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SHORT CONTRIBUTION

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Single cell oil production by *Yarrowia lipolytica* growing on an industrial derivative of animal fat in batch cultures

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Abstract The growth of an oleaginous strain of *Yarrowia lipolytica* on an industrial fat composed of saturated free fatty acids (stearin) was studied. Lipid accumulation during primary anabolic growth was critically influenced by the medium pH and the incubation temperature. This process was independent of the nitrogen concentration in the culture medium, but was favored at a high carbon substrate level and at a low aeration rate. At pH 6 and a temperature of 28–33°C, 9–12 g/l of dry biomass was produced, whereas significant quantities of lipids were accumulated inside the yeast cells (0.44–0.54 g of lipid per gram of biomass). The strain showed the tendency to degrade its storage lipids, although significant amounts of substrate fat, rich in stearic acid, remained unconsumed in the culture medium. *Y. lipolytica* presented a strong fatty acid specificity. The fatty acids C12:0, C14:0, and C16:0 were rapidly incorporated and mainly used for growth needs, while C18:0 was incorporated with reduced rates and was mainly accumulated as storage material. Reserve lipids, principally composed of triacylglycerols (55% w/w of total lipids) and free fatty acids (35% w/w), were rich in stearic acid (80% w/w), while negligible amounts of unsaturated fatty acids were detected. When industrial glycerol was used as co-substrate, together with stearin, unsaturated fatty acid concentration in the reserve lipid increased.

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Introduction

Microbial lipophilic compounds, called single cell oils (SCO), are of potential industrial interest due to their specific characteristics (Ratledge 1994; Steinbüchel 1991). Oleaginous bacteria accumulate mostly specialized lipids [i.e., polyhydroxyalkanoates (Steinbüchel 1991)]. In contrast, oleaginous yeasts and molds accumulate triacylglycerols rich in polyunsaturated fatty acids or having specific structure (Kavadia et al. 2001; Matsuo et al. 1981; Ratledge 1994). Various hydrophobic substances have been used as substrates for SCO production, such as vegetable oils (Aggelis and Sourdis 1997; Bati et al. 1984), fatty esters (Matsuo et al. 1981), crude oils (Füchtenbusch et al. 2000), soaps (Montet et al. 1985), and hydrocarbons (Alvarez et al. 1996; 1997). Animal fat, a by-product of the meat industry, could become a competitive substrate for SCO production. Few investigations, however, have been carried out on the biotechnological valorization of this low added-value material (Ashby and Foglia 1998; Desphande and Daniels 1995).

Although the conversion of sugars to lipids by oleaginous microorganisms has been studied in detail (Ratledge 1994), information concerning the biochemistry of these organisms growing on fats is restricted. Physiological properties of industrial interest, such as the degradation of the accumulated lipid, routinely observed after fat depletion from the growth medium (Aggelis and Sourdis 1997), and the microbial fatty acid specificity, are not sufficiently understood.

The present investigation is a part of work aiming to study the biochemical behavior of an oleaginous *Yarrowia lipolytica* strain growing on various by-products used as substrates. Recently, this strain has been reported to produce significant amounts of lipid (up to 43% w/w of dry weight), rich in oleic and linoleic acid, grown on industrial glycerol (Papanikolaou and Aggelis 2001). The aim of the present study was to investigate the influence of culture conditions on growth and lipid production when an industrial derivative of animal fat composed of satu-

rated free fatty acids was used as substrate. Biochemical interpretations of the yeast behavior are discussed.

Materials and methods

Microorganism and medium

Yarrowia lipolytica ACA-DC 50109 (Greek Coordinated Collections of Microorganisms project MINE) was maintained on potato dextrose agar (Fluka) at 4°C. The culture medium contained (g/l): KH_2PO_4 (Prolabo), 7; Na_2HPO_4 (Merck), 2.5; $MgSO_4{\cdot}7H_2O$ (Merck), 1.5; $CaCl_2$ (Fluka), 0.15; $FeCl_3{\cdot}6H_2O$ (Prolabo), 0.15; $ZnSO_4{\cdot}7H_2O$ (Prolabo), 0.02; $MnSO_4{\cdot}H_2O$ (Fluka), 0.06. $(NH_4)_2SO_4$ (Fluka) at various amounts and yeast extract (Fluka), at 2 g/l, were the nitrogen sources. An industrial fat composed of 100% free fatty acids of animal origin, named stearin, was used as carbon source. The fatty acid composition of stearin was (% w/w): C12:0 10; C14:0 10; C16:0 25; C18:0 52; *cis*-9-C18:1 2. Stearin was provided by Papoutsanis AS, Athens, Greece. Industrial glycerol (by-product of fat saponification process), provided by the Hellenic Industry of Glycerin and Fatty Acids (glycerol content 85%), was used in some experiments as co-substrate. Its impurities (% w/w) were potassium and sodium salts (5%–6%), non-glycerol organic materials (2%), and water (7%).

Culture conditions

Cultures were performed in an oil/water emulsion. Emulsifiers were Tween 80 (OSI) and PEG 20000 (Fluka) at 10% w/w each, against the stearin content. A T25 Ultra Turrax (Janke and Kunkel, Germany) allowed fat dispersion into the aqueous phase. Flask cultures were performed in 250-ml conical flasks containing 50 ml of medium, inoculated with 10^8 cells and incubated in a Certomat (RB Braun Descharmes, France) orbital shaker (agitation rate 185 rpm). Reactor cultures were performed in a 1.5-l MiniJar fermentor (Rikakikai, Japan), with an active volume of 1.25 l, inoculated with 0.05 l of a 24-h preculture. Reactor cultures were achieved at two dissolved oxygen (DO) levels, corresponding to oxygen saturation of 5%–15% and 60%–70%, respectively. Silicone (Sigma) was periodically added as antifoam agent. The pH was adjusted to 6.0±0.1 and automatically controlled by addition of 1 M NaOH. DO was determined off-line by a selective electrode (OXI 96, B-SET, Germany).

Growth and lipid determination

Cell concentration was determined from dry weight (80°C/24 h). Biomass was harvested by centrifugation (7,000 *g*/10 min) and washed with ethanol (95%) (Carlo Erba) and hexane (OSI), in order to remove extracellular fat from the cell surface (Montet et al. 1985; Papanikolaou 1998). Cellular lipids were extracted according to Folch et al. (1957). Unconsumed lipids were extracted from the culture medium twice using petroleum ether (Lambert Rivière) and chloroform (Carlo Erba) as solvents. The organic phase was dried on anhydrous Na_2SO_4, before being eliminated in a Büchi R-144 (Switzerland) flash evaporator.

Chemical analyses

Ammonium was determined in the culture medium after biomass removal, by an ammonia-selective electrode (Orion Inc 95-12, USA). Glycerol and organic acids were determined in a Jasco (Japan) HPLC equipped with a Polypore H 25 cm×7 mm column (Brownlee). The eluant used was H_2SO_4 (Prolabo) 0.04 N. Glycerol was detected by a RI detector (RI-930, Jasco), whereas organic acids were detected by a UV detector (UV-1575, Jasco). Thin layer chromatography analysis of lipids was performed on 60 G silica gel plates (Merck) by using petroleum ether-diethyl ether (Prolabo)-acetic acid (Fluka) (70:30:1, v/v/v) as developing system. Quantification of the different fractions was conducted by gas chromatography (GC) using heptadecanoic acid (Fluka) as an internal standard. Lipids were converted to methyl esters in a two-step reaction with methanolic sodium and hydrochloric methanol (AFNOR 1984), and analyzed in a Delsi-Nermag DI 200 gas chromatograph (USA) equipped with a COT silica capillary column Carbowax (J and W Scientific) (30 m×0.32 mm, film thickness 0.25 µm) and a FID (Delsi-Nermag). Quantification and identification of methyl esters, by GC and GC-MS analyses respectively, were performed as described elsewhere (Papanikolaou 1998).

Results

The effect of several culture conditions on growth and lipid production of *Y. lipolytica* on stearin is given below. Since emulsifiers (PEG 20000 and Tween 80) and yeast extract contain organic carbon compounds, their influence on cell growth was studied in stearin-free media. It was demonstrated that carbon components besides stearin, in the amounts used in the present study, had little or no effect on cell growth.

Effect of the medium pH

The influence of initial pH was studied in media with stearin 10 g/l, $(NH_4)_2SO_4$ 5 g/l, and temperature 28°C. Kinetics were measured at pH 5, 5.5, 6, 6.5, and 7. Substantial growth was observed at pH 6 and 6.5 (x_{max} 9.5 and 8 g/l, respectively; x = total biomass), whereas lipid (L) accumulation was favored at pH 6 [L_{max}=2.7 g/l, $Y_{L/x}$ (lipid produced per total biomass) =0.28 g/g]. At pH 5 and 7, restricted microbial growth was observed. The pH changed slightly during growth, as low amounts of organic acids (mainly citrate at 0.2–0.8 g/l) were produced. An initial pH of 6 was chosen for all following experiments.

Effect of the ammonium sulfate concentration

Kinetics were measured in media with constant stearin (10 g/l) and various $(NH_4)_2SO_4$ concentrations (0–6 g/l) (pH=6, temperature 28°C). Noticeable growth (x_{max}= 7.1–9.5 g/l) and lipid accumulation ($Y_{L/x}$=0.25–0.44 g/g) were observed in all cases. The highest amount of lipid was observed in media with $(NH_4)_2SO_4$ 0.5 g/l (L_{max}=3.8 g/l, $Y_{L/x}$=0.44 g/g). Except for trials with 0 and 0.25 g/l $(NH_4)_2SO_4$, $Y_{L/x}$ was inversely correlated with the nitrogen in the medium (data not shown).

Effect of the incubation temperature

Runs were carried out at 19, 24, 28, 33, and 39°C in media with stearin 10 g/l, $(NH_4)_2SO_4$ 0.5 g/l, and pH=6. Temperatures of 19 and 39°C did not allow high growth, whilst significant growth was observed at temperatures

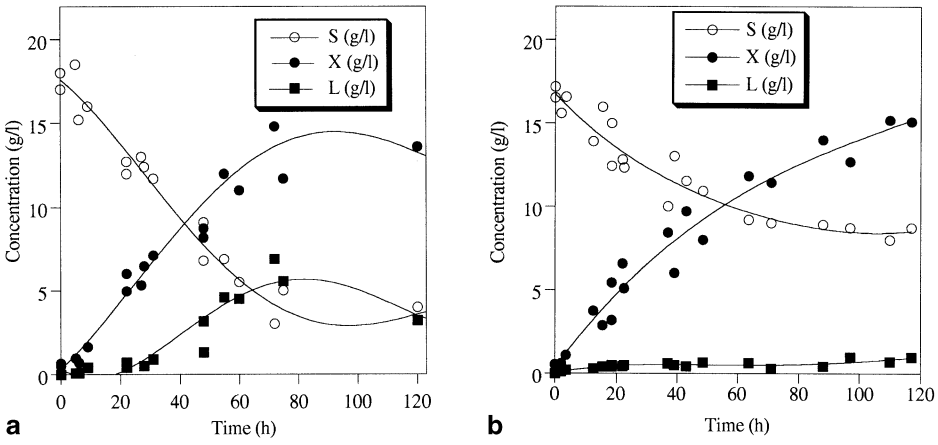

Fig. 1a, b Kinetics of biomass (*x*), stearin (*S*), and cellular lipids (*L*) of *Yarrowia lipolytica* during growth in a bioreactor. Culture conditions: stearin concentration 15 g/l, pH 6, stearin/ammonium sulfate ratio 20 g/g, incubation temperature 28°C. **a** Agitation rate 300 rpm, aeration rate 0.1 VVM. **b** Agitation rate 500 rpm, aeration rate 0.3 VVM. Kinetics measured in duplicate by using different inocula (SE<10%)

Table 1 *Yarrowia lipolytica* growth at various initial stearin concentrations. Representation of biomass (*x*), remaining substrate fat (S_f), and yields ($Y_{x/S}$, $Y_{L/x}$) when maximum concentration of cellular lipids (L_{max}) was observed. Culture conditions: initial pH=6, temperature=28°C, stearin/ammonium sulfate 20 g/g, incubation time 65–85 h. Kinetics measured in duplicate by using different inocula (SE<10%)

Initial stearin (g/l)	*x* (g/l)	L_{max} (g/l)	S_f (g/l)	$Y_{x/S}$ (g/g)	$Y_{L/x}$ (g/g)
4	3.8	1.0	0.4	1.06	0.26
10	8.7	3.8	1.0	0.97	0.44
14	11.3	5.7	3.2	1.04	0.50
15	12.5	6.8	4.5	1.19	0.54
16	10.2	5.0	5.0	0.93	0.49
20	10.3	5.1	9.9	1.02	0.50

of 24–33°C (x_{max}=7.5–8.7 g/l). μ_{max}, calculated in the exponential phase, was 0.26±0.1/h. The highest lipid production was obtained at 28°C (L_{max}=3.8 g/l, $Y_{L/x}$= 0.44 g/g), whereas at 24 and 33°C lower amounts of lipid were accumulated ($Y_{L/x}$=0.22–0.35 g/g). A temperature of 28°C was selected for all following trials.

Effect of stearin concentration in constant fat/ammonium ratios

Kinetics were measured in media with a stearin/$(NH_4)_2SO_4$ ratio of 20 g/g. Except for the experiment with stearin at 4 g/l, significant amounts of lipids were produced (0.44–0.54 g/g) (Table 1). Stearin, at concentrations higher than 10 g/l, was an adequate substrate for lipid accumulation in *Y. lipolytica*.

Effect of the aeration rate

At an oxygen saturation of 5%–15%, remarkable growth occurred (*x*=13.3±1.5 g/l) and large amounts of lipids were accumulated inside the yeast cells ($Y_{L/x}$=0.44± 0.4 g/g) (Fig. 1a). At the end of growth, the unconsumed fat was 3 g/l, while $Y_{x/S}$ (total biomass yield on stearin, *S*) was 1.1±0.1 g/g. In the highly aerated culture (oxygen saturation 60%–70%), however, remarkable growth was observed (15 g/l), but lipid synthesis was insignificant (Fig. 1b). Substantial amounts of stearin remained unconsumed in the culture medium (8 g/l), whereas $Y_{x/S}$ was 1.6±0.1 g/g, which was much higher than in the previous experiment. Low amounts of citric acid (0.5–1 g/l) were produced by both cultures.

Cellular lipid accumulation and degradation – fatty acid specificity

In the first growth step (0–80 h), high substrate fat uptake and lipid accumulation were observed. Degradation of the reserve lipid then occurred, although remarkable amounts of fat remained unconsumed (Fig. 1a). In all cases, media were not depleted of nitrogen, as around 5 mM of ammonium were found at the end of growth (initial concentrations 16–19 mM). The cessation of the substrate fat uptake did not result from nutrient limitation. In a relevant experiment, supplementation with concentrated yeast extract or salts was carried out when the exocellular fat uptake was low; even in the absence of nutrient limitation, this uptake was not improved (data not presented). Storage lipid degradation was always accompanied by an increase of the synthesis of fat-free material. The observed yield of fat-free biomass was 0.6–0.8 g/g of storage lipid consumed.

Fatty acid analysis of both substrate and reserve fat at all growth phases showed that C12:0 and C14:0 rapidly disappeared from the medium and were mainly used for growth needs, as low amounts (2%–4% w/w) were detected in the cellular lipid. C16:0 was found at 13%–14% (w/w) in the storage lipid. C18:0 was incorporated with reduced rates, since it was the principal fatty acid remaining unconsumed during the late growth phase (around 75% w/w) in the medium. It was also the dominant fatty acid of the reserve lipid (up to 80% w/w), whilst negligible amounts of unsaturated fatty acids (1%–2% w/w) were detected. Storage lipid was mainly composed of triacylglycerols (55% w/w) and free fatty acids (35% w/w). Other components were polar lipids and sterols.

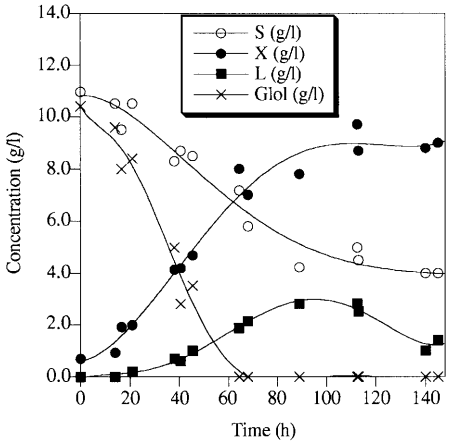

Fig. 2 Kinetics of biomass (*x*), stearin (*S*), glycerol (*Glol*), and cellular lipids (*L*) during growth of *Y. lipolytica* on a stearin/industrial glycerol mixture, in a bioreactor. Culture conditions: stearin concentration 10 g/l, glycerol concentration 11 g/l, pH 6, stearin/ammonium sulfate ratio 20 g/g, incubation temperature 28°C, agitation rate 300 rpm, aeration rate 0.1 VVM. Kinetics measured in duplicate by using different inocula (SE<10%)

Table 2 Storage lipid composition of *Y. lipolytica* during growth on an industrial glycerol-stearin mixture. Culture conditions as in Fig. 2 (SE<10%) (*SCO* single cell oil)

	Fatty acid composition of stearin (%, w/w)			
	C16:0 25	C18:0 52	*cis*-9-C18:1 2.0	*cis*, *cis*-9, 12-C18:2 Traces
	Fatty acid composition of SCO produced (%, w/w)			
Time (h)	C16:0	C18:0	*cis*-9-C18:1	*cis*, *cis*-9, 12-C18:2
68	14.5	71.5	7.0	2.0
89	15.5	72.0	6.5	2.5
112.5	16.0	73.0	6.0	2.0
113	16.0	72.0	5.0	1.5
140	17.0	76.0	4.5	1.0

Growth of *Y. lipolytica* on a medium with stearin and industrial glycerol as co-substrates

Growth on stearin was accompanied by the production of a highly saturated lipid. However, it has recently been reported that growth of *Y. lipolytica* on industrial glycerol (a by-product of the fat saponification process) was accompanied by the production of a lipid rich in *cis*-9-C18:1 and *cis*, *cis*-9, 12-C18:2 (Papanikolaou and Aggelis 2001). Therefore, the use of industrial glycerol as co-substrate, together with stearin, is an interesting possibility to produce a fat with a particular fatty acid composition (e.g., a cocoa butter analogue). Indeed, growth of *Y. lipolytica* on a stearin/glycerol mixture at an oxygen saturation of 5%–15% was followed by high growth and lipid accumulation (x_{max}=9 g/l, L_{max}=2.8 g/l) (Fig. 2), whilst reserve lipid analysis showed that some unsaturated fatty acids (5.5%–9% w/w) were stored in the yeast cells (Table 2).

Discussion

Y. lipolytica exhibited impressive cell growth and reserve lipid production when industrial saturated fats were used as substrate. The yield $Y_{x/S}$ obtained was excellent (1.1±0.1 g/g) (in accordance with Aggelis et al. 1997 and Bati et al. 1984). Lipid yield, with remarkable values ($Y_{L/x}$=0.44–0.54 g/g) in some cases, was critically influenced by the medium pH and the incubation temperature. These findings are in agreement with Bati et al. (1984) and Tan and Gill (1985). Lipid accumulation was a primary anabolic process, being entirely independent of nitrogen exhaustion in the medium (in accordance with Aggelis and Sourdis 1997). This process occurred regardless of the nitrogen availability of the medium, but in media rich in nitrogen, lipid production was lower than that observed in low-nitrogen media. Hence, it seems that in nitrogen-rich media stearin was absorbed mainly by anabolic activities other than lipogenesis. This was also supported by the fact that increased lipid production occurred in media with high stearin and constant fat/ammonium ratio. In addition, lipid synthesis was favored in low aerated media, but in highly aerated media noticeable synthesis of fat-free material occurred and lipid production was low. Similarly, *Candida lipolytica* 1094 growing on corn oil accumulated 55% (w/w) of lipids at 0%–5% saturation, but at 80% saturation, accumulated lipids were only 37% (w/w) (Bati et al. 1984).

The fatty acids C12:0, C14:0, and C16:0 were rapidly incorporated into the cells and used for growth, while C18:0 was slowly incorporated and accumulated in high percentages, up to 80% (w/w), in the reserve lipids. Negligible amounts of unsaturated fatty acids were detected, indicating that the strain did not show desaturase activity under these conditions. Similarly, *C. lipolytica* 1094 and *Langermania gigantia* growing on fats assimilated the polyunsaturated fatty acids and accumulated the monosaturated and saturated fatty acids (Aggelis et al. 1997; Bati et al. 1984). The strong fatty acid specificity of *Y. lipolytica* was probably due to the different fatty acid carriers or to differences in the fatty acid solubility in the aqueous medium.

Lipids of *Y. lipolytica* contained mainly triacylglycerols (55% w/w of total lipids), but also considerable amounts of free fatty acids (35% w/w). Growth of oleaginous yeasts and bacteria on aliphatic compounds showed that triacylglycerols were the major lipid fraction (60%–80% w/w), whilst relatively lower amounts of free fatty acids were found (Alvarez et al. 1996; 1997; Bati et al. 1984).

In the present study, 80±15 h after inoculation, cessation of extracellular fatty acid incorporation and storage lipid degradation was observed, although large amounts of substrate fat, rich in stearic acid, remained unconsumed. Hence, it seems that *Y. lipolytica* discriminated against this fatty acid, the low incorporation rate of which could not saturate the cell metabolic requirements. Similar fatty acid discrimination has been observed during growth of *Saccharomycopsis lipolytica*, *Apiotrichum*

curvatum, and *Pseudomonas oleovorans* (Füchtenbusch et al. 2000; Lee 1992; Tan and Gill 1985).

Storage lipid consumption has already been observed in oleaginous microorganisms growing on glucose (Holdsworth and Ratledge 1988; Kavadia et al. 2001) or fats (Aggelis and Sourdis 1997). Key enzymes regulating storage lipid turnover are carnitine acetyltransferase and isocitrate lyase, the activities of which considerably increased during lipid degradation period (Holdsworth et al. 1988). The activity of isocitrate lyase was low during growth on glucose, but increased considerably in cells growing on C2 compounds (acetic acid, ethanol) or on substrates leading to C2 unit formation (i.e., lipids or *n*-alkanes) (Ermakova et al. 1986; Holdsworth et al. 1988). Therefore, storage lipid consumption in cultures growing on glucose is unlikely to be a common phenomenon. In contrast, it should be considered as common in microorganisms growing on fats, since during the storage lipid uptake period, conditions are generally favorable for cell growth, and synthesis of isocitrate lyase had been previously induced (Holdsworth et al. 1988). Fat-free biomass yield on storage lipid consumed was around 0.6–0.8 g/g (in accordance with Aggelis and Sourdis 1997 and Kavadia et al. 2001).

This study allowed the identification of parameters affecting the production of lipid by *Y. lipolytica* growing on saturated free fatty acids. The degree of unsaturation of the substrate fat was not significantly altered. However, the use of industrial glycerol as co-substrate together with stearin offers the possibility of producing a SCO with a higher degree of unsaturation. More research work should be carried out with this type of experiment, in order to produce a microbial cocoa-butter equivalent with this strategy.

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