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MORPHOLOGY AND RHEOLOGICAL BEHAVIOUR OF YARROWIA LIPOLYTICA DURING PRODUCTION OF INTRA-CELLULAR ENERGETIC MOLECULES: IMPACT OF LIPID ACCUMULATION AND GENETIC MODIFICATIONS

L. Fillaudeau1, J. Cescut1, D. Anne-Archard2, J.M. Nicaud3, J-L.Uribelarrea1, C. Molina-Jouve1

(1) Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés, CNRS UMR5504, INRA UMR792, INSA, 135, avenue de Rangueil F-31077 Toulouse, France
(2) Université de Toulouse, INP, UPS, CNRS UMR5502, Institut de Mécanique des Fluides de Toulouse, Allée du Professeur Camille Soula, F-31400 Toulouse, France
(3) Microbiology and Molecular Genetic Laboratory, CNRS UMR2585, INRA UMR1238, AgroParisTech, INRA centre de Grignon BP 01, F- 78850 Thiverval-Grignon, France

Abstract: Efficient microbial conversion of carbohydrates from plant biomass into energetic molecules such as specific lipids is a real challenge in bioprocess. Our study focussed on the evolution of yeast morphology and rheological behaviour of cell broth during production of intra-cellular lipids. Three Yarrowia lipolytica strains (wild and genetically engineered strains) were investigated under controlled fed-batch culture including 3 steps (growth, nitrogen limitation and lipid accumulation). Physical (microscopy, rheology, particle size distribution, concentration) and biological (cell dried matter, lipid content) parameters were simultaneously characterised or quantified. Culture strategy enabled to reach up to 111gCDW/L in biomass and 0.36g/gCDW lipid. Results demonstrate that yeast strains are characterised by different morphologies. Lipid synthesis is a complex and vital metabolism which affects morphology and physical properties. Broth rheology depends on biomass concentration and morphology and affects bioprocess performances.

Keywords: yeast culture, Yarrowia lipolytica, genetically engineered strain, intra-cellular lipids, morphology, rheology.

1. INTRODUCTION

Negative environmental consequences of fossil fuels combustion have stimulated the research for renewable biofuels production. Recently biodiesel has become more attractive because of its benefits on actual problem linked to crude oil: depletion of resources, political instability in producing countries and greenhouse gas emissions. The conventional method for biodiesel production is the transesterification of plant oil with methanol. However, the cost of biodiesel is still more expensive than that of conventional diesel due to high cost of the raw material. Moreover utilization of oil crops, palm oil plants or animal fats may create alimentary competition as there is a balance between productions and demands (food supply and oleochemical industry). Research for specific fatty acids production processes is a major challenge for a viable biodiesel industry. An alternative route to produce biodiesel with constant and given composition anywhere on the earth with low cost could be the conversion of carbohydrates from plant biomass into lipids by micro-organisms. The oleaginous yeasts are able to accumulate lipids into lipid bodies as triglyceride and sterol ester (Moreten, 1988, Papanikolaou and Aggelis, 2002, Nicaud et al, 2007). Microbial lipids may be a prospective alternative feedstock of classical oil feedstock. Fatty acids are interesting precursor molecules for chemical or enzymatic reactions to produce compounds with relevant characteristics linked to carbon chain length and insaturation degree. With a possible conversion of carbohydrates substrates to triacylglycerol, microbial production of fatty acids constitutes an alternative to vegetable oil production. During cell culture in bioreactor, physical parameters (aeration, mixing, temperature, pH, feeds) and micro-organism physiology and activity closely interact and evolve. Irreducible couplings between heat transfer, mass transfer and fluid mechanics result in a complex and evolving system (Cascaval et al, 2003). Rheological behaviour of culture broth stands as a fundamental parameter in bioprocess performances because it affects simultaneously heat and mass transfer as well as flow pattern. Then, the understanding of rheological behaviour is determinant to drive cell culture
up to a defined goal (biomass production, extra or intra cellular metabolite production, substrate biodegradation, etc.) and to optimize bioprocess (Pamboukian and Facciotti, 2005, Petersen et al., 2008). The present investigation is a part of a work aiming to study biochemical response (growth rate substrate assimilation, lipid accumulation considering accumulation and conversion yields) of Yarrowia lipolytica under controlled operating conditions. Our study focussed on the evolution of yeast morphology and rheological behaviour of cell broth during production of intra-cellular lipids. Three Yarrowia lipolytica strains were cultivated (one wild and two genetically modified strains) in fed-batch culture mode including 3 steps (growth, nitrogen limitation and lipid accumulation). Physical (microscopy, rheology, particle size distribution, concentration) and biological (cell dried matter, lipids contents) parameters were simultaneously measured and discussed. Results provide useful information by answering to both scientific locks:

1. Does lipid accumulation contribute to modify cell morphology?
2. Do genetic modifications of lipid metabolism may affect bioprocess performances?

2. MATERIALS and METHODS

2.1 Microorganism

Yarrowia lipolytica strains were provided by Microbiology and Molecular Genetic Laboratory (MMGL, CNRS UMR2585, INRA UMR1238, Thiverval-Grignon, France). Yarrowia lipolytica is a non-pathogenic ascomycetous yeast. It is one of the most studied ‘non-conventional’ yeast species, in terms of its genetics, molecular biology and biotechnological applications. Yarrowia lipolytica is identified as oleaginous yeast which accumulates lipids into lipid bodies mainly as triglycerides and small amount as sterol ester. Lipid synthesis is a complex and vital metabolism and its synthesis can be highly increased with particular culture conditions (Moreten, 1988). Proteins involved in lipid bodies biogenesis and lipid accumulation have been partially identified (Nicaud et al, 2007) and genetically engineered strains were built by MMGL in order to introduce modification in lipids metabolism.

In this work, three Yarrowia lipolytica strains were investigated: (i) wild strain (WS), (ii) first genetically engineered strain (GES-1) built to over accumulate lipids, and (iii) second genetically engineered strain (GES-2) built to over accumulate lipids with a specific profile (C18:2) and to suppress the consumption of intra-cellular lipids.

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**Fig. 1. Overview of experimental set-up and inlet and outlet flows.**

2.2 Operating conditions and culture strategies

Fed-batch cultures were performed in a 20L bioreactor using the Braun Biostat E fermenting system (Braun, Melsungen, Germany) under highly aerated and agitated conditions. The temperature was regulated at 25 and 28°C, and the pH at 5.6 with the addition of 10mol.L^-1 NH_3 solution or KOH solution. The reactor was supervised with home-made software enabling on-line acquisition (air flow-rate, mixing rate and counter-pressure, pH, temperature dissolved oxygen, gas analyses, mass) and the control and regulation of feed flow-rates (Figure 1). During culture, the bioreactor was fed with sterile solutions (carbon source: glucose and/or glycerol solutions, mineral medium, 5M ammonia solution as nitrogen source, pH regulation solutions, anti-foam) using peristaltic pump (Masterflex and Gilson). Feeds allow optimal growth and optimal lipids accumulation. Feed flows are calculated in order to control micro-organism activity. Oxygen transfer was performed thanks to air flow and mixing (3 Rushton turbines) as well as a regulated counter-pressure around 300mbar.
Three cultures (experiments A, B and C) were performed with the different strains under stable and controlled physical and chemical parameters. Each experiment included three phases, (1) growth, (2) Nitrogen limitation and (3) lipid accumulation in which growth and lipids accumulation kinetics were controlled by nutritional feeds. During phase 1, an exponential profile was used for substrate flow-rate in order to obtain a constant specific growth rate. During phase 2, pH regulation solution was switched from NH₃ to KOH and nitrogen concentration was measured up to nitrogen limitation was reached. During phase 3, substrate was fed with controlled ratio C/N according to nitrogen flux. The bioreactor was supplied with a mineral medium feed at a flow rate in agreement to given ratio (data not shown) of the substrate feed during the growth phase and during the lipids accumulation phase.

### Table 1 Overview of experimental conditions and identification of the three phases.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Exp. A</th>
<th>Exp. B</th>
<th>Exp. C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conditions</td>
<td>Temperature [°C]</td>
<td>25-26</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>5.6</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>Pressure [mBar]</td>
<td>300</td>
<td>300-600</td>
</tr>
<tr>
<td>Phase 1</td>
<td>Time [h]</td>
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<td>0</td>
</tr>
<tr>
<td>Carbon source</td>
<td>glucose</td>
<td>glucose</td>
<td>glucose</td>
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<tr>
<td>Phase 2</td>
<td>Time [h]</td>
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<td>14.8</td>
</tr>
<tr>
<td>Carbon source</td>
<td>glucose</td>
<td>glucose</td>
<td>glucose</td>
</tr>
<tr>
<td>Phase 3</td>
<td>Time [h]</td>
<td>27</td>
<td>20.5</td>
</tr>
<tr>
<td>Carbon source</td>
<td>glucose/glycerol</td>
<td>glucose</td>
<td>glucose/glycerol</td>
</tr>
</tbody>
</table>

### 2.3 Analyses
Broth was sampled along experiment in order to quantify macroscopic parameters as:
- Yeast concentration: spectrophotometric measurement at 600nm (Spectrophotometer Hitachi U-1100, range 0.1-0.6UOD) and cell dry weight (catalytic biomass with lipids, X+lipids [gCDW/L]) measurement (filtration with 0.45µm membrane, drying 60°C, 200mmHg during 48h),
- Lipids content: methanol / chloroform solvent extraction (ASE),
- Cell concentration and size distribution: laser diffraction analyse (Mastersizer 2000 Malvern Instruments Ltd. range from 0.02 to 2000µm),
- Cell morphology: optical microscopy (Nikon, x100, in oil immersion, phase contrast mode),
- Rheological behaviour: rheometer (Bohlin C-VOR 200 Malvern Instruments Ltd, geometry: cone-plate 2°/60mm, shear rate: 0.1 to 100s⁻¹).

A data treatment of experimental biomass (cell dried weight) consisted in curve smoothing by home made software based on a polynomial sliding regression (Lirec software, LISBP). It enabled to interpolate biomass values, Biomass (liss) [gCDW/L], and avoid experimental deviation.

### 3. RESULTS & DISCUSSION

3.1 Overview of cell culture.

*Yarrowia lipolytica* cultures were conducted in fed-batch mode under perfectly controlled conditions in agreement with previously defined culture strategy. The evolution of biomass concentration (catalytic biomass with lipids) and lipid content in function of time and biomass concentration are shown in Figure 2. It is known that lipid production requires medium with an excess of carbon source (e.g.: polysaccharide, glycerol, ethanol, etc.) and limited other nutrients, usually nitrogen (Moreten, 1988).

In experiment A, the wild strain was investigated. Operating conditions enabled to reach biomass concentration of 111gCDW/L with a maximum lipid content of 0.36g/gCDW. Lipid accumulation was performed with glucose/glycerol feed and biomass concentration increased from 50 up to 111gCDW/L during phase 3. In experiment B, GES-1 strain was cultivated and a concentration around 104gCDW/L with a lipid accumulation up to 0.35g/gCDW were achieved. During lipid accumulation, concentration is maintained constant (around 100gCDW/L) in order to evaluate the impact of lipid accumulation without any other perturbation. In experiment C, GES-2 strain was tested; concentration was limited to 57.9gCDW/L due to transfer limitations connected to rheological and morphological aspects. During phase 3, lipid accumulation was controlled by a glucose/glycerol feed. In a first step, a poor lipid accumulation (0.27g/gCDW) was observed probably due to genetic mutation. In a second step (t>47h), intra-cellular lipid consumption was investigated by decreasing carbon source flux.
Fig. 2. Evolution of biomass concentration, X+lipids [gCDW/L], lipid contents [g/gCDW] and rheological parameters, n [] and k [Pa.s] in function of time [h] and biomass (liss) concentration, [gCDW/L].

3.2 Morphology.
Yeast strains were characterised by highly different morphological profiles and genetic type (WS, GES-1 and GES-2) strongly affected strain morphology: WS and GES-1 exhibited mono-dispersed spherical / ovoid populations whereas GES-2 showed bimodal distribution including ovoid and filamentous particles. In Figure 3, pictures illustrating shape and dimension of microorganism, are shown, and the evolution of particle size distribution weighted by equivalent biomass concentration is plotted for different biomass concentrations. Figure 4 shows the evolution of mean volume weighted diameter, D[4,3] and population span, (D(0.1)-D(0.9))/D(0.5) versus biomass in experiments A (WS) and B (GES-1). From growth phase up to lipid accumulation phases, D[4,3] evolved respectively from 5 to 3.5µm and from 7 to 4µm for WS and GES-1. Meantime, WS and GES-1 distribution narrowed as span decreased. For GES-2, D[4,3] was an inaccurate parameter because an ovoid population with a mean diameter around 3-4µm was mixed with a filamentous population with a mean diameter around 50-70µm.

Yarrowia lipolytica strains exhibited similar behaviour during successive phases in regard with morphological changes. A narrowing of particle distribution with a higher proportion of ovoid cells was observed in each culture. Pictures and laser diffraction analyses provided useful and important information about population distribution and evolution. However, the complex particle shape required other dimensional criteria in addition to mean diameter.
Fig. 3. Microscopy of fermentation broth samples (x100, oil immersion, phase contrast mode) and particle size distribution x equivalent cell concentration (estimated with Mastersizer2000) for experiments A, B and C.

### 3.3 Rheological behaviour.

Rheological behaviour of *Yarrowia lipolytica* cell broth was investigated for a shear rate ranging from 0.1 to 100s⁻¹ and described by a power-law model. Figure 2 shows the evolution of index behaviour, n [⁻¹] and consistency index, k [Pa.sⁿ] as functions of time and biomass along growth, nitrogen limitation and lipid accumulation. In all experiments, the rheological behaviour of supernatant revealed a Newtonian behaviour with a viscosity ranging between 3 and 7mPa.s (25°C). During growth and nitrogen limitation phases, experiments A, B and C demonstrated that rheological behaviour was closely correlated to cell concentration. A shear-thinning behaviour quickly appeared with biomass concentration increase and structure index tended to 0.4. Apparent viscosity (25°C) for 1s⁻¹ shear rate was respectively of 0.016, 0.12 and 0.9Pa.s⁻¹ for experiments A, B and C related to a biomass concentration close to 30gCDW/L showing a morphological effect. Morphology constituted a limiting factor to heat and mass transfer as well as power consumption if intensification of bioprocess is aimed. Filamentous (length) particles appeared as major difficulty for scale-up when genetic modification of lipid metabolism were studied. During phase 3, in experiments A and B lipid accumulation seemed to affect broth rheology. A lost of shear-thinning properties was noticeable in experiments A and B, with an increasing structure index. This phenomenon was precisely illustrated in experiment B within constant biomass concentration (t>35h) when lipid accumulation occured. Physical properties (elasticity, deformation) of particles are assumed to be modified during lipid accumulation (Trepat et al, 2007). In experiment C, filamentous particles were assumed to modify rheological behaviour (Petersen et al, 2008) but none
The impact of lipid accumulation seems to be observed. This point is in agreement with culture strategy within lipid accumulation occurred between t=35 and 47h (slight structure index increase), after t>47h intracellular lipid consumption was controlled.

Fig. 4. Evolution of the mean volume weighted diameter, $D_{4,3}$ and span versus biomass (liss) concentration, X+lipid [gCDW/L] for experiments A, B.

4. CONCLUSION

In present work, the evolution of yeast morphology and rheological behaviour of *Yarrowia lipolytica* cell broths was scrutinised during production and accumulation of intra-cellular lipids. Cultures were conducted in fed-batch mode under perfectly controlled conditions in agreement with a culture strategy including three phases: growth, nitrogen limitation and lipid accumulation. Three *Yarrowia lipolytica* strains were investigated: (i) wild strain (WS), (ii) first genetically engineered strain (GES-1) built to over accumulate lipids, and (iii) second genetically engineered strain (GES-2) built to over accumulate lipids with a specific profile and to suppress the consumption of intra-cellular lipids. Culture strategy enabled to reach a biomass concentration up to 111gCDW/L with a maximum lipid content of 0.36g/gCDW. The morphology of yeast strains in relation with their genetic type (WS, GES-1 and GES-2) were characterised: WS and GES-1 exhibited mono-disperse spherical / ovoid populations whereas GES-2 showed bimodal distribution including ovoid and filamentous cells. During successive phases, a narrowing of particle distribution with a higher proportion of ovoid cells was noticeable. Results clearly demonstrated that lipid synthesis is a complex and vital metabolism and its synthesis affected cell morphology and physical properties. Genetic engineered *Yarrowia lipolytica* strains dedicated to introduce modification in lipids metabolism should carefully consider consequences on cell morphology. Various consequences of genetic modification on cell morphology were observed that could strongly reduce bioprocess performances. Broth rheology is closely related to biomass concentration, lipid accumulation and cell morphology.

In the future, our works will focus on morphological characterisation and will aim to propose rheological models for *Yarrowia lipolytica* cell broth as a function of cell concentration, population morphology and lipid accumulation.

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