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Monitoring *Rhodotorula glutinis* CCMI 145 Stress Physiological Response during Fed-Batch Fermentations Using Multi-Parameter Flow Cytometry

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Abstract

Multi-parameter flow cytometry was used to monitor *R. glutinis* stress response during a fed-batch fermentation, through cell viability, lipid content and intrinsic light scatter. During the yeast fermentation, the proportion of cells with permeabilized membrane (dead cells) increased when nutrients and/or oxygen became limiting. Yeast cells showed a higher injury level when grown under other nutrient limitation than under oxygen limiting conditions, as the dead cells reduced their internal content and size in the former situation, suggesting drastic cells lysis.

The maximum yeast lipid content was 8% (w/w) at t=38.3 h. Such low lipid content was attributed to oxygen limitation, which highlights the importance of the oxygen transfer rate when producing lipids from aerobic yeast cultures.

Changes in Forward and Side scatter light signals were detected during the yeast growth, which can provide a useful and fast way to identify the yeast growth phase.

The multi-parameter approach here reported represents a better control system based at the individual cell level that can be used for optimization of yeast bioprocess performance, and may also be used for quick screening of yeast strains for single cell oil production.

Keywords: *Rhodotorula glutinis*, Multi-parameter flow cytometry, light scatter, Propidium iodide, Nile Red, fed-batch fermentation

Introduction

Nowadays there is an increasing interest in the development of more efficient and less time-consuming methods to assess the presence of microorganisms, as well as their viability for bioprocess control and improvement [1]. Rapid detection of microorganisms in samples is one of the key questions to obtain real-time data for the development of more accurate quality control programs, but also for monitoring microbial population during fermentation stages, in order to achieve a better control over processes [1-2]. In fact, throughout the course of any microbial process, it is essential to monitor cell viability, as a high proportion of dead cells present in any part of the bioprocess will be also detrimental as such cells will not participate in the biotransformation process, thus decreasing the process yield. It is therefore important to have information on cell viability in real time; with a high statistical resolution as such information allows decisions on process control in order to reduce the dead cells proportion in the culture. This allows products to be harvested at optimum concentrations, and the activation of inducible systems to be initiated at the correct time, so that high product yields can be achieved. Multi-parameter flow cytometry is capable of providing such information, rapidly, with a high degree of accuracy, providing a control system based on measurements made at the individual cell level for optimization of bioprocess performance [2].

Yeasts are microorganisms of industrial importance. Due to its industrial implication, *Saccharomyces cerevisiae* has been used in most of works using Flow Cytometry [3]. However, there are many other yeast species with significant commercial importance such as *Rhodotorula glutinis* that have been reported as a source of carotenoids of high commercial interest which are used as natural food colorants and feed additives in aquaculture [4-5]. *Rhododotula glutinis* has also been reported as an oil producer microorganism that can be used to produce biodiesel [6-8]. It is important to monitor cell lipid content during the microbial lipid production process but, at present, most of the published works studying the production of biodiesel from yeasts used traditional microbiological techniques to monitor the yeast lipid content [6-9] which are time consuming and generate high amounts of waste (organic solvent) which are harmful to the environment if not recycled by distillation. In addition, enough amounts of biomass must be obtained for subsequent lipid extraction and derivatization [10]. Importantly, lipid content data is usually only available a considerable time after the sample is taken, too late for alterations to be made to process control. Therefore, it is important to measure accurately and quickly microbial oil content when selecting potential oil producers yeast strains or optimizing microbial oil production or scaling-up biofuel production bioprocesses, so that informed decisions on process control can be made.

Multi-parameter flow cytometry can monitor total cell lipid content, near real time, and with a high degree of statistical resolution, during the yeast growth. Lipid measurement has been previously proposed using the Nile Red fluorescent stain for quantification of lipids [10-14]. Its fluorescence is produced in highly hydrophobic environments and quenched in hydrophilic ones.

Fed-batch is the most common cultivation mode used in bioprocess industry. Nutrient limitation, particularly oxygen-limiting condition, is a bottleneck that often occurs during microbial fermentations development, reducing process yield. It is therefore important to have

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information, in real time, on the cell stress physiological states as a response to different environmental conditions that cells experience during process development, so that informed decisions can be made to process control.

Glucose has been used as carbon and energy source in most of the documented commercial cultivation processes as it represents an easily, inexpensive and accessible feedstock being usually obtained in the form of glucose syrups resulting from the hydrolysis (chemical and enzymatic) of agro-industrial residues (lignocellulosic biomass, starches and sugarcane) [15-16]. Therefore the use of glucose as carbon source in yeast lab-scale fed-batch fermentations is of crucial importance when envisaging process scaling-up to commercial scale.

The present work describes the stress physiological response of *R. glutinis* CCMI 145 cells grown in a fed-batch fermentation using glucose as carbon source. Cell viability, light scatter changes and lipid content were monitored using multi-parameter flow cytometry. As far as we are aware, this is the first work reporting the use of multiparameter flow cytometry to monitor yeast fed-batch fermentations by measuring light scatter, cell viability and lipid content.

Materials and Methods

Organism

The yeast *Rhodotorula glutinis* was deposited in the Industrial Micro-organisms Culture Collection (Instituto Nacional de Engenharia, Tecnologia e Inovação, Lisbon, Portugal), with the reference code CCMI 175. Lyophilized cell cultures of the yeast were also used to provide control data.

Growth conditions

The yeast *R. glutinis* was maintained in Yeast Malt Agar. Cultures were grown in 1000 ml baffled shake flasks containing 100 mL of a basal medium based on Easterling et al., [9] with modifications, containing KH_2PO_4 0.8 g/L, NH_4Cl 4 g/L, yeast extract 2 g/L, glucose 14 g/L, maintained at 30ºC, 180 rpm, in darkness. After 1 day (exponential growth phase) this culture was used to inoculate the 2L-fermenter with a 600 mL starting working volume of the same medium composition. Glucose was sterilised separately and mixed with the other components after cooling to make up the fermentation medium. The medium pH was then adjusted to 6 by adding 1 M NaOH or 1 M HCl solutions. The pH of the fermentation medium was measured with a Mettler Toledo steam-sterilizable pH electrode (Columbus, USA) and was controlled with a pH controller (SGI, Mountain View, USA). The dissolved oxygen tension (DOT) in the medium was measured with a Mettler Toledo oxygen electrode (Columbus, USA) and recorded in a recorder (SGI, Mountain View, USA). The stirring rate (100–600 rpm) was manually increased whenever the DOT was below 20%. Pulses (100 mL) containing concentrated basal medium (at the same proportion to the starting medium, referred above) or concentrated basal medium and glucose (14g/L) were added, whenever the dissolved oxygen increased above 80%, or the residual glucose concentration in the broth decreased below 2 g/L, except at the end of the fermentation, when a concentrated glucose pulse (117 g/L) without other nutrients was added to the culture, in an attempt to increase the lipid content assessed by flow cytometry.

Growth evaluation

Growth was periodically evaluated by optical density at 540 nm in duplicate, which was converted into dry cell weight per litre of culture by a regression equation.

Specific growth rate: Specific growth rate was determined by plotting the natural logarithm of biomass concentration against time. Readings within the exponential phase were then used to obtain correct values of the specific growth rate by linear regression.

Residual glucose concentration: The residual glucose concentration in filtered (0.45 µm) samples was determined using the 3,5-dinitrosalicylic acid (DNS) method [17].

Lipid content: A correlation between the total yeast cell lipid content assayed by the traditional soxhlet method [6] and the Nile Red (NR) fluorescence intensity measured by flow cytometry was previously established as reported by Lopes da Silva et al., [18]. Samples were collected at different times of a batch growth developed in shake flasks. In this way the NR fluorescence was converted into cellular lipid content (% dry cell weight) by the regression equation.

Multi-parameter flow cytometry used NR (Riedel de Haën, Buchs SG, Switzerland) according to the method described by Lopes da Silva et al. (2010). 10 µl of a working solution of NR and acetone (33 µg/mL) were added to 1 ml of a cell suspension (~ 10^6 cells/mL). This mixture was gently vortexed and incubated for 2 min at 37ºC in darkness (for this stain concentration, it was observed that *R. glutinis* cells stained with NR displayed the highest fluorescence after incubation for 2 min at 37ºC. After 2 min, the fluorescence faded. Therefore 2 min staining period was chosen for further experiments).

NR florescence was determined using a FACScan flow cytometer (Becton-Dickinson Instruments, Erembodegem, Belgium) equipped with a 488 nm argon laser. Upon excitation by a 488 nm argon laser, NR exhibits yellow-gold and red fluorescence when dissolved in neutral and polar lipids which are detected by the FL2 and FL3 channels respectively. Non-stained cells were used as an auto fluorescence control measured in FL2 and FL3 (AF2 and AF3 respectively) which were always set to the same pre-fixed fluorescence value, in all experiments. The total normalized fluorescence corresponding to total cellular lipids was determined as the sum of the ratios FL2/AF2 and FL3/AF3.

Cell viability: Propidium iodide (PI) is known to stain nucleic acids in cells characterised by a defective membrane integrity and is widely utilized to measure membrane permeability [19-23]. Flow cytometry coupled with PI (Invitrogen, Carlsbad, USA) was used in this study in order to monitor *R. glutinis* cytoplasmic membrane integrity. PI binds to DNA and cannot cross an intact cytoplasmic membrane. Samples taken from the culture were immediately diluted (at least 1:2000 v/v) with phosphate buffer solution (PBS, pH 7.0) and stained with PI. PI stock solution was made up at 2 mg/mL in distilled water. The working concentration of PI was 10 µg/mL. PI was excited at 488 nm and measured at 585 nm (FL2).

All solutions used in flow cytometry were passed through a 0.2 µm filter, immediately prior to use, to remove particulate contamination. In addition, the control software was set on both the light scattering properties in the forward angle direction (FS) signal and the right angle direction (SS) signal.

Results and Discussion

Flow cytometry controls

Cell viability using propidium iodide: In order to demonstrate that it is possible, using multi-parameter flow cytometry, to characterize the cell membrane integrity of individual cells of *R. glutinis* fermentations, it was necessary to establish a positively control to check the performance of the dye. This was then used for comparison with data produced from cultivation experiments.

R. glutinis cells collected from a 48 h slant and resuspended in PBS were stained with PI (Figure 1a). One major population (A) (88%), no staining, could be seen corresponding to cells with an intact cytoplasmic membrane, and a minor population (B) (11%), PI stained, which was identified as dead cells. Such proportion of PI negative cells showed that most of the yeast cells removed from the slant were still viable.

R. glutinis cells removed from the slant and ressuspended in PBS were heat treated in a water bath at 100ºC during 10 min and stained with PI. One major population (B) (90.6%), PI staining, corresponding to cells with permeabilised cytoplasmic membranes could be identified (Figure 1 b).

Lipid content using NR: The maximum microbial lipid productivity is usually obtained at the late exponential phase, when cells start producing storage materials. This was observed for *R. glutinis* cells grown on batch fermentations [8, 10]. *R. glutinis* CCMI 145 cells taken at the late exponential phase (from a bath culture grown in shake flasks) were analyzed by flow cytometry without and with NR (Figure 2a and Figure 2b). The fluorescence intensity of cells stained with NR, detected in the FL2 and FL3 channels, were greatly increased when compared with the mean NR fluorescence intensity of no staining cells.

R. glutinis fed-batch fermentation

Figure 3 shows the fermentation profiles for *R. glutinis* fed-batch

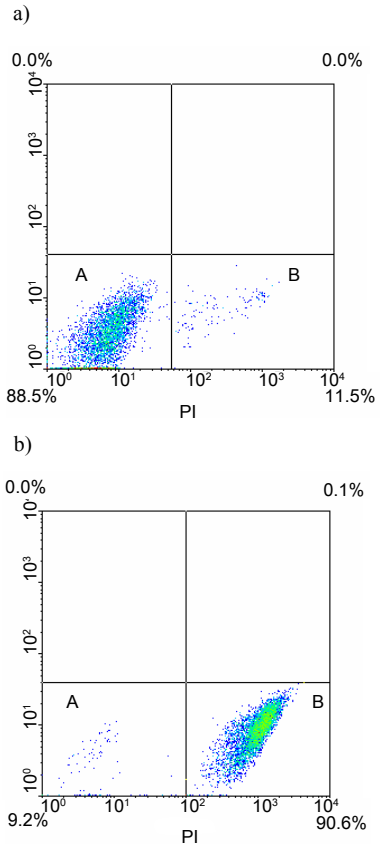

Figure 1: a) Cells collected from a 48 h slant, ressuspended in PBS and stained with PI. One major population exists (A), corresponding to cells with intact cytoplasmic membrane, no staining.

b) *R. gluinis* cells collected from a 48 h slant, ressuspended in PBS and killed in a water bath at 100ºC for 10 min and stained with PI. One main subpopulation (B) could be distinguished, corresponding to cells with permeabilised cytoplasmic membrane.

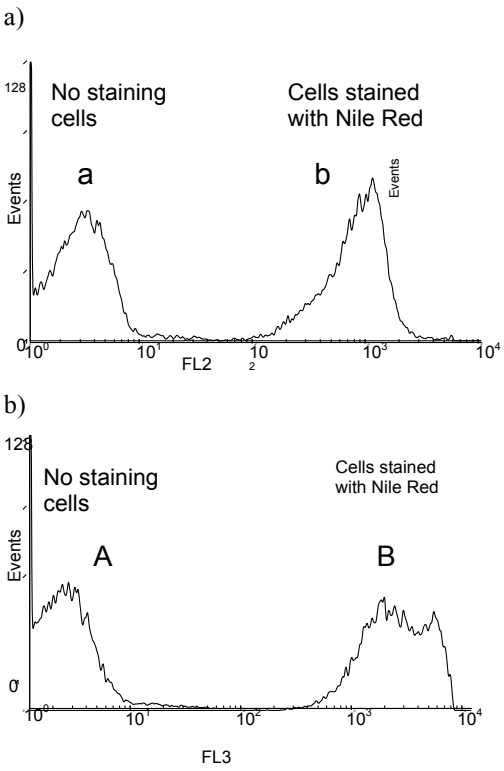

Figure 2: *R glutinis* CCMI 145 cells taken at the late exponential phase (from a bath culture grown in shake flasks) analysed by flow cytometry without and with NR. The fluorescence intensity of cells stained with NR, detected in the FL2 (a) and FL3 (b) channels were greatly increased when compared with the mean NR fluorescence intensity of no staining yeast cells.

fermentation. Whenever the glucose concentration dropped below 2 g/L, a pulse of nutrients was added to the yeast culture. Whenever DOT decreased below 20%, the stirring rate and/or aeration rate were manually increased in order to avoid oxygen limitation.

Biomass started increasing immediately after the inoculation but at t=20.3 h the dissolved oxygen tension (DOT) increased [80.2% at t=14.5 h to 90.4% at t=20.3 h, Figure 3 b)] indicating that growth was ceasing probably due to nutrient limitation other than oxygen or glucose (at t=20.3h glucose concentration was 10.9 g/L). The total Nile Red fluorescence, an indicator of the total lipid content, also dropped from 426 at t=14.5 h to 367 at t=20.3 h (Figure 3 c). The proportion of PI+ cells (with permeabilised membranes) sharply increased from 35% at t=14.5 h to 66% at t=20.3 h (Figure 3 d), supporting that the yeast cells were experiencing nutrient starvation conditions at that time.

Figure 4shows the density plots of PI fluorescence for *R. glutinis* cells, taken at different fermentation times. A pronounced increase in the proportion of dead cells [subpopulation (B)] was observed at t=20.3h [35% at t=14.5h and 67% at t=20.3h, (Figure 4a and Figure 4d)].

PI fluorescence versus SS or FS density plots are also shown. FS is measured in the plane of the laser beam and gives information mainly on cell size, while SS is measured at 90º and provides information on complexity (granularity or internal structure of the cell) [1]. Therefore, PI fluorescence versus SS or FS plots showed that, under certain growth conditions, intact (PI-) and permeabilized cells (PI+) differed in size and complexity. At t=20.3 h, 46% of the cells (PI+) [subpopulation (C), Figure 4e] showed a lower SS signal compared to the SS signal

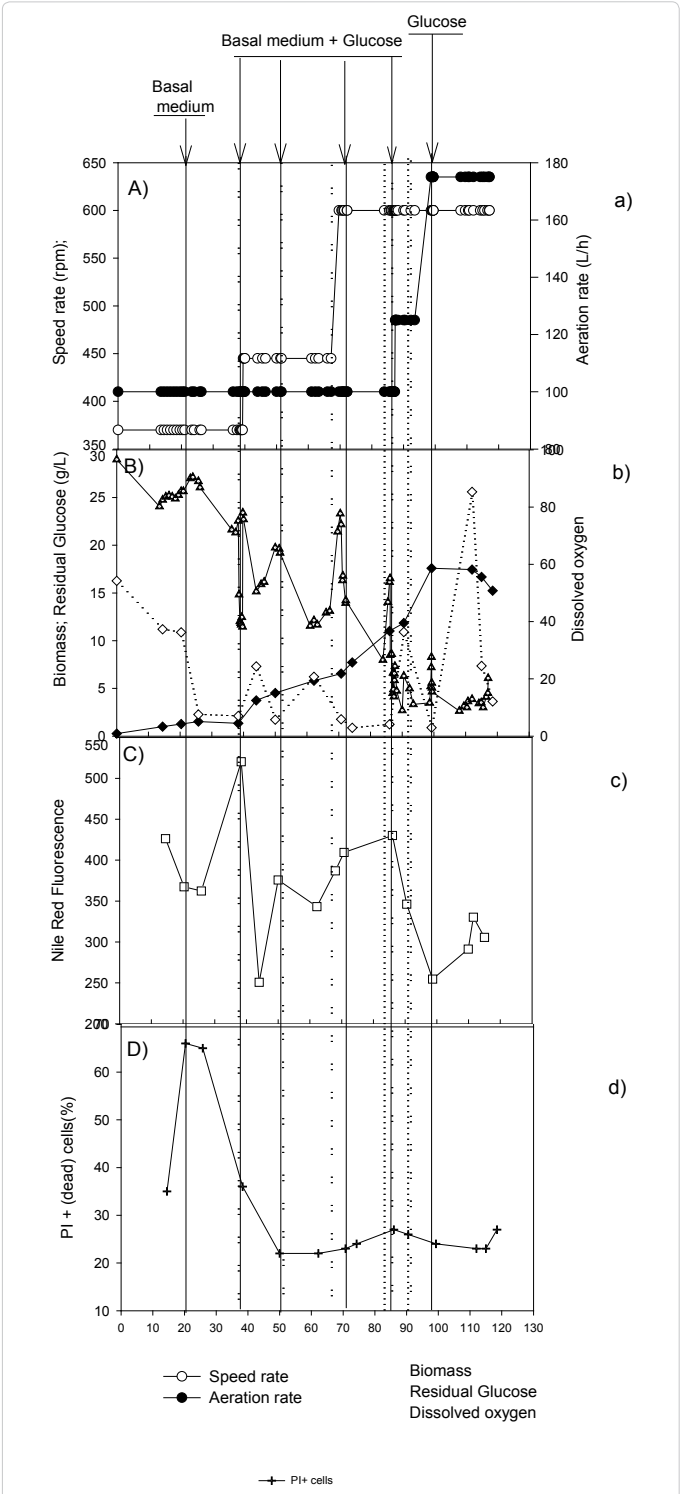

Figure 3: Fermentation profiles for a fed-batch *R. glutinis* yeast culture.
a) Speed and aeration rates
b) Biomass, residual glucose and dissolved oxygen
c) NR fluorescence
d) PI+ (dead cells)

Biomass, residual glucose concentrations are affected by a relative error not exceeding 10% (n=2).
NR fluorescence are affected by a relative error not exceeding 10% (n=2).

Speed rate — Biomass — Nutrient pulses
Aeration rate — Residual Glucose — — — Speed rate increase
Dissolved oxygen ····· Aeration rate increase

of the (PI-) intact cells (subpopulation (E), Figure 4e]. At that time, 30% of the yeast cells PI+ [subpopulation (G), Figure 4f] also displayed lower FS signal, compared to the FS signal of the (PI-) intact cells (subpopulation (I), (Figure 4f). These results suggest an extensive cell size and complexity reduction, which could be due to the severe starvation conditions that the yeast cells were experiencing at t=20.3h as already referred, inducing cell lysis (cell complexity reduction) and shrinkage (cell size reduction). Such behavior was not observed in the previous sample, taken at t=14.5 h [Figure 4b) and c)] as PI+ cells [subpopulations (C) and (D) in (Figure 4b) and subpopulations (G) and (H) in (Figure 4c)] show the approximately same FS and SS signals as those displayed by PI- cells [subpopulation (E) in (Figure 4b) and subpopulation (I) in (Figure 4c)]. These observations were also supported by the optical microscope micrographs (Figure 5b), where a higher proportion of shrunken cells with reduced internal content, presumably dead cells [24-25] can be seen, as compared to the previous sample (Figure 5a).

At t=62.2 h, glucose concentration was 6.2 g/L, biomass concentration attained 6.5 g/L and DOT was 40%. Such growth conditions allowed an increase in the proportion of subpopulation (A) composed of intact cells (78%) [Figure 4g)]. In addition, most of the PI+ cells [subpopulations (C) and (D) in (Figure 4h), and subpopulations (G) and (H) in Figure 4i], did not show significant changes in the FS and SS signals compared to the FS/SS signals of PI- subpopulations [subpopulation (E) in (Figure 4h) and subpopulation (I) in Figure 4i], suggesting that, under such conditions, the dead cells were not in an advanced state of lysis, as they maintained their structure. In fact, at this fermentation time (t=62.2 h) the yeast cells were growing under more favorable growth conditions, which allowed their recovery from the previous adverse growth conditions (t=20.3 h).

The proportion of dead cells attained approximately 23% at t=49h, remaining approximately constant until t=118 h [Figure 3d and Figure 4j].

The NR fluorescence also increased after the nutrient pulse addition (t=20.3 h) attaining its maximum at t= 38 h (520), which corresponded to 8% (w/w) lipid content (Table 1). Afterwards the NR fluorescence decreased, increasing whenever nutrients and glucose were added to the culture, but never reaching higher readings, which could be due to the low DOT values observed particularly after t=86 h (DOT<30%), attaining 9% at t=90 h.

The specific growth rate $\mu_{(33\ h\ <t<96\ h)}$ was 0.02 /h, lower than that reported by Yoon and Rhee [26] (0.13 /h), who studied *R. glutinis* NRRL Y-1091 growth in a batch cultivation. Such difference may be due to the oxygen limitation observed during the yeast growth.

At t=99.2 h, the yeast growth attained the stationary phase and the biomass concentration reached 17.8 g/L, glucose concentration was 0.9 g/L and DOT was 27.6%. Since microbial lipids are synthesized when carbon is in excess [21], a pulse of glucose (without other nutrients)

Fermentation time (h)	NR Fluorescence	Lipids (%w/w)
14.5	426	7.1
20.3	367	6.2
38.3	520	8.4
62.2	343	5.9
118	305	5.4

Table 1: Normalised NR fluorescence of R. *glutinis* cells taken at different fermentation times, detected by flow cytometry, and yeast oil content calculated through the correlation [Lipid content (% w/w dry cell weight) = 0.014× Fluorescence +1.14; r^2 = 0.833].

was added to the culture (t=99.2 h) in an attempt to increase the yeast lipid content. However, biomass concentration remained unchanged after the pulse probably because the oxygen supply was not enough for the yeast cells requirements at that biomass concentration, as DOT remained almost below 10% during the carbon pulse uptake. Regarding the yeast lipid content, the NR fluorescence slightly increased after the

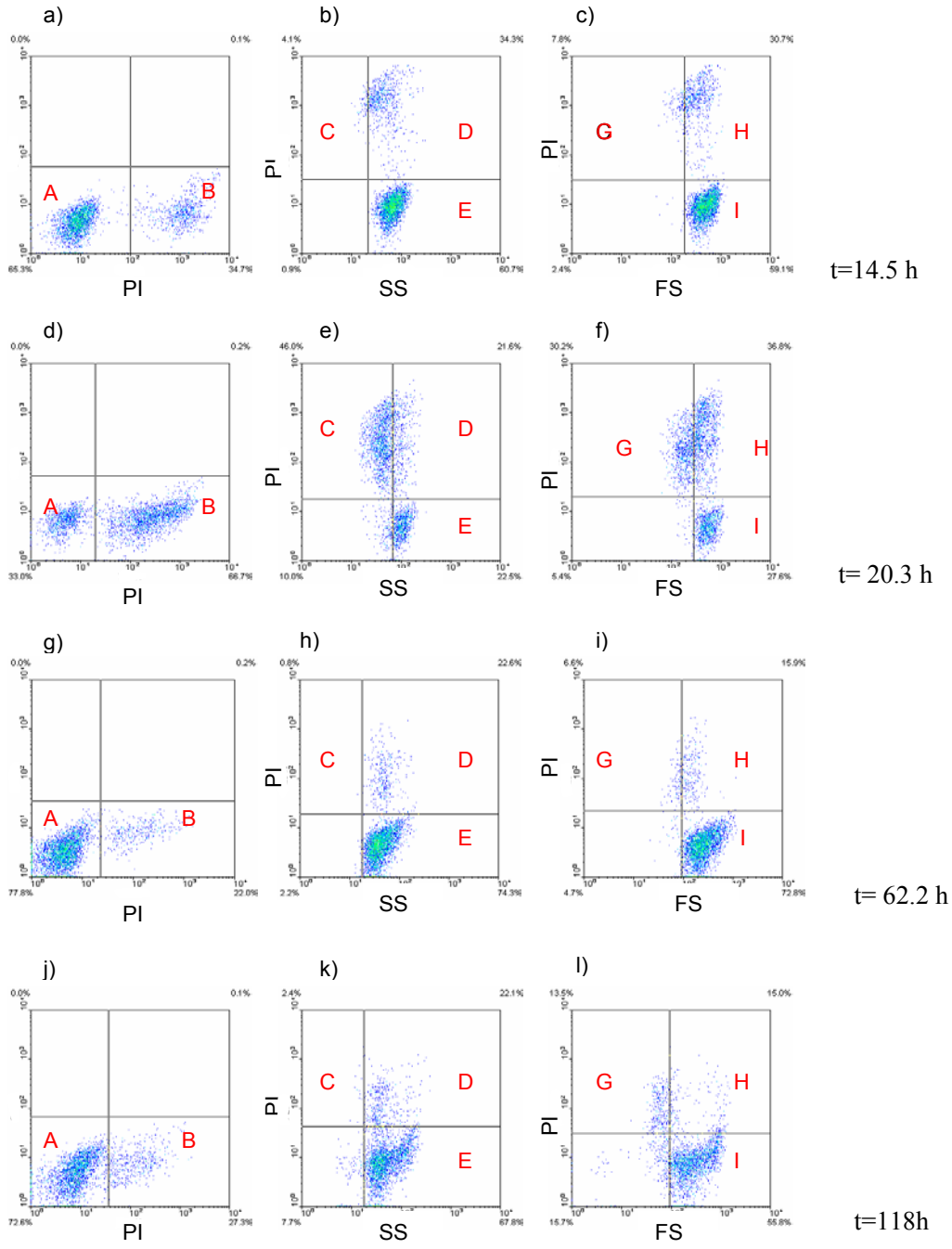

Figure 4: PI fluorescence density plots of *R. glutinis* CCMI 145 cells growing on a fed-batch fermentation taken at t=14.5 h (a), 20.3 h (d) , 62.2 h (g) and 118h (j). Subpopulation A corresponds to cells with intact membrane, no staining (PI-), while subpopulation (B) corresponds to cells with permeabilised membrane, stained with PI (PI+).

PI fluorescence versus side scatter light (SS) [b), e), h) and k)] and forward scatter light [c), f), i) and j)] density plots for *R. glutinis* CCMI 145 cells growing on a fed-batch fermentation taken at t= 14.5 h, 20.3 h, 62.2 h , and 118 h, respectively.

Subpopulation (C) corresponds to permeabilised cells (PI+), depicting lower SS signal compared to the SS signal of subpopulation (E) composed of intact cells (PI-).

Subpopulation (D) corresponds to permeabilised cells (PI+) displaying the same SS signal as subpopulation (E).

Subpopulation (G) corresponds to permeabilised cells (PI+) depicting lower FS signal compared to the FS signal of subpopulation (I) composed of intact cells (PI-).

Subpopulation (H) corresponds to permeabilised cells (PI+) displaying the same FS signal as subpopulation (I).

last carbon pulse addition. As glucose was depleting (t=118 h) the NR fluorescence decreased attaining the reading of 306 (Figure 3c). Since *R. glutinis* is an obligate aerobe, is strongly dependent on oxygen for its energy metabolism and synthesis of cellular components including lipids [27]. This observation highlights the importance of the oxygen availability when producing microbial lipids from aerobic yeast cultures.

As expected, at t=118 h the proportion of polarized healthy cells slightly decreased as compared to the previous sample (73 % at t=118 h, 78% at t=62.2 h, Figure 3d, Figure 4g and Figure 4j). Most of the PI+ yeast cells [subpopulations (C) and (D) in Figure 4k)] showed the same SS signal as the PI- cells [subpopulation (E), Figure 4k], and the proportion of PI+ cells [subpopulation (G), Figure 4l)] that reduced their size (FS signal) relatively to PI- intact cells [subpopulation (J)] was only 14% (Figure 4l) as opposed to the physiological yeast cell response observed at t=22.3h (Figure 4e and Figure 4f), suggesting that at that time (t=22.3h) the growth conditions were especially severe.

Light scatter measurements

It has been reported that bacteria, yeast and microalgae can be detected from the background on the basis of their intrinsic light scattering properties in forward angle light scatter (FS) and right angle light scatter (SS) [28-31,18].

In the present work, light scatter measurements of *R. glutinis* were carried out during the time course of the fed-batch fermentation. Figure 6 shows the FS/SS 3D plot obtained at different fermentation times, at different growth phases. In all cases, a broaden distribution with respect to FS and SS density plot was observed, suggesting cell size and shape heterogeneity, which could be due to the heterogeneity of the asynchronous exponential growing yeast population [29]. At t=62.2 h, two peaks A and B could be seen and according to Hewitt and Nebe-von-Caron [2], it is thought that peak A should correspond to young cells that have been recently separated from their mother cells while peak B most probably correspond to cells that were about to divide and budding cells, larger in size and internal complexity (higher FS and SS signals) due to their higher DNA content. This profile was not observed at t=14.5 h and t=118 h (Figure 6 a, Figure 6b and Figure 6c), suggesting that at that times, the proportion of cells that were dividing were lower than at t=62.7 h.

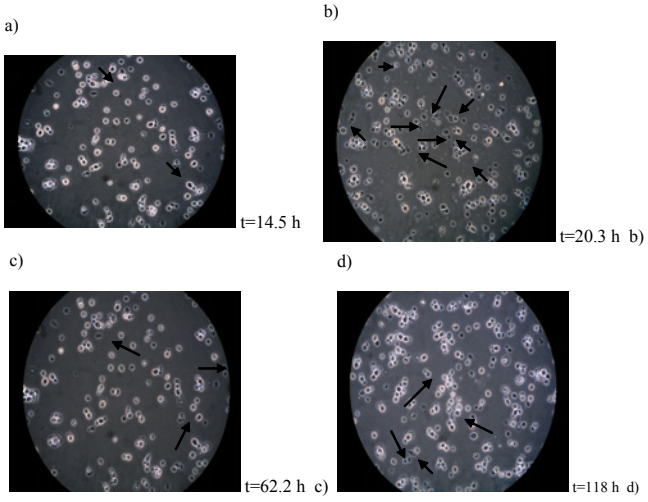

Figure 5: Optical microscope observations of *R. glutinis* CCMI 145 cells grown in a fed-batch fermentation, taken at t= 14.5 h (A), 20.3 h (B), 62.2 h (C) and 159 h (D).Magnification 1000×. The black arrows show empty and shrunk yeast cells, supposedly dead.

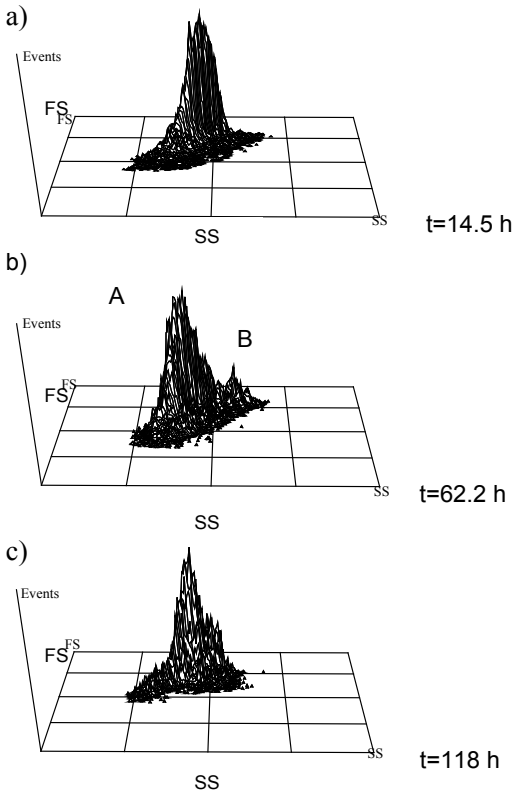

Figure 6: Intrinsic light scattering data for *R. glutinis* cells grown on a fed-batch fermentation taken at t=14.5 h (a), 62.2 h (b) and 159 h (c).

Conclusion

The multi-parameter flow cytometry approach was useful for monitoring at-line *R. glutinis* CCMI 145 stress physiological response during a fed-batch fermentation, providing important physiological information at the individual cell level that is difficult to obtain in any other way.

The low yeast lipid content reported here highlights the importance of the yeast growth conditions, particularly the oxygen and nutrient availability, when producing single cell oil. For this reason, it is envisaged that on-line flow cytometric analysis coupled to a control strategy based on nutrient feeding, aeration rate and speed rate according to Nile Red fluorescence intensity and cell viability could be used as a mean of enhancing the overall yeast lipid production process efficiency.

In addition, as flow cytometric analysis can provide the yeast lipid content near real time, such approach when applied to a larger scale (pilot, pre-commercial and industrial) allows choosing the optimal fermentation time to harvest the yeast cells with the maximum lipid content so that highest productivities can be achieved.

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