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IMPACT OF CELL PHYSIOLOGY AND DENSITIES DURING OXIDATIVE AXENIC CULTURES OF *YARROWIA LIPOLYTICA* ON PHYSICO-CHEMICAL PROPERTIES OF BROTH

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1. INTRODUCTION

Accurate measurements of cell properties (concentration, shape, dimension, density) are fundamental to understand microorganism physiology and they strongly affect physico-chemical properties of broth. These characteristics interact with bioprocess performances (specific rates, yields...) with an improvement or, more generally, a decrease of yields. Experiments were conducted with a singular set-up based on a bioreactor (20L) with a derivation loop including in-situ physical and biological measurements (viscosity, specific density, mass flow rate, electrical conductivity, pH, pO₂ and temperature). Three axenic cultures with *Y. Lipolytica* were investigated in pure oxidative culture in order to produce biomass up to high cell concentration with a control of growth rate by carbon feed (synthetic mineral medium). Cell size and morphology, intracellular water and salts contents are scrutinized along fed-batch cultures. Our strategy aims to investigate the evolution of bioperformance related to physical properties of broth (rheometry, densimetry, conductimetry, cell morphology) versus cell concentration (ranging from 0.1 to 110 gCDW/L), growth rate ($\mu=0,1$ to $0,2$ h⁻¹, cultures #1 and #2) and dimorphism generation by pH changes (pH=5,5 to 7; $\mu=0,1$ h⁻¹; culture #3).

2. MATERIALS AND METHODS

2.1 *Experimental set-up and instrumentation*

The experimental set-up, described by Manon et al. 2011, consisted of a 20 L bioreactor (Chemap-Fermenter, Chemap AG, CH-8601 Volketswil), a displacement pump (TUTHILL DSG 1.3EEET, Q_{max}: 350 l h⁻¹, P_{max}: 5 bars) and a fully instrumented derivation loop (Fig.1).

2.2 *Specific analyses*

Thermogravimetry allowed a quick and accurate estimation of intra- and extracellular water of yeast cells, dry matter, volume and density of a single cell (Illmer et al, 1999). The assumption of the method is that drying of microbial cells occurs during two phases. First, the more volatile extracellular water is lost. Not before this fraction is completely evaporated, the intracellular water starts to vaporise. A 15 ml culture broth is sampled, centrifuged (10min, 4800 tr/min, 4°C), supernatant is removed, weight are controlled at each step. The cell sediment is then diluted with a known weight of distilled water (2/3 of sediment

weight). Drying of 2-4g sample is conducted at 75°C during 120 minutes with a balanced equipped with oven (Metler LJ16 moisture analyser, 160g max, ± 1 mg).

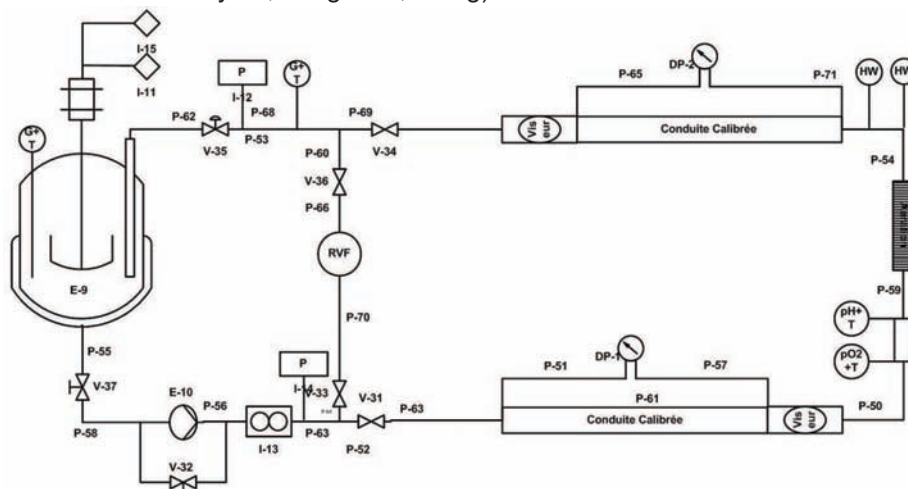


Fig.1. Overview of experimental set-up

Densimetry: Sample density was measured with densimeter (DE40 Mettler Toledo, 0- 3g/cm³, ± 0.0001 g/cm³) following the oscillating body method. Measurement is based on the electromagnetically induced oscillation of a glass U-tube. In our case, 5ml of broth and supernatant samples were analysed at 28°C (cell culture temperature) after 5 minutes rest in order to stop biological activity and to remove gas phase. Optical microscopy: Cells were observed during cultures using an optical microscope (Olympus BH2, objective: DA po 100 UV PL) equipped with a CCD camera (DXM 1200, Nikon). The images are digitized under 975x715 pixel size with the graphics board Matrox 975-0201. The resolution of the system allows observing details with the size of 0.5 μ m.

Cell granulometry : particle size distribution and cell concentration were determined through laser diffraction analyses (Mastersizer 2000 Hydro, Malvern Instruments Ltd. SN: 34205-69, range from 0.02 to 2000 μ m). Samples were diluted between 0.5 up to 5/100 and 150mL and were analysed at room temperature (20°C \pm 2). The batch mode was used under constant circulating and gentle stirring to avoid cell decantation within measurement cell. The linear evolution of obscuration rate (red λ =632.8nm and blue λ =470nm lights), as function of concentration was controlled and enabled to carry out analyses between 10 and 50%. Volume particle distribution and associated cumulative curve as a function of particle diameter was determined.

2.3 Culture conditions and strategy

Y. lipolytica (wild strain W29) is a non-pathogenic ascomycetous yeast and one of the most studied 'non-conventional' yeast species, in terms of its genetics, molecular biology and biotechnological applications. Axenic cultures of this eukaryote microorganism under pure oxidative fed-batch conditions are considered in order to evaluate biomass and dimorphism effect on physic-chemical properties of broth. *Yarrowia lipolytica* cultures were investigated with a control of growth rate by carbon feed under synthetic mineral medium and controlled physical and chemical parameters in experimental set-up with highly aerated (<3.3 VVM), agitated conditions (<1500 RPM) and 250 l/h as nominal flow rate within derivation loop.

3. RESULTS & DISCUSSIONS

3.1 Study of the physiology of microorganisms during cultures

Cell concentrations and growth rates are reported in Table 1. For cultures #1 and #2, growth rates are 0.18 and 0.085h⁻¹. The change in optimal culture conditions (pH5.5 and T=28°C) affects cell physiology and during culture #3, the growth rate is estimated to 0.09h⁻¹ under initial conditions (pH5.5, T_p=28°C) but slightly decrease to 0.075h⁻¹ when pH is increased to 7 in order to generate mycelial transition.

Cell physiology was investigated along cultures through microscopic observation (Fig 2), laser granulometry and morphogranulometric measurements. For the first two cultures (#1, #2), an extension of cell was observed at the beginning of the culture, but these morphological changes are negligible comparing to the dimorphism generation during the culture #3. In initial step, a reduction of mean diameter and sharp decrease of size span was observed. This phase correspond to the adaptation of inoculum to new culture conditions and physico-chemical environment. Over 3gcdw/L, cell populations became homogeneous (#1 and #2) with a mean diameter close to 5µm. In culture #3, over 5-10gcdw/L a pH change was imposed to induce a mycelial transition. An increase of population fraction whose diameter is greater than 10µm was observed, which grows from 1.5 up to 12%vol/vol (Fig. 3).

Table 1: Performances overview and cell morphology

Cultures	#1	#2	#3
Growth rate, μ [h ⁻¹]	0.180	0.085	0.09 and 0.075 (stress)
Cell concentration, X [gms/L]	0.46 → 63	0.42 → 114	0.38 → 4.7 → 56
Shape	Ovoidal	Ovoidal	Ovoidal/Filamentous
Size D[4,3] (D0.1, D0.9) [µm]	5.0 (2.1/9.4)	3.8 (2.1/5.9)	4.5 (2.1/7.6)

Surface measurements (surface diameter, d_s or equivalent circle diameter) obtained by morphogranulometry laser were performed for culture #3. In accordance with the laser granulometry, a slight decrease of equivalent circle diameter was observed and reflects the initial state of the inoculums.

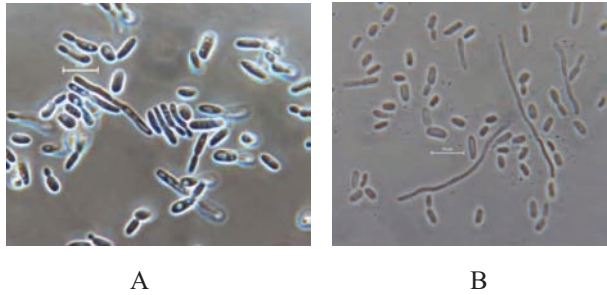


Fig.2. Cell observations (x100, oil, 10gcdw/L) for cultures #1 (A) and #3 (B).

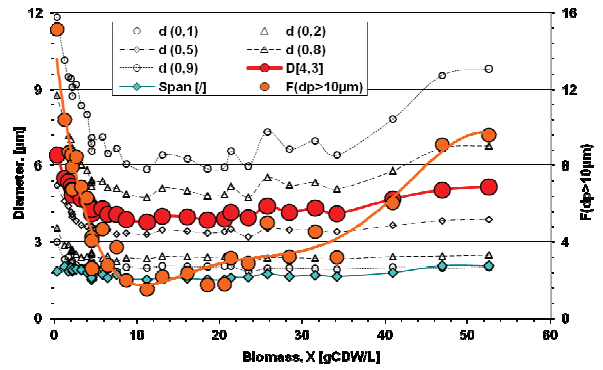


Fig. 3. Diameters evolution $d(0.1)$, $d(0.5)$, $d(0.9)$, $D[4.3]$, dispersion (span) and Fraction $F(dp > 10\mu m)$ versus biomass (Culture # 3)

3.2 Intrinsic cell density

The analysis of drying curve (Fig. 4) leads to deduce the absolute and relative water contents of biomass, n and x , along cultures #1, 2 and 3. The relative humidity of biomass ranges between 80 and 70% (equivalent to an absolute humidity between 2.3 and 4) and substantially decreases with increasing concentration. Average values of absolute and relative humidities respectively equal to 73% (± 4) and 2.80 (± 0.58) can be taken as a reference for *Yarrowia lipolytica*.

The mass and volume balances lead to establish relations between water content and density:

Density: $\rho_{mout}^{Ex-situ} = \frac{m_{Total}}{V_{Total}}$, $\rho_{XH} = \frac{m_{XH}}{V_C}$ and $\rho_X = \frac{m_X}{V_C}$ with $x = \frac{m_{eau-X}}{m_{XH}}$ and $n = \frac{x}{1-x}$
 with $\rho_X = \rho_{XH} \cdot (1-x)$ et $m_X = X \cdot V_{Total}$

With: $\rho_{mout}^{Ex-situ} = \rho_s + X \cdot \left(n + 1 - \frac{\rho_s}{\rho_X} \right) = \rho_s + X \cdot \left(\frac{1}{1-x} - \frac{\rho_s}{\rho_X} \right)$

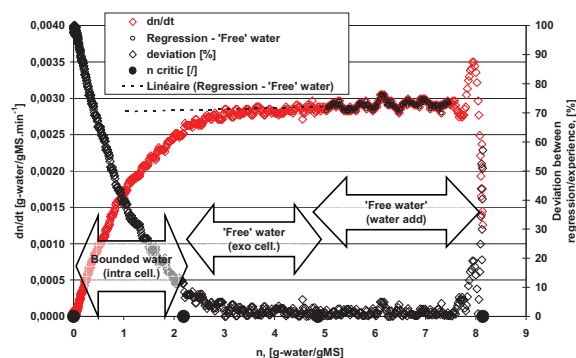


Fig. 4. Example of drying curve— Culture #3, E29

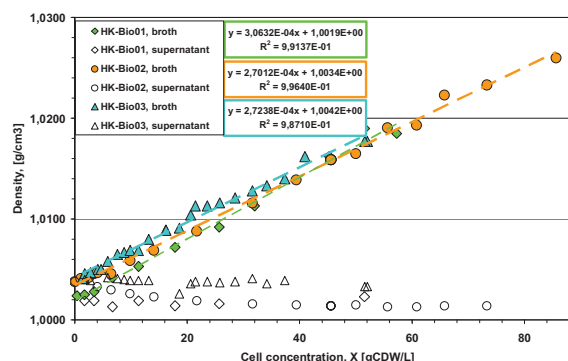


Fig. 5. Broth and supernatant ex-situ density evolution versus cell concentration - Cultures # 1, 2 and 3.

The ex-situ (broth) density will proportionally increase as a function of cell concentration, X . Slope enables to determine intrinsic cell density assuming a constant water content, origin correspond to supernatant density (Fig. 5). The ex-situ density of supernatants remains constant for cultures #1 (1.0017g/mL ± 0.0003), #2 (1.0019g/mL ± 0.0007) and #3 (1.0037g/mL ± 0.0003). The absence of drift of the supernatant density confirms the lack of cell lysis or co-products in the cultures. The broth density evolves linearly with the biomass concentration in agreement with theory. The intrinsic densities of biomass (assuming: $x=0.73$ g-water/gXH) were $\rho_{XH}=1092$ g/L ϕ ($\rho_X=294$ gms/L ϕ), $\rho_{XH}=1081$ g/L ϕ ($\rho_X=292$ gms/L ϕ) and $\rho_{XH}=1083$ g/L ϕ ($\rho_X=292$ gms/L ϕ) for culture #1, #2 and #3 respectively. Wet and dry biomass densities, ρ_{XH} and ρ_X , are close to values reported for *Saccharomyces cerevisiae* by Moll (1994) and Bryan et al. (2010). However, data reported by Jarzebski et al (1989) for dry density will correspondant to other estimated wet density assuming $n=0.73$ g-water/gXH. For *Yarrowia lipolytica*, the mean values of wet and dry biomass densities are estimated to be 1085g/L ϕ and 293gms/L ϕ respectively. It seems that mycelial transition induced in culture #3 does not affect the biomass density.

4. CONCLUSION

In-situ and ex-situ density (broth, supernatant) exhibit opposite tendency. Ex-situ density increases and is correlated with cell concentration considering population balance (size, water and salt contents). Intrinsic cell density of *Yarrowia lipolytica* has been properly identified and compared to litterature. Yeast mycelial transition (morphological change) does not affect intrinsic cell density. On the opposite, in-situ density decreases along fed-bath culture and could be significantly affected by cell physiology (oxygen consumption specific rate) and cell concentration.

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