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Produkcja związków bioaktywnych z wykorzystaniem drożdży *Yarrowia lipolytica*

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Praca wykonana pod kierunkiem

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Spis treści

Wykaz publikacji wchodzących w skład rozprawy doktorskiej

1. **Patrycja Szczepańska**, Piotr Hapeta, Zbigniew Lazar (2021). Advances in production of high-value lipids by oleaginous yeasts. *Critical Reviews in Biotechnology*, 42:1, 1-22.

2. **Patrycja Szczepańska**, Magdalena Rychlicka, Paweł Moroz, Tomasz Janek, Anna Gliszczyńska, Zbigniew Lazar (2022). Elevating phospholipids production by *Yarrowia lipolytica* from crude glycerol. *International Journal of Molecular Sciences*, 23:18, 10737.

3. Qin He /**Patrycja Szczepańska**, Tigran Yuzbashev, Zbigniew Lazar, Rodrigo Ledesma-Amaro (2020). De novo production of resveratrol from glycerol by engineering different metabolic pathways in *Yarrowia lipolytica*. *Metabolic Engineering Communications*, 11, e00146.

4. **Patrycja Szczepańska**, Magdalena Rychlicka, Sylwia Groborz, Angelika Kruszyńska, Rodrigo Ledesma-Amaro, Andrzej Rapak, Anna Gliszczyńska, Zbigniew Lazar (2023). Studies on the anticancer and antioxidant activities of resveratrol and long chain fatty acid esters. *International Journal of Molecular Sciences*, 24(8), 7167.

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Wykaz skrótów stosowanych w pracy

LB - ciałka lipidowe (ang. *lipid bodies*)

TAG – triacyloglicerole

NADPH - Dinukleotyd nikotynoamidoadeninowy

FFA - wolne kwasy tłuszczowe

TFA - zawartość kwasów tłuszczowych

PDC - dehydrogenaza pirogronianowa

Acetylo-CoA - acetylokoenzym A

acylo-CoA - acylokoenzym A

LA - kwas linolowy

OA - kwas oleinowy

PUFA - wielonienasycone kwasy tłuszczowe

FAEE - estry etylowe kwasów tłuszczowych

FFA - wolne kwasy tłuszczowe

CYP1A1 – ludzki cytochrom P450

PL - fosfolipidy

TAL - amoniakalna liaza tyrozyny

4CL - ligaza 4-kumaranu-CoA

STS - syntaza stilbenowa

PAL - liaza fenyloalaninowa

C4H - 4-hydroksylaza cynamonowa

DPPH – rodnik 1,1-difenylo-2-pikrylohydrazylu

PPP - szlak pentozofosforanowy

PE - fosfatydyloetanolamina

PI - fosfatydyloinozytol

CDS - syntaza CDP-DAG

SPO14 - fosfolipaza D

OPI3 - metylotransferazy fosfolipidowa

LDC - koniugaty lipid-lek (*ang. Lipid Drug Conjugate)*

PA - kwas fosfatydowy

CAGR - średnia roczna stopa wzrostu

EGCG - galusan epigallokatechiny

Tyr - tyrozyna

Phe - fenyloalanina

YNB - podłoże minimalne

GUT1 - kinaza glicerolowa

DGK1 - kinaza diacyloglicerolowa

DAG - diacyloglicerole

A549 - komórki ludzkiej linii raka płuc

HT29 - komórki ludzkiej linii raka jelita grubego

BxPC3 - komórki ludzkiej linii raka trzustki

BJ - komórki ludzkiej linii fibroblastów

SOD - dysmutaza ponadtlenkowa

ROS - reaktywne formy tlenu

PS - fosfatydyloseryna

PC - fosfatydylocholina

RES - resweratrol

EPA - kwas eikozapentaenowy

DHA - kwas dokozaheksaenowy

ARA - kwas arachidonowy

CLA - sprzężony kwas linolowy

GLA - kwas γ-linolenowy

Streszczenie

Drożdże *Yarrowia lipolytica* to mikroorganizmy oleiste znane ze swoich zdolności do syntezy cennych metabolitów, znajdujących zastosowanie w przemyśle spożywczym, kosmetycznym, farmaceutycznym i paliwowym. Drożdże te zdolne są do asymilacji szerokiej gamy różnych źródeł węgla potrzebnych do wzrostu i syntezy pożądanych związków, takich jak substraty hydrofilowe (cukry, glicerol) i hydrofobowe (węglowodory, tłuszcze), które mogą być także pozyskiwane z surowców odpadowych. Niniejsza rozprawa doktorska skupia się na badaniu zdolności drożdży *Y. lipolytica* do syntezy dużych ilości fosfolipidów, wśród których główny nacisk położono na fosfatydylocholinę, oraz resweratrolu, związku polifenolowego o udowodnionych właściwościach prozdrowotnych, z zastosowaniem taniego substratu jakim jest glicerol. Praca posiada także aspekt interdyscyplinarny, w którym skupiono się na uzyskaniu lipidowych pochodnych resweratrolu oraz ocenie ich właściwości przeciwnowotworowych i antyoksydacyjnych względem nowotworowych i zdrowych ludzkich linii komórkowych.

W pierwszej fazie badań podjęto się charakterystyki szlaku biosyntezy fosfolipidów u drożdży *Y. lipolytica* i zwiększenia ich produkcji z odpadowego substratu, glicerolu. W tym celu wykonano serię manipulacji genetycznych uzyskując szczep (PS08), charakteryzujący się 6-krotnym zwiększeniem produkcji fosfatydylocholiny (27,8 mg/g) i prawie 3-krotną poprawą produkcji fosfolipidów ogółem (60,2 mg/g) w porównaniu do szczepu kontrolnego. W celu jeszcze większej poprawy produkcji tych związków, przeprowadzono optymalizację warunków hodowli i składu podłoża a następnie przeskalowano proces do warunków bioreaktorowych z zastosowaniem technicznego i odpadowego glicerolu. Uzyskane wyniki wykazały, że produkcja fosfolipidów była na podobnym poziomie niezależnie od rodzaju użytego glicerolu. Finalnie szczep PS08 zdolny był do biosyntezy 653,7 mg/L fosfolipidów, wśród których 352,6 mg/L stanowiła sama fosfatydylocholina.

W kolejnych badaniach skupiono się na syntezie polifenolowego związku jakim jest resweratrol. W tym celu skonstruowano szczepy *Y. lipolytica* zdolne do jego produkcji, w których testowano wydajność dwóch heterologicznych szlaków – szlaku katabolizmu tyrozyny i szlaku katabolizmu fenyloalaniny. Dalsze zwiększanie ilości uzyskiwanego resweratrolu prowadzono poprzez zwiększanie ilości kopii genów kodujących kluczowe

enzymy, jak również przez optymalizację składu podłoża oraz warunkó prowadzenia procesu. Wyniki wykazały, że najlepszym producentem resweratrolu okazał się szczep łączący w sobie oba szlaki katabolizmu wspomnianych aminokwasów, z podwójną kopią genów (szczep T2P2), który zdolny był do sekrecji 0,104 g/L resweratrolu podczas hodowli w kolbach. Następnie, produkcję tego metabolitu z zastosowaniem glicerolu przeprowadzono w bioreaktorze, uzyskując 4-krotnie wyższe stężenie, wynoszące 0,430 g/L resweratrolu.

Kolejna, interdyscyplinarna część badań dotyczyła estryfikacji resweratrolu z wybranymi kwasami tłuszczowymi: palmitynowym, oleinowym i sprzężonym linolowym, a uzyskane koniugaty analizowane były następnie analizy w kierunku ich właściwości przeciwnowotworowych i antyoksydacyjnych względem wybranych ludzkich linii komórkowych raka płuc (A549), gruczolakoraka jelita grubego (HT29) oraz gruczolakoraka przewodowego trzustki (BxPC3). Nadanie charakteru lipidowego cząsteczce resweratrolu miało na celu rozszerzenie jego możliwych zastosowań dzięki zwiększonej bioprzyswajalności i stabilności nowych koniugatów. Zbadano kilka parametrów: żywotność komórek i apoptozę, w tym ekspresję głównych markerów pro- i antyapoptotycznych, a także ekspresję dysmutazy ponadtlenkowej, kluczowego enzymu bariery antyoksydacyjnej organizmu. Uzyskane wyniki pozwoliły na wybór trzech związków: mono-RES-OA, tri-RES-PA oraz mono-RES-CLA, które w porównaniu z innymi estrami wyraźnie wykazywały obniżenie żywotności komórek nowotworowych, bez wpływu na komórki prawidłowe. Ponadto, wybrane estry wykazywały właściwości antyoksydacyjne wobec prawidłowej linii komórkowej, wpływając na regulację ekspresji głównych genów pro-antyoksydacyjnych bez wpływu na ich ekspresję w komórkach nowotworowych, a tym samym zmniejszając obronę komórek nowotworowych przed nasilonym stresem oksydacyjnym indukowanym wysoką akumulacją ROS. Uzyskane wyniki wykazały, że estry resweratrolu i długołańcuchowych kwasów tłuszczowych pozwalają na zwiększenie ich aktywności biologicznej, a tym samym na ich duży potencjał zastosowania klinicznego.

Streszczenie w języku angielskim

The *Yarrowia lipolytica* yeast is an oleaginous microorganism known for its ability to synthesise valuable metabolites with applications in the food, cosmetic, pharmaceutical and fuel industries. These yeasts are capable of assimilating a wide range of different carbon sources needed for growth and synthesis of desired compounds, such as hydrophilic (sugars, glycerol) and hydrophobic (hydrocarbons, fats) substrates, which can also be extracted from waste materials. This dissertation focuses on the study of the ability of the *Y. lipolytica* to synthesise large quantities of phospholipids, among which the main focus is on phosphatidylcholine, and resveratrol, a polyphenolic compound with proven health-promoting properties, using a low-cost substrate such as glycerol. The work also has an interdisciplinary aspect, with a focus on obtaining lipid derivatives of resveratrol and evaluating their anticancer and antioxidant properties against cancerous and healthy human cell lines.

The first phase of the study was undertaken to characterise the phospholipid biosynthetic pathway in the yeast *Y. lipolytica* and to enhance its production from a waste substrate, glycerol. For this purpose, a series of genetic manipulations were performed obtaining a strain (PS08) characterised by a 6-fold increase in the production of phosphatidylcholine (27.8 mg/g) and an almost 3-fold improvement in the production of total phospholipids (60.2 mg/g) compared to a control strain. To further improve the production of these compounds, the culture conditions and medium composition were optimised and the process was then scaled up to bioreactor conditions using technical and waste glycerol. The results showed that the production of phospholipids was at a similar level regardless of the type of glycerol used. Ultimately, strain PS08 was able to biosynthesise 653.7 mg/L of phospholipids, among which 352.6 mg/L was phosphatidylcholine.

Subsequent studies focused on the synthesis of the polyphenolic compound resveratrol. To this end, *Y. lipolytica* strains capable of producing it were constructed, in which the efficiency of two heterologous pathways - the tyrosine catabolism pathway and the phenylalanine catabolism pathway - was tested. Further increases in the amount of resveratrol obtained were carried out by increasing the copy number of genes encoding key enzymes, as well as by optimising the medium composition and process conditions. The results showed

that the best resveratrol producer was a strain combining both pathways of catabolism of the aforementioned amino acids, with a double copy of the genes (strain T2P2), which was able to secrete 0.104 g/L of resveratrol in flasks culture. Subsequently, production of this metabolite using glycerol was carried out in a bioreactor, yielding a 4-fold higher concentration of 0.430 g/L resveratrol.

The interdisciplinary part of the study concerned the esterification of resveratrol with selected palmitic, oleic and conjugated linoleic fatty acids, and the resulting conjugates were then analysed for their anticancer and antioxidant properties against lung carcinoma (A549), colorectal adenocarcinoma (HT29), and pancreatic ductal adenocarcinoma (BxPC3) cell lines. The lipophilic versions of resveratrol molecule was aimed at extending its possible applications through increased bioavailability and stability of the new conjugates. Several parameters were investigated: cell viability and apoptosis, including the expression of major pro- and anti-apoptotic markers, as well as the expression of superoxide dismutase, a key enzyme of the body's antioxidant barrier. The results obtained allowed the selection of three esters: mono-RES-OA, tri-RES-PA and mono-RES-CLA, which, compared to the other esters, clearly showed a reduction in tumour cell viability, with no effect on normal cells. In addition, the selected esters exhibited antioxidant properties towards the normal cell line, affecting the up-regulation of the expression of major pro-antioxidant genes without affecting their expression in cancer cells, thereby reducing the defence of cancer cells against the increased oxidative stress induced by high ROS accumulation. The results showed that the resveratrol and long-chain fatty acid esters allow for an increase in their biological activity and thus their high potential for clinical application.

1. Wstęp

Nutraceutyki stanowią ważną klasę związków, które przy regularnym spożywaniu mogą powodować długoterminowe korzyści dla organizmu człowieka, w tym: zapobiegać chorobom związanym z procesami starzenia, chorobom układu pokarmowego, chorobom układu krążenia, zapaleniu stawów, osteoporozie, cukrzycy, nowotworom czy nawet depresji. Związki te w większości izolowane są z roślin (np. karotenoidy, polifenole czy witaminy), zwierząt (np. polisacharydy), mikroorganizmów (np. aminokwasy) czy ze źródeł morskich, jak algi czy ryby (np. glukozamina czy długołańcuchowe, wielonienasycone kwasy tłuszczowe) [1–4]. Globalny rynek nutraceutyków w 2021 roku wynosił prawie 290 miliardów dolarów a oczekuje się, że wzrośnie do prawie 440 miliardów dolarów do 2026 roku [5].

Niedobory nutraceutyków są problemem powszechnym i mogą przyczyniać się do powstawania poważnych chorób zagrażających zdrowiu a nawet życiu człowieka. Nutraceutyki przyjmowane w podstawowej diecie nie zaspokają zapotrzebowania organizmu na te związki, dlatego konieczna jest ich suplementacja. To z kolei spowodowało dynamiczny rozwój ich przemysłowej produkcji, wykorzystując metody ekstrakcji ze źródeł naturalnych czy ich syntezy chemicznej. Produkcja związków bioaktywnych na drodze syntezy chemicznej, pomimo dobrze poznanych szlaków, które często są reakcjami wieloetapowymi, napotyka wiele trudności, jak chociażby: niestabilne reagenty lub produkty pośrednie, złożona kontrola całego procesu czy powstawanie niepożądanych produktów ubocznych. Z kolei, pozyskiwanie nutraceutyków z surowców naturalnych spotyka się z jednej strony z koniecznością ochrony gruntów rolnych i zapewnienia bezpieczeństwa żywnościowego a z drugiej z koniecznością chronienia środowiska naturalnego [6]. Z tego względu przemysłowa produkcja związków bioaktywnych musi być prowadzona w sposób zrównoważony, naprzeciw czemu wychodzi ich produkcja z wykorzystaniem nowatorskich metod biotechnologicznych.

Metody biotechnologiczne pozyskiwania cennych związków bioaktywnych, zwłaszcza nutraceutyków, są szeroko badane już od czasu opracowania hodowli kultur komórkowych czy udowodnienia, że mogą one być produkowane przez mikroorganizmy, jednakże ich dynamiczny rozwój nastąpił wraz z rozwojem biologii syntetycznej i biologii systemów

pozwalających na precyzyjne manipulacje metabolizmem komórkowym [7]. Stosowane obecnie procesy biosyntezy produktów docelowych wykorzystują zazwyczaj genetycznie modyfikowane mikroorganizmy, linie komórkowe lub tkanki hodowane w bioreaktorach w ściśle kontrolowanych warunkach procesu. Wśród gospodarzy, będących swego rodzaju mikrofabrykami, mikroorganizmy przyciągają szczególną uwagę, przede wszystkim ze względu na ich wyróżniające właściwości, takie jak; szybki wzrost a zatem i krótki proces produkcji, łatwość manipulacji genetycznych i stabilność genetyczna, jak również możliwość wykorzystania tanich i odpadowych związków jako substratów hodowlanych. Przykładowo, przemysłowa biotechnologiczna produkcja glutaminianu sodu, prowadzona w ten sposób od kilkudziesięciu lat, odbywa się z wykorzystaniem zmodyfikowanych bakterii *Corynebacterium glutamicum* [8]. Ponadto, wiele z używanych w przemysłowej produkcji nutraceutyków mikroorganizmów zostało uznanych za bezpieczne a opracowane z ich udziałem procesy pozwoliły na osiągnięcie znaczącej produkcji pożądanych związków [10]. Obecnie, dynamiczny rozwój metod sekwencjonowania kwasów nukleinowych, jak również metod analizy metabolitów wewnątrzkomórkowych (metabolimika), pozwoliły na dogłębne poznanie szlaków biosyntezy produktów naturalnych, z kolei rozwój narzędzi inżynierii genetycznej i łatwość manipulacji mikroorganizmami przyspieszyły prace związane z konstrukcją mikrobiologicznych gospodarzy do produkcji niezliczonych nowych nutraceutyków. Tworzenie modeli metabolicznych w skali genomu oraz wysokoprzepustowej analizy uzyskiwanych transformantów, jak również optymalizacja warunków hodowli dla konkretnych szczepów sprawiły, że przemysłowa mikrobiologiczna produkcja związków bioaktywnych stała się bardziej wydajna, opłacalna i konkurencyjna dla tradycyjnych metod ich produkcji [9]. Inżynieria metaboliczna umożliwiła nie tylko powiększenie gamy możliwych do uzyskania nutraceutyków oraz skali ich produkcji, ale poszerzyła również możliwości wykorzystywania nie tylko prostych źródeł węgla jako substratów, ale również związków złożonych, w tym odpadowych, jak chociażby: melasy z buraków cukrowych [10], odpadowego oleju spożywczego [11], makulatury [12] czy odpadów rolno-przemysłowych [13], co przyczynia się w znacznym stopniu do zrównoważonej gospodarki odpadami [14]. Nie zmienia to faktu, że popyt na związki bioaktywne stale rośnie, jak również odkrywane są nowe związki, których aktywność wydaje się obiecująca i jest stale badana. W związku z tym, poszukuje się ciągle nowych szczepów

mikroorganizmów, w tym w środowiskach, które wydają się trudne do życia (gejzery, czy wieczna zmarzlina) czy o dużym stopniu zanieczyszczenia. Szczególne miejsce wśród takich drobnoustrojów zajmują mikroorganizmy olejogenne, które z czasem stały się jedną z najszerzej analizowanych i wykorzystywanych przemysłowo grup producentów związków bioaktywnych.

1.1. Mikroorganizmy olejogenne

Wszystkie organizmy żywe syntetyzują związki lipidowe, jednakże ich ilość w komórce wynosi zwykle nie więcej niż 5% suchej masy. Substancje te są bezwzględnie potrzebne do budowy i właściwego funkcjonowania błon biologicznych [15]. Z kolei mikroorganizmy oleiste, znajdujące swoich reprezentantów zarówno wśród bakterii, drożdży, grzybów strzępkowych jak i alg, są definiowane jako zdolne do biosyntezy tłuszczy wewnątrzkomórkowych w ilościach przekraczających 20% suchej masy komórki [16]. W zależności od gatunku, zawartość lipidów różni się znacznie i może sięgać nawet do 70% suchej masy komórki, w szczególności w warunkach organiczonej podaży azotu przy zdecydowanym nadmiarze źródła węgla (wysoki stosunek C/N) [17]. Lipidy gromadzone są w tak zwanych ciałkach lipidowych (LB, ang. *lipid bodies*) zlokalizowanych w cytozolu, gdzie w przeważającej większości występują w postaci triacyloglicerolі (TAG), z kolei w mniejszych ilościach jako fosfolipidy (PL), glikolipidy, sterole, diacyloglicerole (DAG), monoacyloglicerole i wolne kwasy tłuszczowe (FFA).

Wśród drobnoustrojów oleistych to drożdże wydają się być najlepiej przystosowanymi gospodarzami do produkcji tłuszczy wewnątrzkomórkowych. Dotychczas zidentyfikowano i scharakteryzowano około 70 z 1600 gatunków drożdży potencjalnie oleistych, a ich liczba wciąż rośnie [18]. Do najlepiej poznanych i zdolnych do gromadzenia największych ilości lipidów w postaci TAG należą drożdże: *Rhodotorula toruloides*, *Cutaneotrichosporon curvatus*, *Yarrowia lipolytica* i *Lipomyces starkeyi*. Badania pokazują, że drożdże są bardziej efektywne niż bakterie czy mikroalgi pod względem akumulacji lipidów [19]. Ich przewaga nad fototroficznymi algami wynika m.in. z faktu, że drożdże nie podlegają wpływom warunków klimatycznych. Ponadto, drożdże wykorzystują bardziej zróżnicowaną gamę źródeł węgla, w tym cukry obecne w biomasie ligninocelulozowej jak glukoza i ksyloza

(np. *L. starkeyi*), oraz wykazują zdecydowanie wyższe tempo wzrostu w porównaniu do mikroalg heterotroficznych [20]. Z kolei w porównaniu do grzybów strzępkowych, drożdże wykazują większą tolerancję na jony metali oraz niską dostępność tlenu. W przeciwieństwie do bakterii, w tym poddawanych szeroko zakrojonym procesom inżynierii metabolizmu bakteriom *Escherichia coli*, drożdże zdolne posiadają kompartmenty komórkowe pozwalające na rozdzielenie przeciwstawnych procesów konkurujących o substraty, jak również mają zdecydowanie większy rozmiar komórek, co ułatwia procesy ich separacji z podłoży hodowlanych [21,22]. Ponadto, drożdże charakteryzują się wyższą tolerancją na potencjalnie niepożądane produkty uboczne prowadzonych procesów (np. kwasy organiczne) przy równocześnie dużej zdolności do biosyntezy wysokich stężeń produktów pożądanych. Stosunkowo duża łatwość manipulacji genetycznych w komórkach drożdży, krótki czas powielania (<1 h), łatwość powiększania skali procesów do warunków przemysłowych [23], jak również zdolność do wzrostu przy wysokich stężeniach cukru oraz akumulacji dużej ilości lipidów zawierających wielonienasycone kwasy tłuszczowe we frakcji TAG, stawiają drożdże w czołówce coraz chętniej wykorzystywanych przemysłowo mikroorganizmów oleistych [22].

Lipidy produkowane przez oleiste gatunki drożdży charakteryzują się podobnym składem i wartościami energetycznymi jak oleje roślinne czy zwierzęce [24]. Z tego względu olej mikrobiologiczny może stanowić alternatywę dla wielu zastosowań opartych tradycyjnie o oleje roślinne (np. jako dodatek w kosmetykach czy żywności) przy jednoczesnym braku konkurencji dla produkcji żywności. Uzyskane na drodze inżynierii genetycznej szczepy drożdży olejogennych mogą gromadzić aż do 90% lipidów w suchej masie, a frakcja magazynowanych TAG może składać się w ponad 80% z nienasyconych kwasów tłuszczowych, takich jak kwas linolowy (LA) i oleinowy (OA) [25]. Biosynteza i akumulacja lipidów ma miejsce w komórce drożdży w wyniku niezrównoważonego (niestabilnego) metabolizmu, który w warunkach ograniczonego dostępu do składników odżywczych, zwłaszcza źródła azotu czy fosforu, przy nadmiarze źródła węgla prowadzi do procesu lipogenezy. Z kolei kiedy komórki umieszczone zostaną w środowisku, w którym mają dostęp do dużej ilości składników odżywczych, zaczynają się szybko dzielić i w efekcie dochodzi do zwiększonego wzrostu tych mikroorganizmów [26].

Synteza i magazynowanie lipidów u drożdży oleistych może zachodzić dwoma drogami: I) *de novo*, która polega na wykorzystaniu substratów hydrofilowych (np. cukry czy glicerol) w warunkach ograniczonej podaży azotu lub II) *ex novo*, polegającej na wykorzystaniu substratów hydrofobowych (np. lipidy czy węglowodory) [27]. Biosynteza lipidów *de novo* u mikroorganizmów oleistych jest procesem następującym po fazie logarytmicznego wzrostu, prowadzonym w fazie stacjonarnej właśnie ze względu na wyczerpanie azotu z podłoża hodowlanego. Metabolitem kluczowym w procesie lipogenezy *de novo* jest acetylokoenzym A (acetylo-CoA), będący prekursorem biosyntezy kwasów tłuszczowych. Powstający w procesie glikolizy kwas pirogronowy trafia do mitochondriów gdzie przekształcany jest w acetylo-CoA w reakcji katalizowanej przez dehydrogenazę pirogronianową (PDC). W dalszej kolejności acetylo-CoA zostaje włączony do cyklu Krebsa lub trafia ponownie do cytoplazmy, gdzie stanie się substratem do biosyntezy kwasów tłuszczowych [28]. Ponadto, pula acetylo-CoA u drożdży oleistych powiększana jest w wyniku działania liazy cytrynianowej (ACL), która rozkłada trafiający do cytoplazmy, w warunkach organiczonej ilości azotu, kwas cytrynowy właśnie do acetylo-CoA i kwasu szczawiooctowego. Acetylo-CoA w wyniku działania karboksylazy acetylo-CoA (ACC) przekształcany jest do malonylo-CoA, który jest bezpośrednim związkiem zapoczątkowującym biosyntezę kwasów tłuszczowych.

Szlak biosyntezy lipidów *ex novo* uruchamiany jest w odpowiedzi komórki na obecność w środowisku substratów hudrofobowych. Związki takie, np. lipidy, najpierw hydrolizowane są do wolnych kwasów tłuszczowych przez zewnątrzkomórkowe lipazy, a następnie trafiają do wnętrza komórki za pomocą transportu aktywnego. W kolejnych etapach tego procesu kwasy tłuszczowe mogą być degradowane do acylo-CoA na drodze β-oksydacji zaspokajając zapotrzebowanie komórek na energię niezbędną do wzrostu i syntezy metabolitów komórkowych [28] lub wykorzystywane są do syntezy nowych TAG i magazynowane w ciałkach lipidowych [29]. Tłuszcze wewnątrzkomórkowe u organizmów olejogennych stanowią materiał zapasowy, który u organizmów nieoleistych magazynowany jest w postaci polisacharydów [30].

1.2. Drożdże *Yarrowia lipolytica*

Jednym z najczęściej analizowanych gatunków drożdży olejogennych, który stał się organizmem modelowym procesu lipogenezy, jest gatunek *Y. lipolytica* [31]. Drożdże te należą do gromady *Ascomycota*, rodziny *Dipodascaceae* i królestwa *Fungi* [32]. Nazwa rodzajowa '*Yarrowia*' została zaproponowana przez van der Walta i von Arxa po zidentyfikowaniu nowego rodzaju przez Davida Yarrowa z Delft Microbiology Laboratory [33,34]. Nazwa gatunkowa '*lipolytica*' pochodzi od zdolności tych drożdży do hydrolizy lipidów. Pod koniec lat 60. XX wieku drożdże te znane były pod nazwą *Candida lipolytica*, następnie zostały przeklasyfikowane na *Endomycopsis lipolytica*, *Saccharomycopsis lipolytica* i wreszcie *Yarrowia lipolytica* [35].

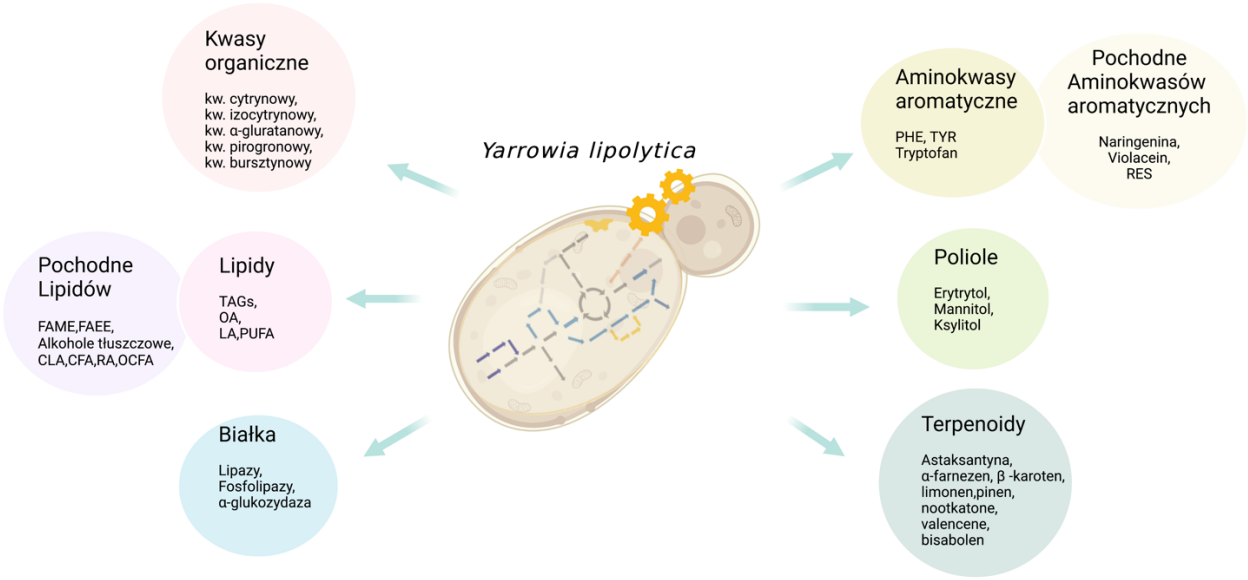
Pierwotnie szczepy *Y. lipolytica* izolowane były ze środowisk bogatych w lipidy lub białka, takich jak różne produkty spożywcze (szczególnie produkty mleczne i mięso), ale także z wody, gleby czy odpadów, zwłaszcza ścieków, jak i ze środowisk zanieczyszczonych ropą naftową [30,36]. Od ponad 50 lat te niekonwencjonalne drożdże cieszą się niesłabnącym zainteresowaniem głównie ze względu na sekrecję wielu metabolitów do medium hodowlanego (kwasy organiczne czy alkohole wielowodorotlenowe), które mają ogromny potencjał do produkcji przemysłowej. Gatunek ten jest również organizmem modelowym w badaniach dotyczących szlaku sekrecyjnego, ze względu na bardzo dużą ilość wydzielanych pozakomórkowo białek (lipazy, proteazy, RNazy), co czyni go również idealnym kandydatem do biosyntezy białek heterologicznych (np. interleukina 2, fucoidanaza) [37,38]. Szczegółowa charakterystyka możliwości aplikacyjnych drożdży *Y. lipolytica* zostanie przedstawiona poniżej (rozdział 1.3). Nieocenioną cechą charakterystyczną drożdży *Y. lipolytica* jest możliwość wykorzystywania przez nie substratów hydrofobowych (kwasy tłuszczowe, triacyloglicerole i alkany), które mogą posłużyć do opracowania wydajnych procesów biotechnologicznych produkcji cennych, biologicznie aktywnych związków, przyczyniając się jednocześnie do ochrony środowiska. Również wykorzystywane przez nie substraty hydrofilowe wpisują się w trendy współczesnej gospodarki wykorzystyjącej odpady do ich waloryzacji w cenne związki. Wśród hydrofilowych źródeł węgla, poza cukrami prostymi - glukozą i fruktozą, gatunek ten potrafi korzystać również z glicerolu czy krótkołańcuchowych kwasów tłuszczowych – octowego

czy masłowego. Związki te stanowią odpad z produkcji biodiesla czy mydeł (odpadowy glicerol) czy produkty fermentacji odpadów komunalnych lub odpadów z przemysłu spożywczego (lotne kwasy tłuszczowe)[39]. Wysoka produktywność biosyntezy cennych metabolitów z wykorzystaniem tanich substratów (odpadowy glicerol lub cukry pochodzące z hydrolizy biomasy ligninocelulozowej) czyni procesy oparte o drożdże *Y. lipolytica* ekonomicznie opłacalnymi [39,40]. Ponadto, zdolność *Y. lipolytica* do wykorzystywania związków ropopochodnych spowodowała zainteresowanie wykorzystaniem tych drożdży w procesach bioremediacji gleb i wód skażonych tymi związkami [41].

1.3. Biotechnologiczne zastosowania drożdży *Y. lipolytica*

Jedną z krytycznych kwestii podczas wdrażania procesów biotechnologicznych do produkcji przemysłowej, szczególnie w przypadku przemysłu spożywczego czy farmaceutycznego, jest bezpieczeństwo związane z zastosowaniem konkretnych mikroorganizmów. Drożdże *Y. lipolytica* uważane są za organizm 'bezpieczny w użyciu' i wielu procesom z ich udziałem, prowadzonym na skalę przemysłową, Amerykańska Agencja Rządowa Żywności i Leków (*Food and Drug Administration - FDA*) przyznała status GRAS (*Generally Recognized As Safe*) [36]. Ponadto, *Y. lipolytica* otrzymały również status QPS (*Qualified Presumption of Safety*) nadany przez Europejski Urząd ds. Bezpieczeństwa Żywności (*European Food Safety Authority - EFSA*) oraz Międzynarodową Federację Mleczarską (*International Dairy Federation - IDF*), co ułatwia ich zastosowanie w przemyśle spożywczym i paszowym [42–44]. W wielu badaniach przeprowadzonych na zwierzętach udowodniono skuteczność i bezpieczeństwo stosowania zarówno biomasy drożdży *Y. lipolytica*, jak i innych produktów uzyskanych z ich udziałem, zarówno oczyszczonych jak i zawartych w biomasie [36,45,46].

Dzięki bogatemu wachlarzowi badań związanych z metabolitami produkowanymi przez drożdże *Y. lipolytica* oraz w świetle informacji związanych z ich bezpiecznym stosowaniem, drożdże te stały się mikrofabryką służącą otrzymywaniu ogromnej gamy cennych biologicznie związków od skali laboratoryjnej po przemysłową, a ich podsumowanie zestawiono na Rysunku 1.



Rysunek 1. Związki bioaktywne syntetyzowane przez drożdże *Y. lipolytica*. Związki są skategoryzowane i zilustrowane według podziału na białka, alkohole wielowodorotlenowe, lipidy, aminokwasy, kwasy organiczne i terpenoidy, na podstawie dostępnych artykułów naukowych i patentów [opracowanie własne na podstawie [47]].

Szybko powiększająca się wiedza dotycząca funkcjonowania i regulacji szlaków metabolicznych u drożdży *Y. lipolytica*, ich zsekwencjonowany genom oraz dynamiczny rozwój wydajnych narzędzi inżynierii genetycznej, szczególnie opartych o metody Golden Gate cloning i system CRISPR/Cas9 [48,49] zaoferowały nowe perspektywy w inżynierii szlaków metabolicznych, umożliwiając wykorzystanie tych drożdży jako efektywnych fabryk do produkcji szerokiej gamy cennych bioproduktów. Udało się opracować procesy biotechnologiczne wydajnej produkcji kwasu cytrynowego i izocytrynowego [50,51], α-ketoglutarowego [52], kwasu pirogronowego [53], kwasu bursztynowego [54], 2-fenyloetanolu [55] czy kwasu itakonowego [56]. Gatunek *Y. lipolytica* został wykorzystany również do opracowania wydajnej produkcji polioli, takich jak mannitol czy erytrytol, powszechnie stosowanych jako dodatki do żywności z uwagi na ich cechy poprawiające smak, słodzące, stabilizujące, zagęszczające i nawilżające (humektanty)[69,70].

Wspomniany powyżej wydajny szlak sekrecyjny drożdży *Y. lipolytica* wydziela do środowiska hodowlanego wiele cennych białek, jak: lipazy [57] i proteazy [58] jak również

pozwala na produkcję białek heterologicznych [36,59–61] mających zastosowanie w przemyśle. Przykładowo, lipazy (EC 3.1.1.3), których drożdże *Y. lipolytica* produkują wiele, m.in. *Lip2p*, *Lip7p* i *Lip8p,* hydrolizują TAG do FFA i glicerolu. Wśród wspomnianych lipaz, to *Lip2p* wykazuje najwyższą aktywność hydrolityczną i może być wykorzystana jako narzędzie terapeutyczne w leczeniu zewnątrzwydzielniczej niewydolności trzustki [62]. Ponadto, wśród ponad 100 rekombinowanych białek wyprodukowanych z wykorzystaniem transformantów *Y. lipolytica* [63], znajduje się kilka ważnych z punktu widzenia farmaceutycznego. Białka te są biokompatybilne z organizmem człowieka i uzyskano je poprzez wklonowanie genów ludzkich w genom szczepów *Y. lipolytica* z wcześniej humanizowanym aparatem N-glikozylacji białek [64]. Przykładem produkcji takiego białka jest cytochrom P450, odgrywający ważną rolę w metabolizmie oksydo-redukcyjnym, który uzyskali Nthangeni i współpracownicy poprzez wklonowanie ludzkiego cytochrom P450 (CYP1A1) [65]. Innymi przykładami białek terapeutycznych mogą być: interferon alfa 2b [66], naskórkowy czynnik wzrostu [67], N-glikoproteiny [68], czynnik krzepnięcia krwi XIIIa [69], proinsulina i insulinotropina [70], receptor estrogenowy α [71] oraz anafilatoksyna C5a, która posiada właściwości zarówno spazmogenne, jak i związane z aktywacją leukocytów [71].

Ze względu na wydajny proces lipogenezy, drożdże *Y. lipolytica* stały się gospodarzem do biosyntezy TAG, również o modyfikowanym składzie kwasów tłuszczowych oraz związków będących lipidowymi pochodnymi. Kwas linolowy (LA) jest głównym wielonienasyconym kwasem tłuszczowym (PUFA) syntetyzowanym przez dzikie szczepy *Y. lipolytica*. Jednakże możliwości jakie daje inżynieria genetyczna umożliwiły opracowanie rekombinowanych szczepów zdolnych do biosyntezy innych kwasów tłuszczowych o zdecydowanie prozdrowotnych właściwościach, jak np. kwas eikozapentaenowy (EPA), kwas dokozaheksaenowy (DHA), kwas arachidonowy (ARA), sprzężony kwas linolowy (CLA) czy kwas γ-linolenowy (GLA) [24,47]. Produkcja oleju bogatego w PUFA jest pierwszym procesem wykorzystującym modyfikowane genetycznie drożdże *Y. lipolytica*, który osiągnął etap komercjalizacji: olej bogaty w EPA [72] znalazł zastosowanie jako suplement diety, a komórki drożdży bogate w EPA jako pasza w hodowli ryb [36,73]. Wśród lipidowych pochodnych produkowanych z udziałem genetycznie modyfikowanych szczepów *Y. lipolytica* wymienić należy: cyklopropan C19 [74],[75] estry metylowe i etylowe kwasów

tłuszczowych - biodiesel (FAME, FAEE) [76], alkohole tłuszczowe [76] czy kampesterol [77].

1.4. Fosfolipidy

Dotychczasowe strategie inżynierii metabolicznej drożdży *Y. lipolytica* w celu produkcji cennych związków lipidowych koncentrowały się głównie na biosyntezie TAG i wolnych kwasów tłuszczowych. Pomimo dużych predyspozycji tych mikroorganizmów do biosyntezy również innych grup lipidów neutralnych, dotychczas nie podejmowano prób ich nadprodukcji. Jednakże, ze względu na ich liczne właściwości biologiczne i szerokie spectrum zastosowania, szczególnie interesujące wydają się związki należące do grupy fosfolipidów.

PL stanowią główny składnik strukturalny błon biologicznych i odgrywają kluczową rolę w ich integralności, przepuszczalności i płynności [78]. Ich obecność warunkuje utrzymanie prawidłowej homeostazy organizmu. PL są lipidami złożonymi o charakterze amfifilowym. Składają się ze szkieletu gliceryno-3-fosforanowego, w którym hydrofobowe grupy acylowe są zestryfikowane w pozycjach sn-1 i sn-2 [79] z kolei pozycja sn-3 zajęta jest przez grupę fosforanową, która przyczynia się do hydrofilowego charakteru całej cząsteczki. Najprostszym związkiem z klasy PL jest kwas fosfatydowy (PA). Ponadto, do PL zaliczają się: fosfatydyloetanolamina (PE), fosfatydyloinozytol (PI), fosfatydyloseryna (PS) i fosfatydylocholina (PC) [80].

PL wykazują wiele funkcji prozdrowotnych, m.in. regulują funkcje wielu narządów, obniżają poziom cholesterolu i trójglicerydów we krwi, pomagają w naprawie uszkodzonej tkanki wątroby czy zapobiegają rozwojowi chorób neurologicznych [81]. Ze względu na swoją rolę fizjologiczną, fosfolipidy charakteryzują się bardzo niską toksycznością i mogą być podawane w każdej z form: pozajelitowo, doustnie i miejscowo [82]. Różnorodność strukturalna fosfolipidów i wynikająca z niej zmienność właściwości chemicznych, biofizycznych i technologicznych, prowadzi do szerokiego zastosowania fosfolipidów w różnych formulacjach farmaceutycznych [82–85]. Po wprowadzeniu do środowiska wodnego, PL samoczynnie tworzą różne struktury nadcząsteczkowe, m.in. mają skłonność do tworzenia liposomów, dzięki czemu mogą być wykorzystywane jako nośniki leków [86].

Ponadto, PL posiadają dobre właściwości emulgujące, dzięki czemu mogą stabilizować emulsje [87]. Dzięki swoim właściwościom związków powierzchniowo czynnych, PL mogą powlekać powierzchnię hydrofobowych mikrokryształów leków w celu zwiększenia ich hydrofilności i stabilności [88]. PL są także powszechnie stosowane w lipidowej terapii zastępczej, w celu „uzupełnienia" błon komórkowych, które ulegają uszkodzeniu w przewlekłych chorobach neurodegeneracyjnych, nowotworach czy procesach starzenia [89].

Obecnie naturalne fosfolipidy otrzymywane są ze źródeł roślinnych, takich jak soja, słonecznik, nasiona rzepaku, kiełki pszenicy i nasiona lnu; jak również ze źródeł pochodzenia zwierzęcego, takich jak żółtko jaja kurzego, mleko lub z kryla. Jednakże modyfikowane genetycznie drożdże *Y. lipolytica*, o zwiększonej zdolności do biosyntezy PL mogłyby stanowić doskonałą alternatywę, która równocześnie wyeliminowałaby konkurencję pomiędzy produkcją żywności ze wspomnianych surowców a zapotrzebowaniem przemysłowym na PL.

1.5. Resweratrol

Oleisty charakter drożdży *Y. lipolytica* i wynikająca z tego obecność dużej ilości acetylo-CoA i malonylo-CoA będących prekursorami bardzo wielu związków, czyni z drożdży *Y. lipolityca* idealnych kandydatów do biosyntezy karotenoidów, terpenów, flawonoidów i poliketydów [90–94]. Paleta związków powstających we wspomnianych szlakach metabolicznych ma duże zastosowanie w produkcji dodatków do żywności, kosmetyków, chemikaliów, paliw czy farmaceutyków.

Jednym z takich związków jest resweratrol (RES), będący naturalnie występującym polifenolowym stilbenem (3,5,4'-trihydroksystilben). Bogatym źródłem tego związku w diecie ludzi są winogrona oraz produkowane z nich czerwone wino. RES został jednak zidentyfikowany w około 70 różnych rodzajach roślin, takich jak borówki, żurawina, rabarbar, passiflora czy orzeszki ziemne [95]. RES należy do grupy antybiotyków - fitoaleksyn i jest produkowany przez rośliny w odpowiedzi na urazy, infekcje, patogeny czy stres środowiskowy [95]. W ciągu ostatnich dwóch dekad opublikowano ponad 2000 badań dotyczących wpływu RES na zdrowie ludzi [PubMed, 2023]. Badania kliniczne udowodniły,

że RES korzystnie wpływa na biomarkery chorobowe cukrzycy, chorób sercowo-naczyniowych i zaburzeń neurologicznych [96,97]. Obecnie RES jest przemysłowo wykorzystywany jako składnik żywności i kosmetyków oraz sprzedawany jako suplement diety. Rynek RES wynosi około 97,7 mln USD i oczekuje się, że będzie rósł ze średnią roczną stopą wzrostu (CAGR) wynoszącą 8,1% od 2018 do 2028 roku [98]. Na obecną chwilę głównym źródłem komercyjnego RES są ekstrakty roślinne, głównie z korzenia rdestowca japońskiego (*Polygonum cuspidatum)* [99], jednakże jego produkcja na drodze syntezy chemicznej przez firmę DSM jak i na drodze biotechnologicznej przez firmę Evolva wykazują tendencję wzrostową [100,101].

Jak wspomniano, RES jest otrzymywany głównie na drodze ekstrakcji z roślin przy użyciu złożonych procesów, których wadami są: wysoki koszt produkcji, wieloetapowa izolacja i oczyszczanie, sezonowość jak również niskie stężenia, co zdecydowanie utrudnia zaspokojenie światowego zapotrzebowania na ten związek [102]. Biosynteza RES przy użyciu mikroorganizmów, oferuje atrakcyjną i zrównoważoną alternatywę, umożliwiając zwiększenie wydajności oraz mniejszy ślad ekologiczny i energetyczny w porównaniu z procesami ekstrakcji roślinnej. W komórkach roślinnych biosynteza RES zachodzi dwoma głównymi szlakami - metabolizmu tyrozyny (Tyr) i fenyloalaniny (Phe) jako związków pośrednich. W przypadku szlaku metabolizmu tyrozyny, w pierwszym etapie amoniakalna liaza tyrozyny (TAL) deaminuje L-Tyr do kwasu p-kumarowego, który przekształcany jest następnie do kumarylo-CoA przez enzym ligazę 4-kumaranu-CoA (4CL). W kolejnym etapie, kumarylo-CoA kondensowany jest z wykorzystaniem trzech jednostek malonylo-CoA w celu utworzenia RES przez syntazę stilbenową (STS) [103]. Z kolei, biosynteza RES w szlaku metabolizmu Phe rozpoczyna się od powstawania kwasu cynamonowego w wyniku deaminacji Phe przez amoniakalną liazę fenyloalaninową (PAL). Następnie, kwas cynamonowy jest hydroksylowany do kwasu p-kumarowego przez 4-hydroksylazę cynamonową (C4H). W ostatnim etapie, podobnie do szlaku metabolizmu Tyr, kwas cynamonowy może być przekształcony w RES za pośrednictwem 4CL i STS. Mikrobiologiczna produkcja RES poprzez heterologiczną ekspresję szlaku roślinnego została z powodzeniem przeprowadzona w kilku gatunkach [104], z których jednym były drożdże *Y. lipolytica*. Gatunek ten charakteryzuje się naturalnie wydajnie funkcjonującymi szlakami prowadzącymi do malonylo-CoA i szlakiem pentozofosforanowym (PPP), dostarczającym

siły redukcyjnej, potrzebnej w procesie lipogenezy w postaci NADPH. Obie cechy metaboliczne są szczególnie istotne dla produkcji związków pochodzących ze szlaku szikimowego oraz metabolitów roślinnych, wymagających aminokwasów aromatycznych oraz malonylo-CoA i udało się opracować szczepy wydajnie produkujące takie roślinne metabolity jak: naryngenina, eriodictyol, taksifolina czy inne związki aromatyczne [105–107]. Również wspominany RES został wyprodukowany w *Y. lipolytica* przez firmę DuPont [107,108].

1.6. Lipidowe pochodne resweratrolu – aktywność biologiczna i biodostępność

RES, podobnie jak inne polifenole, charakteryzuje się wysoką absorpcją po podaniu doustnym oraz szybkim metabolizmem. Może się to wiązać ze słabą biodostępnością tego związku. W efekcie, w krążeniu systemowym można znaleźć jedynie śladowe ilości niezmienionego RES, co ogranicza jego efektywne wykorzystanie praktyczne w medycynie. Ponadto, wykorzystanie RES może być utrudnione przez jego hydrofilowość przy jednoczesnym zastosowaniu w układach lipofilowych [109].

Coraz częściej w światowej literaturze naukowej pojawiają się badania wskazujące na kilka koncepcji poprawiających właściwości farmakokinetyczne polifenoli w układach biologicznych. Jedna z teorii sugeruje wykorzystanie nośnika lipidowego jako formy sprzyjającej zwiększeniu biodostępności związków aromatycznych. Lipidy po przejściu do jelita wiążą się z lipoproteinami, które transportują je kolejno do układu limfatycznego, z którego przedostają się one do krążenia systemowego dopiero na wysokości żyły piersiowej. Związki polifenolowe po dotarciu do jelita transportowane są do naczyń krwionośnych, następnie żyłą wrotną do metabolicznie aktywnej wątroby, a dopiero potem do krążenia systemowego. Dlatego też nadanie związkom polifenolowym charakteru hydrofobowego, może pozwolić na uniknięcie/ominięcie metabolizmu pierwszego przejścia przez wątrobę, co w efekcie zwiększy ich stężenie w przewodach i węzłach chłonnych, które mogą być miejscem ich terapeutycznego działania [110]. Jednym z przykładów zastosowania nośników lipidowych dla substancji bioaktywnej może być dostępny na rynku Siliphos®. Jest to kompleks sylibiny (głównego składnika czynnego sylimaryny) i fosfatydylocholiny, który wykazuje większą biodostępność po podaniu doustnym. Taka forma podania substancji

czynnej zwiększyła jej aktywność farmakologiczną w porównaniu z czystą sylibiną [111]. Dostarczanie związków aktywnych za pomocą nośników lipidowych polega głównie na zamknięciu związków hydrofilowych w wodnym rdzeniu lub na uwięzieniu cząsteczek hydrofobowych w dwuwarstwie lipidowej. Chociaż podejście to jest skuteczne, ma pewne ograniczenia, m.in. możliwość przedwczesnego wycieku substancji aktywnej na skutek różnych czynników fizycznych [112]. Jednym z rozwiązań jest opracowanie koniugatu lipid-lek (*Lipid Drug Conjugate - LDC*), polegającego na utworzeniu wiązania kowalencyjnego pomiędzy substancją czynną, a związkiem lipidowym, dzięki czemu uwolnienie cząsteczki czynnej zachodzi w tkankach docelowych w wyniku działania enzymów endogennych [112].

W światowej literaturze istnieje kilka dostępnych badań naukowych dotyczących lipidowych pochodnych związków fenolowych. Badania te wykazały, że nadanie charakteru lipidowego poprzez modyfikacje struktury związków fenolowych znacząco wpłynęło na poprawę ich aktywności biologicznej i lepszą wydajność w biologicznych układach modelowych. Przykładem mogą być badania Zhong i Shahidi, którzy przeprowadzili estryfikację galusanu epigallokatechiny (EGCG) z kwasem stearynowym, EPA i DHA. Lipidowe pochodne EGCG wykazywały większą aktywność antyoksydacyjną w zmiataniu rodnika 1,1-difenylo-2-pikrylohydrazylu (DPPH) niż sam EGCG [113]. Większą aktywność antyoksydacyjną otrzymano również poprzez estryfikację kwasu rozmarynowego z kwasem eikozanowym [114]. Ponadto, RES zestryfikowany kwasami tłuszczowymi o różnej długości łańcucha (C3:0-C22:6) charakteryzował się wyższą aktywnością antyoksydacyjną w porównaniu z czystym RES. W szczególności dwa estry RES z EPA i DHA wykazywały lepszą aktywność zmiatania nadtlenku wodoru (H_2O_2). Ponadto, badane związki hamowały wydajnie rozszczepienie DNA indukowane przez rodnik hydroksylowy [115]. Ostatnie badania wykazały także, że chemicznie zsyntetyzowany ester RES z kwasem masłowym ma wyższą zdolność do zmniejszania akumulacji tłuszczu w wątrobie oraz wyższą pojemność antyoksydacyjną niż czysty RES [116,117]. W przypadku RES wykazano, że jego acylowanie może zwiększyć stabilność oksydacyjną i opóźnić szybką degradację w przewodzie pokarmowym, a także metabolizm i wydalanie RES *in vivo*, ponieważ metabolizm fazy I/II jest trudniejszy ze względu na częściowe lub całkowite zablokowanie grup hydroksylowych, które w związku z tym wykazują lepszy profil farmakokinetyczny i lepszą dystrybucję w wątrobie, śledzionie, sercu i płucach szczurów, w porównaniu

z dystrybucją czystego RES [118]. W związku z tym, otrzymywanie nowych pochodnych resweratrolu i poprawa ich właściwości farmakokinetycznych są ostatnio bardzo ważnym tematem badań. Co więcej, dotychczas prowadzone badania nad lipofilowymi pochodnymi RES skupiają się głównie na ich właściwości antyoksydacyjnych. Interesującym wydaje się poszerzenie badań o inne aktywności biologiczne, jakie mogą wykazywać uzyskiwane związki.

2. Cel pracy

Interdyscyplinarny charakter prezentowanej pracy wpłynął na wyróżnienie dwóch głównych celów prowadzonych badań. Pierwszym celem głównym badań była wydajna mikrobiologiczna synteza wybranych związków charakteryzujących się aktywnością biologiczną z wykorzystaniem transformantów drożdży *Y. lipolytica*. Wybrano związki biologicznie aktywne z dwóch różnych grup – fosfolipidy oraz resweratrol. Drugim celem głównym była ocena właściwości przeciwnowotworowych i antyoksydacyjnych estrów resweratrolu i wybranych kwasów tłuszczowych na wybranych ludzkich liniach nowotworowych.

Cele główne realizowane były poprzez cele szczegółowe, wśród których wymienić należy:

1. uzyskanie transformantów *Y. lipolytica* o zwiększonej zdolności do produkcji fosfolipidów poprzez inżynierię metabolizmu natywnego szlaku biosyntezy tych związków;
2. uzyskanie transformantów drożdży *Y. lipolytica* wydajnie syntetyzujących resweratrol w szlaku katabolizmu tyrozyny i fenyloalaniny;
3. optymalizację warunków hodowli transformantów drożdży *Y. lipolytica* w celu wydajnej biosyntezy zarówno resweratrolu jak i fosfolipidów w warunkach bioreaktorowych;
4. uzyskanie estrów resweratrolu z kwasami: palmitynowym, oleinowym i sprzężonym kwasem linolowym metodą syntezy chemicznej;
5. ocenę wpływu lipidowych pochodnych resweratrolu na żywotność ludzkich komórek nowotworowych linii BxPC3, A549, HT29 oraz ich wpływu na zdrowe komórki linii BJ, stanowiące grupę kontrolną, poprzez analizę cytotoksyczności, apoptozy i ekspresji głównych genów apoptotycznych;
6. ocenę właściwości antyoksydacyjnych lipidowych pochodnych resweratrolu poprzez pomiar ekspresji dysmutazy ponadtlenkowej, będącej kluczowym enzymem ochrony antyoksydacyjnej organizmu.

3. Komentarze do publikacji

3.1. Publikacja 1

Publikacja przeglądowa stanowi rozszerzony wstęp teoretyczny do badań wykonanych w ramach pracy doktorskiej. Jej głównym celem było przedstawienie ostatnich osiągnięć w zakresie inżynierii szlaku biosyntezy lipidów u drożdży olejogennych. Ponadto, omówiono postępy w syntezie pochodnych lipidów oraz przedstawiono ich znaczenie dla zdrowia człowieka oraz możliwości zastosowań przemysłowych. Omówione zostały możliwości optymalizacji warunków hodowli w celu poprawy wydajności i stężenia uzyskiwanych wartościowych związków. Dodatkowo, przedyskutowano aspekty ekonomiczne obecnej produkcji olejów pochodzenia mikrobiologicznego.

Biorąc pod uwagę spójność tematyczną cyklu publikacji składających się na niniejszą rozprawę doktorską, za najistotniejszą część pracy przeglądowej uznano rozdziały przedstawiające drożdże jako atrakcyjnych, alternatywnych producentów cennych związków lipidowych oraz ich przewagę nad innymi źródłami pozyskiwania lipidów (zwierzęta i rośliny). Obszernie temat ten omówiono we wstępie rozprawy doktorskiej, gdzie częściowo wykorzystano zawartość pracy przeglądowej (rozdziały 1.1 – 1.3). W w publikacji zwrócono uwagę na problem związany z kosztami mikrobiologicznej produkcji związków lipidowych, które często utrudniają ich przemysłowe zastosowanie. W analizie techniczno-ekonomicznej stwierdzono, że aż 80% kosztów produkcji lipidów z udziałem drożdży zależy od zastosowanego źródła węgla, którym w wielu przypadkach jest glukoza. W celu obniżenia nakładów finansowych, które należy ponieść w biotechnologicznej produkcji lipidów, literatura światowa donosi o szeroko zakrojonych badaniach nad bardziej opłacalnymi źródłami węgla, takimi jak gliceryna odpadowa czy hydrolizaty biomasy ligninocelulozowej. Stąd w badaniach prowadzonych w ramach pracy doktorskiej, jako główne źródło węgla wykorzystano glicerol, zarówno kosmetyczny jak i odpadowy.

Pomimo tego, że praca przeglądowa skupia się na produkcji związków lipidowych i ich pochodnych, należy pamiętać, że drożdże oleiste dzięki dużym ilościom prekursorów wykorzystywanych w innych szlakach metabolicznych, którymi są malonylo-CoA oraz acetylo-CoA, stanowią atrakcyjną platformę do syntezy szerokiej gamy nowych wartościowych związków o aktywności biologicznej.

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REVIEW ARTICLE

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Advances in production of high-value lipids by oleaginous yeasts

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ABSTRACT

The global market for high-value fatty acids production, mainly omega-3/6, hydroxy fatty-acids, waxes and their derivatives, has seen strong development in the last decade. The reason for this growth was the increasing utilization of these lipids as significant ingredients for cosmetics, food and the oleochemical industries. The large demand for these compounds resulted in a greater scientific interest in research focused on alternative sources of oil production - among which microorganisms attracted the most attention. Microbial oil production offers the possibility to engineer the pathways and store lipids enriched with the desired fatty acids. Moreover, costly chemical steps are avoided and direct commercial use of these fatty acids is available. Among all microorganisms, the oleaginous yeasts have become the most promising hosts for lipid production - their efficient lipogenesis, ability to use various (often highly affordable) carbon sources, feasible large-scale cultivations and wide range of available genetic engineering tools turns them into powerful micro-factories. This review is an in-depth description of the recent developments in the engineering of the lipid biosynthetic pathway with oleaginous yeasts. The different classes of valuable lipid compounds with their derivatives are described and their importance for human health and industry is presented. The emphasis is also placed on the optimization of culture conditions in order to improve the yield and titer of these valuable compounds. Furthermore, the important economic aspects of the current microbial oil production are discussed.

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Introduction

The majority of the world's supply of cosmetics, food and pharmaceutical oils and fats is derived from plants and animals. About 350 species of crop plants have been found suitable for lipid production, of which the most commonly used species for extraction are shown in Figure 1. Their sustainability is still challenged due to food crises around the world [1]. Over the last few decades, the oleochemical industry has been developing technologies that utilize plant and animal oils and fats as renewable sources, an example of which is the wide range of products from companies such as: Emery Oleochemicals, Univar Solutions, Oleon and others, with a share of over 50% renewable resources [2–6]. As a result of the growing demand for oleochemicals, the production of oilseed producing crops and vegetable oils have been constantly increasing (by 14%; from 148.96 million tons in 2010–2011 to 169.56 million tons in 2013–2014) and further growth is expected (up to 28% by 2023 – based on the average for 2011–2014) [2]. Out of the 157 million tons of fats and oils used in 2012–2013, 77% was used to produce food for humans and animals, 12% for the production of biodiesel and the remaining 11% for the production of oleochemicals. Moreover, the demand is currently experiencing unprecedented growth due to the rapidly growing human population [7].

Due to the increased demand for lipids and limitations in lipid production based on traditional sources, alternative methods of lipid production have been broadened to include biotechnological methods using oleaginous microbes. Microbe-derived lipids are similar in composition to those that can be obtained from plants and animals, while the main advantage of microbial-based manufacturing processes is the independence from season, climate and location. Moreover, they can be synthesized using a wide range of carbon sources, including organic waste or renewable carbon sources. More advantages of these oils over traditional oil sources are tabulated in Table 1.

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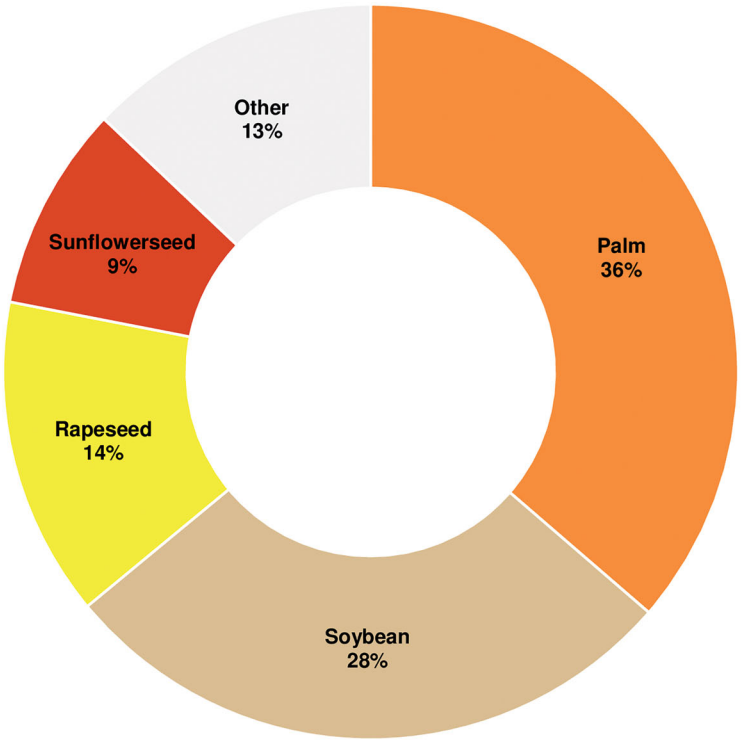

Oil type	Mln tons
Palm	72.69
Soybean	55.33
Rapeseed	28.10
Sunflowerseed	18.07
Other	25.83
TOTAL	200.02

Figure 1. Global oil production in 2018/2019. Other oils include: palm kernel, peanut, cottonseed, coconut and olive oil.

Table 1. Advantages of microbial over plant- and fish-based oil production [114,200,201].

Advantage of microbial oil	Justification
Possibility to use numerous carbon sources as substrates	Flexible metabolism and readily utilized waste products such as waste glycerol or lignocellulosic materials
Wide range of microorganisms	Oleaginous bacteria, yeast and filamentous fungi accumulate at least 20% of lipids in dry weight
Rapid growth	Doubling time of 1–3 h for yeasts results in higher yields (faster division rate = faster nitrogen utilization = shorter time to reach the stationary – lipogenic phase)
High oil content	20–80% compared to 5–35% in plants and 2–30% in fish
High quality of oil composition	Up to 50% of EPA in total lipid content from glucose during fed-batch fermentation
Independence of seasonal weather conditions and climatic changes	Bioreactor cultures allow to avoid extreme weather conditions; light is not required for growth
Less water needed	No issues with transpiration, leakages and evaporation of water
No need to use fertile land	No competition with food production or agriculture; especially important in dry climate regions.
Strictly controlled cultivation	Bioreactor cultures allow for precise control of cultivation conditions

This review presents the oleaginous microorganisms as being promising microbial oil factories, with a particular emphasis put on yeast. The first part of this review describes the chemical nature of oils produced by microbes and their importance for the development of a sustainable economy. Furthermore, microbial species suitable for lipid and lipid derivatives production as well as a brief summary of the effort invested in developing efficient lipid cell factories are presented. The second part of this review focuses on the production of high-value lipids and their derivatives using microorganisms. Moreover, recent developments in lipid biosynthesis pathway engineering are discussed. The work is summed up with a short presentation of possible directions for future developments in the field of microbial oil production.

Microbial oil

Oils produced by microorganisms are called microbial oils (MO) or single-cell oils (SCOs). They include triacylglycerols (TAG) - energy stores, sterol esters, as well as

phospholipids and glycolipids - membrane constituents. Among these lipids, the TAG fraction, which is formed by linking fatty acids (mostly monounsaturated and saturated) with an ester linkage to three alcohol groups in glycerol has been recognized as the main component of SCO, which are chemically similar to vegetable oils, e.g. rapeseed oil [8]. Moreover, TAGs are also the main target for biotechnological product development. The storage lipids are accumulated in specialized cell organelles - lipid bodies (LB) located in the cytosol. TAGs are utilized as an intracellular source of energy, particularly during environmental stress caused by – among others – limitation of the carbon source in the growth medium [9]. The yield and type of lipid depend on several factors, such as the genotype of the microorganism, culture conditions or the substrate used [10].

Importance and economic considerations of microbial oil

In addition to technological interest, there has been a notable increase in the economic interest in oil produced by microorganisms such as bacteria, yeasts and filamentous fungi. Despite higher manufacturing costs of MO due to the cost of feedstock and maintaining aseptic conditions, which are not the issue in the current vegetable oil or animal fat industries, MO as an alternative for large-scale oils production is still significant. The first large-scale SCO production operations have been reported in New Zealand at the end of the 1980s, where *Apiotrichum curvatum* (= Cryptococcus curvatus) ATCC 20509 growing on cheese-whey in pilot-scale operations (viz. 500-L bioreactors) was used [11,12]. Following a techno-economic evaluation of the process, the price of the produced SCO was c. l.0 US $per kg [12]. The present market for natural fats is particularly diverse. The price of oils, depending on their fatty acid (FA) composition, ranges from $0.30/kg to over $100/kg [13,14]. Therefore, novel microorganisms, producing large amounts of lipids with a composition and structure resembling that of the valuable oils, are still in demand [13]. The concept of large-scale production of microbial unusual fatty acids, especially those essential in healthcare, nutrition and cosmetic industries, is now of exceptionally high priority. In particular, the production of microbial oils containing omega-3 fatty acids, such as: eicosapentaenoic (EPA) and docosahexaenoic (DHA), omega-6 acids, such as γ-linolenic acid (GLA), which are used in food, other fatty acids used in cosmetics, such as conjugated linoleic acid (CLA), ricinoleic acid (RA) and certain waxes are of keen interest as they provide the microbial platforms a definitive advantage over corresponding plant platforms [15]. The commercial production of some of these compounds from microbes demonstrates that large-scale production of microbial oils is feasible, given an appropriate process and market. Varying costs of production depend on the species, cultivation method, extraction strategy and the carbon source used. Table 2 presents examples of commercialized lipid products manufactured by microorganisms.

However, despite promising prospects to produce microbial lipids for nutraceuticals, production costs often hinder their industrial implementation. In a techno-economic analysis concerning high-value lipids production from microbes, it was found that as much as 80% of the production cost was dependent on the carbon source used, which in many cases was glucose [16]. In order to reduce the production cost, more cost-effective carbon sources such as agricultural, forest residues or waste glycerol have been extensively studied in the literature [17–19].

Omega-3 fatty acids - EPA and DHA show numerous positive effects - in particular anti-inflammatory, anticancer and anti-thrombotic activity [20]. CLA and GLA-rich microbial lipids possess the ability to inhibit tumor growth and metastasis, while RA exerts analgesic and anti-inflammatory effects [21]. Moreover, CLA and RA are often used in cosmetics to promote hydration and are commonly added to such products as lotions, make-up and cleansers [22]. Producing such high-value fatty acids from microbes can reach a market value ranging from $40,000 to 120,000 per ton [23].

Table 2. Commercialized microbial-derived fatty acids.

	Microorganism	Product	Trade name	Company
Filamentous fungi	*Mucor circinelloides*	GLA-rich oil	Oil of Javanicus, GLA-Forte	J & E Sturge
	Mortierella alpina	ARA-rich oil	SUNTGA40S	Suntory Ltd
			n/a	Gist-brocades/Martek BioSciencesCorp./DSM
			n/a	Wuhan Alking Bioengineering Co. Ltd/Cargill Inc.
Algae	*Crypthecodinium colinii*	DHA-rich oil	DHASCO, life'sDHA™	Martek BioSciences Corp./DSM
	Schizochytrium sp.		DHASCO-S	
	Ulkenia sp.		DHA CL, DHAid™	Lonza
	Thraustochytrium sp. ONC-T8		Schizo-ONC	Ocean Nutrition Canada Ltd/DSM
	Schizochytrium sp.		n/a	Tiankai Biotechnology Co. Ltd
Yeast	*Yarrowia lipolytica*	EPA-rich oil	NewHarvest™	DuPont

The cost of plant-derived lipids containing high-grade GLA is estimated at $45–50/kg [24]. However, the number of vegetable oils containing this acid is narrowly limited. Cocoa butter, the main ingredient of chocolate used to manufacture solid and semi-solid forms of medicines is a very expensive raw material, with price and availability depending on weather and the cocoa-growing conditions [25]. The microbiological production of cocoa butter lipids was economical in the 1980s when the price of cocoa butter was greater than $8/kg; then the price dropped to $4.5/kg, while currently, the cocoa butter is $8/kg again [26]. Moreover, the prices of cocoa supplies are increasing due to growing hindrances in cocoa plant cultivation. These problems include global warming (shortage of water in West African countries), inefficient cultivation methods and the necessity for large tracts of land, which results in the loss of priceless rainforests. There is even a risk of cocoa product disappearance (including cocoa butter, sal fat, shea fat, etc.) [13].

Aquatic organisms are sources of omega-3 fatty acids (DHA and EPA) for functional foods. Adherence to the recommended daily intakes entails high consumption of fish and other seafood [27]. About 282,000 tons of fish oil was consumed by the human population in 2014 and it is estimated that in 2025 this consumption will increase by more than 250 percent and be in excess of 711,000 tons. This indicates a composite annual growth rate of 8% per year, based on polynomial growth forecast [28,29]. If trends continue, the fish oil market will grow from $1.69 billion in 2013 to $5 billion in 2025 [29]. In addition to limited seafood resources, some fish species may be contaminated with dioxins, methylmercury or polychlorinated biphenyls – compounds resulting from environmental pollution. Most of these are hydrophobic compounds that accumulate in the marine food chain, mainly in lipid bodies, which indirectly results in a concentration of these contaminants during the oil extraction process [30]. According to current statistics, human consumption of omega-3 fatty acids will have increased by almost 80% in 2025 [31,32]. The growing global demand for omega-3 fatty acids has a significant impact on the environment - the production of these acids using animal raw materials consumes an ever greater amount of the limited natural resources. According to FAO [28] and Grand View Research [29], global demand for omega-3 amounted to 21,900 tons in 2012 but is expected to grow to over 135,500 tons in 2025, which means an annual growth rate of omega-3 amounting to 16% in 2015–2025. Thus, the utilization and application of various substitutes of those lipids will be highly significant for human nutrition.

Some of the aforementioned challenges may be overcome by carrying genetic modifications and directed evolution of oleaginous microorganisms, process design as well as implementing advanced lipid extraction protocols.

Oleaginous microorganisms

With respect to lipid production, there are some microorganisms known as 'oleaginous' that are able to accumulate over 20% of their biomass in the form of lipids [33]. Only some species of filamentous fungi, microalgae, bacteria and yeast are considered oleaginous, and their oleogenicity is extremely valuable [34]. An overview of the composition of lipids present in various microorganisms is summarized in Table 3.

Lipid accumulation in the oleaginous yeast occurs *via de novo* (*e.g.* from sugar substrates under nitrogen limitation) or *ex novo* (from lipid substrates) routes [34–36]. During *de novo* lipid biosynthesis, acetyl-CoA, constituting a basis for fatty acid synthesis, is formed as a result of the reaction of pyruvate dehydrogenase (PDH) complex after glycolysis. Briefly, the pyruvate resulting from glycolysis is transferred from the cytoplasm into the mitochondria, where it is transformed to acetyl-CoA by a PDH complex. This can be incorporated into the Krebs cycle or exported to the cytoplasm, becoming a substrate for fatty acid biosynthesis [14]. On the other hand, *ex novo* lipid synthesis takes place when hydrophobic substrates are utilized. These carbon sources, including fatty acids, are transported into the cell and can be used as an energy source or undergo enzymatic modifications [37]. Comprehensive reviews on this subject are available elsewhere [13,14,34–36,38,39]. In contrast to oleaginous microorganisms, non-oleaginous species tend to store polysaccharides and produce secondary metabolites [37].

So far, about 70 of the 1600 species of oleaginous yeasts have been identified and have been characterized by their oleaginous character, and the number is still growing [40]. Depending on the species, the lipid content varies and can reach up to 70% of cell dry weight (DCW) under specific nutrient limitations and with an excess of carbon [41]. Most of these species are susceptible to improvement through genetic modification and suitable for large-scale fermentation [42]. Furthermore, most of these species can utilize cheap waste materials including sugar beet molasses [43], waste cooking oil [44], waste paper [45] and agro-industrial wastes [46] thus becoming economical biofactories

Table 3. Lipid content and fatty acid composition in various microorganisms.

Microorganism	Total lipid content (% CDW)	% Fatty acid						References
		C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	
	Non-oleaginous							
Bacteria								
Bacillus subtilis	n/a	5.28	8.6	3.83	8.59	36.48	11.84	[178]
Yeast								
Debaryomyces hansenii	7.0	18.9	1	10.5	43.7	18.7	4.7	[179,183]
Geotrichum candidum	4.8	11.1	6.7	7.1	30.2	26	0.1	[186]
Kluyveromyces marxianus	5.2	14.1	26.1	3.8	37.2	14.1	2.9	[181,182]
Saccharomyces cerevisiae	7.2	14.3	31.2	6.8	39.0	n/a	n/a	[179,180]
Schizosaccharomyces pombe	4.65−9.1	11.07	3.21	5.26	77.94	0	n/a	[68,180]
Filamentous fungi								
Aspergillus niger	13.3	5.5	0.24	1.89	2.21	n/a	n/a	[184,185]
	Oleaginous							
Bacteria								
Gordonia sp.	13-52	n/a	n/a	n/a	n/a	n/a	n/a	[33]
Rhodococcus opacus	4-70	27.7	10.8	4.1–4.8	20.7–24.7	n/a	n/a	[33,187]
Yeast								
Candida curvata	58	26–32	n/a	6–15	44–67	5–10	n/a	[188,190]
Cryptococcus albidus	65	16.1	n/a	5.14	17.7	61.1	<1	[188,191]
Lipomyces starkeyi	61−64	33–56	2-6	5–14	26–55	0.1-3	0.12	[183,188,189]
Rhodotorula glutinis	72	12.22–38.24	n/a	20.96–49.54	7.37–27.62	0.26–15.91	0–2.71	[188,192]
Rhodosporidium toruloides	48-67.5	18-37	1	3–36	19-60	2–13	0–3.5	[189]
Trichosporon fermentans	50.4−65.6	27.5	n/a	5.8	54.2	10.1	n/a	[189,193]
Yarrowia lipolytica	36	11	6.0	1.0	28.0	51.0	1.0	[36]
Filamentous fungi								
Fusarium equiseti	56	26.06	1.77	9.66	28.97	31.12	0.73	[195]
Fusarium oxysporum	22–49	22	0.7	11	42	20	0.1	[194]
Mortierella alpina	31	n/a	n/a	n/a	n/a	n/a	n/a	[197]
Mortierella isabellina	50.1	28.2	5.8	1.0	55.5	5.8	2.4	[199]
Microsphaeropsis sp.	22	0.73	14.4	48.7	1.71	4.5	n/a	[199]
Sarocladium kiliense	33	35.13	n/a	5.93	51.08	n/a	n/a	[196]

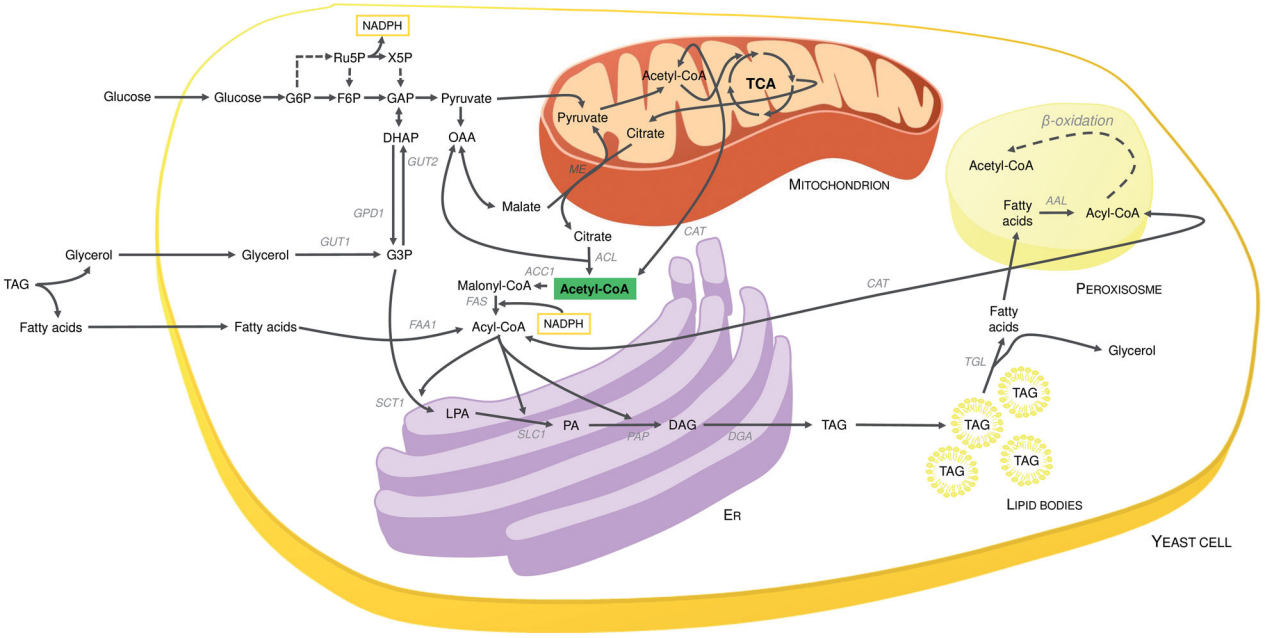

Figure 2. Lipid synthesis and degradation pathways in yeast. Abbreviations: *ACL*: ATP-citrate lyase; *ACC1*: acetyl-CoA carboxylase; *AAL*: acyl/aryl-CoA ligase; *CAT*: carnitine acetyl-transferase; *DAG*: diacylglyceride; *DGA*: diacylglycerol acyltransferase; *DHAP*: dihydroxyacetone phosphate; *ER*: endoplasmic reticulum; *F6P*: fructose-6-phosphate; *FAA1*: acyl-CoA synthetase; *FAS*: fatty acyl synthetase; *G3P*: glycerol-3-phosphate; *G6P* glucose-6-phosphate; *GAP*: glyceraldehyde phosphate; *GUT1*: glycerol kinase; *GUT2*: glycerol 3-phosphate-dehydrogenase; *GPD*: glycerol-3-phosphate dehydrogenase (NAD($^{+}$)); *LPA*: lysophosphatidic acid; ME: malic enzyme; OAA: oxaloacetate; PA: phosphatidic acid; *PAP*: phosphatidic acid phosphatase; Ru5P: ribose-5-phosphate; SCT1: glycerol-3-phosphate acyl transferase; *SLC1*: 1-acyl-sn-glycerol-3-phosphate acyltransferase; TAG: triacylglyceride; TCA: tricarboxylic acid cycle; *TGL*: triacylglycerol lipase; *X5P*: xylulose-5-phosphate.

playing a significant role in waste management. Oleaginous yeasts can accumulate up to 90% of their DCW as TAGs are composed in more than 80% of unsaturated fatty acids, such as linoleic and oleic acids. The most studied species belong to the division *Basidiomycota* (e.g. *Cutaneotrichosporon curvatum*, *Trichosporon oleaginosus*, *Rhodotorula toruloides*) and *Ascomycota*; subdivision *Saccharomycotina* (e.g. *Yarrowia lipolytica*, *Lipomyces starkeyi*). Some *Candida* species (*C. viswanathii, C. tropicalis, C. oleophila*) also exhibit oleaginous phenotypes. The main fatty acids present in microbial SCOs are: palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), and linoleic (C18:2) acids [10].

Enhancing lipogenesis for production of high-value lipids

Oleaginous microorganisms are able to accumulate large amounts of intracellular lipids. Nevertheless, their lipogenesis could be further improved using genetic engineering (Figure 2). The strategies include: enhancing fatty acid synthesis through overexpression of malic enzyme [47] or acetyl-CoA carboxylase [48]; improving TAG accumulation by deletion of glycerol dehydrogenase [49] or overexpression of diglyceride acyltransferase [48]; inhibition of lipid degradation pathways by disruption of β-oxidation [49]. Other approaches focus on increasing cytoplasmic acetyl-CoA pool [50], redox engineering [51] and modulating cellular oxidative stress defense pathways [52]. Comprehensive reviews on the subject are available elsewhere [34,53,54].

Engineering of oleaginous yeasts to produce high-value lipids

Oleaginous yeasts seem to be the best-adapted hosts for lipid production due to their physiological properties. They are able to grow using various carbon sources, display high lipogenesis and are relatively straightforward in large-scale cultivations. Moreover, a wide range of tools available for genetic manipulation has encouraged researchers to develop or improve the lipid biosynthesis pathways. Recent developments in genetic engineering have enabled the emergence of a yeast factory for unusual lipids (Figure 3). These valuable fatty acids are much closer to the final commercial product, thus reducing or completely avoiding chemical processes during their production [55].

Hydroxy fatty acids

Hydroxy fatty acids (HFAs) are saturated or unsaturated fatty acids, including one or more hydroxyl functional

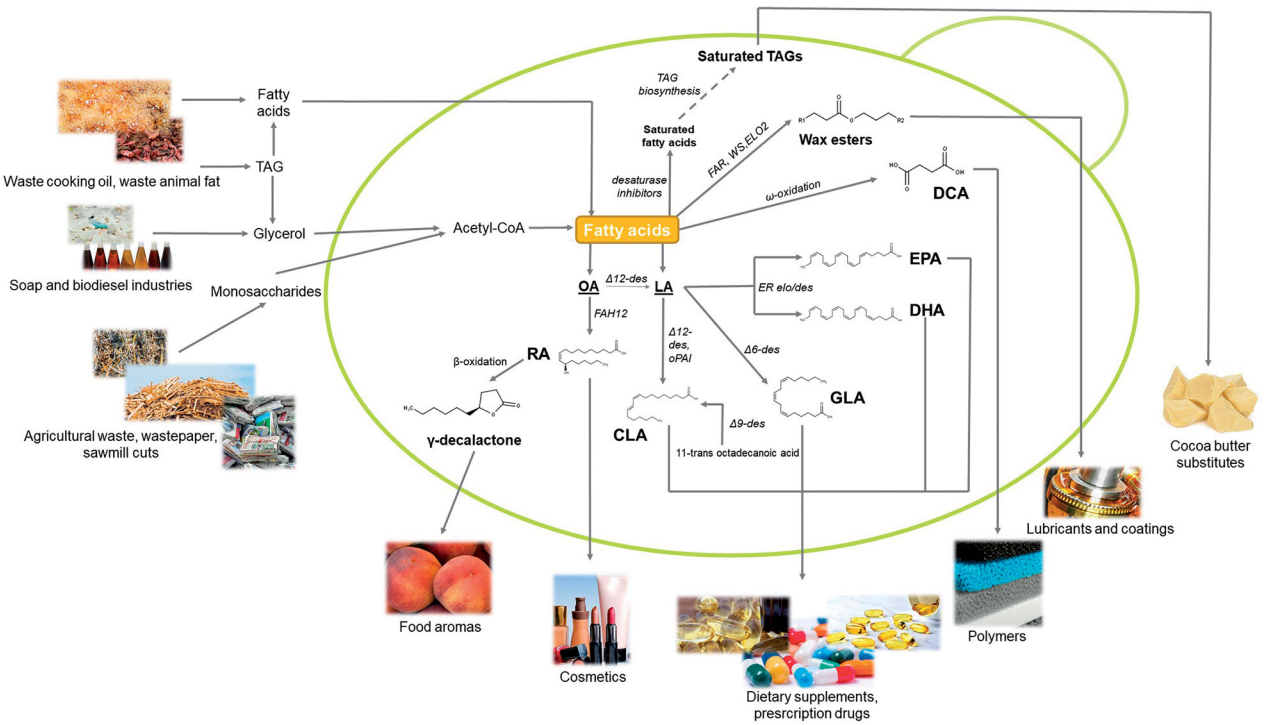

Figure 3. Production of high-value lipids using yeast cell factory. Abbreviations: CLA: conjugated linoleic acid; DCA: dicarboxylic acids; DHA: docosahexaenoic acid; Δ6, Δ9, Δ12-des - desaturases; EPA: eicosapentaenoic acid; *ELO2*: elongase; ER *elo/des*: endoplasmic reticulum elongases and desaturases; *FAH12*: oleate hydroxylase; *FAR*: fatty acid reductase; GLA: γ-linoleic acid; LA: linoleic acid; OA: oleic acid; *oPAI*: linoleic acid desaturase; RA: ricinoleic acid; TAG: triacylglyceride; *WS*: wax synthase.

groups attached to the main carbon chain. HFAs are common in nature and gained attention due to their properties supporting human health, e.g. they are highly significant in the prevention of colorectal cancer. Moreover, they have high viscosity, polarity and reactivity resulting in their application in polymers, surfactants, lubricants, coatings and cosmetics [21]. Nevertheless, most of them are not commercially available due to high production costs and environmentally harmful methods of their synthesis. The chemical production of HFAs also has little benefits due to poor selectivity or the time-consuming technology. Therefore, an interesting alternative for the production of these compounds are microorganisms [56] – microbial biosynthesis offers mild biosynthesis conditions, high specificity and reduced manufacturing costs [57].

Ricinoleic acid

The most studied HFA is ricinoleic acid (RA; C18:1) produced by the castor oil plant (*Ricinus communis*), in which it represents 90% of the total fatty acid (TFA) of the seed oil [58]. Due to the high content of ricin, an extremely toxic protein found in the seeds and the long-strenuous refining process as well as its high price, this oilseed plant is not considered an efficient and suitable source for RA production. The limited number of natural resources has led scientists to seek an alternative – more economical and efficient – sources through genetic engineering techniques.

The molecular mechanisms of RA synthesis are known and are allowed to engineer various species such as the *Arabidopsis thaliana* plant and *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* yeasts in order to produce this unusual fatty acid. However, these yeasts are non-oleaginous and are not able to accumulate large amounts of lipids. As a result, *A. thaliana* was used for cloning the appropriate genes for approved ricin-free oilseed crops, whereas yeasts were used to elaborate metabolic engineering strategies using heterologous genes to produce this fatty acid [58].

The metabolic pathway leading to RA is composed of a reaction catalyzed by Δ12 (oleate) hydroxylase gene (*FAH12*), which adds a hydroxyl group to the 12th carbon of oleic acid (OA, $^{\Delta 9}$ C18:1) [59], esterified to the *sn*-2 position of phosphatidylcholine (PC) in the endoplasmic reticulum (ER) [60]. The first hydroxylase gene was cloned from *R. communis* and expressed in tobacco and *A. thaliana*. However, in both, the amount of RA was still significantly lower than in the native castor seeds [61–63]. In another study, *FAH12* was expressed in *S. cerevisiae* and *S. pombe* with the *GAL10* promoter, but RA represented only 0.8% of TFA [64]. Meesapyodsuk and Qiu isolated the first, non-plant Δ12-oleate hydroxylase from the ergot fungus *Claviceps purpurea* (*CpFAH12*) [65]. It displays high sequence similarity to the fungal desaturases but the low similarity to plant fatty acid hydroxylases. Authors expressed *CpFAH12* in *S. cerevisiae* H1246 under the *GAL1* promoter and grew the transformant in an oleate medium reaching 19% RA of TFA. The same group co-expressed *CpFAH12* and fungal diacylglycerol O-acyltransferase *CpDGAT2*, increasing the production to 30% RA of the TFA [66]. In contrast to *S. cerevisiae*, the fission yeast *S. pombe* are able to accumulate 75% of OA that is the main substrate for *FAH12*. This fact encouraged Holic and coworkers to introduce *CpFAH12* controlled by the *NMT1* inducible promoter into *S. pombe* ARC010-1, resulting in 137.4 μg/ml of RA (52.6% of TFA) [67]. In addition, the toxic effect of RA was observed as the transformants were not able to grow at 30 °C. The problem was solved by applying 37 °C for the preliminary growth cycle followed by incubation at 20 °C for 5 days. Subsequently, Yazawa and colleagues improved RA content 1.8-times by the deletion of the triglyceride lipases (*TGL3*, *TGL4*, *TGL5*) involved in storage-lipid remobilization [68]. The same group identified also *pgl7* encoding phospholipase A2 as the multicopy suppressor, which showed a possibility to synthesize RA more effectively while avoiding cell growth disturbance in the *CpFAH12* genetic background [69]. It is likely that *pgl7* activity is responsible for releasing RA moieties from phospholipids and directing them to TAGs, thereby suppressing the toxic effect. Further extension of this study revealed that the overexpression of *plg7* causes secretion of free RA into culture media [70]. Increased copy number of the overexpressed phospholipase resulted in the production of 200.1 μg/ml of intracellular RA and 184.5 μg/ml of extracellular RA. Co-expression with *ptl2* lipase (that also showed phospholipase activity) improved intracellular accumulation and secretion of RA 1.2- and 1.3-fold, respectively, compared to the strain overexpressing *plg7* alone [71].

Despite the research efforts to produce RA by *S. pombe*, the total lipids in this yeast pose merely 5% of DCW and therefore cannot be considered as a good alternative to *R. communis*. Hence, the research is now focused on the well-known oleaginous yeast *Y. lipolytica*, able to accumulate large amounts of lipids and in which the main synthesized fatty acid is OA, the direct precursor of RA.

In 2014, Beopoulos and coworkers constructed a *Y. lipolytica* JMY2556 strain capable to accumulate RA up to 42% of total lipids and 63 mg/g of DCW [58]. This

strain was unable to carry out β-oxidation due to the deletion of *POX1-6* genes, which led to increased lipid accumulation. Additionally, native Δ12- desaturase was removed to avoid competition for the substrate. The strain was further engineered by the introduction of the Δ12- hydroxylase gene (*RcFAH12*) from *R. communis,* but the resulting strain produced RA representing 7% of all lipids. Expression of the hydroxylase gene (*CpFAH12*) from *C. purpurea,* in the same strain, accounted for 29% of RA of total lipids, and the co-expression of an additional copy of *CpFAH12* increased RA levels up to 35% of total lipids. Moreover, it was noted that overexpression of the native *Y. lipolytica* PDAT acyltransferase (*LRO1*) attained higher levels of both TAG and RA accumulation, while the opposite effect was observed for the co-expression of the *C. purpurea* or *R. communis* type II diacylglycerol acyltransferase (*CpDGAT2* or *RcDGAT2*), when RA levels declined to below 14% of total lipids. Finally, in the best RA-producing strain, three copies of *CpFAH12* and two copies of *LRO1* were introduced, leading to the production of 12 g/L RA (60% of total lipids) in a medium with 160 g/L glucose supplemented with 24 g/L oleic acids in a 10 L bioreactor [72]. The presented results show that *Y. lipolytica* is a suitable host for RA production. However, to achieve satisfactory yields, heavy genetic engineering strategies must be employed.

Polyunsaturated fatty acids

Polyunsaturated fatty acids (PUFAs) display a wide range of health benefits. Essential and non-essential PUFAs are integral components of cell membrane phospholipids, as well as being necessary to maintain brain function. PUFAs may also alleviate the symptoms of Alzheimer's disease as well as cancer and chronic intestinal disorder [73].

Due to the high market demand for nutraceutical supplements, PUFAs production has been the subject of recent research. Daily consumption of foods with high PUFAs content is critical for human health. According to a report by FAO/WHO [74], it appears that adequate PUFAs intake should be between 6% and 11% of the total caloric value of the human diet in adults. As a result of the growing interest in PUFAs, its main source - fish oil - is not able to keep up with the constantly increasing global demand [75].

Conjugated linoleic acid

The conjugated linoleic acids (CLA) are a group of 28 isomers of linoleic acid (LA; 18:2) that are all characterized by a conjugated pair of double bonds separated by a methylene group. CLA is produced naturally in the digestive tract of ruminants by the fermentative bacteria, *Butyrivibrio fibrisolvens*. The mechanism of CLA synthesis consists of the isomerization of LA into CLA by bacterial isomerases or by synthesis *via* α9-desaturase of 11-*trans* octadecanoic acid. Nowadays, commercial CLAs are mainly produced through chemical synthesis, which results in the formation of many by-products, undesirable for medical and nutritional purposes [76]. Since the production of pure isomers with nonselective chemical processes is highly challenging, the biotechnological production of CLA, which has been widely studied in recent years, may become an alternative. A number of microorganisms have been identified as potential CLA producers; nevertheless, their CLA levels are relatively low (265 μg/mL) [77]. So far, LA isomerases derived from *Lactobacillus reuteri*, *Clostridium sporogenes* (*LAI*) and *Propionibacterium acnes* (*PAI*) have been fully characterized. The first two isomerases are *cis*-9, *trans*-11 CLA producing isomerases and the other one is capable of transforming LA to *trans*-10, *cis*-12 CLA [78,79]. Expression of *PAI* in *Escherichia coli* BL21(DE3)pLysS showed that LA in free fatty acid form seems to be the only substrate of this enzyme [80]. The use of only free fatty acids as substrates, which amounts are low in most eukaryotic organisms, poses a major challenge, limiting CLA production by microbiological synthesis [81].

Yeast-based CLA production was first investigated in 2005 by Hornung's group, which expressed the codon-optimized *coPAI* gene in *S. cerevisiae*, achieving up to 5.7% of 10,12-CLA of total FFA [80]. Subsequently, they expressed the *coPAI* gene in tobacco plants, resulting in a CLA yield of up to 15% of total FFA. More recently, Zhang and coworkers have successfully constructed a *de novo trans*-10, *cis*-12 CLA biosynthesis platform by transforming the oleaginous yeast *Y. lipolytica* Po1h, with *PAI* isomerase [82]. They dramatically increased the level of *PAI* using a multi-copy vector. Expression of the codon-optimized *PAI* gene in *Y. lipolytica* resulted in a 6-fold higher yield of CLA, while its multi-copy amplification allowed an increase in the CLA production by almost 30 times. The best-constructed strain was capable of producing up to 5.9% of CLA of TFA in the glucose-based medium. Nevertheless, the strain was deemed unstable and the yield of CLA was not high enough for the prospective development. The same group increased the amount of trans-10-cis-12-CLA with up to 10% (0.4% DCW) in *Y. lipolytica* Po1h [83], ameliorating CLA-producing strain by co-expressing the *Mortierella alpina* Δ12-desaturase gene (*FADS12, d12*) collectively with the *coPAI* multi-copy integration.

Moreover, when the recombinant strain was growing in a medium supplemented with soybean oil, *trans*-10 *cis*-12 CLA was detected up to 44% of total fatty acids (30% DCW), after 38.5 h of cultivation. Interestingly, around 90% of the detected CLA occurred in cells in free fatty acid form. Another study sought to improve the conversion of LA provided with a medium into single isomer CLA by the development of the whole-cell catalysis system displaying *PAI* on the *S. cerevisiae* EBY100 cell surface [84]. Even though the codon optimization of *PAI* resulted in a 35% increase of CLA biosynthesis, the maximum production of *trans*-10, *cis*-12 CLA was only 25.4 mg/L under the optimal conditions. Shortly after, a much more efficient LA conversion to CLA using a permeabilized whole-cell catalyst of *Y. lipolytica* Po1h-pINA1292-spopaid12–16 was presented [85]. The yeast cells were permeabilized by freeze/thawing methods and cultivated under optimal conditions; 28 °C, pH 7, 200 rpm with 1.5 g/L sodium acetate, 100 g wet cells and 25 g/L LA. After 40 h incubation, the permeabilized cells were able to produce up to 15.9 g/L of *trans*-10, *cis*-12-CLA with a conversion yield of 62%. In addition, the extracellular production of *trans*-10, *cis*-12-CLA exceeded 10 g/L, while with LA supplementation the CLA yield was around 22 g/L. Since *PAI* isomerase prefers free fatty acids as substrates, another study demonstrated a different route to increase the amount of intracellular LA in free fatty acid form, using the oleaginous fungus *M. alpina* CCFM 501 [86]. Consequently, they decided to inhibit the long-chain acyl-coenzyme A synthetases by adding a specific inhibitor, triacsin C, which increases cellular free fatty acid levels [87]. Then, after the heterologous expression of the *coPAI* gene in *M. alpina* with free LA and Triacsin C addition, the amount of *trans*-10, *cis*-12-CLA increased approximately 24-fold to 29 mg/L, reaching up to 1.2% of the TFA. In 2017 the largest production of *trans*-10, *cis*-12-CLA isomer by yeast was reported [88]. The best-producing strain was derived from *Y. lipolytica* W29. This recombinant strain (*Y. lipolytica* JMY3479) contains many genetic modifications to produce CLA; the elimination of β-oxidation genes (*pox1–6*Δ), the inability to store lipids as triglycerides (*dga1*Δ, *dga2*Δ, *are1*Δ, *lro1*Δ) and the overexpression of the native Δ12-desaturase gene (*YlFAD2*). Additionally, *coPAI* gene was expressed, yielding 302 mg/L of CLA in a bioconversion medium supplemented with soybean oil during bioreactor cultivation. Finally, a *Y. lipolytica* Po1g strain derivative using inexpensive and renewable raw material, glycerol, as the sole carbon source was able to overproduce CLA [89]. It was made possible by modifying the lipid accumulation pathway, integrating overexpression of diacylglycerol transferase (*DGA1*) with heterologous genes *FADS12/PAI* and optimizing the culture conditions. The maximum CLA content reached 132.6 mg/L.

γ-Linolenic acid

γ-Linolenic acid (GLA, $^{\Delta 6,9,12}$ C18:3) is the valuable intermediate emerging during the biosynthesis of the active prostaglandin, as a consequence of the LA conversion by △6-desaturase (△*6des*). It belongs to the omega-6 family of fatty acids and shows essential clinical and pharmaceutical values.

Not many natural sources of GLA are known. Commercially available oils rich in GLA are derived from borage, black currant and evening primrose, containing up to 25% GLA of total seed oil. Since these sources are not ample enough to meet the growing market demand, microorganisms have proved to be an alternative source of GLA. The microbiological production of GLA has been extensively studied for several years. However, the cost of producing microbial oil still exceeds plant-oil production. For that reason, more efficient strains capable of accumulating large amounts of lipids with adequate GLA composition are constantly being sought.

The GLA biosynthesis pathway consists of two steps: OA is Δ12-desaturated to LA and then converted to GLA by the action of Δ6-desaturase (Δ*6des*). GLA production was first reported in *S. cerevisiae* [90]. Two desaturases – Δ6, Δ12, were isolated from *M. alpina* and simultaneously expressed in *S. cerevisiae* SC334. GLA yields were at levels of up to 8% of TFA without the requirement for supplementation of additional fatty acid substrates. Moreover, their results have shown that two independent promoters (*GAL1, TPI1*) were necessary for the coinstantaneous expression of both desaturases – otherwise the production of GLA was very low. That way, these enzymes were able to successfully convert up to 50% of their respective substrates into products. Subsequent studies to improve production in *S. cerevisiae* INVScI relied on heterologous expression of Δ*6des* from *Rhizopus nigricans* R31.6 under *GAL1* promoter and demonstrated 6.25% of GLA of TFA [91]. The transformants were induced with 2% galactose and supplemented with LA as a substrate. It was also observed that the GLA yield showed a tendency to increase with a decrease in the fermentation temperature from 30°C to 15°C (13.69% at 30°C; 22.23% at 15°C), suggesting that Δ*6des* was likely to play a role in *R. nigricans* during adaptation to low temperatures. Meanwhile, yet another Δ*6des* gene involved in the formation of GLA was identified in the genome of *Mucor rouxii* and introduced into the methylotrophic yeast

Ogataea polymorpha KYC625 [92]. With this mutant strain, the GLA production reached up to 10% of TFA, while using methanol 2% (v/v) as the only carbon source. The following study was focused on the optimization of GLA production by the high-cell-density cultivation of *O. polymorpha* KYC625 in the bioreactor [93]. The fermentation process was optimized by adapting the specific growth rate, which had an effect on the fatty acid composition and the lipid content of the GLA-producing strain. The highest amount of GLA (697 mg/L) was obtained with a specific growth rate of 0.08/h, using glycerol as the sole carbon source. The engineered strain was also expressed Δ*6des* from *M. rouxii*. In the meantime, an interest in the production of GLA by *Y. lipolytica* emerged. Originally, *Y. lipolytica* Po1g was transformed with Δ6, Δ12-desaturases genes from *M. alpina*, under the hybrid *hp4d* promoter [94]. By the simultaneous expression of both desaturases using one plasmid, the recombinant strain was able to produce up to 20% of GLA from endogenous LA and OA (4.9% from TFA), with a high conversion yield of 60%. At last, Sun and coworkers (2017) improved the synthesis of GLA in *Y. lipolytica* by optimizing fermentation conditions, using a temperature-shift strategy to redistribute carbon flux from saturated to unsaturated fatty acids [95]. The temperature system was based on two phases: preliminary growth at 28 °C (biomass production) followed by 6-day incubation at 20 °C (lipid production with a preference for unsaturated fatty acids). Optimization improved the proportion to 6.1% GLA of TFA.

Eicosapentaenoic acid and docosahexaenoic acid

Long-chain ($C_{20–22}$) polyunsaturated fatty acids (LC-PUFAs) such as eicosapentaenoic (EPA; $C_{20:5n-3}$) and docosahexaenoic acid (DHA; $C_{22:6n-3}$) belong to an omega-3 fatty acids group and support essential biological functions in animals, including humans. They were proved to reduce the TAG concentration and blood pressure [20,96], and as components of phospholipids (PL), they provide flexibility, fluidity and selective membrane permeability [97]. Omega-3 fatty acids are necessary for metabolism – mammals are not able to synthesize them on their own, so the acids must be provided with the diet.

The traditional source of EPA and DHA are ocean fish oils [76]. Due to a growing interest in omega-3 fatty acids, this traditional source is not sufficient to keep up with a constantly increasing global demand. To overcome this limitation, the industry started to produce omega-3 fatty acids using microorganisms. Some microalgae, such as *Crypthecodinium cohnii* and

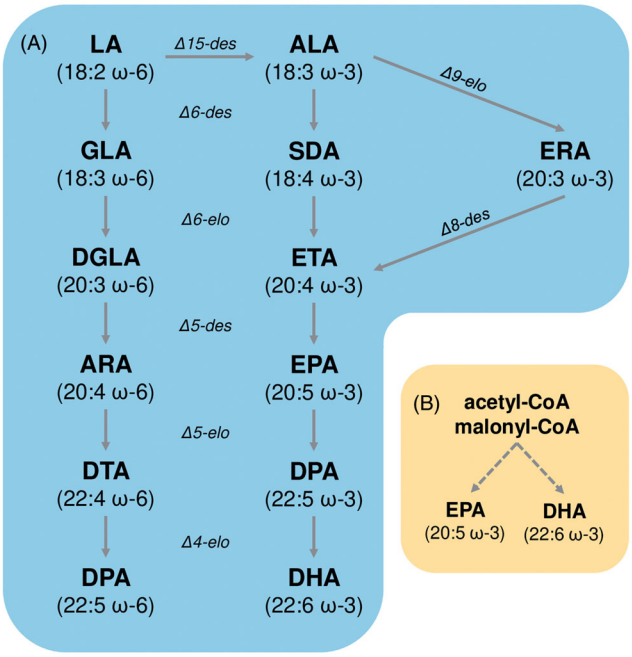

Figure 4. The biosynthesis of LC-PUFAs through the aerobic desaturase and elongase pathway (A) and the anaerobic polyketide synthase pathways; (B) Abbreviations: ALA: α-linoleic acid; ARA: arachidonic acid; DGLA: dihomo-γ-linoleic acid; DHA: docosahexaenoic acid; DPA: docosapentaenoic acid; DTA: docosatetraenoic acid; Δ*5, 6, 8, 15-des*: desaturases; Δ-*4, 5, 6, 9-elo*: elongases; EPA: eicosapentaenoic acid; ERA: eicosatrienoic acid; ETA: eicosatetraenoic acid; GLA: γ-linoleic acid; LA: linoleic acid; SDA: stearidonic acid.

Schizochytrium sp. turned out to be worthy producers of DHA [98]. The production of DHA by these microorganisms has moved into the industrial scale and several market products are already available [99]. In contrast, there is no efficiently large-scale EPA production process by non-adapted native organisms that would result in yields sufficient to meet commercial criteria. Moreover, the cultivation of wild-type microorganisms often turns out to be time-consuming, non-cost-effective and cumbersome.

The biosynthesis of EPA and DHA occurs anaerobically through the polyketide synthase (in marine microalgae) or the aerobic desaturase and elongase pathway (Figure 4) [100,101]. The aerobic pathway is divided into two branches; Δ-6 desaturase pathway (algae, mosses and fungi) or a Δ-9 elongase/Δ-8 desaturase pathway (euglenoids), in which the main precursor is LA. For conversion of LA into EPA and DHA, two enzymatic processes are necessary: 1) elongation of the carbon chain *via* the addition of carbon atoms and 2) desaturation *via* the addition of double bonds. These reactions are catalyzed by a series of specific elongases and desaturases residing in the ER [102]. With the advent of genetic engineering, it became possible to

modify microorganisms for the enhanced production of specific LC-PUFAs. The growing number of reports describing effective heterologous expression of the DHA/EPA biosynthetic pathways in several oleaginous hosts shows the availability of a set of molecular tools with the use of which the synthesis of these important fatty acids became a reality.

To date, reconstruction of the omega-3 pathway in yeast has been reported by several research groups. At first, C18-PUFA specific Δ6-elongase from *Caenorhabditis elegans* was co-expressed with Δ5-, Δ6-fatty acid desaturases in *S. cerevisiae* W303-1A. When C18:3 was supplied as a substrate, the recombinant strain produced low levels of EPA, which represented 0.2% of TFA [103]. Next, the group of Kajikawa attempted to reconstitute the PUFA biosynthetic pathway in *Komagataella pastoris* PPY1 by multiple expression of three genes: Δ6-desaturase, an elongase-like enzyme involved in Δ6 elongation and Δ5-desaturase derived from the liverwort *Marchantia polymorpha* [104]. This strain also resulted in a weak accumulation of EPA, which posed merely 0.03% of TFA. Then, EPA production was slightly improved to 0.1% of TFA by introducing additional copies of Δ6-desaturase, Δ6-fatty acid elongase and Δ5-desaturase genes from the alga *Phaeodactylum tricornutum* [105]. Domergue et al., constructed a *S. cerevisiae* INVSc1 strain carrying Δ5-desaturase from *P. tricornutum*, Δ6-elongase from *Physcomitrella patens* and Δ6-desaturase from *Ostreococcus tauri* [106]. Heterologous expression in this strain resulted in accumulation of EPA levels up to 4.5% of TFA when α-linoleic acid (C18:$3^{9,12,15}$) was used as substrate (71% conversion rate). This result was approximately 20 times higher than the levels obtained in similar reconstitution experiments using analogous Δ6-desaturases [103,107]. Further research proved that Δ5-desaturase from the ciliate *Paramecium tetraurelia* was more efficient than those originating from *O. tauri*, *M. alpina* and *Leishmania major*, resulting in the production of up to 0.49% of EPA of the TFA [108].

To date, the best results in EPA production were achieved using the yeast *Y. lipolytica* [109]. Due to its high LA content, *Y. lipolytica* was investigated for *de novo* synthesis and accumulation of EPA. The research, conducted by DuPont, resulted in the development of two commercial products: oil rich in EPA ("NewHarvest"), which is a dietary supplement for humans, as well as EPA enriched fodder for salmon "Verlasso". First, by screening forty different *Y. lipolytica* strains for lipid production, the strain ATCC 20362 was selected, based on its suitable fermentation characteristics: DCW >100 g/L, lipid content >30% DCW and lipid productivity >1 g/L/h. The selected strain was then transformed with four heterologous genes, encoding *a* Δ6-desaturase, a C18/20-elongase, *a* Δ5-desaturase (from *M. alpina*), and *a* Δ17-desaturase (from *Saprolegnia diclina*), thereby restoring Δ-6 pathway. All genes were introduced under the control of constitutive p*TEF* promoter, resulting in the synthesis of EPA comprising about 3% of the total fatty acid methyl esters (FAME) with 34% of all fatty acids derived from the engineered pathway. On the one hand, such result proved that *Y. lipolytica* could be engineered to produce EPA, but on the other - indicated that additional engineering efforts were needed, e.g. increasing the carbon flux through the pathway, improving elongase efficiency, boosting substrate conversion rate and increasing the expression of auxiliary genes. Therefore, the EPA pathway was further enhanced by using a set of strong promoters, introducing a codon-optimization system and increasing the gene copy number. In the early engineering stages of the Δ6 pathway, the strain *Y. lipolytica* Y9027 was constructed by increasing the copy number of the overexpressed genes, producing EPA at 40% of total FAME under nitrogen-limiting conditions [101]. The carbon flux through the engineered Δ6-pathway was increased by overexpressing the C16/18-elongase gene from *M. alpina* [110], which transforms palmitic acid (C16) into stearic acid (C18), and the Δ12-desaturase gene from *Fusarium moniliforme* [111], which transforms OE to LA. Moreover, the substrate conversion of the overexpressed Δ5-, Δ6- and Δ17-desaturases in this strain, were about 90, 86 and 97%, respectively, of the TFAs entering the triglycerides. The constructed *Y. lipolytica* Y2097 strain had 19 copies of 10 different heterologous genes integrated into its genome in total. One of the main obstacles of the Δ-6 pathway modification was the accumulation of high amounts of GLA (21% of TFA), which inhibits carbon exchange among the PL and acyl-CoA pools. The problem was solved by the introduction of the Δ-9 pathway, ceasing the simultaneous production of GLA. First, such generated *Y. lipolytica* strain (Y4305) contained multiple copies of the codon-optimized genes (Δ-9 elongase, Δ-8 desaturase, C16/18-elongase, Δ-12 desaturase, Δ-17 desaturase, Δ-5 desaturase and choline-phosphotransferase) under control of strong promoters, as well as the disrupted peroxisome biogenesis factor gene *PEX10* (repression of lipid catabolism). Moreover, the bifunctional fusion genes of Δ-9 elongase and Δ-8 desaturase with doubled activity were constructed and introduced [112]. As a result, the *Y. lipolytica* Y4305 strain containing 30 copies of 9 different heterologous genes was able to produce lipids with an EPA of 56.6% of the TFA

from 80 g/L glucose and accumulated EPA at 15% of the DCW [113]. Subsequently, the strain was further engineered to create a Z1978 strain with 35 copies of 17 different genes, which produced EPA of over 58% of TFA and 20% DCW [114]. Finally, the best EPA producer, the strain Z5567, was constructed using Y4305 and Z1978 genetic backgrounds. The Z5567 strain, containing 41 copies of 19 different genes, produced EPA at 50% of total lipids and 25% DCW during fed-batch fermentation [115]. Then, in 2017, a two-stage continuous fermentation process was developed, improving the overall EPA efficiency by 80% and EPA concentration by 40%, while achieving similar EPA titers in biomass and similar conversion rate from glucose, as compared with the standard fed-batch fermentation process [116]. It was also noticed that during long-term cultivation, *Y. lipolytica* evolved to reduce the byproduct generation (mainly organic acids) and increase the lipid content [116].

DHA was first produced using genetic engineering in *S. cerevisiae* YPH499 through the co-expression of an elongase from *Pavlova* sp. and a Δ4-desaturase from *Isochrysis galbana*, with EPA, used as a substrate [117]. In a recent study, it was found that the expression of *Trypanosoma brucei* acyl- CoA synthetase (*ACS1*) in *S. cerevisiae* significantly improves the uptake of exogenous PUFAs [118]. Co-expression of *ACS1* with *ELO5* elongase from *L. major* or Δ-4desaturase from *T. brucei* led to a remarkable improvement in the incorporation of their substrate and the amount of obtained product. This strategy could be applied in the endogenous generation of the desired PUFA, as a substrate for co-expressed enzymes, or as a final product. More recently, Gemperlein and colleagues (2019), proposed the use of artificial biosynthetic PUFA gene clusters encoding multifunctional polyketide synthase (PKS)-like PUFA synthases from myxobacteria for LC-PUFA synthesis in *Y. lipolytica* Po1h [119]. Compared to the existing DHA or EPA bioproduction methods, by the aerobic pathways, using many iteratively acting heterologous elongases and desaturases, PKS enable *de novo* LC-PUFA biosynthesis from acyl-CoA precursors in a multistep process [120]. These studies led to the highest DHA concentration (16.8%) of TFA among all published PUFA-producing *Y. lipolytica* strains.

Cocoa butter substitutes

Cocoa butter (CB) is a pure vegetable fat used to produce chocolate, cosmetics and pharmaceuticals [121]. It is sourced from cocoa beans of *Theobroma cacao*, cultivated in some of the African and Central American countries. CB consists mostly of three different kinds of TAGs – P–O–P, P–O–S and S–O–S (P – palmitic acid; O – oleic acid; S – stearic acid), which allows it to remain solid at room temperature and liquid at 37°C. Saturated fatty acids constitute the major fraction of all fatty acids in CB (55–67% w/w). The average fatty acid profile is: C16:0 (23–30% w/w), C18:1 (30–37% w/w) C18:0 (32–37% w/w) and C18:2 (2–4% w/w) [121,122] and is dependent largely on growing conditions [122].

As mentioned in section 2, the current supply of CB is insufficient due to inefficient production methods and growing demand [123]. Although some vegetable oils, including Illipe and kokum butter, can be used as CB equivalents, their production is limited [124,125]. This fosters an interest in the development of other sources of CB-like lipids, *i.e.* cocoa butter substitutes that can be used as stable, sustainable and economically viable alternatives to CB [123]. One such alternative is the CBS production by the oleaginous yeasts [14,126,127]. However, these microorganisms produce mostly unsaturated lipids, while CB is in essence saturated [13]. In order to increase the degree of saturation, many strategies have already been developed, such as the cultivation of wild-type strains in optimized conditions (including reduced oxygenation), the use of desaturase inhibitors and random mutagenesis. For a comprehensive review, see [128]. Here, we will briefly present recent advancements in microbial CBS production, including the use of waste materials and culture optimization methods.

The cultivation of *Y. lipolytica* ACA-DC 50109 on agro-industrial residues [129] and industrial fats [130,131] yielded saturation levels up to 89% and 64%, respectively. Moreover, Zhao et al.et al. (2016) obtained results suggesting that the optimum conditions for *Y. lipolytica* CICC1778 to synthesize CBS was 30°C with 0.6 mg/L of $CoCl_2{\cdot}6H_2O$ or 0.03 ml/L sterculic acid methyl ester in the medium with glycerol and ammonium tartrate-yeast extract as carbon and nitrogen sources, respectively [132]. Interestingly, Xiong et al. (2015) investigated the efficient conversion of mutton fat to CBE (cocoa butter equivalent) by the fermentation of *Y. lipolytica* CICC1778 which acts as an "Sn-2 specific lipase" [133]. In another study, Wu et al. (2011) investigated lipid production by *Rhodotorula toruloides* Y4 under sulfate-restricted conditions, where 60% of the fatty acids produced were saturated. Compared to the reported control (47% saturation), greater amounts of C16:0 and less C18:1 were produced [134]. The authors also found that lipid samples produced from N-acetylglucosamine by *C. curvatum* ATCC 20509 were appropriate as CBE [135]. A similar shift from C18:1 to C16:0

(from 37% to 50%) in *R. toruloides*, was also obtained using the electrode-fermentation (EF) method with the redox mediator Neutral Red (NR) [136]. The supplementation of NR appears to offer two interesting advantages for the production of CBE: 1) it can shift the fatty acid ratio from C18:1 to C16:0, which cannot be achieved solely by the manipulation of desaturation reactions, and 2) it can potentially be used with different media and strains and is nontoxic [136]. In 2017, Wei et al. proposed an oleaginous yeast strain *Trichosporon oleaginosus* DSM11815 to be a potential CBS producer after further metabolic engineering in the future [137]. They cultivated six different yeasts (*S. cerevisiae* CEN.PK113-7D, *T. oleaginosus* DSM11815, *R. graminis* DSM 27356, *L. starkeyi* DSM 70296, *R. toruloides* DSM 70398 and *Y. lipolytica* CBS 6124) in a nitrogen-limited medium and compared their CBS production ability. Under the same growth conditions, they found that TAGs were the main lipids in all six yeasts and that *T. oleaginosus* DSM11815 can produce more TAGs than the other five yeasts. Less than 3% of the total TAGs were identified as potential S–O–S in the six yeasts. However, *T. oleaginosus* DSM11815 produced 27.8% potential P–O–P and P–O–S at levels of 378 mg TAGs/g of DCW.

The potential for a significant increase in the price of cocoa butter as well as the already existing high price of other saturated exotic fats (e.g. shea butter, sal fat) renders the utilization of several oleaginous yeast strains amenable to produce substitute SCOs of these high-value fats as very promising. It is also surprising that no genetic-engineering efforts were undertaken to generate robust CBS-producing microorganisms. In recent years there were not many attempts to address alternative CBS production methods, which might have been caused by the gradual decline of palm oil prices [138], which can be readily used in CBS production [139,140].

Other exotic fatty acid derivatives

Wax esters

Wax esters (WE) are widely used in various commercial applications, including personal care products, lubricants and coatings. Their synthesis is mediated by fatty acyl reductase (FAR) and wax ester synthase (WS). WEs are mainly obtained from *Simmondsia chinensis* (jojoba tree), but the lipid extraction costs and high environmental requirements for the plant render this process unprofitable [141]. Furthermore, the estimated demand for jojoba oil reaches up to 200,000 tons per year, while the current supply is around 4000 tons per year [141]. According to the current state of knowledge some bacteria belonging to *Acinetobacter*, *Psychrobacter*, *Marinobacter* and *Rhodococcus* genera naturally accumulate wax esters in certain conditions [142]. Unfortunately, research has come to a standstill due to the limited availability of appropriate genetic engineering tools [143].

Efforts have been made to create a robust yeast platform for WE production. The engineering of *S. cerevisiae* included functional expression of five WS genes from different bacteria species (namely: *Acinetobacter* sp., *Marinobacter* sp., *Psychrobacter* sp., and *Rhodococcus* sp.). In addition, their preferred *in vitro* substrates were identified and showed the highest preference for ethanol by WS from *Marinobacter hydrocarbonoclasticus* to other WSs, enabling engineered *S. cerevisiae* CEN.PK 113-5 D to produce biodiesel [144]. The use of different WSs in combination with FARs in order to achieve a synthesis of jojoba-like WEs in bacteria and yeast has been reported previously, but the products were restricted to C28–C36 WEs, which are only a small fraction of all WEs present in the jojoba oil [145]. In 2017, Wenning et al., demonstrated, for the first time, the synthesis of longer chain WEs (up to C42) in *S. cerevisiae* CEN.PK 113-5 D without substrate feeding, by heterologous expression of *FAR* from *Marinobacter aquaeolei* together with the WS from *S. chinensis* and native elongase (*ELO2)* [146]. The maximum yield was 12.24 ± 3.35 mg/g DCW after 48 h cultivation. The study elucidated that the pool of very-long-chain monounsaturated fatty acids (VLCMUFAs) needs to be enhanced in *S. cerevisiae* in order to synthesize jojoba-like diunsaturated wax esters (DUWEs). Therefore, the strain was improved by combined overexpression of a heterologous fatty acid elongase (*CaKCS)* from *Crambe abysinica* with native fatty-acid desaturase (*OLE1*). This generated a strain producing high amounts of monounsaturated fatty alcohols (FOHs) as well as diunsaturated WEs [147]. At the same time, Zhao et al., constructed *Y. lipolytica* Mh-Po1f strain able to produce 570 mg/L of WE [148]. It is the highest wax ester production in microbes reported so far and about 7-fold higher than the wax esters produced by the former best producer *Acinetobacter* ADP1 (80 mg/L). The Mh-Po1f strain was constructed by the introduction of a cytosolic *MhFAR* from *M. hydrocarbonoclasticus* and WS from *Acinetobacter* sp. ADP1. Furthermore, the study showed that the wax ester content and titer increase at nitrogen-limited conditions [143].

Dicarboxylic acids

Dicarboxylic acids (DCA) are a group of valuable organic compounds that can be used as monomers to produce

polyesters, polyamides and polyurethanes as well as intermediates to synthesize perfumes, hot-melting adhesives or lubricants [149]. Currently, the main production of industrial DCA is dependent on petroleum multistage chemical conversion, but this technology is limited by reactant availability and high environmental costs [150]. Moreover, the production costs rise with the increase of the carbon chain length of the DCA.

In microorganisms, DCA are formed by the oxidation of fatty acids in a ω-oxidation process. Briefly, fatty acids are first oxidized to omega-hydroxy fatty acids, further modified to ω-aldehyde fatty acids by fatty-alcohol dehydrogenase/oxidase and finally oxidized to DCA by fatty-aldehyde dehydrogenases (*FALDH1-4*). Then, DCA can enter β-oxidation pathway and serve as an energy source.

One of the first cases of using microorganisms to make DCA was reported by a group of scientists working on the yeast *Candida tropicalis*. The group reported on metabolically engineered strain capable of producing significant amounts (130–140 g/L) of DCA from *n*-alkanes (C13), fatty acids (C18:1, C22:1) or fatty acid methyl esters (C14:0, C16:0, C18:1) [151,152]. This strain was developed by disrupting *POX4* and *POX5* genes coding for the acyl-CoA oxidases and resulting in the high conversion of alkanes to DCA. Interestingly, Hara et al. (2001) demonstrated that repression of *POX* gene activity did not increase DCA production in *Candida maltosa* [153]. Correspondingly, Werner et al., (2017) created a *Meyerozyma guilliermondii* KU131F1 strain with deletions in the main two *POX* genes [154]. Although growth on oleic acid was clearly improved, the produced DCA was degraded, this suggested that additional degradation pathways might exist in this yeast as it was described for the yeast *Candida lusitaniae* 6936 [155]. While β-oxidation is an important spot on the map of improvement of DCA production, it is certainly not the only one. Picataggio et al., (1992) overexpressed cytochrome P450 monooxygenase and reductase genes in *C. tropicalis* H5343 [151]. This resulted in a 30% increase of long-chain DCA yield (DC12 and DC14). It is also known that yeast *Y. lipolytica* has a functional ω-oxidation pathway, therefore it is capable of producing DCA [156]. Most of known cases of engineering *Y. lipolytica* to produce DCA, avail on the knowledge obtained during studies on *C. tropicalis*. For instance, overexpression of some of the cytochrome P450 monooxygenases and reductase enhanced DC18:1 production [157]. Moreover, overexpression of the *FAO1* gene in *Y. lipolytica* H222ΔP strain resulted in a higher ω-hydroxy-fatty acid yield, which is an intermediate in DCA biosynthesis [157]. The strain with such modifications produced 330 mg/L of DCA [158]. A higher concentration was obtained in bioreactor cultures (3.49 g/L of DCA), under nitrogen-limited conditions with glycerol as the only carbon source [159]. The wide use of *Y. lipolytica* to produce lipid compounds encouraged scientists to explore its lipid metabolism [21,48,160]. In 2006, Nicaud et al., proved that disruption of some *POX* genes leads to increased accumulation of DCA. Furthermore, metabolic engineering can be applied to other key enzymes of the β-oxidation downstream of *POX* such as *MFE2*, which in contrast to *POX* is not represented in yeasts by several isozyme variants [161]. Deletion of *MFE2* in *Y. lipolytica* C-00365 improved the production of short-chain DCAs (C9), whereas long-chain DCAs were still consumed and no accumulation was achieved [160]. Although some processes for the fermentative production of long-chain DCAs have been commercialized, the fermentation is still challenging [162]. Further development of optimization of bioprocesses and their transport on an industrial scale should be developed in order to maintain a high DCA titer and controlled production process.

Lactones

Lactones are a special subgroup of esters resulting from the cyclization of hydroxy fatty acids. They are valuable compounds with aromatic properties that can be used by: food and beverage, cosmetic, chemical and pharmaceutical industries. They can be obtained directly from fruits through chemical methods or biotechnological processes, which have been investigated with interest [163–165]. Out of all lactones, γ-decalactone, which has an aroma of peach, is the most commonly produced in the past few years. Some yeast strains can naturally convert RA into γ-decalactone by a 4-stage process of β-oxidation cycles [163]. The intermediate product that is formed as a result of RA oxidation is γ-hydroxydecanoic acid - a precursor of γ-decalactone, which is then cyclized into decalactone form [166]. *Y. lipolytica*, as well as the yeast *Lindnera saturnus* have been tested for their ability to produce γ-decalactone from exogenous RA, castor oil or crude glycerol, which is considered as the second alternative source of this lactone [167,168]. The *L. saturnus* yeast has been recently shown to produce isoamyl acetate (banana flavor) by isoamyl alcohol biotransformation in beet molasses [169].

In the case of *Y. lipolytica*, γ-decalactone production has been enhanced through a number of genetic modifications. First, it was shown that the strain can be improved by disrupting some and upregulating other

POX genes (POX2-5 gene disruption and POX2 overexpression). The constructed strain, *Y. lipolytica* MTLY40-2P, produced 7 g/L of γ-decalactone when castor oil was used as a substrate [170]. Later, *Y. lipolytica* strains were subjected to UV irradiation and genome shuffling, this resulted in 6.5-fold higher γ-decalactone production (3.75 g/L) compared to the wild-type strain [171]. The yield was then increased to 7.51 g/L through immobilization of *Y. lipolytica* cells on attapulgite (ATG) and γ-decalactone biosynthesis in an ionic liquid (IL) containing co-solvent system [171]. More recently, research conducted by Pereira de Andrade *et al.,* in 2017 showed the ability of *Y. lipolytica* CCMA 0242 strain to produce γ-decalactone also from crude glycerol, but the amount of γ-decalactone was negligible (3.5 mg/L) [167]. Efforts to develop a full synthetic pathway along with new approaches to metabolic engineering may improve yeast lactones production in the next few years.

Concluding remarks

This review describes in detail the latest trends and challenges regarding metabolic and genetic engineering strategies of yeast cell factories in order to improve the production of valuable lipids such as EPA, DHA, GLA and CLA but also more unusual and exotic derivatives of fatty acids such as waxes, lactones or dicarboxylic acids. The presented research demonstrates recent advances in synthetic biology that enabled direct production of lipid compounds without the chemical steps required previously, making these processes more economical. This has already resulted in several commercial or pilot-scale products. However, the way to fully economically viable microbiological lipid production processes still require further evaluation of lipid efficiency and productivity through various culture modes or genetic engineering techniques, extraction, and valorization of many by-products, which is a common practice for vegetable oil processing.

Moreover, the constantly developing field of genetic engineering also leads to the identification of new enzymes that can be used to modify fatty acids and TAGs, enriching their properties by combining them with other valuable compounds or membrane lipids, increasing their bioavailability. As it could be seen, the use of oleaginous yeasts as a lipid factory is a field with promising potential for future exploration, which can bring significant benefits not only to the environment but also in expanding the range of high-value lipids with new lipogenic compounds. In addition, genomes of novel oleaginous yeast species, including *Trichosporon fermentans, Saitozyma podzolica* and *Sporobolomyces pararoseus,* have recently been sequenced [172–175]. This, together with the growing number of genetic engineering tools available for their transformation, such as in the case of *Candida hispaniensis, Blastobotrys adeninivorans* or *B. raffinosifermentans* [176,177] could open new possibilities for the microbial production of tailored lipid molecules.

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3.2. Publikacja 2

Jest to pierwsza publikacja prezentująca oryginalne badania, w ramach których opracowano platformę do wydajnej produkcji fosfolipidów przez drożdże *Y. lipolytica*. Dotychczasowe strategie inżynierii metabolizmu tego gatunku drożdży koncentrowały się głównie na intensyfikacji procesu syntezy TAG lub FFA. Nie podejmowano natomiast żadnych prób intensyfikacji biosyntezy PL. Szlak biosyntezy tych związków u *Y. lipolytica* jest dość słabo poznany i angażuje wiele organelli. Celem pracy była identyfikacja genów kodujących kluczowe enzymy związane z metabolizmem PL u *Y. lipolytica* oraz zwiększenie biosyntezy PL poprzez nadekspresję natywnych genów pod kontrolą silnego, konstytutywnego promotora TEF. Jako substrat do hodowli transformantów *Y. lipolytica* wykorzystano odpadowy glicerolu w celu obniżenia kosztów produkcji PL. Szczególną uwagę poświęcono poprawie biosyntezy PC.

Badania rozpoczęto od konstrukcji szeregu transformantów *Y. lipolytica*, charakteryzujących się nadekspresją genów zaangażowanych w szlak biosyntezy PL w różnych kombinacjach, jak również delecją genów odpowiedzialnych za ich degradację. Kluczowym metabolitem pośrednim w szlaku biosyntezy PL jest kwas fosfatydowy (PA), który u drożdży *Y. lipolytica* kierowany jest głównie do produkcji TAG, a w mniejszych ilościach do szlaku CDP-DAG, w którym produkowane są PL (**Rysunek 1**). Z tego względu, podstawą wszystkich skonstruowanych transformantów była nadekspresja genu kodującego syntazę CDP-DAG (*CDS*), której zadaniem było przekierowanie zwiększonej puli PA w szlak syntezy PL. Wszystkie uzyskane szczepy analizowano w podłożu minimalnym (YNB) przy niedoborze źródła azotu, gdzie jako źródło węgla zastosowano glukozę lub glicerol (**Rysunek 2**). Przeprowadzone modyfikacje genetyczne pozwoliły znacząco poprawić produkcję PL przez dwa szczepy PS05 oraz PS07 z wykorzystaniem obu analizowanych substratów, jednak poprawa biosyntezy PL z glukozy była zdecydowanie wyższa. Oznaczało to, że zastosowane źródło węgla jest ważnym aspektem syntezy PL. Szczep PS05 stanowił przykład opisanej wcześniej przez Tai i Stephanopoulos [119] strategii „push and pull", polegającej na skierowaniu maksymalnej ilości strumienia węgla w szlak syntezy PL, poprzez nadekspresję genów kodujących pierwszy i ostatni etap tego szlaku.

Z tego względu szczep PS05 charakteryzował się nadekspresją genu *CDS* w celu nakierowania strumienia węgla do szlaku syntezy PL w połączeniu z nadekspresją genu metylotransferazy fosfolipidowej (*OPI3*), kodującego ostatni etap szlaku, zwiększając przepływ strumienia węgla do produktu końcowego – PC. Z kolei szczep PS07, poza nadekspresją genów *CDS* i *OPI3* posiadał jednocześnie delecję genu fosfolipazy D (*SPO14*), odpowiadającej za rozkład PC.

Ponieważ głównym celem badań była synteza PL z wykorzystaniem taniego i odnawialnego substratu, jakim jest glicerol, wybrane w pierwszym etapie badań szczepy zostały wzbogacone o: I) nadekspresję genu kodującego kinazę glicerolu (*GUT1*), której zadaniem było zwiększenie asymilacji glicerolu przez drożdże *Y. lipolytica*, a także II) nadekspresję genu kodującego kinazę DAG (*DGK1*) przekształcającego DAG w PA, główny produkt pośredni biosyntezy PL. Strategia ta umożliwiła kolejne zwiększenie produkcji PL, gdzie najlepszy producent – szczep PS08, wykazywał prawie 6-krotny wzrost produkcji PC (27,8 mg/g) i prawie 3-krotny wzrost zawartości PL (60,2 mg/g) w porównaniu do szczepu kontrolnego (**Rysunek 3A**). Wprowadzone modyfikacje szlaku biosyntezy PL umożliwiły także zmiany we wzajemnym stosunku poszczególnych frakcji PL. Wynik taki był trudny do uzyskania w komórkach drożdży, gdyż określone PL pełnią w komórce ważne funkcje budulcowe i stabilizujące błony biologiczne. Nadekspresja genu *OPI3* spowodowała oczekiwany efekt wzrostu zawartości frakcji PC, obniżając jednocześnie ilość PS i PE w komórce (**Rysunek 3B**). Analizowane transformanty charakteryzowały się także ponad 2-krotnym obniżeniem całkowitej zawartości kwasów tłuszczowych (TFA) (**Rysunek 3C**). Ponadto, wcześniejsze doniesienia literaturowe wykazały, że PL u *Y. lipolytica* w większości zawierają nasycone kwasy tłuszczowe zarówno w pozycji sn-1, jak i sn-2 [120]. Wyniki te zostały potwierdzone również w prezentowanej rozprawie doktorskiej (**Rysunek 3D**).

W przypadku akumulacji lipidów przez drożdże olejogenne, ograniczenie azotu wpływa na obniżenie stężenia biomasy, natomiast stężenie źródła węgla decyduje o ilości lipidów [121]. Z tego powodu stosunek molowy C/N jest istotny w opracowaniu wydajnego procesu biosyntezy lipidów przez drożdże olejogenne. Według wielu badań, stosunek ten jest czynnikiem zależnym od użytego szczepu *Y. lipolytica* . Dlatego, w celu osiągnięcia wysokiej ilości PL, przeprowadzono badania związane z optymalizacją początkowego stosunku molowego C/N do ich syntezy. Przetestowano C/N wynoszące 10, 35 i 99 - używając

glicerolu jako źródła węgla i chlorku amonu jako źródła azotu (**Rysunek 4A**). Ponadto, ze względu na fakt, że PL stanowią czynnik strukturalny błon komórkowych, a więc ich synteza zachodzi w każdej fazie wzrostu komórki, analizowano ich stężenie w różnym czasie hodowli (po 3 i 6 dniach procesu). Jedna z hipotez zakładała, że w późnej fazie stacjonarnej, tzn. fazie lipogenezy, komórka gromadząc lipidy skieruje większość substratu w syntezę FA i TAG a nie w biosyntezę PL. Jednakże uzyskane wyniki udowodniły, że intensywna biosynteza PL, podobnie jak TAG, rozpoczyna się w fazie stacjonarnej wzrostu i jest taka sama zarówno przy stosunku C/N 35 jak i C/N 99. Niemniej jednak, synteza PL z glicerolu, mimo iż związek ten stanowi szkielet PL, zachodzi mniej wydajnie niż z glukozy. Doprowadziło to do postawienia kolejnej hipotezy, iż geny zaangażowane w szlak biosyntezy PL zależne są od NADPH, którego generowanie u *Y. lipolytica* odbywa się głównie w szlaku PPP, który podczas wzrostu z glicerolu jest osłabiony [122]. Z tego względu druga strategia optymalizacji składu podłoża produkcyjnego dotyczyła suplementacji hodowli niewielkimi stężeniami glukozy lub glukonianu jako substratów "domieszkowych" w celu zwiększenia metabolizmu redukcyjnego i biosyntezę NADPH (**Rysunek 4B-C**). Uzyskane wyniki udowodniły, że ani dodatek glukozy ani glukonianu nie wpłynął na poprawę syntezy PL.

W końcowym etapie badań wykorzystano najwydajniejszego producenta PL, szczep PS08 oraz kontrolny szczep W29, do biosyntezy PL w skali bioreaktorowej, z wykorzystaniem czystego i odpadowego glicerolu jako źródła węgla (**Rysunek 5**). Niezależnie od rodzaju użytego glicerolu, stężenie uzyskanych PL było na podobnym poziomie. Wynik ten świadczy o braku negatywnego wpływu śladowych ilości zanieczyszczeń występujących w odpadowym glicerolu na syntezę PL. Finalnie, szczep PS08 był w stanie wyprodukować 653,7 mg/L PL z zawartością PC wynoszącą 352,6 mg/L.

Uzyskane wyniki wykazały, że u *Y. lipolytica* nadekspresja już minimalnego zestawu enzymów szlaku biosyntezy PL prowadzi do zdecydowanej poprawy biosyntezy tych związków, a odpadowy glicerol może być z powodzeniem zastosowany jako substrat do opracowania taniego i wydajnego procesu ich produkcji.

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Elevating Phospholipids Production *Yarrowia lipolytica* from Crude Glycerol

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Abstract: Phospholipids (PLs) are a class of lipids with many proven biological functions. They are commonly used in lipid replacement therapy to enrich cell membranes damaged in chronic neurodegenerative diseases, cancer, or aging processes. Due to their amphipathic nature, PLs have been widely used in food, cosmetic, and pharmaceutical products as natural emulsifiers and components of liposomes. In *Yarrowia lipolytica*, PLs are synthesized through a similar pathway like in higher eukaryotes. However, PL biosynthesis in this yeast is still poorly understood. The key intermediate in this pathway is phosphatidic acid, which in *Y. lipolytica* is mostly directed to the production of triacylglycerols and, in a lower amount, to PL. This study aimed to deliver a strain with improved PL production, with a particular emphasis on increased biosynthesis of phosphatidylcholine (PC). Several genetic modifications were performed: overexpression of genes from PL biosynthesis pathways as well as the deletion of genes responsible for PL degradation. The best performing strain (overexpressing CDP-diacylglycerol synthase (*CDS*) and phospholipid methyltransferase (*OPI3*)) reached 360% of PL improvement compared to the wild-type strain in glucose-based medium. With the substitution of glucose by glycerol, a preferred carbon source by *Y. lipolytica*, an almost 280% improvement of PL was obtained by transformant overexpressing *CDS*, *OPI3*, diacylglycerol kinase (*DGK1*), and glycerol kinase (*GUT1*) in comparison to the wild-type strain. To further increase the amount of PL, the optimization of culture conditions, followed by the upscaling to a 2 L bioreactor, were performed. Crude glycerol, being a cheap and renewable substrate, was used to reduce the costs of PL production. In this process 653.7 mg/L of PL, including 352.6 mg/L of PC, was obtained. This study proved that *Y. lipolytica* is an excellent potential producer of phospholipids, especially from waste substrates.

Keywords: phospholipids; oleaginous yeast; *Yarrowia lipolytica*; neutral lipids; membrane lipids; glycerol; crude glycerol; metabolic engineering

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1. Introduction

Phospholipids (PLs) are the main structural components of cell membranes and are essential for vital cellular processes, such as vesicle formation, membrane fusion, and cell division [1]. PLs belong to the class of complex lipids with amphiphilic nature. Their structure is composed of the glycerine-3-phosphate backbone in which hydrophobic fatty acyl groups are esterified to the sn-1 and sn-2 positions [2]. The sn-3 position consists of a phosphate group that contributes hydrophilicity. The simplest PL is phosphatidic acid (PA) and all others are named after the hydrophilic residue attached to the phosphate group. Four main groups of PL have been identified: ethanolamine, inositol, serine, and choline. These groups form the most biologically important phospholipids, which are phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylcholine (PC) [3].

PLs show many health benefits; e.g., they regulate the function of many organs, decrease the level of cholesterol and triglycerides in blood, repair damaged liver tissue, and prevent neurological diseases [4]. Moreover, PLs are commonly used in lipid replacement therapy to enrich cell membranes that are damaged in chronic neurodegenerative diseases, cancer, or aging processes. Due to their amphipathic nature, PLs have been widely used in food, cosmetic, and pharmaceutical products as natural emulsifiers and components of liposomes [5].

With the advent and rapid development of metabolic engineering and synthetic biology, the number of microbe-based cellular factories is growing rapidly, promising the ability to convert renewable carbon sources into a wide range of chemicals, biofuels, pharmaceuticals, and natural bioactive compounds [6]. Oleaginous yeasts are characterized by complex inner membranes that allow the storage of large amounts of neutral lipids (mainly triacylglycerols-TAGs). These yeasts grow rapidly and produce lipids at high rates [7], thus becoming a viable platform for the production of valuable lipid compounds. The model oleaginous yeast *Yarrowia lipolytica* has been reported to enhance lipid production [8,9]. Engineering strategies undertaken to date have mainly focused on the biosynthesis of TAGs or free fatty acids. However, there have been no efforts to overproduce PL by engineering their metabolism, which is quite poorly understood and characterized by structural diversity, tight genetic regulation, or involvement of multiple organelles. The well-characterized *Y. lipolytica*, having a GRAS (generally recognized as safe) status, being amenable to genetic engineering due to the availability of various genome editing tools, and being resistant and tolerant to harsh fermentation conditions, may become a promising platform for the synthesis of PL. Moreover, *Y. lipolytica* has the ability to utilize crude glycerol, which contains many impurities including heavy metals, methanol, and salts [10], resulting in a low market price for this carbon source.

This study aimed to decipher PL metabolism in *Y. lipolytica* and increase their production through a series of genetic engineering manipulations. Special attention was put on the improvement of PC biosynthesis. First, strains of *Y. lipolytica* characterized by the increased production of PL were constructed, which were then used in research related to the optimization of the production of these compounds. The influence of the carbon source (glucose, glycerol), nitrogen availability, cultivation time, and the dependence of enzymes in the PL biosynthesis pathway on the availability of NADPH were analyzed. Finally, bioreactor scale cultures were carried out using raw glycerol as a carbon source.

2. Results and Discussion

2.1. Establishing the Y. lipolytica as a Phospholipid-Producing Platform Relative to Different Carbon Sources

Several genetic modifications were performed to construct strains for enhanced phospholipid production, mainly for phosphatidylcholine: the overexpression of genes involved in the PL biosynthesis pathway (shown in blue) and deletion of genes responsible for PL degradation (shown by red crosses; Figure 1). *De novo* lipid synthesis begins with the acylation of Gly-3-P by glycerol-3-phosphate acyltransferase encoded by *SCT1* (YALI0C00209g), resulting in the formation of lysophosphatidic acid (LysoPA), which is acylated to phosphatidic acid (PA) by lysophospholipid acyltransferases encoded by *SLC1* (YALI0E18964g) and *ALE1* (YALI0F19514g). A key intermediate in phospholipid biosynthesis is PA, which in *Y. lipolytica* is mainly directed to TAG production and, in smaller amounts, to the cytidine diphosphate diacylglycerol (CDP-DAG) pathway, in which membrane phospholipids are synthesized [Figure 1].

Therefore, we first constructed strains in which the overall amount of PA would be increased. It was achieved through the overexpression of the individual genes *SCT1*, *SLC1*, and *ALE1,* and redirecting a larger pool of PA toward the CDP-DAG pathway by overexpressing CDP-DAG synthase (*CDS*), which encodes the conversion of PA to liponucleotide CDP-DAG. However, the phospholipid profile of strains PS01, PS02, PS03, and PS04, in both glucose and glycerol based cultures, was the same or slightly changed

(PL lowered) compared to the wild-type strain W29 [Figure 2A,B]. This may suggest that PA levels are tightly regulated in the cell by phosphatidic acid phosphatase *PAH1* (YALI0D27016g), which catalyzes the production of diacylglycerol (DAG) from PA in a reaction that is thought to be a major step in TAG biosynthesis [11]. During increased PA production, the Pah1 enzyme becomes more active and efficiently dephosphorylates larger amounts of substrate into DAG production. PA is also an important signaling molecule in the regulation of lipid metabolism. High levels of PA lead to the reduced translocation of the transcription regulator Opi1 to the nucleus [12], preventing its binding to the transcription factor Ino2. Because Ino2 is an activator of many fatty acid and phospholipid biosynthesis genes, an increase in PA directly upregulates fatty acid biosynthesis mechanisms [4,13]. Thus, modifying the pathway to increase the amount of PA in the cell does not affect PL production.

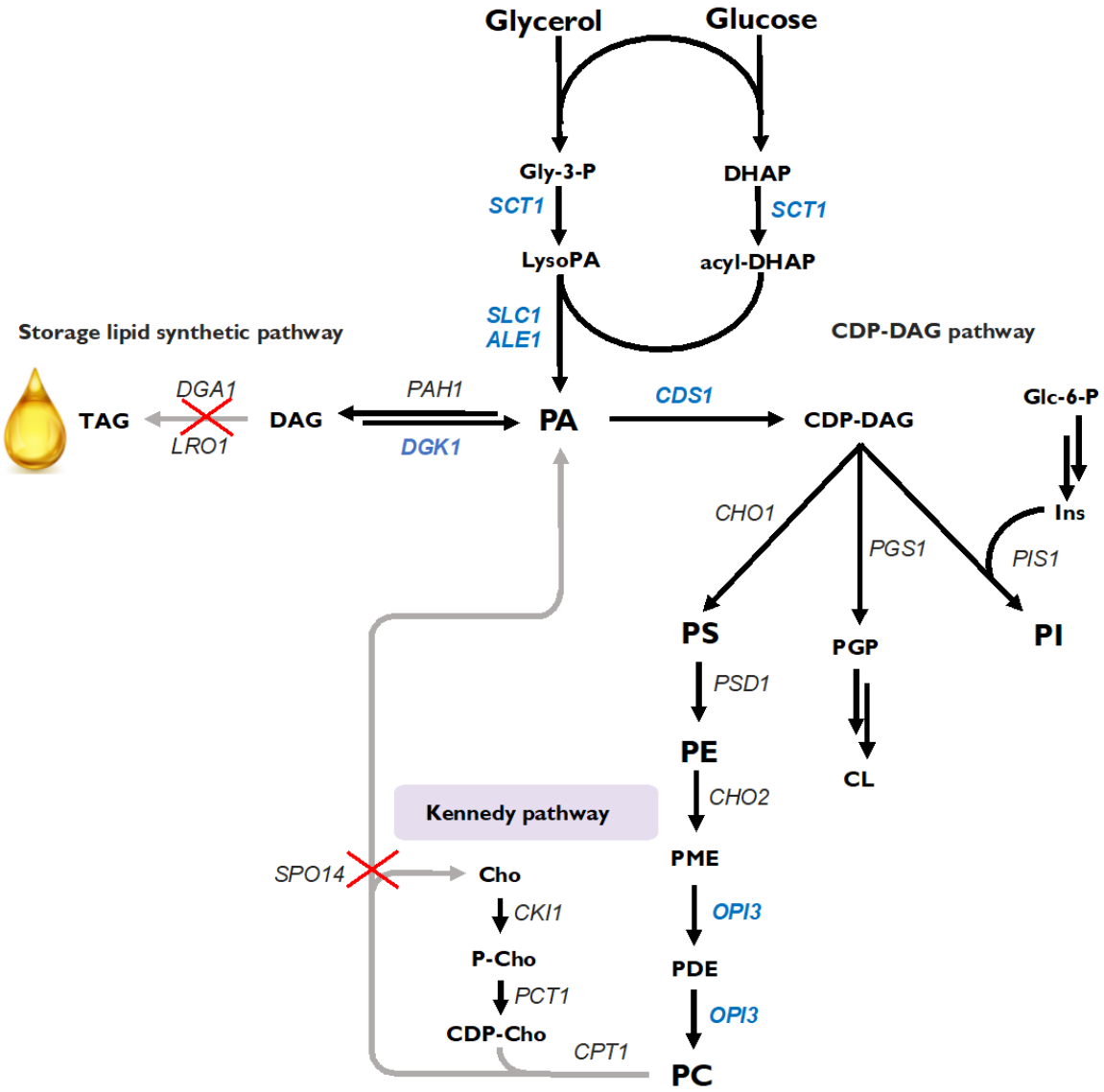
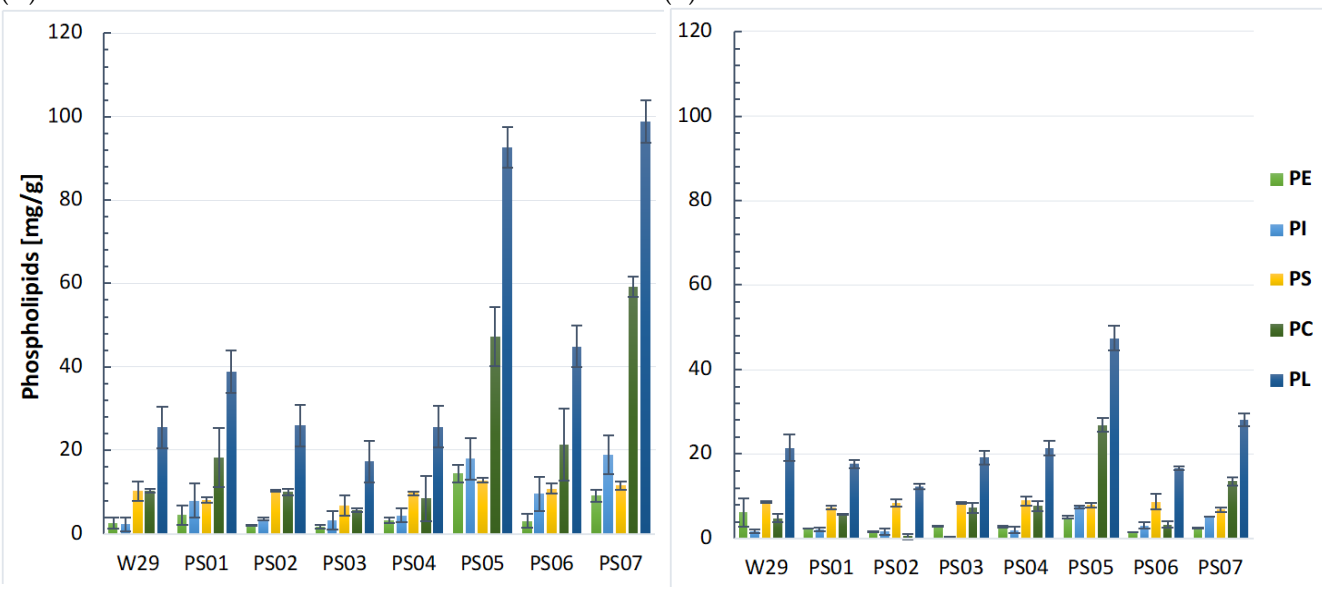


Figure 1. Engineering a phospholipid platform in *Yarrowia lipolytica*. Marked in blue, overexpressed genes; grey arrows marked with X, deleted genes. DHAP, dihydroxyacetone phosphate; Gly-3-P, glycerol 3-phosphate; DAG, diacylglycerol; TAG, triacylglycerol; PME, phosphatidylmonomethylethanolamine; PDE, phosphatidyldimethylethanolamine; PGP, phosphatidylglycerophosphate; CL, cardiolipin; Cho, choline; Ins, inositol; PME, phosphatidylmonomethylethanolamine.

(**A**) (**B**)



(**C**)

	W29	PS01	PS02	PS03	PS04	PS05	PS06	PS07
CDS	-	+	+	+	+	+	+	+
SCT	-	-	+	-	-	-	-	-
SLC	-	-	-	+	-	-	-	-
ALE	-	-	-	-	+	-	-	-
OPI	-	-	-	-	-	+	+	+
ΔLRO1	-	-	-	-	-	-	+	-
ΔSPO14	-	-	-	-	-	-	-	+

Figure 2. Production of phospholipids by engineered *Y. lipolytica* strains. Error bars represent standard deviation from at least three biological replicates. (**A**) Phospholipid levels of engineered strains in shake flasks with YNB minimal medium and 60 g/L glucose, C/N 99. Titers obtained after 144 h cultivation at 160 rpm and 28 °C. (**B**) Phospholipid levels of engineered strains in shake flasks with YNB minimal medium and 60 g/L glycerol, conditions as when cultured on glucose. (**C**) Summary table of genes overexpressed/deleted in each of the evaluated strains.

Subsequently, the pull and push strategy was tested. The strain PS05 was constructed by the overexpression of the *CDS* gene to pull C-flux into the PL biosynthesis pathway in combination with the overexpression of the phospholipid methyltransferase gene (*OPI3*) responsible for the final step of the phospholipid synthesis pathway, pushing the C-flux to the final product—phosphatidylcholine [14]. This strategy led to an increase in the total phospholipid content to 360% compared to the wild-type strain W29 when grown on glucose (W29—25 mg/g DCW, PS05—92.6 mg/g DCW), and to 190% when grown on glycerol as a carbon source (W29—21.45 mg/g DCW, PS05—47.5 mg/g DCW) [see Figure 2A–C]. Furthermore, the level of PC was improved almost four-and-a-half-fold to reach 47.3 mg/g on glucose and five-and-a-half-fold to reach 26.95 mg/g on glycerol.

In the next step, the strain PS05 was a subject of gene deletion, to remove genes responsible for partial phospholipid degradation. This step included genes encoding phospholipid diacylglycerol acyltransferase *LRO1* (YALI0E16797g) and phospholipase D *SPO14* (YALI0E18898g), creating the strains PS06 and PS07, respectively. The product of the *LRO1* gene converts DAG to TAG by transferring the acyl chain from the sn-2 position of glycerophospholipids [15]; hence, the deletion of this gene seemed quite important in increasing PL synthesis. In contrast, the role of *SPO14* has so far been well studied in the

yeast *S. cerevisiae* where it is responsible for the continuous hydrolysis of phosphatidylcholine to choline and PA. However, the genes encoding phospholipases in *Y. lipolytica* have not been functionally characterized. Therefore, the aim of creating the strain PS07 was to investigate the effect of the *SPO14* deletion on phospholipid accumulation. The obtained results indicate that the PS06 strain with a *LRO1* deletion showed a decrease in phospholipid content by more than half compared to the PS05 strain using both glucose and glycerol (GLC—44.9 mg/g of PL, GLY—16.65 mg/g of PL). However, we also observed an increased number of TAGs on TLC plates [Figure S1A,B]. In turn, the strain PS07 showed the highest PL production when cultured on glucose. Compared to W29, the level of PC exhibited a nearly sixfold increase to 59.1 mg/g DCW. Furthermore, the total phospholipid content increased to 98.80 mg/g DCW, representing almost 390% in comparison to the strain W29 [Figure 2A]. When cultured on glycerol, the strain PS07 showed no significant improvement in total phospholipid content; only the level of PC increased almost threefold to 13.6 mg/g DCW compared to the W29 strain.

In summary, the performed genetic modifications allowed us to significantly improve the production of phospholipids by the corresponding transformants on both of the analyzed substrates, glucose and glycerol; however, the improvement on glucose was more pronounced. This means that in *Y. lipolytica*, the carbon source is an important aspect for phospholipids biosynthesis. Since the main objective of this study was to synthesize phospholipids from cheap and renewable substrate such as glycerol, two strains, PS05 and PS07, showing the highest phospholipid production on that substrate, were chosen for further experiments.

2.2. Enhancing Phospholipid Production by Overexpression of DGK1 and GUT1 Genes

To increase phospholipid production by the strains PS05 and PS07, we further modified these strains through the overexpression of DGA kinase *DGK1* (YALI0F19052g) and glycerol kinase *GUT1* (YALI0F00484g) genes, creating the strains PS08 and PS09, respectively. The product of the *DGK1* gene converts DAG, formed by TAG hydrolysis in the stationary phase, into PA, the major substrate of phospholipids [16]. Furthermore, this gene may also counteract the product of *PAH1*, causing the inhibition of TAG synthesis, thereby increasing the PA pool in the cell, as observed in *S. cerevisiae* [17]. In contrast, the overexpression of *GUT1* was carried out to increase the efficiency of glycerol assimilation by *Y. lipolytica*.

The resulting strains were grown in YNB glycerol medium at C/N 99. Compared to W29, the level of PC showed a nearly sixfold increase, reaching 27.8 mg/g in the strain PS08. Furthermore, the PL content increased to 60.2 mg/g DCW, representing an almost 280% improvement compared to the control strain [Figure 3A]. A slightly lower production of PL was observed for the PS09 strain, which led to a 198% increase in PL produced (41.5 mg/g DCW) compared to the wild type. The level of PC was improved by fourfold to 19.4 mg/g DCW. The titer of PLs in both strains, PS08 and PS09, reached 409.36 mg/L and 290.5 mg/L, respectively. However, an almost twofold decrease of the dry biomass of the modified strains compared to the wild-type strain was observed [Figure S2]. In 2020, Ti Liu and co-workers developed a *Saccharomyces cerevisiae* platform for *de novo* production of oleoylethanolamide, a phospholipid derivative [18]. The research began with the construction of a strain overproducing phospholipids, which had a number of genetic modifications: an improved acyl-CoA supply resulting from the installation of a citrate shuttle, an enhanced fatty acid synthase system alongside the removal of acyl-CoA-degrading fatty acyl-CoA oxidase, the overexpression of the two main endogenous fatty acyl-CoA synthetases (*Faa1p* and *Faa4p*), and the deletion of the monoglyceride lipase-encoding gene *YJU3* and the *PAH1* gene. This strain produced 470 mg/L PL including 260 mg/L of PC. Similar modification would likely further improve the titers that can be achieved in *Y. lipolytica*, and they should be tested in the future.

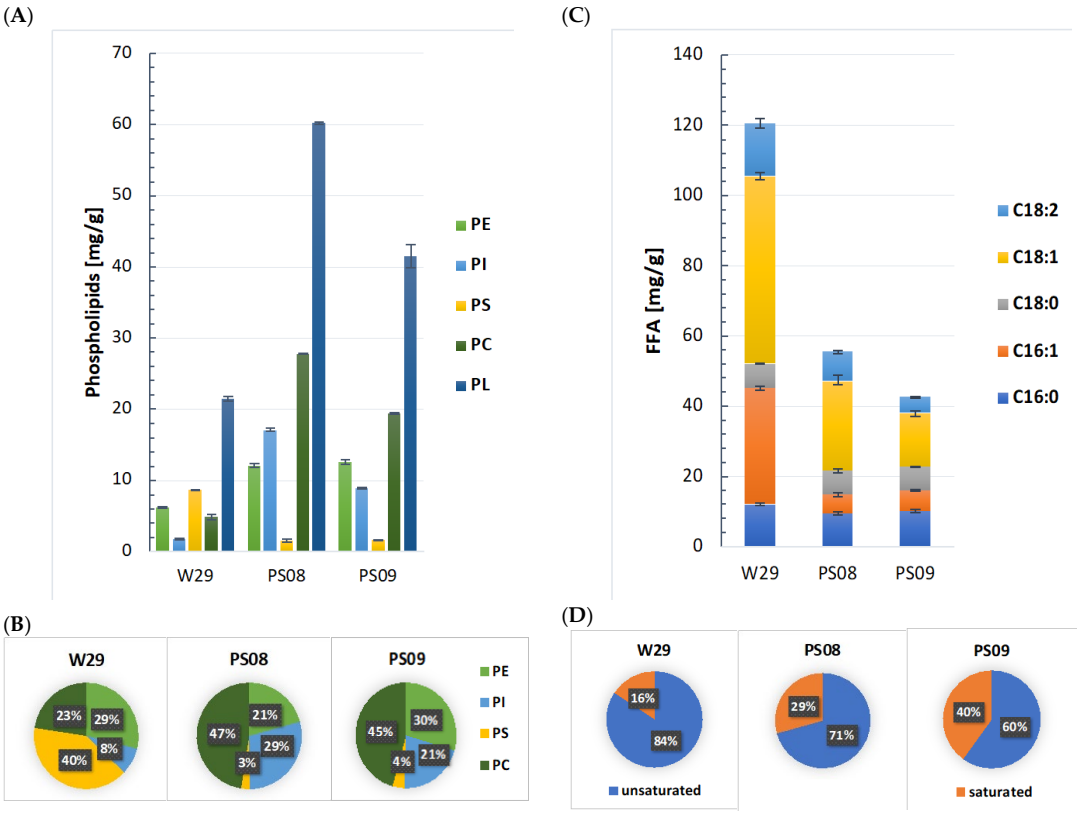


Figure 3. (**A**) Synthesis of phospholipids by final engineered *Y. lipolytica* strains. (**B**) Phospholipid classes distribution in strains W29, PS08, PS09. Cultivations were carried out for 144 h in shake flasks containing YNB with 60 g/L glycerol, C/N 99. (**C**) Total FA quantification of PS08 and PS09 compared with strain W29. (**D**) Distribution of saturated and unsaturated C16 and C18 FAs in the three strains.

The phospholipid composition changed due to the genetic modifications in the strains PS08 and PS09 compared to the control strain; PC content highly increased in both strains, up to 47% and 45%, and PS strongly decreased, to 3% and 4%, respectively [Figure 3B]. This was an expected effect caused by the overexpression of the *OPI* gene. The expected results were also confirmed by the decreased level of PE (21%) in the strain PS08. In contrast, in the PS09 strain, the PE level increased to 30% (compared to PS08), which may indicate a higher preference of *SPO14* toward PE hydrolysis. Unexpectedly, the proportion of PI in both strains increased (to 29% in PS08 and to 21% in PS09); however, the phenotype and consequences of this observation need further investigation.

Analysis of the total fatty acid (TFA) content in the cell showed more than a twofold decrease; PS08 and PS09 strains produced 55.5 mg/g and 42.5 mg/g of TFA, respectively, compared to the wild-type W29 strain, which produced 120.5 mg/g [Figure 3C]. This may indicate that, in *Y. lipolytica*, most of the fatty acids comes from the hydrolysis of TAGs, and in the case of the PS08 and PS09 strains, where TAG production is limited, a decrease in TFA content was noted. Furthermore, previous studies have shown that phospholipids in *Y. lipolytica* mostly incorporate saturated fatty acids at both the sn-1 and sn-2 positions [19]. This observation is consistent with the results shown in Figure 3D, where the percentage of saturated FAs between W29 and PS08, or PS09, increased from 16% to 29% and 40%, respectively.

2.3. Optimization of Flask Culture Conditions

For the lipid accumulation process in yeast, nitrogen limitation usually determines the biomass content, whereas the carbon source concentration usually determines the lipid

amount [20]. Therefore, the C/N ratio is important in determining the lipid content and biomass number of oleaginous microorganisms. According to many studies conducted in this field, the C/N ratio not only affects lipid accumulation but is also a strain-dependent factor for the *Y. lipolytica* [21]. Related to strain differences, the choice of a good carbon source is also of high importance [22]. Therefore, to achieve high lipid accumulation, the initial C/N molar ratio for PL biosynthesis was optimized. We tested three different C/N ratios—10, 35, and 99—using glycerol as a carbon source. Moreover, since phospholipids are a class of lipids that enter the membrane structures of cells, and thus their synthesis is required during each phase of cell growth, we analyzed their synthesis at different time-points during the cultivation (after 3 and 6 days of the process). Initially, our hypothesis assumed, that in the late stationary phase, when the cell begins to accumulate lipids, the main lipid substrates are directed at the synthesis of FAs and TAGs—not PL. As shown in [Figure 4A], nearly the same PL yield in both modified strains was obtained with a C/N ratio of 35 and 99 at day 6, reaching about 60 mg/g in the strain PS08 and about 41 mg/g in the strain PS09. At C/N 99, PL production starts earlier than at C/N 35, which is evident after day 3. These results indicate that the optimal C/N molar ratio is in the range between 35 and 99 promoted phospholipid accumulation when *Y. lipolytica*, with the modified phospholipid pathway, was cultured on glycerol.

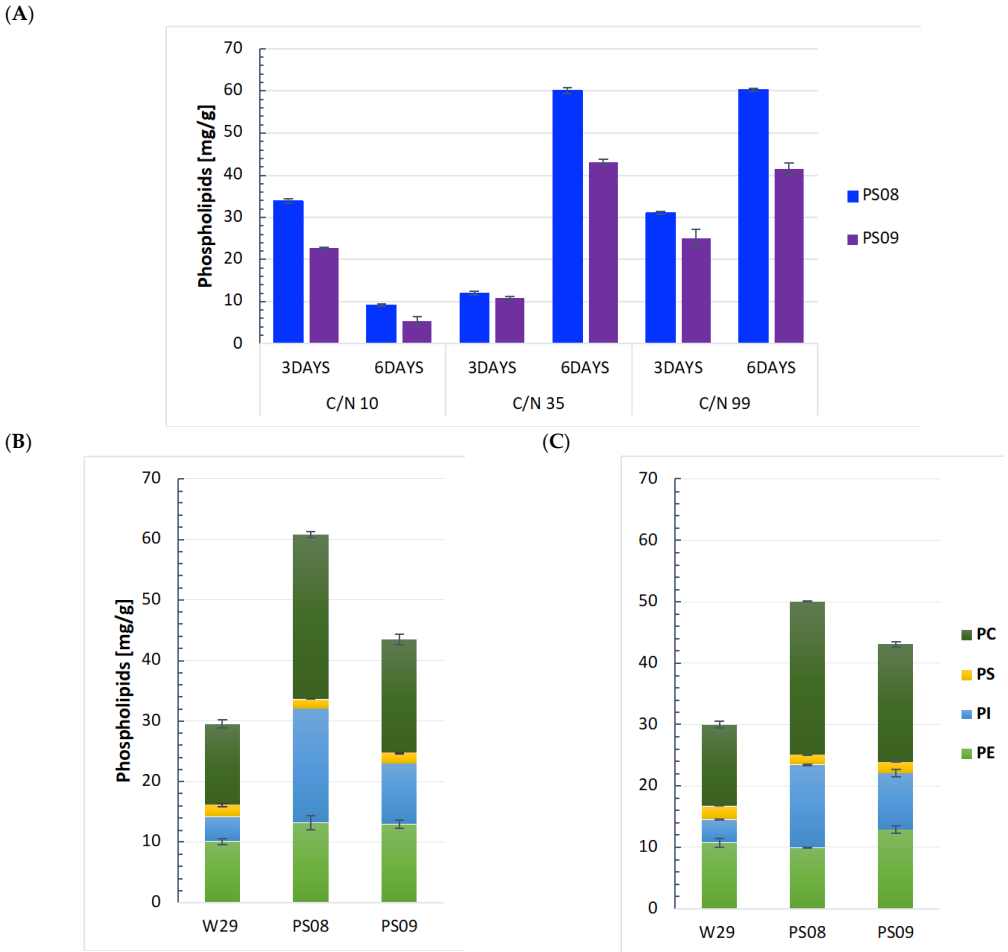


Figure 4. Effects of different culture conditions on *Y. lipolytica*. (**A**) Effects of different time of cultivation (72 h/144 h) and C/N ratios on PLs production. (**B**,**C**) Production of PLs after 144 h with the twofold addition of glucose (**B**) and gluconate (**C**). Supplementation of these two substrates accounted for approximately 5% of the total carbon consumed by the cells and was added at the beginning and middle of the culture; the primary carbon source was glycerol. Error bars represent standard deviation from at least three biological replicates.

Furthermore, as describe above [Figure 2A,B], the synthesis of PL from glycerol, despite glycerol being the phospholipid backbone, is less efficient than from glucose. This led us to speculate that genes involved in the phospholipid biosynthetic pathway may be partially dependent on NADPH, whose generation in *Y. lipolytica* is mainly through the oxidative pentose phosphate pathway (PPP), which is somewhat attenuated when utilizing only glycerol [23]. Thus, our second strategy to optimize the medium for PL production was to culture *Y. lipolytica* on glycerol and feed limiting quantities of glucose or gluconate as 'dopant' substrates to augment the reductive metabolism of lipogenesis, through obligate NADPH synthesis. This method worked well in a study by Park et al. (2019), who used gluconate dosing in *Y. lipolytica* to accelerate acetate-driven lipogenesis and glucose dosing in *Moorella thermoacetica* to stimulate the reduction of CO_2 to acetate by increasing pyruvate kinase-mediated ATP synthesis [24]. Our results show that neither glucose nor gluconate addition affected PL synthesis by the modified strains [Figures 3A and 4B,C]. All of the above-described results raised many questions about the PL biosynthesis by *Y. lipolytica* using different carbon sources, which need further studies for their elucidation.

2.4. Production of Phospholipids on Bioreactor Scale

The biotechnological approach in designing an efficient process needs to be evaluated on a larger scale. Thus, further experiments were performed using the PS08 strain showing the highest PL synthesis and the wild-type strain W29 as a control, which were cultured in a bioreactor using pure and waste glycerol as a carbon sources. Biomass and phospholipid content were determined at the moment of partial (50 g/L) and complete (0 g/L) substrate consumption. The obtained results are shown in Figure 5.

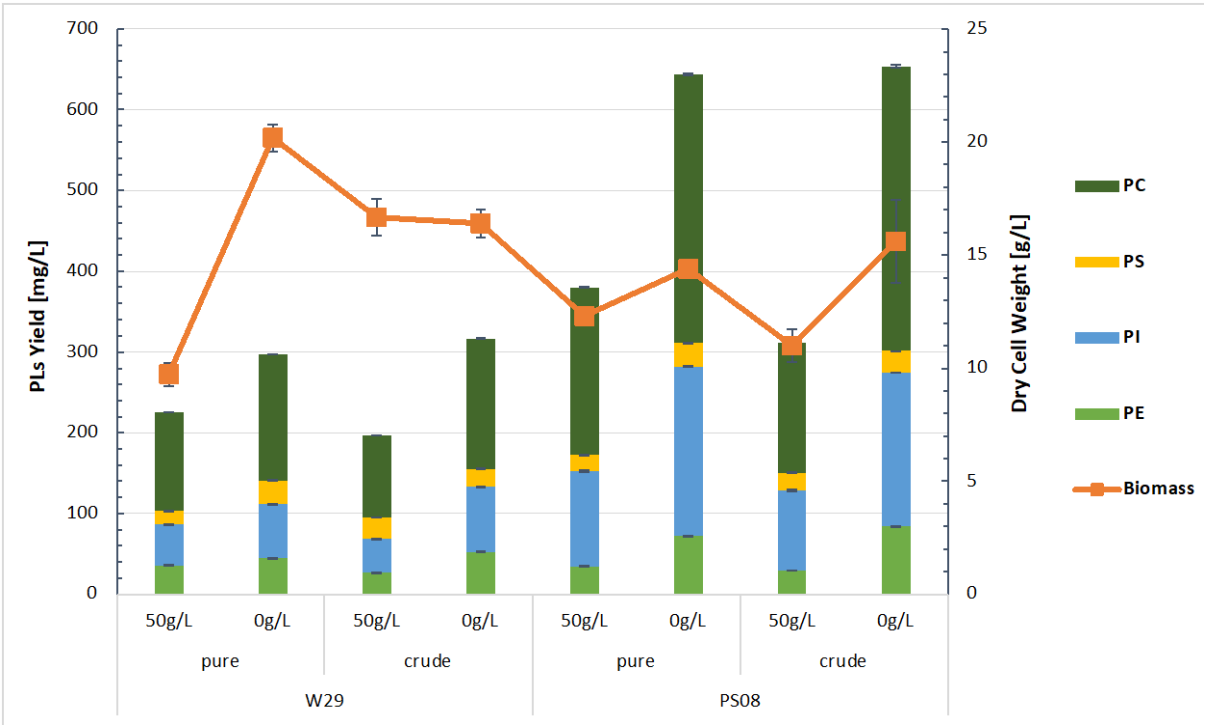


Figure 5. Phospholipids production by control strain and PS08 using pure/crude glycerol as a substrate in bioreactor. Cultures containing YNB medium with 100 g/L glycerol were carried out until complete substrate consumption.

PL production was at a similar level regardless of the type of glycerol used as a carbon source (pure or crude). This observation was made for both W29 and PS08 strains. It demonstrates that there is no negative effect of trace amounts of impurities found in crude glycerol on phospholipid synthesis. After the completion of the bioreactor culture, the PS08

strain's PL content was almost 160% higher than the PL content observed during growth in flasks. At the same time, the PC content increased by nearly 180% (PL—643.7 mg/L, PC—332.6 mg/L from pure glycerol, PL—653.7 mg/L, PC—352.6 mg/L from crude glycerol) [Figure S3]. In the case of the wild-type strain, there was no increase in the production of PL compared to the flask cultures (PL—296 mg/L, PC—155 mg/L from pure glycerol, PL –316 mg/L, PC—160,7 mg/L from crude glycerol) [Figure S3]. Moreover, this strain maintained phospholipid synthesis at a fairly constant level regardless of the growth phase, assuming that 50 g/L glycerol corresponds to the early stationary phase and total glycerol consumption corresponds to the late stationary phase of growth. In contrast, for the strain PS08, one can see an approximately twofold increase in PL content at the time of total substrate consumption, indicating that intensive phospholipid accumulation occurs during the late stationary phase along with the typical accumulation of other lipid fractions characteristic of oleaginous microorganisms [8].

These results indicate that *Y. lipolytica*, with just the overexpression of the minimal set of enzymes, was already able to produce almost 650 mg/L of phospholipids, using technical glycerol, as well as waste glycerol, as a carbon source.

3. Materials and Methods

3.1. Strains, Media, and Culture Conditions

The strains used in this study are shown in Table S1 (Supplementary Materials). The strains of *Y. lipolytica* were maintained in a YPD medium consisting of 10 g/L yeast extract, 10 g/L peptone, and 20 g/L glucose with 10 g/L agar (for plates) at 28 °C. Minimal (YNB) medium for the selection of the *Y. lipolytica* transformants was prepared using 1.7 g/L yeast nitrogen base (without amino acid and ammonium sulfate, Sigma-Aldrich, Saint Louis, MI), 20 g/L glucose, 5 g/L NH_4Cl, and 50 mM phosphate buffer pH 6.8 with 20 g/L agar. The *Escherichia coli* strains harboring plasmids were cultured overnight in LB medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl with 20 g/L agar in plates and 0.05 mg/L kanamycin) at 37 °C. For long-term storage, the strains were kept at −80 °C in 500 g/L glycerol.

3.2. Plasmid Preparation

In this study, two types of plasmids were used. Gene disruption-carrying cassettes were constructed using the pCR-Blunt II TOPO vector (Invitrogen, Carlsbad, CA, USA), whereas plasmids used for gene overexpression were based on a JMP62 plasmid [25]. The disruption cassettes were prepared as described by Fickers and co-workers [26]. Briefly, 1 kb fragments representing promoter (P) and terminator (T) sequences of *SPO14* (*YALI0E18898g*) and *LRO1* (*YALI0E16797g*) were amplified by PCR from *Y. lipolytica* W29 genomic DNA and subsequently fused using the PCR-fusion technique. The obtained PT fragments were cloned into the pCR-Blunt II TOPO vector. Resulting plasmids were then digested with the I-*Sce*I restriction enzyme, and the I-*Sce*I digested *URA3ex* (Ura) or *LEU2ex* (Leu) marker was inserted to obtain the disruption cassettes. The overexpression cassettes were constructed by the PCR amplification of selected genes involved in the PL synthesis pathway from the genomic DNA of *Y. lipolytica* W29. PCR fragments obtained with the appropriate restriction enzyme sites were then digested and ligated into the *Bam*HI/*Bgl*II and *Xma*JI (*Avr*II) digested JMP62 vector carrying a strong constitutive TEF promoter. All constructs were verified using PCR and DNA sequencing (Genomed S.A, Warsaw, Poland). Plasmids and primers used in this study are listed in Tables S2 and S3 (Supplementary Materials).

3.3. Cloning and Transformation Protocols

All restriction enzymes, the Phusion high-fidelity DNA polymerase, and the T4 DNA ligase were purchased from ThermoScientific (Waltham, MA, USA). The reactions followed standard protocols as described by the manufacturers. Plasmids from *E. coli* were extracted using the Plasmid Mini Kit (A&A Biotechnology, Gdańsk, Poland). DNA purifi-

cation from the gel was carried out with the Gel Out extraction kit (A&A Biotechnology, Gdańsk, Poland).

Competent *E. coli* DH5α cells were transformed by the thermal shock protocol. Yeast transformation was carried out following the lithium-acetate method [27]. Transformants were selected on YNB-Leu, YNB-Ura, or YNB-Hygro media, depending on their genotype.

3.4. Flask Cultivation

Three transformants of each strain were tested. The preculture was conducted in 50 mL of rich YPD medium at 28 °C with 250 rpm of agitation for 48h. After that time, cells were washed twice with sterile distilled water and used for inoculation. The initial OD_{600} was set at 0.5 for each strain. Shake flask cultivations were performed in minimal medium containing 60 g/L glucose or glycerol, 1.3 g/L $(NH_4)_2SO_4$, 1 g/L $MgSO_4x7H_2O$, 1.7 g/L YNB, and 50 mM phosphate buffer pH 6.8 and cultivated at 160 rpm at 28 °C for 144 h. Experiments were conducted in three biological replicates.

3.5. Bioreactor Cultures

The precultures were prepared as described for flask cultures. The initial OD_{600} was set to 0.5. The batch cultures were conducted in lipid synthesis medium, as described above, containing 100 g/L of pure or crude glycerol. Crude glycerol was derived from biofuel production from the Trzebinia refinery (LOTOS Group) with 86% (*v*/*w*) glycerol content. All cultures were performed in a 5 L stirred-tank reactor (BIOSTATB-PLUS, Sartorius, Germany) with a working volume of 2 L at 28 °C. The aeration was set to 0.8 vvm with a stirring rate of 600 rpm. The pH 6.8 was maintained automatically by the additions of 30% (*w*/*v*) NaOH solution. The cultures were terminated when the carbon source was completely consumed. The experiments were performed in biological duplicates.

3.6. Lipids Extraction

The lipids were extracted from lyophilized biomass using a modified method, as described before [28]. Briefly, 100 mg of biomass was weighed and mixed with a 2:1 *v*/*v* chloroform-methanol solution (5 mL) and left overnight at 80 °C with 600 rpm of rotation. Then, $MgCl_2$ was added to the suspension at a concentration of 0.034% and vortexed for 10 min. Samples were centrifuged at 1000× *g* for 3 min. The aqueous upper phase was discarded. The artificial upper phase, methanol-water-chloroform (48:47:3), was added to the samples and mixed. Samples were centrifuged at 1000× *g* for 3 min. The aqueous upper phase was again discarded, and the organic lower phase containing the lipid fraction was transferred to a new tube. The solvent was evaporated, and the collected lipids were stored at −20 °C for further analysis.

3.7. Quantification of Phospholipids

The quantitative content analysis of individual phospholipid fractions (phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and phosphatidylcholine) in the obtained extracts was determined by high-performance liquid chromatography using DIONEX UltiMate 3000 chromatograph from Thermo Scientific (Waltham, MA, USA) equipped with a UV/CAD detector (at 300 nm) and a BetaSil DIOL column (Thermo Scientific, 150 × 4.6 mm, 5 µm). In this analysis, the injection volume was 10 µL, whereas the autosampler and column temperature were 20 °C and 30 °C, respectively. The elution was set at a constant flow (3 mL/min) and performed in a gradient: solvent A (1% HCOOH, 0.1% triethylamine (TEA) in water), solvent B (hexane), and solvent C (2-propanol). The elution program was as follows: 3/40/57 (%A/%B/%C (*v*/*v*/*v*)), in 5 min = 4/40/56, in 6 min = 5/40/55, in 7 min = 6/40/54, in 8 min = 7/40/53, and then constant for up to 18 min. The total analysis and conditioning time was 20 min.

3.8. Quantification of Fatty Acids

Samples for total fatty acid analysis were taken as 5 mL of culture at the end of shake flask or bioreactor cultivations. The 5-mL culture volume was kept at −80 °C and then freeze dried. The total lyophilized culture was processed for fatty acid extraction and derivatization to methyl esters (FAMEs), using the method described before [29]. In short, 10 mg of biomass was mixed with 2 mL of solvent solution: 2.5% H_2SO_4, 97.5% methanol, 50 µg/mL of C17:0 as an internal standard, in Pyrex glass tubes (Sigma-Aldrich, Saint Louis, MI, USA). Then all samples were thoroughly mixed and incubated at 80 °C overnight to form FAMEs. FAMEs were extracted by hexane and 0.9% NaCl. The organic phase was collected and stored at −20 °C until use. FAME analysis was performed by gas chromatography on a GC-MS instrument (Shimadzu, Kyoto, Japan) equipped with a Zebron ZB-FAME capillary column (30 m × 0.25 mm × 0.20 µm). The samples (1 µL at 250 °C) were injected in splitless mode using helium (1 mL min^{-1}). The identification of fatty acids was carried out by the comparison of retention times with reference compounds (Supelco 37 Component FAME Mix, Sigma-Aldrich).

3.9. Thin-Layer Chromatography

The qualitative estimation of phospholipids and neutral lipids extracted from *Y. lipolytica* was carried out by the thin layer chromatography method [30] with a solvent system of chloroform-methanol-water (32.5:12.5:2) and petroleum ether/diethylether/acetic acid (32:8:0.8), respectively.

4. Conclusions

In this study, we investigated the potential of *Y. lipolytica* to produce phospholipids, especially phosphatidylcholine. Interestingly, we found that increasing the amount of phosphatidic acid by manipulating the upstream lipid pathway and presumably targeting it in the phospholipid pathway does not lead to an increase in their synthesis. The very wide optimal C/N ratio ranging from 35 to 99 for phospholipid production is also interesting. Finally, the best strain (PS08), containing only a few overexpressed genes, was able to produce, under the best fermentation conditions, almost 650 mg/L PL, including 335 mg/L PC using waste glycerol, as well as technical glycerol, as the sole carbon source. This study shows that relatively simple pathway engineering in *Y. lipolytica* can lead to an increased number of phospholipids, which can most likely be further improved by manipulating the remaining parts of the lipid pathways, the gene copy numbers, and precursor availability. These results also suggest that *Y. lipolytica* may be a promising host for the production of other interesting phospholipid-derived compounds.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms231810737/s1. References [31,32] are cited in the supplementary materials.

Author Contributions: P.S.: conceptualization, investigation, formal analysis, validation, visualization, methodology, writing—original draft. M.R. and A.G.: investigation, formal analysis, methodology, writing—review & editing. P.M.: investigation. T.J.: formal analysis, methodology, review & editing. Z.L.: conceptualization, resources, supervision, investigation, writing—review & editing. All authors have read and agreed to the published version of the manuscript.

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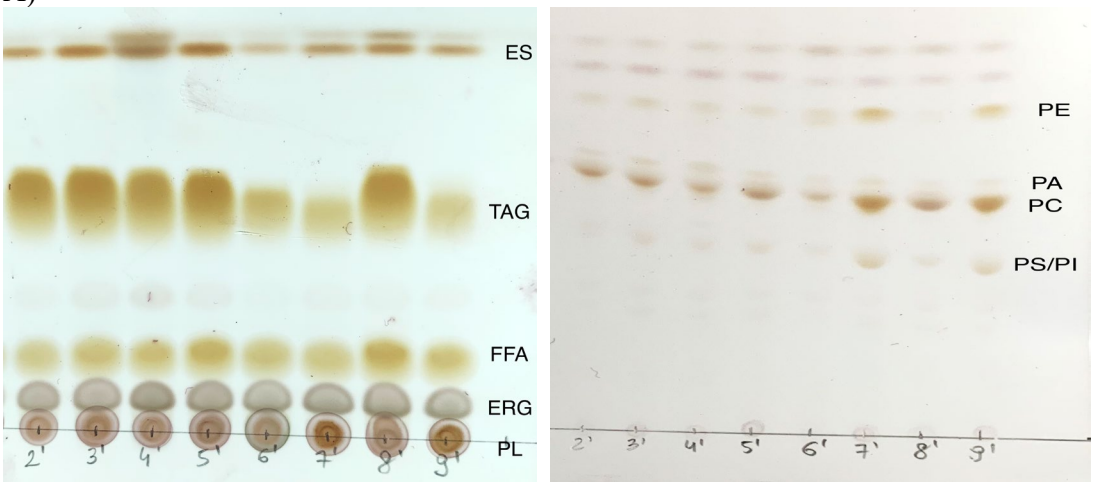
SUPPLEMENATRY MATERIALS

FIGURES

A)



B)

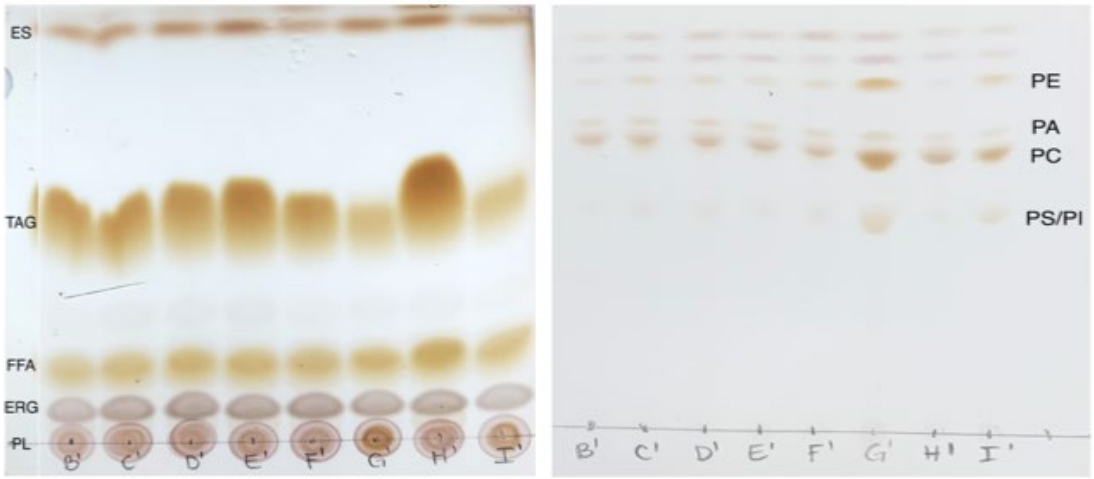


Figure S1. A) TLC separation of neutral lipids (left side) and phospholipids (right side) from *Y. lipolytica* strains grown on glucose. 2'-W29, 3'-PS01, 4'-PS02, 5'-PS03, 6'-PS04, 7'-PS05, 8'-PS06, 9'-PS07 B) TLC separation of neutral lipids (left side) and phospholipids (right side) from strains grown on glycerol. B'-W29, C'-PS01, D'-PS02, E'-PS03, F'-PS04, G'-PS05, H'-PS06, I'-PS07. FFA, free fatty acid; ERG, ergosterol; ES, sterol esters.

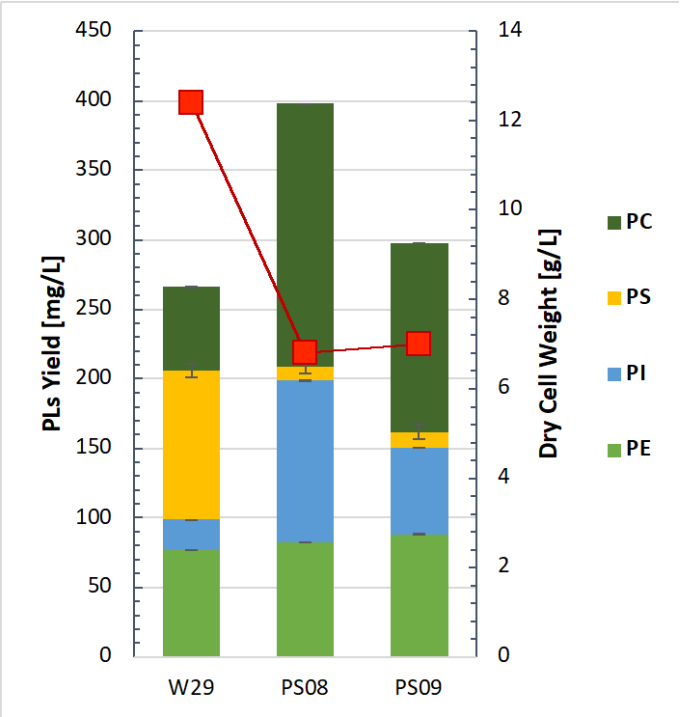


Figure S2. Titers of phospholipids in final engineered *Y. lipolytica* strains. Cultivations were carried out for 144 h in shake flasks containing YNB with 60 g/L glycerol, C/N 99.

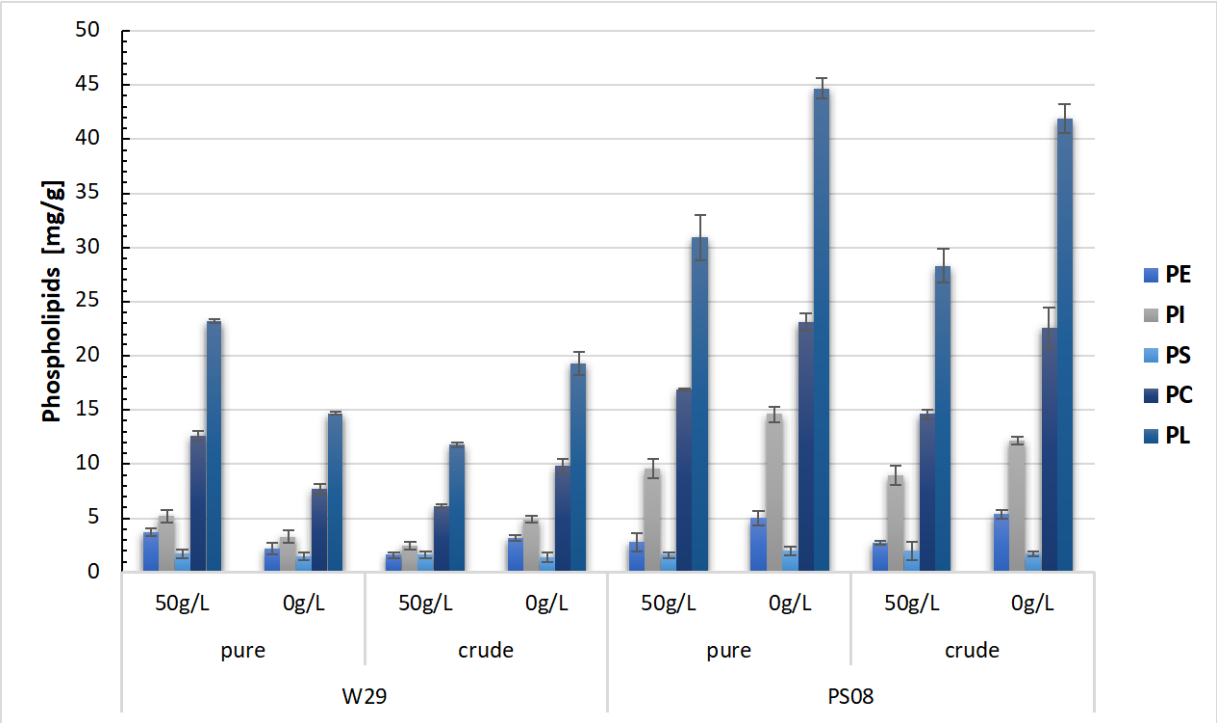


Figure S3. Phospholipids production by control strain and PS08 using pure/crude glycerol as a substrate in bioreactor. Cultures containing YNB medium with 100 g/L glycerol, were carried out until complete substrate consumption.

TABLES

Table S1. Strains of *Y. lipolytica* used in this study.

Strain	**Genotype**	**Source or reference**
W29	*MATa WT*	Culture collection of the Department of Biotechnology and Food Microbiology
Po1d	*MATa ura3-302 leu2-270 xpr2-322*	CLIB139
PS01	*MATa ura3-302 leu2-270 xpr2-322* pTEF-*CDS*	This study
PS02	*MATa ura3-302 leu2-270 xpr2-322* pTEF-*CDS* pTEF-*SCT*	This study
PS03	*MATa ura3-302 leu2-270 xpr2-322* pTEF-*CDS* pTEF-*SLC*	This study
PS04	*MATa ura3-302 leu2-270 xpr2-322* pTEF-*CDS* pTEF-*ALE*	This study
PS05	*MATa ura3-302 leu2-270 xpr2-322* pTEF-*CDS* pTEF-*OPI*	This study
PS06	*MATa ura3-302 leu2-270 xpr2-322* pTEF-*CDS* pTEF-*OPI ⊿LRO1*	This study
PS07	*MATa ura3-302 leu2-270 xpr2-322* pTEF-*CDS* pTEF-*OPI ⊿SPO14*	This study
PS08	*MATa ura3-302 leu2-270 xpr2-322* pTEF-*CDS* pTEF-*OPI* pTEF-*DGK*, pTEF-*GUT1*	This study
PS09	*MATa ura3-302 leu2-270 xpr2-322* pTEF-*CDS* pTEF-*OPI* pTEF-*DGK*, pTEF-*GUT1 ⊿SPO14*	This study

Table S2. Plasmids used in this study.

Plasmid	**Description**	**Reference**
JME1046	JMP62-URA3ex, TEFp, KanR	[31]
JME1046	JMP62-LEU2ex, TEFp, KanR	[32]
JMP62-CDS	JMP62-URA3ex-pTEF-*CDS*	This study
JMP62-ALE	JMP62-URA3ex-pTEF-*ALE*	This study
JMP62-SCT	JMP62-URA3ex-pTEF-*SCT*	This study
JMP62-SLC	JMP62-LEU2ex-pTEF-*SLC*	This study
JMP62-OPI3	JMP62-LEU2ex-pTEF-*OPI3*	This study
JMP62-DGK	JMP62-LEU2ex-pTEF-*DGK1*	This study
JMP62-GUT	JMP62-*Hygro*ex-pTEF-*GUT1*	This study
PUT-LRO	pCR™-Blunt II TOPO™ PUT-*LRO1*	This study
PLT-SPO14	pCR™-Blunt II TOPO™ PLT-*SPO14*	This study

Table S3. Primers used in this study.

Primer	Sequence (5' -> 3')	Aim
P1-LRO1-F	ATTAGCGGCCGCACCAGACTTGCTCCACATTC	Knock-out of the LRO1 and SPO14 genes
P2-LRO1-R	CGATTACCCTGTTATCCCTACCACCGGAAAAAAGCCGATTAC	
T1-LRO1-F	GGTAGGGATAACAGGGTAATCGGATGGAGAAGGGCGTTCG	
T2-LRO1-R	ATTAGCGGCCGCCACGGCTTGCTTTCAGATTC	
P1-SPO14-F	ATTAGCGGCCGCAGCTTTCCTGCAGCATGAG	
P2-SPO14-R	CGATTACCCTGTTATCCCTACCTTTGTGGCGACTCAAGAGTG	
T1-SPO14-F	GGTAGGGATAACAGGGTAATCGGCGACAAACACGTGCTAAG	
T2-SPO14-R	ATTAGCGGCCGCAAACTTGTTCGACGCCATTC	
LRO1-ver-F	GAGTCGCACTTCAGAAAGC	Verification of the knock-out
LRO1-ver-R	CATGGAGCCGGAAATGTC	
SPO14-ver-F	CTCCACTCCCCAGATGAAC	
SPO14-ver-R	CGAGTTGAAGGGGTTCTTG	
OPI3-BamHI-F	GAGAGGATCCATGTCCTTTCCTGACAAGATTGTTG	Overexpression cassetes
OPI3-AvrII-R	TCTCCCTAGGTTACTTCTGCTTGGCAGCGAG	
DGK1-BamHI-F	GAGAGGATCCATGTCTGCTGCATCTACTGGAG	
DGK1-AvrII-R	TCTCCCTAGGTTACTTCTTGAAGATGTCCAGCAG	
CDS1-BamHI-F	GAGAGGATCCATGTCTGAAAAAATTGACGCCCACAC	
CDS1-AvrII-R	TCTCCCTAGGTTATTCAAGAACGGCGCAGAAGC	
ALE1-BamHI-F	GAGAGGATCCATGGCCTTTCCATGGGCAGATAAG	
ALE1-AvrII-R	TCTCCCTAGGTTACTTGGTCTTGATGGTGTCCTTCTTC	
SCT1-BamHI-F	GAGAGGATCCATGTCCGAAACCGACCATC	
SCT1-AvrII-R	TCTCCCTAGGTTATTCCTCATCCTGCTCTCGTC	
SLC1-BamHI-F	GAGAGGATCCATGTCCGTTGCATCCAAGCTC	
SLC1-AvrII-R	TCTCCCTAGGCTACTGAGTCTTCTGGCCAGCGTAG	
GUT1-BamHI-F	GAGAGGATCCATGTCTTCCTACGTAGGAGC	
GUT1-AvrII-R	TCTCCCTAGGTTACTCAAGCCAGCCAAC	
JMP62-pTEF-START	GGGTATAAAAGACCACCGTCC	Verification of overexpression cassetes integration
JMP62-61STOP	GTAGATAGTTGAGGTAGAAGTTG	

3.3. Publikacja 3

Oleisty charakter drożdży *Y. lipolytica*, o którym wspomniano powyżej, i związana z tym dostępność malonylo-CoA [103], stała się hipotezą do badań nad zdolnością tych drożdży do biosyntezy innego cennego metabolitu – resweratrolu. Biosynteza RES wymaga kondensacji aż 3 cząsteczek malonylo-CoA, a jego duża ilość u tego gatunku drożdży powinna być zaletą do osiągnięcia dużych stężeń RES. Badania te prowadzone były równolegle do konstrukcji szczepów zdolnych do zwiększonej produkcji fosfolipidów, ze względu na duże ryzyko niepowodzenia, gdyż RES może okazać się toksyczny dla komórek drożdży. Głównym celem pracy było skonstruowanie szczepów zdolnych do produkcji RES, wykorzystujących zarówno metabolizm Tyr jak i Phe (**Rysunek 1**). W kolejnym etapie, w uzyskane transformanty wprowadzano dodatkowe kopie genów celem poprawy funkcjonowania szlaków metabolizmu obu aminokwasów, przez zwiększenie ilości potrzebnych enzymów (**Rysunek 2**). Ponadto, połączono obie ścieżki biosyntezy RES w jednym szczepie poddając nadeskpresji geny obydwu szlaków metabolicznych jego produkcji (**Rysunek 3**). W ostatnim etapie, produkcja RES z preferowanego substratu przemysłowego, glicerolu, została oceniona w hodowlach bioreaktorowych.

Integracja wielu kopii genów kodujących kluczowe enzymy danego szlaku jest strategią, która według dostępnej literatury światowej okazała się przydatna do zwiększenia strumienia węgla w pożądany szlak metaboliczny u drożdży *Y. lipolytica* [123]. W niniejszej pracy, uzyskane transformanty zdolne do biosyntezy RES testowano w hodowlach w kolbach analizując dwa źródła węgla: glukozę i glicerol. Ponadto, badaniom poddana była konieczność suplementacji podłoża aminokwasami: Tyr i Phe. Uzyskane wyniki udowodniły, że szlak metabolizmu Phe pozwalał na wyższą produkcję RES w pożywce z glukozą w porównaniu do szlaku metabolizmu Tyr. Niemniej jednak, gdy pożywkę hodowlaną suplementowano aminokwasami, najlepszą produkcję RES uzyskano dla szczepu z dwiema kopiami genów szlaku Tyr. Zaskakujące okazały się wyniki produkcji RES w szczepach z dwiema kopiami genów szlaku Phe, zarówno w szczepach P2 (metabolizm Phe), jak i T2P2 (łączony metabolizm obydwu aminowkasów), gdzie zaobserwowano znaczne zmniejszenie produkcji RES w pożywkach suplementowanych aminokwasami. Również zastąpienie

glukozy preferowanym substratem do produkcji RES – glicerolem, faworyzowało szlak Tyr, w którym uzyskano wyższe stężenia RES zarówno w podłożach bez jak i z suplementacją aminokwasem (**Rysunek 4**). Niemniej jednak, najlepszym producentem RES okazał się szczep łączący oba szlaki biosyntezy tego związku (T2P2), dla którego uzyskano 0,104 g/L RES w podłożu z dodatkiem aminokwasów. Wynik ten potwierdza wcześniejsze obserwacje, że zwiększenie strumienia węgla w pożądany szlak metaboliczny poprzez zwiększenie ilości kopii genów kodujących kluczowe enzymy danego szlaku zaowocowało wydajniejszą syntezą RES niezależnie od zastosowanego źródła węgla.

Najlepszego producenta RES w hodowlach w kolbach, szczep T2P2, analizowano również w skali powiększonej do procesów bioreaktorowych (**Rysunek 5**). Podjęto się również wstępnej optymalizacji zarówno składu podłoża jak i warunków hodowlanych. Najbardziej sprzyjający syntezie RES okazał się wariant 3, w którym zastosowano podłoże YNB z glicerolem bez dodatku aminokwasów, szybkość napowietrzania wynoszącą 0.8 vvm oraz szybkość obrotów mieszadła 800 rpm. W tych warunkach szczep T2P2 wyprodukował 4-krotnie wyższe stężenie RES w porównaniu do hodowli w kolbach osiągając 0,43 g/L tego metabolitu. Co ciekawe, okazało się, że dodatek prekursorów – Tyr i Phe do podłoża hodowlanego ma negatywny wpływ na produkcję w warunkach hodowli bioreaktorowych. Obserwacja ta związana jest najprawdopodobniej z dostępnością tlenu, gdyż hodowle w kolbach i hodowle bioreaktorowe znacząco różnią się stężeniem tlenu rozpuszczonego w podłożu, jednak fenomen ten musi być poddany analizie.

Uzyskiwane wyniki jednozdanie wskazują na przewagę oleistych drożdży *Y. lipolytica* nad innymi gatunkami drożdży jako potencjalnych producentów RES. Osiągnięcie wysokiego stężenia tego metabolitu wymagało jedynie integracji dwóch kopii genów szlaków metabolizmu Tyr i Phe, podczas gdy u drożdży nieoleistych, konieczna jest bardzo zaawansowana inżyniera ich metabolizmu. Wyniki te wskazują również, że *Y. lipolytica* może okazać się dobrym producentem również innych metabolitów wtórnych o strukturze poliketydowej.

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De novo production of resveratrol from glycerol by engineering different metabolic pathways in *Yarrowia lipolytica*

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ABSTRACT

Resveratrol is a polyphenol with multiple applications in pharma, cosmetics and food. The aim of this study was to construct *Yarrowia lipolytica* strains able to produce resveratrol. For this purpose, resveratrol-biosynthesis genes from bacteria and plants were expressed in this host. Since resveratrol can be produced either via tyrosine or phenylaniline, both pathways were tested, first with a single copy and then with two copies. The phenylalanine pathway resulted in slightly higher production in glucose media, although when the media was supplemented with amino acids, the best production was found in the strain with two copies of the tyrosine pathway, which reached 0.085 g/L. When glucose was replaced by glycerol, a preferred substrate for bioproduction, the best results, 0.104 g/L, were obtained in a strain combining the expression of the two synthesis pathways. Finally, the best producer strain was tested in bioreactor conditions where a production of 0.43 g/L was reached. This study suggests that *Y. lipolytica* is a promising host for resveratrol production from glycerol.

1. Introduction

Resveratrol (trans-3,5,4′-trihydroxystilbene), a natural polyphenolic compound of the stilbene class, is naturally found in grapes, blueberries, peanuts, etc., and their processed products (Li et al., 2015). Resveratrol showed beneficial effects in various preclinical test (Jeandet et al., 2012; Mei et al., 2015), aimed to provide benefits against tumor, inflammation, diabetes, thrombosis and aging. Therefore, resveratrol has gained an increased interest by pharmaceutical, food and cosmetic industries. At present, the major source of commercial resveratrol is plant extracts such as Japanese knotweed *Polygonum cuspidatum* (Mei et al., 2015) and grapes.

Biotechnological production by recombinant strains provides a potential approach for resveratrol biosynthesis (Borodina and Nielsen, 2014). As shown in Fig. 1, there are two main pathways for resveratrol production, one using tyrosine and the other phenylalanine as intermediates. For the tyrosine pathway, first, a tyrosine ammonia lyase (TAL) deaminates L-tyrosine to *p*-coumaric acid. Then a 4-Coumarate: CoA ligase (4CL) forms coumaroyl-CoA from coumaric acid. Finally, coumaroyl-CoA is condensed by three malonyl-CoA units to form resveratrol by a stilbene synthase (STS) (Wu et al., 2017). On the other hand, through the phenylalanine pathway, first, cinnamic acid is generated by deamination of phenylalanine via a phenylalanine ammonia lyase (PAL). Next, cinnamic acid is hydroxylated to *p*-coumaric acid by a cinnamic acid 4-hydroxylase (C4H). Finally, similarly as in the tyrosine pathway, cinnamic acid can be converted in resveratrol via 4CL and STS.

Presently, most research aimed to produce resveratrol in heterologous host has focused on synthesis via tyrosine (Shin et al., 2012) and mainly in recombinant *E. coli* and *Saccharomyces cerevisiae* (Li et al., 2015). Resveratrol produced through tyrosine by recombinant *S. cerevisiae* reached 531 mg/L resveratrol (Li et al., 2015) while via phenylalanine reached 812 mg/L resveratrol in fed-batch fermentation from glucose (Li et al., 2016).

Yarrowia lipolytica is an oleaginous yeast with high industrial potential (Larroude et al., 2018; Ledesma-Amaro and Nicaud, 2016a; Wang et al., 2020; Xu et al., 2020). It provides several advantages as a eukaryotic host, including its ability to utilize vast raw substrates like glycerol (Ledesma-Amaro and Nicaud, 2016b). *Y. lipolytica* is well

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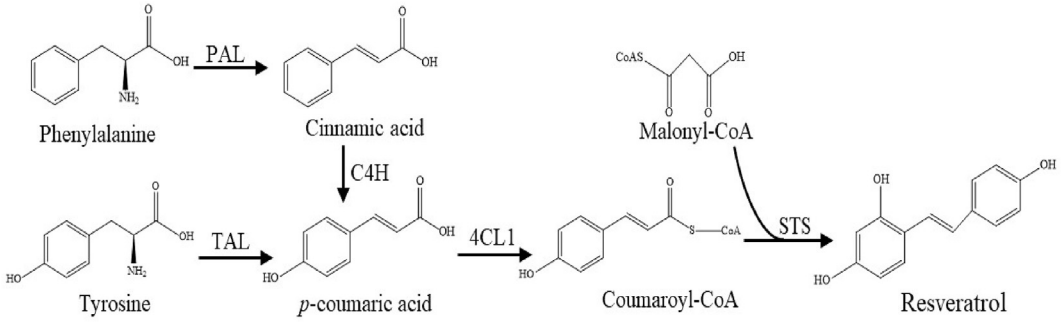

Fig. 1. Biosynthesis paths for resveratrol. PAL: Phenylalanine ammonia lyase; C4H: Cinnamic acid 4-hydroxylase; TAL: Tyrosine ammonia lyase; 4CL1: 4-coumaroyl-CoA ligase; STS: Stilbene synthase.

known for its capacity to overproduce lipids which are produced from malonyl-CoA (Wang et al., 2020). Since resveratrol production requires the condensation of 3 malonyl-CoA molecules, the high availability of this molecule in *Y. lipolytica* could be advantageous to produce high titers of resveratrol. This organism has been recently engineered to produce different molecules from the shikimate pathway such as 2-phenylethanol, *p*-coumaric acid, violacein, and deoxyviolacein (Gu et al., 2020), naringenin (Lv et al., 2020; Palmer et al., 2020; Wei et al., 2020) or arbutin (Shang et al., 2020). *Y. lipolytica* has also been reported to produce small amount of resveratrol when engineered with such purpose. To our knowledge, there are four published reports on the production of resveratrol by *Y. lipolytica*. By introducing the genes downstream of L-phenylalanine, 1.46 mg/L of resveratrol was obtained in minimal medium supplemented with 2 mM tyrosine (Huang et al., 2010). In another report, 1) after relieving the feedback regulation of the shikimate pathway by eliminating amino acids formation and engineering feedback-insensitive DAHP synthases, 2) overexpressing of heterologous phosphoketolase and 3) deleting of pyruvate kinase, 12.67 mg/L of resveratrol was produced from tyrosine (Gu et al., 2020). Also from tyrosine, Palmer et al. (2020) produced 8.8 mg/L resveratrol de novo, exploiting beta-oxidation as a source of malonyl CoA. The titer increased to 48.7 mg/L when 2 mM-coumaric acid was supplemented. During the review process of this article, Sáez-Sáez et al. (2020) reported a resveratrol titer of 52.1 mg/L by a strain harboring FjTAL. After engineering with feedback-insensitive alleles of *ARO4* and *ARO7*, and with five additional copies of the heterologous biosynthetic genes, resveratrol production reached 409.0 mg/L in batch and 12.4 g/L in fed-batch.

In this work, we have created resveratrol-producing strains of *Y. lipolytica* exploiting both, the tyrosine and the phenylalanine branches of the pathway. Further optimization was achieved via the expression of multiple copies of the relevant genes as well as with combinations of the two biosynthesis pathways. Finally, the production from the preferred industrial substrate, glycerol was evaluated in bioreactor.

2. Materials and methods

2.1. Strains and culture conditions

The NEB® Turbo Competent *Escherichia coli* cells were used for all the cloning and plasmid propagation work. The *E. coli* transformants were selected and maintained on plates, and grown on broth of LB medium containing ampicillin (100 μg/ml) or spectinomycin (100 μg/ml) at 37 °C. *Y. lipolytica* Pold (wt), derived from W29 (ATCC20460), was used as parental strain to construct resveratrol-producing strains. were selected on hygromycin (0.2 g/L) when necessary (Larroude et al., 2018). Propagation of *Y. lipolytica* strains were performed using YPD medium (see composition below). Chemical reagents were purchased from Sigma-Aldrich.

2.2. Genes and plasmids

Phenylalanine ammonia lyase (PAL) from Vitis vinifera *(GeneBank accession number*: X75967) (Dubrovina et al., 2010), cinnamic acid 4-hydroxylase (C4H) from Arabidopsis thaliana *(No. NM128601)* (Shin et al., 2012), tyrosine ammonia-lyase (FjTAL) from *Flavobacterium johnsoniae (No. KR095306.1)* (Li et al., 2015), 4-coumaroyl-CoA ligase (4CL1) from *Arabidopsis thaliana (No. NM104046)* (Shin et al., 2012; Li et al., 2015), resveratrol synthase (VvVST) from *Vitis vinifera (No. NM_001281010)* (Li et al., 2015). The genes were codon-optimized for *Y. lipolytica* as in GeneArt. Constructs were complemented by pre-designed 4-nt overhangs and BsaI restriction sites based on requirements of golden-gate method (Celinska et al., 2017) and synthesized by GeneArt (Life Technologies).

All restriction enzymes were purchased from New England Biolabs (NEB). Plasmids from *E. coli* were extracted using the QIAprep Spin Miniprep Kit (Qiagen). Competent *E. coli* cells were transformed by thermal shock protocol. The correct genomic insertions of the expression cassettes in *Y. lipolytica* were verified from genomic DNA and PCR using primers listed in Table S1. Yeast transformation was carried out following lithium-acetate method (Barth and Gaillardin, 1996). Transformants were selected on YNB-Leu, YNB-Ura, or YNB-Hygro media, depending on their genotype. Plasmids used in this study are listed in Table S2.

2.3. Expression cassettes construction

DNA constructions

DNA cassette was constructed using the recently developed Golden Gate Assembly (GGA) method for *Y. lipolytica* (Celinska et al., 2017; Larroude et al., 2018). The synthesized genes were introduced into the donor vector pYTK001. Promoter *TEF*, terminator *Lip2*, and the pYTK001/genes mixed equimolarly were then introduced into vectors pB(Z)US1.1 (2/3) in one-pot reaction together with BsaI (0.5 μl), T4 DNA ligase (0.5 μl), T4 DNA ligase buffer (1 μl) and ddH_2O up to 10 μl. The enzymatic reaction conditions were as follows: [42 °C for 2 min, 16 °C for 5 min] × 25 cycles, 60 °C for 10 min, 80 °C for 10 min, and stored at 4 °C. After reaction, the mixture was used for *E. coli* transformation. White colonies were selected as the complete GGA candidates and identified through plasmid isolation, restriction digestion and colony PCR.

The successfully constructed plasmids were combined together in appropriate order to build DNA cassettes using similar enzymatic reaction system and condition except that BsmBI (0.5 μl) was used instead of BsaI. The ultimate GGA was subsequently linearised using NotI and transformed into *Y. lipolytica* after confirmation. The transformants were screened by nutrient deficient medium and verified PCR using genomic DNA.

2.4. Strains construction

4CL1 and STS are the common enzymes to synthesize resveratrol. In this experiment, the two pathways were constructed independently.

Strain T contained *FjTAL*, *4CL1*, and *VvVST* genes to produce resveratrol from tyrosine. Strain P contained phenylalanine path coding genes *PAL*, *C4H*, *4CL1*, and *VvVST*. Intermediate strains were obtained by removing the marker from strains T and P respectively, using Cre/lox method (Fickers et al., 2003). The second copy of the expression DNA cassettes responsible for the specific path were introduced into these strains, using lithium-acetate method to study the high-expression of resveratrol. All the constructed yeast strains used in this study were listed in Table S3.

2.5. Resveratrol production in flasks

Two transformants of each strain were tested. The preculture was conducted in 50 mL of rich YPD medium at 28 °C with 250 rpm agitation for 48 h. After that time, cells were washed twice with sterile distilled water and used for inoculation of the production medium. An initial OD_{600} was set at 0.5 for each strain. The main culture was conducted in 50 mL YNB minimal medium (6.8 g/L yeast nitrogen base without amino acids containing 5 g/L of ammonium sulfate (YNBww); 20 g/L glucose/glycerol; and 50 mM phosphate buffer pH 6.8), containing 0 or 2 mM tyrosine and/or phenylalanine in 300 mL flasks. After 72 h cultivation at 28 °C with 250 rpm agitation, the OD_{600}, dry biomass as well as concentration of the substrate and resveratrol and coumaric acid were measured. Coumaric acid and resveratrol were extracted by mixing equal volume of absolute ethanol with the culture and centrifuged at 2272 *g* for 30 min. The supernatants were used to analyze *p*-coumaric acid and resveratrol by HPLC. For glycerol as carbon source, 20 g/L of this compound was used instead of glucose.

2.6. Resveratrol production in bioreactor

The inoculum for selected strain (T2P2) was grown 48h in YPD medium at 28 °C, 250 rpm. After that time, cells were washed twice with sterile distilled water. The initial OD600 in bioreactor was set to 0.5. The cultures for T2P2 were performed in a 5-L stirred-tank bioreactor (BIOSTAT B-PLUS, Sartorius, Germany) with a working volume of 2 L at 28 °C. Aeration and stirring rate were set for 0.8 vvm and 800 rpm, respectively. The pH was maintained automatically at 6.8 by the addition of 30% NaOH solution. Five different conditions were tested using YNB medium with 100 g/L of glycerol as a substrate, 1.7 g/L of YNB and 5 g/L of ammonium sulfate; 1) addition of 5 mM of both Phe and Tyr at the beginning of the culture, 2) addition of 2 mM of both Phe and Tyr at the beginning of the culture, 3) without addition of amino acids, 4) lower aeration and stirring rate (0.6 vvm/500 rpm) with addition of 2 mM of both Phe and Tyr 5) limited aeration and stirring rate (0.6 vvm/500 rpm) without addition of amino acids. All the cultures were conducted until complete exhaustion of the carbon source. The bioreactor with appropriate medium was autoclaved at 121 °C, 30 min, 1 atm.

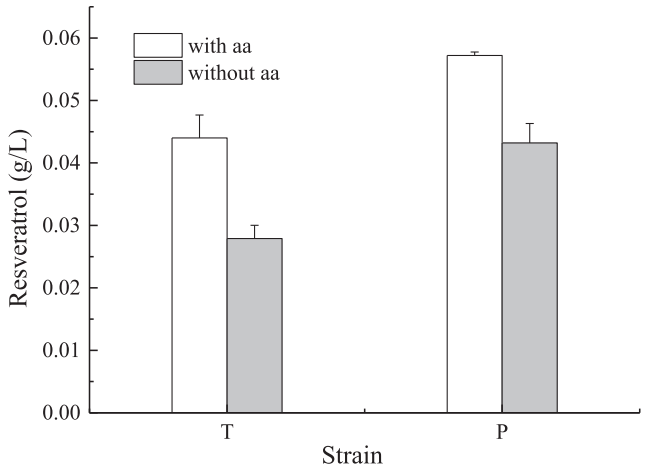

Fig. 2. Resveratrol production by strain T and P using minimal glucose media (YNB) with and without addition of amino acids 2 mM of Phe and 2 mM of Tyr.

2.7. Analytical methods

Resveratrol and *p*-coumaric acid were quantified on HPLC (UltiMate3000, ThermoScientific) equipped with a Hypersile GOLD TM 150 × 4.6 (particle size 5 μm). The eluent (70% acetonitrile, 0.1% formic acid) flow rate was set to 1.0 mL min^{-1}. Resveratrol was detected by absorbance at 304 nm with a retention time of 6.4 min and *p*-coumaric acid at 280 nm of 4.7 min. Resveratrol and *p*-coumaric acid concentrations were calculated from the standard curves, and both resveratrol and *p*-coumaric acid standards were purchased from Sigma-Aldrich.

Dry biomass was analyzed using 10 mL of the culture, washed twice with sterile distilled water and filtered through 0.22 μm membrane. Dry biomass was analyzed gravimetrically by drying it at 105 °C.

3. Results and discussion

3.1. Resveratrol biosynthesis via tyrosine

In order to enable the synthesis of resveratrol in *Yarrowia lipolytica*, we first overexpressed the genes involved in the conversion of tyrosine into the product of interest. The tyrosine ammonia-lyase gene from *F. johnsoniae* (*FjTAL*), the 4-coumarate:CoA ligase gene from *A. thaliana* (*4CL1*) and the stilbene synthase gene from *V. vinifera* (*VvVST*) were introduced under the control of strong and constitutive *TEF* promoter. The resulting strain (strain T) was grown in YNB glucose minimal media with or without the addition of 2 mM of tyrosine and phenylalanine (362 mg/L Tyr; 330 mg/L Phe). Resveratrol concentration reached 0.044 g/L when phenylalanine and tyrosine were added, and 0.028 g/L when no amino acid were added to the medium (Fig. 2). It was also noticed that in the presence of external amino acids the strain accumulated small amounts of coumaric acid (2.7 mg/L), which was not found in the non-supplemented media. Interestingly, we also noticed that biomass was reduced by 60% when amino acids were added to the media coinciding with a higher production of resveratrol (Supplementary Fig. S1). This could indicate that either resveratrol or other intermediates in the pathway may be toxic to the cells. Interestingly we only found weaker growth when resveratrol was added to the media at higher concentrations than 0.1 g/L (data not shown). Further experiments would be necessary to elucidate what is causing the reduced growth in this strain.

3.2. Resveratrol biosynthesis via phenylalanine

In order to create a strain able to produce resveratrol using phenylalanine as intermediate we overexpressed a phenylalanine ammonia lyase (PAL) gene from Vitis vinifera, a Cinnamic acid 4-hydroxylase (C4H) from Arabidopsis thaliana as well as the genes *4CL1* and *VvVST*. The resulted strain (strain P) was able to produce 0.043 g/L of resveratrol from glucose and 0.057 g/L when the media was supplemented with amino acids. The obtained results clearly indicate that the phenylalanine pathway for resveratrol production performs better on glucose compared to the tyrosine pathway, in both conditions (with and without amino acids supplementation; Fig. 2). According to the previous observations, higher resveratrol secretion in supplemented medium reduced the produced biomass (12% that of the P strain without supplements) (Supplementary Fig. S1). In this case, the accumulation of coumaric acid was not observed in any of the conditions, also suggesting a higher flux via phenylalanine.

To our knowledge, there is only one published report on the production of resveratrol by *Y. lipolytica* via phenylalanine (Huang et al., 2010) and three via tyrosine (Gu et al., 2020; Palmer et al., 2020; Sáez-Sáez et al., 2020). They obtained 1.46 mg/L from phenylalanine, and 12.67 mg/L, 8.8 mg/L and 52.1 mg/L from tyrosine, respectively,

which is 39-fold lower than the strain P and similar levels to the strain T generated in this work. Such differences can be explained by the use of different parental strain, different genes, promoters and expression cassettes.

3.3. Enhanced resveratrol biosynthesis using multi-copy integration

Multi-copy integration is a strategy that has proven useful for enhancing metabolic fluxes for bioproduction in *Y. lipolytica* (Larroude et al., 2018). Therefore, here we decided to test whether further copies of the genes used to produce resveratrol either from tyrosine or phenylalanine could help to increase the metabolic flux through the pathway. Accordingly, we generated 3 new strains, T2 (with 2 copies of TAL, 4CL1 and STS), P2 (with 2 copies of PAL, C4H, 4CL1 and STS) and T2P2 (with 2 copies of PAL, C4H, TAL, 4CL1 and STS).

After growing the strain in the same two media as T and P (with and without amino acids supplementation), we observed that the best production in the media without amino acids was 0.078 g/L by the strain T2P2 while in the media with amino acids was T2 with 0.085 g/L (twice more than the T strain) (Fig. 3). Surprisingly, the addition of 2 copies of the genes from the phenylalanine pathway, in both P2 and T2P2, reduced significantly the production of resveratrol in media supplemented with amino acids, which was accompanied by a slight accumulation of coumaric acid, 17.9 and 21.7 mg/L respectively, which is an order of magnitude higher than in the T and P strains. These two strains also showed a very reduced biomass production in both media (Supplementary Fig. S2).

3.4. Resveratrol production from glycerol in Y. lipolytica

Since glycerol is a preferred substrate for biotechnology (Da Silva et al., 2009) we decided to test the capacity of the generated strains to produce resveratrol from glycerol as a sole carbon source. Wild strains of *Y. lipolytica* use glycerol very efficiently (Ledesma-Amaro and Nicaud, 2016b). Due to that, resveratrol biosynthesis from glycerol was first investigated in flask (Fig. 4).

Unlike the glucose medium, in the glycerol based medium without amino acid supplementation the strain T (0.057 g/L) produced higher amount of resveratrol compared to the P strain (0.033 g/L). The P strain also showed reduced growth using glycerol as carbon source, and, similarly to glucose, the addition of amino acids to the media further reduced biomass formation (Supplementary Fig. S3). Multi-copy integration showed better performance than single copy and T2 strain produced more than T, same as P2 produced more than P. Furthermore,

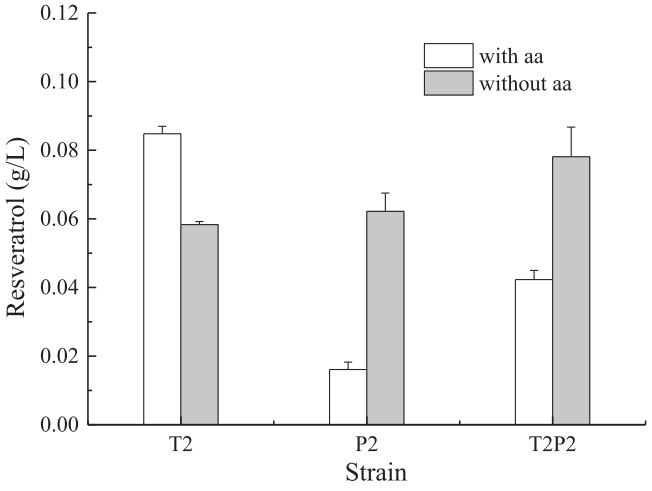

Fig. 3. Resveratrol production by strain T2, P2 and T2P2 using glucose media with and without addition of amino acids 2 mM of both Tyr/Phe.

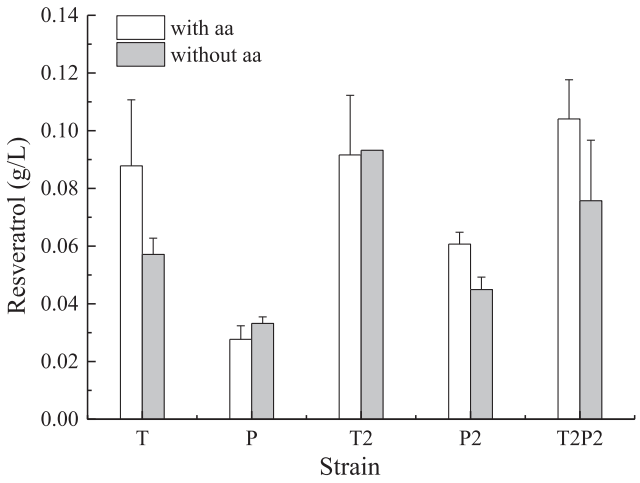

Fig. 4. Resveratrol production by recombinant strains using glycerol as carbon source with and without addition of amino acids 2 mM of Phe and 2 mM of Tyr.

T2P2 strain turned out to perform even better than T2 and P2 strains. In accordance to the results in glycerol the best performing strains were T2 and T2P2 with a production of 0.093 and 0.076 g/L without amino acids supplementation, 0.092 and 0.104 g/L with amino acids, respectively.

Summarizing this part of the study, strains T2 and T2P2 were the best strains regardless whether amino acids were added or not. We therefore selected the strain T2P2 to carry out experiments in bioreactor.

3.5. Resveratrol production in bioreactor

First, we tested five conditions using 100 g/L of glycerol on the strain T2P2 in bioreactor; 1) addition of 5 mM of both Phe and Tyr at the beginning of breeding, 2) addition of 2 mM of both Phe and Tyr at the beginning of breeding, 3) without addition of amino acids, 4) limited aeration and stirring (0.6 vvm/500 rpm) with addition of 2 mM of both Phe and Tyr 5) limited aeration and stirring (0.6 vvm/500 rpm) without addition of amino acids. As shown in Fig. 5 (Supplementary Fig. S4, S5 and S6), the maximum resveratrol was produced in condition 3, where no amino acid was added, the production reached 0.43 g/L. In comparison, with the addition of 5 mM and 2 mM amino acids, 0.17 and 0.31 g/L of resveratrol were secreted, respectively (Fig. 5). The oxygen limited conditions also reduced the production titers to 0.32 g/L with no addition of amino acids and 0.21 g/L when the amino acids were added (Fig. 5).

After 72 h, all the glycerol was consumed for all the conditions with

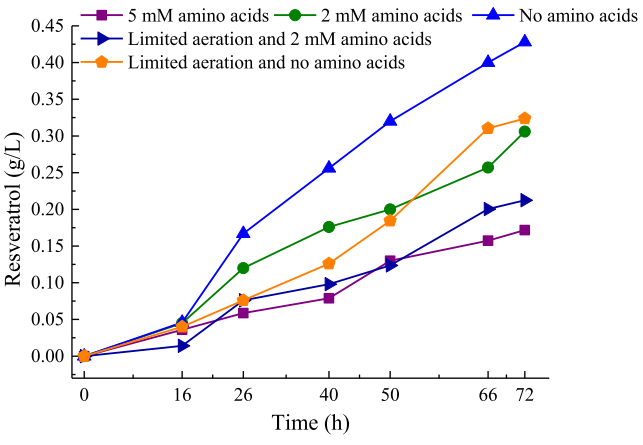

Fig. 5. Resveratrol production by strain T2P2 using glycerol as a substrate in bioreactor.

the exception of condition 4 (Supplementary Fig. S4). The best condition among the analyzed turned out to be condition 3, where all the substrate was consumed within 66 h and the produced biomass reached 18.4 g/L (Supplementary Fig. S6). In the worst performing condition, where only 0.17 g/L of resveratrol was secreted, condition 1, glycerol was consumed the fastest (within 40 h), however, the highest amount of biomass 40 g/L was also reached (Supplementary Fig. S4 and Fig. S5).

These results indicate that *Y. lipolytica* could be a good host for producing shikimate derived molecules, as it has been previously proposed for other compounds derived from aromatic amino acids (Gu et al., 2020), arbutin (Shang et al., 2020), bisdemethoxycurcumin (Palmer et al., 2020), etc. *Y. lipolytica*, with just the overexpression of the minimal set of enzymes was already able to produce de novo 430 mg/L of resveratrol, while similar modifications in other hosts such as *E. coli* and *S. cerevisiae* produced, for example, about 57.77 mg/L (Zhao et al., 2018) and 3.3 mg/L (Shin et al., 2012), respectively. Further production of 200 mg/L resveratrol was achieved by *S. cerevisiae* in batch and 415.65 and 531.41 mg/L in fed-batch bioreactor cultures using glucose or ethanol as substrate, respectively (Li et al., 2015). In order to achieve that they used a strain engineered to over-express feedback-insensitive alleles of *ARO4* and *ARO7*, post-translationally de-regulated acetyl-CoA carboxylase (*ACC1* gene), and the integration of multiple copies of the genes of these pathways. In another report, Li et al. (2016) did further improvement in resveratrol biosynthesis by optimizing the electron transfer to the cytochrome P450 monooxygenase, increasing precursors supply, and decreasing pathway intermediates degradation, which resulted in a production of 812 and 755 mg/L of resveratrol in fed-batch from glucose and ethanol feed, respectively. Similar modification would likely further improve the titers that can be achieved in *Y. lipolytica* and they should be tested in the future.

4. Conclusions

In this work, we explored the potential of *Yarrowia lipolytica* to produce resveratrol. We found that significant production was achieved either from tyrosine or phenylalanine as precursors and we observed that the increase in the copy numbers of the genes of the pathway enhanced the synthesis of resveratrol. Interestingly, we found that the addition of the precursors tyrosine and phenylalanine to the culture broth have a negative effect in production in bioreactor. Finally, the best strain (T2P2) combining the two pathways and multiple gene copies was able to produce, in the best fermentation conditions, 0.43 g/L using glycerol as sole carbon source. This work demonstrates that relatively simple pathway engineering in *Y. lipolytica* can lead to significant amount of resveratrol, which most likely can be further improved by manipulating the upstream part of the pathway and the availability of precursors. These results seem to also suggest that *Y. lipolytica* can be a promising host to produce compounds of interest derived from the shikimate pathway.

CRediT authorship contribution statement

Qin He: Investigation, Formal analysis, Validation, Visualization, Writing - original draft. **Patrycja Szczepańska:** Investigation, Formal analysis, Validation, Methodology, Writing - review & editing. **Tigran Yuzbashev:** Investigation, Methodology. **Zbigniew Lazar:** Resources, Supervision, Investigation, Writing - review & editing. **Rodrigo Ledesma-Amaro:** Conceptualization, Resources, Supervision, Investigation, Writing - review & editing.

Declaration of competing interest

On behalf of the editors I declare that there is no conflict of interest for this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mec.2020.e00146.

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De novo production of resveratrol from glycerol by engineering different metabolic pathways in *Yarrowia lipolytica*

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Table S1 List of primers used in the study

Name	Sequence (5'-3')
pZUA2.3/FjTAL/4CL/VvVST-F	GAATGAACACCATCAACGAGTAC
pZUA2.3/FjTAL/4CL/VvVST-R	CACAGACACTAGATTAGTTGGTCACG
pBUA2.2/4CL/VvVST-F_1	GACGTGATCTTCCGATCTAAG
pBUA2.2/4CL/VvVST-F_2	GTCTATCCCCAAGGCTCCCTC
pBUA2.2/4CL/VvVST-R_1	CGGATAGAGGACAGGTCGTAC
pBUA2.2/4CL/VvVST-R_2	CGATAGACACGTCGGGGTCA
pZLA2.2/PAL/C4H-F_1	CCTTCTGTGTGTCTGACCCC
pZLA2.2/PAL/C4H-F_2	AGGACTTCGACAAGGTGTTC
pZLA2.2/PAL/C4H-R_1	TAGACTTGGTGGAAGCTCGG
pZLA2.2/PAL/C4H-R_2	TGTCCTCGTTAATCTCTCCC
pZLA 2.2/PAL/C4H-F :2 copy	GGCGGGCTTCATGTTAAGAG
pZLA2.3/FjTAL/4CL/VvVST-F :2 copy	GGCGGGCTTCATGTTAAGAG
pZLA 2.2/PAL/C4H-R :2 copy	GAAAGGTAATTCGGGGACGG
pZLA2.3/FjTAL/4CL/VvVST-R :2 copy	GAAAGGTAATTCGGGGACGG
pBUA2.2/4CL/VvVST-F :2 copy	GCTCAATGGTCTGCTTGGAG
pZUA2.3/FjTAL/4CL/VvVST-F :2 copy	GCTCAATGGTCTGCTTGGAG
pBUA2.2/4CL/VvVST-R :2 copy	GAAAGGTAATTCGGGGACGG
pZUA2.3/FjTAL/4CL/VvVST-R :2 copy	GAAAGGTAATTCGGGGACGG

Table S2 List of plasmids used in the study

Plasmids	**Genotype**
pYTK001/FjTAL	FjTAL
pYTK001/4CL1	4CL1
pYTK001/VvVST	VvVST
pYTK001/PAL	PAL
pYTK001/C4H	C4H
pZUS1.1/FjTAL	pTEF-FjTAL, U^+/L^-
pZUS1.2/4CL1	pTEF-4CL1, U^+/L^-
pZUS1.3/VvVST	pTEF-VvVST, U^+/L^-
pZUS1.1/4CL1	pTEF-4CL1, U^+/L^-
pZUS1.2/VvVST	pTEF-VvVST, U^+/L^-
pBUS1.1/PAL	pTEF-PAL, U^+/L^-
pBUS1.2/C4H	pTEF-C4H, U^+/L^-
pBUA2.2/4CL1/VvVST	pTEF-4CL1, VvVST, U^+/L^-
pZLA2.2/PAL/C4H	pTEF-PAL, C4H, U^+/L^-
pZUA2.3/FjTAL/4CL1/VvVST	pTEF-FjTAL, 4CL1, VvVST, U^+/L^-

Table S3 *Y. lipolytica* strains constructed for resveratrol production

	Strain	Parent strain	Genotype
Tyrosine pathway	T	Pold	p*TEF-FjTAL*, p*TEF-4CL1*, p*TEF-VvVST*, Ura^+, Leu^-
Phenylalanine pathway	P	Pold	p*TEF-PAL*, p*TEF-C4H*, p*TEF-4CL1*, p*TEF-VvVST*, Ura^+, Leu^+
Double copy	T2	Po1d	p*TEF-FjTALx2*, p*TEF-4CL1x2*, p*TEF-VvVSTx2*, Ura^-, Leu^+
	P2	Po1d	p*TEF-PALx2*, p*TEF-C4H x2*, p*TEF-4CL1x2*, p*TEF-VvVSTx2*, Ura^+, Leu^+
	T2P2	Po1d	p*TEF-FjTALx2*, p*TEF-PALx2*, p*TEF-C4H x2*, p*TEF-4CL1x2*, p*TEF-VvVSTx2*, Ura^+, Leu^+

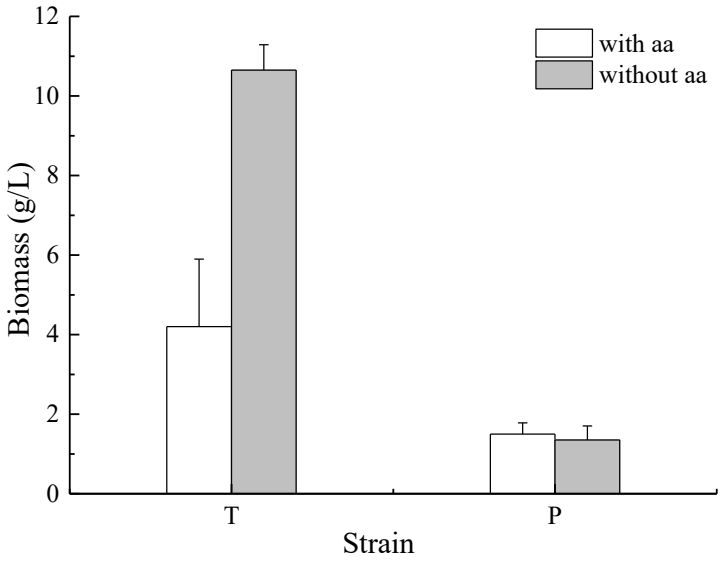

Fig. S1 Biomass of strain T and P using glucose media with and without addition of amino acids

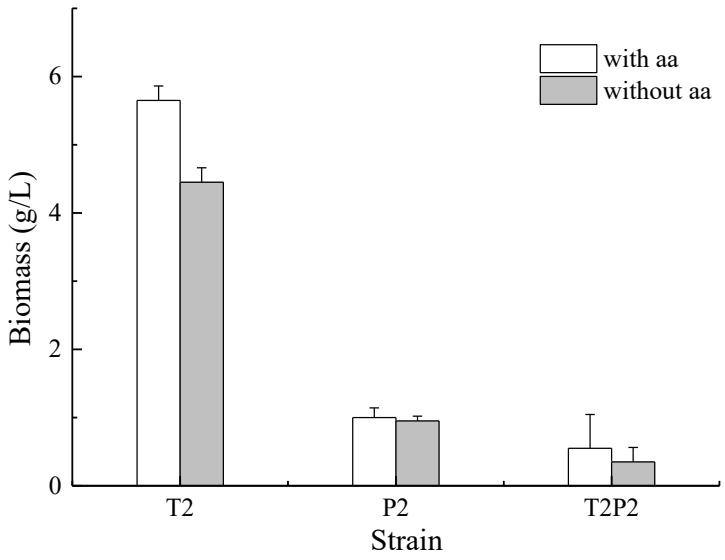

Fig. S2 Biomass of strain T2, P2 and T2P2 using glucose media with and without addition of amino acids

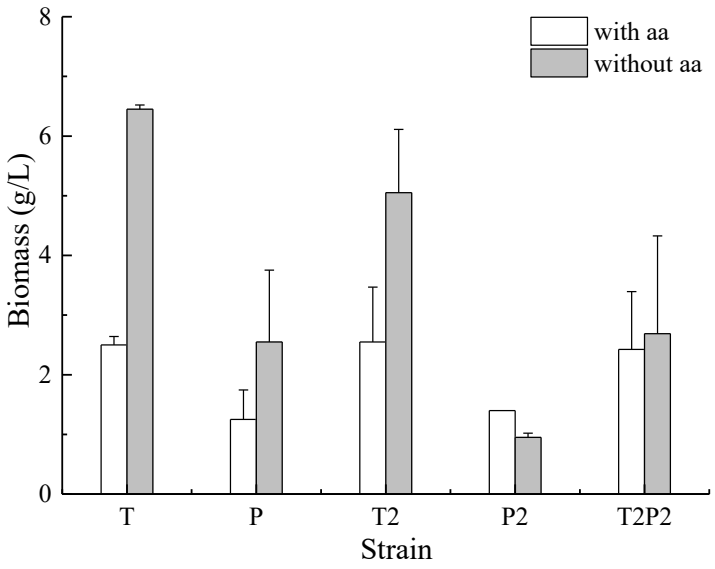

Fig. S3 Biomass of recombinants using glycerol

with and without addition of amino acids

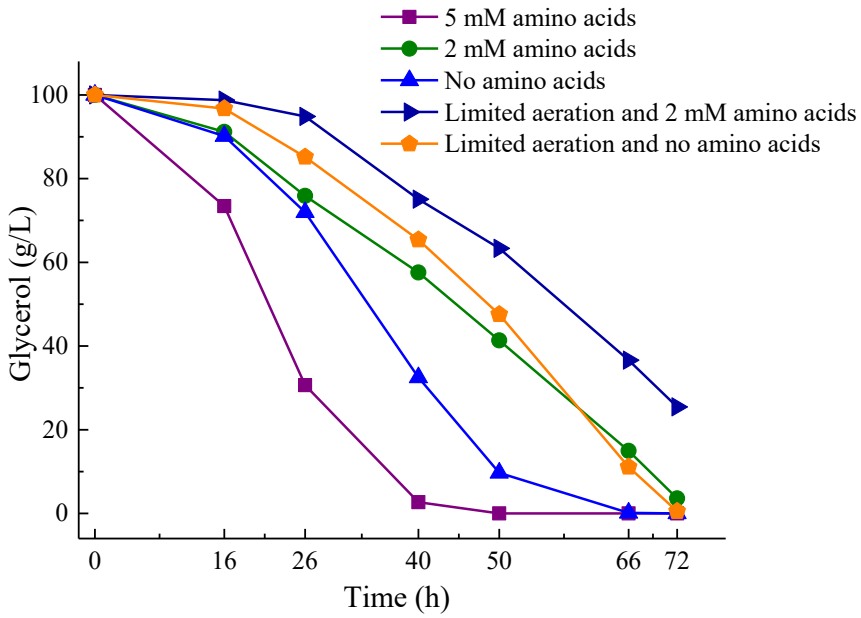

Fig. S4 Glycerol consumption under different fermentation conditions in bioreactor

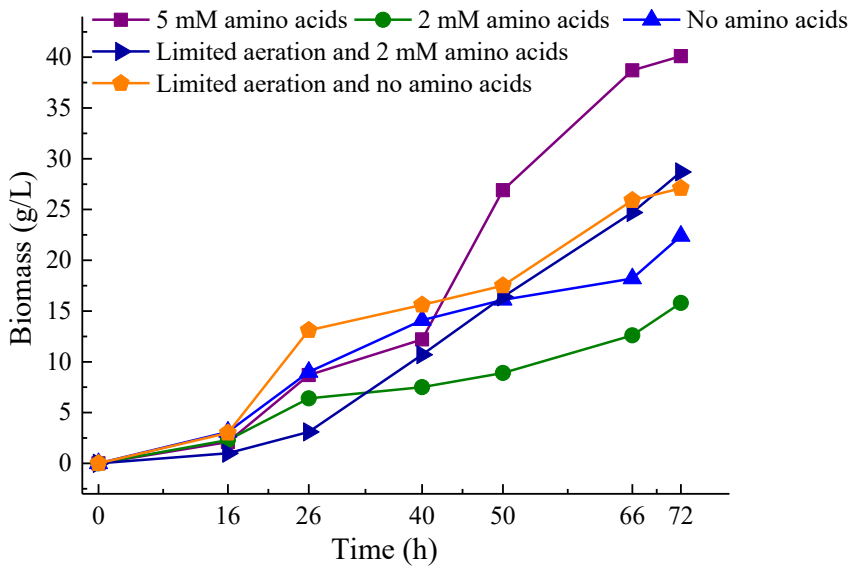

Fig. S5 Biomass production under different fermentation conditions in bioreactor

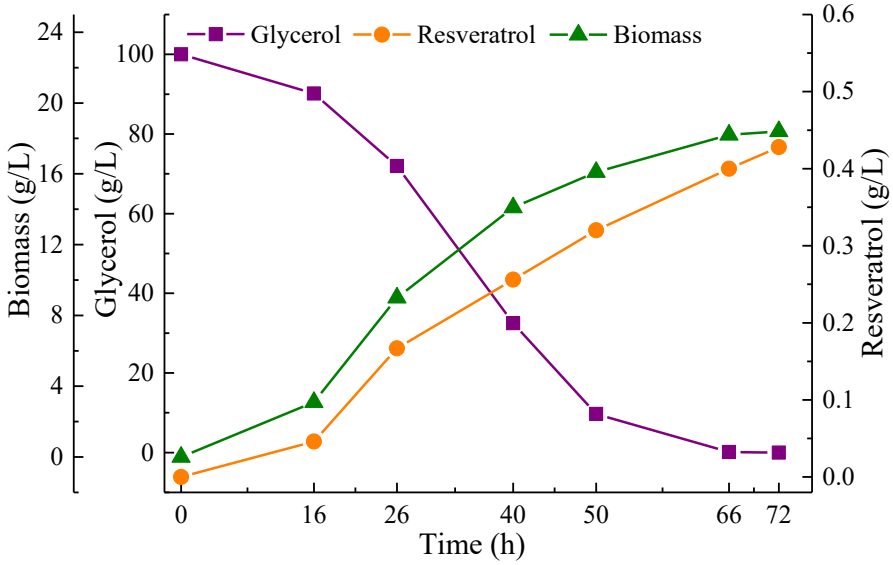

Fig. S6 Biomass, glycerol consumption and resveratrol production by strain T2P2 using glycerol as a substrate in bioreactor under condition 3 (without amino acids added and normal aeration)

3.4. Publikacja 4

Ostatnim artykułem stanowiącym cykl prac składających się na rozprawę doktorską są studia nad badaniem właściwości przeciwnowotworowych i antyoksydacyjnych lipidowych pochodnych RES na wybranych ludzkich liniach nowotworowych. Inspiracją leżącą u podstaw tej pracy był problem z biodostępnością RES, który został szczegółowo opisany we wstępie (rozdział 1.6). Zdecydowano się przeprowadzić lipofilizację RES z wykorzystaniem dwóch najczęściej występujących kwasów tłuszczowych w drożdżach *Y. lipolytica*: kwasem palmitynowym (PA) i kwasem oleinowym (OA). Ponadto, do badań wybrano również sprzężony kwas linolowy (CLA), który charakteryzuje się udowodnionymi właściwościami prozdrowotnymi, a którego produkcja w drożdżach *Y. lipolytica* stanowi przedmiot wielu badań. Ponadto, dotychczas uzyskiwane lipiowe pochodne RES analizowane były jedynie w kierunku ich właściwości antyoksydacyjnych, podczas gdy w niniejszej pracy skupiono się również na ocenie ich właściwości przeciwnowotworowych.

Badania rozpoczęto od przeprowadzenia procesu estryfikacji RES z wykorzystaniem trzech wspomnianych kwasów tłuszczowych: PA, OA oraz CLA (**Rysunek 1**). Początkowo zakładano zwiększenie biodostępności RES poprzez jego sprzęganie z PC, jednakże w trakcie badań okazało się, że sam proces opracowania odpowiedniej metodyki do wykonania tej reakcji jest zbyt długotrwały i nie przynosił pożądanych rezultatów. Stąd do nadanie lipidowego charakteru cząsteczce RES wybrano wspomniane kwasy tłuszczowe. Otrzymane mono-, di- i tri-estry RES oceniano pod kątem ich właściwości przeciwnowotworowych i antyoksydacyjnych względem linii komórkowych raka płuc (A549), gruczolakoraka jelita grubego (HT29), gruczolakoraka przewodowego trzustki (BxPC3) oraz fibroblastów (BJ) jako linii kontrolnej. Linie komórkowe użyte w tym badaniu zostały wybrane ze względu na dużą częstotliwość występowania tych nowotworów, ich dużą oporność na chemioterapię i trudność w leczeniu. Zbadanych zostało kilka parametrów: żywotność komórek i apoptozę, w tym ekspresję głównych markerów pro- i antyapoptotycznych, a także ekspresję dysmutazy ponadtlenkowej, kluczowego enzymu bariery antyoksydacyjnej organizmu.

Uzyskane wyniki pozwoliły wyróżnić trzy estry: mono-RES-OA, tri-RES-PA i mono-RES-CLA, które wykazywały największą redukcję żywotności komórek nowotworowych

(**Rysunek 2**). Mono-RES-OA działał na wszystkie linie nowotworowe lepiej niż czysty RES, powodując zahamowanie żywotności komórek począwszy już od najniższej dawki 5 µg/ml. To samo zaobserwowano dla tri-RES-PA w przypadku komórek HT29 oraz dla mono-RES-CLA w przypadku linii komórkowej A549. Ponadto, większość z tych związków nie hamowała żywotności ludzkich prawidłowych komórek BJ. Jedynym wyjątkiem był mono-RES-OA, który wykazywał silne obniżenie ich linii BJ jednakże dopiero w stężeniu wyższym niż 25 µg/ml. Pozostałe uzyskane lipidowe pochodne RES nie miały istotnego wpływu na żywotność komórek nowotworowych.

W kolejnych badaniach, wyróżnione estry RES podobnie indukowały apoptozę komórek nowotworowych, poprzez modyfikację aktywności kaspaz szlaków proapoptotycznych (*p21*, *p53* i *Bax*) (**Rysunek 3 i 4**). Co więcej, spośród wymienionych estrów, mono-RES-OA najsilniej indukował apoptozę badanych linii nowotworowych, zmniejszając liczbę żywych komórek nawet do 48% dla linii HT29. Nie odnotowano żadnego wpływu na apoptozę w przypadku zdrowej linii BJ, co potwierdza, że testowane związki w użytych stężeniach nie są toksyczne dla komórek zdrowych.

Następnie, przeprowadzono wstępną ocenę aktywności antyoksydacyjnej uzyskanych estrów, którą mierzono poprzez poziom ekspresji dysmutaz ponadtlenkowych (*SOD1* i *SOD2*), będących ważnym wskaźnikiem wewnątrzkomórkowej siły antyoksydacyjnej (**Rysunek 5**). Wyniki wykazały, że wszystkie związki powodowały znaczący wzrost poziomu ekspresji genów *SOD1* i *SOD2* na zdrowych komórkach BJ, potwierdzając właściwości antyoksydacyjne badanych związków. Wynik ten potwierdza inne badania, w których badano właściwości antyoksydacyjne zestryfikowanego RES po dodaniu do mięsa mielonego [115]. W przypadku komórek nowotworowych, estry RES nie miały wpływu lub miały niewielki wpływ na ekspresję genów *SOD*. Zwiększoną ekspresję tych genów autorzy zauważyli jedynie po potraktowaniu ich czystym RES. Komórki nowotworowe zawierają wyższe poziomy reaktywnych form tlenu (ROS) w porównaniu z komórkami prawidłowymi, głównie z powodu ich przyspieszonego metabolizmu wymaganego do utrzymania wysokiego tempa proliferacji. Ze względu na powstawanie nadmiernych ilości ROS, charakteryzujących procesy nowotworowe, większość komórek nowotworowych wykazuje zwiększoną nadekspresję SOD. Ponadto, istnieje wiele badań donoszących, że RES i jego pochodne, jak również same kwasy tłuszczowe, powodują zwiększoną akumulację ROS w komórkach

nowotworowych [124,125]. Uzyskane w niniejszej pracy wyniki wskazały, że estry RES nie miały wpływu na ekspresję głównych genów pro-antyoksydacyjnych w komórkach nowotworowych, zmniejszając szanse obrony tych komórek przed zwiększonym stresem oksydacyjnym wywołanym wysoką akumulacją ROS. Z kolei podwyższoną ekspresję SOD wywołaną traktowaniem czystym RES można tłumaczyć faktem, że związek ten działa negatywnie na komórki nowotworowe. Komórki chroniąc się, nadekspresjonują geny SOD (zaburzenie homeostazy), jednak poziom ekspresji nie jest wystarczający, aby uchronić je przed apoptozą.

Uzyskane wyniki wykazały, że estry RES z długołańcuchowymi kwasami tłuszczowymi tworzą związki o zwiększonej aktywności biologicznej. Pochodne RES mają potencjał do ich zastosowania w profilaktyce i leczeniu nowotworów, a także w supresji stresu oksydacyjnego.

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Studies on the Anticancer and Antioxidant Activities of Resveratrol and Long-Chain Fatty Acid Esters

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Abstract: Resveratrol (RES) is gaining recognition as a natural bioactive compound. To expand the possible applications of RES with its enhanced bioactivity as well as to increase the health benefits of long-chain fatty acids, a lipophilization process of RES was performed using three fatty acids: palmitic acid (PA), oleic acid (OA), and conjugated linoleic acid (CLA). The obtained mono-, di-, and tri-esters of RES were evaluated for their anticancer and antioxidant properties against lung carcinoma (A549), colorectal adenocarcinoma (HT29), and pancreatic ductal adenocarcinoma (BxPC3) cell lines. Human fibroblast (BJ) cells were used as a control. Several parameters were investigated: cell viability and apoptosis, including the expression of major pro- and anti-apoptotic markers, as well as the expression of superoxide dismutase, a key enzyme of the body's antioxidant barrier. Three of the obtained esters: mono-RES-OA, mono-RES-CLA, and tri-RES-PA, which significantly reduced the tumor cell viability up to 23%, at concentrations 25, 10, 50 µg/mL, respectively, turned out to be particularly interesting. The above-mentioned resveratrol derivatives similarly increased the tumor cells' apoptosis by modifying their caspase activity of pro-apoptotic pathways (p21, p53, and Bax). Moreover, among the mentioned esters, mono-RES-OA induced apoptosis of the analyzed cell lines most strongly, reducing the number of viable cells up to 48% for HT29 cells versus 36% for pure RES. Furthermore, the selected esters exhibited antioxidant properties towards the normal BJ cell line by regulating the expression of major pro-antioxidant genes (superoxide dismutases—*SOD1* and *SOD2*) without the effect on their expression in the tumor, and therefore reducing the defense of cancer cells against increased oxidative stress induced by high ROS accumulation. The obtained results indicate that the esters of RES and long-chain fatty acids allow enhancement of their biological activity. The RES derivatives have the potential for being applied in cancer prevention and treatment, as well as for oxidative stress suppression.

Keywords: resveratrol esters; resveratrol; long-chain fatty acids; anticancer properties; antioxidant properties

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1. Introduction

The synthesis of resveratrol derivatives and their novel enriched properties have been a hot research topic recently. Resveratrol (RES; 3,5,4′-trihydroxystilbene) is a naturally occurring polyphenolic stilbene, found mainly in the skin of grapes; however, it can also be found in other fruits such as blueberries, cranberries, rhubarb, passion fruit, and in

their processed products [1]. RES belongs to the phytoalexin group of antibiotics and is produced by several plants in response to injury, infections, pathogens, and environmental stress [2]. The biological effects of RES are associated with human health benefits and have been extensively studied in vitro and in vivo [3]. Some of the described effects include its anti-inflammatory, antioxidant, antidiabetic, neuroprotective, cardioprotective, and anticancer properties [3]. Furthermore, a large number of preclinical studies indicate that RES is a promising drug for the prevention and treatment of cancer [4]. Nevertheless, RES, like other polyphenols, has a high oral absorption and rapid, extensive metabolism, which can be associated with poor bioavailability. As a result, only trace amounts of unchanged RES can be found in the systemic circulation, limiting its effective use in practical medicine [5]. Moreover, utilization of RES may be hindered by its hydrophilicity while being used in lipophilic systems [6]. Therefore, structural modification of RES may provide an opportunity to address the above-mentioned concerns. In fact, there are several available scientific studies on lipophilic derivatives of phenolic compounds. These studies were focused on the maximized antioxidant activities of polyphenols as well as their higher performance in biological model systems, especially via structure modification, such as those for epigallocatechin gallate (EGCG) [6] or rosmarinic acid [7]. Moreover, EGCG esterified with docosahexaenoic acid (DHA) provided a novel bioactive product being a composite of two bioactive compounds, since DHA itself possesses health benefits [8]. Likewise, RES esterified with fatty acid chlorides of different chain lengths (C3:0–C22:6) resulted in higher antioxidant activity for RC20:5n-3 (REPA) and RC22:6n-3 (RDHA) esters and better hydrogen peroxide scavenging activity (RC3:0–RC14:0) compared to pure RES [9]. The RES analogue 4,4′-dihydroxy-trans-stilbene, containing two hydroxyl groups in the *para*-positions 4 and 4′, showed higher antiproliferative activity than pure RES [10]. In mouse and zebrafish lung cancer models, RES derivatives significantly inhibited tumor volume, cell proliferation, and tumor angiogenesis and caused significantly reduced liver metastatic lesions. In similar experiments, RES with hydroxyl groups in positions 3′, 4, and 5′ showed even higher cytotoxicity in HL-60 cells compared to RES, indicating an increased antitumor activity [11]. Recent studies showed that chemically synthesized RE-butyric acid (RE-B) ester has a higher ability to decrease liver fat accumulation and antioxidant capacity than RES itself [9,12].

In the case of oral administration of ester compounds, the ester bond may be rapidly hydrolyzed. In 2007, Biasutto and coworkers reported that some quercetin esters are partially hydrolyzed, while passing through a monolayer of MDCK-1, MDCK-2, and Caco-2 cells. The hydrolysis of the ester was dependent on the type of compound which was examined [13]. According to Pokorski and colleagues (2003), ascorbyl palmitate-treated cats showed more recovery of ascorbate in their brain tissues compared to ascorbic acid-treated cats [14]. Therefore, it can be hypothesized that RES esters may exhibit beneficial effect even if hydrolyzed.

In addition, lipophilic phenolic derivatives also offer several advantages over the original phenols, including improved pharmacological profiles and bioavailability. Polyphenols prevent fatty acid oxidation, assisting their intestinal uptake, and preserve their bioactivity [15], while fatty acids influence the metabolism and bioaccessibility of polyphenols [16]. The latter, when conjugated with fatty acids (as lipophenols) show increased lipophilicity, cell penetration, as well as bioavailability of specific polar phenolic drugs, reaching appropriate solubility of hydrophobic drugs and can become tissue/tumor-specific [17,18].

Collectively, all the information gathered above suggests that lipophilic esters of RES and long-chain fatty acids may represent novel drug candidates with improved anticancer and antioxidant properties; however, their mechanism of action is not yet fully understood and requires further research. In this study, RES was esterified with selected fatty acids; palmitic acid (C16:0), oleic acid (C18:1), and conjugated linoleic acid (C18:2) and mono-, di-, and tri-esters were obtained. The synthesized lipophilic RES derivatives were then tested to identify and evaluate their effect on viability, apoptosis, and antioxidant properties on various cancer lines.

2. Results and Discussion

2.1. Preparation of Esters of Resveratrol and Fatty Acids

In the presented work, the lipophilization of RES to improve its therapeutic potential as an anticancer agent was performed. RES is known for its cancer chemo-protective activity. It acts on three major stages in carcinogenesis, namely anti-initiation, anti-promotion, and anti-progression [19]. It was hypothesized that the increase in lipophilicity of this natural stilbenoid will allow for obtaining derivatives with higher antiproliferative and antioxidant activity. Therefore, we carried out the process of lipophilization using selected fatty acids: palmitic acid (PA) (16:0), oleic acid (OA) (18:1), and conjugated (10*E*,12*Z*)-linoleic acid (CLA) (18:2). For this purpose, chemical acylation of hydroxy groups at 3, 5, and 4′ carbon atoms of RES using chlorides of PA, OA, and CLA was performed. The acyl groups reacted with different numbers of hydroxy groups located in different positions of RES depending on the nature of the electrophile and the reaction conditions. After 3 h of reaction mixtures of mono-, di-, and tri-esters of RES, palmitic or oleic acid were obtained, whereas the reaction of RES with CLA chloride resulted in only mono-*O*-(conjugated)linoleoylresveratrol (Figure 1). The obtained products were separated and purified by column chromatography and their structures were determined using spectroscopic analysis and compared to the literature data. The partial esterification of the above-mentioned groups as well as incorporation of long-chain saturated or unsaturated fatty acids into RES allowed us to examine the structure–activity relationship.

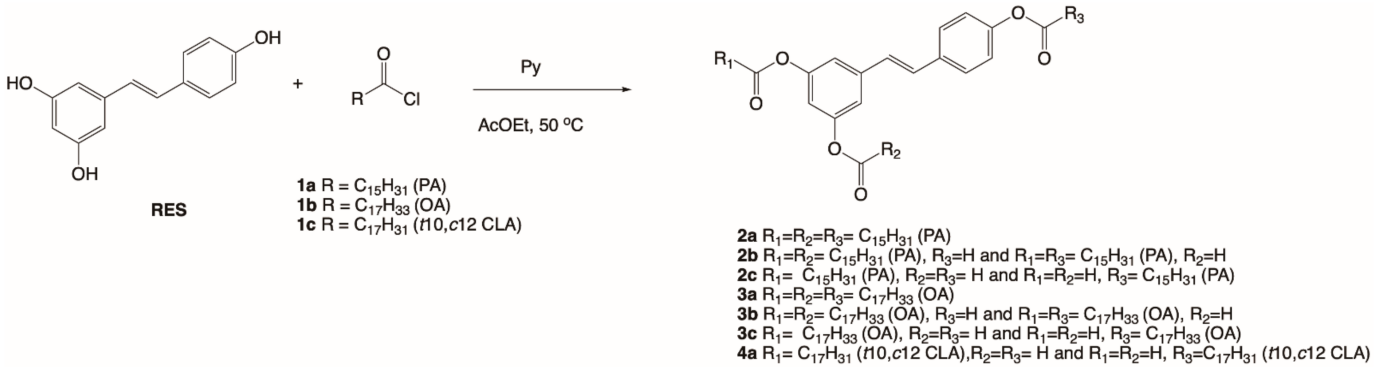

Figure 1. Scheme of chemical acylation of resveratrol.

2.2. RES Esters Inhibit Growth of Various Cancer Cell Lines

We evaluated the effect of all LCFAs-RES esters on the viability of three cancer cell lines, lung adenocarcinoma cell line A549, pancreatic ductal adenocarcinoma cell line BxPC3, and colorectal cancer cell line HT29, and normal cell line BJ as a control. We examined the effects of these compounds on the growth of cells using MTS assay. Cell lines used in these studies were chosen due to the high incidence of these cancers, their high resistance to chemotherapy, and difficulties in their treatment. The cells were treated for 48 h with a wide range of concentrations of RES derivatives ranging from 5 to 75 µg/mL for each ester. The cytotoxicity results are presented in Figure 2.

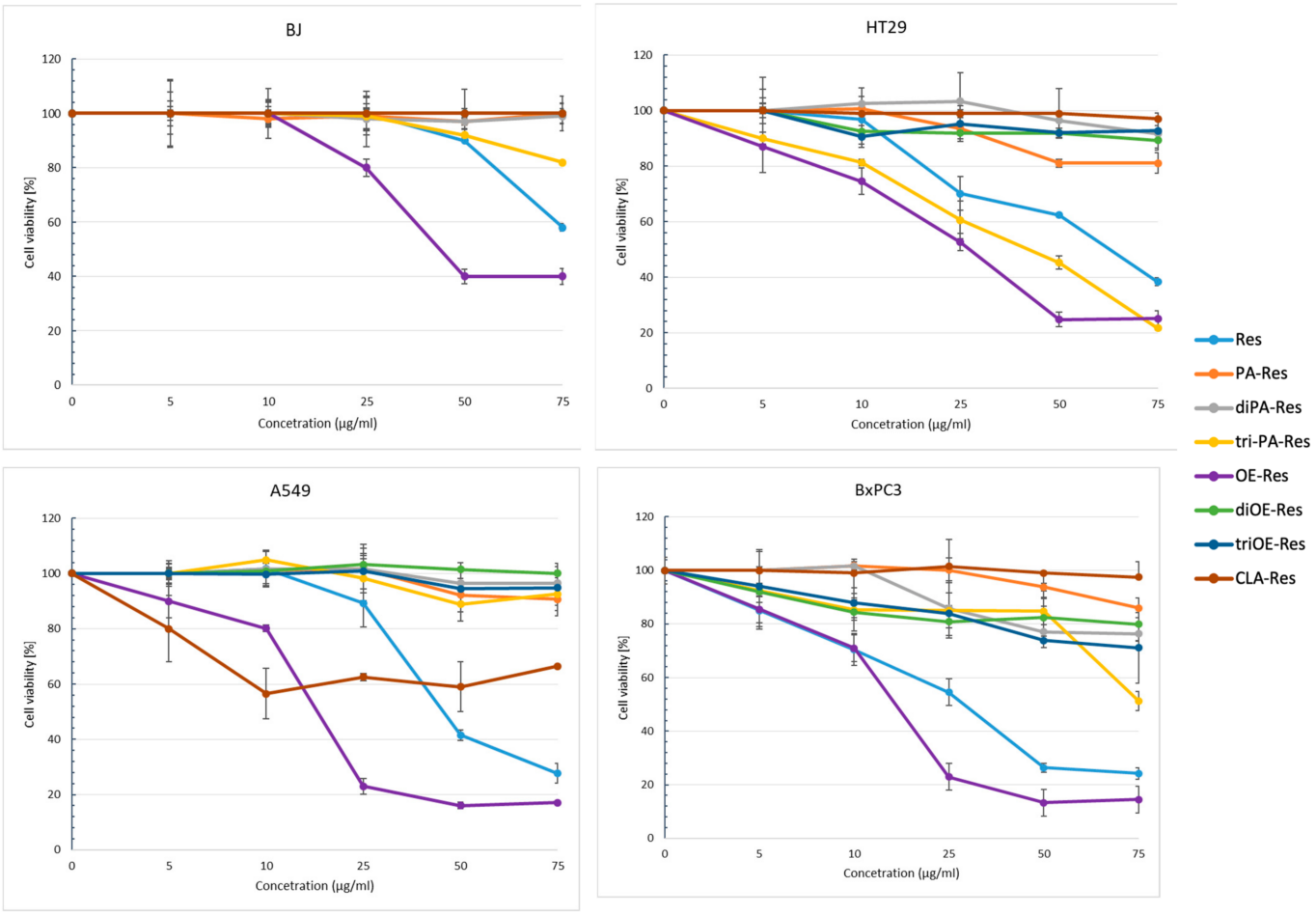

Figure 2. Effects of LCFAs-RES esters on cancer cell viability. A549, BxPC3, HT29, and BJ cells were treated with RES derivatives at different concentration (5–75 µg/mL). Cell viability was assessed by MTS assay after 48 h exposure. Results are expressed as mean ± SE of three separate experiments.

We observed mono-RES-OA (**3c**), tri-RES-PA (**2a**), and mono-RES-CLA (**4a**) esters exhibiting the highest cell viability reduction. The mono-RES-OA acted on all cancer lines better than RES, causing inhibition of cell viability starting from the lowest dose of 5 µg/mL. Similar results were observed for tri-RES-PA in the case of HT29 cells and for mono-RES-CLA acting on the A549 cell line. Moreover, most of these compounds did not inhibit the viability of the human normal BJ cells, except the mono-RES-OA, which showed a strong reduction in their viability at concentration higher than 25 µg/mL.

Significant inhibition was observed for cells treated with mono-RES-OA at a concentration of 25 µg/mL, equal to 23% for the A549 and BxPC3 cells and 53% for HT29 cells. This cell viability reduction was significantly higher compared to their treatment with RES at the same concentration, reaching 90% for A549, 55% for BxPC3, and 70% for HT29 lines, respectively. For HT29 cells, strong viability inhibition was also shown by tri-RES-PA ester reaching 25% of cell viability when cells were treated with 50 µg/mL, while the inhibition reached by the RES treatment was about 2 times lower. In turn, mono-RES-CLA ester reduced cell viability to 55% for A549 cells at a concentration of 10 µg/mL, whereas no effect on these cells treated with 10 µg/mL RES was observed. Interestingly, the rest of the esters had no significant effect on cancer cell viability.

Several studies have demonstrated anticancer activity of RES and its derivatives. Won Young Oh et al. [20] investigated the anticancer activity of RES and its mono-esters, resveratryl propionate (RC3:0) and resveratryl docosahexaenate (RDHA), using liver cancer (HepG2), colon cancer (HT-29, A431), breast cancer (MCF7), and gastric cancer (AGS) cell

lines. According to their results, RES with RC3:0 at a concentration of 50–75 μg/mL reduced cell viability of most lines below 40%; in contrast, RDHA showed the lowest cytotoxicity at all concentrations. Zhu et al. [21] investigated the antiproliferative properties of a series of RES-based aspirin prodrugs. The authors synthesized RSV–aspirin hybrid (RAH), by esterification of the 4′-phenolic hydroxyl of RES with aspirin (acetylsalicylic acid), also obtaining its regioisomer, formed by esterification of the 3-phenol hydroxyl of RES. The anticancer properties of the two compounds were evaluated in human colorectal cancer cells HCT-116 and HT-29 using the MTT assay. As a result, both compounds inhibited the growth of cancer cells in a dose-dependent manner. The observed effect was superior to that demonstrated by administration of RES and acetylsalicylic acid alone or simultaneously. Notably, RAH turned out to be more efficient than its regioisomer, suggesting that the position of esterification influences antitumor activity. Similar results were obtained in our study, confirming that the two free hydroxyl groups at C-3 and C-5 in ring A of the RES moiety play an important role for its biological activity. Moreover, the esterification of RES with OA, PA, and CLA at the C-4 position also favors the anticancer activity of the corresponding esters. More recently, Peterson et al. [22] investigated the effect of ester-derived 4′-RSV on calcium dynamics in triple-negative breast cancer (TNBC), a highly aggressive subtype of breast cancer. Compounds were obtained by esterification of the 4′-hydroxy function of RSV with a pivalate, isobutyrate, or butyrate group, respectively. These compounds were investigated for their ability to reduce cell viability in MDA-MB-231 cancer cell lines using the MTT assay. As a result, both compounds, RSV 4′-pivalate and RSV 4′-isobutyrate, were more active than RES, reducing the cell viability to 14.14% and 7.70% vs. 58.45%, respectively.

2.3. Apoptosis

In the next step, the apoptosis level in A549, BxPC3, HT29, and BJ cell lines was analyzed (Figure 3) to verify if the inhibition of cell growth demonstrated in the MTS assay was related to apoptosis. The induction of apoptosis was investigated using Annexin V-FITC/PI double staining as well as by measuring gene expression of apoptosis master regulators (Figure 4). All tested cell lines were treated with different concentrations of every RES ester (RES, mono-RES-OA—10 μg/mL, tri-RES-PA—50 μg/mL, and mono-RES-CLA—25 μg/mL). The most appropriate concentrations were chosen after the MTS test, where the significant effect on cell viability was noted.

For the BJ line, no effect on apoptosis was noted after treatment with RES esters, which is consistent with the results obtained in the MTS test. These results proved that RES derivatives at the analyzed concentrations have no impact on the normal cell line. In contrast, HT29 and BxPC3 cancer cells were prone to highly increased apoptosis after treatment with RES esters, as evidenced by the decrease in the number of living cells and the consequent increase in the number of apoptotic cells (Figure 3). As in the MTS test, the mono-RES-OA most significantly induced apoptosis in both cell lines, reducing the number of live cells by 48% and 31%, respectively. This result confirms the stronger cytotoxic effect of this ester against the investigated cancer cell lines compared to pure RES (36% in HT29, 27% in BxPC3). The apoptotic effect in both lines was also visible in samples treated with tri-RES-PA, where the number of live cells was reduced to 61.3% for HT29 and to 70.4% for BxPC3, and for cells treated with mono-RES-CLA to 70.4% (HT29) and 73.6% (BxPC3), respectively. The A549 cell line was the most resistant, where we expected increased apoptosis when cells were treated with the mono-RES-CLA; however, only a slight decrease (less than 10%) in the number of live cells was recorded. A similar effect was shown for mono-RES-OA.

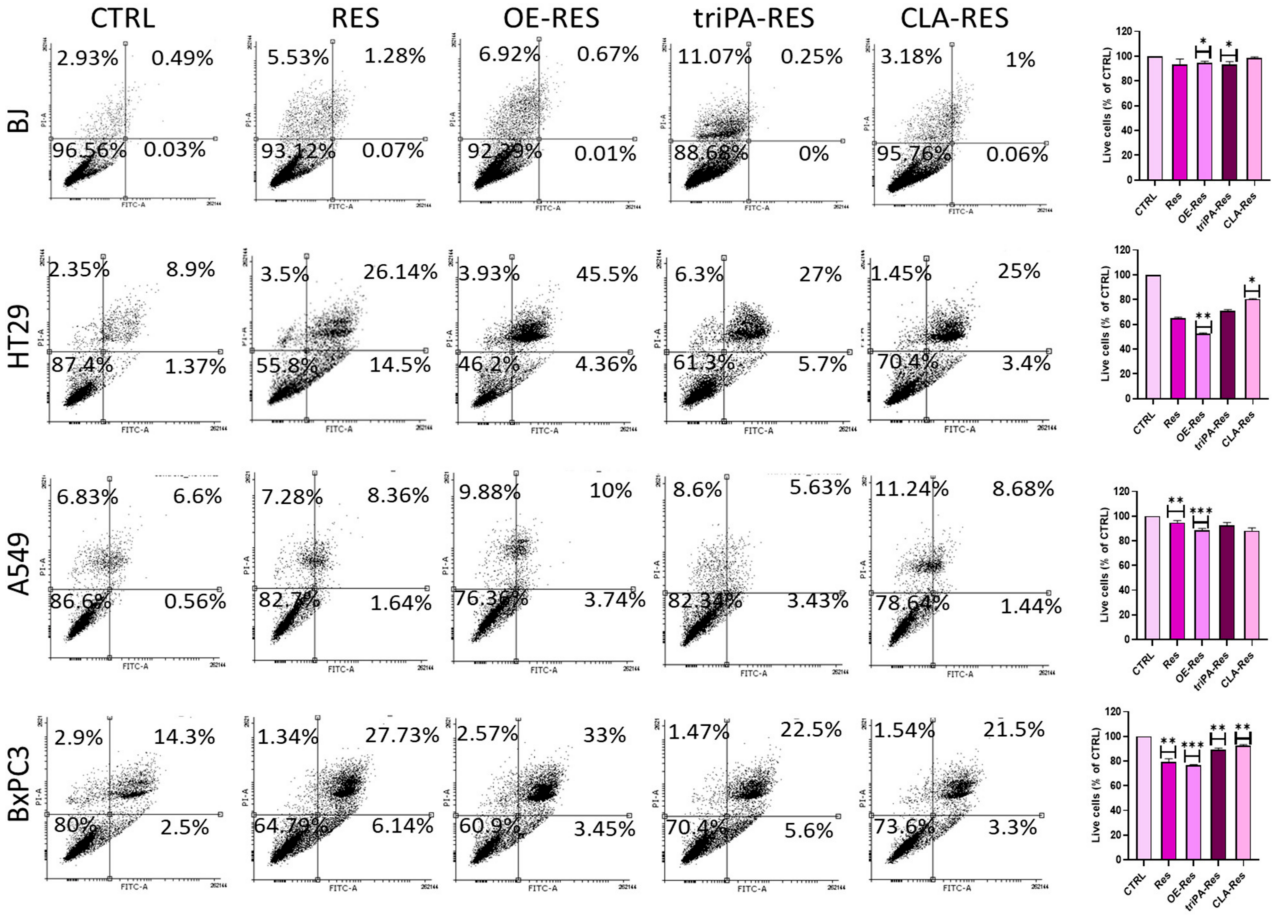

Figure 3. Induction of apoptosis in human cancer cells, HT29, A549, and BxPC3, after treatment with RES derivatives. Normal BJ cells were used as a control line. Representative FACS dot plots showing the effect of treatment with RES and mono-RES-OA (10 µg/mL) tri-RES-PA (50 µg/mL) and mono-RES-CLA (25 µg/mL) on phosphatidylserine exposure and plasma membrane integrity after 48 h of incubation with the cells, as determined by annexin V-FITC/PI staining. A comparison of treatment groups and untreated cells is shown by an asterisk (*). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Similarly, the relative expression of pro- and anti-apoptotic markers was assessed at the mRNA level using the RT-qPCR approach. The obtained data showed that treated HT29 and BxPC3 cancer cells were characterized by a significant upregulation of key pro-apoptotic factors, including *p21*, *p53*, and *Bax*, compared to the untreated cells (Figure 4). In contrast, the apoptosis inhibitor *BCL-2* showed no significant difference in expression in either HT29 or BxPC3 cells. Surprisingly, increased expression of pro-apoptotic genes was also observed in the A549 cells, especially during mono-RES-CLA treatment, which was accompanied by downregulation of the *BCL-2* gene. In the normal BJ cell line, the expression of investigated genes showed no significant changes.

The results suggest that the mono-RES-OA ester showed the strongest cytotoxic activity against the investigated tumor cell lines. Treatment of HT29 and BxPC3 cells with mono-RES-OA enabled a significant increase in apoptosis by enhancing the overexpression of *p21*, *p53*, and *Bax*, suggesting that mono-RES-OA has a very strong pro-apoptotic effect without simultaneously affecting human normal cells.

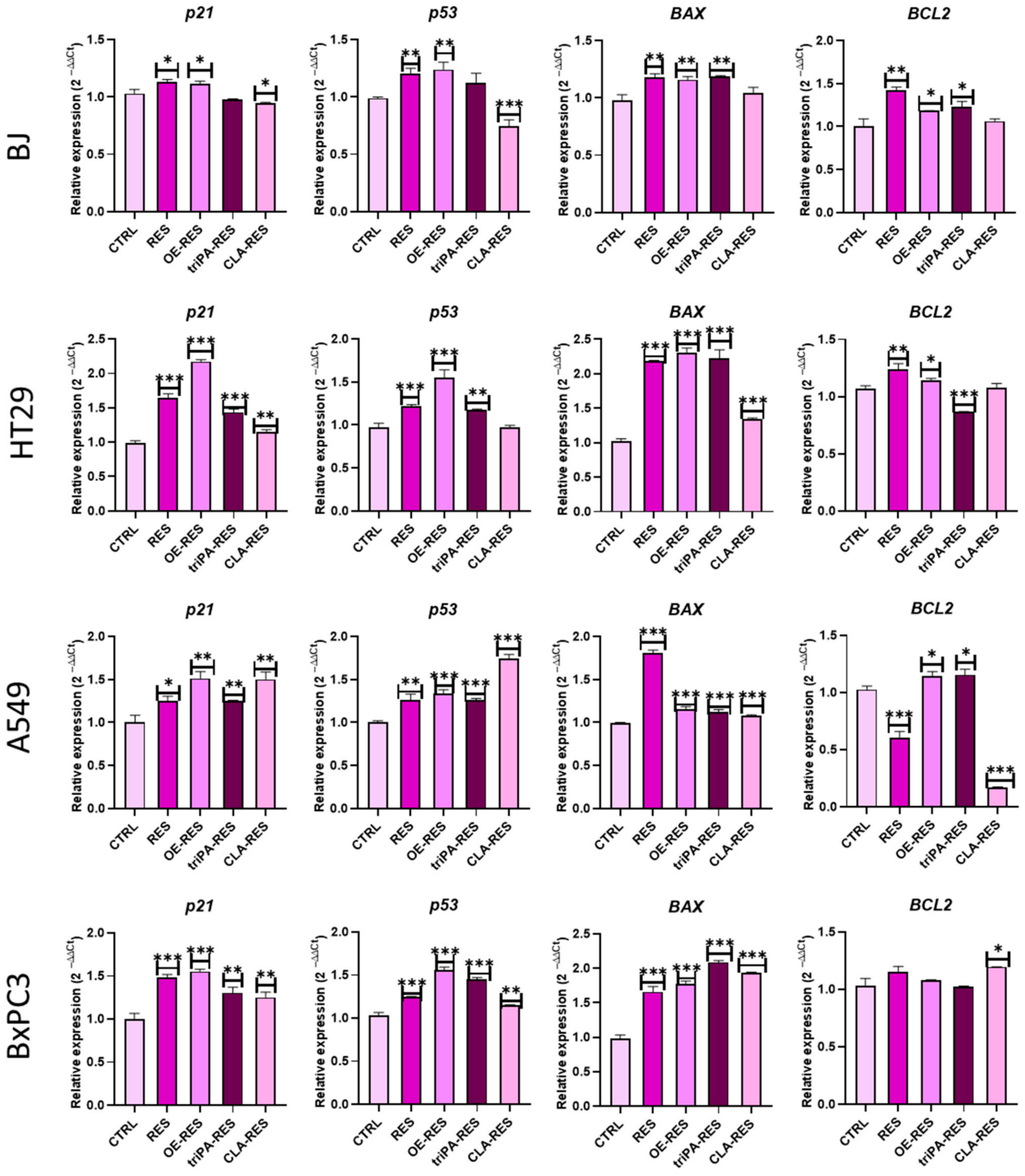

Figure 4. Expression of pro- and anti-apoptotic genes after treatment with RES esters in normal and cancer cells. Bar charts illustrating the relative expression of major apoptotic markers: *p21*, *p53*, *BAX*, and *BCL2* genes. A comparison of treatment groups and untreated cells is shown by an asterisk (*). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.4. Antioxidant Activity

The activity of superoxide dismutase (*SOD*) is an important indicator of intracellular antioxidant power, which converts superoxide anions into H_2O_2 and O_2 [23]. The most common *SOD* isomerases are the cytoplasm-localized copper–zinc SOD1 (CuZnSOD) and the mitochondria-localized manganese SOD2 (MnSOD). In this study, the preliminary

assessment of the antioxidant activity measured by the expression of the *SOD1* and *SOD2* genes was carried out using RT-qPCR (Figure 5). The results showed that all of the compounds caused a significant increase in the expression level of *SOD1* and *SOD2* genes on the healthy BJ cells, confirming the antioxidant properties of the tested compounds. This result is consistent with previous studies, where the properties of the esterified RES in food were investigated [9]. According to that study, RES derivatives showed better antioxidant activity compared to RES alone in a bulk oil system. The RES esters RC20:5n-3 and RC22:6n-3 showed the highest antioxidant activity when added to ground meat. Moreover, they inhibited copper-induced LDL oxidation and hydroxyl radical-induced DNA breakdown. In another study by Ming-Kuei Shih and colleagues [24], authors reported higher oxidative capacity of RES derivatives as well as indicated that their activity is related to the number and position of butyrate esterification sites. In our study, in the case of cancer cells, RES esters had either no or a slight effect on the expression of *SOD* genes. A significant dysregulation of the *SOD1* gene expression was noted on the A549 line with mono-RES-OA treatment and of the *SOD2* gene on the BxPC3 line with tri-RES-PA treatment. In contrast, tumor cells treated with RES showed increased expression of both genes.

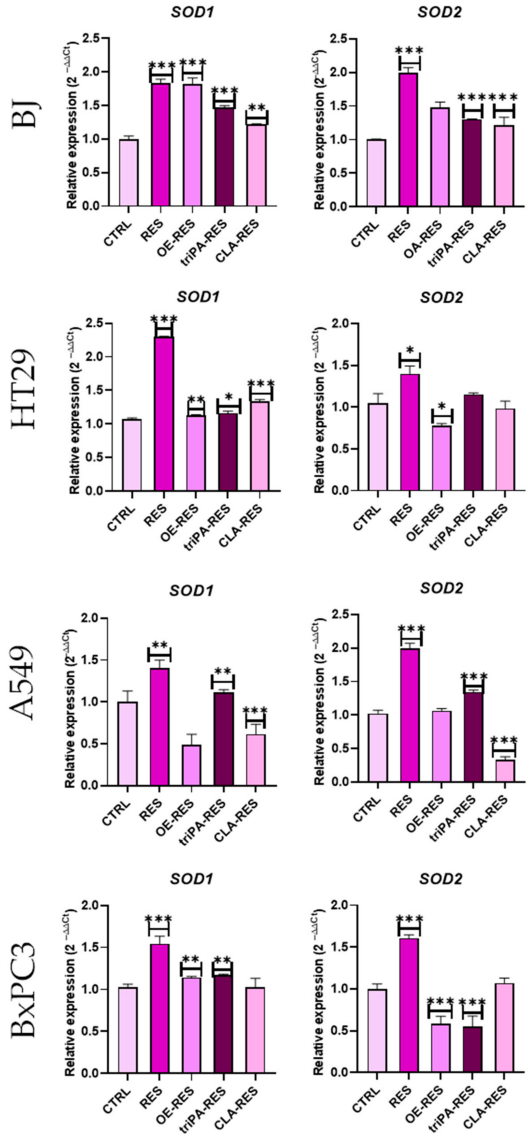

Figure 5. Effect of RES esters on oxidative stress in the normal and cancer cells. Relative gene expression of *SOD1* and *SOD2* transcripts. A comparison of treatment groups and untreated cells is shown by an asterisk (*). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The activity of *SOD1* and *SOD2* genes in cancer cells often depends on the stage of the tumor, as well as its cellular location [25]. Tumor cells contain higher levels of reactive oxygen species (ROS) compared to normal cells, mostly due to their accelerated metabolism, which is required to maintain their high proliferation rate. Due to the formation of excessive amounts of ROS characteristic of tumorigenic processes, most cancer cells show increased overexpression of *SOD*. Furthermore, there are many studies reporting that RES and its derivatives, as well as fatty acids themselves, cause increased ROS accumulation in cancer cells [15,24,26–28].

The obtained results show that RES esters had no effect on the expression of the main pro-antioxidant genes in cancer cells, decreasing the chances of defense of these cells against the increased oxidative stress induced by high ROS accumulation. In contrast, the elevated SOD expression caused by treatment with pure RES can be explained by the fact that the compound has a negative effect on the cells. Cells, protecting themselves, overexpress SOD (disruption of homeostasis) genes; however, the expression level is not enough to prevent apoptosis.

3. Materials and Methods

3.1. Materials

Substrates and solvents were of reagent grade and were used without further purification. Resveratrol was a gift from Hansen Supplements. Conjugated (10*E*,12*Z*)-linoleic acid (CLA), acyl chlorides (palmitoyl chloride and oleoyl chloride), pyridine, propidium iodide (PI), penicillin, streptomycin, and neomycin were obtained from Sigma-Aldrich (St. Louis, MO, USA). All organic solvents were purchased from Merck. RPMI 1640, DMEM, and Fetal Bovine Serum (FBS) were obtained from Thermofisher Scientific (Grand Island, NY, USA).

3.2. Methods of Analysis

Thin layer chromatography (TLC) analysis was carried out on a 0.2 mm pre-coated aluminum sheet 60 F_{254} plate (Merck Ltd., Darmstadt, Germany) with the mixture of hexane/ethyl acetate/formic acid (80:20:2, $v/v/v$). The compounds were detected using a solution of 10 g of $Ce(SO_4)_2$ and 20 g of phosphomolibdenic acid in 1 L of 10% H_2SO_4 followed by heating. The products were purified using column chromatography (CC) on silica gel (230–400 mesh). The solvent ratio was gradients of hexane/ether (2:1 next 9:1), hexane/ethyl acetate/formic acid (30:1:1.2 then 30:20:1). Each fraction was collected and monitored by TLC. Each pure product was procured following solvent removal. Spectroscopic analysis in the range of Nuclear Magnetic Resonance (^{1}H NMR, ^{13}C NMR, COSY, HSQC) was recorded on a Bruker Advance II 600 MHz spectrometer (Bruker, Rheinstetten, Germany). Samples were dissolved in $CDCl_3$ and the chemical shifts of detected signals were referenced to the signals of residual solvent (δH = 7.26, δC = 77.00).

3.3. Synthesis of CLA Chloride

Conjugated linoleic acid (400 mg, 1.43 mmol) was dissolved in anhydrous methylene chloride solution (10 mL) and oxalyl chloride (1.23 mL, 14.1 mmol) was added dropwise to the mixture. Next the reaction mixture was stirred at room temperature. After 1 h the reaction was stopped. Excess oxalyl chloride and the solvent were evaporated under reduced pressure to make the product, which was next used for synthesis without purification.

3.4. Synthesis of Lipid Derivatives of Resveratrol (2a-c-3a)

Resveratrol (200 mg, 0.876 mmol) was dissolved in ethyl acetate (20 mL) and then pyridine (300 µL, 3.702 mmol) was added. Esterification of resveratrol was carried out with acyl chlorides (PA-Cl, OA-Cl, CLA-Cl) at a mole ratio of 1:1. They were added dropwise to a solution of resveratrol in ethyl acetate. The reaction proceeded for 3 h under reflux and a blanket of nitrogen at 50 °C. Upon completion of the reaction, the mixture was allowed to stand until it reached room temperature and was washed 3 times with distilled water (60 °C). The ethyl acetate layer was then passed through a layer of anhydrous sodium

sulfate, followed by the removal of the solvent. Crude products were purified by column chromatography on silica gel, as described in Section 2. The yields and the spectroscopic data of the products are given below and were confirmed by comparing the chemical shifts of resveratrol and its derivatives as well as with the literature data [9].

3.4.1. Tri-O-palmitoylresveratrol (tri-RES-PA) (**2a**)

Colourless solid (60 mg, 9% yield, R_f = 0.9);

^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$ 2:1 (*v*/*v*), δ: 0.81 (t, *J* = 6.7 Hz, 9H, $CH_3(CH_2)_{13}CH_2C(O)$), 1.19–132 (m, 72H, $CH_3(CH_2)_{12}CH_2CH_2C(O)$), 1.68 (m, 6H, $CH_3(CH_2)_{12}CH_2CH_2C(O)$), 2.48 (t, *J* = 7.5 Hz, $CH_3(CH_2)_{12}CH_2CH_2C(O)$), 6.73 (m, 1H, H-4), 6.90 (d, *J* = 16.0 Hz, 1H, H-7), 6.98 (d, *J* = 16.0 Hz, 1H, H-8), 7.01 (m, 2H, H-3′and H-5′), 7.03 (m, 2H, H-2 and H-6), 7.30 (m, 2H, H-2′ and H-6′).

3.4.2. Di-O-palmitoylresveratrol (di-RES-PA) (**2b**)

Colourless solid (148 mg, 28% yield, R_f = 0.5);

^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$ 2:1 (*v*/*v*), δ: 0.81 (t, *J* = 7.0 Hz, 6H, $CH_3(CH_2)_{13}CH_2C(O)$), 1.19–1.32 (m, 48H, $CH_3(CH_2)_{12}CH_2CH_2C(O)$), 1.56 (m, 4H, $CH_3(CH_2)_{12}CH_2CH_2C(O)$), 2.28 (t, *J* = 7.5 Hz, $CH_3(CH_2)_{12}CH_2CH_2C(O)$), 6.53 (m, 1H, H-4), 6.61 (d, *J* = 16.0 Hz, 1H, H-7), 6.87 (d, *J* = 16.0 Hz, 1H, H-8), 6.61–6.87 (m, 4H, H-3′, H-5′, H-2 and H-6), 7.15–7.29 (m, 2H, H-2′ and H-6′).

3.4.3. Mono-O-palmitoylresveratrol (mono-RES-PA) (**2c**)

(132 mg, 37% yield, R_f = 0.2);

^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$ 2:1 (*v*/*v*), δ: 0.76 (t, *J* = 6.8 Hz, 3H, $CH_3(CH_2)_{13}CH_2C(O)$), 1.15–1.31 (m, 24H, $CH_3(CH_2)_{12}CH_2CH_2C(O)$), 1.64 (m, 2H, $CH_3(CH_2)_{12}CH_2CH_2C(O)$), 1.93 (t, *J* = 6.8 Hz, $CH_3(CH_2)_{12}CH_2CH_2C(O)$), 6.31 (m, 1H, H-4), 6.81 (d, *J* = 16.2 Hz, 1H, H-7), 6.87 (d, *J* = 16.2 Hz, 1H, H-8), 6.69-6.98 (m, 4H, H-3′, H-5′, H-2 and H-6), 7.23–7.39 (m, 2H, H-2′ and H-6′).

3.4.4. Tri-O-oleoylresveratrol (tri-RES-OA) (**3a**)

(32 mg, 3.6% yield, R_f = 0.88);

^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$ 2:1 (*v*/*v*), δ: 0.88 (t, *J* = 7.0 Hz, 9H, $CH_3(CH_2)_7CHCH\ (CH_2)_7C(O)$), 1.26 (m, 60H, $CH_3(CH_2)_7CHCH\ (CH_2)_7C(O)$), 1.74–1.76 (m, 6H, CH_2-3″), 2.01–2.03 (m, 12H, CH_2-8″ and CH_2-11″), 2.55 (t, *J* = 7.5 Hz, 6H, CH_2-2″), 5.35–5.36 (m, 2H, H-9″ and H-10″), 6.80 (m, 1H, H-4), 6.97 (d, *J* = 16.0 Hz, 1H, H-7), 7.05 (d, *J* = 16.0 Hz, 1H, H-8), 7.07 (m, 2H, H-3′and H-5′), 7.10 (m, 2H, H-2 and H-6), 7.48 (m, 2H, H-2′ and H-6′).

3.4.5. Di-O-oleoylresveratrol (di-RES-OA) (**3b**)

(188 mg, 28% yield, R_f = 0.5);

^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$ 2:1 (*v*/*v*), δ: 0.88 (t, *J* = 6.8 Hz, 6H, $CH_3(CH_2)_7CHCH\ (CH_2)_7C(O)$), 1.27 (m, 40H, $CH_3(CH_2)_7CHCH\ (CH_2)_7C(O)$), 1.61–1.65 (m, 4H, CH_2-3″), 2.00–2.04 (m, 8H, CH_2-8″ and CH_2-11″), 2.35 (t, *J* = 7.5 Hz, 4H, CH_2-2″), 5.34–5.36 (m, 4H, H-9″ and H-10″), 6.49 (m, 1H, H-4), 6.79–6.80 (m, 2H, H-3′and H-5′), 6.84 (d, *J* = 16.0 Hz, 1H, H-7), 6.90 (d, *J* = 16.0 Hz, 1H, H-8), 6.98–7.07 (m, 2H, H-2 and H-6), 7.35 and 7.46 (2m, 2H, H-2′ and H-6′).

3.4.6. Mono-O-oleoylresveratrol (mono-RES-OA) (**3c**)

(224 mg, 52% yield, R_f = 0.26);

^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$ 2:1 (*v*/*v*), δ: 0.88 (t, *J* = 7.0 Hz, 3H, $CH_3(CH_2)_7CHCH\ (CH_2)_7C(O)$), 1.27 (m, 20H, $CH_3(CH_2)_7CHCH\ (CH_2)_7C(O)$), 1.74–1.77 (m, 2H, CH_2-3″), 2.01–2.03 (m, 4H, CH_2-8″ and CH_2-11″), 2.55 (t, *J* = 7.6 Hz, 2H, CH_2-2″), 5.35–5.36 (m, 2H, H-9″ and H-10″), 6.46 (m, 1H, H-4), 6.77 (d, *J* = 16.0 Hz, 1H, H-7), 6.92 (d,

J = 16.0 Hz, 1H, H-8), 6.74–6.78 (m, 2H, H-3′and H-5′), 7.03–7.21 (m, 2H, H-2 and H-6), 7.30 and 7.43 (2m, 2H, H-2′ and H-6′).

3.4.7. Mono-O-(conjugated)linoleoylresveratrol (mono-RES-CLA) (**3a**)

(100 mg, 23% yield, R_f = 0.16);

^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$ 2:1 (v/v), δ: 0.79 (t, J = 7.0 Hz, 3H, CH_3-18″), 1.17–1.24 (m, 16H, CH_2-4-8 and CH_2-15-17), 1.52–1.54 (m, 2H, CH_2-3″), 1.77–2.08 (m, 4H, CH_2-9″ and CH_2-14″), 2.19 (t, J = 7.2 Hz, 2H, CH_2-2″), 6.16 (d, J = 15.0 Hz, 1H, H-10″), 6.47 (m, 1H, H-13″), 6.70–6.76 (m, 5H, H-4, H-12″, H-11″, H-3′and H-5′), 6.84 (d, J = 16.0 Hz, 1H, H-7), 6.88 (d, J = 16.0 Hz, 1H, H-8), 7.26–7.32 (m, 4H, H-2, H-6, H-2′ and H-6′).

3.5. Cell Lines and Cell Culture

The human cancer cell lines A549 (lung carcinoma) and HT29 (colorectal adenocarcinoma) were cultured in DMEM high-glucose culture medium with 10% fetal bovine serum (FBS) and Antibiotic-Antimycotic Solution, and BxPC3 (pancreatic ductal adenocarcinoma) was maintained in RPMI 1640 culture medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% FBS. As a control, the BJ (human fibroblasts) cell line was used, maintained in modified Eagle's MEM medium with 10% FBS. All cell lines were cultured at 37 °C in a humidified atmosphere of 5% CO_2. The cells were seeded at densities of 5 × 103 cells/0.1 mL (0.32 cm^2) (cell viability assay) and 5 × 104 cells/0.5 mL (1.9 cm^2) (flow cytometry). All cell lines were obtained from the collection of the Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland.

3.6. Determination of Cell Viability

Cell viability was determined using CellTiter-Glo® One Solution Assay (Promega, Madison, WI, USA). For determination of cell viability, cells were seeded in a 96-well plate (NUNC, Roskilde, Denmark) at a density of 8 × 103 cells overnight. Next, all cells were treated with Rev or Rev-LCFAs esters at 10, 25, 50, and 75 μg/mL and incubated in 200 μL of the above culture medium for 48 h. Following the incubation, 20 μL of MTS solution was added to each well for 2 h; next, absorbance at 490 nm was recorded by a plate reader. Each treatment within a single experiment was performed in triplicate.

3.7. Analysis of Apoptosis

Apoptosis was assessed by Annexin V Apoptosis Detection Kit (Santa Cruz Biotechnology, Dallas, TX, USA) according to the manufacturer's protocol. Briefly, the cells were stained with Annexin V-FITC (8 μg/mL) and PI (5 μg/mL) for 15 min at RT in the dark. In between steps, the cells were washed with cold PBS (with Ca^{2+} and Mg^{2+}) containing 2.5% FBS. Data were acquired on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analyzed using Flowing Software 2.5.1 software (Perttu Terho, Turku, Finland). Apoptosis was quantified as a percentage of both Annexin V-positive and Annexin V/PI-double-positive cells.

3.8. RNA Extraction and Real-Time Reverse Transcription PCR (qRT-PCR)

Total RNA was extracted from each cell line using EXTRAzol reagent (Blirt, Gdańsk, Poland) according to the manufacturer's instructions. The concentration of RNA, quality, as well as purity were measured using a nanospectrophotometer (BioTek, Winooski, VT, USA). Transcription of RNA into cDNA was prepared using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Gdańsk, Poland) by means of a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions.

The gene expression levels were evaluated by real-time reverse transcription polymerase chain reaction (RT-qPCR) using SensiFAST SYBR Green Kit (Bioline, London, UK) in a CFX Connect™ Real-Time PCR Detection System (Bio-Rad). Briefly, 10 μL total volume of each reaction consisted of 5 μL of SensiFAST SYBR Master mix, 2.5 μL of targeted

primer, and 2.5 µL of tested cDNA. The Real-Time PCR program was conducted as follows: 95 °C for 2 min, then 41 cycles at 95 °C for 15 s, annealing for 30 s in temperature specified for tested primers, and elongation at 72 °C for 15 s. The qPCR results were replicated in 3 independent experiments, and then the statistics were determined. Relative gene expression was normalized by the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta CT}$ method. Primers used are shown in Table 1.

Table 1. Sequences of primers used in qPCR.

Gene	Primer Sequence (5′–>3′)
BAX	F: CGAGTGGCAGCTGAGATGTT
	R: AAGGAAGTCCAGTGTCCAGC
BCL2	F: ATCGCCCTGTGGATGACTGAG
	R: CAGCCAGGAGAAATCAAACAGAGG
p21	F: TGCCGAAGTCAGTTCCTTGT
	R: GTTCTGACATGGCGCCTCC
p53	F: TTTCGACATAGCGTGGTGGT
	R: CTCAAAGCTGTTCCGTCCCA
SOD1	F: CATTCCATCATTGGCCGCAC
	R: GAGCGATCCCAATCACACCA
SOD2	F: GGACAAACCTGAGCCCCAAT
	R: TTGGACACCAGCCGATACAG

BAX: BCL-2-associated X protein; BCL-2: B-cell lymphoma 2; p21: cyclin-dependent kinase inhibitor 1A; p53: tumor suppressor p53; SOD1: Superoxide dismutase [Cu-Zn]; SOD2: Superoxide dismutase [Mn].

3.9. Statistical Analysis

The obtained results were analyzed by a one-way variance analysis (ANOVA) using GraphPad Software 8 (San Diego, CA, USA) and post hoc Tukey's test. Statistically significant results were marked with an asterisk: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). Results are presented as statistical mean SD from at least three independent experiments.

4. Conclusions

The presented work focused on the anticancer and antioxidant properties of RES esterified with selected fatty acids. To date, this is the first report presenting studies on the anticancer properties of lipophilic esters of RES. The obtained results enabled the selection of three esters, mono-RES-OA, tri-RES-PA, and mono-RES-CLA, which clearly showed efficient reduction in cancer cell viability compared to other esters without affecting the normal cells. In particular, the mono-RES-OA showed the highest antitumor properties, and its effect was stronger than that of pure RES at the same concentration. Furthermore, expression assays including the main pro-antioxidant *SOD* genes showed that the selected esters exhibited antioxidant properties towards the normal BJ cell line without a similar effect in cancer cells, thus reducing the chances of defending these cells against increased oxidative stress induced by high ROS accumulation. These results prove that hydrophobization of RES improves its biological activity. Despite numerous reports concerning the influence of fatty acids on the metabolism and bioavailability of polyphenols, it can be assumed that the obtained RES esters may exhibit the above-mentioned characteristics. RES modified with fatty acids has poorer solubility in water, but when administered orally, it should have higher stability and bioavailability in the intestines, especially being nanoencapsulated, which will also improve its pharmacological properties. However, in order to confirm these assumptions, in vivo studies using various animal models are needed. Further studies are also required to gain an in-depth understanding of the mechanisms of action of selected RES esters against specific cancer cell lines.

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4. Podsumowanie

Starzejące się społeczeństwa, rozwój chorób cywilizacyjnych, konsumpcjonizm wymuszają coraz większy popyt na wiele produktów, w tym również na leki czy nutraceutyki. Jednoczenie, konieczność produkcji dużych ilości żywności, co wymaga zagospodarowywania szerokich areałów gruntów rolnych pod uprawy, przy jednoczesnej konieczności ochrony środowiska naturalnego, sprawia, że mikrobiologiczna produkcja cennych związków o aktywności biologicznej zyskuje ogromne znaczenie i cieszy się coraz szerszym zainteresowaniem. Ideą badań zaprezentowanych w niniejszej rozprawie doktorskiej, było przedstawienie biotechnologicznej platformy do biosyntezy związków o potencjale terapeutycznym, którymi są drożdże *Y. lipolytica.* Ich ogromną zaletą jest również możliwość utylizacji odpadowych produktów z innych gałęzi przemysłu (glicerol, odpady tłuszczowe) czy związków pochodzących z fermentacji odpadów komunalnych (lotne kwasy tłuszczowe) jako substratów w procesach biosyntezy cennych biologicznie metabolitów.

Przeprowadzone badania udowodniły ogromny potencjał drożdży *Y. lipolytica* do produkcji zwiększonych ilości zarówno związków lipidowych - fosfolipidów, jak i związków polifenolowych - resweratrolu. Po raz pierwszy przeprowadzono inżynierię szlaku syntezy fosfolipidów u *Y. lipolytica,* gdzie już nadekspresja minimalnego zestawu enzymów tego szlaku pozwoliła na 2-krotne zwiększenie ilości tych związków do prawie 650 mg/L, również z glicerolu odpadowego. Z kolei najwyższe stężenie resweratrolu uzyskano z wykorzystaniem szczepu T2P2, posiadającym wklonowane dwa heterologiczne szlaki biosyntezy tego metabolitu: szkal katabolizmu tyrozyny i szlak katabolizmu fenyloalaniny. Ponadto, potwierdzono, że zwiększanie kopii genów kodujących kluczowe enzymy pozytywnie wpływa na poprawę wydajności procesu biosyntezy resweratrolu. Z kolei optymalizacji składu podłoża i warunków prowadzenia procesu 4-krotnie zwiększyła ilość uzyskiwanego metabolitu do 0,43 g/L. Z kolei badania związane z lipofilizacją resweratrolu kwasami tłuszczowymi udowodniły, że uzyskane lipidowe pochodne, mono-RES-OA, tri-RES-PA oraz mono-RES-CLA, charakteryzują się zarówno zwiększoną aktywnością przeciwnowotworową jak i antyoksydacyjną w porównaniu do czystego resweratrolu.

Szczególnie koniugat mono-RES-OA wykazywał najwyższe właściwości przeciwnowotworowe względem wszystkich testowanych linii nowotworowych. Uzyskane wyniki wskazują, że zsyntetyzowane nowe pochodne resweratrolu mają potencjał zastosowania ich w profilaktyce i leczeniu nowotworów oraz jako związków chroniącychc przed stresem oksydacyjnym.

W ramach kontynuacji badań zapoczątkowanych w ramach pracy doktorskiej należałoby przeprowadzić inżynierię dalszych etapów szlaku biosyntezy fosfolipidów jak również przekierować strumień węgla z biosyntezy triacylogliceroli. Z kolei, celem zwiększonej biosyntezy związków fenolowych, należałoby wzmocnić funkcjonowanie szlaku szikimowego w celu podniesienia podaży głównych prekursorów w syntezie wybranych związków bioaktywnych. Oprócz tego, pomocne byłyby dalsze badania mające na celu dogłębne poznanie mechanizmów działania wybranych estrów RES i kwasów tłuszczowych względem wybranych linii komórek nowotworowych. Estry te charakteryzują się gorszą rozpuszczalnością w wodzie, jednakże ich podanie doustne powinno poprawić ich stabilność i biodostępność w jelitach. Jednakże żeby potwierdzić te hipotezy, konieczne są badania *in vivo* z wykorzystaniem różnych modeli zwierzęcych.

5. Wnioski końcowe

Najważniejsze wnioski wynikające z przedstawionych danych są następujące:

- uzyskano transformanty *Y. lipolytica* wydajnie syntetyzujące fosfolipidy, które przy zastosowaniu strategii „push and pool" zdolne były wytworzyć prawie 650 mg/L fosfolipidów, w tym 350 mg/L fosfatydylocholiny, z zastosowaniem glicerolu technicznego i odpadowego jako źródła węgla;
- wzrost stężenia kwasu fosfatydowego, prekursora biosyntezy fosfolipidów, nie prowadzi do zwiększonej biosyntezy tych związków;
- źródło węgla odgrywa kluczową rolę w procesie biosyntezy fosfolipidów, a glukoza okazała się substratem zdecydowanie poprawiającym wydajność tego szlaku w porównaniu do glicerolu;
- intensywna akumulacja fosfolipidów zachodzi w późnej fazie stacjonarnej wraz z typową akumulacją innych frakcji lipidowych charakterystycznych dla mikroorganizmów oleistych;
- szczep *Y. lipolytica* T2P2, łączący dwa szlaki - katabolizmu tyrozyny i katabolizmu fenolyalaniny, jak również posiadający podwójne kopie genów kodujących kluczowe enzymy tych szlaków, zdolny był do wyprodukowania najwyższego stężenia resweratrolu, wynoszącego 430 mg/L w hodowli bioreaktorowej przy zastosowaniu podłoża bez suplementacji aminokwasami, przy wyższym natlenieniu;
- uzyskano pochodne lipidowe resweratrolu: tri-RES-PA, mono-RES-OA, mono-RES-CLA wykazujące lepsze właściwości przeciwnowotworowe względem testowanych linii nowotworowych: A549, BxPC3, HT29 niż sam resweratrol;
- estry RES i długołańcuchowych kwasów tłuszczowych wykazywały dużo wyższe właściwości antyoksydacyjne względem zdrowych komórek linii BJ niż sam resweratrol nie wpływając jednocześnie znacząco na ochronę przed stresem oksydacyjnym komórek nowotworowych.

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Dorobek naukowy

Publikacje

1. Qin He /**Patrycja Szczepańska**, Tigran Yuzbashev, Zbigniew Lazar, Rodrigo Ledesma-Amaro (2020). De novo production of resveratrol from glycerol by engineering different metabolic pathways in *Yarrowia lipolytica*. *Metabolic Engineering Communications*, 11, e00146.
2. **Patrycja Szczepańska**, Piotr Hapeta, Zbigniew Lazar (2021). Advances in production of high-value lipids by oleaginous yeasts. *Critical Reviews in Biotechnology*, 42:1, 1-22.
3. Piotr Hapeta, **Patrycja Szczepańska**, Tadeusz Witkowski, Jean-Marc Nicaud, Anne-Marie Crutz-Le Coq, Zbigniew Lazar (2021). The role of hexokinase and hexose transporters in preferential use of glucose over fructose and downstream metabolic pathways in the yeast *Yarrowia lipolytica*. *International Journal of Molecular Sciences*, 22:17, 9282.
4. Piotr Hapeta, **Patrycja Szczepańska**, Cécile Neuvéglise, Zbigniew Lazar (2021). A 37-amino acid loop in the *Yarrowia lipolytica* hexokinase impacts its activity and affinity and modulates gene expression. *Scientific Reports*, 11, 6412.
5. **Patrycja Szczepańska**, Magdalena Rychlicka, Paweł Moroz, Tomasz Janek, Anna Gliszczyńska, Zbigniew Lazar (2022). Elevating phospholipids production by *Yarrowia lipolytica* from crude glycerol. *International Journal of Molecular Sciences*, 23:18, 10737.
6. **Patrycja Szczepańska**, Magdalena Rychlicka, Sylwia Groborz, Angelika Kruszyńska, Rodrigo Ledesma-Amaro, Andrzej Rapak, Anna Gliszczyńska, Zbigniew Lazar (2023). Studies on the anticancer and antioxidant activities of resveratrol and long chain fatty acid esters. *International Journal of Molecular Sciences*, 24(8), 7167.

Staże naukowe

1. 01.06.2019-30.09.2019 Imperial College London, Londyn, Wielka Brytania. *Temat: Production of secondary metabolites by Yarrowia lipolytica.* Opiekun naukowy: Dr Rodrigo Ledesma-Amaro.
2. 01.11.2022-31.01.2023 Monash University, Melbourne, Australia. Temat: Production of high valuable fatty acids by *Yarrowia lipolytica*. Opiekun naukowy: Dr Victoria Haritos.

Doniesienia konferencyjne

1. **Patrycja Szczepańska**. Produkcja wartościowych związków lipidowych przez drożdże *Yarrowia lipolytica*. Ogólnopolska Konferencja Młodych Naukowców nt. Nowe Trendy w Badaniach Naukowych – Wystąpienie Młodego Naukowca, 20-21.06.2020, Kraków, Polska.
2. Patryk Kupaj, **Patrycja Szczepańska**, Agata Rot, Piotr Hapeta, Tomasz Janek, Zbigniew Lazar Z. Volatile Fatty Acid Utilization for Lipid Production by Species Belonging to *Yarrowia* Clade. International Congress on Yeast 15 meets 30 International Conference on Yeast Genetics and Molecular Biology, 23-27.08.2021, Wiedeń, Austria.
3. **Patrycja Szczepańska**, Agata Rot, Patryk Kupaj, Piotr Hapeta, Tomasz Janek, Zbigniew Lazar Z. Regulation of Phospholipid Metabolism in Terms of Phosphatidylcholine Synthesis in *Yarrowia lipolytica* yeast. International Congress on Yeast 15 meets 30 International Conference on Yeast Genetics and Molecular Biology, 23-27.08.2021, Wiedeń, Austria.
4. **Patrycja Szczepańska**, Magdalena Rychlicka, Patryk Kupaj, Tomasz Janek, Anna Gliszczyńska Zbigniew Lazar. Establishing the yeast *Yarrowia lipolytica* as a phospholipid-producing platform. Yeast Lipid Conference, 1-3.06.2022, Goteborg, Szwecja.
5. Patryk Kupaj, **Patrycja Szczepańska**, Tomasz Janek, Jean-Marc Nicaud, Zbigniew Lazar. Volatile fatty acids as carbon sources for lipid biosynthesis using *Yarrowia lipolytica* clade species. Yeast Lipid Conference 1-3.06.2022, Goteborg, Szwecja.
6. **Patrycja Szczepańska**, Patryk Kupaj, Tomasz Janek, Rodrigo Ledesma-Amaro, Zbigniew Lazar. Production of resveratrol from glycerol by engineering different metabolic pathways in *Yarrowia lipolytica*. Yeast in the genomics era, 12-15.07.2022, Vancouver, Kanada.
7. Patryk Kupaj, **Patrycja Szczepańska**, Tomasz Janek, Jean-Marc Nicaud, Zbigniew Lazar. Volatile fatty acids as carbon sources for lipid biosynthesis using *Yarrowia lipolytica* clade species. Yeast in the genomics era, 12-15.07.2022, Vancouver, Kanada.

Granty i projekty badawcze

1. Wykorzystanie surowców odpadowych do syntezy lipidów o znaczeniu terapeutycznym przez drożdże *Yarrowia lipolytica.* Innowacyjny Doktorat nr B020/0007/19; 01.2019-12.2020; kierownik.
2. Wykorzystanie lotnych kwasów tłuszczowych do biosyntezy wosków przez drożdże *Yarrowia lipolytica*. OPUS 19 nr B080/0002/21; 01.02.2021-31.07.2022; wykonawca.

Załączniki

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Wrocław, 13.04.2023

Oświadczenie

Oświadczam, że jestem współautorem prac będących cyklem publikacji, wchodzących w skład mojej rozprawy doktorskiej:

Patrycja Szczepańska, Piotr Hapeta, Zbigniew Lazar (2021). Advances in production of high-value lipids by oleaginous yeasts. *Critical Reviews in Biotechnology*, 42:1, 1-22.

Patrycja Szczepańska, Magdalena Rychlicka, Paweł Moroz, Tomasz Janek, Anna Gliszczyńska, Zbigniew Lazar (2022). Elevating phospholipids production by *Yarrowia lipolytica* from crude glycerol. *International Journal of Molecular Sciences*, 23:18, 10737.

Qin He /**Patrycja Szczepańska**, Tigran Yuzbashev, Zbigniew Lazar, Rodrigo Ledesma-Amaro (2020). De novo production of resveratrol from glycerol by engineering different metabolic pathways in *Yarrowia lipolytica*. *Metabolic Engineering Communications*, 11, e00146.

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Szczepańska

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KATEDRA BIOTECHNOLOGII I MIKROBIOLOGII ŻYWNOŚCI

prof. dr hab. inż. Zbigniew Lazar

Wrocław, 13.04.2023

Oświadczenie

Oświadczam, że jestem współautorem prac będących cyklem publikacji, wchodzących w skład rozprawy doktorskiej pani mgr. inż. Patrycji Moniki Szczepańskiej:

Patrycja Szczepańska, Piotr Hapeta, **Zbigniew Lazar** (2021). Advances in production of high-value lipids by oleaginous yeasts. *Critical Reviews in Biotechnology*, 42:1, 1-22.

Patrycja Szczepańska, Magdalena Rychlicka, Paweł Moroz, Tomasz Janek, Anna Gliszczyńska, **Zbigniew Lazar** (2022). Elevating phospholipids production by *Yarrowia lipolytica* from crude glycerol. *International Journal of Molecular Sciences*, 23:18, 10737.

Qin He /Patrycja Szczepańska, Tigran Yuzbashev, **Zbigniew Lazar**, Rodrigo Ledesma-Amaro (2020). De novo production of resveratrol from glycerol by engineering different metabolic pathways in *Yarrowia lipolytica*. *Metabolic Engineering Communications*, 11, e00146.

Patrycja Szczepańska, Magdalena Rychlicka, Sylwia Groborz, Angelika Kruszyńska, Rodrigo Ledesma-Amaro, Andrzej Rapak, Anna Gliszczyńska, **Zbigniew Lazar** (2023). Studies on the anticancer and antioxidant activities of resveratrol and long chain fatty acid esters. *International Journal of Molecular Sciences*, 24(8), 7167.

Mgr inż. Patrycja Szczepańska przygotowując powyższe publikacje opracowała metodologię, prowadziła badania laboratoryjne oraz analizy ilościowe i jakościowe, brała udział w merytorycznym opracowywaniu wyników i ich dyskusji oraz napisała manuskrypty.

Moja rola polegała na tworzeniu koncepcji badań realizowanych w ramach pracy doktorskiej, koordynowaniu pracy doktorantki, analizie i interpretacji wyników, a także ich dyskusji z danymi literaturowymi oraz merytorycznemu redagowaniu treści manuskryptów.

Zbigniew Lazar

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dr inż. Piotr Hapeta

Wrocław, 28.03.2023

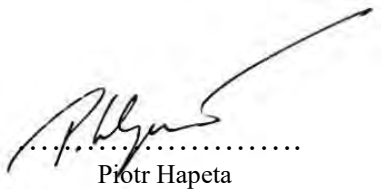
Oświadczenie

Oświadczam, że jestem współautorem pracy będącej częścią cyklu publikacji, wchodzących w skład rozprawy doktorskiej pani mgr. inż. Patrycji Szczepańskiej:

Szczepańska Patrycja, **Hapeta Piotr**, Lazar Zbigniew (2021). Advances in production of high-value lipids by oleaginous yeasts. *Critical Reviews in Biotechnology*, 42:1, 1-22.

Mgr inż. Patrycja Szczepańska przygotowując powyższą publikację dokonała analizy doniesień naukowych i wyników przedstawionych w dostępnej literaturze oraz ich dyskusji, a także napisała manuskrypt.

Mój wkład polegał na przygotowaniu schematów, tabel, a także redagowaniu treści manuskryptu.



.....

Piotr Hapeta

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KATEDRA CHEMII ŻYWNOŚCI I BIOKATALIZY

prof. dr hab. inż. Anna Gliszczyńska

Wrocław, 28.03.2023

Oświadczenie

Oświadczam, że jestem współautorem pracy będącej częścią cyklu publikacji, wchodzących w skład rozprawy doktorskiej pani mgr. inż. Patrycji Szczepańskiej:

Patrycja Szczepańska, Magdalena Rychlicka, Paweł Moroz, Tomasz Janek, **Anna Gliszczyńska**, Zbigniew Lazar, (2022). Elevating phospholipids production by *Yarrowia lipolytica* from crude glycerol. *International Journal of Molecular Sciences*, 23:18, 10737.

Mgr inż. Patrycja Szczepańska przygotowując powyższą publikację opracowała metodologię, prowadziła badania laboratoryjne w zakresie inżynierii metabolicznej drożdży, badania ich zdolności do produkcji fosfolipidów, optymalizacji warunków hodowlanych. Dokonała także analizy wyników, brała udział w merytorycznym opracowywaniu wyników i ich dyskusji oraz napisała manuskrypt.

Mój wkład polegał na opracowaniu metodologii badań dotyczącej analizy jakościowej i ilościowej frakcji fosfolipidowej, analizie uzyskanych wyników chromatografii cieczowej oraz redagowaniu treści manuskryptu.

A. Gliszczyńska

HR EXCELLENCE IN RESEARCH

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Mgr inż. Paweł Moroz

Wrocław, 28.03.2023

Oświadczenie

Oświadczam, że jestem współautorem pracy będącej częścią cyklu publikacji, wchodzących w skład rozprawy doktorskiej pani mgr. inż. Patrycji Szczepańskiej:

> Patrycja Szczepańska, Magdalena Rychlicka, **Paweł Moroz**, Tomasz Janek, Anna Gliszczyńska, Zbigniew Lazar, (2022). Elevating phospholipids production by *Yarrowia lipolytica* from crude glycerol. *International Journal of Molecular Sciences*, 23:18, 10737.

Mgr inż. Patrycja Szczepańska przygotowując powyższą publikację opracowała metodologię, prowadziła badania laboratoryjne w zakresie inżynierii metabolicznej drożdży, badania ich zdolności do produkcji fosfolipidów, optymalizacji warunków hodowlanych. Dokonała także analizy wyników, brała udział w merytorycznym opracowywaniu wyników i ich dyskusji oraz napisała manuskrypt.

Mój wkład polegał na pomocy w prowadzeniu hodowli bioreaktorowych drożdży *Y. lipolytica*.

HR EXCELLENCE IN RESEARCH

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dr inż. Magdalena Rychlicka

Wrocław, 28.03.2023

Oświadczenie

Oświadczam, że jestem współautorem pracy będącej częścią cyklu publikacji, wchodzących w skład rozprawy doktorskiej pani mgr. inż. Patrycji Szczepańskiej:

> Patrycja Szczepańska, **Magdalena Rychlicka**, Paweł Moroz, Tomasz Janek, Anna Gliszczyńska, Zbigniew Lazar, (2022). Elevating phospholipids production by *Yarrowia lipolytica* from crude glycerol. *International Journal of Molecular Sciences*, 23:18, 10737.

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Mój wkład polegał na opracowaniu metodologii i wykonaniu badań dotyczącej analizy ilościowej i jakościowej frakcji fosfolipidowej oraz redagowaniu treści manuskryptu.

Rychlicka

HR EXCELLENCE IN RESEARCH

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dr Tomasz Janek

Wrocław, 28.03.2023

Oświadczenie

Oświadczam, że jestem współautorem pracy będącej częścią cyklu publikacji, wchodzących w skład rozprawy doktorskiej Pani mgr inż. Patrycji Szczepańskiej:

Patrycja Szczepańska, Magdalena Rychlicka, Paweł Moroz, **Tomasz Janek**, Anna Gliszczyńska, Zbigniew Lazar, (2022). Elevating phospholipids production by *Yarrowia lipolytica* from crude glycerol. *International Journal of Molecular Sciences*, 23:18, 10737.

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Mój wkład polegał na opracowaniu metodologii badań dotyczącej analizy ilościowej i jakościowej frakcji lipidowej.

Tomasz Janek

HR EXCELLENCE IN RESEARCH

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Imperial College London

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Dr Rodrigo Ledesma Amaro
Imperial College Research Fellow in Synthetic Biology and Metabolic Engineering

13.04.2023, London

TO WHOM IT MAY CONCERN

I hereby certify that I am the co-author of a publication being a part of a series of publications included in the doctoral dissertation of MSc Eng. Patrycja Szczepańska:

Qin He /Patrycja Szczepańska, Tigran Yuzbashev, Zbigniew Lazar, **Rodrigo Ledesma-Amaro** (2020). De novo production of resveratrol from glycerol by engineering different metabolic pathways in *Yarrowia lipolytica*. *Metabolic Engineering Communications*, 11, e00146.

Patrycja Szczepańska, Magdalena Rychlicka, Sylwia Groborz, Angelika Kruszyńska, **Rodrigo Ledesma-Amaro**, Andrzej Rapak, Anna Gliszczyńska, Zbigniew Lazar (2023). Studies on the anticancer and antioxidant activities of resveratrol and long chain fatty acid esters. *International Journal of Molecular Sciences*, 24(8), 7167.

When preparing the above publications, MSc Eng. Patrycja Szczepańska developed the methodology, conducted laboratory research, participated in the substantive preparation of the results and their discussion, and wrote the manuscripts.

My contribution consisted of conceptualization, supervision and investigation of the performed research as well as reviewing and editing the manuscript.

Yours sincerely,

Dr Rodrigo Ledesma Amaro Centre for Synthetic Biology and Innovation Department of Bioengineering Imperial College, London

南京農業大學
NANJING AGRICULTURAL UNIVERSITY

29.03.2023, China

TO WHOM IT MAY CONCERN

I hereby certify that I am the co-author of a publication being a part of a series of publications included in the doctoral dissertation of MSc Eng. Patrycja Szczepańska:

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When preparing the above publication, MSc Eng. Patrycja Szczepańska developed the methodology, conducted laboratory research, participated in the substantive preparation of the results and their discussion, and wrote the manuscript.

My contribution consisted of developing the methodology, conducting laboratory research, participating in the substantive preparation of the results and their discussion, and writing the manuscript.

Yours sincerely,

Dr Qin He

Qin He

30.03.2023, London

TO WHOM IT MAY CONCERN

I hereby certify that I am the co-author of a publication being a part of a series of publications included in the doctoral dissertation of MSc Eng. Patrycja Szczepańska:

Qin He /Patrycja Szczepańska, **Tigran Yuzbashev**, Zbigniew Lazar, Rodrigo Ledesma-Amaro (2020). De novo production of resveratrol from glycerol by engineering different metabolic pathways in *Yarrowia lipolytica*. *Metabolic Engineering Communications*, 11, e00146.

When preparing the above publication, MSc Eng. Patrycja Szczepańska developed the methodology, conducted laboratory research, participated in the substantive preparation of the results and their discussion, and wrote the manuscript.

My contribution consisted of developing the methodology and investigating part of the laboratory research.

Yours sincerely,

Tigran

Tigran Yuzbashev, PhD
Research Scientist - Molecular Biochemist
Plant Sciences and the Bioeconomy
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Dr hab. Andrzej Rapak prof. nadzw. PAN

Wrocław 14.04.2023

Oświadczenie

Oświadczam, że jestem współautorem pracy będącej częścią cyklu publikacji, wchodzących w skład rozprawy doktorskiej pani mgr. inż. Patrycji Szczepańskiej:

Patrycja Szczepańska, Magdalena Rychlicka, Sylwia Groborz, Angelika Kruszyńska, Rodrigo Ledesma-Amaro, **Andrzej Rapak**, Anna Gliszczyńska, Zbigniew Lazar (2023). Studies on the anticancer and antioxidant activities of resveratrol and long chain fatty acid esters. *International Journal of Molecular Sciences*, 24(8), 7167.

Mgr inż. Patrycja Szczepańska przygotowując powyższą publikację prowadziła badania na ludzkich liniach komórkowych, badała wpływ lipidowych pochodnych resweratrolu na komórki nowotworowe w kontekście analizy ich właściwości przeciwnowotworowych i antyoksydacyjnych. Dokonała także analizy wyników, merytorycznego opracowania wyników i ich dyskusji oraz napisała manuskrypt.

Mój wkład polegał na tworzeniu koncepcji oraz metodyki badań dotyczących pracy na liniach komórkowych, koordynowaniu pracy doktoranta, interpretacji wyników oraz merytorycznemu redagowaniu manuskryptu.

Rapak

Doc. dr hab. Andrzej Rapak
Laboratorium Immunobiologii
Molekularnej Nowotworów
Instytut Immunologii i Terapii
Doświadczalnej PAN we Wrocławiu

mgr. inż. Angelika Kruszyńska

Wrocław, 13.04.2023

Oświadczenie

Oświadczam, że jestem współautorem pracy będącej częścią cyklu publikacji, wchodzących w skład rozprawy doktorskiej pani mgr. inż. Patrycji Szczepańskiej:

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Mój wkład polegał na pomocy w badaniach prowadzonych na ludzkich komórkach nowotworowych.

Kruszyńska Angelika

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prof. dr hab. inż. Anna Gliszczyńska

Wrocław, 13.04.2023

Oświadczenie

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Patrycja Szczepańska, Magdalena Rychlicka, Sylwia Groborz, Angelika Kruszyńska, Rodrigo Ledesma-Amaro, Andrzej Rapak, **Anna Gliszczyńska**, Zbigniew Lazar (2023). Studies on the anticancer and antioxidant activities of resveratrol and long chain fatty acid esters. *International Journal of Molecular Sciences*, 24(8), 7167.

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Mój wkład polegał na opracowaniu metodologii badań dotyczącej reakcji estryfikacji resweratrolu z długołańcuchowymi kwasami tłuszczowymi oraz oczyszczania uzyskanych produktów, współudziale w syntezie lipidowych pochodnych resweratrolu, wykonaniu analizy spektroskopowej wszystkich uzyskanych produktów oraz współudziale w pisaniu manuskryptu.

A. Gliszczyńska

HR EXCELLENCE IN RESEARCH

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Mgr Inż. Sylwia Groborz
13.04.2023 r.

Wrocław,

Oświadczenie

Oświadczam, że jestem współautorem pracy będącej częścią cyklu publikacji, wchodzących w skład rozprawy doktorskiej pani mgr. inż. Patrycji Szczepańskiej:

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Mój wkład polegał na wykonaniu badań dotyczących serii reakcji Real Time PCR w celu określenia poziomów ekspresji genów odpowiadających za apoptozę oraz stres oksydacyjny.

Groborz Sylwia

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dr inż. Magdalena Rychlicka

Wrocław, 13.04.2023

Oświadczenie

Oświadczam, że jestem współautorem pracy będącej częścią cyklu publikacji, wchodzących w skład rozprawy doktorskiej pani mgr. inż. Patrycji Szczepańskiej:

> Patrycja Szczepańska, **Magdalena Rychlicka**, Sylwia Groborz, Angelika Kruszyńska, Rodrigo Ledesma-Amaro, Andrzej Rapak, Anna Gliszczyńska, Zbigniew Lazar (2023). Studies on the anticancer and antioxidant activities of resveratrol and long chain fatty acid esters. *International Journal of Molecular Sciences*, 24(8), 7167.

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Mój wkład polegał na opracowaniu metodologii i wykonaniu badań dotyczących estryfikacji resweratrolu z długołańcuchowymi kwasami tłuszczowymi.

HR EXCELLENCE IN RESEARCH

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