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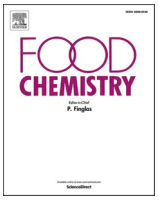

Precursor direct biosynthesis of odd-chain polyunsaturated fatty acids by oomycete *Pythium*

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ABSTRACT

The growing demand for improving the nutritional value of food has driven efforts to enhance the production of polyunsaturated fatty acids (PUFAs) through microbial cultivation, mainly using low-cost substrates in closed-loop systems. One promising approach is precursor-driven biosynthesis, which enables the controlled production of odd-chain PUFAs. In this study, we cultivated two oomycetes from the genus *Pythium* (ATCC 38472 M1 and X42) under 20 different conditions in the presence of propionic acid and TWEEN 80 to stimulate the production of odd-chain PUFAs. This approach led to an increased yield of specific odd-chain PUFAs, including C17:2ω6, C17:3ω3, C17:4ω3, C19:2ω5, C19:3ω5, C19:4ω5, and C19:5ω2. The microorganisms were grown in two different media: potato dextrose broth (PDB) and waste brewery yeast, either alone or supplemented with propionic acid and/or TWEEN 80. The addition of propionic acid facilitated the biosynthesis of odd-chain PUFAs without the need for genetically modified strains, yielding quantities suitable for fermenter-scale production.

1. Introduction

The dietary intake of essential fatty acids (EFAs) is often insufficient in the general population, regardless of whether their diet is primarily plant-based or animal-based. Many individuals fail to meet the recommended intake levels of polyunsaturated fatty acids (PUFAs), particularly omega-3 and omega-6 fatty acids, which are crucial for maintaining optimal health (Neufingerl & Eilander, 2022).

Essential fatty acids (EFAs) are polyunsaturated fatty acids that the body requires for proper physiological functions but cannot synthesize in sufficient amounts de novo, making dietary intake essential. The primary EFAs include linoleic acid (LA, an omega-6 fatty acid) and alpha-linolenic acid (ALA, an omega-3 fatty acid), which serve as precursors for the biosynthesis of longer-chain polyunsaturated fatty acids, such as arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). These fatty acids play a crucial role in various biological processes, including cellular membrane integrity, inflammation regulation, and the production of bioactive lipid mediators such as prostaglandins, thromboxanes, and prostacyclins (Burdge, 2004; Burdge & Wootton, 2002).

Deficiencies in essential FAs cause complications, mainly consisting of an increased incidence of cardiovascular diseases, cancer risk, autoimmune diseases, psychological disorders, memory disorders such as Alzheimer's disease, developmental retardation, vision disorders, muscle weakness and tremors, dyslipidemia, insulin resistance, high blood pressure, thrombosis, diabetes, asthma, obesity, and others, as described by Simopoulos (Simopoulos, 2016).

Fatty acids with odd-numbered straight chains (odd-chain FAs) are commonly found in nature; however, their total proportion in most biological systems typically accounts for only a small fraction, usually less than a few tenths of the total fatty acid content. There are exceptions; mammalian milk and dairy products (cheeses, yogurts, etc.) contain odd-chain FAs (Kallio et al., 2017). These FAs are produced by biosynthesis carried out by symbiotic bacteria in the digestive tract and by de novo biosynthesis in the mammary gland (Kalo et al., 2009; Vlaeminck et al., 2006). Odd-chain triacylglycerols (TAGs) containing at least one odd-chain FA, e.g. margaric (C17:0) or margaroleic (C17:1ω8) acids, whose concentrations in TAGs did not exceed 1 % of total TAGs, were also identified in vegetable oils (Holčapek et al., 2003).

A lower risk of type 2 diabetes mellitus (T2DM) has been associated

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with a higher proportion of saturated odd-chain FAs, e.g., pentadecanoic acid (C15:0) or margaric acid in phospholipids (PL) (Forouhi et al., 2014; Furse et al., 2022; Krachler et al., 2008). A positive correlation was also observed between even FAs - myristic (C14:0), palmitic (C16:0), and stearic (C18:0) and T2DM, while a negative correlation was found between odd-chain FAs (C15:0 and C17:0) and T2DM (Forouhi et al., 2014). Furthermore, phospholipid levels with acids C15:0 and C17:0 were inversely correlated with nonalcoholic fatty liver disease (NAFLD) (Yoo et al., 2017). A recent European Prospective Investigation of Cancer and Nutrition (EPIC) cohort analysis confirmed that total ratios of C15:0 and C17:0 are the most robust metabolic markers of a healthy lifestyle and a lower risk of colon cancer (Venn-Watson et al., 2020).

The situation is completely different with odd-chain polyunsaturated fatty acids (PUFAs). Protists, such as those of the genera *Schizochytrium* and *Thraustochytrium* contain odd-chain PUFAs in unusually high qualitative (from C19:1 to C19:4 and from C21:1 to C21:6) and quantitative amounts (C21:4ω7 constitutes 7.2 % of total FA) (Chang et al., 2011; Chang et al., 2012). Also, another protist, *Khawkinea quartana,* also contains odd-chain PUFAs, e.g., C17:4, C19:5, C21:4, etc. (Řezanka et al., 2015). The filamentous fungus *Saprolegnia* produced odd-chain PUFAs (C17:2ω5, C19:4ω5, and C19:5ω2) when grown with saturated fatty acids with 13, 15, or 17 carbons in the amount of up to units percent of total FAs (Shirasaka et al., 1995). Chytrid fungi grown in a complex medium produced odd-chain PUFA (C17:2ω8 or C17:3ω5) in a maximum of approximately 3 % of total FAs (Akinwole et al., 2014). Precursor-directed PUFA biosynthesis is one way to improve the levels of these important substances in the diet. The industrially cultivated fungus *Mortiella* also produces odd-chain PUFAs after adding odd-numbered precursors. *Mortiella alpina* strain 1S-4 produces and accumulates odd-chain PUFAs in the amount of units of percent of total FAs (Shimizu et al., 1991). However, only odd-chain hydrocarbons (C15, C17, C19), methyl pentadecanoate, or methyl nonadecanoate were precursors. However, only the genetically modified strain produced a significant amount of PUFAs. The strain was defective in its Δ^{12} desaturase activity and was grown in odd-chain alkanes (C11-C19) (Zhang et al., 2006). The amount of C19:2ω8 and C19:3ω8 was more than 11 % of the total FAs. Current research focuses on improving yields through genetic engineering, using waste materials as substrates, and combining microorganisms with improved fermentation systems (Chun-Xiao et al., 2024; Gasovic et al., 2023).

Oomycetes of the genus *Pythium* have been investigated as potential producers of PUFA, including the use of precursor-driven biosynthesis. However, most commercial applications are based on other microorganisms (e.g. yeasts, algae, or fungi) (Chun-Xiao et al., 2024). Although *Pythium oligandrum* is already utilized in plant protection, animal husbandry, and cosmetic applications, further studies are needed to evaluate its safety and regulatory aspects of its use in food production. Another potential use is in the production of fatty acids (FAs). In addition to optimizing PUFA biosynthesis, it is essential to consider microbial growth performance under different cultivation conditions. Alternative carbon sources, such as waste brewery yeast, present an attractive, low-cost, and sustainable option for microbial fermentation. Understanding the impact of these substrates on both biomass yield and lipid composition is crucial for evaluating the feasibility of large-scale production. The fatty acid content of *P. oligandrum* has already been published several times, and analyses have shown that this microorganism is a rich source of polyunsaturated fatty acids (PUFAs), some of which are essential (Athalye et al., 2009; Cantrell & Walker, 2009; Lio & Wang, 2013; O'Brien et al., 1993; Russo et al., 2023; Wu et al., 2013).

For the identification of PUFAs, gas chromatography–mass spectrometry (GC–MS) of suitable derivatives, such as 3-pyridylcabonyl esters (formerly called picolinyl esters), is primarily used (Harvey, 1984). In the analysis of fatty acid methyl esters (FAMEs), the identification of the mass spectrum by comparison with a computer database is often unreliable. The positions of double bonds (DBs) in the aliphatic chain can rarely be determined unambiguously through GC–MS analysis of FAMEs using the electron impact (EI) ionization. Although molecular weight and GC retention times provide useful analytical data, they cannot pinpoint DB positions.

A more effective approach involves derivatizing the carboxyl group with a nitrogen-containing reagent, such as a 3-pyridylcarbinol ester. When ionized in a mass spectrometer, the nitrogen atom—rather than the alkyl chain—carries the charge, minimizing ionization-induced double bond migration. This leads to uniform radical-induced cleavage along the chain, generating a series of high-mass ions corresponding to the cleavage of each C=C bond. Diagnostic ions typically appear at the locations of DBs (Christie, 2024).

When a suitable derivative for mass spectrometry is selected, good chromatographic properties and ease of preparation are crucial factors. Although 3-pyridylcarbinol esters require column temperatures approximately 50 °C higher than those used for FAMEs, they allow a precise determination of double bond positions. Silver ion chromatography is based on a distinctive property of unsaturated organic compounds, i.e., the ability to form complexes with transition metals, e.g., silver. A bond is formed between the Π orbitals of the double bond and the free orbitals of the silver ion. The formation of these complexes thus enables the separation of fatty acids according to the number of double bonds, i.e. the more complexes (essentially the number of DBs), the longer the retention time during elution from the column. Commercially available cartridges based on cation exchanger in Ag^+ form, where SCX is a strong cation exchange extraction cartridge based on silica gel with a bound benzenesulfonic acid functional group, thus enable a simple and rapid separation of FAs derivatives according to the number of DBs (Momchilova & Nikolova-Damyanova, 2012).

Similarly, reverse-phase high-performance liquid chromatography is based on the polarity of the eluted compounds. If one of the most common phases, i.e., C18, is used as the stationary phase, then the compounds are eluted according to their chain lengths. If there is a double bond in the molecule, then the elution time is shortened, and the DB contribution is almost equal to the two CH_2 groups. This creates "critical pairs", e.g. palmitic and oleic acids. As shown below, if a sufficiently efficient column is used, these critical pairs can also be separated (Rao et al., 1995).

This article describes the potential use of *Pythium* sp. for producing odd-numbered PUFAs by precursor cultivation on different carbon substrates (propionic acid or organic waste from the brewing industry). This study explores the potential of using precursor-driven biosynthesis to enhance the production of odd-chain PUFAs in *Pythium* sp. Propionic acid was selected as a precursor due to its role in metabolic pathways that facilitate the incorporation of odd-carbon units into fatty acids, enabling the production of odd-chain PUFAs without the need for genetically modified strains. TWEEN 80 was included as a surfactant to improve substrate availability and enhance microbial growth, thereby optimizing the cultivation conditions. The findings provide insights into the feasibility of using microbial fermentation to produce these bioactive lipids efficiently.

2. Materials and methods

2.1. Chemicals and standards

2-propanol, acetonitrile, dichloromethane, hexane, tetrahydrofuran (THF), ewen-PUFAs, i.e., C12:0; C14:0; C20:5ω-3; C18:3ω-3; C18:3ω-6; C16:1ω-7; C20:4ω-6; C18:2ω-6; C20:3ω-6; C16:0; C18:1ω-9; C18:0; C20:1ω-9; C20:0 were bought from Sigma-Aldrich (now Merck, Prague Czech Republic). Odd-PUFAs, i.e., C19:5ω-3 (4(Z),7(Z),10(Z),13(Z),16(*Z*)-nonadecapentaenoic acid); C17:3ω-3 (8(Z),11(Z),14(Z)-heptadecatrienoic acid); C19:3ω-3 (10(Z),13(Z),16(Z)-nonadecatrienoic acid); C19:3ω-6 (7(Z),10(Z),13(Z)-nonadecatrienoic acid); and C19:2ω6 (10(Z),13(Z)-nonadecadienoic acid) were purchased from Larodan AB, Solna, Sweden. The preparation of ethyl esters from free fatty acids is

Table 1
Amount of lyophilized biomass, total lipids and triacylglycerols obtained by 20 cultivations on different media.

Cultivation[a]	dry weight (mg)[b]	total lipids (mg)	TAGs (mg of total lipids)	total lipids (%)[c]	TAGs (% of total lipids)[c]
14/X42PDB	2533	795.4 ± 17.9	479.6 ± 21.8	31.4	60.3
14/X42PDB + Pr	86	21.1 ± 5.2	9.5 ± 3.9	24.5	44.9
14/X42PDB + Pr + TW	1371	474.4 ± 23.8	245.7 ± 14.5	34.6	51.8
14/X42Y	778	239.6 ± 11.2	137.3 ± 9.7	30.8	57.3
14/X42Y + Pr + TW	725	245.8 ± 18.9	123.1 ± 12.1	33.9	50.1
24/X42PDB	3756	743.7 ± 23.4	363.7 ± 16.3	19.8	48.9
24/X42PDB + Pr	329	60.9 ± 3.8	24.2 ± 4.7	18.5	39.7
24/X42PDB + Pr + TW	1071	256 ± 14.2	109.3 ± 5.9	23.9	42.7
24/X42Y	5311	1566.7 ± 16.8	756.7 ± 11.2	29.5	48.3
24/X42Y + Pr + TW	588	183.5 ± 9.3	82.4 ± 7.9	31.2	44.9
14/M1PDB	4756	1374.5 ± 23.9	586.9 ± 13.6	28.9	42.7
14/M1PDB + Pr	1643	359.8 ± 6.4	107.2 ± 6.7	21.9	29.8
14/M1PDB + Pr + TW	2314	851.6 ± 16.7	286.1 ± 17.4	36.8	33.6
14/M1Y	1644	457 ± 15.7	189.7 ± 9.4	27.8	41.5
14/M1Y + Pr + TW	2463	773.4 ± 23.8	353.4 ± 10.2	31.4	45.7
24/M1PDB	667	164.1 ± 12.8	44 ± 3.8	24.6	26.8
24/M1PDB + Pr	157	31.9 ± 6.7	7.6 ± 2.6	20.3	23.8
24/M1PDB + Pr + TW	1600	438.4 ± 13.6	132 ± 12.3	27.4	30.1
24/M1Y	2089	513.9 ± 14.1	188.6 ± 11.0	24.6	36.7
24/M1Y + Pr + TW	525	139.7 ± 12.4	55.6 ± 5.9	26.6	39.8

[a] Explanations: the number before the slash is the cultivation temperature in °C, X42 or M1 are two different strains (*Pythium* sp. X42 or *Pythium oligandrum* ATCC 38472 M1), PDB is medium potato dextrose broth or waste brewery yeast, Pr is medium with propionic acid and Pr + TW is medium with propionic acid, and TWEEN 80, see experimental section.
[b] the difference between individual weighings is maximum ±0.5 mg.
[c] additional data, calculated from the values of "total lipids" and "TAGs".

described in Section 2.4.

2.2. Cultivation of Pythium sp. and experimental conditions

The cultivation of *Pythium* sp., *Pythium oligandrum* ATCC 38472 (designated as strain M1), and *Pythium* sp. X42 (Biopreparaty s.r.o., Uherce, Czech Republic) was conducted under agitation at 150 rpm at 14 °C and 24 °C using various growth media. These included Potato Dextrose Broth (PDB, 24 g/L), PDB supplemented with propionic acid (3 g/L), PDB supplemented with both propionic acid (3 g/L) and TWEEN 80 (0.5 % *v/v*), waste brewery yeast (20 g/L), and waste brewery yeast supplemented with propionic acid (3 g/L) and TWEEN 80 (0.5 % *v/v*). Cultures were incubated for 14 days at 14 °C and for 7 days at 24 °C until biomass accumulation reached a steady state. The biomass was harvested, centrifuged, washed, and lyophilized. Table 1 (first column) shows the amount of lyophilized cells. Volumes of the solvent mixture were always ~10× higher than the weight of lyophilized cells (e.g., weight was 2553 mg; see Table 1a, the volume of the mixture was 28 mL, etc.). Biological replicates (three independent cultivations) and technical replicates (three chromatographic analyses) were analyzed. The data (Tables 1Sa and 1Sb) revealed that biological variability, particularly the biomass yield of strain X42 across different substrates, is at least one order of magnitude greater than the technical variability.

2.3. Lipid extraction and triacylglycerols isolation

The extraction of lipids was performed according to the method of Bligh and Dyer (1959) with modifications (Řezanka et al., 2010; Řezanka et al., 2016). Different classes of lipids were separated using Sep-Pak Vac Silica cartridges. Frozen cells were suspended in a dichloromethane-methanol (2:1) mixture and stirred for 30 min. Dichloromethane and water were added, and the dichloromethane phase was evaporated under reduced pressure. Total lipids were fractionated according to established protocols (Saunders & Horrocks, 1984). Lipid classes were separated using Sep-Pak Vac Silica cartridges (Waters, MA, USA; 10 g silica) and eluted with chloroform (neutral lipids), acetone (glycolipids), and methanol (phospholipids) as described previously (Kolouchová et al., 2015). Solvents were evaporated under nitrogen, and triacylglycerols (TAGs) were subjected to further analysis.

2.4. Preparation and analysis of fatty acid esters

Preparation of ethyl esters: Approximately 3 mg of the sample was dissolved in 1 mL diethyl ether and 20 mL ethyl acetate, followed by adding 250 μL of 1 M sodium ethoxide in ethanol. The reaction mixture was incubated at room temperature for 50 min before being neutralized with 0.5 mL of saturated oxalic acid solution. Solvents were evaporated under nitrogen, and the residue was dissolved in hexane to achieve the required concentration for analysis. Ethyl esters were analyzed as a neat film using a Perkin-Elmer Model 1310 infrared spectrophotometer (Norwalk, CT).

Splitless gas chromatography injections (0.5 μL) were performed at 100 °C using a Supelcowax 10 fused silica capillary column (60 m × 0.25 mm i.d., 0.25 mm film thickness; Supelco, Prague). The temperature program consisted of an initial hold at 100 °C for one minute, a ramp of 20 °C/min to 180 °C, followed by a ramp of 2 °C/min to 280 °C, held for one minute. Helium was the carrier gas (linear velocity: 60 cm/s). Fatty acid ethyl esters (FAEEs) were identified via mass spectra (Dembitsky et al., 1993; Řezanka & Podojil, 1986) and reference standards from Merck.

Nicotinyl alcohol (1 mL) was mixed with 0.5 mL of 1.0 M potassium tert-butoxide in THF. Approximately 0.5 mg of fatty acid ethyl esters (FAEEs) dissolved in 1 mL dichloromethane was added, and the reaction proceeded at 40 °C for 30 min in a sealed vial. After cooling, the reaction mixture was extracted with water and hexane, and the organic phase was dried over anhydrous sodium sulfate and evaporated.

A mixture of 3-pyridylcarbonyl esters of fatty acids was analyzed using the previously described instrument. The analysis was conducted with a splitless injection at an injection temperature of 100 °C, employing an Ultra-1 capillary column (Supelco, Prague, Czech Republic). The temperature program was initiated at 100 °C for one minute, followed by an increase at a rate of 20 °C/min to reach 180 °C, and subsequently at 2 °C/min until 280 °C, where it was held for one minute. Helium served as the carrier gas, maintaining a linear velocity of 60 cm/s. Mass spectra were acquired across the *m/z* range of 50–500, as detailed in Řezanka et al. (2015). Additional data can be found in Fig. 2 and in the Supplementary Materials (Fig. 1S).

2.5. Silver-ion chromatography (Ag^+-SPE) for PUFA separation

The fractionation procedure has been adapted from the method described by Christie (1989) with a few minor modifications. Bond Elut SCX cartridges (10 g, 60 mL) (Agilent Technologies, Inc., USA), wrapped in aluminum foil, were prepared by washing with a solution of 100 mg

Table 2
Fatty acid content from twenty cultivations after isolation of triacylglycerols.

Fatty acid	14:0	16:0	16:1ω7	17:2ω6	18:0	18:1ω9	17:3ω3	17:4ω3	18:2ω6	18:3ω6	18:3ω3	19:2ω5	20:0	20:1ω9	19:3ω5	19:4ω5	19:5ω2	20:3ω6	20:4ω6	20:5ω3
14/X42PDB[a]	4.2[b]	14.5	8.1	0.0	3.9	19.7	0.0	0.0	12.5	1.1	1.2	0.0	0.4	1.1	0.0	0.0	0.0	0.0	12.9	20.4
14/X42yeast	1.6	10.5	7.3	0.0	2.4	15.7	0.0	0.0	15.6	1.3	2.4	0.0	0.4	0.8	0.0	0.0	0.0	0.7	16.8	24.5
24/X42PDB	5.2	19.4	6.6	0.0	4.9	23.7	0.0	0.0	11.2	0.4	3.6	0.0	1.0	1.3	0.0	0.0	0.0	0.0	9.5	13.2
24/X42yeast	3.1	19.5	11.7	0.0	4.0	8.9	0.0	0.0	20.3	0.6	2.4	0.0	0.0	0.9	0.0	0.0	0.0	1.3	12.5	14.8
14/M1PDB	0.9	11.6	6.8	0.0	1.8	16.3	0.0	0.0	11.3	1.5	3.7	0.0	1.0	1.7	0.0	0.0	0.0	0.9	18.5	24.0
14/M1yeast	2.3	10.0	5.8	0.0	2.3	13.4	0.0	0.0	10.7	1.7	4.3	0.0	0.2	1.3	0.0	0.0	0.0	1.7	19.3	27.0
24/M1PDB	3.4	13.4	13.0	0.0	3.1	16.0	0.0	0.0	17.6	0.9	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	12.5	19.1
24/M1yeast	5.6	18.1	6.2	0.0	2.5	20.5	0.0	0.0	14.2	0.8	2.4	0.0	0.0	0.0	0.0	0.0	0.0	1.2	13.6	14.9
14/X42PDB + Pr	1.6	7.1	5.5	4.2	2.1	14.2	3.0	3.7	10.6	0.6	0.9	3.3	0.5	0.1	4.5	5.2	9.3	0.4	9.5	13.7
14/X42PDB + Pr + TW*	0.8	8.4	4.8	3.6	2.3	11.6	5.5	7.6	7.4	0.8	0.8	2.9	0.2	0.7	5.2	6.8	10.7	0.5	7.6	11.8
14/X42yeast + Pr + TW	2.1	7.9	5.2	4.1	1.9	11.8	4.6	6.5	7.7	0.9	0.9	3.0	0.5	0.6	6.2	7.3	11.4	0.3	6.9	10.2
14/M1PDB + Pr	0.9	10.5	4.9	5.1	2.0	15.4	3.2	4.1	9.5	0.8	1.0	2.6	0.6	0.2	5.0	6.2	8.9	0.5	8.2	10.4
14/M1PDB + Pr + TW	0.7	9.5	4.2	4.8	2.1	12.4	4.8	6.8	7.7	0.5	1.0	2.7	0.3	0.6	5.6	7.1	12.3	0.4	6.9	9.6
14/M1yeast + Pr + TW	3.3	8.4	4.7	3.7	2.0	9.8	4.2	5.8	6.4	0.7	1.0	3.2	0.4	0.7	7.3	8.1	12.8	0.6	7.2	9.7
24/X42PDB + Pr	1.1	10.6	1.3	2.5	1.5	14.6	3.3	3.6	13.6	0.6	0.8	1.7	0.4	3.1	4.9	3.9	8.9	1.5	10.6	11.5
24/X42PDB + Pr + TW	2.6	11.9	3.6	3.3	2.0	9.9	3.8	4.6	14.4	0.7	0.8	2.4	1.8	3.0	4.2	3.3	9.9	1.1	8.8	7.9
24/X42yeast + Pr + TW	4.3	17.4	5.0	2.8	3.8	16.9	3.9	5.4	8.0	0.4	0.7	1.7	0.5	1.0	3.6	2.8	9.4	0.0	4.2	8.2
24/M1PDB + Pr	2.7	7.9	4.6	3.6	1.5	23.9	3.8	4.4	9.6	0.8	4.7	2.1	0.0	0.0	4.9	5.9	8.1	0.9	2.4	8.2
24/M1PDB + Pr + TW	2.9	11.6	4.2	4.2	0.8	11.6	4.2	5.1	10.3	0.5	0.9	2.5	0.3	0.9	5.2	4.2	8.9	1.3	7.8	12.6
24/M1yeast + Pr + TW	1.4	14.7	4.6	4.0	7.7	18.1	3.7	5.3	7.8	0.3	0.0	2.8	0.0	0.0	4.2	5.3	8.7	3.0	3.0	5.4

[a] Abbreviations see Table 1.
[b] For statistics, see Tables 1Sb.

$AgNO_3$ dissolved in 2.5 mL of acetonitrile-water (10:1, *v/v*). Before use, the cartridges were conditioned sequentially with 5 mL of acetonitrile, 5 mL of acetone, and 10 mL of dichloromethane. Approximately 1 mL of FAEE solution, containing 5–10 mg of FAEEs prepared as detailed in the preceding section, was loaded onto the column. Elution was performed with solvents as outlined in Table 2S (see Supplementary Data; Christie, 1989). Each collected fraction was subsequently analyzed using GC–MS or RP-HPLC/MS.

2.6. Semipreparative RP-HPLC for PUFA purification

The semipreparative RP-HPLC setup comprised a 1090 Win system equipped with a PV5 ternary pump capable of handling pressures up to 400 bar (5801 psi), an automated injector (HP 1090 series, Hewlett Packard, Palo Alto, CA, USA), and a Supelcosil™ LC-18 column with 5 μm particle size, measuring 25 cm in length and 10 mm in internal diameter. Chromatographic separation was achieved at a flow rate of 4.0 mL/min using a linear gradient elution. The mobile phase transitioned from an aqueous solution of 1 mM ammonium acetate-ammonia (pH 7) to a mixture of acetonitrile and ethanol (50:50) containing 1 mM ammonium acetate over 25 min. The column temperature was maintained at 35 °C, with a re-equilibration interval of 30 min between runs. The column efficiency was approximately 11,000 plates per 25 cm. Ethyl laurate was employed as an internal standard. A splitter diverted 2 % of the column effluent to the APCI-MS detector, while the remaining 98 %, containing the ethyl ester fractions, was manually collected.

Under the conditions below, APCI-MS analysis was conducted using a high-resolution hybrid mass spectrometer (LTQ Orbitrap Velos, Thermo Fisher Scientific, Bremen, Germany).

The LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was operated in positive FT ionization mode for APCI-MS analysis. Mass spectra were acquired at a resolution of $R = 90{,}000$ at m/z 400, with diisooctyl phthalate (391.2842 Da) serving as the lock mass. The ion spray voltage was set to +2500 V, with a scan range of m/z 70–700. The spray current was 4.0 μA, while the ion source and capillary temperatures were maintained at 350 °C and 300 °C, respectively. Nitrogen was used as both the sheath gas (40 arbitrary units) and auxiliary gas (10 arbitrary units). Helium was utilized as the collision gas for collision-induced dissociation (CID) experiments, with a normalized collision energy of 35 %. Fragment ions were detected in high-resolution FT mode. The mass spectrometer was calibrated using Pierce LTQ Orbitrap negative ion calibration solution (Thermo Fisher Scientific, San Jose, CA, USA), achieving a mass accuracy better than 0.9 ppm. Chemical structures were identified by matching with spectra from the LIPID MAPS® Lipidomics Gateway database (http://www.lipidmaps.org/). Full experimental details are provided in the Supplementary Data.

2.7. Determination of geometric configuration of double bonds

A Perkin–Elmer Model 1310 (Perkin–Elmer, Norwalk, CT) infrared spectrophotometer was used for scanning infrared spectroscopy of ethyl esters as neat film.

2.8. Statistical analysis

Multivariate statistical analysis was performed using the CANOCO 5 software (Microcomputer Power, USA). Principal component analysis (PCA) was employed to visualize data set variability. Detailed explanations and visualizations are provided in Figs. 2S, 3S, and 4S in the Supplementary Data.

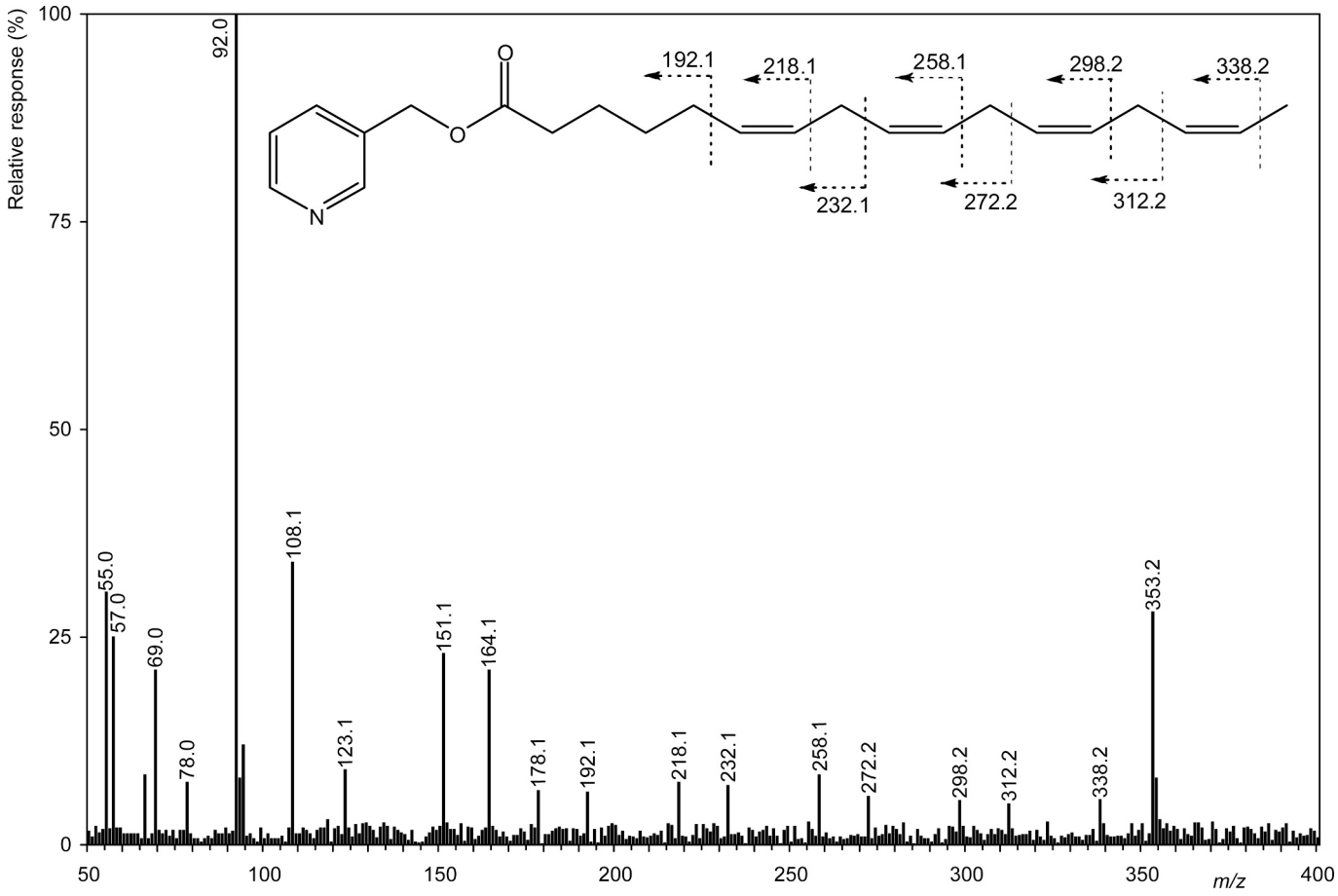

Fig. 1. The mass spectrum of the 3-pyridyl carbonyl ester of 6Z,9Z,12Z,15Z-heptadecatetraenoic acid (17:4ω2).

3. Results and discussion

3.1. Total lipid and triacylglycerol content

The content of total lipids, including most neutral lipids, is shown in Table 1. Compared to previously published data (Bhatia et al., 1972; Russo et al., 2023; Stinson et al., 1991), our cultivation using PDB and yeast had comparable biomass yields. However, with the addition of propionic acid, biomass production or total lipids, including TAGs, decreased (Table 1). After adding propionic acid and TWEEN 80, biomass production increased, as confirmed by statistical analysis (Figs. 2S, 3S, and 4S), which shows the dependence of biomass weight on added propionic acid and TWEEN 80. However, comparing the yields of both total lipids and TAGs is completely different. Although it is clear from Table 1 that the absolute values, i.e., how many total lipids and total TAGs, differ depending on the type of culture by two orders of magnitude, the percentage representation of both total lipids and TAGs is essentially the same. For example, in 14/X42PDB cultivation (biomass yields 2533 mg/L and 795.4 mg of total lipids, that is, 31.4 %) versus 14/X42PDB + Pr (biomass yields 86 mg/L and 21.1 mg of total lipids, which is 24.5 %). Published data on the amount of both total lipids and TAGs vary significantly, mainly depending on the strain and cultivation conditions used. A yield of 55.4 % (Ghasemi et al., 2012), 54.2 % (Bhatia et al., 1972), or 60.4 % (Bhatia et al., 1973) TAGs based on total lipids have been published.

The principal component analysis of the FA composition clearly separated the samples along the first axis according to the type of cultivation media used. More specifically, the addition of propionic acid (and TWEEN 80), had a great influence on the FA composition. In particular, the proportion of C17 and C19 FA increased markedly compared to the samples originating from media without propionic acid (and TWEEN 80, see Table 2). The second axis, which explained a significantly lower percentage of the total variability compared to the first axis, seems to be associated with cultivation temperature. Higher temperature (24 °C) enhanced the proportion of saturated and mono-unsaturated FA (except for C20:0 and C20:1ω-9, which followed an opposite trend). On the other hand, there was no discernable effect of strain, as their samples were fairly intermixed in the analysis (Fig. 2S).

To uncover in more detail, if the addition of TWEEN 80 specifically influences FA composition, only the samples cultivated in media with propionic acid (Pr) and propionic acid and TWEEN 80 (PrTW) were subject to the principal component analysis. The separation of most samples with TWEEN 80 from those with propionic acid only along the first axis confirmed the effect of TWEEN 80, which further increased the proportion of C17 and C19 FA. However, some PrTW samples had the same scores on the first axis as samples with propionic acid only. The second axis was associated with slightly different proportion of two C20 polyunsaturated FA (20:4 and 20:5) and C18:0 on the other hand (Fig. 3S).

It is possible to exchange these media for further cultivations, which is advantageous for large-volume cultivations. The addition of propionic acid alone significantly reduces the production of biomass, as well as lipids and triacylglycerols. On the contrary, after the addition of TWEEN 80, biomass, lipids, and triacylglycerols production is comparable to cultivation only on PDB medium or waste yeast. All these data were used to analyze further and identify fatty acids; see below.

Using alternative carbon sources, such as waste brewery yeast, offers economic and environmental benefits. Brewery by-products are rich in nutrients that can support microbial growth and lipid synthesis, making them a promising low-cost substrate. Moreover, utilizing such waste

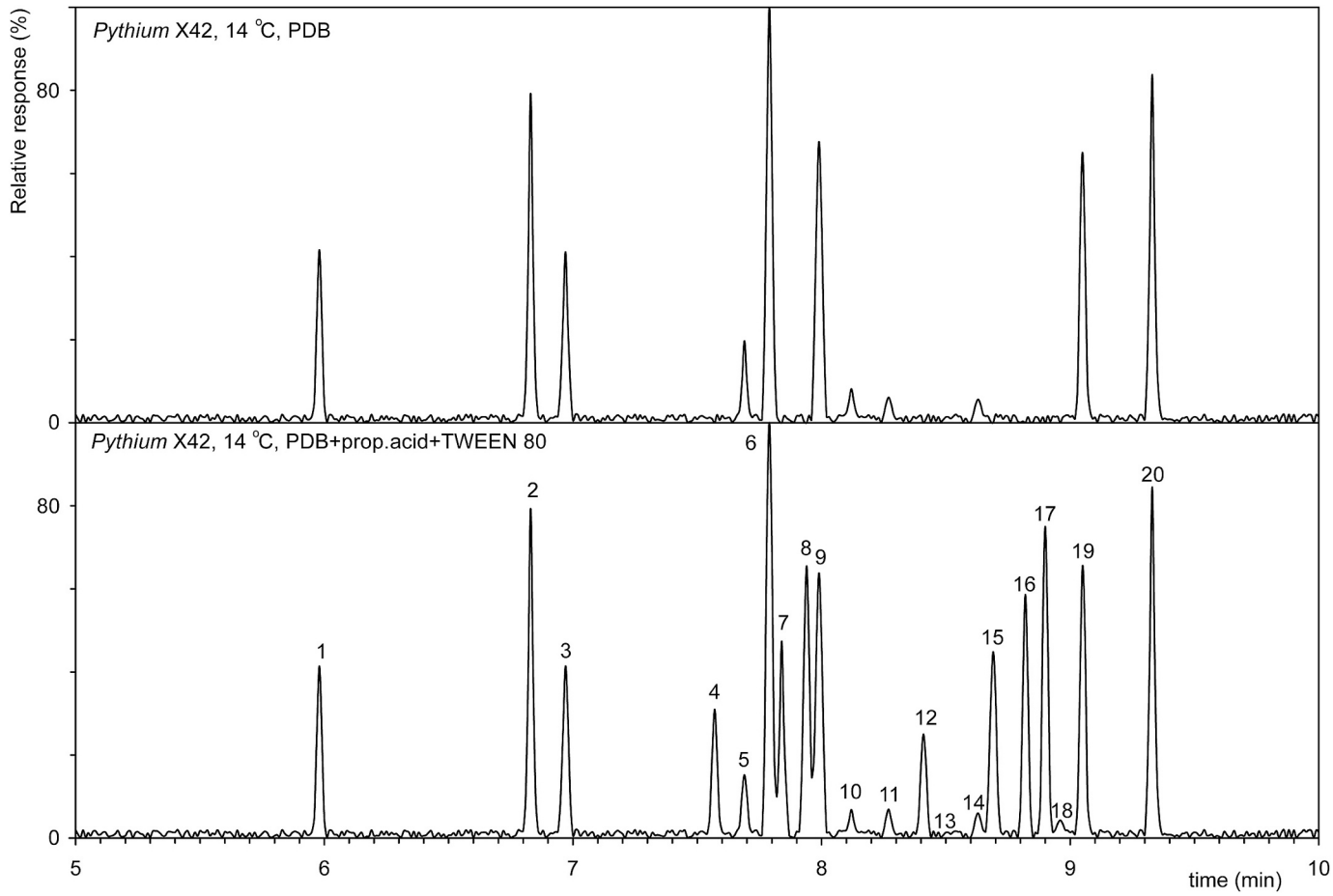

Fig. 2. GC-MS separation of mixtures. Above: FAEEs of the *Pythium* X42 strain cultured at 14 °C, PDB medium, below the FAEEs of the strain X42 cultured at 14 °C, PDB medium with propionic acid and TWEEN 80.

materials aligns with circular economy principles by reducing industrial waste and promoting sustainability. Notably, the application of food industry by-products for microbial fermentation can contribute to producing high-value bioactive lipids with potential nutraceutical applications. Odd-chain PUFAs have gained attention for their possible health benefits, including anti-inflammatory properties and metabolic regulation. Therefore, harnessing microbial biosynthesis from sustainable sources could facilitate the development of novel functional ingredients for human consumption while simultaneously addressing waste management challenges in the food industry. Recent techno-economic analyses have demonstrated that microbial fermentation using industrial by-products, such as brewery waste, is a cost-effective and scalable strategy for producing high-value compounds (Ryu et al., 2013; Serra et al., 2023). Studies on thraustochytrids cultivated on brewery waste have shown that such substrates can maintain high biomass yields while significantly reducing production costs (Russo et al., 2022). This approach has the potential to be adapted for *Pythium* sp., making the biotechnological production of odd-chain PUFAs not only environmentally sustainable but also economically viable. Future research should focus on optimizing fermentation conditions and performing life-cycle assessments to fully evaluate the feasibility of integrating food waste into large-scale microbial lipid production.

3.2. Fatty acid composition and identification

Table 2 shows the fatty acid content of the two cultivated oomycetes *Pythium oligandrum* ATCC 38472 M1 and *Pythium* sp. X42. Fatty acids, particularly odd-chain PUFAs were identified by GC-MS of fatty acid ethyl esters on a polar capillary column (Fig. 1). The PUFA structures were confirmed using 3-pyridylcarbinol esters (formerly called picolinyl esters). The mass spectra of these derivatives feature abundant ions at *m/z* 92, 108, 151 (the McLafferty rearrangement), and 164 Da. The structure can be determined based on characteristic fragment ions, where the typical 14 Da interval corresponds to sequential losses of -CH_2 groups along the fatty acid chain. In the presence of a double bond, the fragmentation pattern shifts, resulting in interval changes to 26 Da (loss of -CH=CH-) or 40 Da (loss of -CH_2-CH=CH-).

The mass spectrum of the peak with RT 7.94 min, the 3-pyridylcarbinol ester of all-*Z*-6,9,12,15-heptadecatetraenoic acid (C17:4ω2) (Fig. 2), showed a gap of 26 Da between *m/z* 338.2 and 312.2, which locates the double bond at position 15 while the gap between *m/z* 298.2–272.2 indicates the double bond at position 12. Other gaps at *m/z* 258.1–232.1 and 218.1–192.1 unambiguously confirm the structure C17:4ω2.

Another example of the 3-pyridylcarbinol ester of all-*Z*-5,8,11,14-nonadecatetraenoic acid (C19:4ω5) is shown in Fig. 1S. The position of the double bonds was determined in the mass spectrum of the 3-pyridylcarbinol ester. The 26 and 40 Da values are apparent in the mass spectrum of this compound, showing similarity to the mass spectrum of the longer homologue, that is, 3-pyridylcarbinol-5,8,11,14-eicosatetraenoate (C20:4ω6) (Christie, 2024; Wolff et al., 1999).

Other PUFAs, as seen in Table 2, were similarly identified. All double bonds have a *cis* (more recently *Z*) geometric configuration, as determined by infrared (IR) spectra (Christie, 2003; Christie & Han, 2010). The IR spectrum completely lacks absorption of the CH bond in the range of 960–980 cm^{-1}, i.e., absorption of the double bond with geometric *trans* configuration (more correctly *E*). For further cultivations and analyses, two cultivations were selected based on the principal component analysis; see Figs. 2S and 3S. The addition of propionic acid,

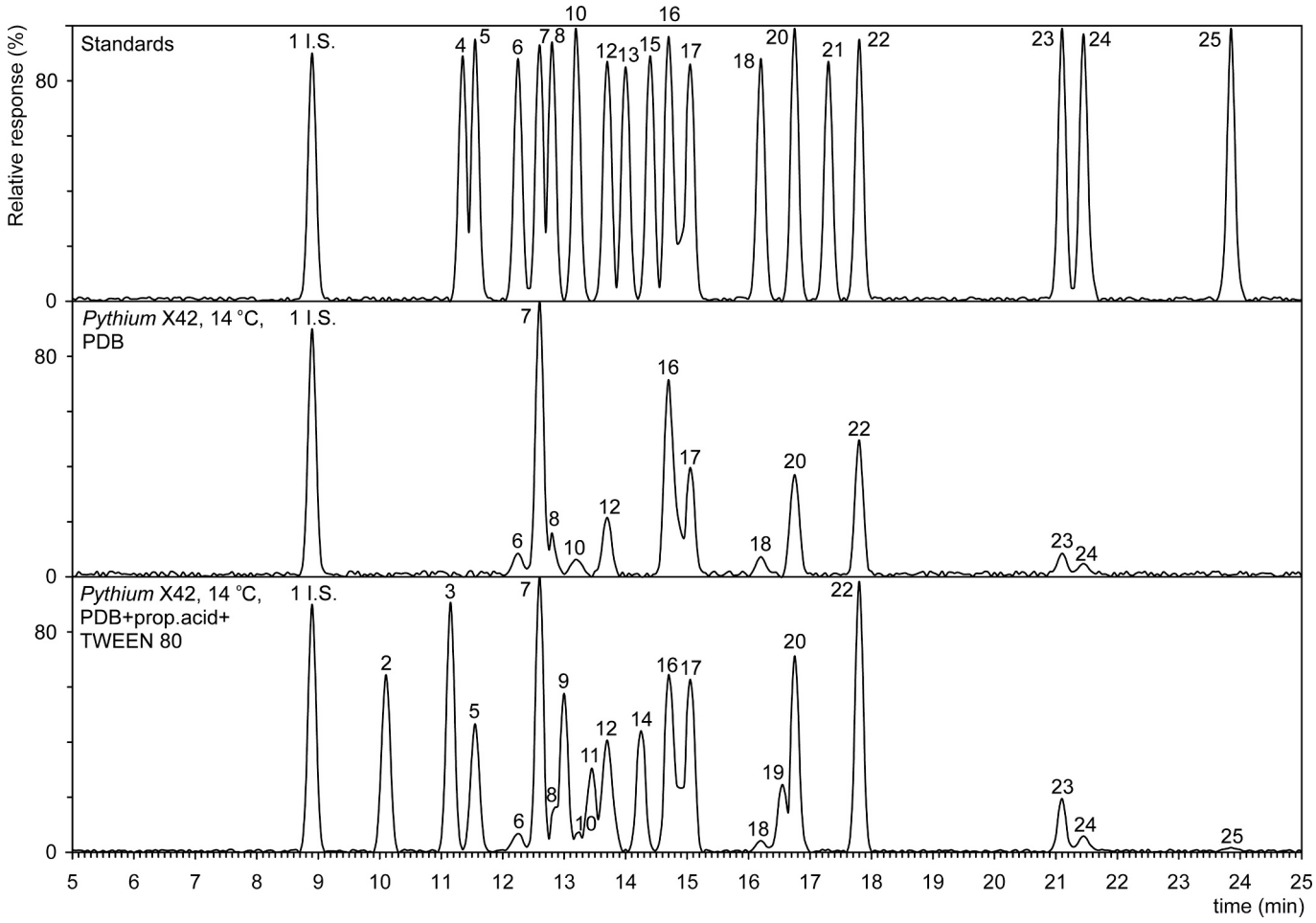

Fig. 3. RP-LC/MS-APCI$^+$ separation of mixtures. Top: FAEEs standards, middle: FAEEs of the *Pythium* X42 strain cultured at 14 °C, PDB medium, bottom: FAEEs od strain X42 cultured at 14 °C, PDB medium with propionic acid and TWEEN 80.

possibly even with TWEEN 80, has a great influence on the composition of FAs. This does not apply to strain or cultivation temperature.

RP-HPLC is most suitable for preparing at least tens of mg of odd-chain PUFAs. This method separates fatty acids or their derivatives by the chain length and degree of unsaturation. The first double bond reduces the effective chain length by a little less than two carbon units, so the oleic acid elutes just after the palmitic acid. The second and subsequent double bonds have less effect on retention; for example, a linolenic acid elutes just before a myristic acid. The stationary phases used are almost exclusively of the octadecylsilyl type. The mobile phase is either acetonitrile or methanol, containing some water. Atmospheric pressure chemical ionization (APCI) with the connection of RP-HPLC (RP-LC/MS-APCI) can be extremely sensitive, providing structural information about the components. A change in the order of elution of specific components is possible with different gradients of acetonitrile and water, which also applies to a mixture of methanol and water or to a ternary mixture of methanol-acetonitrile-water or acetonitrile-tetrahydrofuran-water. Furthermore, reversed-phase HPLC of fatty acid derivatives is an extremely useful technique, particularly for micropreparative (semipreparative) separations of fatty acids. The analysis of ethyl esters of fatty acids (FAEEs) from two cultures of *Pythium* by RP-HPLC is shown in Fig. 3. The separation of 19 FAEEs standards is also shown in Fig. 3 (top), and almost all were separated at the baseline. Their identification was performed by tandem MS/APCI. As already described (Cai & Syage, 2006; Garlito et al., 2019; Li et al., 2020), the base peak in the spectrum of all FAEEs is the $[M + H]^+$ ion, the other majority of ions are of the $[M + H\text{-}46]^+$ type, i.e., loss of EtOH and additional water, i.e., ions of the type $[M + H\text{-}EtOH\text{-}H_2O]^+$.

In the case of the real mixture, i.e., FAEEs after cultivation in PDB medium (14/X42PDB), complete separation was observed for peaks 7 and 8 (C20:5ω3 vs. C18:3ω3). If FAEEs were collected from biomass after cultivation with propionic acid and TWEEN 80 (14/X42PDB + Pr + TWEEN 80), then separation was not sufficient to obtain individual PUFAs, which was the objective of this publication. Therefore, in the next stage, the separation of PUFAs on the number of double bonds and subsequent semipreparative RP-HPLC was carried out by argentation chromatography.

Commercially available ion exchange columns containing chemically bound benzenesulfonic acid, such as Bond Elut SCX columns, can be converted to silver ions and used to achieve the separation of esters of FAs. Based on the published procedure (see also Materials and Methods), FAs were used to divide ethyl esters according to the number of double bonds. A satisfactory resolution of FAEEs with zero to six double bonds was achieved, as seen in Fig. 4. Since substantial changes in solvent composition are used in each step, little cross-contamination occurs, again evident in Fig. 4.

The use of *Pythium* strain to produce PUFAs has already been described many times. Table 3S lists a dozen publications in which FAs were identified under different conditions, i.e., using different strains, media compositions, and cultivation temperatures. As can be seen from the attached table, the representation of individual FAs, especially PUFAs, varies greatly. No odd-chain PUFAs were identified in either of the papers.

Although many dozens of papers have been published on the topic

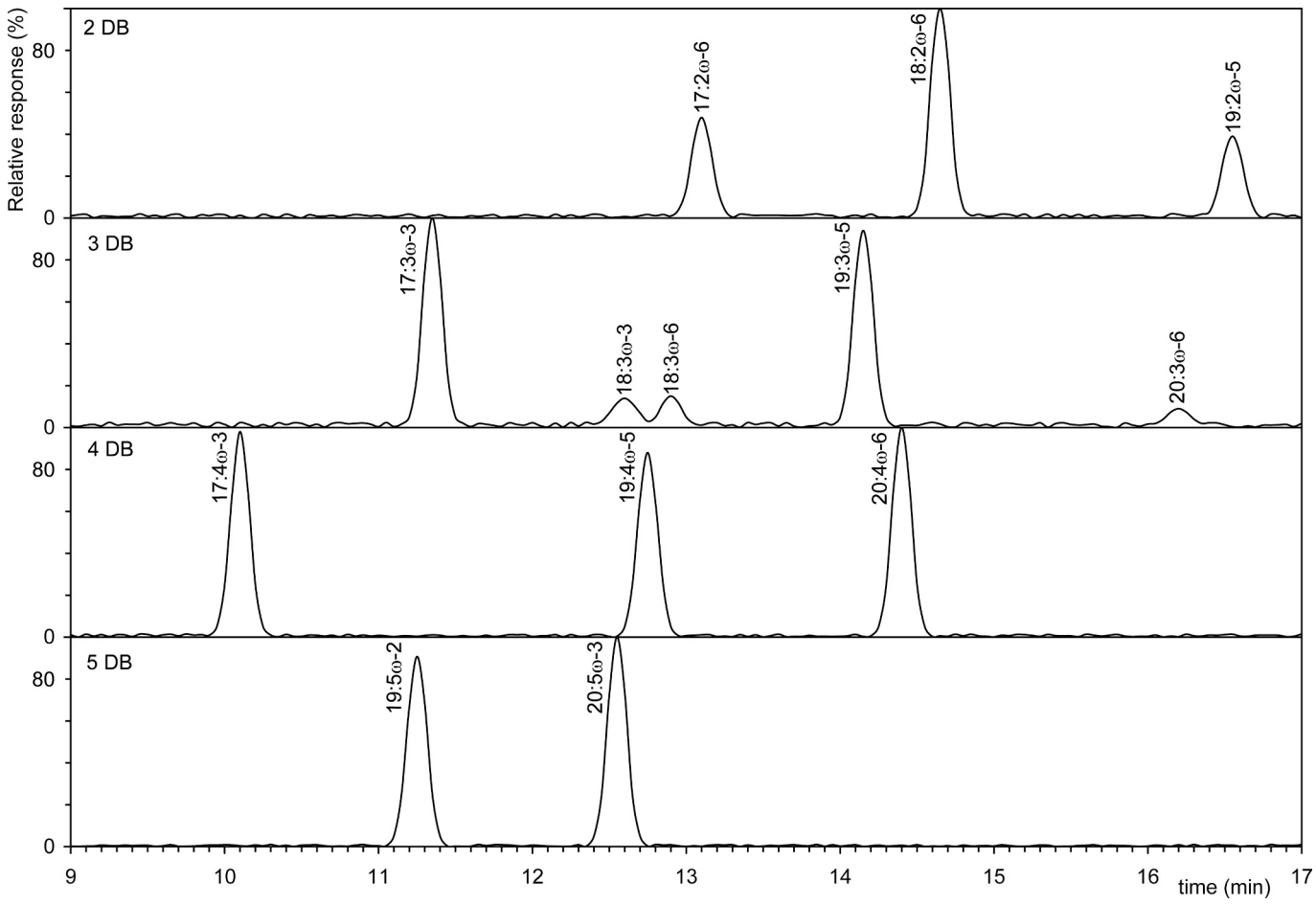

Fig. 4. Separation of PUFAs after Ag^{+}-SPE fractionation. RP-LC/MS-APCI^{+} separation conditions, see Fig. 3.

describing the occurrence of odd-chain PUFAs, including several reviews (Chang et al., 2011; Diedrich & Henschel, 1990), their occurrence in real natural samples is generally limited to the lower percentage units of total FAs. Only in a few papers was the content of odd-chain PUFAs determined as a percentage of total FAs. So far, the only method that can be used to increase the content of odd-chain PUFAs is precursor direct biosynthesis, which includes the addition of odd-chain FA (propionic acid and/or C15:0 or C17:0) or odd-chain hydrocarbons to the medium.

The TAGs (C16:0/C17:2/C20:4, C19:1/C17:2/C19:1, or C19:4/C20:4/C20:4) containing odd-chain PUFAs of the genus *Mortierella* are capable of synthesizing odd-chain TAGs, even if a precursor with an odd-number of carbon atoms is not used (Lu et al., 2019). Several publications have addressed the change in the content of PUFAs in *Mortiellera* fungi after adding odd-chain precursors. For example, Shimizu et al. (Shimizu et al., 1991) added methyl pentadecanoate or n-pentadecane to the culture medium. This change affected the biosynthesis of PUFAs, where even odd-chain PUFAs were identified, e.g., C17:2, C17:3, C19:3, or C19:4. Similarly, a mutant of *Mortierella alpina*, which is defective in its Δ^{12} desaturase activity, produced a high proportion of odd-chain FAs, predominantly 6,9-C17:2, 8,11-C19:2, and 5,8,11-C19:3, when grown on 3 % n-heptadecane (Zhang et al., 2006).

Our production of odd-chain PUFAs is comparable to already published results, e.g., with the production of mutant *Mortierella alpina* (Zhang et al., 2006). The highest yield of 19:3, i.e., 10.1 mg/L g dry mycelia, is comparable to our culture (9.3 mg/L g dry mycelia). If we add the yield of the three odd-chain PUFAs (C17:2, C19:2, and C19:3) as the authors, we again obtain comparable yields, i.e., the published 29.7 vs. 21 mg/L g dry mycelium. Furthermore, in our case, the sum of all odd-chain PUFAs is about 76 mg/L g of dry mycelia. Similarly, it is also the case in another described publication (Shimizu et al., 1991), where the yield of C19:4ω5 reached 19.8 mg/L dry mycelia, and our yield was 12.2 mg/L g dry mycelia.

Another fungus, *Saprolegnia* sp., grown with n-pentadecanoic acid methyl ester, produced 9,12-heptadecadienoic acid (C17:2ω5), 5,8,11,14-nonadedecatetraenoic acid (C19:4ω5) or 5,8,11,14,17-nonadecapentaenoic acid (C19:5ω2) (Shirasaka et al., 1995). Odd-chain PUFAs have also been identified in other microorganisms. Odd-numbered very-long-chain polyunsaturated fatty acids from the dinoflagellate *Amphidinium carterae* were identified by atmospheric pressure chemical ionization liquid chromatography–mass spectrometry (Řezanka et al., 2008).

4. Conclusion

To date, oleaginous microorganisms have been extensively studied for the microbial production of PUFAs, including precursor biosynthesis (Chun-Xiao et al., 2024). However, in contrast to the already well-known and extensively published metabolism of odd-chain saturated fatty acids, e.g., pentadecanoic (C15:0) and heptadecanoic acid (C17:0), see e.g., Jenkins et al. (Jenkins et al., 2015) and papers cited there, as well as completely new studies (Jiang et al., 2024; Lei et al., 2024) the metabolism of odd-chain PUFAs has only been investigated to a limited extent. The metabolism, or chain elongation of C19 to C21 odd-chain PUFAs in rat livers, has been published (Nakano et al., 2000). Although many articles have focused on microbial production, as cited in several reviews (Qiao et al., 2023; Qin et al., 2023; Zhang et al., 2020), systematic overproduction of odd-chain PUFAs, to the best of our knowledge, has not yet been thoroughly explored. The potential for

preparing odd-chain PUFAs enables the production of quantities on the order of tens of milligrams of odd-chain PUFAs. When transferred to a fermenter, this can be scaled to tens or even hundreds of grams, making it possible to study its metabolism and potentially use it as a food supplement or additive in reformulated foods.

Another application of odd-chain PUFAs obtained through precursor-directed biosynthesis is their use in the synthesizing standards of the respective TAGs. Recently, it was published (Zhang et al., 2023) that odd-chain PUFAs in TAGs (e.g., C19:2 to C19:5) serve as biomarkers for profiling in clinical colon cancer.

CRediT authorship contribution statement

Andrea Palyzová: Software, Data curation. **Markéta Kulišová:** Methodology, Data curation. **Katarína Majtán:** Resources, Methodology. **Karel Fous:** Formal analysis, Data curation. **Irena Jarošová Kolouchová:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Tomáš Řezanka:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization.

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Peak No. (FA); 1 (14:0); 2 (16:0); 3 (16:1ω-7); 4 (17:2ω-6); 5 (18:0); 6 (18:1ω-9); 7 (17:3ω-3); 8 (17:4ω-3); 9 (18:2ω-6); 10 (18:3ω-6); 11 (18:3ω-3); 12 (19:2ω-5); 13 (20:0); 14 (20:1ω-9); 15 (19:3ω-5); 16 (19:4ω-5); 17 (19:5ω-2); 18 (20:3ω-6); 19 (20:4ω-6); 20 (20:5ω-3).

Peak No. (FA); Peak No (FAEE); 1 (12:0 I.S.); 2 (17:4ω-3); 3 (19:5ω-2); 4 (19:5ω-3); 5 (17:3ω-3); 6 (14:0); 7 (20:5ω-3); 8 (18:3ω-3); 9 (19:4ω-5); 10 (18:3ω-6); 11 (17:2ω-6); 12 (16:1ω-7); 13 (19:3ω-3); 14 (19:3ω-5); 15 (19:3ω-6); 16 (20:4ω-6); 17 (18:2ω-6); 18 (20:3ω-6); 19 (19:2ω-5); 20 (16:0); 21 (19:2ω6); 22 (18:1ω-9); 23 (18:0); 24 (20:1ω-9); 25 (20:0).

Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.144204.

Data availability

Data will be made available on request.

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