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New yeast-based approaches in production of palmitoleic acid

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HIGHLIGHTS

- Six yeasts were cultivated at different medium C/N and C/P ratios.
- The highest production of palmitoleic acid was observed in *Candida krusei*.
- Palmitoleic acid content in *Candida* was close to that in mink oil and macadamia nuts.
- *S. cerevisiae* produced high and stable amount of palmitoleic acid in all conditions.
- Ammonium sulfate increased the amount of omega 6 linoleic acid.

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ABSTRACT

Palmitoleic acid is found in certain dairy products and has broad applications in medicine and cosmetics. We tried to find a suitable producer of this acid among traditional biotechnological yeast species (*Kluyveromyces polysporus*, *Torulaspora delbrueckii*, *Saccharomyces cerevisiae*) characterized by high biomass yield and *Candida krusei*, *Yarrowia lipolytica* and *Trichosporon cutaneum* accumulating large amounts of lipids. The main factor affecting the content of palmitoleic acid was found to be the C/N ratio in the culture medium, with ammonium sulfate as an optimum nitrogen source leading to highest biomass yield with concomitantly increased lipid accumulation, and an increased content of ω6-linoleic acid, the precursor of prostaglandins, leukotrienes, and thromboxanes. We found that *C. krusei* can be conveniently used for the purpose, albeit only under certain cultivation conditions, whereas *S. cerevisiae* can produce high and stable amounts of palmitoleic acid in a broad range of cultivation conditions ranging from conventional to nutrient limitations.

1. Introduction

Palmitoleic acid is a common constituent of the glycerides of human adipose (fat) tissue and is found in the lipid bilayer of the cell membrane in all human tissues where it can participate in several metabolic processes (Shinde et al., 2013).

Palmitoleic acid does not appear to be toxic as it can be readily found in foods. Palmitoleic acid can be obtained in small quantities from animal fat products, vegetable and marine oils (Shinde et al., 2013). Two plant sources having high concentrations of palmitoleic acid are sea buckthorn (*Hippophae rhamnoides*), and macadamia nut oil (*Macadamia integrifolia*), which is native to Australia. Palmitoleic acid concentration of sea buckthorn is about 40% and macadamia nuts consist of approximately 75% fat, of which palmitoleic acid is approximately 12–22%. Another source of palmitoleic acid is mink oil, which contains about 15% palmitoleic acid. All these sources have limited availability and are premium sources. Palmitoleic acid is a component of dairy products such as milk, yogurt and cheese; accordingly, extracts containing palmitelaidic acid may be obtained from these sources (Eldridge, 2006).

Monounsaturated fatty acids are preferred substrates for acyl CoA-cholesterol acyltransferase, which catalyzes the esterification of hepatic free cholesterol to an inert cholesterol ester pool. This in turn reduces the putative regulatory pool of intracellular free cholesterol, increasing LDL receptor activity and subsequently decreasing circulating cholesterol concentrations (Griel et al., 2008). Palmitoleic acid has also been shown to prevent β-cell apoptosis induced by glucose or saturated fatty acids beta-cells or pancreas secrete insulin and their programmed death or apoptosis leads to type-2 diabetes) (Morgan and Dhayal, 2010). Palmitoleic acid-rich diets have also been reported to improve circulating lipid profile, resulting in reduced total and LDL cholesterol (Griel et al., 2008). Increased *cis*-palmitoleic acid level is also observed in the newborn in response to oxidative stress. A topical application of

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sea buckthorn oil on burned, scaled, wounded, and radioactively damaged skins of both humans and experimental animals have shown healing and anti-inflammatory effects (Gao et al., 2003). Wille and Kydonieus (2003) reported that palmitoleic acid could inhibit the growth of Gram-positive bacteria.

Since the isolation of palmitoleic acid concentrates on plant resources, there have been efforts to extend the possibilities of isolating this FA from microbial sources. The FA is part of microbial lipids and can therefore be obtained in larger quantities by modifying the culture conditions that induce an increase in microbial lipid production (da Rosa et al., 2014) or synthesis of specific lipids (Papanikolaou and Aggelis, 2010). Lipid content can be influenced by carbon source (hydrophobic or hydrophilic substrates), limitations by certain elements (usually N or P), culture temperature, pH of the medium, the inoculum size, length and type of cultivation (Braunwald et al., 2013; da Rosa et al., 2014; Chen et al., 2013). To our knowledge there are currently no available studies that focus on the possibility of using yeast for the production of palmitoleic acid which, as stated above, has broad pharmaceutical and cosmetic applications. The only exception is a Japanese patent dealing with the use of *Kluyveromyces polysporus* as a useful producer of antitumor pharmaceuticals, foods, cosmetics, etc. (Nippon Glass Co., Ltd., 1994).

Our work focuses on the possibility of using yeast for the production of palmitoleic acid. Because the medium composition significantly affects the ability of cells for lipid accumulation (Braunwald et al., 2013), we focused on the most frequently used methods to increase lipid accumulation, monitoring concomitantly changes in the content of palmitoleic acid in total FA, changes to the content of palmitoleic acid in total lipids and changes in palmitoleic acid in cell dry matter. Our paper examines six yeast strains (*K. polysporus*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, *Candida krusei*, *Yarrowia lipolytica* and *Trichosporon cutaneum*) that have different capacities to accumulate lipids and have different uses of biotechnology. Our data suggest that, like *K. polysporus*, *S. cerevisiae* can be used for production of palmitoleic acid in a wide range of conditions (unlimited or limited). Under strictly defined conditions the content of palmitoleic acid in *Y. lipolytica* is similar to that in *K. polysporus*. Whatever the culture conditions, *T. cutaneum* failed to reach such content of palmitoleic acid that would warrant its biotechnological use.

Also *C. krusei* can be used for palmitoleic acid production under some conditions; it then appears to be a better producer than *K. polysporus*, better palmitoleic acid source than mink oil and comparable with macadamia nuts. Our work focuses on the possibility of using yeast for the production of palmitoleic acid.

2. Methods

2.1. Microorganisms

The yeast strains used in the present study were *C. krusei* DBM 2163; *Y. lipolytica* CCY 29-26-36; *T. cutaneum* CCY 30-5-10; *K. polysporus* DBM 2171 (CCY 30-5-10); *S. cerevisiae* DBM 2115; *T. delbrueckii* DBM 39; supplied by Culture Collection of Yeast (CCY), Institute of Chemistry, Slovak Academy of Science, Bratislava and by Collection of Yeasts and Industrial Microorganisms (DBM) of 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.

2.2. Cultivation conditions

The pre-cultures 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 (26 h).

For lipid production, 200 ml of mineral medium in 500 ml Erlenmeyer flasks was inoculated with 10 ml of preculture to a final concentration of OD_{600} 0.2 and incubated on a rotary shaker at 150 rpm and 28 °C. The biomass for analysis was harvested by centrifugation (9000*g*, 10 min) in the stationary phase of growth.

The mineral medium composition was (g per liter): Medium with different nitrogen sources – KH_2PO_4 (3.5); Na_2HPO_4 (2); $MgSO_4{\cdot}7H_2O$ (1.5); NH_4Cl (1.5); yeast extract (1.5) and trace element solution 1 ml ($MnCl_2{\cdot}4H_2O$, 20; $FeSO_4$ $7H_2O$, 1; $NaMoO_4{\cdot}2H_2O$, 1; $CaCl_2$ $2H_2O$, 20), pH 6.0. Glucose was added as carbon source to the concentration 30 g/l and nitrogen source was supplemented to achieve C/N ratio 70. Medium with VFA – KH_2PO_4 (3.5); Na_2HPO_4 (2); $MgSO_4{\cdot}7H_2O$ (1.5); NH_4Cl (1.5); yeast extract (1.5) and trace element solution 1 ml ($MnCl_2{\cdot}4H_2O$, 20; $FeSO_4$ $7H_2O$, 1; $NaMoO_4{\cdot}2H_2O$, 1; $CaCl_2$ $2H_2O$, 20), pH 6.0. VFA (acetic acid 4 g/l or propionic acid 4 g/l); VFA and glucose (acetic acid 4 g/l and glucose 20 g/l or propionic acid 4 g/l and glucose 20 g/l) were added as a carbon source. Cultivations with glucose (20 g/l) as the sole carbon source were used as control. All experiments were performed at least in triplicate (acetic acid 4 g/l or propionic acid 4 g/l); VFA and glucose (acetic acid 4 g/l and glucose 20 g/l or propionic acid 4 g/l and glucose 20 g/l) were added as a carbon source. Medium with C/N 30 growth – 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; $FeSO_4$ $7H_2O$, 1; $NaMoO_4{\cdot}2H_2O$, 1; $CaCl_2$ $2H_2O$, 20). The mineral medium composition for C/N30, C/P 1043 (according to Wu et al., 2010) – 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. Cultivations on YPD medium (C/N 3) were used as control. All experiments were performed in triplicate.

2.3. Determination of dry cell weight

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

2.4. Lipid extraction

After harvesting, the yeast biomass was lyophilized and subsequently mixed with 2 ml of 0.1 M Na_2CO_3. The mixture was repeatedly ground with ballotini glass beads (diameter 0.2 mm) in a mortar, overlaid with liquid nitrogen. After 3 cycles, final volume 50 ml of 0.1 M Na_2CO_3 was added. The resulting crushed biomass 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.

2.5. FAMEs analysis

The total lipids (~5 mg) obtained by lipid extraction were saponified in 10% KOH–MeOH at room temperature overnight. The fatty acid fraction was partitioned between diethyl-ether and alkali solution (pH 9) to remove neutral and basic components. The aqueous phase containing fatty acids was acidified to pH 2 and extracted with hexane. The fatty acid fraction was methylated using BF_3/MeOH (14% solution of BF_3 from Sigma–Aldrich).

The FAMEs (~1 mg) were dissolved in dimethyl disulfide (0.2 ml) and a solution of iodine in diethyl ether (3 mg in 0.05 ml) was added. The mixture was stirred for 1 day, then hexane

(5 ml) was added and the mixture was washed with dilute sodium thiosulfate solution, dried over anhydrous sodium sulfate and evaporated. The products were dissolved in hexane and analyzed by GC–MS.

Gas chromatography–mass spectrometry of FAME was performed on a GC–MS system consisting of Varian 450-GC (Varian BV, Middelburg, The Netherlands), Varian 240-MS ion trap detector with electron ionization (EI), and CombiPal autosampler (CTC, USA) equipped with split/splitless injector. A SP-2380 column (Supelco) was used for separation (100 m, 0.25 mm ID, 0.20 μm film thickness). The temperature program started at 60 °C and was held for 1 min in splitless mode. Then the splitter was opened and the oven was heated to 160 °C at a rate of 25 °C min^{-1}. The second temperature ramp was up to 220 °C at a rate of 1.0 °C min^{-1}, this temperature being maintained for 10 min. The solvent delay time was set to 8 min. The transfer line temperature was set to 280 °C. Mass spectra were recorded at 3 scans s^{-1} under electron ionization at 70 eV, mass range m/z 50–600. FAMEs (fatty acid methyl esters) were identified according to their mass spectra and using a mixture of chemical standards obtained from Sigma–Aldrich.

Higher oven temperature was used to separate the dimethyldisulfides; the starting temperature was 180 °C for 1 min, subsequently increasing at 20 °C/min to 220 °C and at 2 °C/min to 260 °C, which was maintained for 1 min. FAMEs were identified according to their mass spectra and using a mixture of chemical standards obtained from Larodan Fine Chemicals, Malmö, Sweden.

2.6. Statistical analysis

The program CANOCO 5 (Microcomputer Power, Ithaca, NY, USA) was used to perform multivariate statistical analyses.

3. 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. Furthermore, the lipid content can also be affected by the quantity of Zn, Fe, Mn or Mg (Chen et al., 2013). Another possibility is to use a high content of carbon source, either glucose or glycerol, or other carbon sources, which are often waste substrates of various processes. Examples of such substrates may include volatile fatty acids (e.g. acetic and propionic acid) (Fei et al., 2011; Chang et al., 2010). In addition, lipid accumulation is also affected by culture conditions such as temperature, pH of the medium, the length of cultivation. The data used in previous studies (Dey and Maiti, 2013; Chen et al., 2013) served as a basis for our culture conditions: culture temperature 28 °C, medium pH 6.0, and cultivation period of 96 h. We examined the effect of variation in medium C/N and C/P ratio on lipid content and composition of the FA. In this context, we investigated the effect of the N source. Cell growth and accumulation of lipids were also monitored in cultivations with acetic acid and propionic acid as sole C sources or in combination with glucose, since these conditions can affect the synthesis of specific lipids (da Rosa et al., 2014). Out of the more than 600 known yeast species only about 5% are able to accumulate amounts of lipids greater than 5% of dry matter. Among these are, e.g. *Candida*, *Rhodotorula*, *Trichosporon*, or *Yarrowia* (Dey and Maiti, 2013). *K. polysporus*, which does not belong to yeast capable of accumulating higher amounts of lipids, is noted for its ability to accumulate palmitoleic acid (Nippon Glass Co., Ltd., 1994). Similarly, *S. cerevisiae* has been noted as a yeast with a high content of palmitoleic acid in fatty acids (Rezanka et al., 2013b) and also ranks among the yeast widely used in biotechnology, its biomass being a waste material (e.g. from brewing). In this it resembles *T. delbrueckii*, which is widely used in winemaking (Belda et al., 2015). We therefore used *C. krusei, Y. lipolytica*, *T. cutaneum*, *K. polysporus*, *S. cerevisiae*, plus the wine yeast *T. delbrueckii*.

The YPD medium is a rich source of nutrients for yeast growth and the initial C/N ratio in the medium we used was 3. The minimum C/N ratio which leads to lipid accumulation is considered to be 20 (Chen et al., 2013; Papanikolaou and Aggelis, 2011). In our work, we first affected the growth by increasing the C/N ratio to 30 at a C/P ratio of 6. Then we increased the C/P ratio to 1043 while maintaining the C/N 30. Table 1 compares the contents of saturated, monounsaturated and polyunsaturated FA. An increase of the C/N ratio to 30 caused in all yeasts increased content of monounsaturated fatty acids; in *C. krusei* this increase was nearly 50%. In contrast, the content of polyunsaturated fatty acids in *K. polysporus*, *S. cerevisiae* and *T. delbrueckii* decreased, in *T. delbrueckii* from 17% to some 3%, in *C. krusei* from 31% to 11%, in *Y. lipolytica* from 19% to 4%. Increasing the C/P ratio to 1043 had no significant effect on the ratios of saturated to unsaturated fatty acids except for *K. polysporus*, which showed an increased content of saturated FA from 8% to 15% whereas monounsaturated FA decreased. Further increase of the C/N ratio to 70 increased the proportion of saturated fatty acids by about 50% in *K. polysporus*, *S. cerevisiae*, *Y. lipolytica* and by 100% in *T. delbrueckii*, accompanied by a decrease in monounsaturated FA. In most cases, the use of different N sources had no substantial effect on the saturation of fatty acid except for ammonium sulfate, which increased significantly the proportion of polyunsaturated FA in all six yeast strains (see also Fontanille et al., 2012). The use of volatile fatty acids as non-traditional carbon sources led in all cases to similar data on the saturated, monounsaturated and polyunsaturated fatty acids as in the control culture on YPD (C/N 3), and at C/N 30. In conclusion, the C/N and C/P ratios are seen to have a profound effect in terms of saturation of fatty acids and their proportion, higher ratios causing more saturated FA. Ammonium sulfate as N source increases the proportion of polyunsaturated fatty acids.

3.1. Effect of altered C/N and C/P ratios on lipid content

As shown in Table 2, changed culture conditions have a major impact on total lipid content (%) compared with the control culture on YPD (C/N 3). These changes were, however, specific to the yeast strain. As reported in the literature, it is suitable to use for the production of lipids depletion of N, P, Zn, Fe, and Mg under excess of carbon source which is converted into lipids (Veen and Lang, 2004). The nitrogen source bringing about the highest lipid content was potassium nitrate. Similar results were reported by (Jadhav et al., 2012) who studied *Y. lipolytica*. We found the lowest lipid content in cultivation on ammonium nitrate; this may be attributed to the dual effect of NH_4^+ ions simultaneously repressing both transport and the catabolic system for dissimilation of amino acids (Middelhoven and Hoogkamerteniet, 1981). *K. polysporus* showed the highest lipid content with a C/N ratio of 30, irrespective of the C/P ratio. Under these conditions, the lipid content increased by almost 100% (from 5% to 9%). *S. cerevisiae* and *T. delbrueckii* had very similar lipid content in the range of 3–5% and 3–6%, respectively, but higher values were obtained at a C/N ratio of 70. *C. krusei* and *T. cutaneum* had the highest lipid content at a C/N ratio of 30. *Y. lipolytica* has similar lipid content, but the highest values were obtained at a C/N ratio of 70 (KNO_3). In all yeasts an increase in the C/P ratio brought about an about 10% rise in the lipid content see also (Granger et al., 1993). The two main pathways of lipid synthesis by cells are de novo lipid synthesis and ex novo lipid accumulation (Papanikolaou and Aggelis, 2011). De novo lipid accumulation is the result of cultivation on hydrophilic substrates – saccharides or short chain fatty acids (e.g. in waste water utilization) (Peng et al., 2013). Cultivation of microorganisms on hydrophobic substrates as the only carbon source leads

Table 1
Proportion of total mono- and polyunsaturated fatty acids in *K. polysporus, S. cerevisiae, T. delbrueckii*, *C. krusei*, *Y. lipolytica* and *T. cutaneum* cultivated at different C/N and C/P ratios and N sources. Substrates: C control (glucose), –N nitrogen limitation, –N–P nitrogen plus phosphorus limitation, A acetic acid, P propionic acid, G + A glucose plus acetic acid, G + P glucose plus propionic acid.

Yeast species		Type of cultivation	Fatty acids produced			Dry weight (g/l)
			Saturated	Monounsaturated	Polyunsaturated	
Kluyveromyces polysporus	C/N 3	C	8.5	88.3	2.7	4.5
	C/N 30 (C/P 6)	–N	7.5	91.6	0.9	2.4
	C/N 30 (C/P 1043)	–N–P	15.3	83.4	1.3	2.6
	C/N 70	$(NH_4)_2SO_4$	10.9	84.8	4.3	1.0
		KNO_3	12.4	86.1	1.5	1.0
		Urea	14.7	84.2	1.1	2.3
		NH_4NO_3	11.8	86.7	1.5	1.6
	C/N 4 (C/P 0.8)	A	6.9	91.3	1.4	0.4
	C/N 5 (C/P 0.9)	P	3.3	61.6	0.2	0.8
	C/N 33 (C/P 7)	G + A	8.9	88.5	2.3	0.5
	C/N 34 (C/P 7)	G + P	7.0	73.2	2.1	0.3
Saccharomyces cerevisiae	C/N 3	C	16.1	78.8	3.6	5.2
	C/N 30 (C/P 6)	–N	14.2	83.8	2.0	5.4
	C/N 30 (C/P 1043)	–N–P	13.0	84.0	3.0	4.8
	C/N 70	$(NH_4)_2SO_4$	18.5	74.1	7.4	6.3
		KNO_3	21.4	77.6	1.0	5.3
		Urea	24.9	74.2	0.9	6.1
		NH_4NO_3	20.2	78.7	1.1	6.1
	C/N 4 (C/P 0.8)	A	16.2	77.8	2.7	3.9
	C/N 5 (C/P 0.9)	P	13.1	75.3	2.8	2.6
	C/N 33 (C/P 7)	G + A	17.1	77.1	3.2	4.4
	C/N 34 (C/P 7)	G + P	16.1	76.1	3.0	3.2
Torulaspora delbrueckii	C/N 3	C	29.6	51.9	16.9	4.5
	C/N 30 (C/P 6)	–N	16.3	82.6	1.1	4.9
	C/N 30 (C/P 1043)	–N–P	16.0	80.5	3.5	3.6
	C/N 70	$(NH_4)_2SO_4$	32.5	40.3	27.2	8.0
		KNO_3	35.6	48.8	15.6	8.8
		Urea	38.0	48.8	13.2	6.8
		NH_4NO_3	36.0	49.9	14.1	7.3
	C/N 4 (C/P 0.8)	A	28.8	54.5	15.3	1.8
	C/N 5 (C/P 0.9)	P	18.9	33.3	14.5	1.9
	C/N 33 (C/P 7)	G + A	31.4	53.1	14.4	4.0
	C/N 34 (C/P 7)	G + P	26.5	39.3	15.1	2.4
Candida krusei	C/N 3	C	22.6	49.0	30.8	4.5
	C/N 30 (C/P 6)	–N	15.4	73.9	11.0	5.7
	C/N 30 (C/P 1043)	–N–P	15.2	74.6	10.7	4.2
	C/N 70	$(NH_4)_2SO_4$	19.3	43.9	36.8	3.1
		KNO_3	25.4	52.8	21.8	1.8
		Urea	29.2	57.6	13.2	1.9
		NH_4NO_3	25.2	57.9	16.9	1.7
	C/N 4 (C/P 0.8)	A	22.0	49.8	28.2	2.2
	C/N 5 (C/P 0.9)	P	11.3	64.8	23.9	0.4
	C/N 33 (C/P 7)	G + A	22.0	49.5	28.5	5.2
	C/N 34 (C/P 7)	G + P	14.8	60.8	24.4	2.4
Yarrowia lipolytica	C/N 3	C	15.8	65.2	19.0	4.4
	C/N 30 (C/P 6)	–N	22.9	72.5	4.6	7.2
	C/N 30 (C/P 1043)	–N–P	27.4	68.7	3.9	4.5
	C/N 70	$(NH_4)_2SO_4$	16.1	48.1	35.8	2.3
		KNO_3	23.9	56.3	19.8	2.1
		Urea	28.1	57.2	14.7	2.6
		NH_4NO_3	24.0	59.2	16.8	2.3
	C/N 4 (C/P 0.8)	A	18.3	57.2	23.2	4.2
	C/N 5 (C/P 0.9)	P	13.1	63.0	21.3	4.3
	C/N 33 (C/P 7)	G + A	18.6	57.2	23.4	3.5
	C/N 34 (C/P 7)	G + P	15.8	60.0	22.4	3.8
Trichosporon cutaneum	C/N 3	C	19.1	31.2	49.7	1.3
	C/N 30 (C/P 6)	–N	22.8	31.6	45.6	5.6
	C/N 30 (C/P 1043)	–N–P	29.4	37.7	32.9	3.6
	C/N 70	$(NH_4)_2SO_4$	20.8	25.8	53.4	0.8
		KNO_3	27.0	41.2	31.8	0.9
		Urea	32.3	40.8	26.9	1.1
		NH_4NO_3	31.1	41.5	27.4	0.8
	C/N 4 (C/P 0.8)	A	20.1	33.4	45.6	2.6
	C/N 5 (C/P 0.9)	P	15.8	59.7	21.2	2.7
	C/N 33 (C/P 7)	G + A	22.0	30.2	47.2	1.6
	C/N 34 (C/P 7)	G + P	17.8	40.8	39.1	1.8

Table 2
Proportion of fatty acids and total lipids in *K. polysporus, S. cerevisiae, T. delbrueckii, C. krusei, Y. lipolytica* and *T. cutaneum* cultivated at different C/N and C/P ratios and N sources.

Yeast species		Carbon source	Nitrogen source	16:1 Po	16:0 P	17:1	18:2 L	18:1 O	18:0 S	20:0 A	Total lipids (%)
Kluyveromyces polysporus	C/N 3	Glucose	YPD	59.2	7.5	0.0	2.7	29.1	1.0	0.5	5.1
	C/N 30 (C/P 6)	Glucose	NH_4Cl	74.5	7.2	0.0	0.9	17.1	0.3	0.0	8.8
	C/N 30 (C/P 1043)	Glucose	NH_4Cl	66.7	14.6	0.0	1.3	16.7	0.7	0.0	9.3
	C/N 70 (C/P 6)	Glucose	$(NH_4)_2SO_4$	57.2	8.6	0.0	4.3	27.6	1.2	1.1	5.3
	C/N 70 (C/P 6)	Glucose	KNO_3	58.0	9.2	0.0	1.5	28.1	1.5	1.7	6.1
	C/N 70 (C/P 6)	Glucose	Urea	55.3	9.6	0.0	1.1	28.9	1.8	3.3	4.8
	C/N 70 (C/P 6)	Glucose	NH_4NO_3	58.1	9.0	0.0	1.5	28.6	1.5	1.3	3.6
	C/N 4 (C/P 0.8)	Acetic acid	NH_4Cl	56.6	5.2	0.4	1.4	34.7	0.8	0.9	3.2
	C/N 5 (C/P 0.9)	Propionic acid	NH_4Cl	34.1	1.8	34.9	0.2	27.5	0.7	0.8	2.1
	C/N 33 (C/P 7)	Glucose + acetic acid	NH_4Cl	55.7	7.1	0.3	2.3	32.8	0.9	0.9	4.8
	C/N 34 (C/P 7)	Glucose + propionic acid	NH_4Cl	42.3	5.2	17.7	2.1	30.9	0.8	1.0	3.6
Saccharomyces cerevisiae	C/N 3	Glucose	YPD	39.3	11.8	0.0	3.6	39.5	4.3	1.5	4.5
	C/N 30 (C/P 6)	Glucose	NH_4Cl	59.8	11.5	0.0	2.0	24.0	2.7	0.0	2.7
	C/N 30 (C/P 1043)	Glucose	NH_4Cl	56.1	11.2	0.0	3.0	27.9	1.8	0.0	2.9
	C/N 70 (C/P 6)	Glucose	$(NH_4)_2SO_4$	38.9	11.5	0.0	7.4	35.2	3.9	3.1	4.5
	C/N 70 (C/P 6)	Glucose	KNO_3	38.5	14.1	0.0	1.0	39.1	4.0	3.3	4.9
	C/N 70 (C/P 6)	Glucose	Urea	35.5	12.4	0.0	0.9	38.7	5.7	6.8	4.2
	C/N 70 (C/P 6)	Glucose	NH_4NO_3	37.7	13.2	0.0	1.1	41.0	3.9	3.1	3.8
	C/N 4 (C/P 0.8)	Acetic acid	NH_4Cl	35.6	9.5	3.3	2.7	42.2	3.7	3.0	3.1
	C/N 5 (C/P 0.9)	Propionic acid	NH_4Cl	34.8	8.2	8.8	2.8	40.5	3.2	1.7	3.2
	C/N 33 (C/P 7)	Glucose + acetic acid	NH_4Cl	36.2	10.4	2.6	3.2	40.9	4.0	2.7	3.7
	C/N 34 (C/P 7)	Glucose + propionic acid	NH_4Cl	35.0	10.3	4.8	3.0	41.1	3.6	2.2	3.4
Torulaspora delbrueckii	C/N 3	Glucose	YPD	22.6	26.8	0.0	16.9	29.3	2.8	1.6	4.7
	C/N 30 (C/P 6)	Glucose	NH_4Cl	52.1	15.9	0.0	1.1	30.5	0.4	0.0	2.9
	C/N 30 (C/P 1043)	Glucose	NH_4Cl	52.0	15.3	0.0	3.5	28.5	0.7	0.0	3.1
	C/N 70 (C/P 6)	Glucose	$(NH_4)_2SO_4$	18.4	21.4	0.0	27.2	21.9	3.0	8.1	4.9
	C/N 70 (C/P 6)	Glucose	KNO_3	22.8	26.2	0.0	15.6	26.0	4.1	5.3	5.7
	C/N 70 (C/P 6)	Glucose	Urea	21.7	24.3	0.0	13.2	27.1	4.5	9.2	4.3
	C/N 70 (C/P 6)	Glucose	NH_4NO_3	21.7	26.6	0.0	14.1	28.2	4.1	5.3	3.8
	C/N 4 (C/P 0.8)	Acetic acid	NH_4Cl	21.7	21.8	1.4	15.3	32.8	2.9	4.1	3.8
	C/N 5 (C/P 0.9)	Propionic acid	NH_4Cl	18.9	14.2	33.3	14.5	14.4	3.1	1.6	2.7
	C/N 33 (C/P 7)	Glucose + acetic acid	NH_4Cl	21.8	22.8	1.1	14.4	31.3	3.2	5.4	4.1
	C/N 34 (C/P 7)	Glucose + propionic acid	NH_4Cl	20.4	20.2	19.4	15.1	18.9	3.1	3.2	3.0
Candida krusei	C/N 3	Glucose	YPD	8.8	17.4	0.0	30.8	40.2	2.4	2.8	35.4
	C/N 30 (C/P 6)	Glucose	NH_4Cl	16.0	14.8	0.0	11.0	57.9	0.3	0.3	47.8
	C/N 30 (C/P 1043)	Glucose	NH_4Cl	14.4	14.2	0.0	10.7	60.2	0.5	0.5	54.2
	C/N 70 (C/P 6)	Glucose	$(NH_4)_2SO_4$	7.9	13.8	0.0	36.8	36.0	3.4	2.1	31.4
	C/N 70 (C/P 6)	Glucose	KNO_3	9.4	17.3	0.0	21.8	43.4	4.5	3.6	35.9
	C/N 70 (C/P 6)	Glucose	Urea	10.9	18.1	0.0	13.2	46.7	4.4	6.7	33.2
	C/N 70 (C/P 6)	Glucose	NH_4NO_3	12.3	16.7	0.0	16.9	45.6	4.5	4.0	28.1
	C/N 4 (C/P 0.8)	Acetic acid	NH_4Cl	5.9	16.4	2.0	28.2	41.9	2.7	2.9	24.2
	C/N 5 (C/P 0.9)	Propionic acid	NH_4Cl	1.4	7.1	37.0	23.9	26.4	2.1	2.1	15.7
	C/N 33 (C/P 7)	Glucose + acetic acid	NH_4Cl	6.6	16.5	1.6	28.5	41.3	2.2	3.3	33.2
	C/N 34 (C/P 7)	Glucose + propionic acid	NH_4Cl	3.5	10.2	25.3	24.4	32.0	1.9	2.7	26.4
Yarrowia lipolytica	C/N 3	Glucose	YPD	14.0	13.9	0.0	19.0	51.2	1.2	0.7	17.9
	C/N 30 (C/P 6)	Glucose	NH_4Cl	16.4	20.8	0.0	4.6	56.1	2.1	0	22.1
	C/N 30 (C/P 1043)	Glucose	NH_4Cl	14.9	21.7	0.0	3.9	53.8	5.7	0	22.0
	C/N 70 (C/P 6)	Glucose	$(NH_4)_2SO_4$	9.4	10.3	0.0	35.8	38.7	2.3	3.5	18.6
	C/N 70 (C/P 6)	Glucose	KNO_3	10.6	14.5	0.0	19.8	45.7	4.1	5.3	26.4
	C/N 70 (C/P 6)	Glucose	Urea	11.2	15.3	0.0	14.7	46.0	3.7	9.1	16.9
	C/N 70 (C/P 6)	Glucose	NH_4NO_3	11.3	14.6	0.0	16.8	47.9	4.0	5.4	14.2
	C/N 4 (C/P 0.8)	Acetic acid	NH_4Cl	6.9	12.4	3.8	23.2	46.5	2.5	3.4	13.3
	C/N 5 (C/P 0.9)	Propionic acid	NH_4Cl	3.2	9.4	28.2	21.3	31.6	1.9	1.8	8.9

Table 2 (*continued*)

Yeast species		Carbon source	Nitrogen source	16:1 Po	16:0 P	17:1	18:2 L	18:1 O	18:0 S	20:0 A	Total lipids (%)
	C/N 33 (C/P 7)	Glucose + acetic acid	NH_4Cl	7.3	12.5	0.7	23.4	49.2	2.9	3.2	16.5
	C/N 34 (C/P 7)	Glucose + propionic acid	NH_4Cl	3.9	10.4	14.6	22.4	41.5	2.6	2.8	10.2
Trichosporon cutaneum	C/N 3	Glucose	YPD	2.6	17.3	0.0	49.7	28.6	1.8	0.0	49.3
	C/N 30 (C/P 6)	Glucose	NH_4Cl	2.6	18.7	0.0	45.6	29.0	4.1	0.0	57.9
	C/N 30 (C/P 1043)	Glucose	NH_4Cl	1.0	24.3	0.0	32.9	36.7	5.1	0.0	59.9
	C/N 70 (C/P 6)	Glucose	$(NH_4)_2SO_4$	1.9	16.0	0.0	53.4	23.9	3.2	1.6	51.6
	C/N 70 (C/P 6)	Glucose	KNO_3	2.3	19.4	0.0	31.8	38.9	4.4	3.2	55.2
	C/N 70 (C/P 6)	Glucose	Urea	2.6	20.5	0.0	26.9	38.2	5.8	6.0	47.6
	C/N 70 (C/P 6)	Glucose	NH_4NO_3	2.6	20.4	0.0	27.4	38.9	5.1	5.6	41.0
	C/N 4 (C/P 0.8)	Acetic acid	NH_4Cl	2.1	16.5	1.3	45.6	30.0	2.4	1.2	33.2
	C/N 5 (C/P 0.9)	Propionic acid	NH_4Cl	2.1	12.7	32.6	21.2	25.0	2.1	1.0	23.9
	C/N 33 (C/P 7)	Glucose + acetic acid	NH_4Cl	2.2	17.0	0.3	47.2	27.7	3.5	1.5	42.8
	C/N 34 (C/P 7)	Glucose + propionic acid	NH_4Cl	2.0	13.5	13.5	39.1	25.3	3.3	1.0	36.1

to ex novo lipid accumulation. The accumulation of lipids ex novo is a primary anabolic process occurring simultaneously with the production of lipid-free material (Fei et al., 2011). Using volatile fatty acids as a sole carbon source resulted in a significant reduction in the total lipid content. Addition of glucose substantially improved the lipid content compared to the cultivation of acetic acid or propionic acid alone (Fei et al., 2011; Fontanille et al., 2012).

3.2. Fatty acid composition and profiles

The percent proportion of individual fatty acids is shown in Table 2. The lowest abundant FAs included stearic acid (18:0) and arachidic acid (20:0) – see also (Wu et al., 2010). Our results are at variance with the claim of Papanikolaou et al. (2004), who stated that FA composition is relatively constant regardless of a simple change in culture conditions. His studies were performed on oleaginous yeasts which included also *C. krusei*, but even in this yeast we observed significant changes in the representation of FA. It is however true that our changes in culture conditions were substantial; for instance, the use of VFA resulted in the formation of heptadecenoic acid (17:1) (Rezanka et al., 2015). As reported in the literature (Rezanka et al., 2013a; Zheng et al., 2012), the synthesis of FA is strongly influenced by the presence of propionic acid as a substrate. Its presence caused an increase in heptadecenoic acid (17:1) to more than 30% of total FA in *K. polysporus, T. delbrueckii*, *C. krusei*, *Y. lipolytica* and *T. cutaneum*. Its proportion in *S. cerevisiae* was under 9% of total FA.

As stated by Sitepu et al. (2013), relative FA composition varies depending on the composition of the medium and cultivation time. Our results show that, in *K. polysporus* under all conditions used, the most represented FA was palmitoleic acid, its contents ranging from 34% to 74% of total FA. The second most abundant FA was oleic acid (18:1). The share of palmitoleic and oleic acid was mostly in the range of 84–94% of total FA, decreasing to 62% or 73% only in the presence of the propionic acid.

The most common fatty acids in cultivations of *S. cerevisiae* as a biotechnologically widely yeast are palmitoleic acid (16:1) and oleic acid (18:1). The proportion of these two FA amounted to 74–84% of total FA. It was relatively constant, around 40% each, in a wide range of culture conditions. They could therefore be used for nutritional use. FA with the highest abundance, almost 60% of total FA, was palmitoleic acid at C/N 30 and C/P 6.

T. delbrueckii, as a wine yeast, had balanced representation of palmitoleic (16:1), palmitic (16:0) and oleic acid (18:1) in a wide range of culture conditions. Changes in the composition of culture media affected most frequently linoleic acid (18:2), the contents of which ranged from 1% to 27% of total FA.

The most abundant fatty acids in *C. krusei* were palmitic, oleic and linoleic acids, the content of which was more than 70% of total FA. These data correspond to published results (Chen et al., 2013). The major FA was oleic acid, whose proportion was within the range of 32–60% of total FA and it could therefore be used for biotechnological production for nutritional use (Sitepu et al., 2013). The conditions best suited for its production were at C/N of 30, ideally even with a higher C/P ratio. A lower content, by about 25% of total FA, of oleic acid was achieved at a C/N ratio of 70, and lipid content at this ratio was also lower by about 35%. However, the use of this higher C/N ratio of 70 can be of interest when the yeast are grown in ammonium sulfate, which supported a threefold increase in the ω-6 FA, linoleic acid (18:2) to 37% of total FA. Palmitoleic acid content was highest at C/N 30 and reached 16% respectively 14% by C/P ratio of 1043.

The most abundant FA in the case of *Y. lipolytica* was oleic acid (about 50%), followed by about equal content of palmitic, palmitoleic and linoleic acids. Like with *C. krusei*, cultivation on ammonium sulfate brought about an increase in the amount of

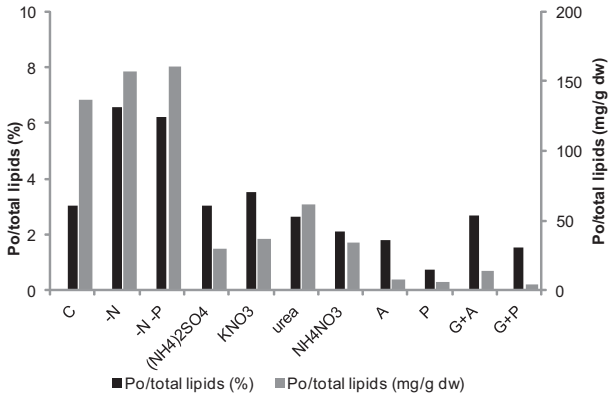

Fig. 1. Content of palmitoleic acid (Po) in total lipids (%) and the yield of the acid per dry matter (mg/g dw) in *Kluyveromyces polysporus*. Substrates: C control (glucose), –N nitrogen limitation, –N–P nitrogen plus phosphorus limitation, A acetic acid, P propionic acid, G + A glucose plus acetic acid, G + P glucose plus propionic acid.

linolenic acid to 36% of total FA. Palmitoleic acid was the highest at C/N 30, reaching 16%.

T. cutaneum featured a high proportion of linoleic acid, which exceeded 53% of total FA in cultivation with ammonium sulfate. Palmitoleic acid content was insignificant, about 2% under all culture conditions.

To sum up, it can be stated that ammonium sulfate as nitrogen source significantly increases the content of ω-6 FA linoleic acid and the use of higher C/N ratios increases the amount of saturated FA (Mondala et al., 2012). The presence of propionic acid greatly increased the content of heptadecenoic acid (17:1). Under all culture conditions the most abundant FA in *K. polysporus* and *S. cerevisiae* was palmitoleic acid (16:1), in *C. krusei* oleic acid (18:1) and in *T. cutaneum* linoleic acid (18:2).

3.3. Palmitoleic acid yield in different yeasts and under different conditions

The suitability of a microbial strain for producing palmitoleic acid or another fatty acid is dictated by several factors, namely a combination of a high yield of biomass, proportion of the fatty acid in total FA, and a high percent lipid content under the chosen culture conditions. These factors can be summarized in the yield of the FA per dry matter (mg/g dw). Figs. 1–6 display this quantity for each yeast together with the proportion of palmitoleic acid in total lipid content. The figures show that the content and yield of palmitoleic acid is dependent on the yeast strain and cultivation conditions. The least suitable to use for all the studied strains are substrates comprising VFA whose presence significantly adversely affects the content and yield of palmitoleic acid. It should be noted, however, that *S. cerevisiae* was able to produce the acid in an amount of about 50 mg/g dw even in the presence of VFA.

The most suitable for *K. polysporus* (Fig. 1), which has been used for the production of palmitoleic acid is the C/N ratio of 30, at which changing the C/P ratio (6 or 1043) did not change the yield (157 and 160 mg/g dw.). Increasing the C/N ratio to 70 there brought a significant decrease in the yield to 30–62 mg/g dw.

S. cerevisiae (Fig. 2) reached lower palmitoleate yields than *K. polysporus* but maintained a relatively stable yield (85–110 mg/g dw) in a wide range of culture conditions. It is therefore possible to envisage the use of waste biomass from biotechnological production, brewing and others processes in which biomass is produced in large quantities, for palmitoleic acid production.

T. delbrueckii (Fig. 3) showed lower yields of palmitoleic acid than *S. cerevisiae* and *K. polysporus*, but if potassium nitrate was used as N source, an almost 100% yield increase occurred from the baseline around 60 mg/g dw to 115 mg/g dw.

C. krusei (Fig. 4) was the best in terms of palmitoleic acid production. The maximum yield was limited to narrowly defined conditions, as in the case of *K. polysporus*, but it reached about threefold higher values, 430 mg/g dw at C/N 30 and C/P 6. These values are close to the values reported for mink oil and reach 70% of palmitoleic acid yield from macadamia nut. Also, the composition of other FA is very close to that of macadamia nut oil – see also Kaijser et al. (2000).

At C/N 30 and C/P 6, *Y. lipolytica* (Fig. 5), reached a production of 260 mg/g dw. Increasing the C/P ratio to 1043 caused about a half drop in the yield to 147 mg/g dw. Increasing the C/N ratio resulted in a huge drop in the subsequent production of palmitoleic acid to about 50 mg/g dw.

T. cutaneum (Fig. 6) reached maximal production of palmitoleic acid (80 mg/g dw) at C/N 30 and C/P 6. Increasing the C/P ratio to 1043 resulted in a reduction of the produced palmitoleic acid to 22 mg/g dw. In other cases, the content was around 10 mg/g dw. Despite the high lipid content, this yeast was the worst producer of palmitoleic acid.

Different lipid accumulation and fatty acid profile in yeast species on different nitrogen sources is for incurred by species-specific differences in nitrogen metabolism (Zhu et al., 2008). Optimization

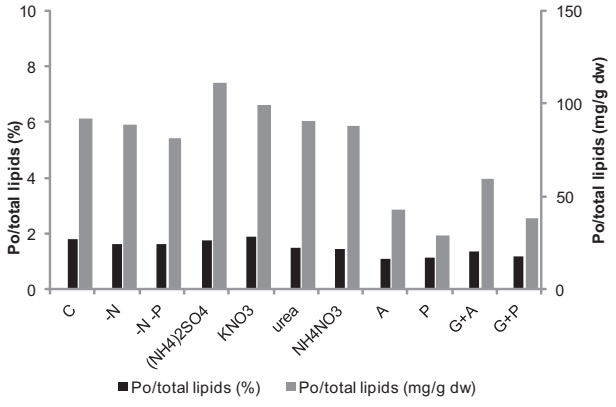

Fig. 2. Content of palmitoleic acid (Po) in total lipids (%) and the yield of the acid per dry matter (mg/g dw) in *Saccharomyces cerevisiae*. Substrates: C control (glucose), –N nitrogen limitation, –N–P nitrogen plus phosphorus limitation, A acetic acid, P propionic acid, G + A glucose plus acetic acid, G + P glucose plus propionic acid.

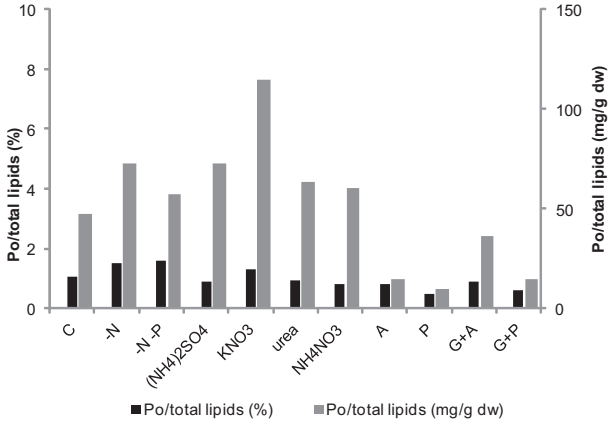

Fig. 3. Content of palmitoleic acid (Po) in total lipids (%) and the yield of the acid per dry matter (mg/g dw) in *Torulaspora delbrueckii*. Substrates: C control (glucose), –N nitrogen limitation, –N–P nitrogen plus phosphorus limitation, A acetic acid, P propionic acid, G + A glucose plus acetic acid, G + P glucose plus propionic acid.

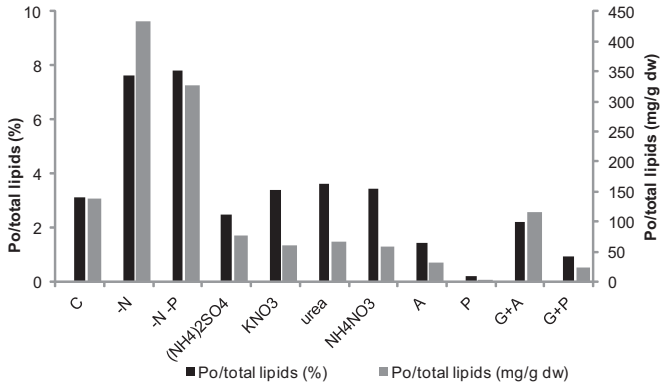

Fig. 4. Content of palmitoleic acid (Po) in total lipids (%) and the yield of the acid per dry matter (mg/g dw) in *Candida krusei*. Substrates: C control (glucose), –N nitrogen limitation, –N–P nitrogen plus phosphorus limitation, A acetic acid, P propionic acid, G + A glucose plus acetic acid, G + P glucose plus propionic acid.

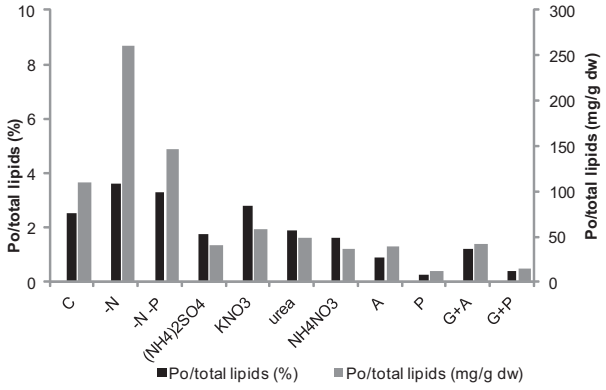

Fig. 5. Content of palmitoleic acid (Po) in total lipids (%) and the yield of the acid per dry matter (mg/g dw) in *Yarrowia lipolytica*. Substrates: C control (glucose), –N nitrogen limitation, –N–P nitrogen plus phosphorus limitation, A acetic acid, P propionic acid, G + A glucose plus acetic acid, G + P glucose plus propionic acid.

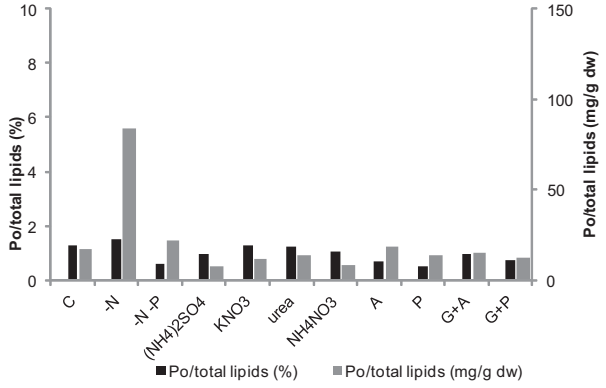

Fig. 6. Content of palmitoleic acid (Po) in total lipids (%) and the yield of the acid per dry matter (mg/g dw) in *Trichosporon cutaneum*. Substrates: C control (glucose), –N nitrogen limitation, –N–P nitrogen plus phosphorus limitation, A acetic acid, P propionic acid, G + A glucose plus acetic acid, G + P glucose plus propionic acid.

of lipid production, higher biomass and lipid yield in terms of total expenses might be achieved by modifying cultivation configuration – batch or fed-batch cultivation in a bioreactor or utilization of waste materials (Dey and Maiti, 2013). In *Saccharomyces* species the lipid accumulating ability might be introduced by genetic manipulation (Yu et al., 2011).

Location of double bonds, especially in monoenoic acids, can be conveniently performed by preparing dimethyl disulfide adducts. This method requires a single reagent and a one-step reaction (see Experimental). The electron impact mass spectra of this adduct of methyl 9-cis- and 9-trans-hexadecenoates (commercially obtained, i.e. palmitelaidic and palmitoleic acids from Larodan, and hexadecenoic acid from yeast) are illustrated in Figs. 1S–3S (Supplements). The molecules are cleaved between the carbon atoms that originally constituted the double bond. This produces two fragment ions, i.e. that containing the terminal methyl part of the molecule at $m/z = 145$ and that with the carboxyl group at $m/z = 217$. Another prominent ion at $m/z = 185$ corresponds to the latter fragment with the loss of methanol. Apart from that, these derivatives give molecular ions at m/z 362. The formation of the adducts is entirely stereospecific, occurring by trans addition, and erythro- and threo-derivatives are therefore formed from cis-and trans-isomers, respectively (Nichols et al., 1986). Although the different geometrical isomers have virtually identical spectra (see Figs. 1S–3S), they are eluted separately from gas chromatography columns (see Fig. 4S), the cis-isomer eluting first, as has been repeatedly described (Nichols et al., 1986).

The use of different media did not affect the proportion of hexadecenoic acid isomers. Hence the representation of palmitoleic acid was always higher than or equal to 99% of all hexadecenoic acid, which is understandable because *Saccharomyces* yeast does not have any other desaturase than Δ9 it also does not have cis–trans (in other words), E-Z isomerase.

4. Conclusion

As the best producer of palmitoleic acid appears *C. krusei*, which was found to achieve values comparable with the composition and yield of mink oil and macadam oil. *Y. lipolytica* reached a 70% yield of palmitoleic acid when compared with *C. krusei*. In terms of microbial biotechnology and wide usage *S. cerevisiae* may be considered for its stable yield of FA over a broad range of culture conditions. *C. krusei* and *Y. lipolytica* could be used for nutritional use due to the massive content of oleic acid and palmitoleic acid, which constitute around 90% of the lipid content.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.06.048.

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