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Lipid accumulation by oleaginous and non-oleaginous yeast strains in nitrogen and phosphate limitation

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Abstract We investigated the possibility of utilizing both oleaginous yeast species accumulating large amounts of lipids (*Yarrowia lipolytica*, *Rhodotorula glutinis*, *Trichosporon cutaneum*, *Candida* sp.) and traditional biotechnological non-oleaginous ones characterized by high biomass yield (*Kluyveromyces polysporus*, *Torulaspora delbrueckii*, *Saccharomyces cerevisiae*) as potential producers of biofuel-utilizable and nutritionally valuable lipids. The main objective was to increase lipid accumulation by increasing C/P ratio together with higher C/N ratio, while maintaining high biomass yield. The C/N ratio of 30 was found to lead to higher biomass content and the total lipid content increased significantly with higher C/P ratio. With higher ratios of both C/N and C/P, the content of monounsaturated fatty acids (FAs) in cell lipids increased while polyunsaturated FAs decreased. Oleaginous yeast species had a lower proportion of unsaturated FAs (approx. 80 %) than non-oleaginous strains (approx. 90 %). At a C/N ratio of 30 and C/P ratio 1043, *T. cutaneum* produced a high amount of ω-6 unsaturated linoleic acid, the precursor of some prostaglandins, leukotrienes, and thromboxanes, while *Candida sp.* and *K. polysporus* accumulated a high content of palmitoleic acid.

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Introduction

Microbial oils, so-called single cell oils (SCO), are produced by microorganisms such as bacteria, yeast, molds, and algae and belong among the possible substitutions for biodiesel production. Their main advantages are easy cultivability, seasonal independence, and favorable fatty acid composition (high ratio of C16 and C18) similar to plant oils (Li et al. 2007; Steen et al. 2010). Industrial utilization of yeasts lies in exploitation of their potential for production of enzymes, essences, proteins, and flavors (Ratledge 1982; Sugano et al. 1986). Genus *Rhodotorula* is utilized for its ability to produce high proportions of linoleic and linolenic acids (Granger et al. 1993a). It is also possible to acquire microbial polyunsaturated fatty acids (PUFA) which are employed in medicine for preventing blood platelet aggregation and for lowering plasma cholesterol (Dyerberg 1986; Lindberg and Molin 1993). PUFA support normal cell functions and influence the fluidity of membranes (Lomascolo et al. 1994; Ratledge 1982; Veen and Lang 2004). Like PUFA, also monounsaturated fatty acids have important uses; for instance, palmitoleic acid (a constituent of human skin) is utilized as an antibacterial agent in medicine (Wille and Kydonieus 2003) and as a protection against skin aging (Hayashi et al. 2003).

Increased lipid accumulation by microbial cells is observed under the conditions of deficiency of nitrogen (N), phosphorous (P), zinc (Zn), iron (Fe), magnesium (Mg), Cu, K, or Mn and under over-abundance of carbon (C), which is under these conditions transformed into storage lipids (Chen et al. 2013; Veen and Lang 2004). The ability of microorganisms to amass lipids can be used in utilization of waste materials such as lignocellulosic wastes, by-products in the industrial manufacture of organic acids, molasses, or glycerol, and for improving the economical balance of various other processes (Gouda et al. 2008; Hu et al. 2009; Papanikolaou and Aggelis 2002).

Medium composition and cultivation conditions play an important role in microbial lipid accumulation (Braunwald et al. 2013; Papanikolaou and Aggelis 2010). Apart from these factors, the ability of lipid accumulation is strain-dependent (Dey and Maiti 2013). Lipid producers are often oleaginous microorganisms that are able to store lipids to as much as 60 % of dry cell mass (Li et al. 2007; Yoon and Rhee 1983), but it is also possible to use non-oleaginous strains, which produce lipids in smaller amounts but with a high proportion of specific fatty acids (e.g., ω-6 unsaturated fatty acids) and are able to grow to high densities and provide high biomass yield on inexpensive substrates. Yeasts accumulate lipids mostly in the form of triacylglycerols (TAGs) whose nutritional (fat digestion, absorption), biochemical, and physical properties are affected by the distribution of fatty acids between the different positions of the TAG. The physiology of their accumulation has been well studied in *Saccharomyces cerevisiae*, which belongs to non-oleaginous yeast (Carman and Han 2011). Because of their high potential nutritive value, we explored various positional isomers of TAGs also in other species including oleaginous ones.

This study is focused on lipid accumulation abilities of seven yeast species, both oleaginous (*Candida* sp., *Rhodotorula glutinis*, *Yarrowia lipolytica*, *Trichosporon cutaneum*) and non-oleaginous (*Saccharomyces cerevisiae*, *Kluyveromyces polysporus*, *Torulaspora delbrueckii*) in conditions of N and N, P limitation. Changes in the ratio of saturated to unsaturated fatty acids, the content of palmitoleic acid (16:1) and linoleic acid (18:2), and the proportions of positional isomers of produced TAGs were monitored.

Materials and methods

Microorganisms

The yeast strains used in the present study were *Candida* sp. DBM 2163, *K. polysporus* DBM 2171 (CCY 30-5-10), *R. glutinis* CCY 20-2-20, *S. cerevisiae* DBM 2115, *T. delbrueckii* DBM 39, *T. cutaneum* CCY 30-5-10, and *Y. lipolytica* CCY 29-26-36 supplied by Culture Collection of Yeast, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, and by Collection of Yeasts and Industrial Microorganisms, University of Chemistry and Technology, Prague. For long-term storage, the stock cultures were maintained in 20 % glycerol at −60 °C. Malt extract agar (23 g/L, pH 7) was employed for short-term storage.

Cultivation conditions

The precultures of yeast strains were cultivated in 200 mL of YPD medium (20 g/L peptone, 10 g/L yeast extract, 20 g/L glucose, initial pH 6.0) in Erlenmeyer flasks on a rotary shaker at 150 rpm at 28 °C to the late exponential growth phase.

For lipid production, 200 mL of mineral medium in 500-mL Erlenmeyer flasks was inoculated with 6 mL of preculture to a final concentration of A_{600} 0.2 and incubated on a rotary shaker at 150 rpm and 28 °C. The mineral medium composition (according to Wu et al. 2010 and Gill et al. 1977) for N-limited growth (C/N 30, C/P 6) was (g/L) glucose (30), NH_4Cl (1.5), KH_2PO_4 (7), Na_2HPO_4 (2), yeast extract (1.5), and trace element solution 1 mL ($MnCl_2{\cdot}4H_2O$, 20; $FeSO_4{\cdot}7H_2O$, 1; $Na_2MoO_4{\cdot}2H_2O$, 1; $CaCl_2{\cdot}2H_2O$, 20). The mineral medium composition for N, P-limited growth (C/N 30, C/P 1043) was (g/L) glucose (30), NH_4Cl (1.5), KH_2PO_4 (0.05), Na_2HPO_4 (0), K_2SO_4 (4), yeast extract (1.5), and 1 mL of a trace element solution. The pH was adjusted to 6.0.

Glucose was added as carbon source to the concentration of 30 g/L. The cultivations were carried out for 96 h (until early stationary phase). After cultivation, the cells were centrifuged (9000 *g*, 10 min) and washed two times. Biomass was frozen at −75 °C and lyophilized.

Determination of dry cell mass

For the dry cell mass determination, 10 mL of culture in stationary phase of growth was filtered using vacuum pump and a pre-dried and weighed nitrocellulose filter (0.45 μm, Millipore) and the sample was washed several times. The dry cell mass was determined after drying the samples to a constant mass at 110 °C.

Lipid extraction

Lyophilized yeast was mixed with 2 mL of 0.1 mol/L Na_2CO_3, and the mixture was briefly ground with ballotini glass beads (diameter 0.2 mm) in a mortar, overlaid with liquid nitrogen, and ground again. This process was repeated three times, and 50 mL of 0.1 mol/L Na_2CO_3 was finally added. The crushed yeast was extracted with a chloroform-methanol mixture according to Bligh and Dyer (1959). The sample was centrifuged and the lower phase was evaporated to dryness.

Chiral analysis of triacylglycerols

Chiral analysis of triacylglycerols was described previously (Rezanka et al. 2013a) Briefly, the triacylglycerols were separated by LC-MS (1090 Win system connected with AB Sciex API 4000 mass spectrometer, AB Sciex, Ontario, Canada) on chiral column Astec CYCLOBOND™ I 2000 DMP (3,5-dimethylphenyl carbamate modified β-cyclodextrin) and identified by Applied Biosystems Analyst version 1.4.2 software.

Statistical analysis

Statistical analysis was performed with Sigma Stat3.5 (USA). In one-way ANOVAs, the statistical significance of differences in mean values of the different measured parameters was calculated and compared with Tukey's test at the 5 % level of probability.

Results and discussion

The ability of cells to accumulate lipids is influenced by changes in the C/N or C/P ratio, i.e., the amount of nitrogen or phosphorous source in the medium. As mentioned above, lipid accumulation is enhanced by limitation of N or other medium components, such as P, Zn, Fe, Mn, or Mg (Gill et al. 1977; Chen et al. 2013; Veen and Lang 2004). In addition, it is also affected by culture conditions such as temperature, pH of the medium, and the length of cultivation. The data used in previous studies (Dey and Maiti 2013; Chen et al. 2013; Liu et al. 2013; Wu et al. 2010) served as a basis for our culture conditions: culture temperature 28 ° C, medium pH 6.0, and cultivation period of 96 h.

The N limitation is one of the most utilized approaches for intensifying lipid accumulation (Galafassi et al. 2012; Chen et al. 2013; Papanikolaou and Aggelis 2011; Razavi et al. 2007). We examined the effect of variation in medium C/N and C/P ratios on lipid content and composition of the FA. We first influenced the growth by N limitation (from original C/N 3 to C/N 30) while the C/P ratio was conserved at 6. A C/N ratio of 20 is considered as the minimum value that leads to induction of lipid production (Chen et al. 2013; Chi et al. 2011; Papanikolaou and Aggelis 2011). The amounts of saturated and unsaturated fatty acids in the lipids are displayed in Fig. 1. The C/N ratio of 30 led in all yeast strains to higher proportion of monounsaturated fatty acids. In *Candida* sp. the increase was from 50 to 70 %, in *R. glutinis* from 67 to 82 %, and in *T. delbrueckii* from 52 to 83 % of total FA. Only *T. cutaneum* had a higher proportion of polyunsaturated FA (46 %) than monounsaturated FA (32 %). At a C/N ratio of 30, all yeasts showed a decrease in the content of polyunsaturated FA. In *T. cutaneum* and *Y. lipolytica*, this was accompanied by a rise in saturated FA whereas *Candida* sp., *R. glutinis*, *K. polysporus*, *S. cerevisiae*, and *T. delbrueckii* exhibited a decrease in saturated FA.

In the next step, apart from N limitation (C/N 30), we also monitored the influence of P limitation. In terms of medium composition, the content of KH_2PO_4 was lowered to 0.05 g/L and Na_2HPO_4 was omitted, similarly as in the work of Gill et al. (1977). While the C/N ratio 30 was maintained, the C/P ratio was increased from 6 to 1043. This increase of C/P ratio did not influence the already observed effect of increased proportion of monounsaturated FA and decrease of polyunsaturated FA (in comparison to control cultivation). Under the influence of increased C/P ratio, the proportion of saturated and unsaturated FA did not change significantly in *Candida* sp., *R. glutinis*, *S. cerevisiae*, *T. delbrueckii*, and *Y. lipolytica*. For instance, at C/N 30 and C/P 1043, the oleaginous yeast *T. cutaneum* increased the content of monounsaturated FA (from 32 % at C/N 3 to 38 %) and decreased the polyunsaturated FA (from 50 % in unlimited conditions to 33 %)—see Fig. 1.

The total lipid content under the conditions of combined N, P limitation increased in oleaginous yeast strains in comparison to cultivation at a C/N ratio of 30 and C/P 6 and control cultivation at a C/N ratio of 3 (Fig. 2). This increase was between 10 and 15 % in favor of C/P 1043 compared with the C/P maintained during growth. There are only a few works

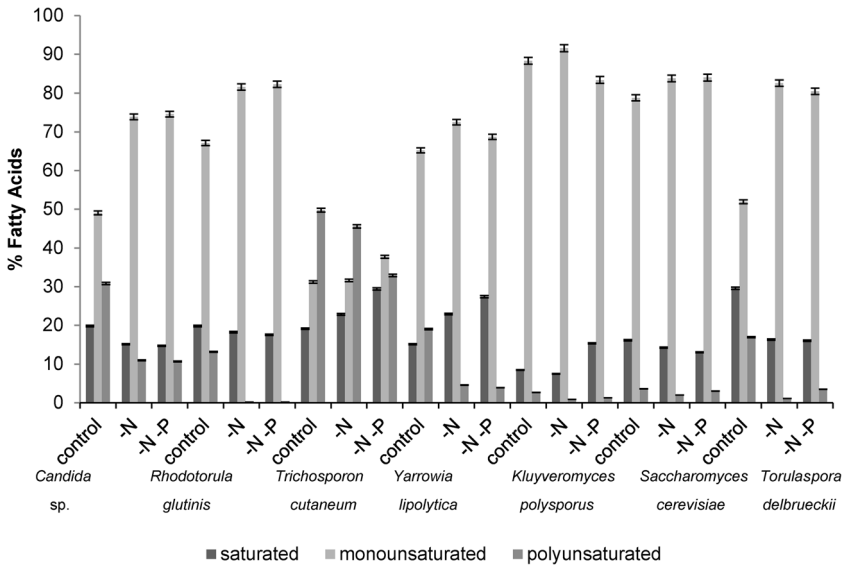

Fig 1 Comparison of saturated, monounsaturated, and polyunsaturated fatty acids produced by oleaginous (*Yarrowia lipolytica*, *Rhodotorula glutinis*, *Candida* sp., *Trichosporon cutaneum*) and non-oleaginous (*Torulaspora delbrueckii*, *Kluyveromyces polysporus*, *Saccharomyces cerevisiae*) yeast strains after 96 h growth in N and N, P limitation conditions

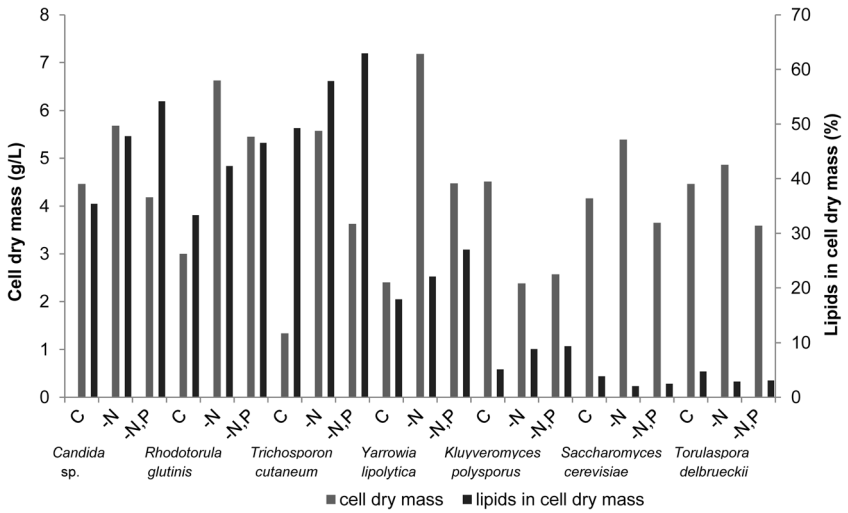

Fig 2 Comparison of lipid content and biomass yield of oleaginous and non-oleaginous yeast strains at C/N ratio 30 and at C/N 30 plus C/P 1043

that describe the cultivation conditions of oleaginous yeast species under phosphorus-limited conditions. Our results correspond with the data of Gill et al. (1977), in which *Candida* sp. in P limitation showed a lesser amount of accumulated lipids than in conditions of N-limitation while the concurrent application of both N and P limitation increased the amount of accumulated lipids above the value obtained by culturing in N-limitation (Gill et al. 1977) Granger et al. (1993a) described an up to 20 % increase of lipids in *R. glutinis* at the C/P ratio of 637. According to Wu et al. (2010) lipid accumulation by *Rhodosporidium toruloides* at a C/P ratio of 2045 reached almost 64 %.

In our work, the content of dry cell mass in N-limited conditions significantly increased in both oleaginous and non-oleaginous yeasts. In the cultivation in both N and P limitations, it slightly decreased, but with simultaneous slight increase of lipid content (Fig. 2). As reported by Wu et al. (2010) after the P source is depleted, the cells continue to accumulate lipid until the carbon source is utilized as well. Wu et al. (2010) used a glucose concentration of 70 g/L, which is more than twice the amount used in our work. It is therefore likely that if we used a similar approach, an increase of the carbon source content would lead to increase in both the lipid content and the amount of dry cell mass.

When Wu et al. (2010) and Ratledge and Wynn (2002) cultivated oleaginous yeasts in a nitrogen limited medium in the presence of excess glucose, cellular adenosine monophosphate (AMP) level dropped significantly due to the activation of AMP deaminase. As the mitochondrial isocitrate dehydrogenases in oleaginous species are allosterically activated by AMP, a reduced AMP level led to a buildup of citrate, which crossed the mitochondrial membrane into the cytosol to fuel lipid accumulation.

Depletion of phosphorus leads to a similar situation in which AMP is broken down to release inorganic phosphate for other cellular processes. Thus, both nitrogen and phosphorus limitation share a common biochemical consequence, a reduced mitochondrial isocitrate dehydrogenase activity due to depletion of the allosteric activator AMP (Wu et al. 2010).

Different strains have diverse lipid yields in different media (Tsigie et al. 2012). The theoretical lipid yield per consumed glucose is approximately 0.33 g/g glucose (Ratledge 1988). In practice, the lipid yield of oleaginous yeasts was reported at 0.15–0.23 g/g glucose (Granger et al. 1993b). The lipid yield found here for oleaginous strains was 0.05–0.09 g/g glucose. These amounts are apparently low, but they correspond to the stress conditions C/N 30 and C/P 1043.

Wu et al. (2010) reached similar values of lipid yield (0.08) in *R. toruloides* at a C/P ratio of less than 2000, which resembles our values observed in *Candida* sp., *R. glutinis*, and *T. cutaneum*. These results can be used for cultivation on waste substrates which usually have a higher nitrogen content and therefore a low C/N ratio and are not as suitable for the production of lipids based on a nitrogen-limited strategy. For example, Hua et al. (2007) cultivated *R. toruloides* on *Jerusalem artichoke* tuber hydrolysate at a C/N ratio of 23, reaching lipid production of 40 %, and (Hua et al. 2007) and Angerbauer et al. (2008) reported lipid content 34 % in *Lipomyces starkeyi* cultivation in C/N ratio 18 on a sewage sludge-based medium. It is easier to remove P from waste substrates than N, since soluble phosphate can be released by rapid hydrolysis using mineral or organic acids such as hydrochloric, sulfuric, or citric (Szogi and Vanotti 2009) and afterwards removed by precipitation using reaction with metal ions such as Mg, Fe, Al, or Ca ions (Liu et al. 2008).

The proportion of individual FA in studied yeast strains is shown in Table 1. An increase in the ratio of oleic acid (18:1) to linoleic acid (18:2) was observed for all oleaginous yeasts at C/N 30 and C/P 6 and also at C/N 30 and C/P 1043. Oleic acid was the most represented FA in oleaginous yeast, where its content ranged from 29 to 82 % (in both N and N, P

Table 1 Fatty acid composition of the total lipid (as relative fatty acid content %, wt/wt) produced by oleaginous (*Yarrowia lipolytica*, *Rhodotorula glutinis*, *Candida* sp., *Trichosporon cutaneum*) and non-oleaginous (*Torulaspora delbrueckii*, *Kluyveromyces polysporus*, *Saccharomyces cerevisiae*) yeasts cultured under N- and N, P limitation condition at 28 °C for 96 h

Yeast	Cultivation	16:0	16:1	18:0	18:1	18:2	Others
Candida sp.	C	17.4	8.8	2.4	40.2	30.8	0.4
	-N	14.8	16.0	0.3	57.5	11.0	0.4
	-N -P	14.2	14.4	0.5	60.2	10.3	0.4
Rhodotorula glutinis	C	12.6	0.2	7.2	66.4	13.1	0.5
	-N	13.0	0.2	5.1	81.0	0.2	0.5
	-N -P	12.1	0.2	5.3	81.7	0.2	0.5
Trichosporon cutaneum	C	17.3	2.6	1.8	28.4	49.5	0.4
	-N	18.7	2.4	4.1	29.0	45.4	0.4
	-N -P	24.3	1.0	5.1	36.7	32.5	0.4
Yarrowia lipolytica	C	13.9	14.0	1.2	51.2	19.0	0.7
	-N	20.5	16.2	2.1	56.1	4.4	0.7
	-N -P	21.4	14.7	5.7	53.8	3.7	0.7
Kluyveromyces polysporus	C	7.5	59.2	1.0	29.1	2.7	0.5
	-N	7.1	74.3	0.3	17.1	0.8	0.5
	-N -P	14.6	66.7	0.5	16.4	1.3	0.5
Saccharomyces cerevisiae	C	11.8	39.3	4.3	39.5	3.6	1.5
	-N	11.5	59.8	2.2	23.0	2.0	1.5
	-N -P	11.2	56.1	1.3	26.9	3.0	1.5
Torulospora delbrueckii	C	26.8	22.6	2.8	29.3	16.9	1.6
	-N	15.3	51.1	0.4	30.5	1.1	1.6
	-N -P	14.7	51.0	0.7	28.5	3.5	1.6

limitations) while the least represented FA was stearic acid, also see (Wu et al. 2010). By contrast, for non-oleaginous yeast the most represented FA was palmitoleic acid, its content being in the range of 52–75 % in both N and N, P limitations.

Among the other represented FAs were palmitic acid (16:0), palmitoleic acid (16:1), and linoleic acid (18:2). The content of unsaturated FA in non-oleaginous strains was 85–93 %; oleaginous genera had slightly lower proportion of unsaturated FA (71–85 %) in N and N, P-limitations. These results on the FA content resemble those shown in the literature (Hu et al. 2009) and are similar to the composition of vegetable oils. Microbial oils used for the production of biodiesel can therefore be produced in phosphate and nitrogen-limited conditions (Vicente et al. 2009).

Interesting results were observed in the content of linoleic acid (18:2), the precursor of some prostaglandins, leukotrienes, and thromboxanes, which belongs to the ω-6-polyunsaturated acids. These fatty acids cannot be synthetized by people and must be received in the diet. The yeast strains that can produce significant amounts of ω-6 FA may be suitable candidates for the commercial production of such acids for nutritional use (Sitepu et al. 2013). The best producer in our work proved to be the oleaginous yeast *T. cutaneum*, in which the linoleic acid content was approximately 50 % of total FA, which is exceptional. In *Trichosporon*, the total lipid content can reach up to 50 % in dry cell mass; therefore, the content of linoleic acid in its total mass can be substantial.

Another interesting oleaginous yeast with high linoleic acid content was *Candida* sp., in which the proportion of the essential FA was below 5 % of total lipids (in both studied limiting conditions).

Another fatty acid that is of great interest in medicine as an antimicrobial agent against bacteria is palmitoleic acid (Eldridge 2006; Wille and Kydonieus 2003). This FA may play a role in regulating melanin content and thereby can have influence on the graying of hair (Hu et al. 2011; Jeon and Cheon 2009) and is proposed for use in treating metabolic and cardiovascular diseases (Hernandez et al. 2013). Higher amounts of palmitoleic acid in the skin lead to increased resistance against the effects of solar radiation (Hayashi et al. 2003). From the oleaginous yeasts that were studied, *Candida* sp. produced palmitoleic acid in ratio more than 7 % of the total amount of lipids.

From the studied non-oleaginous yeasts, *K. polysporus* was exceptional in terms of palmitoleic acid production which reached a quantity exceeding 6 % of the total lipids. The remaining two non-oleaginous strains (*S. cerevisiae* and *T. delbrueckii*) displayed palmitoleic acid content above 50 % of the total FA, which corresponds to a value 1.5 % of the total amount of lipids. This low value corresponds with the low amount of lipids that is produced by these strains. Their great advantage is in that they are widely used in biotechnology (Rezanka et al. 2013b) and their biomass is produced in high quantities as waste material.

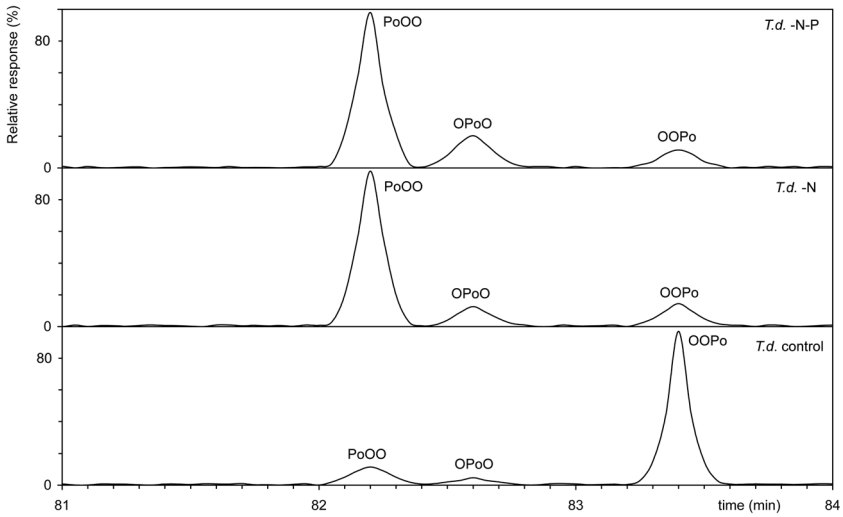

Fig 3 LC/ESI chromatogram of *Torulospora delbrueckii* TAGs at different C/N and C/P ratios

Nutrient limitation also influenced the proportional content of positional isomers of palmitoleic acid in cell triacylglycerols (Figs. 3 and 4). These proportional changes are different for different yeast species.

Analysis of TAGs on a chiral column showed that in *T. delbrueckii* and *K. polysporus*, -N and -N-P starvation brought about changes in the ratios of biosynthesized diastereoisomers and enantiomers. The control culture of *T. delbrueckii* contained an asymmetric diastereoisomer, i.e., the enantiomer *sn*-OOPo, as the major molecular species while under -N and -N-P starvation, it was the *sn*-PoOO enantiomer. Symmetric molecular species, i.e., OPoO, was always present in minor proportions.

Another abundant TAG, namely di-palmitoleine-olein, was studied in *K. polysporus*. All three of its molecular species, i.e., *sn*-PoPo, *sn*-OPoPo, and *sn*-PoPoO, were identified in varying proportions. Figure 4 shows that in this case, -N and -N-P starvation had no substantial influence on the abundance of the major molecular species, i.e., *sn*-PoPoO. These two examples (Figs. 3 and 4) clearly show that, although the two TAGs are structurally close, the acyltransferase activities responsible for their biosynthesis vary and depend not only on the yeast species but also on the physiological conditions of cultivation. Hence -N and -N-P starvation substantially affects the biosynthetic pathway of individual molecular species.

Conclusion

Our work shows that by increasing the medium C/N ratio together with the C/P ratio, one can effectively increase the lipid content of different oleaginous yeast species. Furthermore, to our knowledge, this is the first demonstration that, using such a method, the cultivation of different non-oleaginous yeasts may substantially increase the content of palmitoleic acid, which is used in medicine. Palmitoleic acid can reach up to 70 % of total FAs. We have also proposed the possibility of using *T. cutaneum* as a producer of nutritionally

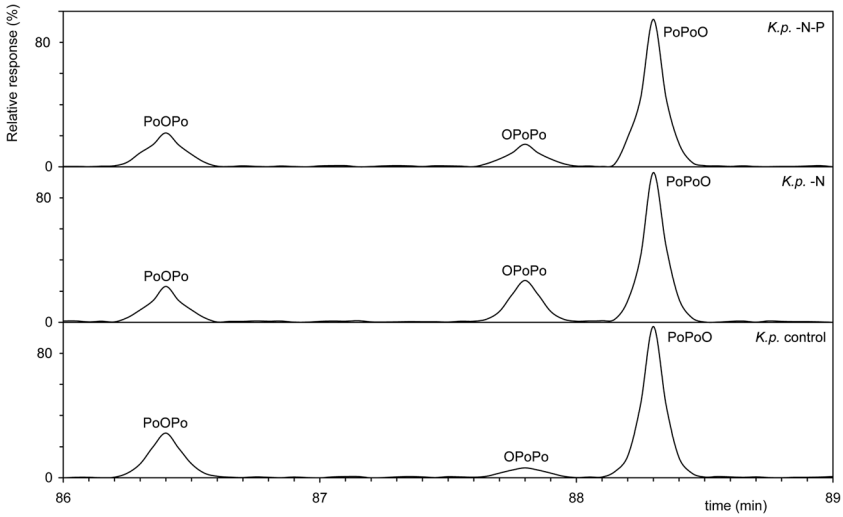

Fig 4 LC/ESI chromatogram of *Kluyveromyces polysporus* TAGs at different C/N and C/P ratios

valuable linoleic acid (ω-6 FA), which is produced to as much as 45 % total FAs. This method of cultivation represents a prospective strategy for the control of microbial lipid accumulation and may facilitate a cost-effective production of microbial lipids using relatively cheap raw materials as substrates.

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