this method are usually made from synthetic polymers or polysaccharides, e.g. MANAE-agarose. In the case of covalent immobilization, the reaction generally occurs between the amino acid part of the enzyme (which does not display any catalytic activity) and a group designated to bind to the support, especially with the affinity for amine or thiol residues [55,65,71,72]. This method ensures a high rigidity of the resulting complex but requires more stringent conditions, which makes it more expensive than physical adsorption. In contrast to other methods, the covalent bond technique mainly uses magnetic nanoparticles, silica gels, and chitosan [65]. Immobilization by entrapment involves the "catching" of the lipase by ordered polymer networks or microcapsules. The retained enzyme shows a limited mobility despite the lack of direct contact with the support [65–68]. The entrapment method provides a high stability of the aggregates and a higher efficiency than physical adsorption. The most known support for encapsulation in the entrapment is κ-carrageenan [73]. The method of immobilization by cross-linking is based on immobilizing lipase by cross binding, which leads to the formation of a spatial structure. The resulting complex, as well as showing high stability in aqueous solvents, has a wide tolerance range for pH and temperature [64,74,76]. Cross-linking can, however, result in a loss of activity because the process is carried out in extreme conditions and can cause irreversible changes in the conformation of the enzyme. An example cross-linker in this method is glutaraldehyde enables the high activity of the biocatalyst [65,74,75]. In our study, immobilization of a commercial APS-BCL onto IB-150A was performed by a combination of covalent bonding (the molecular action occurs between the apolar epoxide groups of support and the nucleophiles (primary amine group) of the proteins of enzyme [16,76]) and (to a lesser extent) physical - according to the manufacturer recommendation. The physicochemical properties are provided in Table 1. As was to be expected based on the literature data [76] and

Table 1
The physicochemical properties of IB-150A support (data achieved from Chiral Vision).

Characteristic	Type	Matrix	Functional group	Particle size [μm]	Moisture content
Crosslinked copolymer of methacrylate carrying oxirane groups.	Covalent	Polyacrylic	epoxide, apolar	150–300	< 5 %

preliminary studies, the apolar support (IB-150A) was shown to promote a better accessibility of the lipase to the substrate than the polar counterpart of this support (IB-150P).

Immobilization of APS-BCL onto IB-150A began to be studied in the literature. However, there are only few reports concerning this subject, therefore, there is strong need for meaningful development of this problem [77,78]. In this paper, an extensive study of the effects of substrate systems (reaction components and media) on the enzymatic properties of the immobilized APS-BCL is reported. The enantioselectivity of APS-BCL immobilized onto the polyacrylic support IB-150A in different reaction conditions (acyl donors, non-aqueous solvents) was investigated. The effects of the substrate on the lipolytic activity of the immobilized APS-BCL, relative to the free lipase, and the effects of the ω6/ω9 PUFAs/MUFAs ratios on enzymatic activity were also examined.

2. Materials and methods

2.1. Chemicals

APS-BCL (≥30,000 U/g) was obtained from Sigma Aldrich. The polyacrylic support Immobead IB-150A was purchased from ChiralVision (The Netherlands). The organic media of high purity (>98–99%) were purchased from Sigma Aldrich (*n*-heptane, *n*-hexane, 2-propanol, isooctane, diisopropyl ether, and *t*-butyl methyl ether) and POCH (Poland) (cyclohexane, dichloromethane). The substrates of kinetic resolution ((*R,S*)−1−phenylethanol, isopropenyl acetate, vinyl acetate) were purchased from Sigma Aldrich. Other organic chemicals (trifluoroacetic acid) were obtained from Sigma Aldrich. Buffer reagents (sodium dihydrogen phosphate, and disodium hydrogen phosphate) were obtained from POCH (Poland). Vegetable oils were obtained from Oleofarm (Poland).

2.2. Instrumentation and conditions

The Shimadzu HPLC system (Japan) used consisted of a pump (LC-20 CE), a UV–VIS detector (SPD-20A), a degasser (DGU-20A5), an autosampler (SIL-20ACHT), and a column oven (CTO-10ASVP, t = 15 °C). As a chiral selector, a Lux Cellulose-3 (LC-3) (4.6 mm × 250 mm) column with cellulose tris(4-methylbenzoate) was used. The column had a 5 μm particle size. The most optimal chromatographic conditions for (*R*)- and (*S*)−1−phenylethanol and their esters were optimized with *n*-heptane/2-propanol/trifluoroacetic acid (98.7/1.3/0.15, v/v/v) mobile phase at a flow rate of 1 mL/min. The UV detection wavelength was set at 254 nm.

The Hitachi spectrophotometer (Japan) was equipped with a UV–VIS detector. The wavelength was set at 595 nm.

2.3. Methods

2.3.1. Immobilization of APS-BCL on the polymeric support IB-150A for the evaluation of enantioselective and lipolytic activity

The method was carried out according to the procedure described in the literature [76] (a modified technique recommended by the ChiralVision supplier was employed). For the enantioselective activity evaluation, 8 mg of APS-BCL (10 mg for the lipolytic activity evaluation) was mixed with 1 mL phosphate buffer (pH 7.0, 100 mM) in an Eppendorf flask (2 mL) to obtain a suspension with enzyme concentration of 8 mg/mL (10 mg/mL for the lipolytic activity evaluation). 50 mg of IB-150A was washed with 0.3 mL (0.005 mM) 2-propanol in an Eppendorf flask (2 mL) and left for 15 min at room temperature (20 °C). After incubation, the solvent was removed by filtration (parchment filters folded on glass funnels) and the supports were added to the free enzyme mixture (lipase in phosphate buffer) in an ice bath (mixed for 5 min) and incubated (without mixing) in a refrigerator for 24 h. The supports were then rinsed by distilled water, and left to dry.

2.3.2. Kinetic resolution of (R,S)− 1-phenylethanol

The kinetic resolution was carried out using a methodology described in a previous study [35] and the literature [36,79], with slight modifications. The amount of APS-BCL immobilized on IB-150A (8 mg/mL), 1 mM (*R,S*)−1−phenylethanol, and 3.12 mM vinyl or isopropenyl acetate were mixed in a glass shell vial (1 mL) with 0.41 mL (0.082 mM) *n*-heptane. The samples were agitated (550 rpm, 37 °C) and collected after 12 h. Then, 50 μL of each sample was transferred to the HPLC vial. The samples were evaporated and 0.9 mL (0.18 mM) of *n*-heptane was added. The mixture was filtrated (using a hydrophobic filter) after 10 min of stirring (300 rpm). 5 μL of the sample was injected into the HPLC column. The experiment was repeated in other reaction media, with different logP values (incubation for 12 h or, in case of diisopropyl ether, for 6 h) using isopropenyl acetate as the acyl donor.

Enantioselective parameters (enantiomeric excesses of the substrate (ee_s) and of the product (ee_p), conversion (C), enantiomeric ratio (E) (also called enantioselectivity)) were calculated [42,80] from the following formulae:

$$E = \frac{\ln[(1-C)(1-ee_s)]}{\ln[(1-C)(1+ee_s)]}$$

$$ee_s = \frac{|S_s - R_s|}{|S_s + R_s|} \times 100$$

$$ee_p = \frac{|S_p - R_p|}{|S_p + R_p|} \times 100$$

$$C = \frac{ee_s}{ee_p + ee_s} \times 100$$

C, ee_p and ee_s values were expressed as percentages.

2.3.3. Evaluation of the lipolytic activity of APS-BCL

2.3.3.1. Determination of the amount of immobilized APS-BCL by Bradford's method. Bradford's method was performed according to the literature [76,81], with essential modifications. Five samples (1 mg/mL, 3 mg/mL, 5 mg/mL, 7 mg/mL, 9 mg/mL, respectively, each with 1 mL phosphate buffer at a pH of 7.0, 100 mM) in Eppendorf flasks (2 mL), to a calibration curve, were prepared. The test sample was composed of 0.5 mL supernatant obtained from an immobilized sample of lipase, 50 μL phosphate buffer (pH 7.0, 100 mM), and 1.5 mL Bradford's reagent. After the addition of Bradford's reagent, the reaction mixture was manually stirred for 15 min. The measurements were carried out in duplicate with a UV–VIS spectrophotometer (at a wavelength of 595 nm), and the arithmetic mean of the measurements of the same sample was taken as the final result. The amount of immobilized APS-BCL was calculated as the difference between 10 mg of free lipase and the amount of APS-BCL calculated from the regression equation.

2.3.3.2. Evaluation of the lipolytic activity of immobilized APS-BCL: The multicomponent unsaturated fatty acids (MC-UFAs) method. The method was described in a previous study [35] and the literature [82–85], with a

few modifications. Evaluation of the lipolytic activity of the lipase was carried out by acid-base titration. The suspension of gum arabic (7 %, w/v) was obtained by suspending gum arabic in distilled water (in a 250 mL conical flask) and incubating for 1 h in a water bath (at 80 °C) to disable the enzymes, present in this emulsifier. After incubation, the suspension was cooled to room temperature and mixed, in equal volumes, with oil containing TG of fatty acids to achieve an emulsion. The assay mixture in the conical flask (50 mL) was composed of the emulsion (5 mL), phosphate buffer (3 mL, 100 mM, pH 7.4), and either free APS-BCL (whose amount had been determined by Bradford's method) or immobilized APS-BCL (7.9–9.3 mg/mL), incubated at 37 °C for 30 min with simultaneous stirring (at 300 rpm), and stopped by adding 10 mL of methanol/acetone at equal volumes (5:5). A different cycle of determination was performed without calculating the amount of immobilized APS-BCL by Bradford's method, while assuming the value of 10 mg APS-BCL.

Immobilization yield (I_y) was calculated from the following formula:

$$I_y = \frac{LA_0}{LA_{10}} \times 100\%$$

where: I_y = immobilization yield, LA_0 = amount of lipase calculated as the difference between the initial amount of lipase and the amount remaining in the supernatant after immobilization onto 50 mg of IB-150A (calculated by Bradford's method), LA_{10} = initial amount of lipase (10 mg) [35].

Activity recovery (A_{rec}) and activity retention (A_{ret}) were calculated from the following formulae [83]:

$$A_{rec} = \frac{U_I}{U_{IN}} \times 100\%$$

$$A_{ret} = \frac{U_I}{U_B} \times 100\%$$

where A_{rec} – activity recovery (the ratio of the activity of immobilized APS-BCL to the activity of the initial amount of free protein in the solution that had been used for immobilization), A_{ret} – activity retention (the ratio of the activity of immobilized APS-BCL to the activity of the same amount of free protein in the solution that had been immobilized onto the support), U_I – activity of immobilized lipase, U_{IN} – activity of the initial amount of APS-BCL that had been used for immobilization, U_B – activity of the amount of free protein in the solution that had been immobilized onto the support.

2.3.4. Kinetic parameters

The kinetic parameters for APS-BCL in the free form and in the form immobilized onto IB-150A were calculated when evaluating lipolytic activity of APS-BCL in various amounts of peanut oil as the substrate by the titration method described in subsection 3.3.3.2, and by Söödmiak et al. [76]. The Michaelis constant (K_m) and the maximum velocity (V_{max}) were determined from Lineweaver-Burk plots, using the initial rate of reaction [82,84,85]:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}$$

where $[S]$ – concentration of the substrate, V – initial velocity of the reaction, V_{max} = maximal velocity of the reaction, K_m = Michaelis constant. The turnover numbers (k_{cat}) of native and immobilized APS-BCL were calculated from the following formula [82,86]:

$$k_{cat} = \frac{V_{max}}{[E_T]}$$

where $[E_T]$ = total amount of the enzyme in the reaction medium.

3. Results and discussion

3.1. Effects of isopropenyl acetate and vinyl acetate on the enantioselectivity of immobilized APS-BCL in the kinetic resolution of (R,S)−1−phenylethanol

Kinetic resolution of (*R,S*)−1−phenylethanol, catalyzed by immobilized APS-BCL, was performed by high-performance liquid chromatography (HPLC). Isopropenyl acetate and vinyl acetate were used as the acyl donor (Fig. 1, Fig. 2). The standards of products are provided in Supplementary Material (Fig. Supp-1, Supp-2). The amount of lipase used for the reaction (8.0 mg) was established based on literature data [35,87–89]. Based on the method optimized in the preliminary studies and on the results obtained in the previous study [35], *n*-heptane was chosen as the optimal reaction medium. The results are provided in Table 2A. To the best of our knowledge, the kinetic resolution of (*R,S*)−1−phenylethanol catalyzed by APS-BCL, immobilized onto IB-150A, with isopropenyl acetate as the acyl donor, has not been examined before. Based on the analysis of our data, it can be concluded that immobilization increased the enantioselectivity of APS-BCL. The time of 12 h was selected as the most suitable for the reaction (as further incubation resulted in a complete conversion of one of the substrates). High values of conversion (*C*) (49 % ± 0.4 for isopropenyl acetate, 50 % ± 0.3 for vinyl acetate), enantiomeric excess of products (ee_p) (99 % ± 0.5 % and 96 % ± 0.5, respectively), enantiomeric excess of substrates (ee_s) (95 % ± 1.0 % and 97 % ± 0.5, respectively), and enantiomeric ratio (*E*) (775.4 and 206.9, respectively) were achieved. The results show high enantioselectivity of immobilized APS-BCL for both acyl donors, at a similar *C* value, although the *E* value for isopropenyl acetate was nearly four times higher than that for vinyl acetate. Compared with the data obtained in the previous study [35], where an *E* of 170 for free APS-BCL with isopropenyl acetate was obtained (Table 2B), in the present study, an over fourfold increase in the *E* value for immobilized APS-BCL was observed. Based on these results, it should be noted that the reaction time for lipase necessary to gain a high conversion, in the same conditions and with the same amount of lipase (8.0 mg), was 48 h for the free form and 12 h for the immobilized form (due to the lack of products for the free lipase after 12 h of the reaction time). Therefore, it can be concluded that immobilization of APS-BCL on IB-150A shortens the reaction time required in the enantioselective resolution of (*R,S*)−1−phenylethanol towards the chirally pure (*R*)−1−phenylethanol. The results were collated with the literature data [87–91], assuming conversion to be the most comparable catalytic parameter (Table 2A, Table 2B). As mentioned above, the kinetic resolution of (*R,S*)−1−phenyletanol catalyzed by immobilized lipases with isopropenyl acetate in the catalytic system with immobilized APS-BCL has been done for the first time while vinyl acetate as the acyl donor is already commonly used. Yan et al. [87] carried out the kinetic resolution of (*R,S*)−1−phenylethanol, catalyzed by a lipase from *Aspergillus oryzae* (AOL) in the native form (bound with mycelium), with various acyl donors, and ee_p values exceeding 99 and those of *E* exceeding 200 were received. However, the most favorable results were obtained for vinyl acetate (*C* =37 %) and isopropenyl acetate (*C* =34 %). On the other hand, Melais et al. [88] tested resolution of (*R,S*)−1−phenylethanol catalyzed by a lipase from *Pseudomonas cepacia* (PCL) and lipase B from *Candida antarctica* (CAL-B) in free form using vinyl acetate and isopropenyl acetate as the acyl donors. As noticed in the cited study [88], the best results from employing isopropenyl acetate and vinyl acetate were achieved in the resolution catalyzed by CAL-B (*C* =50 %, *E* > 200). Li et al. [89] investigated kinetic resolution of (*R,S*)−1−phenylethanol catalyzed by free APS-BCL, and immobilized onto a macroporous resin, using vinyl acetate as the acyl donor. The incubation time was 30 h for the native lipase and 0.5 h for immobilization, *C* was nearly 50 %, and *E* exeeded 200. In turn, Soni et al. [91] carried out the kinetic resolution of (*R,S*)−1−phenylethanol catalyzed by APS-BCL immobilized onto polyaniline nanofibers (PANF), treated previously

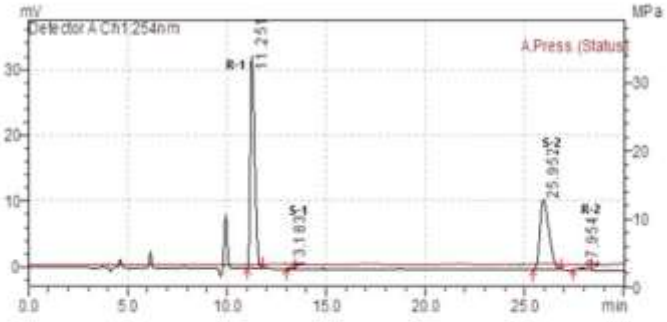

Fig. 1. HPLC chromatogram of (*R,S*)– 1-phenylethanol and its esters: **R-1** – (*R*)–1–phenylethanol acetate; **S-1** – (*S*)–1–phenylethanol acetate; **R-2** – (*R*)–1–phenylethanol; **S-2** – (*S*)–1–phenylethanol; Chromatographic conditions: Lux Cellulose-3 (4.6 mm × 250 mm×5 μm) column; mobile phase: n-heptane/2-propanol/ trifluoroacetic acid (98.7/1.3/0.15 v/v/v), flow rate– 1 mL/min, t– 15 °C, UV– 254 nm. The reaction mixture was composed from APS-BCL (8.0 mg, catalyst), IB-150A (50.0 mg, support), (*R,S*)–1–phenylethanol (9.98 μL, acyl acceptor), *n*-heptane (0.41 mL, reaction medium), isopropenyl acetate (28.25 μL, acyl donor). Incubation time: 12 h.

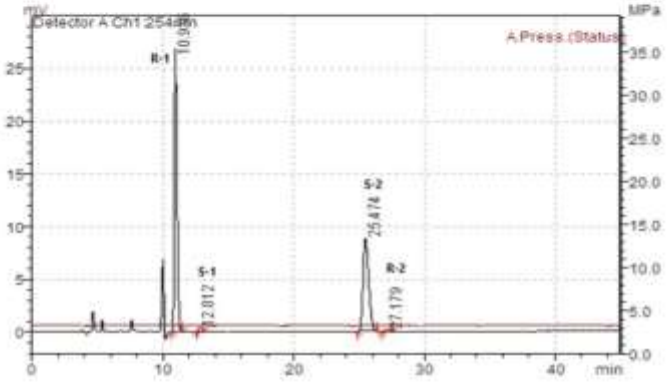

Fig. 2. HPLC chromatogram of (*R,S*)– 1-phenylethanol and its esters: R-1 – (*R*)–1–phenylethanol acetate; S-1 – (*S*)–1–phenylethanol acetate; R-2 – (*R*)–1–phenylethanol; **S-2** – (*S*)–1–phenylethanol; Chromatographic conditions: Lux Cellulose-3 (4.6 mm × 250 mm×5 μm) column; mobile phase: n-heptane/2-propanol/ trifluoroacetic acid (98.7/1.3/0.15 v/v/v), flow rate– 1 mL/min, t – 15 °C, UV– 254 nm. The reaction mixture was composed from APS-BCL (8.0 mg, catalyst), IB-150A (50.0 mg, support), (*R,S*)–1–phenylethanol (9.98 μL, acyl acceptor), *n*-heptane (0.41 mL, reaction medium), vinyl acetate (28.25 μL, acyl donor). Incubation time: 12 h.

Table 2
The results of kinetic resolution of (*R,S*)–1–phenylethanol catalyzed by immobilized APS-BCL (Table 2A) and comparison with free lipase (Table 2B). "x" - Data not available, [a] – Amano lipase PS from *Burkholderia cepacia*, [b] – lipase B from *Candida antarctica*, [c] – lipase from *Pseudomonas cepacia*, [d] – lipase from *Aspergillus oryzae*, [e] – enantiomeric excess of products, [f] – enantiomeric excess of substrates, [g] – conversion, [h] – enantiomeric ratio.

Table 2A

Lipase	Stereopreferention	Immobilized						
		Acyl group donor	Time [h]	ee_p^e [%]	ee_s^f [%]	C^g [%]	E^h	References
APS-BCL[a]	*R*	Isopropenyl acetate	12	99 ± 0.5	85 ± 1.0	49 ± 0.4	775.35	This study
APS-BCL[a]	*R*	Vinyl acetate	12	96 ± 0.5	97 ± 0.5	50 ± 0.3	206.66	This study
APS-BCL[a]	*R*	Vinyl acetate	0.5	99	99	50	> 200	[89]
APS-BCL[a]	*R*	Vinyl acetate	24	97.4	x	49.7	165	[91]
CAL-B[b]	*R*	Vinyl acetate	48	99	99	50	> 200	[90]

Table 2B

Lipase	Stereopreferention	Free						
		Acyl group donor	Time [h]	ee_p^e [%]	ee_s^f [%]	C^g [%]	E^h	References
APS-BCL[a]	*R*	Isopropenyl acetate	48	94	99	51	170	[35]
APS-BCL[a]	*R*	Vinyl acetate	30	99	99	50	> 200	[89]
APS-BCL[a]	*R*	Vinyl acetate	24	97.1	x	37.4	88	[91]
PCL[c]	*R*	Isopropenyl acetate	24	99	11.4	10.3	> 200	[88]
CAL-B[b]	*R*	Isopropenyl acetate	24	99	99	50	> 200	[88]
CAL-B[b]	*R*	Vinyl acetate	24	99	99	50	> 200	[90]
AOL[d]	*R*	Vinyl acetate	48	99	x	37	> 200	[87]
AOL[d]	*R*	Isopropenyl acetate	48	99	x	34	> 200	[87]

with a surfactant (SDS-BCL), with vinyl acetate as the acyl donor. An improvement of the catalytic parameters of lipase after immobilization (C –49.7 %) in comparison with lipase in the free form (C –37.4 %) was observed. In another study, Xing et al. [90] tested the kinetic resolution of (*R,S*)–1–phenylethanol, catalyzed by CAL-B, immobilized onto magnetic nanoparticles (Magnetic CAL-B CLEAs complex). The E value

significantly exceeded 200, and the *C* value was 50 after 48 h of incubation. The results obtained in our study, similarly to the above-mentioned parameters [90], show a high enantioselectivity of immobilized lipase in the kinetic resolution of (*R,S*)−1−phenylethanol. The catalytic parameters obtained could indicate that immobilization onto the polymeric support IB-150A is beneficial to gaining a maximal value of conversion after merely 12 h of reaction time. According to the author's observations, this can be due to the type of the support used. It is worth mentioning that in our study, APS-BCL was bound covalently to the support and slightly adsorbed. In our opinion, the functional group in IB-150A, composed of the polyacrylic matrix (methacrylate carrying oxirane groups) and epoxide functional groups, which are apolar, can decrease the steric hindrance and enhance the availability of lipase for hydrophobic substrates. On the other hand, due to the higher enantioselectivity of immobilized APS-BCL (compared with free lipase), the significant effect of diffusion limitations on lipase activity has not been noted. It was observed that concentration gradients of substrates within the support particles should not decrease the reaction rate, as can be seen from the high selectivity of the reaction. Furthermore, physical adsorption could improve the stability of the lipase-support complex [55,65,89]. Stabilization of the open from of the lipase could have a positive effect on lipase activity. The physical interactions between APS-BCL and the macroporous resin composed of crosslinked polystyrene created a stable complex that was characterized by a high enzymatic activity [89]. The authors observed a relationship between the pore diameter and immobilization degree, with an effect on lipase activity [89]. As regards the acyl donors, in studies reported in the literature [89–91], vinyl acetate has been applied as an optimal agent. However, in the present study, the enantioselective ratio of immobilized APS-BCL in the kinetic resolution of (*R,S*)−1−phenylethanol when using isopropenyl acetate was significantly higher than when vinyl acetate was used. Because of pioneering nature of the studies using isopropenyl acetate in the reaction catalyzed by APS-BCL immobilized onto IB-150A carrier, comparison with other immobilized lipases was not possible. Our results corroborate the conclusion of a previous study [35] that isopropenyl acetate is a valuable alternative when used as the acyl donor. Moreover, the activity of APS-BCL immobilized onto the polyacrylic support IB-150A contributed to a shorter reaction time to obtain (*R*)−1−phenylethanol, along with achieving high catalytic parameters.

3.2. Effects of organic solvents on the enantioselectivity of immobilized APS-BCL in the kinetic resolution of (R,S)−1−phenylethanol

Kinetic resolution of (*R,S*)−1−phenylethanol catalyzed by immobilized APS-BCL in 7 various reaction media after 12 h of incubation was carried out by high-performance liquid chromatography (HPLC) (Fig. Supp-3). Due to the complete conversion of one of the substrates in the diisopropyl ether medium, the incubation time was shortened to 6 h. Analysis of the data (Table 3) revealed high values of catalytic parameters for all the solvents used. The highest values of enantiomeric ratio were obtained for *n*-hexane ($E = 844$), diisopropyl ether ($E = 519$), and *n*-heptane ($E = 497$). As regards the enantiomeric excess of products, for all the solvents, ee_p was in the range of 98–99 %, whereas the enantiomeric excess of substrates was the highest for *n*-hexane ($ee_s = 97$ % ± 0.5), *t*-butylmethyl ether ($ee_s = 89$ % ± 1.0), isooctane ($ee_s = 89$ % ± 2.0), and diisopropyl ether ($ee_s = 82$ % ± 1.0). As regards the conversion, the best results were obtained for *n*-hexane ($C = 49$ % ± 0.1), *t*-butylmethyl ether ($C = 48$ % ± 0.4), isooctane ($C = 48$ % ± 0.7), diisopropyl ether ($C = 45$ % ± 0.4) and *n*-heptane ($C = 45$ % ± 0.3). The reaction media tested had various partition coefficient (logP) values. The enantioselective parameters achieved in this study confirm the trend observed in a previous study [35] that the effect of the logP value on enantioselectivity did not tend to be linear. In the analysis of the enantiomeric ratio obtained in this study, we assumed the influence of the ramification of the solvent's carbon chain on the *E* value in the kinetic resolution of (*R,S*)−1−phenylethanol catalyzed by the immobilized APS-BCL. In the reactions performed in a medium with a lower logP value (1−2), the highest *E* was recorded in the solvent with a branched framework (diisopropyl ether and *t*-butylmethyl ether), while the lowest in dichloromethane, which has an unbranched structure. However, in the case of the reaction tested with more lipophilic solvents (with logP values of 4–5), the lipase catalyzing the kinetic resolution in the reaction medium with an unbranched structure, i.e. *n*-hexane and *n*-heptane, showed a higher enantiomeric ratio for isooctane (2,2,4-trimethylpentane), which has a ramified carbon chain. In the previous study [35], the effect of 8 organic solvents on the enantioselectivity of native APS-BCL in the kinetic resolution of (*R,S*)−1−phenylethanol had been investigated (Table 3). Diisopropyl ether proved to be the most suitable reaction medium. The results of the present study also confirm a high enantioselectivity of lipase in this solvent. Moreover, the reaction time allowing us to achieve the optimal value of the catalytic parameters was merely 6 h. It can therefore be concluded that immobilization of APS-BCL onto IB-150A for the reaction carried out in diisopropyl ether

Table 3
The results of kinetic resolution of (*R,S*)−1−phenylethanol catalyzed by immobilized APS-BCL in organic solvents and juxtaposition with catalytic parameters achieved using free lipase [35]. [a] – enantiomeric excess of products, [b] – enantiomeric excess of substrates, [c] – conversion, [d] – enantiomeric ratio.

	Immobilized						
Solvent	LogP	Time [h]	ee_p[a]	ee_s[b]	*C* [%]	*E*[d]	References
t-butylmethyl ether	1.06	12	98 ± 0.5	89 ± 1.0	48 ± 0.4	300	This study
Dichloromethane	1.25	12	99 ± 0.0	17 ± 2.5	15 ± 1.8	245	
Diisopropyl ether	1.52	6	99 ± 0.5	82 ± 1.0	45 ± 0.4	519	
Cyclohexane	3.44	12	99 ± 0.5	64 ± 1.8	39 ± 0.8	393	
n-Hexane	3.60	12	99 ± 0.0	97 ± 0.5	49 ± 0.1	844	
Isooctane	4.09	12	98 ± 0.5	89 ± 2.0	48 ± 0.7	300	
n-Heptane	4.66	12	99 ± 0.0	80 ± 1.0	45 ± 0.3	497	
	Free						
Solvent	LogP	Time [h]	ee_p[a]	ee_s[b]	*C* [%]	*E*[d]	References
t-butylmethyl ether	1.06	48	95	71	43	83	[35]
Dichloromethane	1.25	48	97	30	24	88	
Diisopropyl ether	1.52	24	98	90	48	309	
Toluene	2.73	48	98	76	44	227	
Cyclohexane	3.44	48	96	92	49	163	
n-Hexane	3.60	24	98	67	41	202	
Isooctane	4.09	48	95	89	48	117	
n-Heptane	4.66	48	94	99	51	171	

allowed us to obtain high values of catalytic parameters during a significantly shortened reaction time. This trend is noticeable in all the solvents used, although, with a lack of proportional growth. However, for *n*-hexane, the incubation time required to obtain high values of catalytic parameters for the immobilized lipase was reduced two-fold compared to the free form. The effects of solvents with various logP values on the enantioselectivity of BCL (native) in biodiesel synthesis was investigated by Liu et al. [92]. The reaction was executed in 5 various solvents. A higher lipase activity was observed in hydrophilic media (*t*-butanol, *t*-amyl alcohol) compared to hydrophobic media (petroleum ether, *n*-hexane, isooctane). To the best of the authors' knowledge [92], the effect of the organic solvent on lipase activity was dependent on the amount of water allowed to be retained in the microenvironment of the lipase active site. Zieniuk et al. [93], on the other hand, examined the effect of a solvent on the optimal hydrolytic activity of CAL-B. For this purpose, 20 reaction media with various logP were screened. The hydrolytic activity of CAL-B was assayed by the hydrolysis of *p*-nitrophenyl laurate. The reaction medium in which the highest CAL-B activity (expressed in U/g) was achieved was isooctane (>200 U/g). Satisfactory results were also obtained for acetone and *t*-butylmethyl ether. These reaction media were characterized by various logP values, which could indicate no linear relationship between lipase activity and partition coefficient. However, this suggestion does not exclude the lack of the influence of logP values on enzyme activity [93]. The authors stated that the reaction medium had a crucial impact not only on the solubility and solvation of the reactants, but also interacted with the active site of the enzyme [93]. Enzymatic activity can also be affected by the dielectric constant, the dipole moment, the ability to form hydrogen bonds, and denaturation capability. In this study, the authors suggested above that the presence of a branched structure in the hydrocarbon chain of the solvent could improve the enantioselectivity of APS-BCL. Our findings confirm the observation made by Zieniuk et al. [93] concerning the positive effect of the presence of branches in a hydrocarbon structure on lipase activity. Dias et al. [94] investigated kinetic resolution of (*R,S*)−1−phenylethanol catalyzed by PCL immobilized in double layered hydroxide of Zn/Al-Cl with *n*-hexane as the reaction medium (Table 4). High catalytic parameters were obtained. Our results corroborate the cited suggestions [94] that *n*-hexane could be used as the most favorable reaction medium. Yan et al. [95], on the other hand, performed kinetic resolution of (*R,S*)−1−phenylethanol catalyzed by surfactant-modified AOL in 8 various solvents (Table 4). Low values of conversion for all the media were obtained, with the exception of *t*-butylmethyl ether for which the conversion value was 46.8 %. Our results confirm that *t*-butylmethyl ether is another useful reaction medium for immobilized lipase.

In this study, the effects of the reaction media on the catalytic properties of immobilized APS-BCL have been investigated. High values of enantioselectivity in all the investigated media have been demonstrated. A reduction of the reaction time compared to the free lipase has been observed. Based on the catalytic parameters, diisopropyl ether, and *n*-hexane have been shown to be the most optimal media for use in this type of reaction. A possible influence of branching of the solvent carbon chain on the enantioselectivity of lipase has also been suggested, although further studies are needed to confirm this hypothesis.

3.3. Effect of the vegetable oils on the lipolytic activity of immobilized APS-BCL

The results presented in Section 3.1 and Section 3.2 described the high enantioselectivity of immobilized APS-BCL. It is worth noticing, that this enzyme is also characterized by other activities that needed to be investigated as part of comprehensive enzymatic studies. Therefore, the lipolytic activity of APS-BCL immobilized onto the polyacrylic support IB-150A using the multicomponent unsaturated fatty acids (MCUFAs) method, with the application of vegetable oils as the source of PUFAs and MUFAs, was tested. Important is that studied oils differ in the content of ω3-, ω6-PUFAs, and ω9-MUFAs, affecting their miscellaneous therapeutic properties (mentioned in the introduction). Additionally, the lipase demonstrates different lipolytic activity toward oils various in acid contents [35]. The assay was performed on 13 vegetable oils, lipolyzed by native or immobilized APS-BCL. The amount of lipase achieved using Bradford's method (LA_B) [76], the lipase loading (L_L), the activity of the amount of the free protein in the solution that had been immobilized onto the support (with the amount calculated by Bradford's method) (U_B, the activity concerns a sample with free lipase, without the support) [76,81], the activity of the amount of lipase immobilized onto IB-150A (U_I, the activity concerns a sample with the support), were determined. The results are shown and superimposed on the data obtained in the previous study [35] in Table 5. Moreover, the immobilization yield, activity recovery, and activity retention of APS-BCL in 13 oils (Table 6) was depicted. The APS-BCL amount, calculated by Bradford's method ranged from 7.9 to 9.3 mg per 10 mg of lipase used in the process, whilst the lipase loading was in the range of 158–186 mg/g of the carrier. The immobilization yield was in the range of 79–93 %. The highest U_I values were obtained during the lipolysis of oils from grape seed (39.3 ± 0.3 U), hemp (39.0 ± 0.3 U), safflower (35.7 ± 0.2 U), and rape (35.7 ± 0.5 U), while the lowest activity of the immobilized APS-BCL lipolyzed oils from peanut (28.8 ± 0.3 U), avocado (29.7 ± 0.3 U), sesame (30.5 ± 0.2 U) and camelina (30.7 ± 0.8 U). Based on the U_B value, the best results were obtained for APS-BCL activity decomposing oils from hemp (49.0 ± 0.3 U), avocado (46.8 ± 0.2 U), black cumin (45.3 ± 0.2 U), and safflower (45.0 ± 0.5 U). The lowest lipolytic activity was obtained for peanut oil (26.2 ± 0.3 U). In the prior work [35] the authors assayed the lipolytic activity of APS-BCL in the free form (10 mg for the test sample). The best results were obtained for oils from hemp (44.3 U), black cumin (42.7 U), safflower (42.2 U), and grape seed (42.2 U). In the case of oils with an ω6/ω9 ratio higher than 2.3, the activity of lipase (U) was markedly higher. In this study, the results have also shown this correlation. However, this relationship was not confirmed with black cumin oil. The activity of immobilized APS-BCL in this substrate was 33.0 ± 0.2 U, which is less than in oils from rape (35.7 ± 0.5 U), pumpkin seed (34.7 ± 0.2 U), and corn (33.8 ± 0.7 U) with a lower ω6/ω9 ratio in the composition. As regards the U_B value, the cut-off seems to be preserved, with the exception of APS-BCL activity from lipolyzed avocado oil (46.8 ± 0.2 U). In all the decomposed substrates, with the exception of peanut oil, the U_I of immobilized APS-BCL decreased relative to U_B. Of note is the fact that in this experiment, the effect of various vegetable oils used as substrates on the lipolytic activity of immobilized APS-BCL in one screening was tested the first time. Siódmiak et al. [76] performed an assay of the lipolytic

Table 4
The results achieved in the kinetic resolution of (*R,S*)−1−phenylethanol in the most suitable reaction medium in the current study and selected literature. "x" - Data not available, [a] – Amano lipase PS from *Burkholderia cepacia*, [b] – lipase from *Aspergillus oryzae*, [c] – Lipase from *Pseudomonas cepacia*, [d] – conversion, [e] – enantiomeric excess of products, [f] - enantiomeric ratio.

Lipase	Stereopreferention	Main solvent	C^d [%]	ee_p^e	E^f	References
APS-BCL[a]	*R*	*n*-Hexane	49 ± 0.1	99 ± 0.0	844	This study
APS-BCL[a]	*R*	Diisopropyl ether	45 ± 0.4	99 ± 0.5	519	This study
AOL[b]	*R*	*t*-butylmethyl ether	46.8	99	x	[95]
PCL[c]	*R*	*n*-Hexane	50	99	x	[94]

Table 5
The results of the enzymatic activities of native APS-BCL and immobilized onto IB-150A support in 13 various vegetable oils.

Oil	ω3[1]	ω6[1]	ω9[1]	ω6/ω9 ratio[2]	U_{in}[μM/min][3]	References	LA_{in} [mg][4]	L_s [mg/g][5]	U_p[μM/min][6]	U_i[μM/min][7]	References
Peanut	0	6.3	69	0.09	27.5	[35]	8.2 ± 0.2	164.0 ± 8.0	26.2 ± 0.3	28.8 ± 0.3	This study
Camelina	29	17	0	0.00	31.0		8.2 ± 0.1	164.0 ± 4.0	35.0 ± 0.2	30.7 ± 0.8	
Rape	7.5	16	56	0.29	39.0		8.8 ± 0.2	176.0 ± 8.0	41.3 ± 0.3	35.7 ± 0.5	
Pumpkin seed	0	43	30	1.43	34.0		8.1 ± 0.1	162.0 ± 4.0	40.7 ± 0.5	34.7 ± 0.2	
Walnut	10	17	17	1.00	27.2		8.1 ± 0.1	162.0 ± 4.0	36.0 ± 0.3	32.7 ± 0.2	
Sesame	0	38	37	1.03	38.3		9.1 ± 0.1	182.0 ± 4.0	38.8 ± 0.7	30.5 ± 0.2	
Avocado	0	9.6	51	0.19	34.2		9.2 ± 0.1	184.0 ± 4.0	46.8 ± 0.2	29.7 ± 0.3	
Rice	0	32	44	0.73	34.2		8.8 ± 0.2	176.0 ± 8.0	39.2 ± 0.2	31.3 ± 0.2	
Corn	0	49.9	28.5	1.75	32.2		9.3 ± 0.2	186.0 ± 8.0	36.7 ± 0.3	33.8 ± 0.7	
Black cumin	0	52	22	2.36	42.7		7.9 ± 0.1	158.0 ± 4.0	45.3 ± 0.2	33.0 ± 0.2	
Hemp	16	51	11	4.64	44.3		8.4 ± 0.2	168.0 ± 8.0	49.0 ± 0.3	39.0 ± 0.3	
Safflower	0	69	13	5.31	42.2		7.9 ± 0.1	158.0 ± 4.0	45.0 ± 0.5	35.7 ± 0.2	
Grape seed	0	62	18	3.44	42.2		8.3 ± 0.2	166.0 ± 8.0	42.0 ± 0.2	39.3 ± 0.3	

1 – The quantity of main PUFA/MUFA (ω3 – α-linolenic acid, ω6 – α-linoleic acid, ω9 – oleic acid) in 100 g of oil. Data were achieved from Oleofarm company (Poland),
2 - The quantitative ratio between ω6 and ω9 PUFAs/MUFAs.
3 – The enzymatic activity of free lipase in the initial amount of 10 mg,
4 – The amount of lipase being the difference between the initial amount of lipase and the amount remaining in the supernatant after immobilization onto 50 mg of IB-150A support (calculated in Bradford's method), 5 – The amount of protein per 1 g of support (Lipase Loading)
6 – the activity of amount of free protein in the solution that had been immobilized onto the support (the amount presented in LAB) 7 – The enzymatic activity of amount of APS-BCL immobilized onto IB-150A support (the amount presented in LAB)

Table 6
The results of immobilization yield (J_y), activity recovery (A_{rec}), activity retention (A_{ret}) of APS-BCL_s in 13 vegetable oil. [a]immobilization yield (in the range of 79–93%), [b]activity recovery [c]acitivity retention. The explanations and calculation methods were shown in subsection 2.3.3.2.

Oil	J_y^a	A_{rec}^b	A_{ret}^c
Peanut	82 %	104.9 %	110.2 %
Camelina	82 %	98.9 %	87.6 %
Rape	88 %	91.5 %	86.3 %
Pumpkin seed	81 %	102.0 %	85.3 %
Walnut	81 %	120.3 %	90.7 %
Sesame	91 %	79.6 %	78.5 %
Avocado	92 %	86.8 %	63.4 %
Rice	88 %	91.7 %	80.0 %
Corn	93 %	105.2 %	92.3 %
Black cumin	79 %	77.3 %	72.8 %
Hemp	84 %	88.0 %	79.6 %
Safflower	79 %	84.6 %	79.3 %
Grape seed	83 %	93.3 %	93.7 %

activity of CAL-B, immobilized onto 12 various polymeric supports (including IB-150A) using olive oil as the substrate. The lipase loading of IB-150A was 147.4 mg/g. Of all the other carriers, the highest amount of lipase was immobilized onto IB-150A. To the best of the authors' knowledge [76], it may have been due to the chemical nature of the support and the type of bond. As regards the activity, it was observed that the low value (1.2 U) may have been caused by the steric hindrance of the short epoxide spacer arms contained in IB-150A. This may have decreased the affinity of the enzyme active site for long chains of fatty acids from TAG as the substrates. Furthermore, as results from the loading of the lipase, a high availability of the enzyme to the support may induce the overloading of lipase onto this carrier [76]. It should be noted that APS-BCL immobilized onto IB-150A is attached both covalently and by adsorption. For this reason, the lipase activity decrease may have been caused by the stiffening of the molecular structure [96, 97]. This, together with the leak of the enzyme from the complex, may have influenced the durability and stability of the lipase and, in consequence, its activity [35]. As mentioned above, olive oil acted as the substrate in the study conducted by Siódmiak et al. [76], therefore, for comparison, the lipolytic activity of APS-BCL in this oil was examined as well. When compared with CAL-B, it seems that APS-BCL is characterized by an undeniably higher activity (1.2 U for immobilized CAL-B versus 35.7 ± 0.3 U for immobilized APS-BCL) The differences in activity were presumably related to the lid, the mobile part of the lipase. In the case of APS-BCL_s, Kim et al. [98] suggested that APS-BCL had no positional specificity. Schrag et al. [99] concluded that the molecular mechanism of substrate specificity of this lipase remains unknown. However, Sanchez et al. [13] reported that APS-BCL had high activity, irrespective of the chain length of the fatty acids. On the other hand, Pleiss et al. [100] suggested that APS-BCL showed specificity towards long-chain fatty acids, in turn, referring to the literature [8], CAL-B showed high activity to short- and medium-, and lower activity with long-chain fatty acids. It is assumed that one of the factors affecting the affinity may have been the fact that the short oligopeptide peptide does not completely cover the active centre, while retaining the ability to be absorbed onto the hydrophobic surface [76,101]. One should recall here the 'interfacial activation' mentioned in the introduction to this paper, i.e. the movement of the lid causing the reaction of the lipase with the insoluble substrate [102]. The hydrophobic groups present in the support strongly reacted with the hydrophobic part of the enzyme next to the active site, which resulted in the shift of the structural equilibrium to an open configuration [103]. The effect of pH of the lipase solution is also an important factor contributing to lipase activity, as it affects the generation of hydroxyl radicals and influences the surface charge and the interface potential properties of the catalyst. The results in our study corroborate that APS-BCL is characterized by a high lipolytic activity with respect to long-chain fatty acids. Nevertheless, the relationship between the length of the fatty acid carbon chain and APS-BCL activity is still unclear and will be investigated in further research.

Based on our results, it can be concluded that the decrease of activity of immobilized APS-BCL could be also reflected in the composition and structure of PUFAs and MUFAs present in the oil. As mentioned in the previous research [35], the variabilities in lipase activity depending on the oil used could be due to the ω6/ω9 ratio of PUFA/MUFA and/or the amount of ω3, ω6, PUFAs, and ω9 MUFAs contained in the substrate. Linoleic acid, the main compound of ω6 PUFAs, is characterized by a less twisted structure than α-linolenic acid in ω3 PUFAs. Moreover, it has two double bonds in contrast to oleic acid in ω9 MUFAs, which only has one double bond. On the other hand, a strong association between the lipase and the carrier may decrease the accessibility to the active site of the enzyme. Taking into account the steric hindrance, caused by the "chain kinks" of PUFAs and MUFAs, the cleavage conditions of substrates drastically decrease, thus, this link could harm lipase activity [104,105]. Our research confirms the suggestions of many authors [104–107] that the physical and chemical properties of PUFAs and MUFAs and the degree of unsaturation had, even after immobilization, a significant effect on lipase activity.

Analysis of our data revealed an interesting phenomenon. The U value achieved with 10 mg of APS-BCL was lower for all the oils tested (except for peanut oil, where it was slightly higher) than the activity achieved with the amount of APS-BCL calculated by Bradford's method. This could be related to the aggregation of the enzyme molecules (mainly near the isoelectric point) as a result of mutual interactions, caused by the high amount of lipase [108–110]. Despite the various influencing factors, it appears that the quantity of the lipase used has an undeniable effect on lipolytic activity. However, it can be suggested, that in the case of APS-BCL, this trend may not always be maintained, as it depends on the reaction conditions. Our results revealed that a suitable amount of APS-BCL led to the maximum activity of the lipase possible in the amount of the substrate used in the particular reaction under specified conditions. The optimal value for each substrate could, however, be different. A more in-depth understanding of this phenomenon can be helpful in the optimization of a suitable catalytic system, thus, it should be tested in a wider spectrum, which will be the topic of further research.

The kinetic parameters determined in this study are shown in Table 7. The K_m value for the free APS-BCL calculated for peanut oil as the substrate was 45.8 ± 1.2 U/mg while that for APS-BCL immobilized onto the polyacrylic support IB-150A was 110.0 ± 6.3 U/mg. As regards the V_{max} value, the maximal velocity for native APS-BCL was 2.508 ± 0.003 U/mg, while that for the immobilized APS-BCL was 2.879 ± 0.003 U/mg. As regards the turnover number (k_{cat}), the value of 0.293 ± 0.000 s^{-1} was obtained in our experiment for the native lipase and 0.336 ± 0.001 s^{-1} for immobilized APS-BCL. The values of V_{max} and k_{cat} for free and immobilized APS-BCL were comparable. In turn, the K_m value for immobilized APS-BCL was significantly higher than that for lipase in the free form. Zdarta et al. [85] determined the catalytic parameters for native and immobilized CAL-B by spectrophotometry, with the hydrolysis of *p*-nitrophenyl palmitate (*p*-NPP) to *p*-nitrophenyl (*p*-NPP). In contrast to our findings, V_{max} decreased after immobilization (V_{max} = 29.4 ± 1.3 U/mg of the immobilized lipase, V_{max} = 33.1 ± 1.4 U/mg of free form). This finding confirmed the results obtained by Yong et al. [109], where the V_{max} decreased after immobilization (138.9 U/mg of free lipase from *Candida rugosa* (CRL) versus 79.6 U/mg of CRL immobilized onto magnetic microspheres). The authors suggested that the decreasing value of maximal velocity may have been caused by a lower affinity for the substrate of lipase after immobilization. However, this difference in kinetic parameters may have been due to the variety of the lipases, supports, substrates and assay methods used. On the other hand, Singh et al. [82] studied the kinetic parameters of CAL-B and CRL immobilized onto silica nanoparticles. Based on the results, both K_m and V_{max} were found to be higher for the free lipase than for the immobilized form (3.6-fold for CAL-B and 1.5-fold for CRL for K_m, and 1.3-fold for CAL-B and 1.2-fold for CRL for V_{max}). According to the authors, the lower K_m could indicate a higher affinity of the immobilized lipase than that of the native enzyme. A reverse relationship was shown in this study: K_m was found to be higher for the immobilized lipase, which may have been due to a lower affinity of the immobilized APS-BCL for the substrate. From that point of view, it can be suggested that immobilization onto IB-150A caused extenuation of the affinity of the lipase for the substrate. It should be mentioned that suggested dependency concerned kinetic studies of APS-BCL immobilized on an appropriate support, concrete, oil (peanut), and conditions. In turn, Alagoz et al. [86] studied the kinetic parameters of formate dehydrogenases from *Candida methylica* (FDH) in the native and immobilized forms. The FDH assay was carried out with two substrates: sodium formate and NAD^+ at various concentrations. The immobilization was carried out onto various support systems: glyoxyl silica (FDH-GS), glyoxyl agarose (FDH-GA), and IB-150 (FDH-IB-150). K_m and k_{cat} were calculated. Analysis of the results for sodium formate revealed that K_m was lower for the immobilized FDH (3.06 ± 0.14 mM of FDH-GS, 3.22 ± 0.18 mM of FDH-GA, and 2.79 ± 0.15 mM of FDH-IB-150, respectively) than for the free enzyme (4.18 ± 0.22 mM), while k_{cat} was higher for FDH-IB-150 (0.434 ± 0.012 s^{-1}) and native FDH (0.182 ± 0.008 s^{-1}) but lower for FDH-GS (0.091 ± 0.006 s^{-1}) and FDH-GA (0.081 ± 0.007 s^{-1}). According to the suggestions in this report [86], the differences in K_m may have been caused by the hydrophobic interactions that changed the acidity constants of the main substrate bindings groups. Therefore, the polymeric properties of IB-150 (which is a copolymer of methacrylate carrying oxirane groups with a short epoxide spacer arm) may have been the reason for the decreasing K_m value and the increasing k_{cat} value. Of note is the fact that the method of determining the kinetic parameters in our study was different than that employed in the assays performed in the cited studies. More detailed studies comparing both methods for the same lipase and the same substrate are being planned.

Our experiment showed that the activity of APS-BCL after immobilization, in terms of the free lipase, changed depending on the oil used as the substrate. Based on the values of such parameters as the activity recovery (A_{rec}) or activity retention (A_{ret}) (or both), the phenomenon of hyperactivation (values above 100 %) was observed for walnut (A_{rec} =120.3 %), peanut (A_{rec} =104.9 %, A_{ret} =110.2), corn (A_{rec} =105.2), and pumpkin seed oil (A_{rec} =102.0 %). The immobilized APS-BCL showed hyperactivation, doubtless, due to the better accessibility to the substrate. Paradoxically, the steric hindrance generated by the short epoxide spacer arms present in IB-150A [76] may have positively affected APS-BCL activity in peanut oil. However, for most of the substrates used, these parameters were lower than 100 %. The results indicated that in most substrates, the lipolytic activity of APS-BCL after immobilization was lower than that in the native APS-BCL. As regards the enzymatic activity, expressed as the U value, the A_{ret} in many cases was lower than A_{rec} because of the decreasing values of native lipase in the amount of 10 mg than the amount calculated by Bradford's method [81]. Many studies [35,76,83,84] investigated the lipolytic activity of lipase determined by titration with olive oil as the substrate. Of note is the fact that in our study, for the peanut oil as the substrate, among others, the immobilized APS-BCL showed the lowest U activity and, on the other hand, hyperactivation in both activities was demonstrated. On the other hand, in recent reports [3,111] the effect of detergents and ionic strength on lipase hyperactivation has been observed. According to these suggestions, detergents could promote the stabilization of the open lipase form. However, high ionic strength could cause deactivation of the active centre of the lipase, and thus, influence on durability and stability of enzyme. This phenomenon undoubtedly requires more extensive research to confirm. However, the selection of the most suitable substrate for lipase activity, among vegetable oils, has not been investigated so far. This investigation could probably serve as an introduction to a significantly wider screening with the testing of lipolytic activity for other lipases immobilized onto various supports. The cut-off limit proposed in a previous study and confirmed in the present one could take various values for different lipases, which is an important aspect in recognizing lipase behavior considering the different reaction conditions. Hereby, the results will be necessary to model catalytic systems and helpful in testing new drug formulations.

Table 7
The kinetic parameters of APS-BCL in native and immobilized form, calculated from Lineweaver-Burk equations. K_m – Michaelis-Menten constant of native and immobilized APS-BCL, V_{max} – maximum velocity of native and immobilized APS-BCL, k_{cat} – turnover number of native and immobilized APS-BCL.

Lipase form	Kinetic parameters			
	K_m [U/mg]	V_{max} [U/mg]	k_{cat} [s^{-1}]	R^2
Native	45.8 ± 1.2	2.508 ± 0.003	0.293 ± 0.000	0.908
Immobilized	111.0 ± 6.3	2.879 ± 0.003	0.336 ± 0.001	0.930

4. Conclusions

The experiments we carried out demonstrated a significant effect of

substrate systems (reaction components and media) on the activities of immobilized APS-BCL. A high enantioselectivity of lipase in the kinetic resolution of (*R,S*)−1−phenylethanol was achieved. The usefulness of isopropenyl acetate as the acyl donor in obtaining chirally pure (*R*)−1−phenylethanol ($C = 49\,\% \pm 0.4$) was demonstrated. Diisopropyl ether ($C = 45\,\% \pm 0.4$, $E = 519$, after 6 h of reaction) and *n*-hexane ($C = 49\,\% \pm 0.1$, $E = 844$, 12 h of reaction), due to the high conversion and enantiomeric ratio, were selected as the most optimal non-aqueous media for immobilized APS-BCL. The lipolytic activity of APS-BCL immobilized onto the polyacrylic support IB-150A, while employing the MC-UFAs method (13 various vegetable oils), as a novel substrate screening, was evaluated. The cut-off limit value for the ω6/ω9 ratio (2.3) was confirmed. Peanut oil, due to hyperactivation ($A_{rel} = 104.9\,\%$, $A_{rel} = 110.2\,\%$), was proposed as the most suitable substrate for the assay of lipolytic activity. In conclusion, the immobilization of APS-BCL on the polyacrylic support IB-150A increased the enantioselectivity of the lipase in obtaining (*R*)−1−phenylethanol in non-aqueous media and reducing the reaction time. Kinetic studies of the lipolytic activity showed that IB-150A was suboptimal for APS-BCL in the aqueous medium. On the other hand, in the enantioselectivity study, a meaningful usefulness of this support in a non-aqueous medium was demonstrated. Our study showed that substrate engineering is a useful tool to modify (increase or decrease) lipase activity. According to the authors' assumptions, these issues could undoubtedly provide important support for the modeling of catalytic systems. Therefore, the behavior of APS-BCL immobilized onto Immobead support in various substrates, poorly described in literature so far, will be widely known and applied in various medical and pharmaceutical branches of science.

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CRediT authorship contribution statement

Jacek Dulęba: Investigation, Formal analysis, Writing – original draft, Methodology, Conceptualization, **Tomasz Siódmiak:** Conceptualization, Methodology, Formal analysis, Writing – original draft. **Michał Piotr Marszałł:** Supervision.

Author agreement

We confirm that the work described was original research that has not been published previously, and not under-consideration for publication elsewhere, in whole or in part. On behalf of all the authors, I confirm that all the authors have seen the current manuscript files and mutually agreed to the submission of this manuscript to "*Enzyme and Microbial Technology*". We further confirm that no conflict of interest exists in the submission of this manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.procbio.2022.06.003.

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Supplementary Material

Fig. Supp-1.

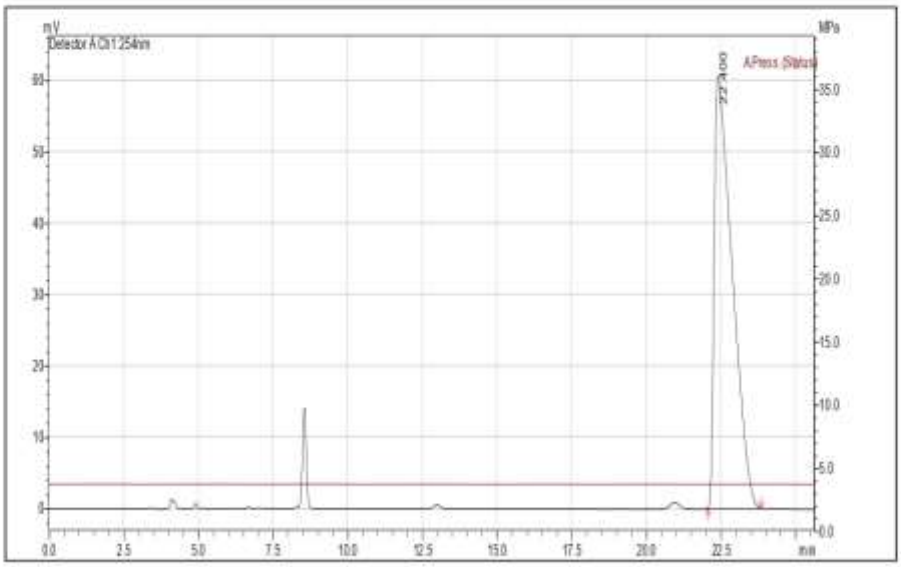


Fig. Supp-2.

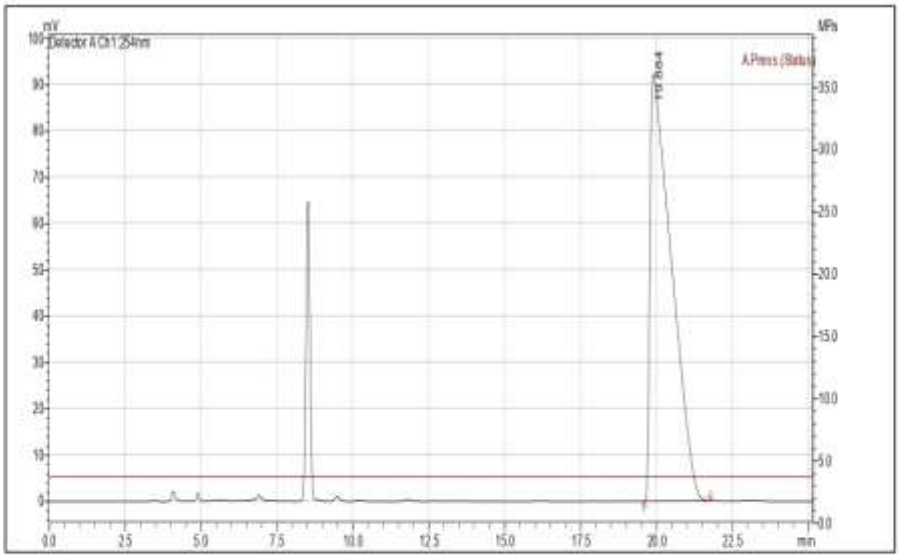
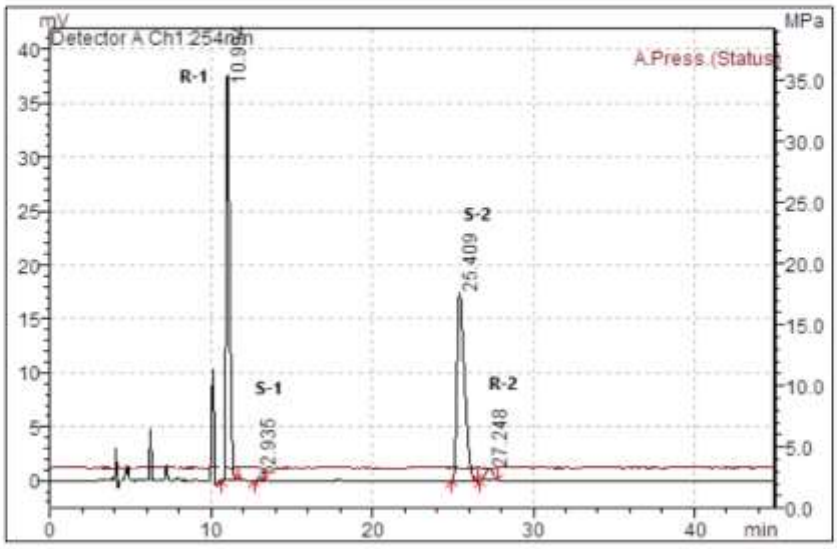


Fig. Supp-3.

I



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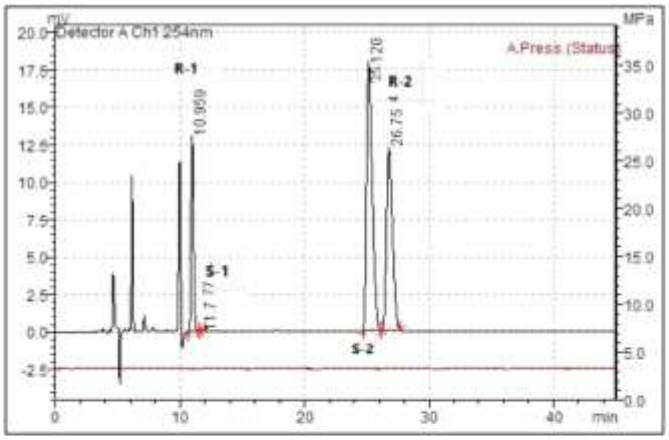

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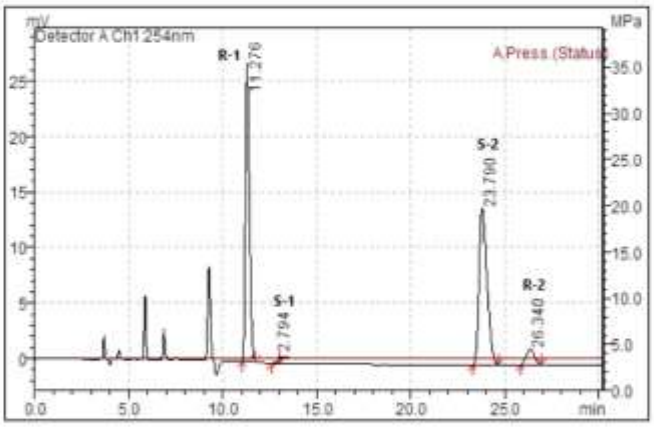
mV
Detector A Ch1 254nm
MPa
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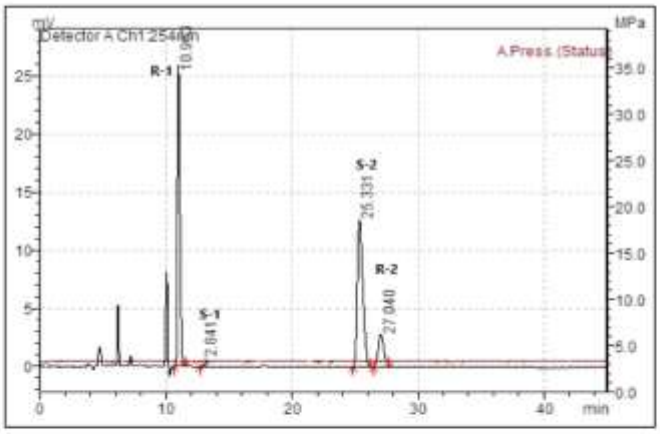
S-1
12.794
S-2
23.790
R-2
26.340
min

IV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MPa
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S-2
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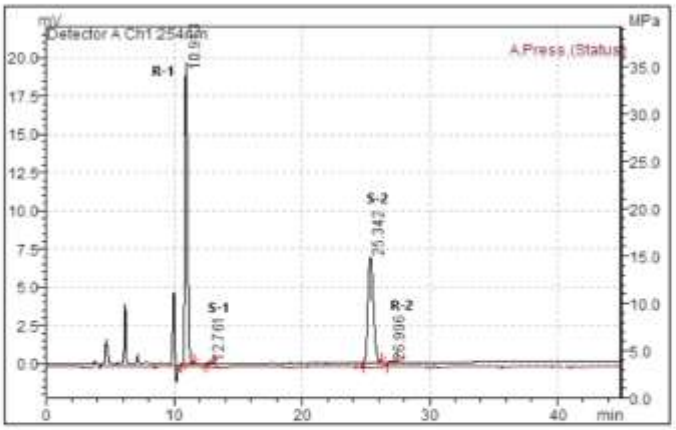
R-2
27.040
min

V


mV
Detector A Ch1 254nm
MPa
A:Press (Status)
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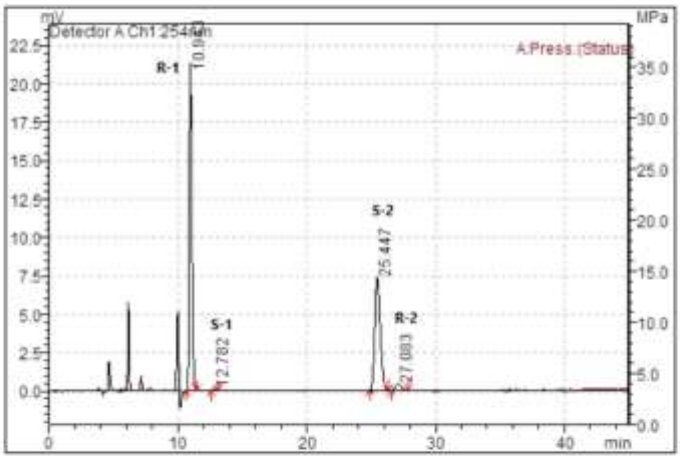
S-1
12.761
S-2
25.342
R-2
26.906
min

VI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MPa
A:Press (Status)
R-1
10.959
S-1
12.782
S-2
25.447
R-2
27.093
min

FIGURE LEGENDS

Fig. Supp-1.

HPLC chromatogram of (*R*)-1-phenylethanol standard. Chromatographic conditions: Lux Cellulose-3 (4.6 mm×250 mm×5 µm) column; mobile phase: *n*-heptane/2-propanol/ trifluoroacetic acid (98.7/1.3/0.15 v/v/v), flow rate=1 mL/min, t=15 °C, UV=254 nm. Reaction mixture was composed from (*R*)-1-phenylethanol (9,98 µL) and *n*-heptane (0,41 mL). Retention time for (*R*)-1-phenyletanol – 22,400 min.

Fig. Supp-2.

HPLC chromatogram of (*S*)-1-phenylethanol standard. Chromatographic conditions: Lux Cellulose-3 (4.6 mm×250 mm×5 µm) column; mobile phase: *n*-heptane/2-propanol/ trifluoroacetic acid (98.7/1.3/0.15 v/v/v), flow rate=1 mL/min, t=15 °C, UV=254 nm. Reaction mixture was composed from (*S*-1-phenylethanol (9,98 µL) and *n*-heptane (0,41 mL). Retention time for (*S*)-1-phenylethanol – 19,884 min.

Fig. Supp-3.

HPLC chromatograms of (*R,S*)-1-phenylethanol and theirs esters: **R-1** – (*R*)-1-phenylethanol acetate; **S-1** – (*S*)-1-phenylethanol acetate; **R-2** – (*R*)-1-phenylethanol; **S-2** – (*S*)-1-phenylethanol; Chromatographic conditions: Lux Cellulose-3 (4.6 mm×250 mm×5 µm) column; mobile phase: *n*-heptane/2-propanol/ trifluoroacetic acid (98.7/1.3/0.15 v/v/v), flow rate=1 mL/min, t=15 °C, UV=254 nm. Reaction mixture was composed from APS-BCL (8.0 mg, catalyst), IB-150A (50.0 mg, support), (*R,S*)-1-phenylethanol (9.98 µL, acyl acceptor), reaction medium (0.41 mL), vinyl acetate (28.25 µL, acyl donor). **I** – *t*-butylmethyl ether, **II** – dichloromethane, **III** – diisopropyl ether, **IV** – cyclohexane, **V** – *n*-hexane, **VI** – isooctane (2,2,4-trimethylpentane). Incubation time: 12 h (6 h for diisopropyl ether)

11.3. Zastosowanie lipazy z *Burkholderia cepacia* w reakcjach o znaczeniu farmaceutycznym – P3

Jacek Dulęba, Tomasz Siódmiak, Rafał Mastalerz, Natalia Kocot, Agnieszka Dębińska, Emilia Suchomska, Mateusz Czeczka, Kamil Cała, Dorota Wątróbska-Świetlikowska, Michał Piotr Marszałł, The application of lipase from *Burkholderia cepacia* in the enantioselective pharmaceutical biocatalysis, *Farm Pol*, 2022, Tom 78 (4), 194-200.

Lipaza z *Burkholderia cepacia* (BCL), ze względu na wysoką aktywność enancjoselektywną, jest powszechnie stosowana w przemyśle farmaceutycznym. Enzym ten charakteryzuje się szeroką specyficznością substratową, niezależnie od długości łańcucha węglowego substratu. Obecność wieczka umożliwia zmianę konformacji z zamkniętej na otwartą, co skutkuje odsłonięciem miejsca aktywnego enzymu [32]. Ta cecha umożliwia lipazie aktywność na granicy faz. Co więcej, BCL poddawano różnym technikom immobilizacji.

Lipaza BCL znalazła zastosowanie w szerokim spektrum reakcji o znaczeniu farmaceutycznym, najczęściej w kinetycznym rozdziale (*R,S*)-1-fenyloetanolu. BCL wykorzystano również do otrzymywania czystych enancjomerów z grupy niesteroidowych leków przeciwzapalnych (NLPZ), takich jak (*S*)-ibuprofen, (*R)*-ketoprofen oraz (*S*)-ketoprofen. Poza (*R,S*)-1-fenyloetanolem, BCL została również użyta w kinetycznym rozdziale innych prekursorów leków, takich jak kwas migdałowy czy pochodne propan-1,3-diolu. Ponadto, lipazę tą badano w reakcjach z udziałem pochodnych glicerofosfolipidów np. *myo*-inozytol oraz substancji pochodzenia naturalnego np. L-mentol.

Przegląd literatury wskazuje na zastosowanie BCL w reakcjach o znaczeniu farmaceutycznym.

6

The application of lipase from *Burkholderia cepacia* in the enantioselective pharmaceutical biocatalysis

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The application of lipase from *Burkholderia cepacia* in the enantioselective pharmaceutical biocatalysis

Lipases are widely applied catalysts in the pharmaceutical and biotechnological industries. They are characterized by high catalytic activity, and stability and are environmentally friendly. These enzymes could be used in free and in immobilized form. The immobilization procedure is exerted to improve enzyme activity and is carried out by different methods (e.g. physical adsorption, covalent binding, ionic interaction, encapsulation, cross-linking, entrapment). The most important lipase activities are enantioselectivity (e.g. the kinetic resolution of the racemic mixture) and lipolytic activity, enabling hydrolysis of triacylglycerol (TAG) to free fatty acids (FFA) and glycerol in an aqueous medium. One of the most commonly investigated is lipase from *Burkholderia cepacia* (BCL), characterized by high hydrolytic activity, regardless of the length of the organic acid chain. The enantioselectivity of BCL allows receiving chirally pure compounds in, among others, the transesterification reactions.

The review aimed to present the application of BCL in enantioselective reactions of pharmaceutical significance. Obtained using this lipase, the (*R*)-1-phenylethanol, is the chiral building block in drug synthesis. However, BCL has been also studied for the synthesis of nonsteroidal anti-inflammatory drugs (NSAIDs) such as (*S*)-ibuprofen and (*S*)-ketoprofen. Moreover, this enzyme has been used in the kinetic resolution of *myo*-inositol derivatives, applied in treating hormonal disorders, and (*R*,*S*)-mandelic acid used in dermatology. It was shown that BCL was also helpful in receiving chirally pure L-menthol, the demulcent and cooling compound in various drug forms. The application of BCL in catalyzing desymmetrization of propan-1,3-diol derivatives, applied as intermediates in various drug synthesis, has been noted. Therefore, BCL seems to be one of the most suitable catalysts for performing pharmaceutical biocatalysis and is of increasing interest to researchers and research consortia.

Keywords: lipase from *Burkholderia cepacia*, immobilization, enantioselectivity, chiral drugs, chiral intermediates.

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List of abbreviations (Wykaz skrótów)

Asp – asparagine (asparagina)
ATP – adenosine triphosphate (adenozynotrifosforan)
BCL – lipase from *Burkholderia cepacia* (lipaza z *Burkholderia cepacia*)
C – conversion (konwersja)
CALB – lipase B from *Candida antarctica* (lipaza B z *Candida antarctica*)
CLEAs – cross-linking enzyme aggregates (sieciowane agregaty enzymu)
CLECs – cross-linked enzyme crystals (sieciowane kryształy enzymu)
CoA – co-enzyme A (koenzym A)
COX – cyclooxygenase (cyklooksygenaza)
COX-1 – cyclooxygenase 1 (cyklooksygenaza 1)
COX-2 – cyclooxygenase 2 (cyklooksygenaza 2)
CRL – lipase from *Candida rugosa* (lipaza z *Candida rugosa*)
DAG – diacylglycerol (diacyloglicerol)
Ee – enantiomeric excess (enancjomeryczny nadmiar)
ee_p – enantiomeric excess of products (enancjomeryczny nadmiar produktów)
ee_s – enantiomeric excess of substrates (enancjomeryczny nadmiar substratów)
FFA – free fatty acid (wolne kwasy tłuszczowe – WKT)
GDM – *gestational diabetes mellitus* (cukrzyca ciążowa)
His – histidine (histydyna)
IP_3 – inositol triphosphate (trifosforan inozytolu)
MAME – mandelic acid methyl ester (ester metylowy kwasu migdałowego)
NLPZ – niesteroidowe leki przeciwzapalne (non-steroidal anti-inflammatory drugs – NSAIDs)
NSAIDS – non-steroidal anti-inflammatory drugs (niesteroidowe leki przeciwzapalne – NLPZ)
PCOS – polycystic ovary syndrome (zespół policystycznych jajników)
Ser – serine (seryna)
TAG – triacylglycerol (triacyloglicerol)
TBME – *t*-butylmethyl ether (eter *t*-butylometylowy)
TG – triglycerides (triglicerydy)
THF – tetrahydrofuran (tetrahydrofuran)
WKT – wolny kwas tłuszczowy (free fatty acid – FFA)
V – velocity (szybkość)

Lipase from *Burkholderia cepacia* (BCL)

Lipases (EC 3.1.1.3) are important players in the biotechnological industry. They belong to hydrolases due to breaking chemical bonds in an aqueous medium. The noteworthy aspect is that the lipases show increased activity at the water-lipid surface, which is named 'interfacial activation' [1-3]. These enzymes, through the hydrolysis of triglycerides (TG), to free fatty acids (FFA) and glycerol, demonstrate a significant role in the digestive metabolism of fats [4]. Lipases are applied in synthesizing and hydrolyzing organic compounds, e.g. esters and amines, involved in the pharmaceutical industry [5].

Moreover, these enzymes are used in the preparation of optically pure compounds, e.g. by a kinetic resolution of a racemic mixture of drugs, when the first of the enantiomers demonstrates preferable pharmacological activity and minor side effects than the second [6, 7]. The lipases show high activity in their free form. However, they can be subjected to various processes aiming to increase their activity significantly. The most common strategy in this regard is immobilization onto, among others, solid carriers [8, 9]. The advantage of such a procedure is an increase in the activity and stability of the enzyme. Nevertheless, it should be mentioned that final lipase activity after immobilization depends on lipase origin, type of carrier, reaction medium, and type of immobilization [10]. However, the catalytic parameters of native lipase could also be improved by adding surfactants. Yan et al. [11] studied the enantioselectivity of lipase from *Aspergillus oryzae* modified by a nonionic surfactant (Tween 40). The achieved results indicate the high activity and enantioselectivity in the kinetic resolution of (*R*,*S*)-1-phenylethanol.

Lipase from *Burkholderia cepacia* (BCL), previously called *Pseudomonas cepacia* (PCL) belongs to the most widely tested and applied lipases in organic synthesis. The structure of the lipase is shown in Figure 1 [12]. BCL is composed of 320 amino acid residues. According to the literature data [13, 14], the optimal thermal condition for BCL was 50°C. Schrag et al. [15] presented the conformation of PCL based on three independent models. The active site of this lipase is composed of Ser87, His286, and Asp264, forming a catalytic triad. During the interfacial activation, the movement of the α-5 helix, accompanied by the relocation of the α-9 helix was observed. Sanchez et al. [13] reported that BCL stood out with high hydrolytic activity to wide spectra of fatty acids, irrespective of acid chain length. Kim et al. [16] demonstrated that BCL reveals a lack of particular positional specificity. BCL shows low free-form stability; thus, it is widely studied in immobilized form. The immobilization of BCL has been so far performed by various techniques (Figure 2). Physical adsorption is technically the simplest method; therefore, among others, is the most commonly used. BCL was adsorbed on, e.g., microporous propylene, mesoporous silicates, or cross-linked polystyrene. Moreover, BCL was immobilized by entrapment, on κ-carrageenan, or covalent binding, on polyphenylene sulfide [13].

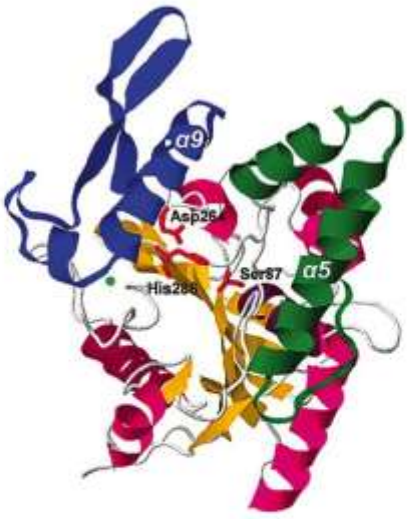

Figure 1. The molecular structure of lipase from *Burkholderia cepacia* (BCL) [12].
Rycina 1. Struktura lipazy z *Burkholderia cepacia* (BCL) [12]

Lipase from *Burkholderia cepacia* – obtaining of enantiopure pharmaceutical compounds

(*R*)-1-phenylethanol

BCL is widely used in compounds' kinetic resolution as chiral drugs or precursors. The racemic mixture of (*R*,*S*)-1-phenylethanol is one of the most commonly investigated model substrates. The resolution of this racemate has been performed to obtain a chirally pure (*R*)-enantiomer [17]. (*R*)-1-phenylethanol is used in the pharmaceutical industry as a chiral building block in drug synthesis. Moreover, according to the literature [18, 19], (*R*)-1-phenylethanol was applied as the ophthalmic preservative and inhibitor of cholesterol intestinal absorption. The kinetic resolution of (*R*,*S*)-1-phenylethanol is based on e.g. transesterification catalyzed by lipase with an acyl donor group to obtain optically pure (*R*)-1-phenylethyl ester. BCL demonstrates an enantiopreference to (*R*)-enantiomer. The methods of improvement of this reaction are widely described in many reports (Table 1). Li et al. [20] performed the kinetic resolution of (*R*,*S*)-1-phenylethanol catalyzed by BCL immobilized onto the macroporous resin, using the vinyl acetate as the acyl donor. The high enantioselective parameters were obtained. The conversion (*C*) was 50%, and enantiomeric excess of the substrate (ee_s) was 99% after 30 h of incubation. Kawakami et al. [21] studied transesterification of (*R*,*S*)-1-phenylethanol with BCL immobilized on modified silicates, with vinyl acetate as the acyl donor. Similarly, the lipase showed high enantioselectivity with the enantiomeric excesses of 98–99%, and *C* of 40%. However, immobilization can also decrease lipase activity. In this case, the immobilized lipase shows lower activity than the free form. Hara et al. [22] performed enantioselective acylation of (*R*,*S*)-1-phenylethanol, catalyzed by BCL in native form and immobilized by two different methods: immobilization in modified sol-gels and cross-linking with glutaraldehyde to form the cross-linked enzyme aggregates (CLEAs). The kinetic resolution was carried out with vinyl acetate as the acyl donor and the *t*-butyl methyl ether (TBME) as the solvent. According to achieved catalytic parameters for free lipase (*C* = 36%) and immobilized form (*C* = 47% for sol-gel complex and *C* = 51% for CLEAs) improvement of the lipase activity after immobilization was demonstrated. In another study, Melais et al. [23] performed kinetic resolution catalyzed by native BCL with vinyl acetate and isopropenyl acetate as the acyl donors, achieving (*R*)-1-phenylethanol. The results were better for vinyl acetate (*C* = 31%,

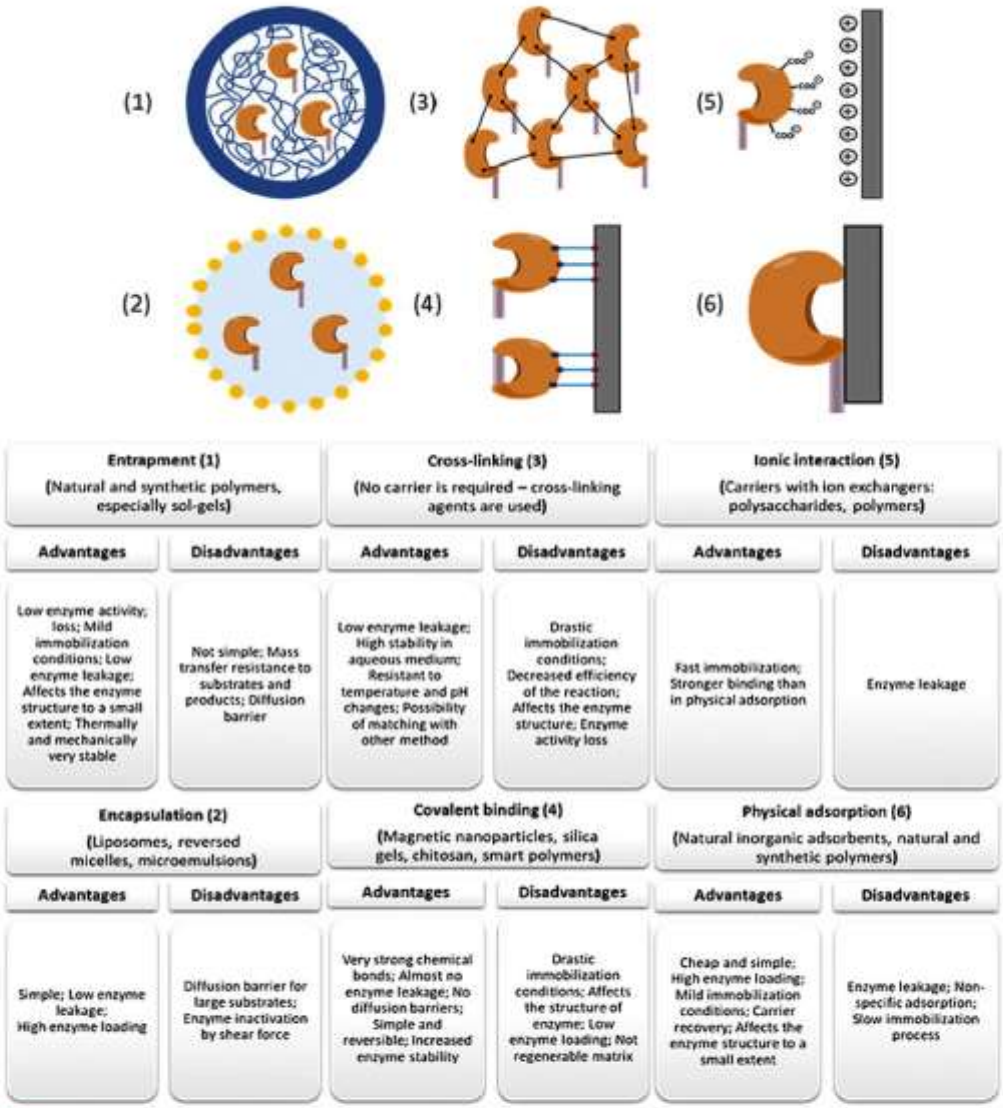

Figure 2. The review of immobilization techniques, including materials, advantages, and disadvantages.
Rycina 2. Przegląd technik immobilizacji, uwzględniający materiały, zalety i wady.

ee_p - 98.5%) than isopropenyl acetate (C - 11.4%, ee_p - 99%). It is worth noting that in the previous paper [24], the kinetic resolution of (*R,S*)-1-phenylethanol with isopropenyl acetate as the acyl donor has been investigated. The gained results (C - 51%, ee_p - 94%) indicate high enantioselectivity of BCL. It should be emphasized that the studies on the new methods of applying BCL in obtaining highly pure (*R*)-1-phenylethanol are ongoing.

(*S*)-ibuprofen and (*S*)-ketoprofen

Lipases are applied in the kinetic resolution of profens, the 2-arylpropionic acid derivatives, which belong to nonsteroidal anti-inflammatory drugs (NSAIDs) [25]. Some compounds from

Table 1. The kinetic resolution of (*R,S*)-1-phenylethanol catalyzed by BCL presented in various publications [20–24].
Tabela 1. Kinetyczny rozdział (*R,S*)-fenyloetanolu, katalizowany przez BCL, zaprezentowany w różnych publikacjach [20–24].

Form of lipase	Support	Acyl group donor	C (%)	ee (%)	Literature
Immobilized	Macroporous resin	Vinyl acetate	50	99	[20]
Immobilized	Silicates	Vinyl acetate	40	98–99	[21]
Immobilized	Sol–gel matrix	Vinyl acetate	47	–	[22]
	CLEAs		51	–	
Native (Free)	–	Vinyl acetate	31	98.5	[23]
		Isopropenyl acetate	11.4	99	
Native (Free)	–	Isopropenyl acetate	51	94	[24]

"–" – data not available

profens group undergo the characteristic phenomenon, known as the chiral metabolic inversion. The reaction is based on the transformation of (*R*)-enantiomer to preferable (*S*)-enantiomer form in the presence of coenzyme A (CoA), adenosine triphosphate (ATP), and Mg^{2+} [26]. In the case of (*R,S*)-ibuprofen, the differences in the therapeutic activities of each enantiomer have been described in the literature, indicating that the (*S*)-ibuprofen in the *in vitro* tests is characterized by 160 times higher activity than (*R*)-ibuprofen in inhibiting prostaglandin synthesis [6]. According to Siódmiak et al. [27], (*S*)-enantiomer inhibits the activity of both types of cyclooxygenases COX (COX-1 and COX-2) while (*R*)-ibuprofen inhibits COX-1 less potently than (*S*)-form and shows lack of inhibition of COX-2. Moreover, the gastrointestinal side effects, typical for this group of drugs, could be reduced using (*S*)-ibuprofen. Rajan et al. [28] showed the formation of the (*S*)-amyl ibuprofen catalyzed by cross-linked BCL (cross-linked enzyme crystals – CLECs) with *n*-amyl alcohol as the acyl acceptor. Furthermore, the kinetic resolution of (*R,S*)-ibuprofen was also studied using other lipases, e.g. *Candida rugosa* (CRL) and lipase B from *Candida antarctica* (CAL-B) [7, 29, 30]. In the case of (*R,S*)-ketoprofen, it was reported that (*S*)-enantiomer demonstrates lower toxicity and minor side effects with essentially better therapeutic activity. Li et al. [31] tested the kinetic resolution of (*R,S*)-ketoprofen catalyzed by BCL immobilized on resin with various alcohols as the acyl acceptors. The (*R*)-ketoprofen ester and the (*S*)-ketoprofen ester were obtained with the best results for 1-octanol ($E = 1.91 \pm 0.07$) and 1-heptanol ($E = 1.61 \pm 0.12$).

Myo-inositol derivatives

Myo-inositol is the organic compound in glycerophospholipids, which are included in neurotransmitters, e.g. inositol triphosphate (IP_3) and diacylglycerols (DAG). Moreover, *myo*-inositol increases the activity of glucose transporter proteins. Therefore, it has been applied as a supplement in the treatment of gestational diabetes (GDM – *gestational diabetes mellitus*) and polycystic ovary syndrome (PCOS) [32]. The derivatives of *myo*-inositol were used to synthesize enzyme inhibitors and building blocks in drug synthesis. Gammon et al. [33] carried out the synthesis of 2-deoxy-2-*C*-alkyl glucosides of *myo*-inositol. These analogs, due to inhibition of an *N*-deacetylase, were applied as the intermediates in mycothiol biosynthesis. Manoel et al. [34] performed the kinetic resolution of racemic 1,3,4-tri-*O*-benzyl-*myo*-inositol catalyzed by BCL immobilized onto Accurel MP 100 with vinyl acetate as acyl donor. The achieved catalytic parameters ($C = 49{,}9\%$, ee = 99% after 24 h of incubation) indicate high enantioselectivity of BCL.

(*R*)-mandelic acid

Mandelic acid is the chiral compound, applied as the building block in pharmaceutical synthesis. In dermatology, mandelic acid, because of its exfoliating and moisturizing activity, is contained in creams for patients with sensitive skin with moderate acne [35]. The (*R*)-mandelic acid methyl ester (MAME) was used in the evaluation of the specificity of acylase from various origins (*Escherichia coli, Kluyvera citrophila, Acetobacter turbidans, Bacillus megaterium*) [36]. These acylases were applied subsequently as the catalysts in the synthesis of crucial β-lactam antibiotics (ampicillin, cephalexin, cephamandol). The high specificity of acylase from *Escherichia coli* and *Kluyvera citrophila* to MAME was demonstrated by the ratio of $V_{MAME}/V_{PGK} > 1$. This selection of enzymes was helpful in the kinetically-controlled syntheses of antibiotics. In another study, Dąbkowska et al. [37] performed the kinetic resolution of racemic (*R,S*)-mandelic acid catalyzed by BCL in free form with vinyl acetate as the acyl donor in various thermal conditions (25–60°C). It was concluded that enzyme affinity constants are regardless of temperature. According to the obtained kinetic

parameters, it was suggested that BCL had a higher affinity to (*R*)-enantiomer than (*S*)-enantiomer; however, (*S*)-enantiomer reacted faster.

L-menthol

BCL in whole-cell form has been applied for obtaining enantiomers of natural compounds. L-menthol has commonly used as a coolant and pain demulcent in various drug forms such as ointments, creams, gels, and slices. Yu et al. [38] studied the hydrolysis of DL-menthyl acetate to obtain L-menthol catalyzed by various lipases. Based on received catalytic parameters (*C* = 43% and ee = 97%), the whole-cell BCL (American Type Culture Collection – ATCC 25416) showed high enantioselectivity. Therefore, according to the author's conclusions, this enzyme was the most suitable biocatalyst for achieving optically pure L-menthol.

Propan-1,3-diol derivatives

The derivatives of propan-1,3-diol have been concerned, due to substitution in position 2 (chiral center), as the important intermediates in achieving antitumor antibiotics, antifungal and immunosuppressive drugs [39]. BCL has been used to catalyze enantioselective desymmetrization. The transesterification of ((1,3-dihydroxypropan-2-yloxy)methyl)-5,6,7,8-tetrahydroquinazoline-2,4(1*H*,3*H*)-dione, was catalyzed by BCL in two solvents: tetrahydrofuran (THF) and *t*-butyl methyl ether (TBME). The catalytic parameters were obtained after 2.5 h of incubation (ee = 94% in THF, ee = 99% in TBME). The efficient enantioselectivity of the reaction showed the high usefulness of BCL in catalyzing these reactions.

Conclusions

Lipase from *Burkholderia cepacia* is characterized by the high utility in the reactions leading to obtaining chiral drugs or building blocks. Due to the immobilization of BCL onto supports by various methods, it is possible to increase the catalytic abilities of lipase. BCL has been used in catalyzing the reaction of obtaining chirally pure (*R*)-1-phenylethanol, (*S*)-ibuprofen, (*S*)-ketoprofen, (*R*)-mandelic acid, L-menthol, and *myo*-inositol and propane-1,3-diol derivatives. Each of the obtained compounds, to a greater or lesser extent, is applied in pharmaceutical reactions. Taking into account the received catalytic parameters, e.g. (ee = 99, *C* >40), it should be added that the new enantiomers were characterized by high optical purity. The comprehensive studies concerning the use of lipase in enantioselective biocatalysis allowed perceiving BCL as the versatile catalyst in obtaining chirally pure compounds, widely applied in medicinal and pharmaceutical areas.

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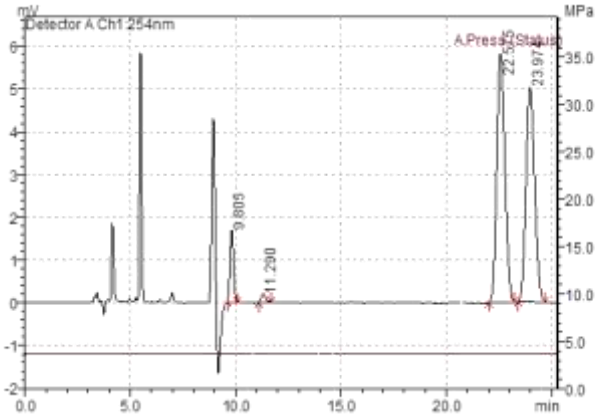
11.4. Ocena aktywności enancjoselektywnej i lipolitycznej lipazy Amano A z *Aspergillus niger* – P4

Tomasz Siódmiak, **Jacek Dulęba**, Natalia Kocot, Dorota Wątróbska-Świetlikowska, Michał Piotr Marszałł, The High 'Lipolytic Jump' of Immobilized Amano A Lipase from *Aspergillus niger* in Developed 'ESS Catalytic Triangles' Containing Natural Origin Substrates, *Catalysts,* 2022, 12, 853.

Lipaza Amano A z *Aspergillus niger* (AA-ANL) należy do najpowszechniej stosowanych komercyjnie dostępnych lipaz z *Aspergillus niger* (ANL). AA-ANL w reakcji hydrolizy, w porównaniu z APS-BCL, wykazuje zwiększoną aktywność w kierunku łańcuchów węglowych substratów średniej długości. Ponadto, cechuje się wysoką aktywnością i stabilnością. Podobnie jak APS-BCL, ze względu na obecność wieczka w strukturze, wykazuje aktywację międzyfazową, co umożliwia wzrost aktywności katalitycznej.

Przeprowadzono kinetyczny rozdział (*R,S*)-1-fenyloetanolu w reakcji transestryfikacji katalizowanej przez AA-ANL w formie wolnej i immobilizowanej na nośniku poliakrylowym IB-150A. Otrzymane wyniki nie wskazały na wysoką aktywność enancjoselektywną lipazy w obu formach (**Rycina 11, dane nieopublikowane w P4**). Z tego powodu, uznano reakcję za nieselektywną – wyniki uzyskane w badaniach nie zostały uwzględnione w publikacji.



Rycina 11. *Chromatogram kinetycznego rozdziału (R,S)-1-fenyloetanolu, katalizowanego przez immobilizowaną AA-ANL. Wysokie piki substratów wskazują na nieselektywność reakcji.*

Oznaczono aktywność lipolityczną immobilizowanej AA-ANL. Badanie zostało poprzedzone spektrofotometrycznym wyznaczeniem ilości immobilizowanego białka metodą Bradforda [85]. Hydrolizę enzymatyczną prowadzono w 22 olejach pochodzenia roślinnego oraz oleju rybnym [86]. Zbadano również wpływ zawartości poszczególnych kwasów ω3/ω6/ω9 w substratach na aktywność lipazy. Wyznaczono parametry katalityczne, takie jak I_y i I_e.

Otrzymane wyniki wskazywały na niską aktywność lipolityczną AA-ANL w formie wolnej. Z drugiej strony, zaobserwowano znaczący „skok" aktywności lipazy w formie immobilizowanej w

porównaniu z postacią natywną. Hiperaktywacji immobilizowanej lipazy nie uzyskano w reakcji hydrolizy triglicerydów kwasów tłuszczowych zawartych w oliwie z oliwek – w pozostałych olejach immobilizowana AA-ANL wykazywała niezwykle wysoką aktywność np. 2400.00% w oleju z pestek dyni, 1433.00% w oleju rybnym czy 1266.67% w oleju kukurydzianym. Na podstawie wyników uzyskanych w badaniach wstępnych, do dalszych oznaczeń wybrano olej arachidowy. Odnotowano wyższą aktywność lipolityczną w olejach z wartością stosunku ω6/ω9 powyżej 3.44 oraz spadek aktywności przy zmniejszeniu się wartości stosunku ω3/ω9.

Zbadano również aktywność lipolityczną AA-ANL immobilizowanej na dwóch innych nośnikach polimerowych, różniących się od IB-150A typem oddziaływań – IB-D152 (oddziaływania kationowe) i IB-EC1 (oddziaływania niejonowe adsorpcyjne). Wykazano znaczący wpływ typu użytego nośnika na ilość immobilizowanego białka. Dane literaturowe wskazują, że mała ilość lipazy unieruchomionej na nośniku można skutkować wyższą aktywnością lipolityczną – fakt ten może wynikać z braku zawady sterycznej, która może nastąpić w przypadku przeładowania nośnika lipazą.

Przeprowadzono badanie wpływu temperatury (25-65°C) i pH (4-9) na aktywność immobilizowanej AA-ANL. Zaobserwowano tzw. skok lipolityczny w całym zakresie warunków reakcji. Otrzymane wyniki sugerują znaczący efekt warunków reakcji na aktywność lipolityczną immobilizowanej AA-ANL. Ponadto, zbadano wpływ dwóch różnych mieszanin olejowych jako substratów na aktywność lipazy. Substraty sklasyfikowano na podstawie aktywności enzymu, uzyskanej w badaniach wstępnych: olej „mocny" (wysoka aktywność), olej „średni" (średnia aktywność), olej „słaby" (niska aktywność). W przypadku mieszanin olejów „mocnych", olejów „średnich" oraz oleju „mocnego" i „słabego" zaobserwowano wysoką aktywność lipolityczną, oraz niską aktywność lipolityczną w przypadku oleju „średniego" i „słabego" oraz średnią przy olejach „słabych". Nie stwierdzono efektu hiperaddycyjnego aktywności w żadnej z zastosowanych mieszanin.

W następnych etapach testowano wpływ ponownego użycia nośnika na aktywność lipolityczną AA-ANL w 4 cyklach, badanie stabilności AA-ANL oraz wyznaczono parametry kinetyczne reakcji. Otrzymane wyniki wskazały na nieznaczny spadek aktywności lipolitycznej AA-ANL względem pierwszego cyklu. Jednakże, badania stabilności wykazały znaczący spadek aktywności po 7-dniowym przechowywaniu immobilizowanej AA-ANL w komorze klimatycznej KBF P240. Biorąc pod uwagę parametry otrzymane w badaniach kinetycznych (wyższe K_m i V_{max}), wyznaczonych z krzywej Lineweavera-Burka, stwierdzono spadek powinowactwa immobilizowanej lipazy do substratu w porównaniu z formą wolną.

W przeprowadzonych badaniach otrzymano niską aktywność enancjoselektywną i wysoką lipolityczną lipazy AA-ANL immobilizowanej na nośnikach polimerowych. Jednakże, natywna AA-ANL wykazywała niską aktywność w obu typach reakcji. W oparciu o wyniki badań aktywności enzymatycznej lipaz, wprowadzono pojęcie „trójkąta katalitycznego", obejmującego układ enzym-nośnik-substrat ze wzajemnymi oddziaływaniami (**Rycina 12**) [87]. Wspomniany układ reakcyjny jako część tzw. biblioteki katalitycznej stanowi ważny aspekt w projektowaniu układów katalitycznych stosowanych w reakcjach o znaczeniu farmaceutycznym.

Rycina 12. *Schemat tzw. trójkąta katalitycznego i jego składowych [87].*

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Article

The High 'Lipolytic Jump' of Immobilized Amano A Lipase from *Aspergillus niger* in Developed 'ESS Catalytic Triangles' Containing Natural Origin Substrates

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Abstract: Lipase Amano A from *Aspergillus niger* (AA-ANL) is among the most commonly applied enzymes in biocatalysis processes, making it a significant scientific subject in the pharmaceutical and medical disciplines. In this study, we investigated the lipolytic activity of AA-ANL immobilized onto polyacrylic support IB-150A in 23 oils of natural origin containing various amounts of polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (MUFAs). The created systems were expressed as an 'ESS catalytic triangle'. A distinct 'jump' (up to 2400%) of lipolytic activity of immobilized AA-ANL compared to free lipase and hyperactivation in mostly tested substrates was observed. There was a 'cutoff limit' in a quantitative mutual ratio of ω-PUFAs/MUFAs, for which there was an increase or decrease in the activity of the immobilized AA-ANL. In addition, we observed the beneficial effect of immobilization using three polyacrylic supports (IB-150A, IB-D152, and IB-EC1) characterized by different intramolecular interactions. The developed substrate systems demonstrated considerable hyperactivation of immobilized AA-ANL. Moreover, a 'lipolytic jump' in the full range of tested temperature and pH was also observed. The considerable activity of AA-ANL-IB-150A after four reuse cycles was demonstrated. On the other hand, we observed an essential decrease in stability of immobilized lipase after 168 h of storage in a climate chamber. The tested kinetic profile of immobilized AA-ANL confirmed the increased affinity to the substrate relative to lipase in the free form.

Keywords: Lipase Amano A from *Aspergillus niger*; Immobead polyacrylic supports; vegetable oils of natural origin; fish oil; PUFAs; MUFAs; 'lipolytic jump'; hyperactivation

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1. Introduction

Biocatalysis has become an increasingly important scientific field in recent years. Given the multidisciplinary interconnections between biotechnology, pharmacology, and biochemistry, the use of enzymes in synthesis reactions and analysis is emerging as an interesting alternative to chemical catalysts. Catalytic reactions are characterized by low cost, mild conditions, and environmental friendliness. Therefore, they are included in the so-called 'green chemistry' domain. A wide range of enzymes with specific properties is applied in biocatalysis. As catalysts, lipases (EC 3.1.1.3) in particular deserve the attention of researchers. These enzymes, which are a significant part of the hydrolase group, show activity such as hydrolysis of triacylglycerols to fatty acids, di- and monoglycerols, and glycerol (Figure 1) [1–6]. The action occurs in the water–oil interface.

Furthermore, they are characterized by substrate specificity and stereoselectivity toward the ester bond [7–9]. An interesting phenomenon is so-called 'interfacial activation', an exposition of the lipase active center by the movement of the characteristic 'lid' [10–13]. The consequence is a change in lipase conformation from closed to open form, resulting in increased enzymatic activity. The 'interfacial activation' is specific for most lipases (e.g., *Aspergillus niger*, *Burkholderia cepacia*, *Candida rugosa*, *Thermomyces languinosis*, and *Rhizomucor miehei*). However, its occurrence has not been observed in some cases (e.g., lipase B from *Candida antarctica*, *Pseudomonas aeruginosa*, and *Burkholderia glumae*) However, the characteristic features of this enzyme group have been commonly applied in medicine, pharmacy, biotechnology, and cosmetology [13–19]. Lipases catalyze a wide range of reactions, e.g., esterification, transesterification, and interesterification, as well as acid-, amino-, and alcoholysis [14,20].

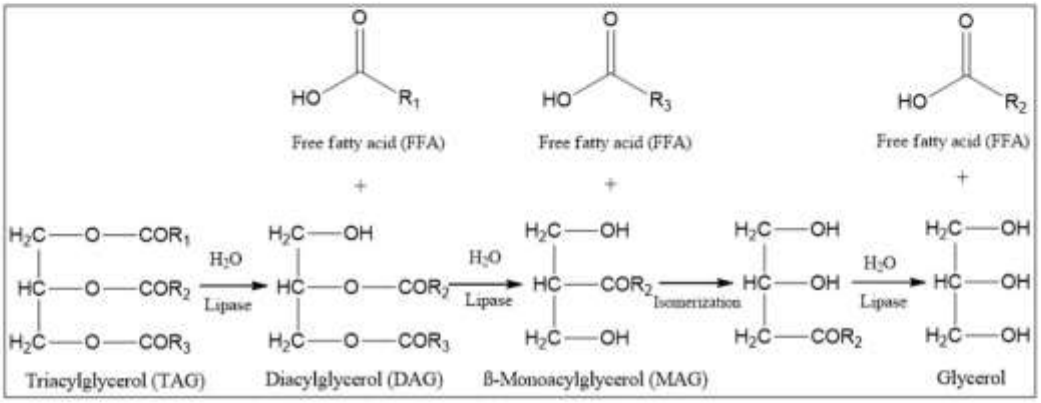

Figure 1. Hydrolysis of triacylglycerols catalyzed by lipase.

Amano lipase A from *Aspergillus niger* (AA-ANL) is a commercially available lipase of *Aspergillus* sp. origin. It is the most commonly applied enzyme in numerous catalytic reactions [21–25]. According to the literature, AA-ANL is characterized by positional specificity toward 1- and 3-glycerol groups and substrate specificity to the medium-length carbon chains. The optimal temperature range of this lipase is 40–50 °C, with an optimal pH of 4–7. Lipase from *Aspergillus niger* (ANL) exhibits high levels of activity, stability, and specificity [21,26,27]. Therefore, it has been used in the pharmaceutical, biotechnological, chemical, food, cosmetics, and agricultural industries [28,29]. Apart from commonly applied commercial preparations, ANL is often acquired in laboratories, e.g., by fermentation or genetic recombination [30–33]. Owing to its properties, AA-ANL has proven its usefulness in catalyzing abundant reactions in both free [30–37] and immobilized forms. Qiao et al. [34] exploited AA-ANL in the native (free) form to hydrolyze triglycerides from soybean oil to obtain biodiesel by efficient and environmentally-friendly means. On the other hand, Yildiz et al. [23] performed enzymatic hydrolysis of 1,3-ketoacetates catalyzed by AA-ANL to obtain chiral β-hydroxy ketones, which are used as building blocks in the synthesis of macrolide antibiotics and antitumor drugs. In another work, Kuboki et al. [35] applied AA-ANL to deacetylation to obtain regioselective cytidine. AA-ANL has also been tested as a catalyst in Henry reactions (nitroaldol reactions) to afford chiral β-nitroalcohols, which are used as intermediates in the synthesis of antibiotics, fungicides, and insecticides. The studies mentioned above [22,23,35] demonstrate the wide applicability of AA-ANL in catalyzing reactions of pharmaceutical significance. Given currently dominant research trends, improvement of the catalytic parameters of lipase (activity, stability, and specificity) is required. Immobilization is one of the most popular processes associated with the modifi-

cation of enzymatic properties [38–42]. The interaction between lipase and immobilization material, so-called 'support', occurs by various mechanisms, as shown in Figure 2.

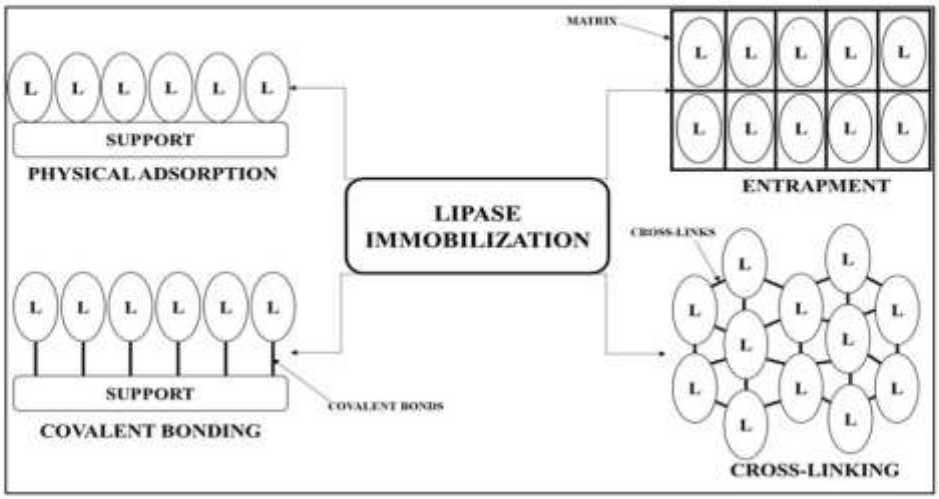

Figure 2. The simplified division of immobilization techniques. "L" indicates a lipase molecule.

Covalent binding should be included in the above-mentioned immobilization methods, among others, due to the remarkably durable connection of lipase support [43–45]. This interaction is stronger than physical adsorption. Therefore, the risk of 'leakage' of lipase from the formed complex is significantly decreased. Compared with crosslinking and entrapment methods, covalent binding is characterized by lower rigidity, which results in increased lipase activity. Furthermore, a sustainable immobilization technique should provide the optimal rotation in enzyme molecular structure towards open conformation exposition. However, in the case of the functional group of the support, containing the so-called 'short spacer arm', this interaction can cause attraction of the substrate too close to the enzyme active center, resulting in their blockade and, ultimately, limited availability, which determines decreased enzyme activity. On the other hand, the stability of the formed bond allows for the reuse of immobilized lipase in subsequent reaction cycles, owing to the strong interaction, preventing enzyme loss. An additional advantage of the above-mentioned technique is low reaction cost compared with crosslinking and entrapment. Moreover, immobilization does not require extreme conditions or aggressive chemicals (disturbing or destroying protein lipase structure), which is considered environmentally friendly. Therefore, this method has been commonly applied in the immobilization of many lipases, such as lipase B from *Candida antarctica* (CAL-B), lipase from *Candida methylica* (CML), *Thermomyces languinosis* (TLL), *Rhizomucor miehei* (RML), or Amano lipase PS from *Burkholderia cepacia* (APS-BCL) [44,46–52]. In the case of supports, the widely tested materials in covalent binding are of polyacrylic origin. These include, among others, commercially available Immobead 150 (IB-150). As materials with various functional groups (IB-150P: polar; IB-150A: apolar), they are used for immobilization of various lipases [46,51,53–55], as well as enzymes from other groups, such as β-galactosidases, pullulanases, dehydrogenases, or laccases [56–59]. IB-150 was investigated for immobilization of β-galactosidase from *Aspergillus oryzae* for the modification of support functional groups. Regarding AA-ANL, immobilization has been performed with several methods. However, the process of applying the Immobead polyacrylic supports for AA-ANL has not been reported in the literature to date. Zdarta et al. [60] immobilized AA-ANL onto the surface

of silica Stöber to improve the immobilization and testing of lipase kinetic parameters. In other studies conducted by the same authors, lipase AA-ANL was immobilized onto sponges from *Luffa cylindrica* in an attempt to improve the thermal and chemical stability of the catalyst. As mentioned above, the application of AA-ANL immobilized onto polymeric support IB-150B has not been studied. Therefore, it was suggested that studies concerning the above issues could be helpful in the modulation and development of a 'catalytic library' composed of a wide range of designed enzymatic models and support materials. To create such a system, and optimal substrate must be selected. The enzymatic activity of lipase is expressed by its lipolytic activity. The application of triglycerides containing fatty acids is determined by the length of the carbon chain appropriate for lipase specificity, e.g., CAL-B prefers short-chain fatty acids. In contrast, lipase APS-BCL shows high activity toward carbon chains, regardless of their length [10,61,62]. In turn, AA-ANL prefers substrates containing medium-length carbon chains [24]. The wide spectrum of substrates used for lipase-catalyzed hydrolysis includes fats with unsaturated fatty acids from both vegetables and animals, such as polyunsaturated fatty acids (PUFAs) ω3 (with three double bonds), ω6 (with two double bonds), and monounsaturated fatty acids (MUFAs) ω9 (with one double bond) of natural origin (not synthetic). α-linolenic (ALA), eicosapentaenoic (EPA), and docosahexaenoic (DHA) are among the most important ω3 PUFAs. They are an important component of a healthy human diet, especially EPA and DHA, the supplementation of which decreases cardiovascular disease risk. Additionally, they exert a slight antidepressive effect and support lipogenesis, which is a promising obesity treatment [62–67]. According to recent reports 60, ω3 PUFA supplementation, especially DHA, can accelerate the regeneration of the organism after COVID-19 infection [68,69]. For the above-mentioned reasons, increasing interest in including ω3 PUFAs in the diet has been observed [68–71]. Moreover, the formulation of cod liver oil, registered as an OTC drug, was recently introduced into the pharmaceutical market. Until recently, only preparations registered as dietary supplements or food for special medical purposes were available [69,70]. Regarding ω6 PUFAs, the main compound from this group is linoleic acid (LA), which, in its coupled form (CLA), despite transconfiguration, shows many health-promoting effects, such as decreasing cholesterol oxidation and increasing β-oxidation in skeletal muscles [63,72]. Another advantage of LA is the possibility of its endogenous production in human organisms compared with EPA and DHA. On the other hand, excessive dietary consumption of LA can cause a fatty liver [73] Oleic acid (OA) is the main representative of ω9 MUFAs. Like LA, OA is an important energetic reservoir for the organism and exhibits antisclerotic activity. However, excess OA can be harmful to the metabolism. Therefore, a PUFA-rich diet should be properly balanced. Hence, unsaturated fatty acids are applied in developed multicomponent (MC-UFA) systems. As suggested by the authors of previous works [61,62], the ω6/ω3 PUFA supplementation system seems to be the most beneficial for the human organism. In contrast, the proportion of ω6 to ω3 PUFAs should not be high due to the previously mentioned risk of obesity associated with fatty liver [73]. With respect to dietary supplements containing PUFAs and MUFAs, MC-UFAs contain both and occur in vegetable oils, which are, together with fish oil, the main sources of PUFAs in the human diet. The ratios of ω-PUFAs and ω-MUFAs in fish and vegetable oils are been shown in Table 1.

Preliminary studies and literature data show that the ratio of ω-PUFA content can influence lipase lipolytic activity in the lipolysis process. Therefore, applying fish and vegetable oils with various amounts of unsaturated fatty acids could be helpful in the development of optimal catalytic systems. An interesting model is the so-called 'catalytic triangle', which is composed of the enzyme, support, and substrate (ESS) related to each other by intramolecular interactions [74]. In this study, we aimed to investigate the effect of the broad spectrum of substrate systems with varying content of ω-PUFAs and ω-MUFAs on the lipolytic activity of AA-ANL in free form and immobilized onto polyacrylic support IB-150A. We tested the influence of six oil mixtures on the enzymatic activity of immobilized AA-ANL. In addition, the effect of temperature, pH, and three supports on the selected substrates was investigated. Moreover, the influence of reusing immobilization complex

AA-ANL-IB-150A on lipase activity was evaluated. We also verified the stability of the enzyme following storage in a climate chamber. Kinetic parameters were determined to establish the profile of the lipase affinity for the substrate in light of the lipolytic activity of immobilized AA-ANL corresponding to various amounts of substrate.

Table 1. The quantitative ratios of ω-PUFA and ω-MUFA acids in fish and vegetable oils. The data were established based on information provided by Oleofarm (Poland) and Sigma-Aldrich (Steinheim, Germany).

Oil	ω3/ω6	ω6/ω9	ω3/ω9	ω6/ω3	ω9/ω3	ω9/ω6
Peanut	-	0.09	-	-	-	10.95
Blackberry	1.71	-	-	0.59	-	-
Rapeseed	0.47	0.29	0.13	2.13	7.47	3.50
Pumpkin seed	-	1.43	-	-	-	0.70
Walnut	0.59	1.00	0.59	1.70	1.70	1.00
Sesame	-	1,03	-	-	-	0.97
Avocado	-	0.19	-	-	-	5.31
Rice	-	0.73	-	-	-	1.38
Corn	-	1.75	-	-	-	0.57
Black seed	-	2.36	-	-	-	0.42
Hemp	0.31	4.64	1.45	3.19	0.69	0.22
Safflower	-	5.31	-	-	-	0.19
Grape seed	-	3.44	-	-	-	0.29
Hazelnut	-	0.12	-	-	-	8.09
Evening primrose	-	10.09	-	-	-	0.10
Argan	-	0.66	-	-	-	1.51
Milk thistle	-	2.04	-	-	-	0.49
Borage	0.02	-	-	58.00	-	-
Apricot kernel	-	0.38	-	-	-	2.61
Olive	-	0.10	-	-	-	9.71
Fish	6.67	0.60	4.00	0.15	0.25	1.67
Sunflower	-	0.04	-	-	-	24.21
Linseed	0.04	-	-	24.23	-	-

2. Results and Discussion

2.1. Effect of Substrates on the Lipolytic Activity of Immobilized AA-ANL

In the present study, we evaluated the lipolytic activity of AA-ANL immobilized onto polyacrylic support IB-150A (U_I) and in its free form in an amount corresponding to the amount of lipase immobilized onto the support (U_B), as determined by the Bradford method (LA_B) [46,75]. We screened 22 vegetable oils and fish oil. Lipase loading (L_L), immobilization yield (I_y), and immobilization efficiency (I_e) were calculated based on the obtained results. The data are juxtaposed in Table 2. The amount of lipase immobilized onto polyacrylic support IB-150A was in the range of 4.0 ± 0.3–6.5 ± 0.1 mg, with an immobilization yield of 40 ± 3.0–65 ± 1.0%, whereas (L_L) was in the range of 80.0 ± 6.0–130.0 ± 2.0 mg on 1.0 g of support. According to the lipase amount corresponding to the amount immobilized onto the support, AA-ANL showed the highest activity in olive (11.33 ± 0.25 U), grape seed (6.83 ± 0.35 U), and rice (5.33 ± 0.51 U) oils. In contrast, the tested lipase was the least active in sesame, corn, and hazelnut oils (0.50 ± 0.10 U). In the case of AA-ANL immobilized onto IB-150A beads, the most activity was observed in milk thistle, linseed (9.17 ± 0.25 and 9.17 ± 0.10 U, respectively), and grapeseed (8.67 ± 0.17 U) oils. The results were compared with literature data [46,61]. We studied the lipolytic activity of the immobilization complex of AA-ANL-IB-150A toward a significantly wide spectrum of substrates for the first time. Dulęba et al. [61] investigated the lipolytic activity of free APS-BCL (initial amount: 10.0 mg) in 13 vegetable oils. High levels of enzymatic activity were achieved in the 27.5–44.3 U range. The highest activity of APS-BCL was shown in black cumin, hemp, safflower, and grapeseed oils, and the lowest levels of activity were observed in peanut and walnut oils. On the other hand, Siódmiak et al. [46] studied the lipolytic activity of free

CAL-B in olive oil (2.69 U). Our studies showed significantly lower (more than tenfold) enzymatic activity of AA-ANL relative to APS-BCL, which was simultaneously higher than that of CAL-B. The results confirmed that AA-ANL prefers fatty acids with a medium-length carbon chain. However, compared with APS-BCL, AA-ANL presents a lower affinity to the long carbon chains of fatty acids. Furthermore, AA-ANL is characterized by higher levels of activity than CAL-B, which favors the short carbon chains of fatty acids. The suggested conclusions concern lipase in its native form. The discussed relations altered in the case of immobilized AA-ANL. Considering the l_e parameters, immobilized AA-ANL, apart from a few cases, presents significant hyperactivation (≥100%) compared to the free lipase in an amount corresponding to the amount immobilized onto the support. In turn, with respect to the immobilization efficiency parameter, immobilized AA-ANL was the most active in pumpkin seed (2400.00%), fish (1433.33%), and corn (1266.67%) oils. A comparison of the obtained results with literature data [62] suggests that immobilization significantly improved the lipolytic activity of AA-ANL. Lipase APS-BCL hyperactivation occurred in four oils (peanut, pumpkin seed, walnut, and corn oils), as expressed by activity recovery, whereas hyperactivation expressed by activity retention was only demonstrated for peanut oil. The hyperactivation value of immobilized APS-BCL slightly exceeded 100%. However, it should be noted that APS-BCL showed high levels of activity in its free form. According to the enzymatic activity value (U), we hypothesized that immobilization decreased the lipolytic activity of APS-BCL. The results achieved in this study indicate a beneficial effect of immobilization on the lipolytic activity of AA-ANL. Immobilization of lipase onto a polyacrylic support was based on covalent binding of distinct groups contained in the support (oxirane group), with a strong nucleophilic group expressed in the lipase molecule (probably the amine group from the catalytic triad in the active enzyme center). In the case of APS-BCL, the so-called short 'spacer arm' formed by this bond is localized too close to the lipase active center. Therefore, the availability of lipase to the substrate was hindered [46]. Alagoz et al. [76] studied the effect of the 'spacer arm' on the activity of pectinase immobilized onto two types of support: Florisil (magnesium silicate) and nanosilicate. The results of the study showed that immobilization using a 3-glioxypropyltriethyloxisilicate (3-GTPMS) 'spacer arm' caused enzyme inactivation. The authors explained the effect of the significant rigidity of the immobilization complex, which contributed to the blockage of the pectinase active center. On the other hand, the 'spacer arm' of the glutaraldehyde had a positive effect on lipase retention. Glutaraldehyde has also been applied as a 'spacer arm' in the immobilization of lipase from *Thermomyces languinosis* on a multicomponent support system (metalorganic structure hydroxyapatite-glycyrrhizin-lithium) [77]. According to our research, regarding AA-ANL, the short 'spacer arm' seems not to block substrate access to the enzyme active site. The immobilization onto the support and stiffening of the complex promotes the substrate, ipso facto increasing lipase reactivity [61]. However, the steric hindrance in these complexes may impede the availability of the substrate to the enzyme active site [42,46,62,78]. The extremal increase in lipolytic activity is dependent on the type of substrate used. Each oil contains a certain amount of unsaturated fatty acids. All used substrates have ω6 PUFA, such as LA. Additionally, blackberry, rapeseed, walnut, hemp, borage, linseed, and fish oils contain ω3 PUFAs, such as ALA (fish oil also contains EPA and DHA). In turn, blackberry, linseed, and borage oils do not contain ω9 MUFAs acids, such as OA. The main representatives of the unsaturated fatty acids group differ in terms of the number of double bonds and chain rotation (so-called "kinks"), which could influence lipase lipolytic activity [61,62,79–81]. The effect of the ratio of ω6/ω9 PUFAs/MUFAs on APS-BCL activity has been described in previous works [61,62]. Authors assumed that in oils characterized by a ratio of ω6/ω9 of more than 2.3 (cut-off limit = 2.3) [61], lipase lipolytic activity is higher than in oils with a ratio below this value. This dependence was observed for both (free and immobilized) lipase forms. In the case of our studies, the cutoff limit (the ω6/ω9 PUFA/MUFA ratio) for AA-ANL lipase was 3.44, above which value it showed higher activity than in the case of other oils. With respect to the ratio of ω3/ω9 PUFAs/MUFAs, as the value declined, the AA-ANL activity decreased. However,

comparison was only possible between oils containing ω3 PUFAs; hence, the comparative spectrum was relatively limited. Taking into account the results of prior research [61,62], the effect of the ω6/ω9 ratio on AA-ANL activity was confirmed, although without linear dependence. Notwithstanding, the investigation showed the extremely beneficial effect of immobilization by the covalent binding method on AA-ANL lipolytic activity. The obtained catalytic parameters are undoubtedly helpful for the development of substrate systems to obtain modified optimal models. Such a system could be applied to in vitro tests with the aim of fat digestion, especially of fats unfavorable to human health. As mentioned in the Introduction, a diet containing unsaturated fatty acids should be properly balanced, as an excess of ω6 PUFAs can be harmful to the organism [73]. Our results showed the high catalytic specificity of AA-ANL to ω6 PUFAs, which can be useful in the future to create a therapeutic system supporting the release of fat excess from the body. However, further studies on wider substrate and support spectra should be conducted to confirm the beneficial lipase activity in the developed substrate systems; we attempted to do so, which will be described in a subsequent report.

Table 2. AA-ANL lipolytic activity in free form and immobilized onto IB-150A support in 22 vegetable oils and fish oil.

Oil	LA_B [1] (mg)	L_L [2] (mg/g)	I_y [3] (%)	I_e [4] (%)	U_B [5] (μmol/min)	U [6] (μmol/min)
Peanut	6.1 ± 0.1	122.0 ± 2.0	61.0 ± 1.0	250.00	0.67 ± 0.10	1.67 ± 0.10
Blackberry	5.8 ± 0.1	116.0 ± 2.0	58.0 ± 1.0	158.82	2.83 ± 0.17	4.50 ± 0.35
Rapeseed	5.9 ± 0.2	118.0 ± 4.0	59.0 ± 2.0	113.33	2.50 ± 0.10	2.83 ± 0.29
Pumpkin seed	4.0 ± 0.3	80.0 ± 6.0	40.0 ± 3.0	2400.00	0.17 ± 0.09	4.00 ± 0.25
Walnut	5.7 ± 0.1	114.0 ± 2.0	57.0 ± 1.0	440.00	0.83 ± 0.17	3.67 ± 0.42
Sesame	6.5 ± 0.1	130.0 ± 2.0	65.0 ± 1.0	566.67	0.50 ± 0.10	2.83 ± 0.25
Avocado	6.3 ± 0.2	136.0 ± 4.0	63.0 ± 2.0	428.57	1.17 ± 0.10	5.00 ± 0.17
Rice	4.5 ± 0.1	90.0 ± 2.0	45.0 ± 1.0	134.38	5.33 ± 0.51	7.17 ± 0.09
Corn	6.1 ± 0.1	122.0 ± 2.0	61.0 ± 1.0	1266.67	0.50 ± 0.10	6.33 ± 0.26
Black cumin	6.2 ± 0.1	124.0 ± 2.0	62.0 ± 1.0	240.00	1.67 ± 0.10	4.00 ± 0.29
Hemp	4.5 ± 0.2	90.0 ± 4.0	45.0 ± 2.0	516.67	1.00 ± 0.10	5.17 ± 0.17
Safflower	5.2 ± 0.2	104.0 ± 4.0	52.0 ± 2.0	377.78	1.50 ± 0.10	5.67 ± 0.25
Grape seed	5.0 ± 0.1	100.0 ± 2.0	50.0 ± 1.0	126.83	6.83 ± 0.35	8.67 ± 0.17
Hazelnut	4.4 ± 0.2	88.0 ± 4.0	44.0 ± 2.0	433.33	0.50 ± 0.10	2.17 ± 0.17
Evening primrose	4.4 ± 0.1	88.0 ± 2.0	44.0 ± 1.0	314.29	1.17 ± 0.17	3.67 ± 0.10
Argan	4.6 ± 0.2	92.0 ± 4.0	46.0 ± 2.0	600.00	0.50 ± 0.10	3.00 ± 0.17
Milk thistle	4.6 ± 0.2	92.0 ± 4.0	46.0 ± 2.0	343.75	2.67 ± 0.10	9.17 ± 0.25
Borage	4.8 ± 0.1	98.0 ± 2.0	48.0 ± 1.0	980.00	0.83 ± 0.09	8.17 ± 0.10
Apricot kernel	5.0 ± 0.1	100.0 ± 2.0	50.0 ± 1.0	310.00	1.67 ± 0.26	5.17 ± 0.35
Olive	4.5 ± 0.2	90.0 ± 4.0	45.0 ± 2.0	38.24	11.33 ± 0.25	4.33 ± 0.17
Fish	4.0 ± 0.3	80.0 ± 6.0	40.0 ± 3.0	1433.33	0.50 ± 0.10	7.17 ± 0.17
Sunflower	4.7 ± 0.1	94.0 ± 2.0	47.0 ± 1.0	120.00	1.67 ± 0.26	2.00 ± 0.25
Linseed	4.4 ± 0.2	88.0 ± 4.0	44.0 ± 2.0	175.00	1.17 ± 0.17	9.17 ± 0.10

[1]—the amount of lipase immobilized onto IB-150A (difference between initial lipase amount and the protein amount) in the supernatant via Bradford's method, [2]—the amount of lipase corresponding to 1.0 g of support, [3]—immobilization yield, i.e., the percentage ratio between LA_B and the initial amount of the lipase (10 mg), [4]—immobilization efficiency, i.e., the percentage ratio of lipolytic activity of immobilized lipase to lipase activity in free form in the amount corresponding to the amount of lipase immobilized onto the support (mean activity values were used for the calculations), [5]—the enzymatic activity of free lipase in the amount corresponding to the amount immobilized onto the support, [6]—the enzymatic activity of lipase immobilized onto the support (on beads). Data are presented as a means ± standard deviations of three analyses ($n = 3$).

2.2. The Effect of the Support on AA-ANL Lipolytic Activity

We evaluated the effect of three polymeric supports (Immobead: IB-150A, IB-D152, and IB-EC1) on the enzymatic activity of immobilized AA-ANL. The determination was performed on a free lipase sample in an amount corresponding to the amount immobilized on the support. The results, along with each support characteristic feature, are juxtaposed

in Table 3. The experiment was carried out using peanut oil as the reaction substrate. According to analysis of the various amounts of lipase immobilized onto the support, the largest amounts of lipase were immobilized onto polyacrylic IB-EC1 (5.0 ± 0.1 mg, I_y = 50.0 ± 1.0), and the smallest amounts were immobilized onto polyacrylic IB-D152 (1.3 ± 0.1 mg, I_y = 13.0 ± 1.0). Taking U_l as the I_e value, increased lipolytic activity of AA-ANL immobilized onto IB-D152 support was observed (U_l = 2.33 ± 0.10, I_e = 233%), compared with AA-ANL immobilized onto other supports. Siódmiak et al. [46] investigated the lipolytic activity of CAL-B immobilized onto 12 polymeric supports (Immobead) with olive oil as the reaction substrate. The results indicated the highest activity retention (A_{ret}) of CAL-B immobilized onto IB-D152 support (A_{ret} = 178.0%). The authors stated that high lipase activity immobilized onto this support may have been caused by electrostatic interactions of the polarized carboxylic group as the characteristic functional group of the support. It should be mentioned that the reaction medium was slightly alkaline (pH 7.4). Therefore, substrate binding and increasing concentration were promoted near the enzyme active center. This phenomenon may have been additionally facilitated by the strong bond between the polarized carboxylic group and the nucleophile (probably amine group) occurring in the active site of the enzyme. This binding (probably peptide), due to its considerable rigidity (conditioned by partially double-bond character) [82], could guarantee the high durability of the complex, i.e., resistant to 'enzyme leakage', with simultaneous blockage of the associated rotation. This phenomenon could allow for optimal location adjustment of lipase localization for high substrate concentrations. Because the immobilization type is characterized by a small amount of immobilized protein, the risk of steric hindrance is decreased. This means that the lipase molecules can hydrolyze more of the substrate. Comparing the activity results of AA-ANL onto IB-D152 support with that of AA-ANL onto IB-EC1 support, it seems that a larger amount of the immobilized lipase did not correlate with increased activity. However, the immobilization technique of lipase onto IB-EC1 was based on adsorption interactions characterized by strong hydrogen bonds [83]. Despite this, lipase molecules after adsorption were significantly less ordered [83] than in the case of covalent bonding, as well as less stable, which made them more susceptible to enzyme leakage. The adsorption of larger lipase molecules on the support surface [46] could increase steric hindrance, as expressed by decreased activity. Nevertheless, in all immobilization complexes, AA-ANL showed hyperactivation without the loss of activity compared to the free form—in the case of AA-ANL-IB-150A and AA-ANL-IB-D152 complexes, the activity increased. On the other hand, in the AA-ANL-IB-EC1 complex, the activity did not change. The obtained results confirm the beneficial effect of immobilization on AA-ANL lipolytic activity in each support. However, the study of lipase for a broader spectrum of supports and substrates is required to investigate, in more detail, the effects of immobilization on AA-ANL activity. Therefore, as mentioned in the previous subsection, this subject will be the aim of further studies.

Table 3. Lipolytic activity of AA-ANL immobilized onto three polyacrylic supports, as well as the characteristic features of each support (data achieved from ChiralVision).

Support	Interaction Type	Functional Group	Molecular Size (μm)	LA_B [1] (mg)	L_L [2] (mg/g)	I_y [3] (%)	U_B [4] (μmol/min)	U_l [5] (μmol/min)	I_e [6] (%)
IB-150A	Covalent	Epoxide, apolar	150–300	3.7 ± 0.1	74.0 ± 2.0	37.0 ± 1.0	0.17 ± 0.09	0.33 ± 0.09	200.00
IB-D152	Cationic	Carboxylic acid	350–700	1.3 ± 0.1	26.0 ± 2.0	13.0 ± 1.0	1.00 ± 0.17	2.33 ± 0.10	233.00
IB-EC1	Non-ionic, adsorption	Carboxylic ester	350–700	5.0 ± 0.1	100.0 ± 2.0	50.0 ± 1.0	0.17 ± 0.09	0.17 ± 0.09	100.00

[1]—the amount of lipase immobilized onto IB-150A (difference between initial lipase amount and the protein amount in the supernatant using Bradford's method), [2]—the amount of lipase corresponding to 1.0 g of the support, [3]—immobilization yield, i.e., the percentage ratio between LA_B and the initial amount of the lipase (10 mg), [4]—the enzymatic activity of free lipase in the amount corresponding to the amount immobilized onto the support, [5]—the enzymatic activity of lipase immobilized onto the support (on beads), [6]—immobilization efficiency, i.e., the percentage ratio of lipolytic activity of immobilized lipase to lipase activity in (its) free form in the amount corresponding to the amount of lipase immobilized onto the support (mean activity values were used for calculations). Data are presented as a means ± standard deviations of three analyses (n = 3).

2.3. The Effect of Temperature on the Lipolytic Activity of the Immobilized AA-ANL

We investigated the effect of temperature on the lipolytic activity of AA-ANL in free form and immobilized onto polyacrylic support IB-150A with peanut oil as the reaction substrate. The results are listed in Table 4. Two dependencies were observed: a change in the activity of free and immobilized AA-ANL (Figure 3) and the effect of immobilization on lipase activity, depending on thermal conditions (Figure 4). The study showed the highest enzymatic activity of immobilized AA-ANL at 45 °C, whereas free AA-ANL was the most active at temperatures of 45 °C and 65 °C. An interesting phenomenon (for both lipase forms) is a significant decrease in activity in the temperature range of 45–55 °C and an increase in activity in the range of 55–65 °C. Siódmiak et al. [46] studied the effect of temperature on the lipolytic activity of CAL-B immobilized onto polyacrylic support IB-D152. The maximum activity of immobilized CAL-B was in the range of 25–55 °C, and that of free CAL-B was 55 °C. At temperatures above 55 °C, the activity decreased. On the other hand, Sun et al. [84] demonstrated the thermal optimum of immobilized CAL-B to be 30 °C, above which a significant activity decrease was noted. Thermal stability studies (temperature range of 30–65 °C) on immobilized lipase from *Aspergillus niger* were performed by Dos Santos et al. [85] The results indicated a slight increase in ANL activity after immobilization and an activity maximum at 55 °C. Our studies demonstrated an essential improvement of activity of immobilized AA-ANL compared with the free form. However, the increase in activity of AA-ANL-IB-150A did not correlate with activity maximum (U). As mentioned above, AA-ANL showed the highest activity at 45 °C (2.67 ± 0.17 U), whereas the maximum increase in activity compared to the free form occurred at 25 °C (I_e = 800.00%). Despite a significant improvement in the catalytic parameters of AA-ANL after immobilization, low enzymatic activity compared to other lipases, such as APS-BCL [61,62], should be noted. However, modified thermal conditions did not seem to abolish the beneficial effects of immobilization on AA-ANL activity. The covalent interactions between IB-150A support and the active lipase center probably stabilized AA-ANL against unfavorable conditions. Therefore, further studies with the application of a broader spectrum of substrates, especially with higher AA-ANL activity, are suggested to further thermally optimize AA-ANL. Given the increase in AA-ANL activity in the temperature range of 55–65 °C, we propose extending the temperature range to reaching the point at which lipase activity decreases (also by denaturation).

Table 4. Effect of temperature on the lipolytic activity of AA-ANL immobilized onto polyacrylic support IB-150A.

Temperature (°C)	I_e [1] (%)	U_B [2] (µmol/min)	U_I [3] (µmol/min)	A_{rel} [4] (%)
25	800.00	0.17 ± 0.09	1.33 ± 0.21	100.00 ± 22.95
35	500.00	0.33 ± 0.09	1.67 ± 0.42	62.50 ± 21.31
45	400.00	0.67 ± 0.10	2.67 ± 0.17	50.00 ± 20.01
55	400.00	0.17 ± 0.09	0.67 ± 0.17	50.00 ± 14.93
65	150.00	0.67 ± 0.10	1.00 ± 0.16	18.75 ± 12.04

[1]—immobilization efficiency, i.e., the percentage ratio of lipolytic activity of immobilized lipase to lipase activity in free form in the amount corresponding to the amount of lipase immobilized onto the support (mean activity values were used for the calculations) [2]—the enzymatic activity of free lipase in the amount corresponding to the amount immobilized onto the support, [3]—the enzymatic activity of lipase immobilized onto the support (beads), [4]—the percentage ratio of the activity of the immobilized lipase under each condition to its activity in under conditions in which it achieved its maximum value. Data are presented as a means ± standard deviations of three analyses (n = 3).

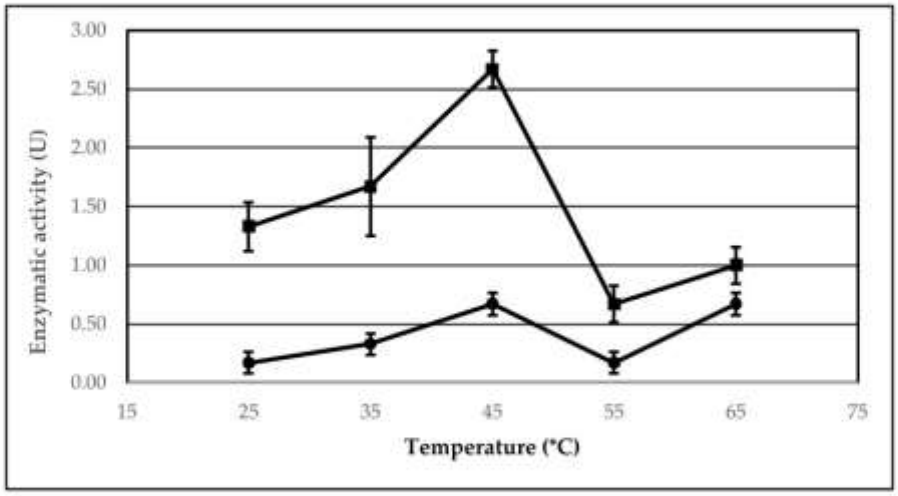

Figure 3. Effect of temperature on the activity of AA-ANL in free form (circles) and immobilized onto IB-150A support (squares). Reaction mixture: free AA-ANL (5.0 ± 0.2 mg) or AA-ANL immobilized on IB-150A beads, phosphate buffer (100 mM, pH 7.4), and an emulsion containing equal volumes of peanut oil and gum arabic suspension. The mixture was incubated for 30 min. The error bars represent the standard deviations of the mean.

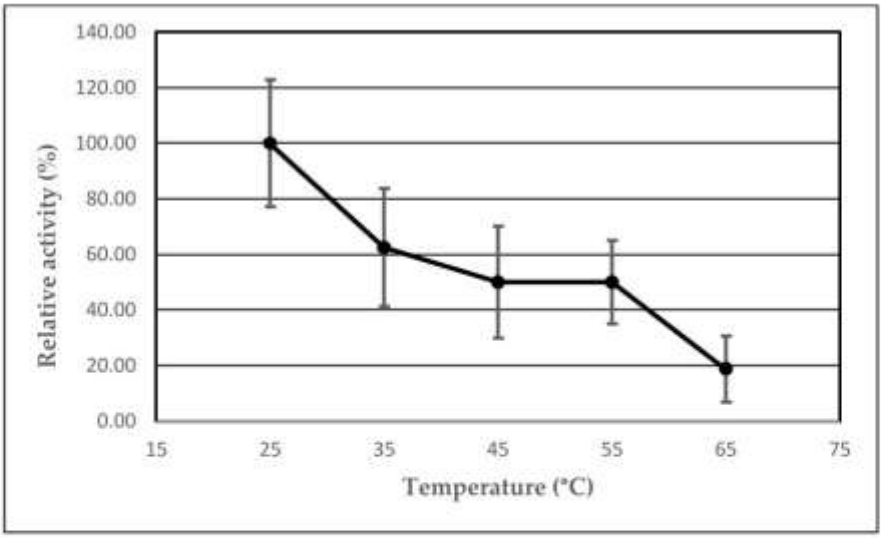

Figure 4. Effect of immobilization on AA-ANL activity under various thermal conditions. Reaction mixture: free AA-ANL (5.0 ± 0.2 mg) or AA-ANL immobilized on IB-150A beads, phosphate buffer (100 mM, pH 7.4), and an emulsion containing equal volumes of peanut oil and gum arabic suspension. The mixture was incubated for 30 min. The error bars represent the standard deviations of the mean.

2.4. The Effect of pH on the Lipolytic Activity of Immobilized AA-ANL

In this experiment, we investigated the effect of pH on the lipolytic activity of AA-ANL in its free form and immobilized on IB-150A polyacrylic support with peanut oil as the reaction substrate. The results are presented in Table 5. Analogous to the temperature studies, the following two dependencies were observed: a change in activity of AA-ANL in free and immobilized form (Figure 5) and the effect of immobilization on lipase activity, depending on the pH conditions (Figure 6). According to analysis of the received data, the maximum activity of immobilized AA-ANL was obtained at pH 7 (3.00 ± 0.51 U), whereas in the case of the free form, maximum activity occurred at pH 4–5, 9 (0.33 ± 0.10 U, 0.33 ± 0.17 U, and 0.33 ± 0.10 U, respectively). With respect to the profile of the free lipase, native AA-ANL demonstrated low activity in the whole range of tested pH values. Moreover, the values remained stable, and the increase occurred with the alkalization of the reaction medium. In the case of immobilized AA-ANL, the activity increased in the pH range of 4–7 and decreased at pH 8, with slight increase at pH 9. Compared with studies performed by other authors [60,86], the activity improvement with alkalization of the medium can be perceived as surprising, as authors had previously noted an activity decrease under these conditions. Our research confirmed the optimal pH for immobilized AA-ANL [60]. Similarly to the temperature studies, the pH experiment demonstrated the beneficial effect of immobilization on AA-ANL activity in the whole range of tested conditions. The most hyperactivation was noted at pH 7 (I_e = 1800%). However, a decrease in this value was observed with the alkalization of the medium. The literature data [62] show that lipases immobilized onto Immobead support demonstrate a consistent profile of catalytic parameters, as in the free form. Our results confirmed that immobilization of AA-ANL is not entirely responsible for increased activity with the neutralization of the medium, and free AA-ANL activity is maintained at a stable level. On the basis of the obtained results, it can be concluded that the immobilized AA-ANL seems more readable for the interpretation of lipase behavior under varying pH conditions because the free AA-ANL showed low activity, practically independent of the tested conditions. However, analogous to temperature studies, testing AA-ANL activity under more extreme pH conditions with a synchronously extended range of the substrate spectrum could help to optimize AA-ANL properties under various medium conditions.

Table 5. Effect of pH on the lipolytic activity of AA-ANL immobilized onto polyacrylic IB-150-A support.

pH	I_e [1] (%)	U_B [2] (µmol/min)	U_I [3] (µmol/min)	A_{rel} [4] (%)
4	250.00	0.33 ± 0.10	0.83 ± 0.33	13.89 ± 2.93
5	550.00	0.33 ± 0.17	1.83 ± 0.19	30.56 ± 17.65
6	1400.00	0.17 ± 0.09	2.33 ± 0.35	77.78 ± 21.33
7	1800.00	0.17 ± 0.09	3.00 ± 0.51	100.00 ± 30.02
8	1300.00	0.17 ± 0.09	2.17 ± 0.25	72.22 ± 34.74
9	700.00	0.33 ± 0.10	2.33 ± 0.51	38.89 ± 5.79

[1]—immobilization efficiency, i.e., the percentage ratio of lipolytic activity of immobilized lipase to lipase activity in free form in the amount corresponding to the amount of lipase immobilized onto the support (mean activity values were used for the calculations) [2]—the enzymatic activity of free lipase in the amount corresponding to the amount immobilized onto the support, [3]—the enzymatic activity of lipase immobilized onto support (on beads), [4]—the percentage ratio of the activity of the immobilized lipase under each condition to its activity under the conditions in which it achieved its maximum value. Data are presented as a means ± standard deviations of three analyses (n = 3).

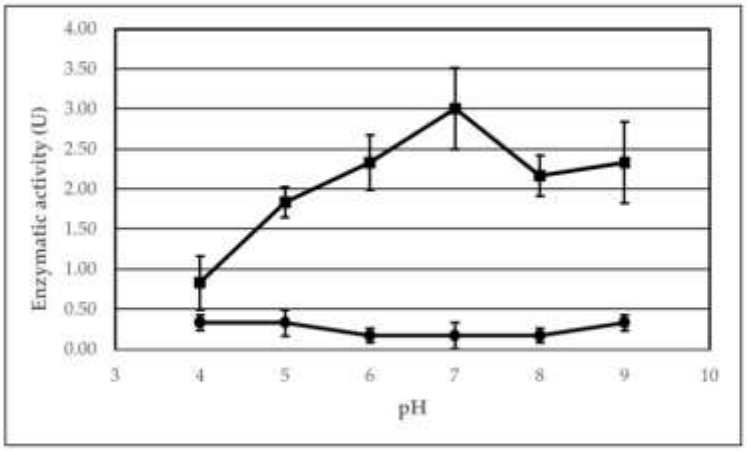

Figure 5. Effect of pH on AA-ANL activity in free form (circles) and immobilized onto IB-150A (squares). Reaction mixture: free AA-ANL (5.0 ± 0.2 mg) or AA-ANL immobilized onto IB-150A beads, phosphate buffer (100 mM, pH in the range of 4–9), and an emulsion containing equal volumes of peanut oil and gum arabic suspension. The mixture was incubated for 30 min. The error bars represent the standard deviations of the mean.

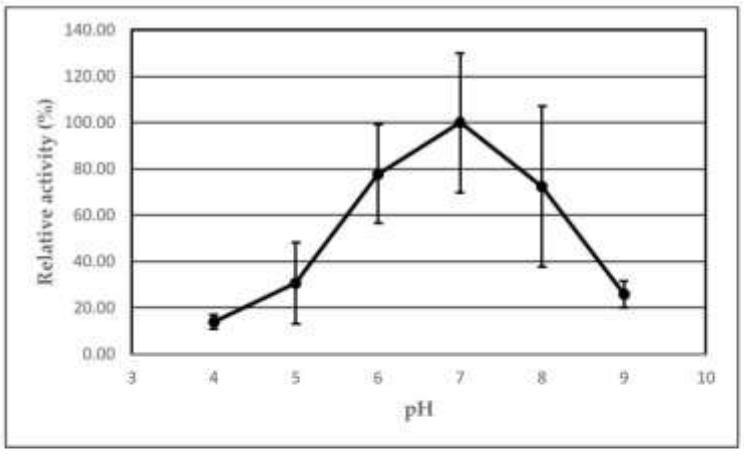

Figure 6. Effect of immobilization on AA-ANL activity under various pH conditions. Reaction mixture: free AA-ANL (5.0 ± 0.2 mg) or AA-ANL immobilized on IB-150A beads, phosphate buffer (100 mM, pH in the range of 4–9), and an emulsion containing equal volumes of peanut oil and gum arabic suspension. The mixture was incubated for 30 min). The error bars represent the standard deviations of the mean.

2.5. The Effect of Substrate Mixtures on the Lipolytic Activity of Immobilized AA-ANL

We investigated, for the first time, the activity of free and immobilized AA-ANL in a substrate mixture containing two oils (in equal volumes). The results are shown in Table 6. The mixtures were tested in five forms: (a) mixture of two oils with medium lipolytic activity of immobilized AA-ANL, (b) and (c) mixture of one oil with medium and one oil with low lipolytic activity of immobilized AA-ANL, (d) mixture of two oils with the

highest lipolytic activity of immobilized AA-ANL, (e) mixture of two oils with the lowest lipolytic activity of immobilized AA-ANL, (f) mixture of one oil with the highest and one oil with the lowest lipolytic activity of immobilized AA-ANL. Oils were selected based on the results obtained in Section 2.1, according to I_e parameters. The hyperactivation of immobilized lipase was observed in all mixtures, except a mixture of avocado and sunflower oils. High levels of hyperactivation (1800% and 1600%) were observed in the formulation containing oils from (a). Despite the essential activity values, they were lower than the lipase activity obtained with a single oil. Therefore, the additive effect of ω-PUFAs/ω-MUFAs, expressed in tested oils, should be excluded. However, AA-ANL-IB-150A in the rapeseed and olive oil mixture showed significantly higher hyperactivation than in the one-substrate mixture. In turn, immobilized AA-ANL showed lower activity in sunflower and avocado oil composition than in single oils. With respect to the activity of immobilized AA-ANL in formulations composed of oil, in which the highest activity of the catalytic system was obtained, and oil, in which the lowest activity of the catalytic system was obtained, the phenomenon of averaging the enzymatic activity was not observed. The dominant influence of the substrate towards which the lipase showed the highest and the medium activity was observed. In addition, the various activities of two mixtures containing oil with medium and low lipolytic activity of AA-ANL were. In terms of divergent dependencies, the additive or inhibiting effect was conditioned by the use of substrates and with ω-PUFAs/ω-MUFAs. Mixtures were selected on the basis of various activity ratios. As this was a novel research method (there is a lack of studies on lipase lipolytic activity in various substrates in one sample in the literature to date), it was not possible to compare our results to references. Moreover, the suggestions and interpretations presented herein are of a of pioneering nature and shed new light on the importance of the given dependencies. Therefore, it is necessary to develop this research in the future by using a wider spectrum of substrate mixtures, along with other lipases and supports, which will be the goal of future experiments.

Table 6. Lipolytic activity of free and immobilized AA-ANL in substrate mixtures. The oils in which (Section 2.1) AA-ANL-IB-150A showed the highest lipolytic activity are indicated by + +, the oils with medium lipolytic activity are indicated by +, and the oils with the low AA-ANL-IB-150A activity are indicated by −.

Oil Mixture 1:1	LA_B [1]	L_L [2]	I_y [3] (%)	I_e [4] (%)	U_B [5] (μmol/min)	U_I [6] (μmol/min)
(a) Milk thistle + Sesame (+)	5.6 ± 0.1	112.0 ± 2.0	56.0 ± 1.0	1800.00	0.17 ± 0.10	3.00 ± 0.10
(b) Sunflower + Avocado (+/−)	5.7 ± 0.1	114.0 ± 2.0	57.0 ± 1.0	42.00	9.17 ± 0.26	3.83 ± 0.33
(c) Milk thistle + Sunflower (+/−)	5.8 ± 0.1	116.0 ± 2.0	58.0 ± 1.0	400.00	1.00 ± 0.25	4.00 ± 0.15
(d) Pumpkin seed + Fish (+ +)	5.5 ± 0.1	110.0 ± 2.0	55.0 ± 1.0	1600.00	0.17 ± 0.10	2.67 ± 0.26
(e) Olive + Rapeseed (−)	5.5 ± 0.1	110.0 ± 2.0	55.0 ± 1.0	500.00	0.33 ± 0.09	1.67 ± 0.09
(f) Pumpkin seed + Olive (+ +/−)	5.3 ± 0.1	106.0 ± 2.0	53.0 ± 1.0	1400.00	0.17 ± 0.10	2.33 ± 0.16

[1]—the amount of lipase immobilized onto IB-150A (difference between initial lipase amount and the protein amount in the supernatant (using Bradford's method)), [2]—the amount of lipase corresponding to 1.0 g of the support, [3]—immobilization yield, i.e., the percentage ratio between LA_B and the initial amount of the lipase (10 mg) [4]—immobilization efficiency, i.e., the percentage ratio of lipolytic activity of immobilized lipase to lipase activity in free form in the amount corresponding to the amount of lipase immobilized onto the support (mean activity values were used for calculations), [5]—the enzymatic activity of free lipase in the amount corresponding to the amount immobilized onto the support, [6]—the enzymatic activity of lipase immobilized onto the support (on beads). Data are presented as a means ± standard deviations of three analyses (n = 3).

2.6. Reuse of Immobilized AA-ANL

We determined the activity of AA-ANL immobilized onto polyacrylic support IB-150A after four reuse cycles. The results are shown in Table 7. A minimal decrease was observed in immobilized AA-ANL activity in the subsequent reaction cycles (13% A_{rel} in four cycles). This phenomenon could be caused by leakage of protein from the support, as well as denaturation, which is probably related to the interaction of lipase molecules interacted with the support via physical adsorption. As it mentioned in the Introduction, this action is

less pronounced than the covalent binding between the lipase and support. The remaining lipase molecules were immobilized on the beads by interactions guaranteeing the activity of the AA-ANL-IB-150A complex, which prevented the protein from being washed out. Therefore, separating supports from the primary reaction mixture by filtration through the polyamide layer enables the efficient separation of solid and fat emulsions. However, a slight loss of carrier occurred (caused by the high viscosity of the emulsion and getting stuck in the pores of the polyamide layer), which may have also contributed to the reduced AA-ANL activity after subsequent cycles. Dos Santos et al. [85] tested the effect of support reuse on the lipolytic activity of ANL, i.e., so-called 'process stability'. In this case, ANL was increased due to residual fermentation of mangaba fruits and immobilization by covalent interactions on silicone matrix sol-gel. Lipase immobilized onto supports was reused for seven cycles. The lipase activity was maintained at a high level for four cycles (10% decrease in A_{rel}). After five cycles, a significant decrease to 50% of activity was observed. The authors separated the immobilized lipase from the substrate (olive oil) by filtration under a vacuum. Then, the immobilized complex was washed with *n*-hexane to remove of the residual substrate and product. The technique of separating Immobead carriers (containing lipase) by filtration with a polyamide layer for reuse presented herein is a novel method for determining lipolytic activity of lipase by acid-base titration. However, this method undoubtedly requires optimization, especially an increased number of reuse cycles, which will be the subject of future studies.

Table 7. Lipolytic activity of immobilized AA-ANL after support reuse (one, two, three, and four cycles). The activity parameter of free AA-ANL is shown as the initial value (one cycle).

Support Reuse Cycle	U_B [1] (µmol/min)	U_I [2] (µmol/min)	I_e [3] (%)	A_{rel} [4] (%)
1	1.83 ± 0.19	4.33 ± 0.25	236.00	100.00 ± 0.06
2	n/a	4.06 ± 0.18	221.00	94.00 ± 1.36
3	n/a	3.78 ± 0.22	206.00	87.00 ± 10.59
4	n/a	3.78 ± 0.12	206.00	87.00 ± 3.78

[1]—the enzymatic activity of free lipase in the amount corresponding to the amount immobilized onto the support, [2]—the enzymatic activity of lipase immobilized onto the support (on beads), [3]—immobilization efficiency, i.e., the percentage ratio of lipolytic activity of immobilized lipase to lipase activity in free form in the amount corresponding to the amount of lipase immobilized onto the support (mean activity values were used for calculations), [4]—percentage ratio of the activity of immobilized lipase in each cycle to the activity value of lipase support complex in one cycle; the I_e parameter was determined for all cycles based on AA-ANL activity in free form in one cycle. Data are presented as a means ± standard deviations of three analyses (n = 3).

2.7. Storage Stability of the Immobilized AA-ANL

We investigated the effect of complex AA-ANL-IB-150A storage on lipase stability. The study was performed under standard storage conditions (24 h at room temperature) and in a KBF P240 climate chamber (168 h, 40 °C). The obtained results (Table 8) demonstrated a significant (nearly ninefold) decrease in lipolytic activity of immobilized lipase after storage in a climate chamber, which suggests that the central factor conditioning the drastic activity loss could be the storage time. After 168 h of AA-ANL storage in a climate chamber, the enzyme leakage probably occurred due to the disengagement of covalent binding between lipase and support or protein denaturation. Therefore, despite the high rigidity, this connection was characterized by high lability during long storage, which was reflected by the essential activity decrease. Da Silva et al. [87] immobilized ANL on Celite diatomaceous earth by physical adsorption. The effect of storage on lipase activity was also studied. As in our study, the lipase was kept at temperature of 40 °C for five days. The investigation demonstrated a decrease in residual activity of 27%. In our research, the comparison concerned another catalytic parameter (I_e). Notwithstanding, the activity decrease was much higher. Therefore, applying immobilization complex AA-ANL-IB-150A is not beneficial in reaction requiring a long duration. Nevertheless, the exact stability profile of immobilized AA-ANL must be determined, which will be studied in future experiments.

Table 8. Lipolytic activity of immobilized ANL stored under the conditions recommended by ChiralVision (24 h, room temperature) and in a climate chamber (168 h).

Storage Time (h)	U_B [1] (µmol/min)	U_I [2] (µmol/min)	I_e [3] (%)
24	2.50 ± 0.10	4.67 ± 0.10	187.00
168	2.17 ± 0.17	0.50 ± 0.17	23.00

[1]—the enzymatic activity of free lipase in the amount corresponding to the amount immobilized onto the support, [2]—the enzymatic activity of lipase immobilized onto the support (on beads), [3]—immobilization efficiency, i.e., the percentage ratio of lipolytic activity of immobilized lipase to lipase activity in free form in the amount corresponding to the amount of lipase immobilized onto the support (mean activity values were used for the calculations). Data are presented as a means ± standard deviations of three analyses (n = 3).

2.8. Kinetic Studies

The kinetic studies of the reaction were performed with the aim of modifying the affinity of immobilized AA-ANL to the substrate. We calculated the following kinetic parameters based on Lineweaver–Burk curves: K_m, V_{max}, and k_{cat}. In addition, the correlation coefficient (R^2) was determined. The results are shown in Table 9. With respect to the K_m parameter, an extremal increase was observed in the affinity of the lipase to the substrate of immobilized AA-ANL (K_m = 1827.95 ± 0.17 mg/mL) compared with free AA-ANL (K_m = 29.20 ± 0.08 mg/mL). That is, an essential increase was observed in the activity of immobilized lipase relative to the free form. Dos Santos et al. [85] studied the kinetic parameters of ANL obtained by fermentation in free form and immobilized onto a sol-gel matrix. The obtained results of immobilized lipase (K_m = 115 ± 4 mM) juxtaposed those of with free lipase (K_m = 77 ± 2 mM) indicate higher affinity of ANL to the substrate, which was confirmed in our study. The authors suggested that lipase immobilization by covalent binding could change the lipase conformation and hinder substrate availability. The catalytic parameters resulting from the evaluation of lipolytic activity were determined by titration. However, olive oil was used as the substrate for the reaction. In our study, peanut oil was used as the substrate. On the other hand, Zubiolo et al. [87], performing a similar experiment investigating catalytic parameters of free and immobilized ANL (encapsulation in a sol-gel matrix), achieved an increase in enzyme affinity to the substrate by limiting mass transfer. Substrate solubility could also influence the behavior of encapsulated lipase [88]. In our study, covalent interactions between lipase and the support seemed to form a rigid complex that prevented protein leakage and steric hindrance due to the rotation limitation of other lipase molecules relative to one another. Therefore, the aggregation risk was limited. However, the catalytic parameters were mainly dependent on the curve course, which differed for each screening. Hence, we propose the development of kinetic studies of AA-ANL based on a wider support spectrum (the effect of intramolecular interactions between enzymes and support on lipase affinity to the substrate). In future studies, other kinetic mechanisms will be investigating considering new factors influencing lipase properties.

Table 9. Kinetic parameters of immobilized AA-ANL compared with lipase in free form. Data are presented as a means ± standard deviations of three analyses (n = 3).

Lipase	K_m (mg/mL)	V_{max} (U/mg)	k_{cat} (s^{-1})	R^2
AA-ANL	29.20 ± 0.08	0.061 ± 0.005	0.003 ± 0.001	0.933
AA-ANL-IB-150A	1827.95 ± 0.17	1.648 ± 0.019	0.082 ± 0.006	0.986

3. Materials and Methods

3.1. Materials

Amano lipase A from *Aspergillus niger*, 2-propanol (IPA), Bradford reagent, fish oil, and TrizmaBase were received from Sigma Aldrich (Steinheim, Germany). Acetone, methanol, disodium hydrogen phosphate, sodium dihydrogen phosphate, concentrated hydrochloric acid, citric acid, and arabic gum were purchased from POCH

(Gliwice, Poland). IB-150A, IB-D152, and IB-EC1 supports were purchased from ChiralVision (Leiden, The Netherlands). Vegetable oils (peanut, blackberry, rapeseed, pumpkin seed, walnut, sesame, avocado, rice, corn, black cumin, hemp, safflower, grape seed, hazelnut, evening primrose, argan, milk thistle, borage, linseed, sunflower, apricot kernel, and olive) were purchased from Oleofarm (Wroclaw, Poland). The water used in the study was purified by Milli-Q Water Purification System equipment (Millipore, Bedford, MA, USA). Lipolytic activity was investigated using a Seven Multi pH meter (Mettler Toledo, Schwerzenbach, Switzerland), a UV-Vis U-1800 spectrophotometer (Hitachi, Tokyo, Japan), and a Unimax 1010 incubator (Heidolph, Germany). The stability of immobilized AA-ANL was determined using a KBF P240 climate chamber (Tuttingen, Germany).

3.2. Immobilization of AA-ANL onto Polyacrylic Supports IB-150A, IB-D152, and IB-EC1

The immobilization process was performed based on the procedure recommended by ChiralVision and literature data [44], with slight modifications. A total of 50.0 mg of IB-150A support was placed in Eppendorf vials (2.0 mL). Then, beads were washed with 0.3 mL of 2-propanol for 15 min, and the solvent was removed by washing the sample with purified water and filtration with fluted filters. The separated supports were then dried. Then, they were transferred to Eppendorf vials (2.0 mL) containing a precisely mixed suspension of AA-ANL in phosphate buffer pH 7.0 (10.0 mg/mL). The samples were mixed with a spatula for 5 min in an ice bath and stored in a fridge (4.0 °C) overnight. The procedure was repeated in the case of polyacrylic supports IB-D152 and IB-EC1.

3.3. Determination of the Amount of Immobilized AA-ANL

The amount of protein immobilized on the support was determined by a modified Bradford method [46,75]. The study was performed using the UV-Vis spectrophotometric method (λ = 595.0 nm), measuring the absorbance of the free lipase remaining in the suspension after the immobilization process (concentration range: 1.0–10.0 mg/mL). The measurement was conducted twice. The amount of lipase immobilized onto the support was calculated with a calibration curve equation ($R^2 = 0.998$). The result was the two-sample mean. The lipase loading (L_L) was determined based on the obtained data. Then, the supports were transferred to a filter for 24 h until they were completely dry.

3.4. Determination of the Lipolytic Activity of Immobilized AA-ANL

The lipolytic activity of immobilized AA-ANL relative to free form was determined by titrimetric method, as described in previous studies and literature data [46,61,62,84,85]. The study was performed using 22 vegetable oils and fish oil. The reaction mixture (emulsion) containing the immobilized lipase was composed of supports with lipase, 3.0 mL phosphate buffer (pH 7.4), 2.5 mL of the appropriate oil (substrate), and 2.5 water suspension containing arabic gum. The reaction mixture was composed of lipase in free form in the amount of native AA-ANL corresponding to the amount embedded onto the support during immobilization (calculated using the Bradford method). An additional sample containing 10 mg AA-ANL (free sample assuming 100% effective immobilization) was added to each substrate. The mixtures were incubated at 37 °C for 30 min with rotation at 600 rpm. Then, the reaction was stopped by adding a 10.0 mL methanol and acetone mixture (volume ratio 1:1). Titration was conducted using 0.05 M NaOH standard solution at temperature, with phenolphthalein as the indicator. A change in mixture color to orange, together breaking of the emulsion, was considered the end point of titration. The activity of free lipase in the amount corresponding with the amount immobilized onto the support (U_B) and the activity of the lipase on the support (U_I) were determined based on obtained results. In addition, the ratio of immobilized lipase activity to free lipase activity, i.e., the immobilization efficiency (I_e) was calculated with the following equation:

$$I_e = \frac{U_I}{U_B} \times 100\% \quad (1)$$

Immobilization yield (I_y) was calculated according to the following formula [62]:

$$I_y = \frac{LA_B}{LA_{10}} \quad (2)$$

where I_y = immobilization yield; LA_B = amount of lipase, defined as the difference between the initial amount of lipase and the amount remaining in the supernatant after immobilization onto 50 mg of IB-150A (calculated by Bradford's method); and LA_{10} = initial amount of lipase (10 mg).

3.5. Effect of the Support on Lipolytic Activity of Immobilized AA-ANL

The experiment was conducted using methods similar to those described in Sections 3.2–3.4. Apart from the immobilization of lipase onto a covalent polyacrylic support, the process was also carried out on polyacrylic supports IB-D152 (cationic) and IB-EC1 (non-ionic). Peanut oil was applied as the reaction substrate. Based on obtained results, we calculated the immobilization yield (I_y), activity of free lipase in the amount corresponding with the amount immobilized onto the support (U_B), the activity of the lipase on the support (U_I), and immobilization efficiency (I_e).

3.6. Effect of Temperature and pH on the Lipolytic Activity of Immobilized AA-ANL

We determined the effect of temperature and pH on AA-ANL lipolytic activity in a similar manner as in Section 3.4. Among the tested oils, peanut oil was chosen for condition optimization. In the case of the temperature study, the mixture was incubated in the temperature range of 25–65 °C for 30 min. The samples were titrated at room temperature. For the pH study, lipase samples in free form (lipase weight) and in immobilized form were incubated by the appropriate buffer (3 mL) in the range of pH 4.0–9.0 (citric buffer 100 mM in the pH range of 4.0–6.0, phosphate buffer 100 mM in the pH range of 7.0–8.0, and tris-based buffer for pH 9.0). The following parameters were determined based on the achieved results,: activity of free lipase in the amount corresponding with the amount immobilized onto the support (U_B), the activity of the lipase on the support (U_I), immobilization efficiency (I_e), and the relative activity (A_{rel}), i.e., the ratio of the enzymatic activity of tested sample to the sample with maximal activity.

3.7. Effect of Substrate Mixtures on the Lipolytic Activity of Immobilized AA-ANL

Based on the results of the immobilization efficiency calculated in Section 3.4, the six substrate systems composed of two oils were developed: (a) oil mixture in which AA-ANL demonstrated medium lipolytic activity (milk thistle oil + sesame oil), (b) and (c) oil mixture in which AA-ANL demonstrated medium and low lipolytic activity (sunflower oil + avocado oil, milk thistle oil + sunflower oil), (d) oil mixture in which AA-ANL demonstrated the highest lipolytic activity (pumpkin seed oil + fish oil), (e) oil mixture in which AA-ANL demonstrated the lowest lipolytic activity (olive oil + rapeseed oil), (f) oil mixture in which AA-ANL demonstrated the highest and the lowest lipolytic activity (pumpkin seed oil + olive oil). The investigation was performed similarly as in Section 3.4 (incubation at 37 °C, lipase suspended in buffer (pH 7.4)) with modifications with respect to preparation of the reaction mixture; the emulsion was composed of 2.5 mL water suspension with arabic gum and 1.25 mL of each oil. Taking into account the obtained results, as in Section 3.4, the following parameters were calculated: the activity of free lipase in the amount corresponding with the amount immobilized onto the support (U_B), the activity of the lipase on the support (U_I), and immobilization efficiency (I_e).

3.8. Reusability of Immobilized AA-ANL

We investigated the reusability of supports with immobilized lipase according to the procedure described in Section 3.4 by introducing a novel reuse method. After incubation, the supports were separated from the reaction mixture by filtration with a polyamide layer to a conical flask with a volume of 50 mL. The fat emulsion, without supports, was

inactivated by adding 10.0 mL of a methanol and acetone mixture (volume ratio 1:1). A volume of 3.0 mL of phosphate buffer (pH 7.4) and 5.0 mL of fat emulsion were added to the flask with lipase immobilized on the supports, and the sample was incubated for 30 min at 37 °C with rotation at 600 rpm. The procedure was repeated twice for a total of four reuse cycles. The immobilized lipase was inactivated in the mixture after four cycles. The lipolytic activity of inactivated mixtures was determined by titration according to the technique described in Section 3.4. Based on the achieved results, the following parameters were calculated: activity of free lipase in the amount corresponding with the amount immobilized onto the support (U_B), the activity of the lipase immobilized on the support (U_I), immobilization efficiency (I_e), and the relative activity (A_{rel}), i.e., the ratio of enzymatic activity of the tested sample to the sample with maximal activity.

3.9. Storage Stability of Immobilized AA-ANL

The effect of the storage of complex AA-ANL-IB-150A determined similarly as in Section 3.4. Lipase was immobilized on supports stored in a KBF P240 climate chamber for 7 days (168 h) at a temperature of 40 °C and 75% rH and compared to a sampled immobilized using the standard procedure recommended by ChiralVision (24 h at room temperature). The free lipase was not stored in the climate chamber. Based on achieved results, we calculated the activity of free lipase in the amount corresponding with the amount immobilized onto the support (U_B), the activity of the lipase on the support (U_I), and immobilization efficiency (I_e).

3.10. Kinetic Studies

The kinetic studies of AA-ANL in free from and immobilized onto polyacrylic support IB-150A were conducted according to the procedure described in Section 3.4 and literature data [46,47,62,89,90]. Peanut oil was used as the substrate in the concentration range of 100–800 mg/mL. Based on obtained results, the kinetic Michaelis-Menten constant (K_m), the maximal velocity of the reaction (V_{max}), and turnover number (k_{cat}) according to the Lineweaver-Burk curve were calculated using the following equations:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}} \quad (3)$$

where V is the initial velocity, V_{max} is the maximal velocity, K_m is the Michaelis-Menten constant, and [S] is the initial substrate concentration.

$$k_{cat} = \frac{V_{max}}{[E_T]} \quad (4)$$

where $[E_T]$ is the total enzyme amount in the reaction mixture.

4. Conclusions

In this work, we described a study of the lipolytic activity of AA-ANL immobilized onto polyacrylic support IB-150A in 22 vegetable oils and fish oil. We tested the effects of three supports, six substrate mixtures, temperature, and pH on the lipolytic activity of immobilized AA-ANL. In most substrates, a significant 'jump' of enzymatic activity of immobilized AA-ANL was observed compared to lipase in free form, the activity of which was very low. High levels of hyperactivation parameters were demonstrated (the highest I_e = 2400% in pumpkin seed oil). A mutual quantitative effect of ratios of ω3 ω6-PUFAs, and ω9 MUFAs on AA-ANL activity was suggested. A 'cutoff limit' of ≥3.44 in the ω6/ω9 PUFAs/MUFAs ratio was proposed, above which increased activity of AA-ANL was observed; when the ratio of ω3/ω9 PUFAs/MUFAs declined, the AA-ANL activity decreased. The beneficial effect of polyacrylic supports on AA-ANL activity (highest activity of complex AA-ANL-IB-D152 — I_e = 233%) was confirmed. The hyperactivation of AA-ANL was demonstrated in the oil mixture. A 'lipolytic jump' of immobilized AA-ANL was

observed in the whole range of tested temperature and pH values (maximal activity at pH 7). On the other hand, high levels of lipase activity were observed under extremal thermal (65 °C) and medium (pH 9.0) conditions. In terms of reuse of the immobilized lipase, considerable activity of this complex was observed after four cycles, indicating the positive effect of covalent interactions between enzymes and the support. However, due to the drastic loss of lipolytic activity, low stability of immobilization complex AA-ANL-IB-150A was observed after storage for 168 h in a climate chamber. The calculated kinetic parameters confirmed a significant affinity of immobilized AA-ANL to the substrate. The obtained results could be used to develop enzymatic systems, so-called 'catalytic triangles of lipase-support-substrate', with the aim of obtaining an optimal model for application in in vitro studies. The investigated wide substrate spectrum, i.e., oils with varying content of PUFAs/MUFAs, enables selection of appropriate oil systems, which positively influence digestive enzymes and can be used in obesity treatment and to balance the fat contained in the diet with the aim of achieving optimal homeostasis. Activation of lipase toward PUFAs/MUFAs can promote 'healthy fats' with multidirectional activity, with beneficial effects not only on lipid metabolism but also on other systems in the human body.

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catalysts

MDPI

Correction

Correction: Siódmiak et al. The High 'Lipolytic Jump' of Immobilized Amano A Lipase from *Aspergillus niger* in Developed 'ESS Catalytic Triangles' Containing Natural Origin Substrates. *Catalysts* 2022, *12*, 853

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The authors wish to make the following corrections to this paper [1]:

Table Legend

In the original publication, there was a mistake in the legend for Table 3. "IB-150A" in Table footnote 1 should be "support". The correct legend appears below.

The corrected legend:

[1]—the amount of lipase immobilized onto support (the difference between the initial lipase amount and the protein amount in the supernatant using Bradford's method).

Error in Table

In the original publication, there was a mistake in Table 2 as published. The error in the value of the L_L in avocado oil and the lack of "$_l$" in "U_l" unit were found. The corrected version of Table 2 appears below.

The corrected version of Table 2:

Oils	LA_B [1] (mg)	L_L [2] (mg/g)	I_s [3] (%)	I_c [4] (%)	U_B [5] (μmol/min)	U_L [6] (μmol/min)
Peanut	6.1 ± 0.1	122.0 ± 2.0	61.0 ± 1.0	250.00	0.67 ± 0.10	1.67 ± 0.10
Blackberry	5.8 ± 0.1	116.0 ± 2.0	58.0 ± 1.0	158.82	2.83 ± 0.17	4.50 ± 0.35
Rapeseed	5.9 ± 0.2	118.0 ± 4.0	59.0 ± 2.0	113.33	2.50 ± 0.10	2.83 ± 0.29
Pumpkin seed	4.0 ± 0.3	80.0 ± 6.0	40.0 ± 3.0	2400.00	0.17 ± 0.09	4.00 ± 0.25
Walnut	5.7 ± 0.1	114.0 ± 2.0	57.0 ± 1.0	440.00	0.83 ± 0.17	3.67 ± 0.42
Sesame	6.5 ± 0.1	130.0 ± 2.0	65.0 ± 1.0	566.67	0.50 ± 0.10	2.83 ± 0.25
Avocado	6.3 ± 0.2	126.0 ± 4.0	63.0 ± 2.0	428.57	1.17 ± 0.10	5.00 ± 0.17
Rice	4.5 ± 0.1	90.0 ± 2.0	45.0 ± 1.0	134.38	5.33 ± 0.51	7.17 ± 0.09
Corn	6.1 ± 0.1	122.0 ± 2.0	61.0 ± 1.0	1266.67	0.50 ± 0.10	6.33 ± 0.26
Black cumin	6.2 ± 0.1	124.0 ± 2.0	62.0 ± 1.0	240.00	1.67 ± 0.10	4.00 ± 0.29
Hemp	4.5 ± 0.2	90.0 ± 4.0	45.0 ± 2.0	516.67	1.00 ± 0.10	5.17 ± 0.17
Safflower	5.2 ± 0.2	104.0 ± 4.0	52.0 ± 2.0	377.78	1.50 ± 0.10	5.67 ± 0.25
Grape seed	5.0 ± 0.1	100.0 ± 2.0	50.0 ± 1.0	126.83	6.83 ± 0.35	8.67 ± 0.17
Hazelnut	4.4 ± 0.2	88.0 ± 4.0	44.0 ± 2.0	433.33	0.50 ± 0.10	2.17 ± 0.17
Evening primrose	4.4 ± 0.1	88.0 ± 2.0	44.0 ± 1.0	314.29	1.17 ± 0.17	3.67 ± 0.10
Argan	4.6 ± 0.2	92.0 ± 4.0	46.0 ± 2.0	680.00	0.50 ± 0.10	3.00 ± 0.17
Milk thistle	4.6 ± 0.2	92.0 ± 4.0	46.0 ± 2.0	343.75	2.67 ± 0.10	9.17 ± 0.25
Borage	4.8 ± 0.1	98.0 ± 2.0	48.0 ± 1.0	980.00	0.83 ± 0.09	8.17 ± 0.10
Apricot kernel	5.0 ± 0.1	100.0 ± 2.0	50.0 ± 1.0	310.00	1.67 ± 0.26	5.17 ± 0.35
Olive	4.5 ± 0.2	90.0 ± 4.0	45.0 ± 2.0	38.24	11.33 ± 0.25	4.33 ± 0.17
Fish	4.0 ± 0.3	80.0 ± 6.0	40.0 ± 3.0	1433.33	0.50 ± 0.10	7.17 ± 0.17
Sunflower	4.7 ± 0.1	94.0 ± 2.0	47.0 ± 1.0	120.00	1.67 ± 0.26	2.00 ± 0.25
Linseed	4.4 ± 0.2	88.0 ± 4.0	44.0 ± 2.0	175.00	1.17 ± 0.17	9.17 ± 0.10

check for updates

Citation: Siódmiak, T.; Dulęba, J.; Kocot, N.; Wątróbska-Świetlikowska, D.; Marszałł, M.P. Correction: Siódmiak et al. The High 'Lipolytic Jump' of Immobilized Amano A Lipase from *Aspergillus niger* in Developed 'ESS Catalytic Triangles' Containing Natural Origin Substrates. *Catalysts* 2022, *12*, 853. *Catalysts* **2023**, *13*, 480. https://doi.org/10.3390/catal13030480

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Missing Citation

1. In the original publication, Reference 14 was not cited. The citation has now been inserted in Section 1 (Paragraph 1) and should read:

The interfacial activation is specific for most lipases (e.g., *Aspergillus niger*, *Burkholderia cepacia*, *Candida rugosa*, *Thermomyces lanuginosus*, and *Rhizomucor miehei*). However, its occurrence has not been observed in some cases (e.g., lipase B from *Candida antarctica*, *Pseudomonas aeruginosa*, and *Burkholderia glumae*) [14].

2. In the original publication, Reference 60 was not cited. The citation has now been inserted in Section 1 (Paragraph 3) and should read:

In other studies conducted by the same authors, lipase AA-ANL was immobilized onto sponges from *Luffa cylindrica* in an attempt to improve the thermal and chemical stability of the catalyst [60].

Error in References Citation

1. In the original publication, the citation of a reference as Reference 60 is wrong. It should be Reference 21. Corrections have been made to Section 1 (Paragraph 3).

The corrected text:

Zdarta et al. [21] immobilized AA-ANL onto the surface of silica Stöber to improve the immobilization and testing of lipase kinetic parameters.

2. In the original publication, the citation of a reference as Reference 87 is wrong. It should be Reference 88. Corrections have been made to Section 2.8 (Paragraph 1).

The corrected text:

Zubiolo et al. [88], performing a similar experiment investigating catalytic parameters of free and immobilized ANL (encapsulation in a sol-gel matrix).

Text Correction

There were errors in the original publication.

1. Corrections have been made to the Abstract (the last sentence): "increased" should be replaced with "decreased".

The corrected text:

The tested kinetic profile of immobilized AA-ANL confirmed the decreased affinity to the substrate relative to lipase in the free form.

2. Corrections have been made to Section 1 (Paragraph 3): "has been" should be replaced with "was".

The corrected text:

Therefore, this method was commonly applied in the immobilization of many lipases, such as lipase B from *Candida antarctica* (CAL-B), lipase from *Candida methylica* (CML), *Thermomyces lanuginosus* (TLL), *Rhizomucor miehei* (RML), or Amano lipase PS from *Burkholderia cepacia* (APS-BCL) [44,46–52].

3. Corrections have been made to Section 1 (Paragraph 3). "IB-150B" should be replaced with "IB-150A".

The corrected text:

As mentioned above, the application of AA-ANL immobilized onto polymeric support IB-150A has not been studied.

4. Corrections have been made to Section 1 (Paragraph 3): "60" should be deleted.

The corrected text:

According to recent reports, ω3 PUFA supplementation, especially DHA, can accelerate the regeneration of the organism after COVID-19 infection [68,69].

5. Corrections have been made to Section 2.1 (the seventh sentence): "fish, argan" was missed.

The corrected text:

In contrast, the tested lipase was the least active in sesame, fish, argan, corn, and hazelnut oils (0.50 ± 0.10 U).

6. Corrections have been made to Section 2.1 (Paragraph 1): "*languinosis*" should be replaced as "*lanuginosus*".

The corrected text:

Glutaraldehyde has also been applied as a 'spacer arm' in the immobilization of lipase from *Thermomyces lanuginosus* on a multicomponent support system (metalorganic structure hydroxyapatite-glycyrrhizin-lithium) [77].

7. Corrections have been made to Section 2.8 (Paragraph 1): "modifying" should be replaced with "evaluating".

The corrected text:

The kinetic studies of the reaction were performed with the aim of evaluating the affinity of immobilized AA-ANL to the substrate.

8. Corrections have been made to Section 2.8 (Paragraph 1): "increase" should be replaced with "decrease", "immobilized" should be added before "lipase", "immobilized AA-ANL" should be placed in brackets, and the sentence after "That is" should be enriched.

The corrected text:

With respect to the K_m parameter, an extremal decrease was observed in the affinity of the immobilized lipase to the substrate (immobilized AA-ANL (K_m = 1827.95 ± 0.17 mg/mL) compared with free AA-ANL (K_m = 29.20 ± 0.08 mg/mL)). That is, an essential effect of immobilization on lipase affinity to the hydrophobic substrate has been observed. It should be mentioned that the previous sections noted a significant increase in the lipolytic activity of the immobilized lipase compared to the free sample. Hence, the linear relationship between lipase activity and its affinity to the substrate has not been shown.

9. Corrections have been made to Section 2.8 (Paragraph 1): "higher" should be replaced with "lower".

The corrected text:

The obtained results of immobilized lipase (K_m = 115 ± 4 mM) juxtaposed those of with free lipase (K_m = 77 ± 2 mM) indicate lower affinity of ANL to the substrate, which was confirmed in our study.

10. Corrections have been made to Section 2.8 (Paragraph 1): "an increase" should be replaced with "a change" and "by limiting mass transfer" should be replaced with "that affected limiting in mass transfer".

The corrected text:

On the other hand, Zubiolo et al. [88], performing a similar experiment investigating catalytic parameters of free and immobilized ANL (encapsulation in a sol-gel matrix), achieved a change in enzyme affinity to the substrate that affected limiting in mass transfer.

11. Corrections have been made to Section 2.8 (Paragraph 1): "and steric hindrance" should be deleted.

The corrected text:

In our study, covalent interactions between lipase and the support seemed to form a rigid complex that prevented protein leakage due to the rotation limitation of other lipase molecules relative to one another.

12. Corrections have been made to Section 3.4 (Paragraph 1). The unit (mL) was missed.

The corrected text:

The reaction mixture (emulsion) containing the immobilized lipase was composed of supports with lipase, 3.0 mL phosphate buffer (pH 7.4), 2.5 mL of the appropriate oil (substrate), and 2.5 mL of the water suspension containing arabic gum.

13. Corrections have been made to Section 4 (Paragraph 1): "significant" should be replaced with "low".

The corrected text:

The calculated kinetic parameters confirmed a low affinity of immobilized AA-ANL to the substrate.

The authors state that the scientific conclusions are unaffected. This correction was approved by the Academic Editor. The original publication has also been updated.

Reference

1. Siódmiak, T.; Dulęba, J.; Kocot, N.; Wątrobska-Świetlikowska, D.; Marszałł, M.P. The High 'Lipolytic Jump' of Immobilized Amano A Lipase from *Aspergillus niger* in Developed 'ESS Catalytic Triangles' Containing Natural Origin Substrates. *Catalysts* **2022**, *12*, 853. [CrossRef]

12. Wnioski

1. W zakresie przeprowadzonych badań, oceniono aktywność enancjoselektywną i lipolityczną lipaz z *Burkholderia sp.* i *Aspergillus sp.* w formie wolnej i immobilizowanej na nośnikach polimerowych.
2. Na podstawie wyników badań wstępnych, wybrano lipazy Amano PS z *Burkholderia cepacia* (APS-BCL) oraz Amano A z *Aspergillus niger* (AA-ANL) jako enzymy o najlepszych właściwościach katalitycznych.
3. Analiza wyników lipaz w formie wolnej pozwala stwierdzić wysoką aktywność enancjoselektywną APS-BCL i niską AA-ANL oraz wysoką aktywność lipolityczną APS-BCL i niską AA-ANL
4. Uzyskano wysoką aktywność enancjoselektywną immobilizowanej APS-BCL i niską immobilizowanej AA-ANL.
5. Odnotowano korzystny wpływ rozpuszczalników oraz donorów grupy acylowej na aktywność immobilizowanej APS-BCL.
6. Wyniki badań aktywności katalitycznej immobilizowanej APS-BCL wykazały spadek aktywności lipolitycznej lipazy w porównaniu z formą wolną.
7. W przypadku immobilizowanej AA-ANL, uzyskano wzrost aktywności lipolitycznej lipazy względem formy natywnej.
8. Zaobserwowano znaczący wpływ temperatury i pH na aktywność lipolityczną immobilizowanej AA-ANL oraz spadek jej aktywności po 4 cyklach reakcji, a także niską stabilność po przechowywaniu w komorze klimatycznej.
9. Zoptymalizowano metodę kinetycznego rozdziału (*R,S*)-1-fenyloetanolu, z zastosowaniem wysokosprawnej chromatografii cieczowej i chiralnych faz stacjonarnych. Otrzymano całkowicie rozdzielone, do linii bazowej, piki produktów i substratów reakcji.

13. Streszczenie

Biokataliza jest procesem, polegającym na wykorzystaniu m.in. enzymów do katalizowania reakcji chemicznych. Powszechnie stosowanymi białkami enzymatycznymi w badaniach katalitycznych są lipazy. Charakteryzują się one wysoką aktywnością katalityczną (np. aktywność enancjoselektywna, aktywność lipolityczna). Aktywność enancjoselektywna umożliwia katalizowanie reakcji m.in. kinetycznego rozdziału racemicznych mieszanin związków chemicznych w celu otrzymania optycznie czystych enancjomerów, natomiast aktywność lipolityczna pozwala na hydrolityczny rozkład triglicerydów kwasów tłuszczowych.

Zaprezentowane w niniejszej pracy doktorskiej badania dotyczyły oceny aktywności enancjoselektywnej (w kinetycznym rozdziale (*R*,*S*)-1-fenyloetanolu) oraz aktywności lipolitycznej (poprzez hydrolizę triglicerydów kwasów tłuszczowych ω3/ω6/ω9) lipaz z *Burkholderia sp.* (Lipaza Amano PS z *Burkholderia cepacia*, APS-BCL) oraz *Aspergillus sp.* (Lipaza Amano A z *Aspergillus niger*, AA-ANL). Przeprowadzono proces immobilizacji na nośnikach polimerowych celem zwiększenia parametrów katalitycznych lipaz.

Otrzymane wyniki wskazują na wysoką aktywność enancjoselektywną i lipolityczną APS-BCL w formie wolnej oraz wysoką aktywność enancjoselektywną i nieznacznie niższą aktywność lipolityczną w formie immobilizowanej, w porównaniu w formą wolną. Z kolei, aktywność enancjoselektywna AA-ANL osiągnęła niską wartość zarówno dla formy wolnej jak i immobilizowanej, natomiast aktywność lipolityczna w formie immobilizowanej znacząco wzrosła w porównaniu z formą wolną. Przeprowadzone eksperymenty wskazały na pozytywną rolę lipaz w katalizowaniu reakcji o znaczeniu farmaceutycznym. Zaprojektowane w ramach badań modele katalityczne, tzw. trójkąty katalityczne mogą stanowić bazę dla badań farmaceutycznych na skalę przemysłową.

14. Summary

Biocatalysis is a process that uses e.g. enzymes to catalyze chemical reactions. Lipases are commonly used enzymatic proteins in catalytic research. They are characterized by high catalytic activity (enantioselective and lipolytic activity). The enantioselective activity enables catalyzing reactions, e.g. kinetic resolution of racemic mixtures of chemical compounds to obtain optically pure enantiomers, while the lipolytic activity allows hydrolytic decomposition of triglycerides of fatty acids.

The research presented in this doctoral thesis concerned the evaluation of enantioselective activity (in the kinetic separation of (R,S)-1-phenylethanol) and lipolytic activity (by hydrolysis of triglycerides of ω3/ω6/ω9 fatty acids) of lipases from *Burkholderia sp.* (Lipase Amano PS from *Burkholderia cepacia*, APS-BCL) and *Aspergillus sp*. (Amano Lipase A from *Aspergillus niger*, AA-ANL). The immobilization process on polymer supports was carried out to increase the catalytic parameters of lipases.

The results indicate high enantioselective and lipolytic activity of APS-BCL in the free form, high enantioselective activity, and slightly lower lipolytic activity in the immobilized form compared to the free form. On the other hand, the enantioselective activity of the AA-ANL reached a low value for both the free and immobilized form, while the lipolytic activity in the immobilized form significantly increased compared to the free form. The conducted experiments showed the positive role of lipases in catalyzing reactions of pharmaceutical importance. The catalytic models designed as part of research, the so-called "catalytic triangles" can be the basis for pharmaceutical research on an industrial scale.

15. Spis rycin i tabel

16. Literatura

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17. Oświadczenia autora i współautorów o udziale w powstaniu publikacji

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Oświadczenie autora o udziale w powstaniu publikacji

Oświadczam, że mój udział w publikacji naukowej:

Jacek Dulęba, Tomasz Siódmiak, Michał Piotr Marszałł, Amano Lipase PS from *Burkholderia cepacia* - Evaluation of the Effect of Substrates and Reaction Media on the Catalytic Activity, *Curr. Org Chem.*, 2020, 24 (7), 798-807. https://doi:10.2174/1385272824666200408092305

obejmował koncepcję projektu, metodologię badań naukowych: przygotowanie próbek, oznaczenie aktywności enancjoselektywnej lipazy metodą wysokosprawnej chromatografii cieczowej (HPLC), oznaczenie aktywności lipolitycznej lipazy metodą miareczkową; analizę i interpretację otrzymanych wyników, redagowanie i edycję manuskryptu, odpowiedzi dla recenzentów oraz zarządzanie projektem.

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Oświadczenie autora o udziale w powstaniu publikacji

Oświadczam, że mój udział w publikacji naukowej:

Jacek Dulęba, Tomasz Siódmiak, Michał Piotr Marszałł

The influence of substrate systems on the enantioselective and lipolytic activity of immobilized Amano PS from *Burkholderia cepacia* lipase (APS-BCL), *Process Biochem.* **2022**, 120, 126-137. https://doi.org/10.1016/j.procbio.2022.06.003

obejmował: koncepcję projektu w zakresie badań aktywności lipolitycznej oraz kinetyki lipazy APS-BCL, metodologię badań aktywności lipolitycznej (również immobilizacji) oraz kinetyki lipazy APS-BCL, analizę i interpretację wyników badań aktywności lipolitycznej i kinetyki, wykonanie badań eksperymentalnych, napisanie publikacji (Abstract, Introduction, Materials and methods (2.1., 2.3.1., 2.3.3., 2.3.4.), Results and discussion (3.3.), Conclusions (fragmenty dotyczące aktywności lipolitycznej i kinetyki lipazy APS-BCL)), odpowiedzi na recenzje dotyczące badania aktywności lipolitycznej i kinetyki lipazy APS-BCL;

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obejmował konceptualizację projektu, dokonanie przeglądu literatury fachowej, redagowanie i edycję manuskryptu oraz zarządzanie projektem.

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obejmował: koncepcję projektu, metodologię badań: immobilizację lipazy Amano A z *Aspergillus niger* (AA-ANL) na nośniku poliakrylowym IB-150A, oznaczenie ilości immobilizowanego białka metodą Bradford'a, oznaczenie aktywności lipolitycznej AA-ANL metodą miareczkową, badanie wpływu warunków reakcji (temperatura, pH), nośników, mieszaniny substratowej na aktywność immobilizowanej AA-ANL oraz badanie stabilności AA-ANL; analizę i interpretację wyników, redagowanie i edycję manuskryptu, odpowiedzi dla recenzentów oraz formalne zarządzanie projektem.

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Catalysts, 2023, 13, 480. https://doi.org/10.3390/catal13030480

obejmował przygotowanie korekty manuskryptu.

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*autor korespondencyjny

obejmował nadzór merytoryczny nad analizą i interpretacją wyników, krytyczną recenzję manuskryptu, nadzór nad redagowaniem odpowiedzi dla recenzentów oraz formalnym zarządzaniem projektem. W niniejszej pracy pełnię rolę autora korespondencyjnego.

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*autor korespondencyjny

obejmował: koncepcję projektu w zakresie badań aktywności enancjoselektywnej lipazy APS-BCL, metodologię badań aktywności enancjoselektywnej (również immobilizacji) lipazy APS-BCL, analizę i interpretację wyników badań aktywności enancjoselektywnej, napisanie publikacji: (Materials and methods (2.2., 2.3.2.), Results and discussion (3.1., 3.2.), Conclusions (fragmenty dotyczące aktywności enancjoselektywnej lipazy APS-BCL)), przygotowanie suplementu; pozyskanie środków finansowych, odpowiedzi na recenzje dotyczące badania aktywności enancjoselektywnej lipazy APS-BCL, korespondencję z wydawnictwem;

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obejmował nadzór merytoryczny nad redagowaniem manuskryptu.

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obejmował: nadzór merytoryczny nad metodologią badań, analizą i interpretacją wyników, krytyczną recenzję manuskryptu, nadzór nad redagowaniem odpowiedzi dla recenzentów oraz formalnym zarządzaniem projektem.

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Catalysts, 2023, 13, 480. https://doi.org/10.3390/catal13030480

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obejmował nadzór merytoryczny nad korektą manuskryptu.

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obejmował konsultacje merytoryczne w toku przygotowania manuskryptu oraz formalny nadzór nad projektem.

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Jacek Dulęba, Tomasz Siódmiak*, **Michał Piotr Marszałł**
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obejmował nadzór merytoryczny nad prowadzonymi badaniami.

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Jacek Dulęba, Tomasz Siódmiak, Rafał Mastalerz, Natalia Kocot, Agnieszka Dębińska, Emilia Suchomska, Mateusz Czeczka, Kamil Cała, Dorota Wątróbska-Świetlikowska, **Michał Piotr Marszałł,** The application of lipase from *Burkholderia cepacia* in the enantioselective pharmaceutical biocatalysis, *Farm Pol*, 2022, Tom 78 (4), 194-200. https://doi/10.32383/farmpol/151576

obejmował nadzór merytoryczny nad redagowaniem manuskryptu.

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obejmował: nadzór merytoryczny nad koncepcją projektu, metodologią badań, oraz formalnym zarządzaniem projektem.

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Catalysts, 2023, 13, 480. https://doi.org/10.3390/catal13030480

obejmował nadzór merytoryczny nad korektą manuskryptu.

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obejmował nadzór merytoryczny nad redagowaniem manuskryptu.

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obejmował nadzór merytoryczny w zakresie metodologii badań oraz zarządzania projektem, a także krytyczną recenzję manuskryptu.

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Catalysts, 2023, 13, 480. https://doi.org/10.3390/catal13030480

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obejmował pomoc w części eksperymentalnej (przygotowanie roztworów buforowych) oraz w redagowaniu manuskryptu.

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obejmował pomoc w redagowaniu manuskryptu.

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obejmował pomoc w przygotowaniu korekty manuskryptu.

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obejmował pomoc w redagowaniu manuskryptu.

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Oświadczenie współautora o udziale w powstaniu publikacji

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