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# D-stat culture for studying the metabolic shifts from oxidative metabolism to lipid accumulation and citric acid production in *Yarrowia lipolytica*

Abril Ochoa-Estopier[a,b,c], Stéphane E. Guillouet[a,b,c,*]

[a] INSA-LISBP, 135 Av. de Rangueil, F-31077 Toulouse, France
[b] INRA, UMR792 Ingénierie des Systèmes Biologiques et des Procédés, F-31400 Toulouse, France
[c] CNRS, UMR5504, F-31400 Toulouse, France

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ABSTRACT

Lipid accumulation in oleaginous yeasts is triggered by nutrient imbalance in the culture medium between the carbon source in excess and the nitrogen source in limiting concentration. However *Yarrowia lipolytica* when cultivated on glucose as the sole carbon source, mainly produces citric acid upon nitrogen limitation over lipid accumulation (only 5–10% triacylglycerol). Therefore for developing bioprocess for the production of triacylglycerol from renewable carbon source as glucose it is of first importance to control this imbalance in order to avoid citric acid production during TAG accumulation.

Using D-stat cultivation system, where the N/C was linearly decreased using a constant change rate we were able to identify the N/C ratio inducing TAG accumulation (0.085 Nmol $Cmol^{-1}$) and citric acid (0.021 Nmol $Cmol^{-1}$). We therefore demonstrated that it was possible to accumulate lipids without excretion citric acid as long as the N/C was within this indicated range. Moreover enzyme specific activities measurement during the D-stat indicated that ATP-citrate lyase, malic enzyme and acetyl-coA carboxylase were strongly induced at the onset of lipid accumulation and showed different patterns when citric acid was excreted.

Our results give relevant information for future industrial bioprocess development concerning the production of lipids using renewable carbohydrate substrates as an alternative way to produce synthons for fuel or chemical industry. By controlling the N/C over the fermentation process on glucose *Y. lipolytica* can accumulate lipids without excreting citric acid.



## 1. Introduction

*Yarrowia lipolytica* is one of the most studied "non-conventional" yeasts isolated from oily environments such as polluted soils, raw poultry or dairy products (Sinigaglia et al., 1994; Deak, 2001; Lanciotti et al., 2005; Yalcin and Ucar, 2009), classified as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA, USA). It has become an important industrial microorganism due to the versatility of its metabolism to produce metabolites such as heterologous proteins (Nicaud et al., 2002; Madzak et al., 2004), lipids (Papanikolaou et al., 2003; Bankar et al., 2009; Beopoulos et al., 2009) or citric acid (Papanikolaou et al., 2002; Moeller et al., 2007; Rymowicz et al., 2007).

*Y. lipolytica* belongs to the so-called oleaginous yeasts due to its ability to accumulate high amounts of lipid mainly in the form of triacylglycerol (TAG) (Rattray et al., 1975). *Y. lipolytica* is indeed able to accumulate lipids up to 43% of its dry mass on glycerol (Papanikolaou and Aggelis, 2002) and over 50% (w/w) of total lipids, on fatty acids (Papanikolaou et al., 2003).

*De novo* lipid accumulation in oleaginous yeasts from osidic substrates is triggered by nutrient imbalance in the culture medium between the carbon source (in excess) and the nitrogen source in limiting concentration (although other nutrients can be identically made limiting, such as phosphorus, zinc, iron) (Granger et al., 1993; Ratledge and Wynn, 2002; Fontanille et al., 2012). Contrarily ex-novo lipid accumulation (i.e. from oily substrates) approximates to a bioconversion process and therefore is independent to any nutrient imbalance (Papanikolaou and Aggelis, 2011).

Ratledge and Wynn (2002) proposed a general pattern of succession of the metabolic events that take place upon a N-limitation in oleaginous microorganisms (*Candida 107* and *C. utilis*, *Rhodotorula glutinis*, *Mucor circinelloides*) leading to lipid accumulation as

* Corresponding author at: INSA-LISBP, 135 Av. de Rangueil, F-31077 Toulouse, France. Tel.: +33 5 61 55 94 47; fax: +33 5 61 55 94 00.
*E-mail address:* stephane.guillouet@insa-toulouse.fr (S.E. Guillouet).

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follows: drop in the adenosine monophosphate (AMP) concentration due to the activation of the AMP deaminase, inhibition of the isocitrate dehydrogenase (ICDH) by the low AMP content leading to the accumulation of isocitrate and citrate and then citrate that can be exported from the mitochondrion by a citrate/malate transporter, citrate is cleaved by the ATP-citrate lyase (ACL) into acetyl coenzyme A and oxaloacetate, the acetyl-coA is used for fatty acid synthesis by the acetyl-coA carboxylase (ACC). One characteristic that differentiates oleaginous yeasts from non-oleaginous yeasts is the presence of ATP citrate lyase (ACL) (Holdsworth et al., 1988; Zhang et al., 2007), several authors have also considered that acetyl coA carboxylase (ACC) (Waite and Wakil, 1962; Hasslacher et al., 1993) and malic enzyme (Zhang et al., 2007) are essential for lipid accumulation

Several studies have shown that the metabolic shift in *Y. lipolytica* is related to a nutrient-limited medium such as nitrogen, phosphorus or magnesium (Gill et al., 1977; Anastassiadis et al., 2002) or to the cultivation conditions such as growth rate, oxygen availability, pH or temperature (Finogenova et al., 1996; Il'chenko et al., 1998; Kamzolova et al., 2003; Moeller et al., 2007). One of the parameter that influences the metabolic shift is the N/C ratio (Sattur and Karanth, 1989; Anastassiadis et al., 2005). It is commonly observed in oleaginous yeasts a transient citrate excretion during the first steps of lipogenesis (Boulton and Ratledge, 1983; Evans and Ratledge, 1983a). However *Y. lipolytica* when cultivated on glucose as the sole carbon source, mainly produces citric acid upon nutrient limitation over lipid accumulation only 5–10% (w/w) of TAG (Papanikolaou et al., 2006). Therefore it is legitimate to wonder whether *Y. lipolytica* can accumulate lipids (TAG) without producing citric acid when cultivated on carbohydrate substrates. Developing a bioprocess for the production of triacylglycerol from renewable carbon source as glucose is of first interest as an alternative way to produce synthons for fuel or chemical industry. However the mechanisms controlling the metabolic shift from oxidative to citric acid production over lipid accumulation still need to be elucidated.

Chemostats have been widely used to study microbial metabolism toward numerous nutrient limitations. Alternative continuous cultivation systems were later developed for physiological studies of various microorganisms such as (i) the accelerostat (A-stat) where the dilution rate was linearly increased at a constant change rate (Paalme et al., 1995, 1997) (ii) the D-stat where the dilution rate was kept constant while one of the cultivation parameter (temperature, N/C) was modulated at a constant rate (Kasemets et al., 2003), (iii) auxostat where a cultivation parameter (pH, $pO_2$, $CO_2$) was kept constant by controlling the medium feeding pump (Kasemets et al., 2003).

We developed, in this study, a slight variant of D-stat cultivation system, where the N/C was linearly decreased using a constant change rate in order to quantify the impact of the modulation of the N/C ratio on the metabolism of *Y. lipolytica* grown on glucose as the sole carbon source. This study aimed at investigating the possibility to accumulate TAG without citric acid production in this yeast on glucose.

## 2. Materials and methods

### 2.1. Microorganism, media and growth conditions

The strain used in this study was *Y. lipolytica* W29 obtained from the Laboratoire Microbiologie et Génétique Moléculaire, INRA, (Paris-Grignon, France). The strain was maintained on YPD/glucose agar medium containing 10 g $l^{-1}$ yeast extract, 20 g $l^{-1}$ bactopeptone, 20 g $l^{-1}$ glucose and 20 g $l^{-1}$ agar. Plates were stored at 4 °C. For preparation of glycerol stocks (for cryopreservation of the cells), pure sterile glycerol was added to the culture broth to reach a final glycerol concentration of 30% (v/v). This cell suspension (glycerol stocks) was then stored at −80 °C.

Precultivations for chemostat were carried out on a 5-ml tube of YPD medium at 28 °C for 16 h at 100 rpm. The culture in growth phase was transferred into a 1-l Erlenmeyer flask containing 170 ml of mineral medium at pH 5.6 prepared as follows (all compounds are expressed in g $l^{-1}$): $(NH_4)_2SO_4$, 3.5; $KH_2PO_4$, 6; $MgSO_4$, 2; EDTA, 0.0375; $ZnSO_4{\cdot}7H_2O$, 0.0281; $MnCl_2{\cdot}4H_2O$, 0.0025; $CoCl_2{\cdot}6H_2O$, 0.00075; $CuSO_4{\cdot}5H_2O$, 0.00075; $Na_2MoSO_4{\cdot}2H_2O$, 0.00005; $CaCl_2{\cdot}2H_2O$, 0.0125; $FeSO_4{\cdot}7H_2O$, 0.00875; $H_3BO_3$, 0.0025; D-biotin, 0.00025; D-L-panthotenic acid, 0.001; nicotinic acid, 0.001; my0-inositol, 0.00625; thiamin, 0.001; pyridoxine, 0.001; para-aminobenzoic acid, 0.0002. Glucose was added to a final concentration of 18 g $l^{-1}$ with chloramphenicol at 10 mg $l^{-1}$. After 16 h of growth at 28 °C and 100 rpm, 170 ml of the broth were used to inoculate 1.7 l of the same mineral medium in a 3-l bioreactor.

### 2.2. Chemicals used

The chemicals glucose, salts, oligo-elements (Carlo Erba) and EDTA (Qbiogène), orthophosphoric acid were provided by VWR, the vitamins by Sigma. All products had the highest analytical grade available.

### 2.3. Chemostat and D-stat cultivation in bioreactor

Chemostat and D-stat experiments were performed in a 3-l stirred tank bioreactor with a working volume of 1.5 l using the Biostat B. Braun Biotech International (Sartorius AG, Germany) with the acquisition software MFCS/win 2.0. The temperature was regulated at 28 °C and the pH at 5.6 by the in-line addition of 5 M NaOH. The fermentation started in batch under full aerobic conditions. The dissolved oxygen was maintained above 20% of saturation by modulating the air flow rate or the stirring rate in order to avoid oxygen limitation. 11 h after inoculation, when the glucose was totally consumed, continuous cultivation was started. Foaming was controlled by periodically adding sterile antifoaming agent (polypropylene glycol, Aldrich) via a peristaltic pump.

For chemostat cultivation, the bioreactor was continuously fed with the mineral medium (except $(NH_4)_2SO_4$) supplemented with 23 g $l^{-1}$ glucose at 0.108 l $h^{-1}$. The bioreactor was fed with a second reservoir containing 60 g $l^{-1}$ $(NH_4)_2SO_4$ at 0.0117 l $h^{-1}$ corresponding to a N/C ratio of 0.1285 molN Cmol$^{-1}$. The working dilution rate was 0.08 $h^{-1}$.

### 2.4. D-stat feeding strategy

When the steady state was reached after 5 residence times ($\bar{\tau} = 1/D$), the D-stat was set up. The feeding rate of the mineral medium supplemented with glucose was maintained constant at 0.108 l $h^{-1}$ while the $(NH_4)_2SO_4$ feeding rate followed a linear and smooth decrease from 0.0117 l $h^{-1}$ to 0.0003 l $h^{-1}$ for 50 h corresponding to a decrease in the N/C ratio from 0.1285 to 0.0033 molN Cmol$^{-1}$ (mole of nitrogen per mole of carbon). All other parameters were maintained unchanged.

### 2.5. Off gas analysis

The inlet and outlet gas compositions in carbon dioxide ($CO_2$) and oxygen ($O_2$) were measured by a fermentation gas monitor system (LumaSense technologies Europe). The system combines a multipoint sampler 1309 with a gas analyzer (INNOVA 1313). Gas analysis was performed every minute during the whole experiment. $CO_2$ production rate ($r_{CO_2}$) and $O_2$ consumption rate ($r_{O_2}$)