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Flow Cytometry as a Tool to Verify Media Influence in Bio-Oil Accumulation by *Yarrowia Lipolytica*

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Yarrowia lipolytica, a non-conventional and strictly aerobic yeast, has been extensively studied due to its ability in producing substances attractive for the industry, such as organic acids, proteins, lipases and lipids. Oleaginous microorganism refers to a group that can present a lipid content higher than 20% of its cell dry weight. *Y. lipolytica* is described by literature as an oleaginous yeast because the presence of a large amount of oil into the cell. Oil is stored usually in a special organelle that is designated by different terms in literature, such as lipid particle, oil body, lipid droplet (LD) or lipid body (LB). The structure comprehends neutral lipids, mainly triacylglycerol and steryl esters, forming a hydrophobic core encompassed by a phospholipid monolayer with some proteins. There are several methods available to determinate intracellular oil; however all of them demand a long time to provide data. Flow cytometry is a technique that provides data almost in real time (at line). Therefore it is possible to evaluate the biological system in a qualitative and quantitative way and faster than the traditional methods. The yeast strain *Yarrowia lipolytica* IMUFRJ 50682 is currently being used in our laboratory as a model to investigate the production of a plurality of desired substances, lipase, citric acid and the like. In the present work, this yeast was cultivated on three different media in flasks. Medium 1: complex mineral medium plus pure glycerol; Medium 2: simple mineral medium plus crude glycerin and; Medium 3 simple mineral medium plus glucose. Lipid accumulation was monitored by flow cytometry associated with the fluorescent stain Nile Red (9-diethylamina-5H-benzo[a]phenoxazine-5-one). By using this technique, it was able to determinate cell total lipid and distinguish polar lipids and neutral lipids into the cell, the last ones related with stored oil particle. Although three media used showed differences in oil accumulation, their growth profiles were very similar. A significant increment in total lipid content was detected in Medium 1 and 3, using pure glycerol and glucose, respectively. The results obtained demonstrate flow cytometry as a successful technique to monitor lipid storage in cultures of *Y. lipolytica*.

1. Introduction

The dimorphic yeast *Yarrowia lipolytica* is a non-conventional and non-pathogenic microorganism intensively studied by our research group due to this ability to produce a plurality of desired substances such as organic acids, proteins, lipases and lipids. Traditionally, this yeast uses glucose as carbon source. However, glycerol can be used instead, and as an additional alternative raw glycerol derived from industrial production can be used as well (Papanikolaou et al., 2002). *Y. lipolytica* is reported by literature as an oleaginous microorganism because of the presence of a large amount of oil into the cell (Rywinska et al., 2013). Oleaginous microorganisms consist in a group able to present lipid content higher than 20% of their biomass (Rossi et al., 2011). Oil from *Y. lipolytica* has truly biotechnological application potential in biodiesel production as an alternative to vegetable oil and/or animal fats. Microbial oil is stored usually in a shape of a special compartment into the cell. The structure is named in literature by different terms, for instance lipid particle, oil body, lipid droplet (LD) or lipid body (LB). Neutral lipids, mainly triacylglycerol and

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steryl esters, comprise the hydrophobic core of lipid body encompassed by a phospholipid monolayer embedded with some proteins (Mlicková et al., 2004, Rossi et al., 2011).
Although there are available several methods to determinate intracellular oil, most of them demand a long time to provide data and usually uses non environmental friendly solvents and residues (Bligh and Dyer, 1959). In order to monitor bioprocesses and obtain faster information, more tools and techniques have been adopted. Flow cytometry is a multiparametric technique that is able to provide data almost in real time (at line). Accordingly, it is possible to evaluate the biological system in a qualitative and quantitative way and faster than traditional methods (Silva et al., 2012). This technology characterized each individual cell that allows a quantitative analysis of a population. Cells intercept a radiation beam and optical signals are generated by radiation, and structural and functional cell parameters are correlated with the intensity of those signals. In addition, by using cytometry associated with fluorochromes, new data can be provided about cell structures and/or elements. For instance, lipid is determined with the fluorescent dye Nile Red that is able to bond with neutral and polar lipids from the cell (Díaz et al., 2010).
The present work purpose is to monitor and determine oil accumulation into *Yarrowia lipolytica* cell grown in three different media by flow cytometry.

2. Materials and Methods

2.1 Microorganism and pre-inoculum Preparation

A wild *Yarrowia lipolytica* strain IMUFRJ 50682 isolated from an estuary of Guanabara Bay in Rio de Janeiro, Brazil and identified by the Institute of Microbiology, Federal University of Rio de Janeiro (Hagler and Mendonça-Hagler, 1981) was maintained in a slant culture at 4 °C in medium YPD-agar (glucose 2 % w/v, yeast extract 1 % w/v, peptone 2 % w/v and agar 2 % w/v).
Pre-inoculum was prepared by growing cells from slant culture in 500 mL Erlenmeyer flasks containing 200 mL of YPD medium (yeast extract 1% w/v, peptone 2% w/v and glucose 2% w/v) for 72 hours in a shaker at 160 rpm and 28 ºC.

2.2 Media and Culture Conditions

Three different media were used in the following conditions: 200 mL of medium in 500 mL erlenmeyer flask in a shaker at 250 rpm and 28 ºC. Media composition is described as follows. Medium 1 (Santos et al., 2012): 7.0g /L KH_2PO_4, 2.5g /L Na_2HPO_4, 1.5g /L $MgSO_4.7H_2O$, 0.2g /L $CaCl_2.2H_2O$, 0.15g /L $FeCl_3$, 0.02g /L $ZnSO_4.7H_2O$, 0.06g /L $MnSO_4.H_2O$, 0.10g /L yeast extract (N content 10%) and 64.0 g/L crude glycerin with 80% w/w of glycerol. Medium 2 (modified from Kovalchuk, 2005) 1.0g /L KH_2PO_4, 0.16 g /L $K_2HPO_4.3H_2O$, 0.70g /L $MgSO_4.7H_2O$, 0.50 g/L NaCl, 0.40 g/L $Ca(NO_3)_2.4H_2O$, 3.0 g/L $(NH_4)_2SO_4$, 0.60 mg/L H_3BO_3, 0.048 mg/L $CuSO_4.5H_2O$, 0.12 mg/L KI, 0.48 mg/L $MnSO_4.4H_2O$, 0.24 mg/L $Na_2MoO_4.2H_2O$, 0.48 mg/L $ZnSO_4.7H_2O$, 2.0 mg/L $FeCl_3.6H_2O$, 0.3 mg/L thiamim hydrochloride, 20.0 mg/L uracil plus 116.2 g/L pure glycerol. Medium 3 (Martins, 2013): 7.0 g/L KH_2PO_4, 2.5 g/L Na_2HPO_4, 1.5 g/L $MgSO_4.7H_2O$, 0.2 g/L $CaCl_2.2H_2O$, 0.09 g/L $FeCl_3.6H_2O$, 0.02 g/L $ZnSO_4.7H_2O$, 0.06 g/L $MnSO_4.H_2O$, and 43.34 g/L glucose plus 1.0 g/L $(NH_4)_2SO_4$.
Biomass initial concentration of 1.0 g cell dry/L from pre-inoculum was used to inoculate the media.

2.3 Biomass quantification

Biomass concentration was determined by optical density measurements at 570 nm. Absorbance values were converted to biomass dry weight (g per liter) by a predetermined factor (Martins et al., 2012).

2.4 Lipid determination by flow cytometry

The yeast lipid content was assessed by multi-parameter flow cytometry in association with fluorescent dye Nile Red (NR). The NR fluorescence was determined using CyFlow Space flow cytometer equipped with a 488 nm argon laser. Upon excitation by a 488 nm argon laser, NR emits fluorescence at 580 nm and 610 nm when dissolved in neutral and polar lipids respectively (Andrade et al., 2012) which are detected by the FL2 (590 ± 25 nm) and FL3 (675 ± 20 nm) channels. Cultured cells were stained with 3.0 x 10^{-3} μM NR in 1 mL of volume reaction, incubated at 37°C for 7 minutes. For each sample, approximately 60,000 cells/mL were analysed using a log amplification of the fluorescent signal.
Flow cytometry data was analyzed using the FloMax© Software version 2.7 (Partec GmbH, Munster, Germany).

3. Results and Discussion

In previous studies (Santos et al., 2012 and Martins, 2013), *Y. lipolytica* was able to accumulate intracellular lipid when it was cultivated in current selected media. Hence, these media were used to evaluate the lipid content and its type in yeast during a batch growth. Each medium shows a

distinguishable biomass concentration profile during fermentation (Figure 1). Medium 3 used glucose as carbon source and it exhibits higher cell concentration (X_F= 12.55 g/L) by the end of assay compared to media that had glycerol as carbon source (Medium 2, X_F= 4.73 g/L and Medium 3, X_F= 10.54 g/L). It is also detectable different specific growth rates among the media used (Medium 3, μ = 0.10 h^{-1}; Medium 2, μ = 0.12h^{-1}, Medium 1, μ = 0.08h^{-1}).

Figure 2A represents fluorescent intensity values detected by FL2 (neutral lipids). It has been found that at 24 h fluorescent intensity was relatively low. This response is probably due to cell metabolism in this phase that is directed towards cell growth. There is a fluorescent intensity increase in following samples. Medium 2 and 3 show their highest values at 96 h and Medium 1 at 168 h. These high intensity values occurred during stationary phase for all media used. According to Raschke and Knorr (2009) LD formation starts during late exponential phase and continues during stationary phase not as soon as the carbon sources in the growth medium start to diminish. Andrade et al. (2011) monitored the yeast *Rhodosporidium toruloides* NCYC 921 growing under carbon and nitrogen limitation by flow cytometry and observed the maximum fluorescence intensity in the stationary phase. Their results support data reported herein.

Detector FL3 refers to fluorescence corresponding to polar lipids (Figure 2B) that usually comprise phospholipid lipid of lipid body involucre and cell membranes (Rossi et al., 2011). FL3 fluorescence profile of each medium seems to have a similar shape to its correspondent FL2 profile, considering occurred an increase in FL3 value (polar lipids) there was an increase in FL2 (neutral lipids) as well. It is possible probably because both lipids are in the lipid body.

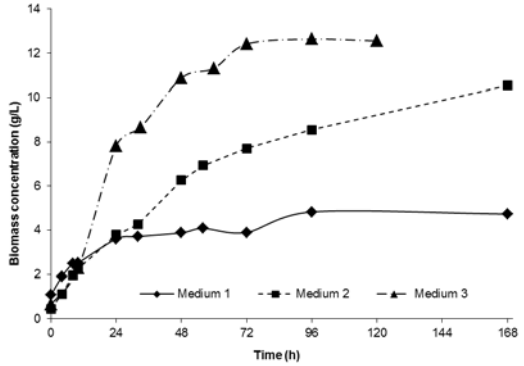
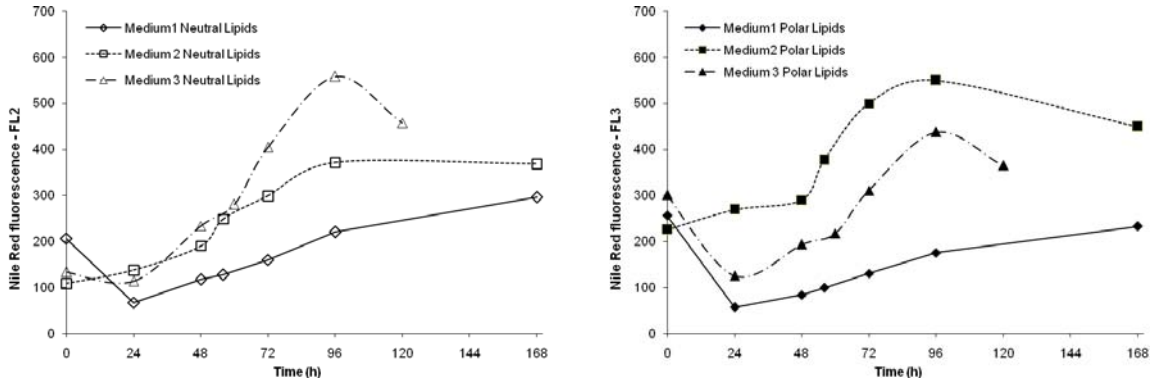


Figure 1: Biomass concentration (g/L) during yeast growth in Medium1, 2 and 3.



(A) (B)

Figure 2: Nile red fluorescence profiles for neutral lipids in FL2 (A) and polar lipids in FL3 (B).

Figure 3 shows FL2 fluorescence histograms in Medium 1, 2 and 3 at 24 h and 96 h. These graphics allow identification of an increase in fluorescence intensity with increasing culture age. Histograms show FL2-Nile Red peak shifting from low fluorescence 100 at 24 h (Figure 3 A, C and E) to high fluorescence 1000 at 96 h (Figure 3 B, D and F). This shift in fluorescence intensity might be due to growing lipid droplets into the cells (Raschke and Knorr, 2009). Figure 3 presents different profiles due to difference in the

composition of each medium, that present distinct C/N ratios and minor elements, like aminoacids, that can influence in such observed behaviour.

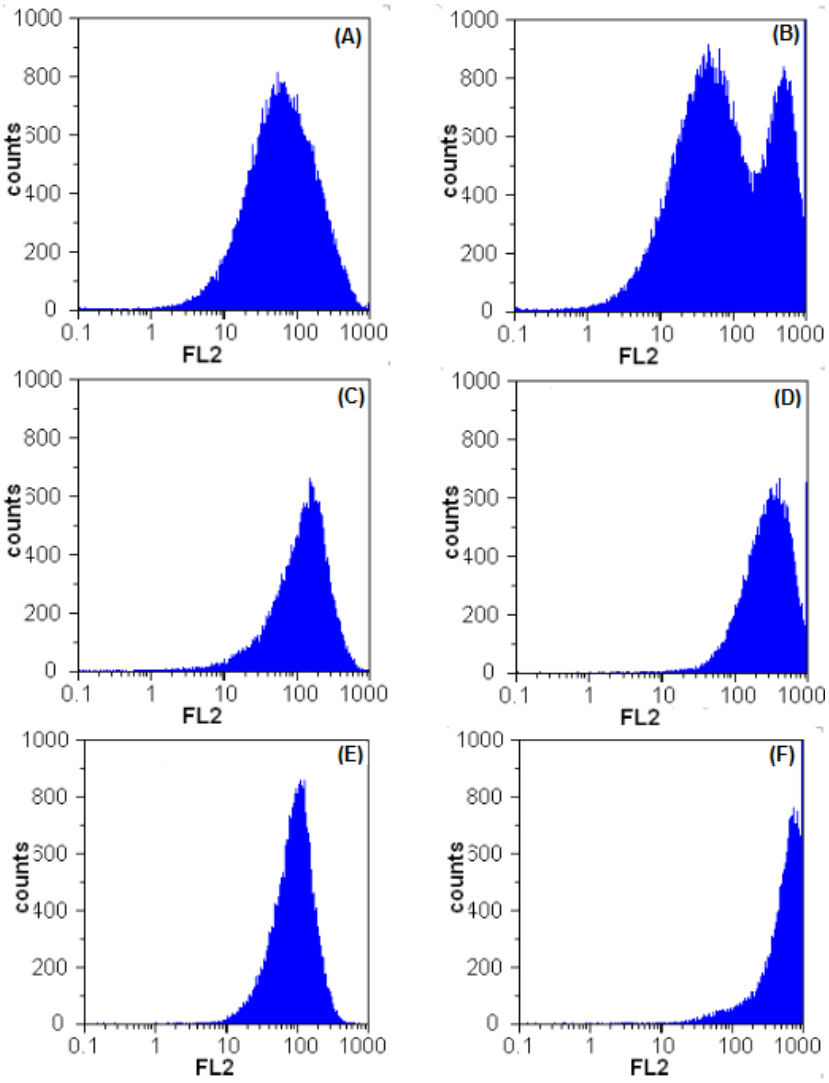


Figure 3: FL2 fluorescence histograms in Medium 1 at 24 h (A) and 96 h (B), Medium 2 at 24 h (C) and 96 h (D), Medium 3 at 24 h (E) and 96 h (F). Y-axis is the number of cells (counts) and X-axis is the fluorescent intensity at FL2 (Díaz et al, 2010).

It has been reported that bacteria, yeast and microalgae can be detected from the background on the basic of their intrinsic light scattering properties in forward angle light scatter (FSC) and right angle light scatter (SSC). The light scatter signals, FSC and SSC, give information on cell size and internal complexity, respectively (Andrade et al., 2012).

In the present work, SSC measurements of *Y. lipolytica* were performed during the time course of fermentation. Figure 4 shows light scatter SSC histograms in Medium 1, 2 and 3 obtained at different fermentation times. In all cases, SSC peak presents a slight displacement towards the X-axis right side of the histograms. According to Silva et al. (2011), these displacements in the histograms indicate an increasing on cells complexity due to increasing of DNA content and also lipid body content.

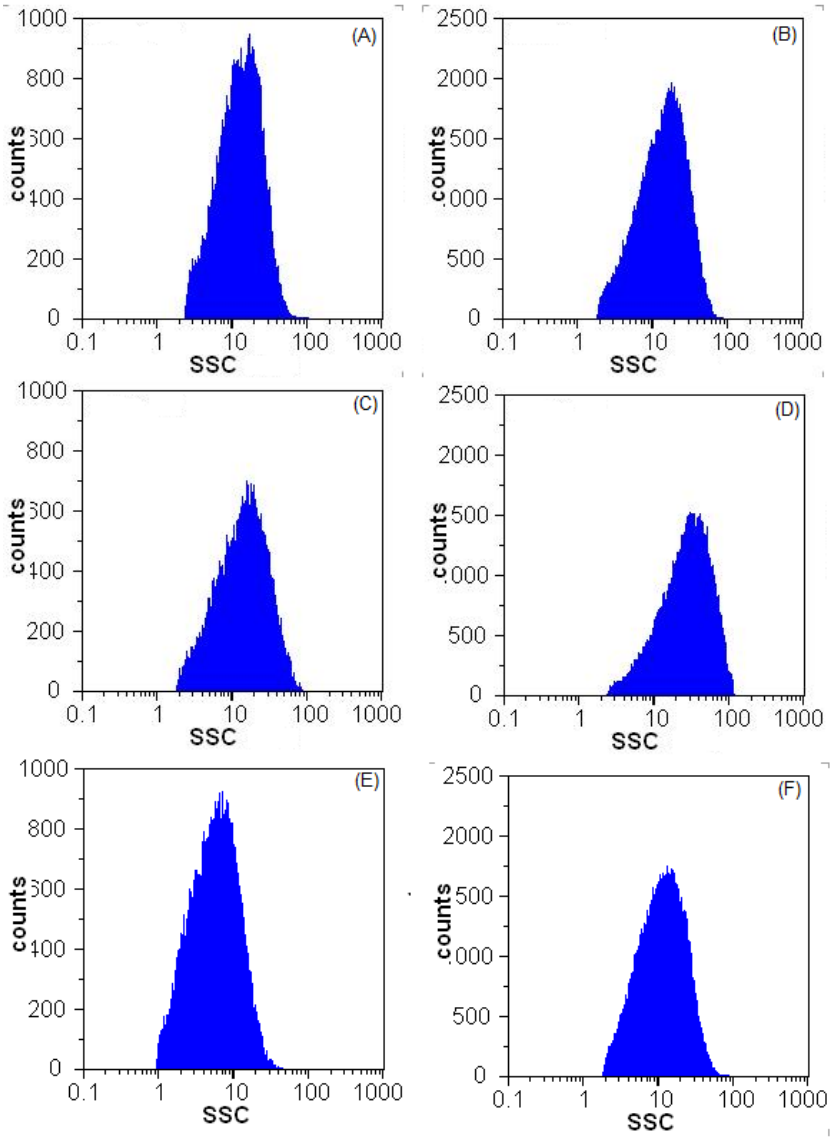


Figure 4: SSC histograms in Medium 1 at 24 h (A) and 96 h (B), Medium 2 at 24 h (C) and 96 h (D), Medium 3 at 24 h (E) and 96 h (F).

4. Conclusion

Highest values of oil accumulation were found in growth stationary phase (Figure 1 and 2) for all media tested, independently of medium composition.

Flow cytometry combined with Nile Red fluorochrome seemed to be able to determinate both neutral and polar lipids (Figure 2) in *Yarrowia lipolytica* yeast in three different media. In addition, through SSC measurement it was possible to observe different levels in the cell complexity with the fermentation progress.

This technique provides a powerful and rapid tool to monitor lipid production in *Yarrowia lipolytica*. Furthermore, flow cytometry can be used in bioprocess monitoring to obtain information from heterogeneous and complex microbial samples faster and more accurately (Figure 2) than with conventional microbiological methods (Bligh and Dyer, 1959).

References

Andrade R., Leal R., Roseiro J., Reis A., Silva T. L., 2012, Monitoring *Rhodosporidium toruloides* NCYC 921 batch fermentations growing under carbon and nitrogen limitation by flow cytometry, W. J. Microbiol. Biotechnol., 28, 1175 -1184.

Bligh, E.G., Dyer, W.J., 1959, A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol., 37:911–917.

Díaz, M., García, L.A., Herrero, M., Quirós, C., 2009, Application of flow cytometry to industrial microbial bioprocesses. Biochem. Eng. J., 48, 387-407.

Haegler, A.N. and Mendonça-Haegler, L.C., 1981, Yeast from marine and stuarine waters with different levels of pollution in the State of Rio de Janeiro, Brazil, Appl. Environ. Microbiol. 41, 173-178.

Kovalchuk, A., 2005 Molecular Analysis of the LTR Retrotransposon Ylt1 from the Genome of Dimorphic Fungus *Yarrowia lipolytica*, Dissertation, Institut für Mikrobiologie, Fakultät Mathematik und Naturwissenschaften der Technischen Universität Dresden, Germany.

Martins F.F., Ferreira T. F., Azevedo D. A., Coelho, M. A. Z., 2012, Evaluation of crude oil degradation by *Yarrowia lipolytica*, Chemical Engineering Transactions, 27, 223-228, DOI:10.3303/CET1227038.

Martins, F.F., 2013, "Physiology and quantification of lipid in *Yarrowia lipolytica* by flow citometry" Dissertation (MSc.), School of Chemistry, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil (in Portuguese)

Mlicková, K., Roux, E., Athenstaedt, K., D'Andre, S., Daum, G., Chardot, T., Nicaud, J., 2004, Lipid accumulation, lipid body formation, and acyl coenzyme A oxidases of the yeast *Yarrowia lipolytica*, Appl. Environ. Microbiol. 70, 3918-3924.

Papanikolaou, S.; Chevalot, I.; Komaitis, M.; Marc, I., Aggelis, G., 2002, Single cell oil production by *Yarrowia lipolytica* growing on an industrial derivative of animal fat in batch cultures, Appl. Microbiol. Biot. 58, 308–312.

Raschke D., Knorr D., 2009, Rapid monitoring of cell size, vitality and lipid droplet development in the oleaginous yeast *Waltomyces lipofer,* J. Microbiol. Methods 79, 178 – 183.

Rossi, M., Amaretti, A. Raimondi, S., Leonardi, A., 2011, Getting Lipids for Biodiesel Production In: Oleaginous Fungi, Biodiesel - Feedstocks and Processing Technologies, Ed. Stoytcheva, M., InTech.

Rywinska, A.; Juszczyk, P.; Wojtatowicz, M.; Robak, M.; Lazar, Z.; Tomaszewska, L.; Rymowicz, W., 2013 Glycerol as a promising substrate for *Yarrowia lipolytica* biotechnological applications. Biomass Bioenerg 48, 148-166.

Santos, R.S., Morais, B.L.B., Masuda, C.A., Ribeiro, B.D., Coelho, M.A.Z., Amaral, P.F.F., 2012 Study of intracellular lipid production in yeast as an alternative feedstock for biodiesel synthesis, In: COBEQ, 2012, Buzios, Brazil (in Portuguese).

Silva, T. L., Feijão, D., Reis, A., 2011, Monitoring *Rhodotorula glutinis* CCMI 145 stress physiological response during fed-batch fermentation using multi-parameter flow cytometry, Microbial Biochem. Technol., 3, 025-031.

Silva, T. L., Roseiro, J.C., Reis, A., 2012, Application and perspectives of multi-parameter flow cytometry in microbial biofuels production processes, Trends Biotechnol., 30, 225-232.