Bioresource Technology 192 (2015) 726–734

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

New yeast-based approaches in production of palmitoleic acid

Irena Kolouchová [a,*], Karel Sigler [b], Olga Schreiberová [a], Jan Masák [a], Tomáš Řezanka [a,b]

[a] Department of Biotechnology, University of Chemistry and Technology Prague, Technická 5, 166 28 Prague, Czech Republic
[b] Institute of Microbiology, CAS, Vídeňská 1083, 142 20 Prague, Czech Republic

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.

ARTICLE INFO

Article history:
Received 24 April 2015
Received in revised form 9 June 2015
Accepted 10 June 2015
Available online 16 June 2015

Keywords:
Oleaginous yeasts
Non-oleaginous yeasts
Palmitoleic acid
Microbial lipids
Fatty acids

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

* Corresponding author at: Technická 5, CZ-166 28 Prague 6, Czech Republic. Tel.: +420 220 444 367; fax: +420 224 311 082.
E-mail address: irena.kolouchova@vscht.cz (I. Kolouchová).

http://dx.doi.org/10.1016/j.biortech.2015.06.048
0960-8524/

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