Development of *Rhodosporidium toruloides* NCYC 921 fed-batch cultures for simultaneous production of lipids and carotenoids

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In this work the yeast *Rhodosporidium toruloides* NCYC 921 was developed in a dual stage pH control fed-batch culture, designed to improve growth and fatty acids production in the first stage, and induce the carotenoid production in the second stage, aiming at implementing a sustainable process for biodiesel production. Two assays (I and II) were performed in a 7 L benchtop bioreactor to evaluate the impact of different pH values (5.0 and 5.5) in the second stage of fed-batch culture. In these assays, a concentrated glucose solution was used as carbon source. Two assays (III and IV) were performed in a 7 L benchtop bioreactor to determine if a sugar solution obtained from carob pulp was a viable alternative to a defined carbon source such as glucose solution, thus aiming at reducing the process costs. The possibility of scaling up *R. toruloides* cultures was evaluated by performing an assay (V) in a 50 L bioreactor, using a concentrated glucose solution as carbon source. The highest biomass concentration was obtained in Assay I, with a value of 118.9 g.L\(^{-1}\), while the highest biomass productivity was recorded in Assay II (1.52 g.L\(^{-1}\).h\(^{-1}\)). The highest carotenoid content (4.44 mg.g\(^{-1}\) DCW) and productivity (4.12 mg.L\(^{-1}\).h\(^{-1}\)) were both obtained in Assay IV, indicating that using carob pulp syrup as carbon source has a beneficial impact regarding carotenoid production. The highest fatty acid content (30.24 % w/w DCW) was obtained in Assay V and the highest fatty acid productivity (0.29 g.L\(^{-1}\).h\(^{-1}\)) was obtained in Assay I. Flow cytometry was used to assess the yeast cell viability and carotenoid content throughout the assays.

Keywords: *Rhodosporidium toruloides* NCYC 921; carob pulp syrup; fed-batch cultures; carotenoids; fatty acids; flow cytometry.

1. Introduction

Energy consumption rate per capita is directly correlated to the quality of life (QL) of a given population [1]. As QL rises in today’s developing countries and as the world’s population increases, the worldwide energy consumption is predicted to increase accordingly [2]. As most of the energy consumed nowadays comes from non-renewable sources like petroleum or coal [3], it is imperative that alternative energy sources are developed in order to sustain population and QL growth. Furthermore, traditional fossil fuels are detrimental to the environment [4] and are often the cause of political and social instability in many parts of the world [2,3]. Due to these constraints, biofuels are becoming an increasingly more attractive alternative to traditional fuels as they are renewable, can theoretically be produced by any country with access to biomass or lignocellulosic wastes and are more environmentally friendly [4-6]. Nevertheless, and despite these advantages, current worldwide biofuel output is still insufficient to meet the growing energetic needs [2], meaning that developing this technology is of the utmost importance.

Biofuels comprise a vast array of renewable fuels derived from biomass, ranging from something as simple as unprocessed wood residues to more technologically advanced fuels like bioethanol and biomethanol, biohydrogen, biomethane and biodiesel [5,7]. According to this technological distinction, biofuels are categorized either as primary or secondary [8].

Primary biofuels encompass all biomass that can be directly used as fuel, without any further processing, like wood and lignocellulosic residues from crops, animal waste or gas generated in landfills [8].

Secondary biofuels are considered the result of processing primary biofuels and can be further classified as being of first, second or third generation. This distinction is made according to the raw materials and technologies used in fuel production [5,6,8].

First generation biofuels are derived primarily from foodstuffs and currently represent the vast majority of biofuels produced worldwide [6]. Sugar and starches extracted from sugarcane or sugar beet can be used to
produce bioethanol via fermentation and vegetable oils from rapeseed, sunflower or soybean, to name some, can be used to produce biodiesel by transesterification [5,6]. Up today, first generation bioethanol remains the most produced biofuel, with Brazil and the United States of America (USA) as the two leading producers (88% of the total produced volume in 2009) [5]. Perhaps the most pressing issue regarding first generation biofuels is the fuel-versus-food debate. As biofuels gain a greater economic importance, the cost of raw materials is expected to rise, just like in any other market. Increased revenues from crops destined to the biofuel market makes then more desirable to farmers, which could in turn raise the prices of food crops due to a diminishing offer [9]. Changing from food to fuel crops also negatively impacts biodiversity as these last ones are sown in detriment of the first [6].

Second generation biofuels are a possible solution to overcome the fuel-versus-food issue as they are derived from non-edible materials. Both biodiesel and bioethanol can be classified as being of second generation, depending on the feedstock used to produce them [6]. However, the technology necessary to efficiently convert lignocellulosic biomass to simple sugars is still in its infancy and biofuels produced in this fashion are not yet commercially viable [5]. And although not derived from foodstuffs, some authors [5] call attention to the fact that non-food crops can still lead to a rise in food prices, due to the allocation of land and water resources to fuel rather than food crops. Some authors [10] consider that, as far as estimating that even if oilseed crops were cultivated in all available arable land, the feedstock generated by said crops would not be sufficient to produce biodiesel to cover half of the world’s energy demand.

Third generation biofuels are currently the most technologically advanced ones and have their origin in microorganisms, either being derived from biomass or being produced directly by them [4,11-13]. Classification is based on the raw material utilized to produce the biofuel and the technological advancement associated with this production. The use of microorganisms as raw materials presents several advantages over traditional substrates. First and foremost, the fuel-versus-food issue is resolved because microorganisms do not compete with food crops in any way. Microbial cultures for biodiesel production still relies heavily on pure substrates as carbon sources, often derived from food crops, however there has been a concentrated effort to use lignocellulosic wastes and industry by-products as substrates in culture media to obtain biofuels and high-value-added products from microorganisms [13-15]. Secondly, the production of microbial fuels is far less labor intensive than traditional crops, as many of the processes can be automated, both in terms of monitoring and control. Microbial cultures are also more easily scalable and have shorter life cycles, and are not dependent on seasons and climate [11-13,15]. On the other hand, third generation biofuels are still significantly more expensive than fossil fuels or first and second generation biofuels, mainly due to high production costs and extraction/conversion of the desired products [13].

Biodiesel is the biofuel resultant from the transesterification of natural triglycerides, from sources such as vegetable oils, animal fats or single cell oils (SCO, lipids from microorganisms that present a lipid content in excess of 20% of their total mass) [6,13,16]. The most common process converts triglycerides into fatty acid alkyl esters and glycerol through reaction between an alcohol and the triglycerides, usually catalyzed; the utilized alcohol, usually methanol or ethanol, dictates which fatty acid alkyl esters are produced: either fatty acid methyl esters (FAMEs) or fatty acid ethyl esters (FAEES), respectively [4,6,13]. The most commonly used technique is transesterification using methanol, so the alkyl esters used for biodiesel production are usually FAMEs [17].

Microbial production of biodiesel is not yet an economically viable process as production costs far surpass the market price of the produced biodiesel [15]. However, if the microbial biomass, beyond its high lipid content, is rich in high-value-added products, such as carotenoids, (which have many different applications in pharmaceutical, nutraceutical, food and feed industries, with a high market value), their commercialization may sustain the microbial biofuel production. This strategy has gained an added importance in more recent years as several species of algae and yeasts have been found to produce meaningful quantities of both fatty acids (convertible to biodiesel) and valuable by-products, such as carotenoids [11,18].

Carotenoids are tetra-terpenoid C₄₀ pigments containing isoprene residues and a polylene chain of conjugated double bounds [19]. These compounds are naturally occurring in nature and although often considered to be higher plant pigments, they are also synthetized by algae and other microorganisms, like some species of fungi [19,20]. Due to their strong anti-oxidative properties, carotenoids have been proven to reduce the incidence of several diseases, such as cancer [21,22] or type 2 diabetes [23]. Additionally, carotenoid consumption has been linked to reduced incidences of cardiovascular diseases, cataracts, age-related macular degeneration and diseases related to low immune function [20]. As interest in carotenoids as high-value-added products increases, so does their commercial value; in 2003, the European carotenoid market was worth US$348.5 million and projections pointed to a yearly increase of 2.7%, to reach US$419.6 million in 2010 [24].
_Rhodosporidium toruloides_ is an oleaginous yeast and a prolific carotenoid producer [15,18,25]. Formally designated either as _Rhodotorula glutinis_ (of which _R. toruloides_ is an anamorph) or _Rhodotorula gracilis_, this yeast belongs to the Fungi kingdom, Basidiomycota phylum, Microbotryomycetes class, Sporidiobolales order and _Rhodotorula_ genus [26,27]. Towards biodiesel production, it has been reported that the yeast _R. toruloides_ can accumulate intracellular lipids up to 70% (w/w dry cell weight, DCW) [11], although most studies report lower lipid contents (from 48% [28] to under 20% [15], with the vast majority of results falling within this range [12,18,25,29]). The aforementioned works indicate that the most prevalent intracellular fatty acids are palmitic (16:0), stearic (18:0), oleic (18:1ω9), linoleic (18:2ω6) and linolenic (18:3ω3). Biodiesel production from yeasts is more advantageous than from microalgae or bacteria because the first require longer cultivation periods, more complex equipment to sustain growth and are more prone to contaminations, and the latter have lower oil contents and there are more cumbersome to extract [11]. In regards to the production of carotenoids, the majority of pigments produced by _R. toruloides_ are β-carotene, torulene and torularhodin [30]. However, information on _R. toruloides_ as a carotenoid producer is still scarce.

Microbial biodiesel production can be made more economically sustainable by reducing production costs through the use of alternative cultivation substrates, such as using lignocellulosic waste materials as a carbon source [15].

The carob tree (*Ceratonia siliqua*) is an evergreen tree belonging to the Leguminosae family, Caesalpinaceae subfamily [31]. The fruit of the carob tree, the carob pod, has a pulp that is rich in easily extractable sugar [32]. Because the seeds of the carob pod currently have a much higher commercial value than the pulp, this latter is considered a by-product and thus a low-cost substrate from which sugar-rich syrups can be obtained [33]. Carob pulp syrup (CPS) preparation has been studied by several authors and has been successfully used as a carbon source in yeast fermentations [33-35]. More recently, Freitas et al. [15] have proposed CPS as a carbon source in _R. toruloides_ cultivations as it appears to have a beneficial effect on the carotenoid production.

Flow cytometry (FC) is a laser-based technology that allows the simultaneous analysis of the chemical and physical characteristics of particles in a fluid. Depending on the FC apparatus and setup, FC can be used for cell counting, cell sorting, protein engineering and biomarker detection, amongst other applications [36]. FC is of particular importance to process modeling as it allows real time process control, a strategy that yields far more reliable and accurate results than traditional analytical techniques, which are usually performed on samples taken during the course of an experiment or process but only give the results some time afterwards [7].

Based on the results published by Freitas et al. [15] and Dias et al. [29], this work aims primarily to evaluate the possibility of culturing _R. toruloides_ NCYC 921 using CPS as carbon source in a dual-stage fed-batch system, designed to first maximize biomass and fatty acids productivities, then carotenoid productivity.

### 2. Methods

#### 2.1. Microorganism

The microorganism used for this work was the oleaginous yeast _Rhodosporidium toruloides_ NCYC 921, supplied by the National Collection of Yeast Cultures (Norwich, United Kingdom). The yeast was kept in malt extract agar slants at 4 °C. Prior to being used to prepare the inocula, the yeast was transferred to fresh malt extract agar slants which were incubated (NIR 252 Incubator, SANYO Electric Co., Ltd., Japan) for 72 h at 30 °C.

The inocula were prepared in baffled Erlenmeyer flasks containing semi-defined culture medium, as described by Pan et al. [37] (g.L⁻¹): KH₂PO₄ 12.5; Na₂HPO₄ 1.0; (NH₄)₂SO₄ 5.0; MgSO₄·7H₂O 2.5; CaCl₂·2H₂O 0.25; yeast extract, 1.9. A trace minerals solution (in g.L⁻¹), FeSO₄·7H₂O 40.0; CaCl₂·2H₂O 40.0; MgSO₄·7H₂O 10.0; AlCl₃·6H₂O 10.0; CoCl₂ 4.0; ZnSO₄·7H₂O 2.0; Na₂MoO₄·2H₂O 2.0; CuCl₂·2H₂O 1.0; H₂BO₃ 0.5) was prepared in 5 N-HCl and added to the culture medium at a concentration of 0.25 mLL⁻¹. The culture medium was sterilized by autoclaving (Uniclave 88, Portugal). To prevent excess caramelization, the carbon source, whether glucose or CPS, was autoclaved separately and then added to the remaining components of the culture medium. The carbon source solution was added to the culture medium so that the final carbon source concentration was 35 g.L⁻¹ before inoculation.

For assays done in the 7 L bioreactor, 150 mL of culture medium were inoculated using two slants and grown for 24 h at 30 °C and 150 rpm (Unitorum Infors, Switzerland).

To prepare inocula for the 50 L bioreactor, two 150 mL pre-inocula were prepared as previously described. These were then used to inoculate baffled 5 L-Erlenmeyer flasks containing 1350 mL each of culture medium with a sugar concentration of 35 g.L⁻¹, resulting in two 1500 mL inocula that were grown for 24 h at 30 °C and 150 rpm (Unitorum Infors, Switzerland). Both inocula were used to inoculate the 50 L bioreactor.

#### 2.2. CPS preparation

Prior to use, crushed carob pulp (Choronando & Filhos Lda., Faro, Portugal), supplied in plastic bags placed inside burlap sacks, was stored at room temperature in the laboratory.

CPS preparation for the first Assay using this carbon source was done with the same technique as described by Freitas et al. [15], with some modifications: (i) fragmented carob pulp was mixed with water in a 1:2 ratio; (ii) extraction was carried out overnight at room temperature; (iii) the following day, the water was collected and the wet carob pulp was pressed with a hand-operated hydraulic press to over 150 bar in order to extract more water; (iv) the
collected water was centrifuged (Sigma 2-16K, Sartorius, Germany) for 10 min at 9000 rpm and at 5 °C to remove any suspended solids. The solution obtained after this step will henceforth be referred to as sugar solution from carob pulp, SSCP; (v) the SSCP was acidified to pH 2.0, placed in open Erlenmeyer flasks and left to hydrolyze overnight at 100 °C (Memmert UM 80, Memmert GmbH+Co.KG, Germany). The product of the hydrolysis is the CPS, which was again centrifuged (Sigma 2-16K, Sartorius, Germany) for 10 min at 9000 rpm and at 5 °C, then sterilized by autoclaving (Uniclave 88, Portugal) after the pH was corrected to 4.0.

For a second Assay using CPS as the carbon source, CPS was prepared as previously described, with one additional step: before hydrolysis, the SSCP was placed in an incubator (Memmert ULE 500, Memmert GmbH+Co.KG, Germany) at 40 °C and left to concentrate until approximately half of the volume had been lost. The concentrated SSCP was acidified to pH 2.0 and left to hydrolyze at 100 °C overnight, then centrifuged (Sigma 2-16K, Sartorius, Germany) for 10 min at 9000 rpm and at 5 °C to remove any precipitates, as done before to the SSCP. The pH of the CPS was raised to 4.0 and it was sterilized by autoclaving (Uniclave 88, Portugal).

A second batch of carob pulp (Chorondo & Filhos Lda., Faro, Portugal), stored under controlled conditions (inside air tight containers at 5 °C) was used to prepare CPS according to the method described above for the CPS used in the first Assay that used this carbon source. The aim of this experiment was to evaluate the impact of storage conditions on CPS composition.

CPS was prepared according to the technique described by Turhan et al. [33], by which the extraction of soluble compounds is done with a 1:4 carob pulp to water ratio for 2 h at 80 °C (Memmert ULE 500, Memmert GmbH+Co.KG, Germany). The resulting solution was then hydrolyzed and autoclaved as previously described. This was done with both carob pulp batches.

Samples gathered during SSCP and CPS preparation were analyzed by high performance liquid chromatography (HPLC) (Agilent 1100 Series, Germany), using an Aminex HPX-87H (Bio-Rad, California, USA) to screen for glucose, sucrose, fructose, galactose, xylose, arabinose, furfural and HMF and an Aminex HPX-87P (Bio-Rad, California, USA) column to screen for acetic acid (CH₃COOH), furfural and HMF. The dimension of both columns was 300 × 7.8 mm. 0.5 mM H₂SO₄ was used as the mobile phase in the HPX-87H columns while water was used as the mobile phase in the HPX-87P column, both at a rate of 0.6 mL.min⁻¹. The columns temperatures were 50 °C and 80 °C, respectively. A refraction index (RI) detector was used to quantify the saccharides and acetic acid. An ultraviolet (UV) radiation detector (280 nm) was used to quantify furfural and HMF.

2.3. Cultivations

Five Assays were performed in total. Assays I through IV were carried out in a 7 L bioreactor (FerMac 310/60 Bioreactor, Electrolab Biotech, United Kingdom) and Assay V was performed in a 50 L bioreactor (C 809, Biolafitte, France).

Assay I was done following the same conditions as described in the 4th assay of the work by Dias et al. [29]: Rhodosporidium toruloides NCYC 921 cells were cultivated in a two-step pH control fed-batch system, designed to first maximize the yeast biomass growth and lipid accumulation and, in a second step, the carotenoid production was induced by changing the feeding nutrient composition and the medium pH. The cultivations began in batch mode, with an initial total sugar content of 35 g.L⁻¹ at the time of inoculation, and the pH fixed at a value of 4.0; once the available carbon source was exhausted, the cultivation proceeded as a fed-batch, with constant composition feeding (although with a variable rate) of a solution containing a carbon source and other nutrients until the end of the growth phase; once the culture reached the stationary phase, the pH was altered to 5.0, and the previous feeding solution was changed to solely a carbon source, i.e. water with dissolved sugar(s) at a concentration of 600 g.L⁻¹, again with a variable feeding rate. Throughout the entire cultivation the temperature was kept at 30 °C.

Assay II was performed under the same conditions of Assay I, except for the pH of the stationary phase (5.5 instead of 5.0). The purpose of this Assay was to evaluate the impact of the medium pH of 5.5 on carotenoid production during the stationary phase, since this value was used in earlier works that also used this strain (Ratlidge and Hall [38] and Yoon and Rhee [39]), being considered optimal. Indeed Dias et al. [29] carried out a yeast cultivation at a constant pH of 5.5. Assay II used a pH of 5.5 for the second stage of fed-batch in a dual stage fed-batch strategy.

In Assay III, the CPS used had a total sugar concentration of 200 g.L⁻¹, one third lower than the one used in previous experiments. It was observed that such a total sugar concentration resulted in the use of higher feeding rates, which led to the end of the cultivation before the carotenoid production stage, due to the maximum level of the vessel had been reached earlier.

In order to overcome this limitation, the CPS was concentrated, so that lower feeding rates could be used. This concentrated CPS was used in Assay IV.

Finally, the possibility of scaling up this process was evaluated by carrying out a fermentation in a 50 L semi-industrial scale fermenter (35 L working volume). The conditions set for this Assay (Assay V) were the same as for Assay I.

Table 2.1 gives a summary of all performed Assays and what parameters were unique to each one. All additional parameters were kept the same throughout the Assays.

Table 2.1 - Conditions of the yeast cultivations performed during this work. SI, semi-industrial; CS, carbon source; Conc. CPS, concentrated CPS; TSC; total sugar concentration.

<table>
<thead>
<tr>
<th>Assay</th>
<th>pH</th>
<th>Scale</th>
<th>CS</th>
<th>TSC (g.L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>4.0/5.0</td>
<td>Laboratory</td>
<td>Glucose</td>
<td>600</td>
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<tr>
<td>II</td>
<td>4.0/5.5</td>
<td>Laboratory</td>
<td>Glucose</td>
<td>600</td>
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<tr>
<td>III</td>
<td>4.0/5.0</td>
<td>Laboratory</td>
<td>CPS</td>
<td>200</td>
</tr>
<tr>
<td>IV</td>
<td>4.0/5.0</td>
<td>Laboratory</td>
<td>Conc. CPS</td>
<td>550</td>
</tr>
<tr>
<td>V</td>
<td>4.0/5.0</td>
<td>SI</td>
<td>Glucose</td>
<td>600</td>
</tr>
</tbody>
</table>

Assays I through IV were performed in a 7 L (5 L working volume) bioreactor, coupled with a dedicated controller to measure and control agitation, dissolved oxygen (DO), temperature, pH and foaming (FerMac 310/60 Bioreactor, Electrolab Biotech, United Kingdom). pH was controlled by adding 5 M sodium hydroxide (NaOH) and 5 M-HCl and...
measured with a pH probe (Mettler Toledo, Switzerland). Polypropylene glycol (PPG) was added in the beginning of the cultivation to prevent foaming at an early stage. Throughout the cultivation the foam level was monitored and controlled by adding drops of a 1:10 aqueous sterilized solution of PPG. Agitation was done by means of two Rushton turbines and temperature was controlled by heating/cooling system comprising an external heating blanket and an internal cold finger. The temperature probe is an integral part of the FerMac 360 Controller. Adequate mixing was further assured by placing a harness with four baffles inside the fermentation vessel. DO levels were monitored with the aid of an oxygen probe (Mettler Toledo, Switzerland).

Assay V was performed in a 50 L (35 L working volume) bioreactor (C 809, BioLafitte, France), coupled with a dedicated control module to measure and control temperature and stirring. pH and DO were controlled with the aid of an external control module (MOD 7F, Setric Genie Industriel, France) connected to the control module or 5 M-HCl manually. Measurements were made with a pH probe (Ingold InFit 764-50, Mettler Toledo, Switzerland) and a DO probe (Mettler Toledo, Switzerland). Foaming was controlled by manually adding drops of PPG. Agitation was done by the means of three Rushton turbines and four lateral baffles and temperature was controlled by the fermenter’s integrated heating/cooling external jacket.

In Assays I through IV, the DO was maintained at 40% of the air saturation by automatically tuning the agitation speed in order to avoid oxygen limitation. In Assay V, all changes in agitation speed were done manually as the BioLafitte bioreactor was not capable of automatically controlling this setting. Additionally, DO was controlled by changing the internal pressure of the vessel.

2.4. Biomass and sugar concentrations

Biomass concentration was calculated from a correlation between the optical density measured at 600 nm (OD_{600}) of a sample and its dry cell weight (DCW) established by Parreira [40]. OD_{600} was measured in duplicate for each sample using a ThermoSpectronic Genesys 20 spectrophotometer (Portugal).

Residual glucose concentration in Assays I, II and V was analyzed by the 3,5-dinitrosalicylic acid (DNSA) method [41]. Residual sugar concentration in Assays III and IV was analyzed by HPLC (Merck-Hitachi LaChrom L-7000 series, USA), using a 6.5 × 300 mm Sugar-Pak® column (Water, USA). A Ca-EDTA solution (50 mg.L⁻¹) was used as the mobile phase, at a rate of 0.5 mL.min⁻¹. The column temperature was set at 75 °C. The chromatograms were analyzed using the Chromelon™ 6.40 SP6 software (Build 783, Dionex, Thermo Fischer Scientific Inc., USA).

Only glucose, fructose and sucrose were quantified with this method, using a RI detector in a time interval between 0 and 20 min. The sample’s injection volume was 20 µL.

2.5. Fatty acid analysis

Gas chromatography (GC) was the technique chosen to identify and quantify the fatty acids present in the yeast biomass. The extraction of fatty acids and their conversion to FAMEs was done according to the protocol described by Freitas et al. [25], which in turn was derived from the work of Lepage and Roy [42].

The samples were analyzed with a SCION GC 436 chromatographer (Buerker, Germany), equipped with a flame ionization detector (FID). Separation was performed on a 0.32 mm × 30 m fused silica capillary column (film 0.25 µm) (Supelcowax 10, Supelco, Bellafonte, Palo Alto, CA, USA) with helium as a carrier gas, at a flow rate of 1.6 mL.min⁻¹.

The column temperature was programmed at an initial temperature of 200 °C then increased at 2 °C.min⁻¹ to 220 °C. Injector and detector temperatures were 250 and 280 °C, respectively, and the split ratio was 1:20 for 5 min and then 1:10 for the remaining time. The column pressure was 13.5 psi. Peak identification and response factor calculation was carried out using the above-mentioned internal standard.

Each sample was prepared in duplicate and injected twice. The amount of each FAME present in the sample was calculated according to Equation 1, where m(FAME), is the mass of a specific FAME, \( A(FAME) \) is the respective peak’s area, \( A(17:0) \) is the area of the peak corresponding to the internal standard and \( RF(FAME) \) is the response factor of the FAME. This factor was considered to be equal to 1.

\[
m(FAME)_i = \frac{A(FAME)_i}{A(17:0)} \times RF(FAME) \tag{1}
\]

2.6. Carotenoid detection and cell viability assessment by flow cytometry

Cellular viability and total carotenoids content were analyzed by FC using a FACScalibur benchtop flow cytometer (Becton-Dickinson Biosciences, Franklin Lakes, New Jersey, USA) equipped with a blue argon laser (488 nm) and a red diode laser (635 nm). The FSC and SSC detectors both worked in the same wavelength as the blue argon laser (488±10 nm). Additionally, this equipment has four fluorescence detectors, FL1 through FL4. Radiation ranges are as follows: FL1, 530±30 nm; FL2, 585±42 nm; FL3, > 670 nm; FL4, 661±16 nm [43].

Gathered data was analyzed with the program FCS Express 4 Flow Research Edition (De Novo Software, USA).

All buffers were filtered prior to use with a 0.22 µm filtration membrane (TPP® Syringe-Filter 0.22 µm, TPP Techno Plastic Products AG, Switzerland).

The yeast total carotenoid content was determined by FC using a correlation between this parameter and the cell’s autofluorescence (AF) levels measured by the FL2 detector, according to the technique established by Freitas et al. [25].

Cell viability was determined by assessing membrane integrity, membrane potential and enzymatic activity, using propidium iodide (PI), 3,3’-dihexyloxacarbocyanine iodide (DiOC6(3)) and diacetate of carboxyfluorescein (CFDA), respectively. This was done according to the methodology described by Freitas et al. [25].
3. Results and discussion

3.1. CPS preparation

The concentrations of sugars (sucrose, glucose, xylose, arabinose and fructose), HMF and furfural of the SSCP and CPS used in Assays III and IV were determined by HPLC and are presented below. The impact of storage conditions and extraction protocol on the composition of SSCP and CPS were also evaluated.

The samples of SSCP and CPS collected at different stages of CPS preparation are presented in Table 3.

Figure 3.1 presents the concentration of sucrose, glucose, xylose, arabinose, fructose, total sugars, HMF and furfural present in the products obtained in each step of CPS preparation for Assay III.

Figure 3.2 shows the concentration of sucrose, glucose, xylose, arabinose, fructose, total sugars, HMF and furfural present in the product obtained in each step of CPS preparation, which was used in Assay IV.

Figure 3.3 shows the concentration of sucrose, glucose, xylose, arabinose, fructose, total sugars, HMF and furfural present in the product obtained in each step of CPS preparation using a second batch of carob pulp which was stored in closed containers at 5 °C. Additionally, a second extraction was performed to determine how much sugar remained in the discarded carob pulp.

Figure 3.4 presents the concentration of sucrose, glucose, xylose, arabinose, fructose and total sugars present in the SSSPs prepared according to the methodology of Turhan et al. [33] (1:4 w/w carob pulp to water ratio, 80 °C, 2 h) vs. the methodology of Freitas et al. [15] (1:2 w/w carob pulp to water ratio, room temperature, overnight), for both batches of carob pulp stored under different conditions.
These experiments lead to the conclusion that sugar extraction from carob pulp benefits from conditions that are more aggressive than those utilized to prepare CPS for Assays III and IV. Protocols like the one suggested by Turhan et al. [33] yield better sugar extractions because the solution is less likely to become saturated due to higher water to carob pulp ratios being employed. Additionally, the application of heat increases solubility and facilitates the release of soluble compounds from the carob pulp. It is expected that this method also increases the extraction of inhibitory compounds like organic acids. Unfortunately this could not be evaluated because of the lack availability of the HPLC apparatus in the time frame in which these experiments were done. This last hypothesis, together with the fact that the application of heat means that the extraction process will have higher energetic costs, means that the conclusion that one method is preferable to the other is not obvious. A complete cost assessment should be made in order to determine which of the processes is more advantageous.

A second conclusion is that the conditions in which the carob pulp is stored play a major role in the composition of the CPS. Carob pulp that is improperly stored loses humidity and becomes degraded, making difficult the sugar extraction process.

Because these experiments were done only after Assays III and IV were concluded, the conditions found to be optimal for CPS preparation (disregarding the higher energy costs) were not applied when preparing CPS for said Assays.

3.2. Cultivations

The summarized results of Assays I through V are present in Table 3.3.

Comparison between Assays I and II reveals that the maximum biomass concentration achieved was 20.03% greater in the first Assay. In both instances, the maximum biomass concentration was reached during the second stage of fed-batch culture, therefore some importance can be attributed to the different pH used in these Assays. Assay II presented a higher maximum biomass productivity, however this value was achieved during the first stage of fed-batch culture in both instances, when culture conditions were the same. In regards to biomass concentration, results of Assay I were 11.64% higher than those reported by Li et al. [28] for a cultivation done in a 15 L bioreactor operating in fed-batch mode, using glucose as the carbon source, and approximately 6.5% lower than the results published by both Zhao et al. [44] and Dias et al. [29], for cultivations performed with similar conditions as those of Assay I.

In Assays III and IV both the maximum biomass concentrations and maximum biomass productivities were lower than those observed for Assays I and II. Assays III and IV also presented higher percentages of cells with either depolarized or permeabilised membranes through the culture, meaning that conditions were less favorable than in previous Assays. This can be partially attributed to the CPS, due to the inhibitory compounds present in it, however in both Assays the culture was subjected to oxygen limitation. This factor cannot be disregarded when analyzing why cellular growth was impaired. Further studies are needed to conclude if oxygen limitation was indeed the main factor behind the lower biomass concentrations and productivities or if this is in fact due to the use of CPS as the carbon source. If this latter situation is true, then the use of CPS preparation strategies that do not require heat to extract the sugars from the carob pulp or to sterilize the CPS might yield better results. In comparison with other works that used alternative carbon sources, Assay IV yielded far greater biomass concentrations [14,15,18].
Despite using the same set of conditions as Assay I and as published by Dias et al. [29], Assay V yielded lower biomass concentrations than those (67.80% lower than the one observed for Assay I and 69.85% than the value reported by Dias et al. [29]). However, this Assay encountered a series of setbacks that severely affected its outcome, from aeration monitoring problems to complications with pH monitoring and controlling, so these results cannot be considered definitive. The way the culture progressed suggests that this two-stage culturing technique can indeed be scaled up and there should be further inquiries into this process.

Results show that using CPS as the carbon source, in opposition to glucose, greatly increases the yeast carotenoid production. Carotenoid content and productivity increased dramatically from Assay I to Assay IV, from 0.20 mg.g\(^{-1}\) DCW and 0.26 mg.L\(^{-1}.h\(^{-1}\) to 4.44 mg.g\(^{-1}\) DCW and 4.12 mg.L\(^{-1}.h\(^{-1}\), respectively. The carotenoid productivity of Assay IV is the highest in the available literature, approximately 23% higher than the highest reported productivity in the works by Saenge’s team [14,18]. Interestingly and despite its problems, Assay V was also successful as the maximum carotenoid productivity increased 120.00% comparing to the value obtained in Assay I and 51.72% comparing to the value reported by Dias et al. [29], which was obtained with the same experimental setup, but using a smaller bioreactor (5 L working volume).

Concerning fatty acid content and productivity, again Assay V presented interesting results. This Assay achieved the highest fatty acid content, at 30.24% w/w, which is a 24.70% increase over the maximum fatty acid content obtained in Assay I. This result might be a consequence of the oxygen limitation experienced by the yeast’s metabolism shifts from division and population growth to the accumulation of storage materials. A similar result can be observed in the work of Dias et al. [29], as their highest fatty acid content (�788% w/w DCW) was achieved in an experiment where the culture suffered oxygen limitation. Results of Assays I and II concerning fatty acid content, were similar to those published by Dias et al. [29]. However, because of lower biomass productivities, fatty acid productivities in Assays III and V were severely affected, being 51.72% and 82.76% lower than those observed for Assay I. The lowest fatty acid content was observed in Assay IV (10.99% w/w DCW), likely due to the presence of high concentrations of inhibitory compounds present in the culture medium.

European Standard EN 14214 dictates that biodiesel must not contain linolenic acid methyl ester over 12% w/w and must not contain PUFA derived methyl esters with four or more unsaturations over 1% w/w [45]. There are of course other criteria, however these two are the ones that are more directly related to
the raw material used to produce the biodiesel. In this sense, results from Table 3.3 show that the fatty acids produced in Assays I, II, IV and V would yield biodiesels that would meet EN 14214 requirements, while biodiesel produced from the fatty acids of Assay III would fail the requirements on account of the percentage of linolenic acid (at specific cultivation times). Fatty acids with four or more unsaturations were not observed in any of the Assays.

4. Conclusions

The conditions under which carob pulp is stored was found to have an impact on the composition of CPS as carob pulp kept refrigerated in airtight containers had more extractable sugars than carob pulp kept in the laboratory at room temperature, despite this later having a larger surface area. It was also found that the extraction procedure used to prepare CPS for the cultivations was not the most adequate, regarding the extraction efficiency. A technique that uses higher water to carob pulp ratios in combination with heating was found to extract more sugars from the carob pulp, while increasing CPS productivity. This result is consistent with others found in the available literature. The employed method had, however, a lower energy requirement which aids in an overall process cost reduction. Additionally, by not using heating during extraction, the amounts of inhibitory compounds in the CPS is minimized. The method by which CPS was sterilized (autoclaving) led to the degradation of a significant part of the extracted sugars, either by caramelization or via the formation of HMF and furfural. Therefore, alternative means of sterilizing the CPS that to not require heating (e.g. filtration) might have yielded better results in regards to cell viability in the cultures that used CPS as the carbon source.

Assays carried out using CPS as the carbon source revealed that this is a suitable substrate to grow R. toruloides NCYC 921, despite cell viability being somewhat compromised. Furthermore, these assays revealed that carotenoid productivity was greatly improved when CPS was used, as the maximum carotenoid content and productivity obtained in these assays (particularly in Assay IV, 4.44 mg.g-1 and 4.12 mg.L-1.h-1, respectively), were significantly higher than the values currently be found in the available literature. Fatty acid content and productivity were comparable to those of assays where glucose was used as the carbon source and are similar to other values found in literature. However, both cultures done with CPS suffered from oxygen limitation, an issue that several authors have found to be determinant in the outcome of R. toruloides cultivations. This was due to an incorrect control of stirring speed, resulting from equipment failure.

Using a pH of 5.5 for the second stage of cultivation was found to yield less preferable results, despite several authors having mentioned this value to be optimal for R. toruloides cultivations.

Using the strategy that was found to yield the best results (two-stage fed-batch culture, first at a pH of 4.0 then at 5.0, using a 600 g.L⁻¹ glucose solution as carbon source), the cultivation was successfully scaled up to a 50 L bioreactor (35 L working volume). However, there were several issues with the used equipment as both DO and pH levels were not adequately monitored and controlled. This greatly compromised biomass productivity, which in turn led to lower fatty acid and carotenoid productivities. Despite these issues, this experiment registered the highest fatty acid content amongst all performed assays and the lowest PUFA and linolenic acid contents, therefore this strategy should be pursued.

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