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Lipidomic analysis of psychrophilic yeasts cultivated at different temperatures

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ABSTRACT

Analysis of polar lipids from eight psychrophilic yeasts (*Cryptococcus victoriae*, *Cystofilobasidium capitatum*, *Holtermaniella wattica*, *Mrakiella aquatica*, *M. cryoconiti*, *Rhodotorula lignophila*, *Kondoa malvinella* and *Trichosporon aggtelekiense*) grown at 4–28 °C by hydrophilic interaction liquid chromatography/high resolution electrospray ionization tandem mass spectrometry determined 17 classes of lipids and identified dozens of molecular species of phospholipids including their regioisomers. Most of the yeasts were able to grow over the whole temperature range, reaching the highest biomass at 4 or 10 °C. On temperature drop to 4 °C, all eight strains showed a significant decrease of MUFA and a simultaneous increase of PUFA such as α-linolenic acid, the content of which in the biomass reached up to 20%. We also found alterations in the proportions of individual phospholipids (PI, PE and PC), the PC/PE-ratio decreasing with decreasing temperature. With increasing temperature the content of PoO-PC rose while that of LL-PC decreased, the drop in the content of LL-PC being nearly 100-fold while the content of PoO-PC increased more than twice.

A change in temperature brought about changes in molecular species of PC (molecular species PO-PC versus OP-PC) as well as PE, i.e. PO-PE and OP-PE. The phase transition temperature of PO-PC differs from OP-PC by 7 °C and the difference between PO-PE and OP-PE is some 10 °C; we thus assume that the cell compensates for the adverse temperature effect by changing the fatty acids in the *sn*-1 and *sn*-2 positions.

1. Introduction

The ability of microorganisms to adapt to different temperatures at which they grow is fascinating. Some microorganisms were found to be capable of growing at extreme temperatures [1]. Many studies in this field that concern yeast [2] deal with ecology and more particularly taxonomy [3] while only a few describe changes of lipids and fatty acids, especially their unsaturation in psychrophilic yeast. Yeast can grow in a broad range of temperatures. This is nicely documented in one of the most common industrial processes using yeasts - production of beer - wherein optimum fermentation temperature for top brewer's yeast can reach more than 20 °C whereas for the closely related bottom brewer's yeast it is around 10 °C [4]. Essential structural components of yeast membranes are phospholipids. The main phospholipids include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA). The major fatty acids (FA) found in these phospholipids are palmitic (P; 16:0), palmitoleic (Po; 9–16:1), stearic (S; 18:0) and oleic (O; 9–18:1). Minor acids include medium length FA such as caprylic (Cy; 8:0), capric (C; 10:0), lauric (L; 12:0) and myristic (M; 14:0) acid, unsaturated acids such as gadoleic (G; 20:1), linoleic (L; 9,12–18:2) and α-linolenic acid (Ln; 9,12,15–18:3) and/or very-long chain FA, i.e. behenic (B; 22:0), lignoceric (Lg; 24:0), cerotic (Ce; 26:0) acid and many others. Among other constituents are odd-numbered FA - pentadecanoic (15:0) or pentadecenoic (15:1) acids [5,6]. Other components of yeast membrane lipids are neutral lipids, particularly sterols, especially ergosterol and its esters with FA [7].

The separation of complex lipid mixtures such as yeast lipids makes use of a number of methods [8,9]. One of the most effective ones is based on the direct entry of the sample into the mass spectrometer, often a high resolution mass spectrometer. Besides the advantages such as speed, accuracy, or low demand for processing and sample preparation the method has some drawbacks. The major advantage of liquid chromatography-mass spectrometry (LC/MS) analysis over shotgun lipidomics is the usage of chromatographic separation to simplify the complex lipid extracts. We therefore used the method of LC/MS, more specifically hydrophilic interaction liquid chromatography (HILIC), which is a variant of normal phase liquid chromatography. Due to the nature of the analyzed lipids, we preferred high resolution electrospray (ESI) mass spectrometry in positive or negative mode, which is commonly used in the analysis of lipids [10,11]. HILIC separates lipids on the basis of their polar head groups whereas separation according to chain length, number and configuration of double bonds, etc., is only

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partial. We used an HILIC column for separating different lipid classes and high resolution mass spectrometry (HR-MS), positive and/or negative ESI for identification of molecular species including regioisomers of appropriate lipid classes in 8 psychrophilic yeasts cultured at various temperatures.

2. Material and methods

2.1. Chemicals and standards

Acetonitrile, 2-propanol, hexane, dichloromethane, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (OP-PE) and ergosterol (Es) were purchased from Sigma-Aldrich (Prague, CR). Cholesteryl oleate (SE), triolein (TG), diolein (DG), oleic acid (FA), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (PC), 1,2-dioleoyl-*sn*-phosphatidylethanolamine (PE), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) (PS), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphatidylcholine (LPC), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphatidic acid, Na-salt (LPA), and 1,2-dioleoyl-*sn*-glycero-3-phosphatidyl-rac-glycerol (Na-salt) (PG), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (sodium salt) (PS) were from Larodan (Malmö, Sweden). 1,2-Dioleoyl-*sn*-glycero-3-phospho-(1′-myo-inositol) (ammonium salt) (PI) and 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-myo-inositol-3′-phosphate) (ammonium salt) (PIP), 1,2-dioleoyl-*sn*-glycero-3-phospho-(1′-myo-inositol-3′,4′,5′-trisphosphate) (ammonium salt) (PIPs), 1-oleoyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (18:1-16:0-PC, respective OP-PC), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (16:0-18:1-PC, respective PO-PC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (PO-PE) were purchased from Avanti (Avanti Polar Lipids, Inc., Alabama, USA).

2.1.1. Cultivation-microorganisms

The yeast strains used in the present study were *Cryptococcus victoriae* (DBVPG 4826), *Cystofilobasidium capitatum* (DBVPG 4845), *Holtermaniella wattica* (DBVPG 5411), *Mrakiella aquatica* (DBVPG 4979), *Mrakiella cryoconiti* (DBVPG 5179), *Rhodotorula lignophila* (DBVPG 7029) and *Kondoa malvinella* (DBVPG 7023). All were supplied by DBVPG - Industrial yeast collection, Department of Agricultural, Food and Environmental Science, University of Perugia (Italy). For long term storage the stock cultures were maintained in 20% glycerol at −70 °C. Malt extract agar (23 g/L, pH 7) was employed for short term storage. *Trichosporon aggtelekiensis* (MycoBank MB814052) was donated by Dr. A. Novakova from the Institute of Microbiology [12].

2.1.2. 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, sterilized at 121 °C for 20 min) in Erlenmeyer flasks at 10 °C on a rotary shaker at 150 rpm to the late exponential growth phase. For lipid production, 200 mL volume of YPD medium in 500 mL Erlenmeyer flasks was inoculated with 10 mL of pre-culture to a final concentration of OD_{600} 0.2 and incubated on a rotary shaker at 150 rpm and different temperatures (4 °C; 10 °C; 20 °C and 28 °C) in triplicate parallels.

The cultivations were carried out from 3 to 8 days (until early stationary phase) depending on cultivation temperature. After cultivation, the cells were centrifuged (9000 g, 10 min) and washed two times. Biomass yield was determined as dry cell weight. Biomass was frozen at −75 °C and lyophilized [13].

2.2. Isolation of lipids

Isolation and pretreatment of lipids was carried out according to Bligh and Dyer [14], with modifications as described previously [15]. For full description of the experiments see the Supplements.

2.3. FAMEs analysis

Analysis of fatty acids in the form of methyl esters (FAME) was described previously [16,17]. A complete description of all conditions of FAME analysis by GC/MS is in the Supplements.

2.4. HILIC-LC/MS-ESI

The total lipids were identified and quantified by LC/MS. A detailed description of negative ESI is again described in the Supplements.

2.5. Statistics

To perform multivariate statistical analyses, we used the program CANOCO 5 (Microcomputer Power, USA). Since the gradient lengths in the data were short, we used a principal component analysis (PCA) to visualize the variability within the dataset.

Three replicates ($n = 3$) of each cultivation, at 4 °C; 10 °C; 20 °C and 28 °C temperatures, were used. The software Statistica for Windows (version 12.0, Dell) was used for analyses. Data were presented as means ± SD.

3. Results

3.1. Identification and production of fatty acids

Table 1 gives the contents of fatty acids (FA) in polar lipids in the 8 yeast strains cultivated at different temperatures (4 °C; 10 °C; 20 °C and 28 °C). Only major FA are given and the data are given as means ± S.D. The more extensive Table 1S (Supplements) contains data on all identified FA; for space constraints the data are given without ± S.D., which in minor FA reach a maximum of ± 20 relative percent. The content of individual acids, as dependent on the temperature of cultivation, was not much different. Analysis and identification of FA, see Table 1, were done routinely. Apart from common major acids such as palmitic, palmitoleic, stearic, oleic, linoleic and α-linolenic acids, the analysis documented the presence of odd-numbered FA, e.g. margaric or margaroleic acids, minor short-chain FA with less than C16 (myristic, myristoleic) and, on the other hand, FA with more than C18 (arachidic, gadoleic, behenic, erucic, and lignoceric acids), which are commonly found in some yeasts. Table 1 and Fig. 1 show that the content of monounsaturated FA, mainly oleic acid, strongly varies in dependence on cultivation temperature and yeast species.

As seen in Fig. 1, the content of monounsaturated FA, predominantly oleic acid, decreases with increasing temperature in several yeasts, i.e. in the genus *Mrakiella*, remains virtually unchanged in *K. malvinella* and drops in *R. lignophila*. In order to verify these trends, we performed the Principal Component Analysis (PCA) of fatty acid composition in all yeast species cultivated at four different temperatures. Fig. 2 shows that of the five major acids (P, S, O, L and Ln) four are in line with the commonly accepted hypothesis about the influence of unsaturation of FA on the fluidity of the membranes under changing temperatures, the only exception being oleic acid where the first two canonical axes are plotted; the first axis explained 54.2% and the second 28.0% of the variability. We therefore focused on lipids containing these acids.

3.2. Separation and identification of polar lipids

We used two approaches, analysis of polar lipids using LC/MS and also analysis of molecular species of phospholipids, including lysophospholipids and their regioisomers (see below). The commonly used methods of analysis of lipids include TLC and HPLC and the connection of HPLC with mass spectrometer is preferred.

The chromatograms in Fig. 1S demonstrate a base-line separation of all major lipid classes (PC, PE, PG, PI, PS, and PA, including