J Ind Microbiol Biotechnol (2013) 40:865–875
DOI 10.1007/s10295-013-1278-2

BIOTECHNOLOGY METHODS

Assessment of β-carotene content, cell physiology and morphology of the yellow yeast *Rhodotorula glutinis* mutant 400A15 using flow cytometry

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Received: 14 November 2012 / Accepted: 21 April 2013 / Published online: 10 May 2013

Abstract Flow cytometry was used to assess β-carotene content, cell membrane permeability, cell size and granularity in *Rhodotorula glutinis* mutant 400A15 grown under different oxygen transfer coefficients (k_La) and carbon to nitrogen ratios (C/N). A Doehlert distribution was used in order to select the best conditions that induced the highest carotenoids production. The highest β-carotene content (0.79 mg g^{-1} DCW) at the lowest k_La and C/N (5×10^{-3} s^{-1} and 11.3 respectively). Under these conditions, the biomass concentration attained 18.60 g L^{-1}. The highest ratio of cells with permeabilised membranes (2.6 %), and the highest cell size and granularity were also obtained under these conditions. It was observed that C/N showed a stronger influence than the k_La on the measured cell parameters.

Keywords Flow cytometry · β-carotene · Cell integrity · Cell size · Cell granularity

Introduction

Carotenoids are of importance in both animals and humans. They promote the enhancement of the immune response, conversion to vitamin A and the scavenging of oxygen radicals [5, 8, 13]. Increasing commercial interest is due to the potential benefits in human and animal health such as anti-carcinogenic and tumor suppression activity as described in the literature [5, 16]. They are also used as natural food colorants and pigment supply in fish diets [14].

Microbial sources of carotenoids have received increasing attention because of the restricted rules and regulations currently applied to chemically synthesized/purified pigments [4, 6, 14, 21]. Among these sources, *Rhodotorula* sp. are well known carotenoid and lipid producers [1]. The major carotenoid pigments produced by this yeast are β-carotene, torulene and torularhodin in various proportions, β-carotene is the most popular in pharmaceutical and food markets, with a high market price. However, this carotenoid in wild strains of *R. glutinis* is relatively low [23].

Traditional methods for assessment of carotenoids in microbial cells are time consuming, use high amounts of toxic organic solvent and require enough amounts of biomass for subsequent carotenoid extraction and quantification. Multi-paramater flow cytometry is a powerful technique that allows monitoring, in real time, and with a high degree of statistical resolution, individual physiological states and several cellular parameters during microbial processes [15] including carotenoid content in microbial cells. An et al. [2] reported the use of flow cytometry and cell sorting to isolate the carotenoid pigment astaxanthin, and found that autofluorescence emission by the red yeast *Phaffia rhodozyma* was related to its carotenoid content. Mendoza et al. [20] reported a flow cytometric method for detecting β-carotene in *Dunaliella salina* strains, using the fluorochrome Nile Red. Compared to the traditional detection of carotenoids, flow cytometry allows their rapid quantification, in real time, at the single cell level, and does not present the disadvantages associated with the traditional methods.

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Moreover, when producing a product of interest obtained from a microbial process such as carotenoids, it is crucial to monitor cell viability, as a high number of dead or injured cells will not participate in the product formation, thus reducing the process performance. On the other hand, reduction in cell size and cell granularity (or internal structure) during the yeast growth has been associated with a cellular stress response to adverse environmental conditions such as prolonged nutrient deprivation [3]. Cell membrane integrity (as an indicator of cell viability), cell size and cell granularity have been monitored by flow cytometry during yeast batch fermentations [3, 7, 15, 19].

Carotenoid content in yeast is strongly affected by growth conditions such as oxygen availability [25], carbon to nitrogen ratio (C/N) [13], and medium composition [26], among other parameters. Indeed, the use of low-cost substrates in the microbial processes allows the reduction of the overall production process costs, representing an additional economic advantage. Glycerol, a by-product of the biodiesel industry, can be used as carbon source in media formulations, reducing the overall production costs. Some strains of *Rhodotorula* species can grow on glycerol producing carotenoids [24].

The present study reports the effect of the oxygen availability and C/N on *Rhodotorula glutinis* mutant 400A15 β-carotene production, grown on glycerol, using the Doehlert distribution experimental design [12]. The use of multi-parametric flow cytometry was of particular interest, introducing an innovative approach in this work, allowing the simultaneous and quick assessment of the yeast β-carotene content, permeabilised cells ratio, cell size and cell granularity changes, under different environmental conditions, in a high number of samples. Such approach is not possible when traditional microbial and analytical methods are used to determine these variables.

Materials and methods

Strain and culture conditions

The yeast strain mutant 400A15, obtained in the Laboratory of Microbiology of the Dipartimento di Agraria (Università degli Studi di Sassari) was used in this study. This strain is a primary mutant of the strain *Rhodotorula glutinis* C2.5t1 which lost the ability to produce torularhodin but increased β-carotene production ability [9].

Culture media were: YEPGLY [yeast extract (Oxoid, Hampshire, UK) 1 %; bacto-peptone (Becton, Dickinson and Company, Sparks, MD, USA) 2 %; Glycerol (87 % aqueous solution, Merck, Darmstadt, Germany) 2 %, added with agar (Oxoid) 2 %, when required] utilized for yeast cultivation and short term storage at 4 °C, and YEPDGLY (yeast extract 1 %, bacto-peptone 2 %, glycerol 20 %) for long term storage, at −80 °C.

Previous batch growth

Before the experimental design, a yeast batch culture was carried out in order to previously establish a flow cytometric protocol for the quick measurement of cellular parameters. Cells were grown aerobically in 1 L baffled shake flasks containing 0.200 L of YEPGLY at 30 °C, 150 rev min^{-1}, in an Infors HT (New York, NY, USA) incubator, for 72 h.

Experimental design

Experimental distribution for two factors, according to the Doehlert uniform design [12] was used to produce response surfaces. Seven experiments in duplicate were carried out within an experimental domain with shake flasks containing working volume ranging from 0.1 to 0.4 L. The C/N, calculated on a molar basis, was varied from 11.3 to 65.0, maintaining a constant glycerol concentration of 80 g L^{-1} (2.6 mol C L^{-1}), and changing the nitrogen concentration, as shown in Table 1. Yeast cells were grown for 72 h under the same culture conditions reported above. Afterwards samples from each experiment were analyzed for carotenoid content, cell size and granularity by flow cytometry. Biomass concentration and residual glycerol were also analyzed.

Coded representation of the factors was used for calculation purposes. The responses studied in this design were: biomass, β-carotene content, cell size (from FSC signals detection) and cell granularity (from SSC signals detection). The model used to express the responses was a second order polynomial model:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{12} X_1 X_2 + \beta_{22} X_2^2$$

where Y is the response from each experiment; β corresponds to the parameters of the polynomial model; X is the experimental factor level (coded units).

Growth evaluation

Samples were collected at regular time intervals for optical density (OD) reading at 600 nm, using a spectrophotometer Genesys 20 Visible (Thermo Scientific Waltham, MA, USA), in duplicate, for each sample. A correlation between the OD and dry cell weight (DCW) was previously established (OD = 2.6 × DCW − 11.2).

Determination of $k_L a$

Oxygen transfer coefficient ($k_L a$) from the headspace of the shake flasks was determined by using a non-fermentative

Table 1 Different growth conditions (medium volume and C/N) for the optimization of β-carotene production in *R. glutinis* 400A15

Test	Volume (L)	C/N	YE (mol N L^{-1})	P (mol N L^{-1})	YE + P (mol N L^{-1})
1	0.33	11.3	0.10	0.13	0.23
2	0.18	11.3	0.10	0.13	0.23
3	0.40	20.0	0.06	0.08	0.13
4	0.25	20.0	0.06	0.08	0.13
5	0.10	20.0	0.06	0.08	0.13
6	0.33	65.0	0.02	0.02	0.04
7	0.18	65.0	0.02	0.02	0.04

Culture medium volume varied from 0.10 to 0.40 L to change the k_La. C/N, calculated on a molar basis, varied from 11.3 to 65.0, maintaining a constant glycerol concentration at 80 g L^{-1} (which contain 2.6 mol C), and changing the nitrogen concentration. All the experiments were carried out in duplicate

C Carbon; *N* Nitrogen, *YE* Yeast extract, *P* peptone

gassing out method [28]. Culture medium volumes of 0.1, 0.2, 0.3, 0.4 and 0.5 L were used for k_La calibration curve.

Flow cytometer

For multi-parameter flow cytometry analysis, a FACSCalibur device (Becton–Dickinson, Franklin Lakes, NJ, USA) equipped with a 15-mW air-cooled argon-ion laser (emission, 488 nm) and a red diode laser (635 nm) was used. Sensors for detection of forward and side light scatter, green (530/30 nm, band pass filter, FL1 channel), yellow (585/42 nm, band pass filter, FL2 channel), orange-red (>670 nm, long pass filter, FL3 channel) and red (660/16 nm, band pass filter, FL4 channel) fluorescences were used. At least, 10,000 events were analyzed per sample on the flow cytometer.

All solutions used in flow cytometry were passed through a 0.2 μm filter, immediately prior to use, to remove particulate contamination. Forward (FSC), side scatter (SSC) light signals and fluorescence, expressed in channel number, were plotted in a logarithmic scale and data were analyzed with WinMDI software (version 2.9). Average FSC and SSC and fluorescence signals were calculated using the same software. Rainbow calibration beads (3.0–3.4 μm) (BD Biosciences Pharmingen, San Diego, California, USA) were used for routine calibration of the flow cytometer. Threshold was set on FSC at 256 V, the sheath fluid speed was 12 μL min^{-1} and the number of events per second was 800.

Carotenoid evaluation

The carotenoid (β-carotene) content in yeast samples was assessed by multi-parameter flow cytometry, after a correlation between the yeast autofluorescences as measured by the flow cytometer, and the yeast carotenoid content was assessed by a traditional method that had been previously established as described below.

The yeast autofluorescences measured in the FL_1, FL_2, FL_3 and FL_4 channels were adjusted to the first logarithmic decade for the first sample collected at the beginning of the yeast growth. Then FL_1, FL_2, FL_3 and FL_4 profiles were monitored, using the same settings, throughout the yeast growth. A correlation between FL_1, FL_2, FL_3 and FL_4 measured by flow cytometry and the total yeast carotenoid content assessed by the traditional method was previously established, analyzing samples taking at different times of a batch growth, as it is known that the carotenoid content increases with the culture age [24]. In this way FL_1, FL_2, FL_3 and FL_4 could be correlated into carotenoid content (% dry cell weight) by the regression equation.

For the traditional carotenoid detection, cells were spun down at 11,000 *g* for 7 min, and lyophilized. 150 mg of dried biomass mixed with 150 mg of sand (0,5 mm of diameter, previously washed with a HCl 50 % solution for 2 h and then washed with distilled water until the was pH 7) were ground in a mortar. Then 3 mL of DMSO were added to the lyophilized biomass and incubated at 40 °C in the darkness, for 10 min. The DMSO phase containing the carotenoids was then recovered by centrifugation at 3,500 *g*, for 5 min. The pellet was re-suspended in 2 mL of DMSO to extract the remaining carotenoids and processed as indicated above. Then 2 mL of acetone, 2 mL of NaCl solution (20 % w/v) and 2 mL of petroleum ether (60–80 °C) were added to 5 mL of DMSO containing the carotenoids. The solution was vortexed and kept in the dark, until formation of the upper phase of petroleum ether, which was collected. Petroleum ether was added until complete extraction of the carotenoids from DMSO, collecting the upper phase. The extracts were combined and evaporated under an inert atmosphere in order to prevent the carotenoids degradation. The residue was re-suspended in acetone and these extracts were injected in a HPLC

system HP-1100 (Hewlett Packard, Waldrom, Germany), using a μ-Bondapack C18 column (250/4.0 mm) (VDS-optilab, Berlin, Germany) and a UV/VIS ($\lambda = 450$ nm) (Hewlett Packard, series 1100, Waldronn, Germany) detector. The mobile phase used was methanol (with 0.2 % water) and acetonitrile (75:25). The pigments were eluted in 16 min, at 1 mL min^{-1}, and identified by comparison with thin layer chromatography analysis [22]. The β-carotene was identified by comparing HPLC retention time of the carotenoid with that of the standard compound (Sigma, 97 % purity), which was also used to obtain a calibration curve for determination of the β-carotene content.

Cell membrane integrity

Propidium iodide (PI) is known to stain nucleic acids in cells characterized by a defective membrane integrity and has been widely used to detect yeast cytoplasmic membrane permeability [3, 7, 17–19]. Flow cytometry coupled with PI (Invitrogen, Carlsbad, CA, USA) was used in this study in order to monitor *R. glutinis* cytoplasmic membrane integrity. PI cannot cross an intact cytoplasmic membrane, but binds to DNA if the membrane is injured. Samples taken from the culture were immediately diluted (at least 1:2,000 v/v) with phosphate buffer solution (PBS, pH 7.0) and stained with PI. Five microliters of PI stock solution (2 mg mL^{-1} in distilled water) were added to 995 μL diluted sample, so that the final PI working concentration was 10 μg mL^{-1}. The PI was excited at 488 nm and measured at FL2 (585/42 band pass filter).

Residual glycerol

The residual glycerol in filtered samples (0.2 μm) was evaluated by HPLC (Agilent 1100, Santa Clara, CA, USA). The column used was Aminex HPX-87H 300 mm × 7.8 mm (Catalog 125-0140, BIO-RAD, Hercules, CA, USA) and the eluent Super pure water H_2SO_4 5 mM.

Results and discussion

Batch growth for carotenoid content, cell size and granularity detection by flow cytometry

Cell samples collected during the yeast batch growth were analyzed for membrane integrity, autofluorescence (measured in the FL1, FL2, FL3 and FL4 channels), FSC and SSC signals by flow cytometry, in order to establish a multiparametric flow cytometry protocol to be used for the analyses of cellular parameters and β-carotene production of a great number of samples, in a short time.

Figure 1a shows *R. glutinis* 400A15 biomass production, cell size (FSC), granularity (SSC) and cell membrane permeability during the batch growth. Biomass concentration increased during the exponential phase, reaching

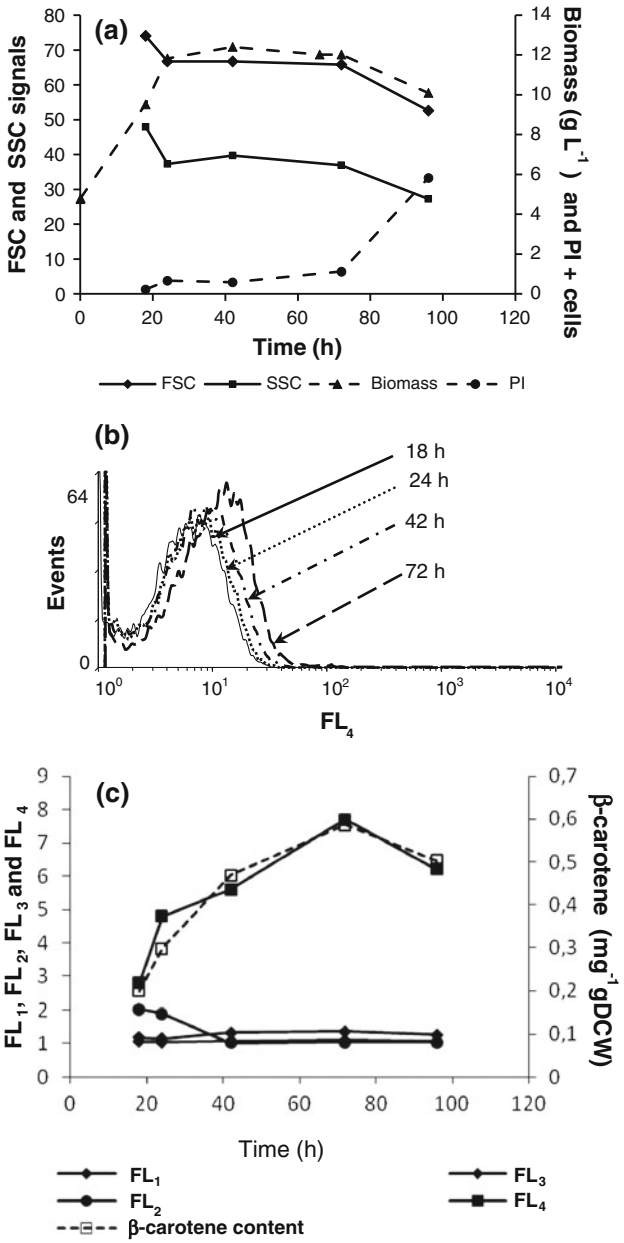

Fig. 1 *R. glutinis* mutant 400A15 batch fermentation profiles. **a** Biomass, PI + cells percentage, FSC and SSC signals profiles during *R. glutinis* mutant 400A15 batch growth. Biomass, PI + cells percentages, FSC and SSC values were affected by a relative error not exceeding 10 % ($n = 2$). **b** *R. glutinis* mutant 400A15 cells FL4 autofluorescence measured during the yeast batch growth. **c** *R. glutinis* mutant 400A15 cells autofluorescences measured in FL_1, FL_2, FL_3, FL_4 detectors, and yeast β-carotene content, assessed by the traditional method, during a batch growth. The FL_1, FL_2, FL_3, FL_4 and β-carotene content values were affected by a relative error not exceeding 10 % ($n = 2$)

11.8 g L^{-1} at $t = 24$ h, then decreasing until the end of the experiment. The FSC is measured in the plane of the laser beam and gives information on cell size, while SSC is measured at 90° to the beam, detecting different cross sectional areas thus providing information on cell granularity or internal structure [10, 15, 17, 18]. The FSC and SSC average signals decreased from the exponential phase (at $t = 20$ h, FSC = 74, SSC = 42) to the stationary phase, leveling off for 48 h. They then started decreasing until the end of the experiment (at $t = 96$ h, FSC = 53, SSC = 27), following the same trend of the biomass profile, during this stage. It was observed that the FSC and SSC signals decreased after $t = 72$ h, as a result of the extended nutrient deprivation period that cells experienced as the number of budding cells (displaying higher FSC and SS signals) decreased. This result is in accordance with those previously described for the yeast *Rhodosporidium toruloides* NCYC 921 [3]. The proportion of PI + cells was below 2 % until $t = 72$ h, reaching 6 %, at the late stationary phase ($t = 96$ h).

The yeast autofluorescence was detected in the four channels FL_1, FL_2 and FL_3 and FL_4. While FL_1, FL_2 and FL_3 did not change during the batch growth, the FL_4 peak, as well as the corresponding average value increased (from 2.5 at $t = 18$ h, to 7.6 at $t = 72$ h, Fig. 1b and c) as the culture aged. It was observed that FL_4 followed the yeast β-carotene production assessed by HPLC (Fig. 1c). When excited at 488 nm, the wavelength most commonly used in laser cytometry, β-carotene fluoresces at 520 nm [27]. However, in the present case, it was observed that the autofluorescence intensity at around 660 nm (FL4) showed the highest values comparing to the autofluorescences read in the remaining detectors (FL1, FL2 and FL3) showing a good correlation with the yeast β-carotene content [$(\beta - \text{carotene content}\,(\text{mg g}^{-1}) = 0.084 \times \text{FL4} - 0.05$, with $R^2 = 0.93$) for FL4, comparing to $R^2 = 0.89$, 0.82. 0.26 obtained for FL1, FL2 and FL3, respectively]. Ukibe et al. [27] also reported that with a 488 nm-laser, the fluorescence intensity emitted by *Xanthophyllomyces dendrorhous* yeast cells detected in the FL4 channel correlated with the astaxanthin content, despite the excitation and emission maxima of astaxanthin in acetone that were found at 350 and 570 nm, respectively. This observation may be due to the fact that the β-carotene molecules, as intracellular components, therefore, linked to other intracellular components, may display a different wavelength from that emitted by the pure compound. On the other hand, unknown cellular substances that fluoresce at the same range of wavelength (FL4) might be positively correlated with the β-carotene cell content, or with the activity of β-carotene biosynthesis [25].

The highest content of β-carotene was detected at the yeast stationary phase ($t = 72$ h) (Fig. 1a, b). This observation is in agreement with Bhosale et al. [6] who reported that the carotenoids accumulation in yeast starts during the late exponential phase, and continues during the stationary phase.

In this way, it was possible to establish a quick flow cytometric protocol to simultaneously detect the yeast cytoplasmic membrane integrity, β-carotene content, cell

Table 2 Data from the experiments varying medium volume (as a way to change the k_La) and nitrogen content (as a way to change C/N ratio) according to a Doehlert distribution for two factors

Test	Volume (L) (k_La) (s^{-1})	Nitrogen content (mol L^{-1}) (C/N)	Cells (g L^{-1})	Permeabilised cells (%)	Cell size (FSC)	Granularity (SSC)	β-carotene content (mg g^{-1}DCW)	β-carotene productivity (mg L^{-1} h^{-1})	Consumed glycerol (g L^{-1})
1	0.33 (5×10^{-3})	11.3 (0.23)	17.3	2.6	60.5	46.5	0.78	0.19	42.0
2	0.33 (5×10^{-3})	11.3 (0.23)	19.9	2.8	71.2	57.5	0.79	0.22	43.4
3	0.18 (12.5×10^{-3})	11.3 (0.23)	24.5	1.9	55.2	44.2	0.50	0.17	55.8
4	0.18 (12.5×10^{-3})	11.3 (0.23)	24.3	1.2	65.0	44.9	0.66	0.22	52.4
5	0.40 (4×10^{-3})	20.0 (0.13)	14.6	0.9	55.9	46.5	0.50	0.10	46.0
6	0.40 (4×10^{-3})	20.0 (0.13)	14.6	0.9	53.0	42.8	0.52	0.11	36.8
7	0.25 (8.3×10^{-3})	20.0 (0.13)	14.9	0.7	50.3	43.2	0.53	0.11	38.9
8	0.25 (8.3×10^{-3})	20.0 (0.13)	15.1	0.5	48.0	32.5	0.41	0.08	44.2
9	0.10 (31.3×10^{-3})	20.0 (0.13)	17.4	1.2	48.9	42.8	0.46	0.11	42.0
10	0.10 (31.3×10^{-3})	20.0 (0.13)	16.7	1.3	38.3	33.6	0.35	0.08	55.3
11	0.33 (5×10^{-3})	65.0 (0.04)	6.5	0.4	41.5	37.3	0.37	0.03	19.6
12	0.33 (5×10^{-3})	65.0 (0.04)	6.3	0.4	41.4	35.9	0.37	0.03	20.1
13	0.18 (12.5×10^{-3})	65.0 (0.04)	4.4	0.3	40.1	29.1	0.38	0.02	15.1
14	0.18 (12.5×10^{-3})	65.0 (0.04)	4.9	0.3	42.3	39.5	0.36	0.02	15.1

The responses under study were biomass production (g L^{-1}), permeabilised cells percentage, FSC and SSC signals (as a cell size and cell granularity measurement) and β-carotene content. Data were organized according to nitrogen concentration (as a way to express C/N)

size and cell granularity, which were used further in the experimental design.

Experimental design

Table 2 shows the data obtained from the experimental design for *R. glutinis* 400A15 cells grown under culture conditions developed according to a Doehlert experimental distribution for two factors: shaken flask working volume (as a means to control oxygen availability) and carbon to nitrogen ratio (C/N). Each volume used in the experiments was characterized by the determination of the specific oxygen transfer coefficient (k_La).

The highest biomass concentration (24.5 g L^{-1}) was observed for the lowest C/N (11.3, corresponding to 0.23 mol nitrogen L^{-1}), at the k_La of 12.5 × 10^{-3} s^{-1}. For this C/N (tests 1–4) the change in k_La from 5 × 10^{-3} to 12.5 × 10^{-3} s^{-1} induced the formation of cells as the values of biomass increased from 18.6 to 24.4 g L^{-1}, a 31 % change. This observation indicated that, in these experiments, the oxygen was limiting cell growth, and its enhanced availability increased the biomass content. The continuous mode of oxygen supply in batch shake flask experiments, allowed the continuous use of nutrients at a rate controlled by k_La, which resulted in the highest glycerol consumption detected in tests 3–4 (Table 2).

For C/N of 20.0 (center of the experimental domain) corresponding to a nitrogen concentration of 0.13 mol L^{-1}, in tests 5–10, the eight-fold change in k_La from 4 × 10^{-3} to 31.3 × 10^{-3} s^{-1} also induced an increase in biomass production from ~14.8 g L^{-1} (tests 5–8) to ~17.0 g L^{-1} (tests 9–10). This observation corresponds to a 14 % step change, with cells growing at a linear rate of 1.42 g L^{-1} per each k_La unit. On the contrary, the highest C/N (65.0, corresponding to a nitrogen concentration of 0.04 mol L^{-1}) was detrimental for growth as biomass attained the lowest concentration (<7 g L^{-1}), even when oxygen supply was increased (tests 11–14), due the nitrogen exhaustion (confirmed by the low glycerol consumption) that halted the yeast growth and division as a result of the lack of this nutrient for *de novo* protein and nucleotide synthesis.

Data in Table 2 also reveals the effect of environmental factors on *R. glutinis* 400A15 cell permeabilization, determined by flow cytometry of PI stained cells. Higher percentages of permeabilised cells (>1 %) were obtained for the tests at lower C/N, wherein biomass concentrations were higher (tests 1–2, 3–4, 9–10). The acid metabolites, known to be produced during active yeast growth, may explain the highest proportion of permeabilised cells in these experiments. Producing more biomass, the yeast cells from these cultures would have produced more acid metabolites, damaging the membranes, which is in accordance with the results reported by Lopes da Silva et al. [18]. Conversely, at the C/N of 6, the biomass concentration did not surpass 7 g L^{-1}, and the proportion of permeabilised cells did not exceed 0.37 % (tests 11–14). Overall and according to data in Table 2, cell permeabilization does not seem to be closely associated with k_La (tests 1–4 and 11–14).

The effect of medium volume (k_La) and C/N on cell size is shown in Table 2. Tests 1–4 show that, for a constant C/N of 11.3, the k_La increased from 5 × 10^{-3} to 12.5 × 10^{-3} s^{-1} led to cell size reduction of 15 %.

At a constant C/N of 20.0 (nitrogen concentration of 0.13 mol L^{-1}), changing k_La from 4 × 10^{-3} to 31.3 × 10^{-3} s^{-1}, cell size decreased again, achieving a 20 % drop within the k_La range under study (tests 7–8, 9–10).

For C/N of 65.0, an increase in k_La from 5 × 10^{-3} to 12.5 × 10^{-3} (tests 11–14) did not lead to a significant variation in cell size as nitrogen was limiting the yeast growth.

At low and constant k_La of 5 × 10^{-3} s^{-1}, an increase in C/N from 11.3 to 65.0 led to a 37 % decrease in cell size (tests 1–2, 11–12). At twice the value of k_La (12.5 × 10^{-3} s^{-1}) the same step change in C/N increase led to a 26 % increase in cell size (tests 3–4, 13–14). Overall, the increase in C/N reduced the yeast cell size. The glycerol consumption also decreased with the C/N increase, due to the nitrogen limiting conditions in these experiments, suggesting that, at higher C/N ratios, cells would have experienced longer starvation periods, resulting in cell size reduction.

The cellular granularity measured by SSC signals followed a similar trend as the cell size (FSC) indicating that cells displaying a higher granularity and internal structure level were larger.

The β-carotene concentration reached the maximum content (0.79 mg g^{-1} DCW) for the lower C/N (11.3), at a k_La of 5 × 10^{-3} s^{-1} (Table 2). This result is in accordance with that reported by Somashekar and Joseph [26], as the carotenoid content produced by the yeast *R. glutinis* CFR-1AU attained the maximum content (26 mg g^{-1} DCW) at the lowest C/N (10) of the range studied. El-Banna et al. [13] also reported that strain *R. glutinis* var. *glutinis* produced the maximum carotenoids content (0.37 mg g^{-1}) at low C/N of 5. From Table 2, it can also be seen that, at the lowest value of C/N (11.3), the β-carotene content decreased from 0.77 to 0.60 mg g^{-1}, as the k_La increased from 5 × 10^{-3} to 12.5 × 10^{-3} s^{-1}, which corresponds to 22.1 % decrease (tests 1–4). For the highest C/N (65.0), the values of β-carotene remained stable with virtually no change when k_La varies from 5 × 10^{-3} to 12.5 × 10^{-3} s^{-1} (tests 11–14).

At the C/N of 20.0, the production of β-carotene by the yeast cells decreased. Again, higher k_La values reduced the yeast β-carotene content (tests 5–6, 7–8, 9–10). This can be

explained by the fact that, under a higher oxygen transfer rate, the biomass increased, resulting from the carbon excess directed towards biomass formation, instead of β-carotene production. Such results are in accordance with those described by Saenge et al. [24] who reported that the experimental conditions that enhanced *Rhodotorula glutinis* TISTR 5159 biomass, resulted in the reduction in the yeast carotenoids content. At the highest C/N (65.0), the β-carotene content did not change with the k_La increase (tests 11–12, 13–14).

Generally, the increase in C/N decreased the production of β-carotene and the k_La increase had a detrimental effect on this parameter. It was also observed that the maximum β-carotene content was detected in the largest yeast cells in size and complexity (tests 1–4).

Like the β-carotene content, the β-carotene productivity (expressed in g L^{-1} h^{-1}, calculated as the product between the biomass concentration and the β-carotene content, divided by the cultivation time) decreased, as the C/N increased (Table 2). However, in almost cases, for a fixed C/N, the oxygen availability increase did not affect the β-carotene productivity, because the biomass concentration increased with the k_La, while the β-carotene content decreased, maintaining approximately constant the product of these two variables.

Overall, the highest β-carotene contents and concentrations were observed when biomass concentration, cell size and granularity were maximums (C/N = 11.3, tests 1–4). It has been reported that the yeast carotenoids and lipids are usually produced during the stationary phase [24]. The yeast cell size and granularity maxima could be associated to the intracellular lipidic storage materials synthesis, known to increase the FSC and SSC signals [3, 10].

On the other hand, the highest proportion of cells displaying permeabilised membranes with higher β-carotene content (tests 1–4) should be related to the highest acid metabolites production, as a result of the highest biomass production observed in these experiments, as above mentioned.

Analysis of the polynomial models parameters

The observations referred above were consistent with the model representation of the experimental domain (Table 3). The relative importance of shake flask working volume (k_La) and carbon to nitrogen ratio (C/N) towards biomass, permeabilised cells, cell size, cell granularity and β-carotene content was given by the relative value of β parameters in the regression equations (Table 3). The β parameters analysis allows a better understanding of the stress introduced by the two factors under study. In fact, increasing culture volumes acts as a measure of the cell stress experienced by *R. glutinis* 400A15 under low oxygen levels and high carbon to nitrogen ratios results in low nitrogen availability.

For biomass, the value of β_1 (medium volume, which determines different k_La values) is negative, meaning that a larger medium volume (low k_La) in the shake flask resulted in low oxygen transfer and, consequently, reduced biomass concentration (Table 3). The value of β_2 (C/N) is also negative and much larger than β_1 meaning that the C/N is much more influential on the final biomass concentration than the medium volume used to control oxygen transfer in the process. This high negative influence is a result of the drastic decrease in nitrogen content in the culture at high C/N ratio. The magnitude of β_{12} (interaction between volume and C/N) provides information about how the two

Table 3 Parameters of the polynomial models representing the studied responses

	Model	Biomass	Permeabilised cells	SSC	Cell size	β-Carotene
Model parameters	β_0	15	0.64	37.86	49.16	0.47
	β_1	−1.48	0.1	3.78	5.32	0.06
	β_2	−9.21	−1.04	−7.4	−11.29	−0.18
	β_{12}	4.32	−0.56	−2.97	−5.6	−0.1
	β_{11}	0.82	0.44	3.57	−0.13	−0.01
	β_{22}	−2.27	0.63	4.15	2.63	0.08
Model validation (Fischer test)	Effectiveness of the parameters	200.26	11.84	3.21	12.86	18.25
	Significance level (%), $F(5.8)$	$\alpha = 0.001$	$\alpha = 0.01$	$\alpha = 0.05$	$\alpha = 0.001$	$\alpha = 0.01$
	Lack of fit	1.71	17.05	0.11	0.01	0.53
	Significance level (%), $F(1.7)$	$\alpha = 0.10$	$\alpha = 0.01$	$\alpha = 0.10$	$\alpha = 0.10$	$\alpha \geq 0.10$

β_0, response at the centre of the experimental domain; β_1 and β_2, parameters of the factors; β_{12}, parameter of the interaction of the factors; β_{11} and β_{22}, self-interaction parameters of the factors. Critical and calculated values in both *F*-tests were used to test the effectiveness of the parameters and the lack of fit.

factors, together, contribute to the response. The interaction term, β_{12}, is positive, which means that both factors do not interact toward the decrease of biomass concentration.

The two factors affect the number of permeabilised cells in distinct forms, supporting the observation made when analyzing Table 2. In fact, the value of β_1 is positive and ten-fold higher than β_2 whereas the interaction term (β_{12}) is negative and small. The overall expression is $\beta_1 > \beta_2 > \beta_{12}$ meaning that a fraction of the response is due to the negative interaction between the two factors.

The two factors affect cell granularity and cell size exactly in the same way: Increasing medium volume (decreasing k_La) acts positively on both responses, expressing the increase in cell internal complexity and cell size, as a result of the lack of oxygen. Conversely, C/N ratio (β_2) influences negatively the responses, meaning that nitrogen depletion induced morphological changes as a stress response. The relative importance of the expression takes the form $\beta_1 >> \beta_2 > \beta_{12}$.

For β-carotene, β_1 and β_2 show opposite signs. Again, β_1 is higher than β_2, indicating that the former parameter has a stronger influence on the β-carotene production. β_{12} is also negative although taking a lower value than β_1 and β_2 being characterized by the expression $\beta_1 > \beta_2 >> \beta_{12}$.

Isoresponses surfaces plots

The meaning of the model factors becomes clearer when assessing the response variation in the whole span of the experimental domain.

Figure 2 shows the response surfaces for biomass concentration, permeabilised cells percentage, cell size and cell granularity when varying C/N and the volume of the culture medium. The response surface shown in Fig. 2a displays the variation of biomass within the experimental domain. The importance of C/N on the response is expressed by the horizontal lines on the response surface: a small variation of C/N produces a response in the biomass concentration, whereas changes in k_La induced only

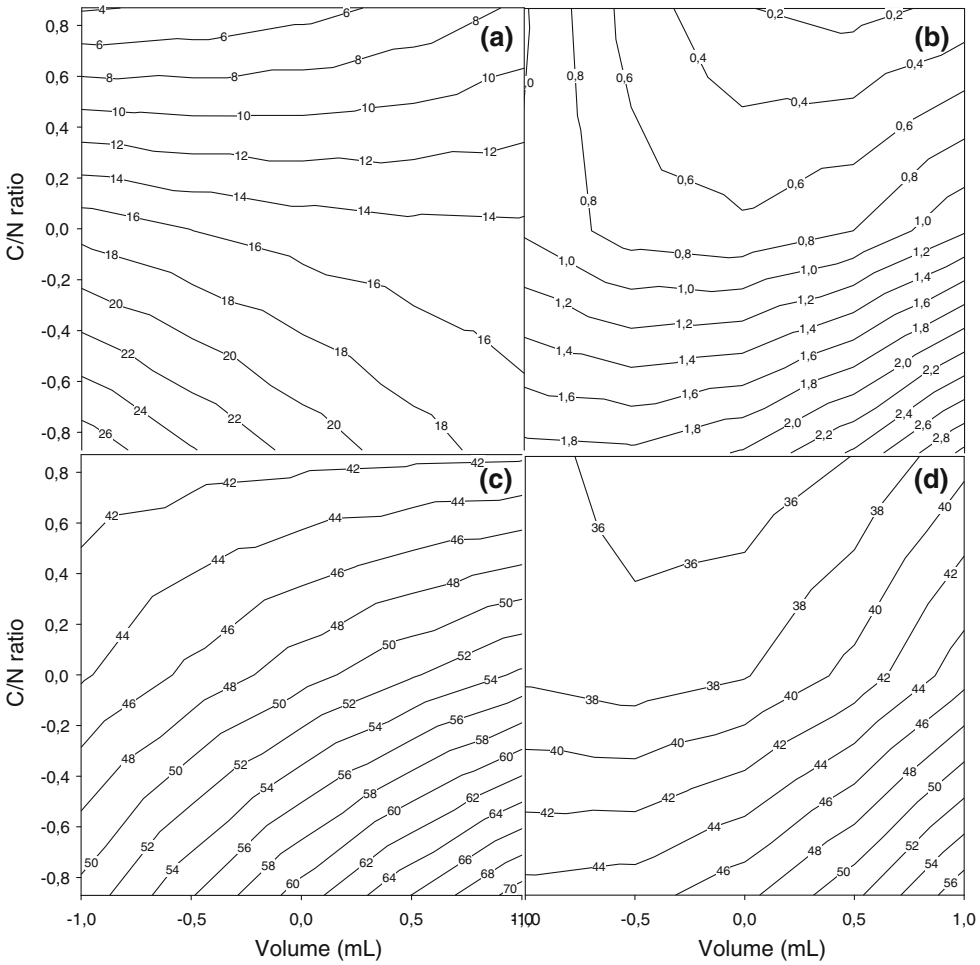

Fig. 2 Isoresponse contours for *R. glutinis* mutant 400A15 **a** final biomass concentration; **b** permeabilised cells percentage; **c** cell size; **d** cell granularity; upon the carbon to nitrogen ratio (changing nitrogen concentration in a constant glycerol content) and medium volume (k_La)

smaller changes in the culture. The horizontal lines are less stressed in the areas built by the higher values of both factors (upper left and right quadrants). The widest response span in the domain varies between 4 and 26 g L^{-1} at the highest k_La value (low medium volume). The response span decreases as k_La takes lower values (higher medium volume). This effect is due to the lack of oxygen in the areas of lower oxygen transfer.

Figure 2b shows that the amount of permeabilised cells within the experimental domain is limited. However, data show that lower C/N (higher nitrogen content) increased the amount of affected cells, possibly due to the production of acid metabolites usually found in active grown yeast cultures, known to induce yeast cell membrane damage. The number of permeabilised cells also increased with the decrease in k_La which leads to a higher stress level in the cells. This effect was more noticeable at low C/N.

Figure 2c and d show that cell size and cell granularity grow simultaneously achieving their maximum value in experiments at low C/N, where the higher nitrogen concentration allowed the yeast growth. Low k_La values induced higher cell size and granularity. Conversely, Fig. 2a shows that, at low C/N, the presence of oxygen allows a high amount of carbon to flow towards biomass formation (down left quadrant) with a parallel increase in cell size and cell granularity (Fig. 2c, d) which increased as volume grows decreasing oxygen availability. Albeit the small percentage of permealilized cells, Fig. 2b shows the area of higher cytoplasmic membrane damage within the experimental domain (down right quadrant) is made of larger and more complex cells (Fig. 2c, d).

Figure 3 pictures the variation of β-carotene within the experimental domain. Again, C/N shows to have more influence on the production of β-carotene than k_La as a small C/N variation produced a noticeable response in the β-carotene concentration. Lower values of k_La increased the accumulation of β-carotene although a step change in k_La and is less effective in the response variation than C/N.

Analysis of experimental data

Statistical validation of the polynomial equations was made by analysis of variance (ANOVA) [11]. The adequacy of the models to fit the sets of data was performed using two

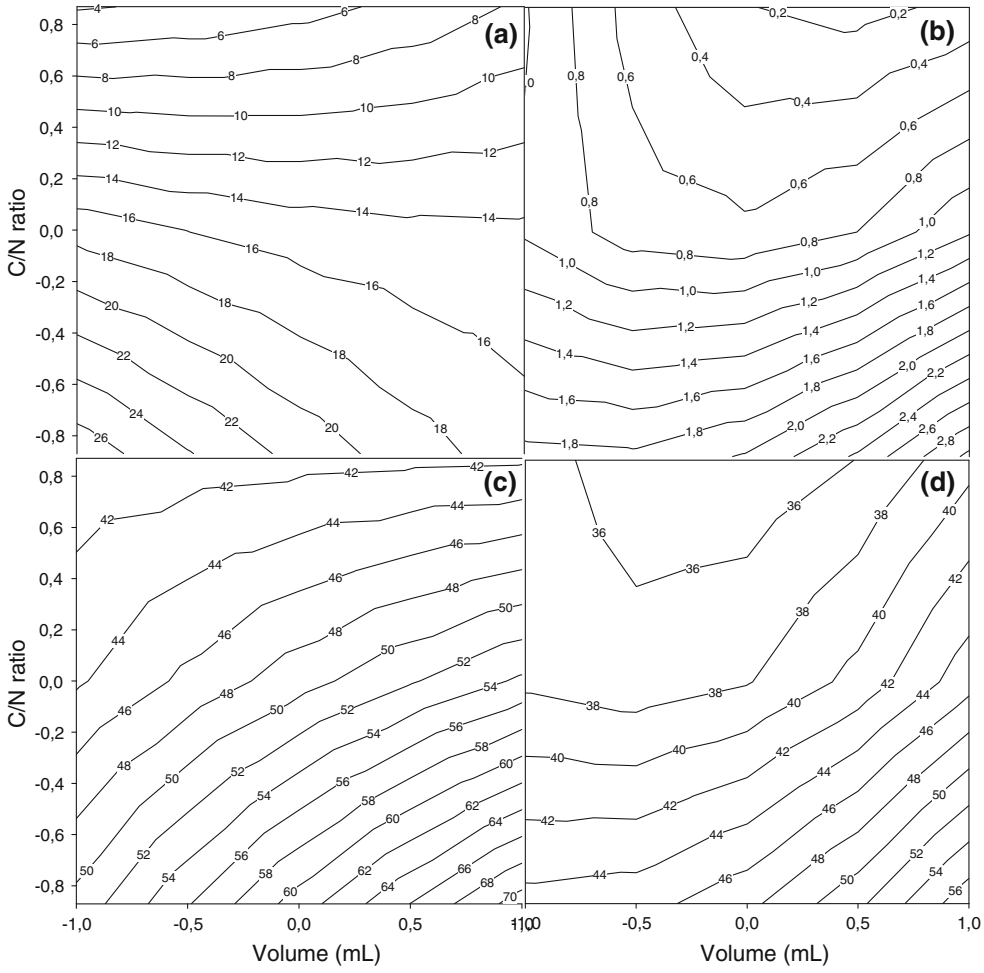

Fig. 3 Isoresponse contours for *R. glutinis* mutant 400A15 final β-carotene content, upon the carbon to nitrogen ratio (changing nitrogen concentration the glycerol concentration) and medium volume (k_La)

statistical tests: (1) the *F*-test for the effectiveness of the factors which detects whether the source of variance, included in the residuals, is due to the inadequacy of the models to reproduce experimental data; and (2) the *F*-test for the lack of fit, that is performed in order to detect if the origin of the variance was due to experimental error. Table 3 shows the Fisher variation ratios and levels of confidence evaluated for each *F*-test.

The *F*-test for the effectiveness of the factors applied to biomass, permeabilised cells and β-carotene concentration showed a level of confidence at which the null hypothesis (H_0) can be rejected of 0.001 % for biomass and cell size, 0.01 % for permeabilised cells and β-carotene concentration, and 0.05 % for cell granularity. Thus, with a good level of confidence it can be assumed that a significant amount of variance in the data has been represented by the factors in the models, i.e., the factors, as they appear in the model, do have an effect upon the responses analyzed.

The *F*-ratio for the lack of fit, also seems to be significant for the responses under study, as the null hypothesis can be rejected with levels of confidence of 0.01 and 0.1 % as shown in Table 3. In these conditions, the alternative hypothesis (H_a) is accepted which means that the lack of perfect prediction of the models is explained by the experimental error. All the models fit the sets of data.

Conclusions

In this work, flow cytometry proved to be a powerful tool for the assessment of *R. glutinis* mutant 400A15 β-carotene production, simultaneously giving detailed information on physiological and morphological changes, when the yeast was grown under different environmental conditions. Such approach, never reported before, allowed the quick assessment of a high number of cellular parameters, in a high number of samples, which is not possible when using traditional microbiological and analytical methods.

Acknowledgments The RC and IM work was partially supported by Ministero delle Politiche Agricole e Forestali (MIPAF) (D.M. 26285/7303/2009), project entitled "I lieviti nel recupero e valorizzazione del glicerolo grezzo derivante dalla produzione di biodiesel" (LIEBIG). Beatriz P. Nobre thanks FCT for the research grant (SFRH/BPD/42004/2007).

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