# Microbial production of carotenoids and lipids by the yeast *Rhodosporidium toruloides* NCYC 921 cultivated in fed-batch cultures

**Ana Sofia Santos Sousa**

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## Abstract

In this work the yeast *Rhodosporidium toruloides* NCYC 921 was cultivated in a 7 L bioreactor (working volume of 5 L, fed-batch regime), for the production of biomass, fatty acids (for biodiesel purposes) and carotenoids with commercial interest. Several different feeding strategies were studied. In the first fed-batch, carried out at a pH of 5.5, pulses of concentrated nutrients + glucose solutions and glucose solutions were added manually. In the second fed-batch (pH 5.5), a concentrated nutrients + glucose solution was continuously added by using a peristaltic pump, and pulses of a concentrated glucose solution were added manually. In the third fed-batch (pH 5.5), both types of solutions were continuously added with the peristaltic pump. The fourth fed-batch also used the same feeding strategy as the previous fed-batch, but the medium pH was changed to 4.0, with the goal of enhancing the biomass productivity, and further increasing the fatty acids and carotenoids productivity in future works. The third fed-batch achieved the maximum fatty acid productivity, reaching 0.34 $g.L^{-1}.h^{-1}$. Both the highest fatty acids content (31.45% w/w) and total carotenoids (0.40 $mg.g^{-1}$) was achieved in the first fed-batch. The maximum total carotenoids productivity was achieved in the second fed-batch, reaching 0.22 $mg.L^{-1}.h^{-1}$. The fourth fed-batch (pH 4.0) reached the highest maximum biomass concentration (119.61 $g.L^{-1}$) and the highest maximum biomass productivity (1.76 $g.L^{-1}.h^{-1}$).


Keywords: *Rhodosporidium toruloides*, biodiesel, carotenoids, fatty acids, fed-batch, flow cytometry.

## 1 Introduction

Fossil fuels are still the major source of energy, as well as the driving force for global economy [1]. However, being a non-renewable resource, with their prices rising over the years [2], and with the environmental problems associated with their use ($CO_2$ emissions), there has been an increasing need for alternative renewable sources. From the current alternatives, biofuels are the most environmentally friendly option [3]. Biodiesel is a renewable fuel, with a biological origin, highly biodegradable and very low toxicity. It can be produced from vegetable oils, animal fat, used oils and from microorganisms.

Chemically is a mixture of methyl esters of fatty acids and can be obtained by transesterification, regarded as the best method for biodiesel industrial production, due to its low cost and simplicity [4].

Third generation biofuels, obtained from oleaginous microorganisms (microalgae, bacteria, fungi and yeast), which produce more than 20% of their oil weight, are a viable alternative energy source. The storage of oils in oleaginous microorganisms occurs when one of the nutrients in the culture is exhausted (usually nitrogen), and the excess of carbon is then converted by the cells into triglycerides. This synthesis occurs during the stationary phase, when cellular growth decreases and the produced lipids are stored inside the cells, as reserve material [4]. The use of microorganisms for the production of biofuel has the following benefits when compared with other sources: it does not compete with the food industry (food vs fuel) [3], shorter life time, easier scale up, and the local environment has a lesser impact on production [5]. Most studies use autotrophic microalgae, however they exhibit a slower growth rate when compared to bacteria or fungi. Additionally, autotrophic cultures cannot achieve high biomass concentrations and high oil productivities, due to light and oxygen limitations [6]. Yeast can grow in low-cost fermentation media, which can lower biodiesel production costs [1]. Although yeasts released $CO_2$, they have the advantage of requiring a lower initial investment, displaying higher growth rates and lipid productivity, when compared with microalgae [1,7].

The yeast *Rhodosporidium toruloides* NCYC 921 (which specie is an anamorph of *Rhodotorula glutinis* specie [2]) has been widely reported as a potential oil producer (for biodiesel production) [2,8] and as a source of carotenoids (with high commercial interest) [2]. Their lipids are composed of the FA palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and γ-linolenic (18:3); their composition and their content, on dry weight, vary widely and depend on the culture conditions. The most common carotenoids produced by *R. toruloides* are β-carotene, torulene, torularrudina and γ-carotene which are interesting compounds for food, pharmaceutical and nutraceutical industries [2,9]. Currently, microbial biodiesel is economically unsustainable, since the costs of production are still higher than the cost of fossil fuels and first generation biodiesel. However, *R. toruloides* is rich in suitable oils (for the production of biodiesel) and carotenoids, whereby the co-extraction of these two products may make the biodiesel production process economically sustainable [2].

Most of the published works studying microbial biodiesel production uses traditional microbiological methods to monitor cell growth. These techniques have several limitations, since results are only obtained sometime after sample collection, or sometimes only after the bioprocess has ended. In addition, they assume that microbial cultures are homogeneous, and do not provide any information on cell physiology [1,7]. Flow cytometry (FC) can provide, in real time, and with a high degree of accuracy, information on cell viability, the content of total carotenoids (TC) and the metabolic activity of the cell [2,7]. It allows the detection of a variety of intermediate cell physiological states, between death and the full metabolic activity of the cell, in a microbial population, using specific fluorochromes or combinations of these [9].

The main objective of the present work was to optimize the microbial biomass production of the yeast *R. toruloides* NCYC 921, using a bioreactor (work volume of 5 L) operating in fed-batch mode, and utilizing glucose as carbon source. FC was used to monitor, in real time, various cell parameters such as TC content and cell viability. Cultures were also monitored in terms of biomass, percentage of dissolved oxygen (DO), residual carbon and nitrogen concentrations, FA and TC content.

## 2 Methods

### 2.1 Experimental strategy

Assays were conducted in a bench bioreactor, in fed-batch mode, in order to characterize the growth, FA and CT productivity for the yeast *R. toruloides*. An experiment in batch mode was previously conducted, for comparison with the growth in fed-batch mode. The operational conditions used for the experiments are described in Table 2.1.

Table 2.1 - Conditions used in the assays of the yeast *Rhodosporidium toruloides* NCYC 921.

| Mode | pH | Feeding strategies | Added solutions | Agitation rate |
|---|---|---|---|---|
| Batch | 5.5 | - | - | Dependent of DO |
| Fed-batch I | 5.5 | Manual pulses | Nutrients + glucose, Minerals, Trace minerals, MgSO4.7H2O and Glucose 600 $g.L^{-1}$ | 600 rpm |
| Fed-batch II | 5.5 | Peristaltic pump (nutrients + glucose) and manual pulses (glucose) | Nutrients + glucose and glucose 600 $g.L^{-1}$ | 600 rpm |
| Fed-batch III | 5.5 | Peristaltic pump | Nutrients + glucose and glucose 600 $g.L^{-1}$ | 600 rpm |
| Fed-batch IV | 4.0 | Peristaltic pump | Nutrients + glucose and glucose 600 $g.L^{-1}$ | Dependent of DO |

All assays were performed with an initial glucose concentration of 35 $g.L^{-1}$, at 30°C and aeration of 2 $L.min^{-1}$ (except the fed-batch IV: ventilation was increased to 3 $L.min^{-1}$ at 73 h). Fed-batch II used a tube with inside diameter (ID) of 2.5 mm and fed-batch III-IV ID of 1.6 mm, to allow better control of the feed rate. The fed-batch IV was performed at pH 4, since parallel assays performed within the CAROFUEL project using the same strain cultivated in baffled shake flasks (Master's Thesis Corália Silva, Instituto Superior de Agronomia, Universidade de Lisboa, 2014), concluded that this was the optimal pH for growth, instead of the pH used in the prior assays described by Yoon and Rhee (1983) and Pan *et al.* (1986) who used the same strain for lipid production [11,12].

### 2.2 Microorganism and Pre-inoculum

The yeast *R. toruloides* NCYC 921 was purchased to the National Collection of Yeast Cultures (Norwich, UK). The strain was stored on slants of Malt Extract Agar, at 4ºC.

For inoculum preparation, *R. toruloides* yeast cells from two slant grown for 72 h at 30ºC were transferred to the growth medium (150 mL) with the following composition (Pan *et al.* (1986)) [10] ($g.L^{-1}$): $KH_2PO_4$, 12,5 $g.L^{-1}$; $Na_2HPO_4$, 1,0; $(NH_4)_2SO_4$, 5,0; $MgSO_4.7H_2O$, 2,5; $CaCl_2.2H_2O$, 0,25; yeast extract (YE), 1,9; Trace minerals 0,25 $mL.L^{-1}$. A solution of trace minerals contained following minerals in gram per L 5N-HCL ($g.L^{-1}$): $FeSO_4.7H_2O$, 40; $CaCl_2.2H_2O$, 40; $MgSO_4.7H_2O$, 10; $AlCl_3.6H_20$, 10; $CoCl_2$, 4; $ZnSO_4.7H_2O$, 2; $Na_2MoO_4.2H_2O$, 2; $CuCl_2.2H_2O$, 1; $H_3BO_4$, 0,5. Glucose was added to the culture at a final concentration of 35 $g.L^{-1}$, and was sterilized separately and mixed with the other components after cooling to make up the culture medium. In order to obtain a culture in exponential phase, 1 L baffled shake flasks were incubated for 24 h at 150 rpm, 30°C and in the absence of light.

### 2.3 Assays

The assays were performed in a 7 L bioreactor (work volume of 5 L) (FerMac 310bioreactor, Electrolab Biotech, United Kingdom (UK)), equipped with a Rushton impeller. The bioreactor was attached to a controller module of agitation, DO, temperature, pH (FerMac 360bioreactor, Electrolab Biotech, UK) and foaming. The pH was measured using a steam sterilizable electrode (Mettler Toledo 405-DPAS-SC-K8S/325, USA) and controlled automatically through the addition of 5M NaOH and 5M HCl, on demand, to 5.5±0.1 or 4.0±0.1. Foaming was controlled by addition of polypropylene glycol (PPG) and the temperature was controlled to 30ºC. The vessel was fitted with four equally spaced baffles. The yeast cultivations took, on average, 7 days. Dissolved oxygen (Broadley James, USA) was maintained above 40%, by automatic control of the agitation rate and controlling the flow of air, to avoid the limitation of the yeast growth caused by deficiency of this nutrient [7,12].

The bioreactor initially contained 2.850 L of growth medium, with a salt concentration corresponding to a final volume of 5 L (except for the batch and the first fed-batch, which contained a mineral concentration corresponding to the 2.850 L). After the bioreactor sterilization, 1.25 mL of trace minerals and glucose solution with a final concentration of 35 $g.L^{-1}$ were added to the growth medium.

For each sample taken from the bioreactor, the optical density (OD) was read at 600 nm (ThermoSpectronic Genesys 20, Portugal) and the yeast cells were analyzed by FC (Becton–Dickinson, Franklin Lakes, NJ, USA). The samples were centrifuged (centrifuge Sigma 2-16K, Sartorius, Germany), for 10 min, at 9000 rpm and at 5ºC, and the biomass was collected and lyophilised (Heto PowerDry LL3000 Freeze Dryer, Thermo Scientific, EUA) for subsequent FA analysis, in order to evaluate the yeast oil as potential feedstock for biodiesel production. The supernatants were frozen (-18ºC) for subsequent sugar and nitrogen analyses. All the experiments were conducted in duplicate. For each sample, the glucose concentration was estimated using strips for rapid detection of glucose (Combur-Test Strips by Roche, Switzerland). The addition of nutrients or carbon source was based on the values of biomass and of residual glucose concentrations.

Quantitation of biomass concentration was carried out through a correlation, previously established by Parreira *et al.* (2014), between the OD and the dry weight of the yeast (Absorbance 600 nm = 0.7062 [Biomass] + 0.4338, $R^2$ = 0.9986) [1]. TC were quantified by measuring the autofluorescence of cells by FC [2], through a correlation previously established between the autofluorescence of the cells and the TC and determined by HPLC.

The fed-batch cultivations started as batch, to allow the culture to reach a certain biomass concentration before the addition of nutrients. After the batch cultivation, the growth phase was prolonged by adding a nutrient solution (YE 20 $g.L^{-1}$, $MgSO_4.7H_2O$ 9 $g.L^{-1}$, and carbon source: glucose 600 $g.L^{-1}$), through manual pulses or continuous feeding (using a peristaltic pump: Watson Marlow 520 Du, UK). Once the culture reached the stationary phase, a glucose concentrated solution (600 $g.L^{-1}$) was added (manual or continuous) to induce the synthesis of carotenoid and lipids [13].

### 2.4 Glucose and nitrogen concentrations

Residual glucose concentration in the samples was analyzed by the 3,5-dinitrosalicylic acid (DNS) method [14]. Residual nitrogen concentration present in the supernatant was quantified using the method of *Kjeldahl*, which determines the nitrogen in the organic matter [1].

### 2.5 Fatty acid analysis

FA's extraction and preparation of methyl esters were carried out according to the following protocol described by Lepage and Roy [1,15] with modifications: Freeze-dried samples of *R. toruloides* (100 mg) were transmethylated with 2 mL of methanol/acetyl chloride (95:5, v/v) and 0.2 mL heptadecanoic acid (5 $mg.mL^{-1}$, Nu-Check-Prep, Elysian, USA) in petroleum ether (80ºC – 100ºC), as an internal standard. The mixture was sealed in a light-protected Teflon-lined vial under nitrogen atmosphere and heated at 80ºC for 1 h. The vial contents were then cooled in the dark, diluted with 1 mL water and extracted with 1 mL of n-heptane. The heptane layer, which contained the methyl esters, was dried over $Na_2SO_4$ and collected under nitrogen atmosphere. The methyl esters were then analyzed by gas–liquid chromatography, on a chromatograph (SCION GC 436 da Bruker, Germany), equipped with a flame ionization detector. Separation was carried out on a 0.32 mm × 30 m Supelcowax 10 capillary column (film 0.25 µm) with helium as a carrier gas, at a flow rate of 1,3 $mL.min^{-1}$. The column temperature was programmed at an initial temperature of 200◦C for 20 min, then increased at 2 $ºC.min^{-1}$ to 220ºC.The column temperature was programmed at an initial temperature of 200ºC for 8 min, then increased at 4$ºC.min^{-1}$ to 240◦C. Injector and detector temperatures were 250 and 280ºC, respectively, and split ratio was 1:50 for 5 min and then 1:10 for the remaining time. Column pressure was 13.5 psi. Peak identification and response factor calculation was carried out using known standards. Each sample was made in duplicate and injected once.

### 2.6 Flow cytometry

For FC analysis, a FACSCalibur (Becton–Dickinson, Franklin Lakes, NJ, USA) device equipped with a argon-ion laser (emission, 488nm), a red diode laser (emission 635nm) and sensors for detection of forward and side light scatter, green FL1 (530±30 nm), yellow FL2 (585±42nm), orange FL3 (>670nm) and red FL4 (600±16nm) fluorescence was used to quantify the TC content of *R. toruloides* cells, assess the cytoplasmic membrane integrity and the mitochondrial and cytoplasmic membranes potential, throughout the experiments [2]. Data were analyzed and treated in FCS Express 4 Flow Research Edition program.

#### 2.7.1 Carotenoid detection by flow cytometry

The TC content in yeast samples was also assessed by FC according to Freitas *et al.* (2014) [2], through a correlation between the yeast autofluorescences measured by FC, and the yeast carotenoid

content assessed by HPLC. Samples taken from the culture were immediately sonicated (Transsonic T 660/H, Elma), for 10 s, and diluted (so that the number of events was between 800-1000 events.$s^{-1}$) with PBS (phosphate buffered saline solution, NaCl, 8.0 g.$L^{-1}$; potassium chloride, 0.2 g.$L^{-1}$; di-sodium hydrogen phosphate, 1.15 g.$L^{-1}$; potassium dihydrogen phosphate, 0.2 g.$L^{-1}$, pH 7.3 ± 0.2) (Oxoid)). The yeast autofluorescences measured in the FL1, FL2 and FL3 channels were adjusted to the first logarithmic decade for the first sample collected at the beginning of the yeast growth. Then FL1, FL2 and FL3 profiles were monitored, using the same settings, throughout the yeast growth. A correlation between the autofluorescences measured by FC, and the total yeast carotenoid content assessed by the traditional method was established *(TC content (mg.$g^{-1}$) = 0.01036 x FL2 – 0,2877; $R^2$ = 0.9214))*. The correlation was achieved by analyzing samples taken at different times of the growth of the yeast, as it is known that TC content increases with the culture age [2].

### 2.7.2 Cell membrane potential and integrity

A mixture of propidium iodide (PI) (Invitrogen, EUA) and 3,3-dihexylocarbocyanine iodide ($DiOC_6(3)$) (Invitrogen, EUA) was used to evaluate *R. toruloides* cytoplasmic membrane integrity and mitochondrial membrane potential during the growth of the yeast. PI binds to DNA but cannot cross an intact cytoplasmic membrane. $DiOC_6(3)$ is a lipophilic carbocyanine stain that accumulates intracellularly in polarised or hyperpolarized cytoplasmic and mitochondrial membranes, due to its positive charge [2]. Stock solutions of each dye were prepared as follows: PI was made up at 1 mg.$mL^{-1}$ in distilled water and $DiOC_6(3)$ was made up at 10 µg.$mL^{-1}$ in dimethyl sulphoxide (DMSO). The working concentrations of PI were 1 µg.$mL^{-1}$ and $DIOC_6(3)$ 0.1 ng.$mL^{-1}$. PI fluorescence was measured at the FL3 channel and $DIOC_6(3)$ fluorescence was measured at the FL1 channel [16]. Since there is a spectral overlap between $DIOC_6(3)$ and PI-emitted fluorescence, the system software compensation was set up in such a way that $DIOC_6(3)$-emitted fluorescence was eliminated from the PI emitted fluorescence detector and vice versa [2]. Samples taken from the culture were immediately sonicated and diluted (number of events between 800-1000 events.$s^{-1}$) with PBS, stained with $DIOC_6(3)$ (1 µL) and incubated in the dark for 5 min. PI (1 µL) was added before cell FC analysis.

## 3 Results and discussion

### 3.1 Growth of yeast *Rhodosporidium toruloides* NCYC 921

Table 3.1 shows the kinetic parameters calculated for *R. toruloides* NCYC 921 batch and fed-batch cultivations

Table 3.1 - Kinetic parameters calculated the growth of *R. toruloides* NCYC 921.

| Parameters | | Batch | Fed-Batch I | Fed-Batch II | Fed-Batch III | Fed-Batch IV |
|---|---|---|---|---|---|---|
| **μ ( $h^{-1}$) (addition step)** | | - | 0.09 ($R^2$=0.95) | 0.03 ($R^2$=0.95) | 0.05 ($R^2$=0.99) | 0.05 ($R^2$=0.99) |
| **Maximum biomass concentration ($g.L^{-1}$)** | | 19.50 (t=41.83h) | 81.12 (t=166.25h) | 102.13 (t=164.50h) | 97.18 (t=97.00h) | 119.61 (t=97.47h) |
| **Maximum biomass productivity ($g.L^{-1}.h^{-1}$)** | | 0.73 (t=22.83h) | 1.74 (t=35.75h) | 1.48 (t=47.00h) | 1.58 (t=50.00h) | 1.76 (t=47.00h) |
| **Maximum FA content (%p/p)** | | 10.74 (t=22.83h) | 31.45 (t=166.25h) | 29.42 (t=184.54h) | 27.57 (t=74.00h) | 26.19 (t=117.67h) |
| **Maximum AF concentration ($g.L^{-1}$)** | | 1.38 (t=22.83h) | 25.20 (t=166.25h) | 28.08 (t=184.54h) | 24.83 (t=74.00h) | 30.65 (t=97.47h) |
| **Maximum FA productivity ($g.L^{-1}.h^{-1}$)** | | 0.08 (t=22.83h) | 0.25 (t=30.42h) | 0.32 (t=64.17h) | 0.34 (t=74.00h) | 0.31 (t=97.47h) |
| **Within European Standard EN 14214 defined limits** | | Yes | Yes | Yes | Yes | Exception: t=19.00h 18:3ω3:14.31% |
| **For Maximum FA productivity** | **%SFA** | 34.74 | 41.08 | 36.51 | 39.68 | 28.83 |
| | **%MUFA** | 38.76 | 35.75 | 36.57 | 35.41 | 40.32 |
| | **%PUFA** | 26.50 | 23.17 | 26.92 | 24.92 | 30.85 |
| **Maximum TC content ($mg.g^{-1}$)** | | 0.13 (t=18.33h) | 0.40 (t=166.25h) | 0.33 (t=184.54h) | 0.28 (t=144.00h) | 0.15 (t=117.67h) |
| **Maximum TC concentration ($mg.L^{-1}$)** | | 1.67 (t=33.83h) | 32.55 (t=166.25h) | 31.30 (t=184.54h) | 27.47 (t=144.00h) | 14.06 (t=117.67h) |
| **Maximum TC productivity ($mg.L^{-1}.h^{-1}$)** | | 0.08 (t=22.83h) | 0.19 (t=166.25h) | 0.22 (t=64.17h) | 0.19 (t=144.00h) | 0.12 (t=117.67h) |

The specific growth rate achieved during the batch, 0.25 $h^{-1}$ ($R^2$ = 0.99) (Table 3.1), was higher those reported by Li *et al.* (2007), which ranged from 0.135 to 0.119 $h^{-1}$. These authors cultivated the yeast *R. toruloides* Y4 in shake flasks, varying the initial glucose concentration from 10 to 150 $g.L^{-1}$, to study the effect of the initial carbon source concentration on the yeast growth [7]. The specific growth rate for this batch was also much higher when compared to then one obtained by Freitas *et al.* (2014) (0.06 $h^{-1}$) who cultivated the same strain in shake flasks containing the growth medium with 35 $g.L^{-1}$ glucose [2]. Higher growth rates are expected when using a bioreactor since this cultivation system allows an efficient aeration and agitation supply and the pH is automatically adjusted. This specific growth rate is also slightly higher than the one achieved by Malisorn *et al.* (2009) (0.20 $h^{-1}$), obtained with a *R. glutinis* DM28 yeast batch (3 L bioreactor), using radish brine with a concentration of 30 $g.L^{-1}$, with the goal of optimizing β-carotene production [17].

The biomass concentration obtained during the batch (19.50 $g.L^{-1}$, Table 3.1), was superior to that obtained by Li *et al.* (2007), 4.7 $g.L^{-1}$ and 18.6 $g.L^{-1}$ for the experiments carried out at 10 and 90 $g.L^{-1}$ of glucose, respectively [7]. This result is also higher than the biomass concentration achieved by Malisorn *et al.* (2009), 2.6 $g.L^{-1}$, for the assay mentioned above [17].The biomass productivity obtained during the batch (0.73 $g.L^{-1}.h^{-1}$) was much higher than those reported by Freitas *et al.* (2014) (0.12 $g.L^{-1}.h^{-1}$), who cultivated the same strain in shake flasks containing the growth medium with 35 g $L^{-1}$ [2]. Malisorn *et al.* (2009) also reported a lower productivity (0.1 $g.L^{-1}h^{-1}$) for the yeast R. glutinis DM28 in batch [17].

During the batch, the carbon source was almost exhausted at the end of the exponential phase (t=22.83h) (final residual glucose concentration: 1.56 $g.L^{-1}$); as a result, there was no accumulation of

FA and TC. The maximum FA content obtained was low (10.74% w/w), as well as the maximum TC concentration (1.67 $mg.L^{-1}$). For the yeast *R. glutinis* CGMCC, developed in shake flasks with the aim of studying the effect of different lighting intensities and temperatures on the growth and lipid and carotenoids production by this yeast, Zhang *et al.* (2014), obtained values between 24.8% and 38.8% for the total lipids (TL), and 0.9 and 2.6 $mg.L^{-1}$ for the maximum TC concentrations, having exhausted the carbon source at t=40.00h [18]. Freitas *et al.* (2014) obtained 32% w/w for FA content and 38.5 $\mu g.g^{-1}$ for TC content, for the same yeast, developed in baffled shake flasks [2]. Anyway, the maximum concentration of TC obtained in the present study lies within the values obtained by Zhang *et al.* (2014) and above the values obtained by Freitas *et al.* (2014) [2,18].

Comparing the maximum biomass concentrations obtained in the different cultivations, it can be concluded that the one conducted at pH 4 (fed-batch IV) resulted in the highest biomass concentration (119.61 $g.L^{-1}$), with an increase of 23.08% compared to fed-batch III, 17.12% compared to fed-batch II and 47, 45% compared to fed-batch I. The maximum biomass concentration obtained in the fed-batch IV was higher than that obtained by Li *et al.* (2007) who cultivated *R. toruloides* Y4 in a 15 L bioreactor in fed-batch mode, attaining 106.5 $g.L^{-1}$ of biomass concentration. Glucose was also used as the carbon source in a growth medium adjusted to 5.5 [7].

The maximum biomass productivity was also higher for the fed-batch IV (1.76 $g.L^{-1}.h^{-1}$), with an increase of 11.39% compared to fed-batch III, 18.92% the fed-batch II and 1.15% compared to compared to the fed-batch I. Compare with the other assays, although fed-batch IV reached the maximum biomass concentrations, it presented lower FA and TC content. Indeed when cells grow under optimal growth conditions, they use the available energy for growth, rather than storage materials such as carotenoids.

Maximum biomass concentration observed in the latter cultivation was higher than in the others. Since the lipids are a product associated to the growth [2] it would be expected that the FA content increased with the biomass concentration increase. However, this did not happen. The limitation of oxygen that was observed in the culture (t=40.00 h) may explain the reduction in FA content compared to previous cultivations. According Saenge *et al.* (2011), increasing the rate of aeration in a culture of *R. glutinis* 5159 TISTR batch developed in the a bioreactor, induced a significant increase of the biomass concentration and the production of lipids, and a slight increase in the production of carotenoids [19]. Simova *et al.* (2002) reported that increasing the aeration rate increased the carotenoid content of the strain *Rhodotorula rubra* GED2, when grown in a batch bench reactor system [20]. Thus, the high biomass concentration obtained in fed-batch IV resulted in a decrease in the DO during the stationary phase, which also explains the low concentration of FA and TC.

Although FA only correspond to a fraction of TL, FA productivities obtained in the fed-batch II-IV are close to the TL productivity obtained by Zhao *et al.* (2011), in a fed-batch cultivation intermittently feeding with glucose (0.36 $g.L^{-1}.h^{-1}$) and continuously feeding (0.39 $g.L^{-1}.h^{-1}$) [12]. The maximum FA and TC productivities obtained in fed-batch IV are similar to those obtained for the other fed-batch cultivations. Although the FA and TC contents decreased in fed-batch IV, the biomass productivity increased, which explains the high FA and TC productivities obtained in this experiment.

The yeast FA composition lies within the European Standard EN 14214 that defined limits for certain FA (except at the point t=19,00h, fed-batch IV, where 18:3ω3 has a percentage 14.31%, higher

than the limit, 12%) [21]. For time cultivation where the FA productivity was maximum, a higher proportion of MUFA (monounsaturated fatty acids) was observed for the fed-batch IV meaning that the FA composition, is best for biodiesel production [22].

Maximum TC concentrations decreased slightly from fed-batch II to IV. These TC concentrations are relatively low when compared with the results obtained by Saenge *et al.* (2011) of 135.25 $mg.L^{-1}$, who used a 2L bioreactor to grow *Rhodotorula glutinus* in a fed-batch mode, using glycerol as a carbon source. However, TL concentrations (6.05 $g.L^{-1}$) obtained by these authors are much lower than the results in these assays. The higher TC concentrations obtained by Saenge *et al.* (2011) may be due to low levels of TL, since the yeast accumulates the excess of carbon mainly to produce carotenoids instead of lipids [19].

### 3.2 Flow cytometry

FC allowed to determine the TC content produced by the yeast *R. toruloides* and allowed to monitor the viability of cells by detecting at-line the integrity of the cytoplasmic membrane and the potential of the mitochondrial membrane. As expected, in general, subpopulations stress (A and C) increased when the residual glucose concentration diminished or depleted, and decreased when the nutrient + glucose solution or glucose solution was added to the culture. Fed-batch III and IV contained more cells with permeabilised cytoplasmic membrane, probably because, in these cultures, the cells were exposed to a carbon limiting condition for extended periods of time, as opposed to what happened in the Fed-batch I and II. Moreover, cells from the fed-batch IV, were exposed to a dual nutrient limitation (carbon and oxygen) which was not found in other cultivations, which resulted in a high proportion of cells with permeabilised cytoplasmic membrane.

## 4 Conclusions

This work established a strategy for optimizing the production of biomass by *R. toruloides yeast* NCYC 921, developed in a bioreactor operating in fed-batch mode.

The strategy developed in this work allowed the development of a method for feeding nutrients and carbon to the bioreactor, leading to high cell density cultures, reaching a maximum biomass concentration of 119.61 g $L^{-1}$ (biomass productivity of 1.76 $g.L^{-1}.h^{-1}$). As a result of having selected conditions that favored the growth of the yeast cell, the production of FA and CT was lower in this assay, compared with previous assays.

FC allowed quantifying, in near real time, the TC content of the yeast. This technique was also used to assess the physiological states of the yeast cells during the cultivations, in order to understand the response of cells growing conditions under different conditions. The mitochondrial activity and the cytoplasmic membrane integrity of *R. toruloides* cells were affected by the lack of nutrients and oxygen (more evident in fed-batch III and IV). Since FC allows a rapid reading of several cell parameters (simultaneously), it is an ideal tool to be used in the process optimization of the lipids and carotenoids production by yeasts, from the laboratory to pilot scales.

## 5 References


[1] T. M. P. Parreira, “Otimização do processo de produção de lípidos e carotenóides a partir da biomassa da levedura *Rhodotorula glutinis* NRRL Y-1091,” Dissertação para obtenção do grau de mestre em Engenharia Alimentar, Universidade de Lisboa, Instituto Superior de Agronomia, 2014.

[2] C. Freitas, B. Nobre, L. Gouveia, J. Roseiro, A. Reis, and T. Lopes da Silva, “New at-line flow cytometric protocols for determining carotenoid content and cell viability during *Rhodosporidium toruloides* NCYC 921 batch growth,” *Process Biochem.*, vol. 49, no. 4, pp. 554–562, Apr. 2014.

[3] P. S. Nigam and A. Singh, “Production of liquid biofuels from renewable resources,” *Prog. Energy Combust. Sci.*, vol. 37, no. 1, pp. 52–68, 2011.

[4] X. Meng, J. Yang, X. Xu, L. Zhang, Q. Nie, and M. Xian, “Biodiesel production from oleaginous microorganisms,” *Renew. Energy*, vol. 34, no. 1, pp. 1–5, Jan. 2009.

[5] T. Lopes, D. Feijão, and A. Reis, “Monitoring *Rhodotorula glutinis* CCMI 145 Stress Physiological Response during Fed-Batch Fermentations Using Multi-Parameter Flow Cytometry,” vol. 3, no. 1, pp. 25–31, 2011.

[6] T. Lopes, D. Feijão, and A. Reis, “Using Multi-parameter Flow Cytometry to Monitor the Yeast *Rhodotorula glutinis* CCMI 145 Batch Growth and Oil Production Towards Biodiesel,” pp. 2166–2176, 2010.

[7] Y. Li, Z. Kent, and F. Bai, “High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-batch culture,” vol. 41, pp. 312–317, 2007.

[8] R. Cutzu, A. Clemente, A. Reis, and T. Lopes, “Assessment of β-carotene content, cell physiology and morphology of the yellow yeast *Rhodotorula glutinis* mutant 400A15 using flow cytometry,” *Ind. Microbiol. Biotechnol.*, vol. 40, no. 8, pp. 865–875, 2013.

[9] T. Lopes da Silva, A. Reis, C. Hewitt, and J. C. Roseiro, “Citometria de fluxo - Funcionalidade celular on-line em bioprocessos,” *Bol. Biotecnol.*, pp. 32–40, 2004.

[10] J. G. Pan, M. Y. Kwak, and J. S. Rhee, “High density cell culture of *Rhodotorula glutinis* using oxygen-enriched air,” *Biotechnol. Lett.*, vol. 8, no. 10, pp. 715–718, 1986.

[11] S. Yoon and J. Rhee, “Lipid from yeast fermentation: effects of cultural conditions on lipid production and its characteristics of *Rhodotorula glutinis*,” *J. Am. oil Chem. Soc.*, vol. 60, pp. 1281–1286, 1983.

[12] X. Zhao, C. Hu, S. Wu, H. Shen, and Z. K. Zhao, “Lipid production by *Rhodosporidium toruloides* Y4 using different substrate feeding strategies,” *J. Ind. Microbiol. Biotechnol.*, vol. 38, no. 5, pp. 627–32, 2011.

[13] C. Ratledge and Z. Cohen, “Microbial and algal oils : Do they have a future for biodiesel or as commodity oils ?,” vol. 20, no. 7, pp. 155–160, 2008.

[14] G. L. Miller, “Use of dinitrosalicylic acid reagent for determination of reducing sugar,” *Anal. Chem.*, vol. 31, no. 3, pp. 426–428, 1959.

[15] C. Lepage, G.; Roy, “Direct transesterificação of all classes of lipids in a one-step reaction,” *J. Lipid Res.*, vol. 27, pp. 114–119, 1986.

[16] C. Roseiro, A. Reis, and T. Lopes, “Applications and perspectives of multi-parameter flow cytometry to microbial biofuels production processes,” pp. 1–8, 2012.

[17]C. Malisorn and W. Suntornsuk, “Improved β-carotene production of *Rhodotorula glutinis* in fermented radish brine by continuous cultivation,” *Biochem. Eng. J.*, vol. 43, no. 1, pp. 27–32, Jan. 2009.

[18] Z. Zhang, X. Zhang, and T. Tan, “Lipid and carotenoid production by *Rhodotorula glutinis* under irradiation/high-temperature and dark/low-temperature cultivation,” *Bioresour. Technol.*, vol. 157, pp. 149–53, 2014.

[19] C. Saenge, B. Cheirsilp, T. Tachapattaweawrakul, and T. Bourtoom, “Potential use of oleaginous red yeast *Rhodotorula glutinis* for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids,” *Process Biochem.*, vol. 46, no. 1, pp. 210–218, 2011.

[20] E. D. Simova, G. I. Frengova, and D. M. Beshkova, “Effect of aeration on the production of carotenoid pigments by *Rhodotorula rubra-Lactobacillus* casei subsp. casei co-cultures in whey ultrafiltrate,” 2003.

[21] “European Standard EN 1421,” 2003.

[22] G. R. Stansell, V. M. Gray, and S. D. Sym, “Microalgal fatty acid composition: implications for biodiesel quality,” *J. Appl. Phycol.*, vol. 24, no. 4, pp. 791–801, 2012.