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Cell lipids of the *Candida lipolytica* yeast grown on methanol

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Abstract

Candida lipolytica yeast, grown on 1% methanol as the only carbon and energy source, synthesized 4.9% of dry cell mass as lipids, 52.3% of which were polar lipids. Polar lipids consisted mainly of phospholipids and sphingolipids as their minor components. The total long-chain bases content has been found to account only for 0.7% of the polar lipids. The long-chain bases composition determined by thin-layer and gas chromatography shows a preponderance of trihydroxy bases and a small amount of dihydroxy bases. The striking finding was the high content of 19-phytosphingosine (90.8% of total long-chain bases). Fatty acid (FA) composition of polar lipids was characterized by the relatively high concentration of unsaturated fatty acids (66.4% of total FA) and by the predominance of fatty acids with 16 carbon atoms (85.0% of total FA).

Keywords: Candida lipolytica; Yeast; Lipids; Methanol; Fatty acids; Phytosphingosines; Sphingolipids

1. Introduction

In recent years interest in yeasts, as sources of oils and fats, has been renewed for their potential application in the production of highly specific fats [1]. Various changes of the growth conditions could alter the fatty acid profile of oil produced by yeasts. Such changes include choice of growth substrate, choice of limiting nutrient (essential for accumulation of lipids), temperature, pH and aeration [2]. With respect to the yeast strain, of all the factors, that may influence the lipid content and fatty acid composition,the carbon source may be the key one.

The present study was undertaken to investigate the conversion of methanol as carbon source to cell lipids of *Candida lipolytica* yeast. Methanol has same advantages over other substrates for the growth of microorganisms. It is water soluble, easily absorbed, available in pure form and economical. The use of methanol as the only carbon source was mainly investigated in connection with the production of yeast biomass or single cell proteins [3]. However, very little information is available on lipids, and the formed lipids were not characterized in details, especially polyunsaturated fatty acids, and sphingolipids and their long chain bases. The former are important in the prevention and treatment of coronary heart diseases [4]. The latter are connected with transmembrane signal transduction [5]. In yeasts, the long-chain base phytosphingosine and to a lesser extent dihydrosphingosine replace sphingosine, the predominant long chain base in mammalian cells. Wells and Lester [6] have isolated one mutant strain of *Saccharomyces cerevisiae* with obligate requirement for a long chain base, phytosphingosine, for growth and viability. This mutant

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lacks serine palmitoyltransferase [7], the first enzyme in sphingolipid long-chain base synthesis.

The utilization of methanol as sole carbon and energy source by *Candida lipolytica* was studied with respect to the content of lipids and main lipid fractions, as well as the composition of fatty acids and sphingolipid long-chain bases of polar lipid fraction. The present paper establish the exclusive presence of 19-phytosphingosine in sphingolipid long-chain base composition of the *Candida lipolytica* yeast.

2. Materials and methods

2.1. Yeast strain and growth conditions

Candida lipolytica strain 33M (deposited in the Collection of Microorganisms of the Faculty of Food Technology and Biotechnology, University of Zagreb) was first cultivated in the inoculation medium (glucose 2%, peptone 0.6%, malt extract 0.3%, yeast extract 0.3%) and then in the production medium with 1% (v/v) methanol as carbon source on the rotary shaker as described previously [8]. For the control purpose the yeast was grown in production medium with 1% (v/v) glucose as carbon source. The cells were harvested in the stationary growth phase by centrifugation at 4000 *g* and washed once with distilled water.

2.2. Extraction of lipids

Total cellular lipids were extracted in three steps with 95% ethanol, chloroform–methanol (2:1, v/v) and chloroform–methanol–HCl (124:65:1, v/v/v) [9]. This last extract was neutralized with 2 *M* NaOH before combination with the two neutral extracts. The combined extracts were then reduced to dryness in vacuum. Total lipids were redissolved in chloroform–methanol (2:1, v/v) and partitioned according to the method of Folch et al. [10] using 0.2 volume of water. The upper non-lipid phase was removed, and the lower lipid phase was washed four times with Folch's upper phase. The lower phase was evaporated to dryness under a stream of nitrogen and the remaining total lipids were determined gravimetricaly. Biomass remaining after lipid extraction was dried at 105°C to constant mass. Dry cell mass was calculated as a sum of the total lipid mass and the mass of dried recovered delipidated residue.

2.3. Isolation and identification of polar and nonpolar lipids

Total lipids were separated into polar and nonpolar fractions by counter-current distribution according to Galanous and Kapoulas [11]. After removing the solvents under the stream of nitrogen, the amount of polar and nonpolar lipids was determined gravimetricaly. Polar and nonpolar lipids were analyzed by thin-layer chromatography (TLC). TLC analyses were performed on silica gel G plates (E. Merck, Darmstadt, Germany). Solvent systems used were: chloroform– methanol–water (65:25:4, v/v) for polar lipids; light petroleum–diethyl ether–acetic acid (90:10:1, v/v) for nonpolar lipids. Polar lipids were detected with ammonium molybdate–perchloric acid spray reagent and identified by comparison with standards (Serdary Research Labs., London, Canada) In addition polar lipids were detected by specific spray reagents: ninhydrin for amino group, silver nitrate–ammonium hydroxide for inositols, Dragendorff reagent for choline, α-naphthol–sulfuric acid spray for reducing sugars [12]. Nonpolar lipids were detected with chrom-sulfuric acid and identified by comigration with standards (Analabs, Nen, New England, USA).

2.4. Preparation and analysis of fatty acids and long-chain bases from polar lipids

Polar lipids were subjected to acid methanolysis according to Gaver and Sweeley [13] to obtain fatty acid methyl esters and long-chain bases. Fatty acid methyl esters were analyzed by gas–liquid chromatography (GLC). They were identified by comparison of their retention times with those of fatty acid methyl ester standards (Supelco, Bellefonte, PA, USA).

Long-chain bases were analyzed by TLC on silica gel plates [14] and identified by comigration with authentic standards. They were analyzed by GLC, as well. For that purpose they were precipitated as neutral oxalates by stepwise addition of small portions of 10% oxalic acid in ethanol. Obtained

oxalates were permethylated with methyl iodide in dimethylsulfoxide [15]. Permethylated bases were determined by comparing their retention times with those of standards. Sphinganine (dihydrosphingosine), sphingosine and 18- phytosphingosine were from Sigma; the mixture of 18-, 19- and 20-phytosphingosine was generously provided by Professor M. Proštenik who prepared these bases in his laboratory (Department of Chemistry and Biochemistry, Faculty of Medicine, University of Zagreb, Croatia). The slope of the straight lines in semilogarithmic plots of retention times versus chain-length of the standards was also used for identification of various bases in the homologous series.

GLC was carried out on Perkin-Elmer Sigma 2 gas chromatograph with flame ionization detector and nitrogen as carrier gas. Two packed columns were used: 3% OV-101 on Gas-Chrom Q (80–100 mesh), temperature programmed from 120 to 270°C at 2°C/min (for bases and hydroxy fatty acids) and 10% FFAP on Chromosorb W HP (80–100 mesh), temperature programmed from 120 to 220°C at 5°C/min (for fatty acids). For the quantitative estimation of the total long-chain bases content of polar lipids, the method of Lauter and Trams [16] was employed.

3. Results

3.1. Yeast lipids

The results of the analysis of lipids isolated from the biomass of the yeast *Candida lipolytica* grown on methanol or glucose as the only carbon source are summarized in Table 1.

Methanol proved to be a less efficient carbon source than glucose for lipid accumulation of the investigated yeast. The amount of total lipids of glucose-grown cells was about three times as large as that of methanol-grown cells. However, total lipids of cells grown on methanol or glucose showed no significant differences in the polar and neutral lipid contents. Polar lipids make more than half of the total cell lipids for both carbon sources (52.3 and 64.2%, respectively). TLC analysis of polar lipids revealed the presence of phospholipids as the major component of polar lipids. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol were identified as principal components by comparing their R_F values with those of standards and by their reactions with various specific reagents. Besides, a cerebroside was detected in polar fraction. Among nonpolar lipid components, triacylglycerols, sterol, sterolester and free fatty acids were detected, as well as trace amounts of mono- and diacylglycerols.

Quantitative determination of the sphingolipid long-chain bases content in yeast grown on two different carbon sources showed that it was low (Table 1). The long-chain bases accounted only for 0.7% of polar lipids or 0.02% of dry cell biomass in methanol-grown cells. However, glucose-grown cells have four times higher quantity of long-chain bases in polar lipid fraction (2.8%) or about ten times higher regarding dry cell biomass (0.26%) in comparison with methanol-grown cells. Long-chain bases are integral part of all natural sphingolipids, from simple ceramide to complex glyco-sphingolipids and phosphoinositol sphingolipids. Accordingly, the value of sphingolipid long-chain bases pointed out that sphingolipids are a minor lipid component in the yeast grown on both carbon sources.

The results of comparing the long-chain bases content in methanol-grown cells with that of glucose-

Table 1
The content and composition of lipids and distribution of sphingolipid long-chain bases of *Candida lipolytica* yeast

Carbon source	Lipids			Sphingolipid–LCB	
	Total (% DC)	Polar (% TL)	Nonpolar (% TL)	LCB (% DC)	LCB (% PL)
Methanol (1%)	4.9±0.4	52.3±1.3	47.1±1.1	0.018±0.004	0.7±0.08
Glucose (1%)	14.3±0.9	64.2±2.1	35.6±1.9	0.26±0.03	2.8±0.05

Abbreviations: LCB, long-chain bases; DC, dry cell mass; TL, total lipids; PL, polar lipids.
Data represent the mean values and ranges of two independent experiments.

grown cells indicate that carbon-source is essential for sphingolipid production.

3.2. Fatty acids and long-chain bases

The fatty acid (FA) and long-chain bases (LCB) composition of the polar lipid fraction, obtained by GLC analysis, are presented in Table 2.

In polar lipids only seven fatty acids of chain lengths from 14 to 18 carbon atoms were detected. They all contained an even number of carbon atoms. Polar lipids were characterized by a preponderance of unsaturated fatty acids. The ratio of unsaturated to saturated fatty acids was 2:1. The main fatty acid was palmitoleic acid (16:1), which amounted to 54.5% of the total fatty acids. Another major fatty acid was palmitic acid (16:0). Fatty acids of 16 carbon atoms accounted for 85% of total FA of polar lipids.

Fatty acids of 18 carbon atoms were present in much lower concentration (about 13% of total FAs), among which oleic acid (18:1) predominated. Hydroxypalmitic acid (h16:0) was the only hydroxylated fatty acid among fatty acids present in the composition of the polar lipid fraction. The presence of hydroxy fatty acids suggested a connection with sphingolipids, where the latter are attached to a long-chain base by amide linkage. Thus, the quantitative data for hydroxy fatty acids may serve as a rough measure of the sphingolipid content.

Table 2
Composition of fatty acids and long-chain bases of *Candida lipolytica* polar lipids

Fatty acid (FA)	% of total FA[a]	Long-chain base (LCB)	% of total LCB[a]
14:0	1.4±0.1	18-Sphingosine	2.0±0.2
16:0	28.4±0.7	18-Dihydrosphingosine	2.0±0.1
16:1	54.5±1.6	18-Phytosphingosine	2.5±0.2
h 16:0	2.1±0.3	19-Phytosphingosine	90.8±2.5
18:0	1.7±0.1	20-Phytosphingosine	2.7±0.3
18:1	7.2±0.3		
18:2	4.7±0.2		
Saturated	31.5±0.9	Dihydroxy	4.0±0.3
Unsaturated	66.4±2.1	Trihydroxy	96.0±3.0
Hydroxy	2.1±0.3		

h=hydroxy fatty acid.
[a] Data represent the mean values and ranges of two independent experiments as in Table 1.

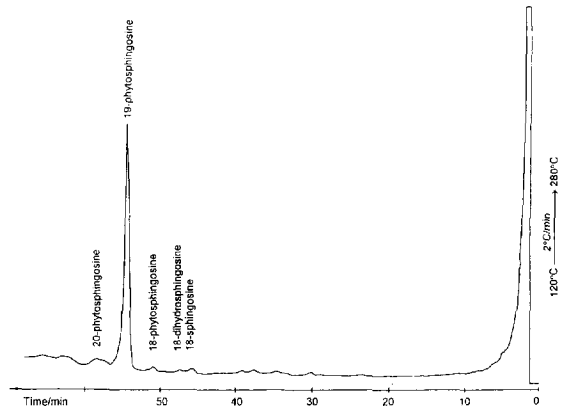

Fig. 1. GLC chromatogram of polar lipid permethylated bases of the *Candida lipolytica* yeast grown on methanol.

The long-chain base fractions obtained by acid hydrolysis were analyzed by TLC. The base fractions from polar lipids gave ninhydrin positive spots corresponding to phytosphingosine, sphinganine (dihydrosphingosine) and sphingosine reference standard and the anhydro derivatives of these long-chain bases. Gas chromatograms of the long-chain bases, shown on Fig. 1, gave results consistent with those from TLC.

Polar lipids contained dihydroxy and trihydroxy bases, among which trihydroxy bases predominated. About 91% of the long-chain bases consisted of 19-phytosphingosine. Other bases, listed in decreasing concentrations, were 18-phytosphingosine, 20-phytosphingosine, 18-sphingosine and 18-dihydrosphingosine (Table 2).

4. Discussion

Growth of the yeast *Candida lipolytica* on methanol as the only carbon and energy source did not result in the accumulation of considerable quantities of lipids. This methanol-utilizing yeast synthesized only 4.9% of the dry cell mass as lipids. This result suggests that lipids are not the only energy-storage form of the methanol-grown cells. The analysis of the total lipid content contributes to this conclusion. The major part of total lipids are polar lipids (52.3%) and not nonpolar lipids whose main components are triacylglycerols. Polar lipids contain phospholipids as the principal components and sphingolipids as their

minor components. These data are in agreement with those obtained for *Candida boidinii* and *Hansenula polymorpha* grown on 1 or 4% methanol. The lipid contents of both yeasts were low (5–6%) and lipid compositions were characterized by low levels of triacylglycerol and high content of phospholipids [17]. In contrast, Jigami et al. [18] found that the yeast *Candida quilliermondii* accumulates even up to 12% of the lipids in its biomass when grown on methanol, 67% being neutral lipids.

Growth of yeast on methanol as the carbon source is accompanied by peroxisomal proliferation [19]. The proliferation of peroxisomes is harmonized with the induction of peroxisomal enzymes, catalase (a marker enzyme of peroxisomes) and the enzymes of the fatty acid β-oxidation [20]. Yeasts contain not only microbodies (peroxisomes), but also many specialized membranes [21]. Based on the recognized occurrence of phospholipid in the biomembranes, a relatively high content of polar lipids in the present study may be associated with the specific membrane development. The amount of sphingolipid long-chain bases, on the other hand, was strikingly low (Table 1). Their small amount suggests that the cellular levels of sphingolipids are low, because long-chain bases are the backbone moieties of all natural sphingolipids. This assessment is consistent with fatty acid composition (Table 2) and TLC data. The fatty acid composition indicated approximately 66% of unsaturated fatty acids along with the negligible amount of hydroxy fatty acids. The presence of hydroxy fatty acids suggests a connection with sphingolipids which, to our knowledge, represent the only source of hydroxy fatty acids. Small amount of glycolipids, whose R_F value was identical with that of standard cerebroside, was detected on thin-layer plates. Investigation of subcellular distribution of yeast sphingolipids showed that they are highly localized in the plasma membrane [22]. This fact may explain why cellular levels of the sphingolipids are low. More detailed comparisons between our findings and the earlier studies are hardly possible, because of the fact that sphingolipids and sphingolipid long-chain bases have not yet been investigated on this carbon source.

The fatty acid composition of polar lipids is characterized by high concentration of unsaturated fatty acids (Table 2). It is twice as the concentration of saturated ones. Higher degree of unsaturation is linked with methanol metabolism. The characteristics of the methanol metabolism in yeasts are the increase in the activity of catalase, marker enzyme of peroxisomes, that converts peroxidatively methanol to formaldehyde, and the formation of NADH in oxidation of methanol to carbon dioxide [23]. Desaturation of fatty acids in yeast, on the other hand, requires NADH (or NADPH) as a reducing cofactor in addition to the molecular oxygen. The high level of unsaturated fatty acids may also be associated with development of specific intracellular structures (peroxisomes). Unsaturated fatty acids are more likely to play a structural role than saturated ones, the latter being claimed to be more often incorporated into storage products. The concentration of unsaturated fatty acids is high, mainly due to palmitoleic acid, that makes up more than one half (54.5%) of total fatty acids in polar lipids. However, the concentration of polyunsaturated fatty acids is very low. The only identified polyunsaturated fatty acid, linoleic acid makes up only 4.7% of total fatty acids in polar lipids. This result indicates that methanol, as the carbon source, does not enable the accumulation of polyunsaturated fatty acids. Another feature of the fatty acid composition of polar lipids is the presence of higher content of C16 fatty acids (85% of total FA). The ratio of C16/C18 fatty acids was 6.25. Intense synthesis of C16 acid is characteristic for the microorganisms that utilize methanol via ribulose monophosphate pathway [24].

The most interesting finding in the present study is undoubtedly the composition of the long-chain bases. Sphingolipids of the investigated yeast showed the unusual long-chain base composition with a very high percentage of C19-phytosphingosine (90.8% of total LCB). This is the first time that these long-chain base is found in such a high concentration in natural source. The presence of these bases in lower concentrations was found in the mixture of cerebrins of *T. utilis* [25] and three separated cerebrins from *S. cerevisiae* (10–12% of total LCB) [26].

It is known that long-chain base synthesis de novo involves condensation of serine and palmitoyl-CoA or stearyl-CoA in order to produce LCB of 18 and 20 C-atoms [27]. Accordingly, 19-phytosphingosine is probably formed by the condensation of threonine and palmitoyl-CoA. Sphingolipids are one of the

most characteristic components of the outer membranes, suggesting that 19-phytosphingosine is also incorporated into yeast membrane that greatly influences the maintenance of the membrane structure.

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