Journal of Biotechnology 234 (2016) 116–126

Contents lists available at ScienceDirect

Journal of Biotechnology

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

High lipid accumulation in *Yarrowia lipolytica* cultivated under double limitation of nitrogen and magnesium

S. Bellou, I.-E. Triantaphyllidou, P. Mizerakis, G. Aggelis*

Unit of Microbiology, Division of Genetics, Cell and Development Biology, Department of Biology, University of Patras, Patras 26504, Greece

ARTICLE INFO

Article history:
Received 1 April 2016
Received in revised form 26 June 2016
Accepted 2 August 2016
Available online 3 August 2016

Keywords:
Yarrowia lipolytica
Double limitation
Nitrogen limitation
Magnesium limitation
Organic nitrogen

ABSTRACT

Yarrowia lipolytica cultivated under double nitrogen and magnesium limitation, but not under single nitrogen or single magnesium limitation, produced 12.2 g/l biomass containing 47.5% lipids, which corresponds to a lipid production 5.8 g/l. These yields are the higher described in the literature for wild strains of *Y. lipolytica*. Transcription of *ACL1* and *ACL2*, encoding for ATP-citrate lyase (ATP:CL) was observed even under non-oleaginous conditions but high activity of ATP:CL was only detected under oleaginous conditions induced by low or zero activity of NAD^+ dependent isocitrate dehydrogenase. The low activity of malic enzyme (ME), a NADPH donor in typical oleaginous microorganisms, indicated that ME may not be implicated in lipid biosynthesis in this yeast, and NADPH may be provided by the pentose phosphate pathway (PPP). These findings underline the essential role of magnesium in lipogenesis, which is currently quite unexplored. The presence of organic nitrogen in low concentrations during lipogenesis was also required, and this peculiarity was probably related with the PPP functioning, being the NADPH donor of lipogenic machinery in *Y. lipolytica*.

1. Introduction

Single cell oils (SCOs) have been considered as potential feedstock for biodiesel production (Bellou et al., 2014a, 2016) but industrial scale implementations are presently prohibitive due to the high cost of the process (Koutinas et al., 2014). A reasonable approach aiming to reduce SCO production cost involves the use of low acquiring cost substrates (Bellou et al., 2012, 2014a,b; Chatzifragkou et al., 2011; Donot et al., 2014; Economou et al., 2010, 2011; Fontanille et al., 2012; Papanikolaou and Aggelis, 2011a; Sestric et al., 2014). However, productivities obtained so far are pretty low for supporting a sustainable industrial SCO production.

The oleaginous microorganisms cultivated in high C:N ratio media can convert the sugars into storage lipid after nitrogen depletion through a secondary metabolic process, notably similar to that accepted for mammalian cell. Specifically, nitrogen starvation triggers a cascade of biochemical events, which include disturbance of the TCA cycle due to the inhibition of the mitochondrial isocitrate dehydrogenase (ICDH), excretion of citrate in the cytosol and finally cleavage of citrate by the ATP-citrate lyase (ATP:CL) into oxaloacetate and acetyl-CoA. Acetyl-CoA together with reducing power in the form of NADPH, which is presumably provided by malic enzyme (ME) activity, both are prerequisites for fatty acid (FA) synthesis. The FAs are esterified with glycerol through the action of various acyl-transferases forming triacylglycerols (TGAs) that are stored as lipid bodies in the cytosol, while after the exhaustion of the carbon substrate the lipid reserves are routinely degraded for maintenance purpose, in a process in which various acyl-CoA oxidases are involved (for details see: Bellou et al., 2014a, 2016; Papanikolaou and Aggelis, 2011a,b). Two general approaches have been employed to maximize lipid accumulation, either through enhancing FA synthesis (e.g. by overexpressing ATP:CL and ME – Blazeck et al., 2014; Li et al., 2013; Zhang et al., 2014) or through preventing lipid degradation (by deleting genes encoding for acyl-oxidases – Dulermo and Nicaud, 2011). However, *Y. lipolytica* seems to be an unusual oleaginous organism since several strains of this yeast cultivated under nitrogen limited conditions convert the substrate (i.e. glucose or similarly metabolized) into storage lipid but this lipid is rapidly degraded after reaching a maximum value without any apparent cause, even if high substrate amounts are still

Abbreviations: ATPCL, ATP-citrate lyase; D, dilution rate; FA, fatty acid; FAMEs, fatty acid methyl-esters; GD, NAD^+-dependent glycerol-3-phosphate dehydrogenase; GK, glycerol kinase; ICDH, NAD^+-dependent isocitrate dehydrogenase; MP, meat peptone; ME, malic enzyme; NL, neutral lipids; P, phospholipids; SCOs, single cell oils; TGAs, triacylglycerols; TE, tomato extract; TL, total lipids; UI, unsaturation index; YE, yeast extract.

* Corresponding author.
E-mail address: George.Aggelis@upatras.gr (G. Aggelis).
URL: http://mailto:George.Aggelis@upatras.gr (G. Aggelis).

http://dx.doi.org/10.1016/j.jbiotec.2016.08.001
0168-1656/

available in the medium. Indeed, during lipid turnover the carbon substrate is converted into citric acid (see for instance Makri et al., 2010).

In the current investigation, the yeast *Y. lipolytica* was cultivated in batch and continuous cultures and studied according to the typical model described above for oleaginous microorganisms. Single (nitrogen) and double (nitrogen and magnesium) limited media were employed and some key enzymes implicated in lipogenesis were studied in both transcriptional level and activity. A wild type strain of *Y. lipolytica* was chosen instead of genetically modified strains in which the effect of nutrient limitation on the liposynthetic machinery would be altered by the inserted mutations. It was demonstrated that double (nitrogen and magnesium) limitation is required for a high lipid accumulation. In addition, the presence of organic nitrogen in low concentrations during lipogenesis and glucose as carbon source instead of glycerol, enhance lipid accumulation.

2. Material and methods

2.1. Microorganism and culture conditions

Experiments were conducted with *Yarrowia lipolytica* ACA-DC 50109. The strain was maintained on potato dextrose agar (PDA, Conda, Madrid, Spain) at 7 ± 1 °C.

Growth media had the following composition (in g/l): KH_2PO_4 (Fluka, Steinheim, Germany), 7.0; Na_2PO_4 (Fluka), 2.0; $CaCl_2{\cdot}2H_2O$ (Carlo Erba, Rodano, Italy), 0.1; $ZnSO_4{\cdot}7H_2O$ (Merck, Darmstadt, Germany), 0.001; $CuSO_4{\cdot}5H_2O$ (BDH, Poole, England), 0.0001; $Co(NO_3)_3{\cdot}3H_2O$ (Merck), 0.0001; $MnSO_4{\cdot}5H_2O$ (Fluka), 0.0001. Inorganic, such as $NH_4C_2H_3O_2$ and $(NH_4)_2SO_4$, and organic, such as meat peptone (MP), tomato extract (TE) and yeast extract (YE), nitrogen sources were used at various combinations and concentrations. TE was obtained after extraction of a semi-solid tomato by-product with deionised water (1:3, w/v) at 121 °C for 2 h and filtration through Whatman no. 1 paper. Magnesium source ($MgSO_4{\cdot}7H_2O$ – Fluka) was used at concentration 1.5 g/l. In magnesium limited media no magnesium was added to the medium, thus YE (containing 0.1%, w/w magnesium) was the only magnesium source. Medium pH was adjusted to 6 with a concentrated NaOH (Merck) solution. Glycerol (purity 98%, Fluka) or glucose (Merck) were used as hydrophilic carbon sources at various concentrations, while commercial refined olive oil (Hellenic fine oils S.A., Athens, Greece) was used as hydrophobic substrate at 25 g/l.

Flask experiments were performed in 250 cc Erlenmeyer flasks containing 50 ml of the growth medium as described by Bellou et al. (2014c). Briefly, the flasks were sterilized at 121 °C for 20 min, inoculated with 1 ml of a mid-exponential phase pre-culture containing 4×10^8 cells, and incubated in a rotary shaker at 180 rpm and 28 ± 1 °C. Dissolved oxygen concentration was determined using a Hana HI9146-04 selective electrode (Hanna Instruments, Cluj-Napoca, Romania) (Papanikolaou et al., 2004).

Bioreactor cultures were performed in a Bioengineering bioreactor (Ralf Plus-System, Wald ZH, Switzerland) of total volume 3.7 l and working volume 1.8 l, equipped with four baffles and two flat-bladed turbines with six blades (Rushton turbine). The bioreactor was sterilized at 121 °C for 120 min and kept at room temperature at least for 48 h in order to ensure medium sterility. The culture vessel was inoculated with 200 ml of a mid-exponential phase pre-culture containing 4×10^8 cells/ml. Dissolved oxygen concentration was kept constant at 20% of saturation value (corresponding to 1.49 mg/l at 28 °C) using a cascade controller varying agitation rate (from 150 to 280 rpm) and incoming gas (mixtures of air and pure oxygen) flow rate and composition. The medium pH was automatically controlled at 6 ± 0.03 by the addition of 1 M NaOH. Incubation temperature was controlled at 28 ± 0.1 °C. Continuous cultures were carried out at dilution rate (D) equal to 0.028 or 0.032 1/h. Fresh medium was added through a peristaltic pump (Peripex G2 IP40, Bioengineering AG, Switzerland) at a constant rate, whereas an overflow weir connected to another pump (REGLO Analog MS-4/6 ISM 828, ISMATEC, Switzerland) was operating at the same rate so that working volume was kept constant in the reactor. Fluctuations of flow rate were less than 1.3%. Steady-state conditions were obtained after continuous flow of at least five working volumes of medium. Antifoam A (Fluka) was added if necessary.

2.2. Analytical methods

2.2.1. Biomass, substrate and lipid determinations

Determinations of dry biomass (dry weight – DW), glycerol, glucose and organic acids were performed as described elsewhere (Bellou et al., 2012, 2014c). Briefly, 50 ml of culture were withdrawn and centrifuged (15,000 rpm, 15 min, 4 °C, Heraeus, Biofuge Stratos, Osterode, Germany) for harvesting the cells. The cells were washed twice with cold distilled water, dried at 80 °C until constant weight and then gravimetrically determined. The supernatant was collected and stored at −20 °C for further analysis. In the cases that olive oil was used as substrate, the culture broth was extracted with 10 ml hexane (Sigma, Steinheim, Germany) in triplicate, followed by further extraction after medium acidification with 4 M HCl solution. The organic phase was collected and dried over anhydrous Na_2SO_4 (Sigma), the solvent was evaporated under vacuum and the oil was gravimetrically determined.

Ammonium nitrogen concentration in the growth medium was determined using an ammonia gas sensing electrode (Model 51927-00, HACH, Düsseldorf, Germany) after alkalification of the broth. Organic nitrogen was determined after sample oxidation to nitrate with peroxodisulphate using the HACH Lange test kit LCK 338.

Extraction of total cellular lipids was performed with chloroform: methanol (2:1) (Folch et al., 1957). The organic phase was then washed with a 0.88% (w/v) KCl (Merck) solution and gravimetrically determined after solvent evaporation under vacuum. Total lipids (approximately 100 mg), dissolved in 1 ml of chloroform (Sigma) were fractionated by using a column (25 × 100 mm) containing 1 g silicic acid (Fluka). The column was washed successively with dichloromethane (Sigma) (100 ml) to obtain neutral lipids- NL, acetone (Fluka) (100 ml) to obtain glycolipids plus sphingolipids- G + S, and methanol (Sigma) (50 ml) for phospholipids- P. The produced fractions (NL, G + S, and P) were quantified both gravimetrically and by using known quantities of margaric acid (C17:0), added as internal standard (see below).

2.2.2. Determination of enzyme activities

Cell free extract preparation was prepared as described by Bellou et al. (2014a,b,c). In detail, cells derived from a known culture volume were harvested by centrifugation (at 15,000 rpm and 4 °C for 15 min), washed twice with a 50 mM Na_2HPO_4/KH_2PO_4 (Fluka) buffer pH 7.5 and re-suspended in a 30 mM Na_2HPO_4/KH_2PO_4 buffer pH 7.5 at a ratio 1 ml buffer per 0.5 g wet biomass. Yeast cells were ruptured at 4 °C by sonication at 70 W using a Sonics Vibra cell CV188 sonicator (Newtown, CT, USA). Cell debris were separated through centrifugation (18,000 rpm, 50 min, 4 °C), and the supernatant was collected and filtered through a Whatman 0.2 μm membrane to remove solidified lipids and remaining cell debris.

The enzymatic activities were determined in a Unicam 5625 UV/VIS (Unicam Ltd., Cambridge, UK) spectrophotometer at 25 °C. For glycerol kinase (GK) (EC 2.7.1.30) determination, the sample was incubated in a solution containing hydrazine-glycine buffer (1 M–0.2 M, pH 9.8), $MgCl_2$ (0.002 M), ATP (0.075 M) and L-α-glycerophosphate dehydrogenase. Thereafter glycerol (0.1 M) and