

Production and extraction of lipids and carotenoids from the yeast *Rhodospiridium toruloides* NCYC 921 and production of biogas by anaerobic digestion of the residues.

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December 2015

Abstract

The present work studies an integrated process that includes (i) the fed-batch cultivation of the yeast *Rhodospiridium toruloides* NCYC 921, towards the production of fatty acids (FA, for biodiesel production) and carotenoids, (ii) the co-extraction of these products and (iii) the anaerobic digestion of the residues generated in these steps. Fermentations were carried in 7 L (fermentation I) and 50 L (fermentations II and III) reactors. The highest biomass ($1.33 \text{ g DCW L}^{-1} \text{ h}^{-1}$), carotenoid ($0.26 \text{ mg L}^{-1} \text{ h}^{-1}$) and FA ($0.29 \text{ g L}^{-1} \text{ h}^{-1}$) productivities were achieved in fermentation I. The highest carotenoid and FA contents were, respectively, $0.44 \text{ mg g}^{-1} \text{ DCW}$ (fermentation II) and 40.89% (w/w) (fermentation III). The highest biomass concentration was $198.2 \text{ g DCW L}^{-1}$ (fermentation III), a value that exceeds those reported in the literature. Extraction and separation of the carotenoids and FA was performed through the direct saponification of the biomass, followed by carotenoid extraction, acidification of the soaps and FA extraction. Relative to the initial biomass contents, the maximum carotenoid and FA extraction yields were, respectively, 96.6% and 93.2%. Carob pulp residue (CR), generated during carob pulp syrup (CPS, a low cost substrate) preparation, supernatants resulting from fermentations where glucose (GS) or CPS (CS) were used as substrates, and yeast residue (YR), resulting from the product extraction steps, were anaerobically co-digested, in 71.5 mL batch digesters. The co-digestion of these residues resulted in biogas volumetric productions higher than the control assay (inoculum self-digestion), reaching methane (CH_4) levels above 60% (v/v). Co-digestion of a GS and YR mixture resulted in the highest volumetric production of biogas (55.4 mL, STP) and highest methane yield ($236.4 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}$).

Keywords: *Rhodospiridium toruloides* NCYC 921, carotenoids, fatty acids, anaerobic digestion, biogas.

1. Introduction

The development and well-being of modern society relies on an economy that depends on the constant access to cheap energetic resources. The growth of the world population in conjunction with the urbanization and industrialization of the emerging economies will be the main driving forces behind the expected 37% increase in the consumption of untransformed energetic natural resources (primary energy [1]) by 2040 [2].

Fossil fuels are currently the dominating primary energy resource, accounting, in 2014, for more than 80% of the world primary energy consumption [3]. This dependency is, however, unsustainable. The combustion of fossil fuels accounts for over 90% of the energy-related carbon dioxide (CO_2) emissions, one of the major greenhouse gases (GHG) believed to be the cause of climate change that, if not reduced, will have a severe and irreversible environmental impact worldwide [3]. More-

over, the finiteness of fossil fuels, with their exhaustion being expected to occur in the next 50 years [4], their uneven geographical distribution and the geopolitical consequences of this, are some of the major factors that make these resources a threat to the world's energy security and equity [5, 6]. These factors urge the need to, not only, implement energy efficiency strategies in the production and consumption of energy [5, 7], but also to develop sustainable and environmentally compatible energy sources that can supersede the usage of fossil fuels.

Secondary biofuels are solid, liquid or gaseous energy carriers that store energy derived from biomass, through its chemical transformation, extending the range of applications of biomass as an energy source [8]. The most common examples are biodiesel and bioethanol. The wide availability of raw materials, the compatibility with existent fuel distribution infrastructures and the pos-

sibility of partially or completely replacing diesel and gasoline in conventional engines, makes biofuels very attractive as immediate replacements for these fuels [9]. Biofuels can also be less polluting, as their combustion generates less emissions of CO₂ and other pollutants [10–12].

Biodiesel, currently the biofuel most widely used as an alternative to petrodiesel, is a mixture of fatty acid alkyl esters produced through the catalyzed transesterification of acylglycerols or direct esterification of free fatty acids (FFA). A small alcohol is used as an acyl acceptor, usually methanol, forming fatty acid methyl esters (FAME), the most common form of biodiesel [13]. Biodiesel is a non-toxic biodegradable substitute of conventional diesel, with better lubricant properties, a higher cetane number, better combustion efficiency and lower sulfur and aromatic contents [14].

While the production of secondary biofuels is becoming increasingly competitive, production costs are still a major obstacle, making subsidization necessary in order to allow biofuels to compete against conventional fuels [15]. 60 to 95% of biodiesel production cost arise from the substrates used, mostly edible vegetable oils [13].

Biodiesel (and biofuels in general) can be classified according to the source of the raw materials used in their production. First generation (1G) biodiesel designates biodiesel derived from edible crops [16]. The direct competition with the food and feed market, raising food prices, and the overall negative environmental impact of its production cycle, makes 1G biodiesel unsustainable [17]. Second generation (2G) biodiesel is derived from non-edible vegetable oils and waste cooking oil [18]. The non-edible crops used in the production of 2G biodiesel can, however, have similar sustainability problems as 1G crops and can compete indirectly with food production [17, 18].

Third generation (3G) biodiesel designates biodiesel derived from the so called single cell oils (SCO), oils produced by oleaginous microbes, namely microalgae and yeasts [19]. Although still in early stages of development, 3G biodiesel is expected to take advantage of the microbes high growth rates, ease of culture and lower spatial requirements, to achieve high productivities and bring down costs [20, 21]. Oleaginous yeasts are particularly promising as they exhibit higher growth rates than microalgae, reaching higher productivities in biomass and lipids with less dependence on external factors such as light exposure [22, 23].

One of the major limitations to the scale-up of SCO production is its economic viability [21, 23]. A suggested strategy is the production of SCO under the concept of biorefinery, combining the production of SCO with the extraction of high added-

value products (polyunsaturated fatty acids, pigments, etc.) and the valorization of the residual biomass as animal feed or through the production of biogas by anaerobic digestion (AD) [19, 22]. The integration of an AD process in the production of microalgae-derived biodiesel has been considered crucial to its sustainability [24, 25].

Rhodospiridium toruloides (or *Rhodotorula gracilis*) is a prominent oleaginous yeast due to its high biomass and lipid productivities and lipid content [26], with biomass concentrations as high as 185 g dry cell weight (DCW) L⁻¹ [27] and lipid contents reaching 79% DCW [26]. This yeast is also often called pink yeast due to its content in carotenoids, mostly β-carotene, torulene and torularhodin [22]. Carotenoids are high added-value products that have increasing demand in areas such as agriculture, aquaculture and in the poultry, nutraceutical, food, pharmaceutical and cosmetic industries [28]. The properties of *R. toruloides* make this yeast a particularly interesting choice for the production 3G biodiesel under a biorefinery concept, combining the production of SCO, for biodiesel manufacture, and carotenoids, a high added-value product that can make the process economically sustainable.

AD is the biological degradation of complex mixtures of biodegradable organic compounds in the absence of O₂, involving a consortium of interdependent heterotrophic bacteria that generate, as the final product of the microbial metabolism, a gaseous mixture, called biogas, consisting mainly of methane (CH₄, 60-80%) and CO₂ (20-40%) [29]. The biogas can be captured and used directly as a fuel, or treated for the removal CO₂ and other impurities, to increase its CH₄ content to over 95% [8]. The production and extraction of FA (and other products of interest) from heterotrophic oleaginous microorganisms generates significant quantities of wastes, such as supernatants and residual cell biomass, with high organic loads. These wastes, that would otherwise constitute an environmental and economic burden for the process, have potential to serve as substrates in AD processes, enabling their energetic valorization through the production of biogas.

Flow cytometry (FC) is an advanced technique that allows the quantitative and qualitative analysis of each individual cell in a sample, *in situ* and almost in real time (at-line), evaluating several physical and biochemical parameters of a large number of cells [30]. The process involves passing individual cells through a light beam of a defined wavelength, and the measurement of the scattered light or of the fluorescence emitted by excitation of endogenous fluorochromes (autofluorescence, AF) or of exogenous fluorochromes added to the sample. FC has shown promise in monitoring the

cultivation of oleaginous microbes for the production of biofuels [30–34]. In particular, a correlation between the AF of *R. toruloides* cells and the cellular total carotenoid content has been established by Freitas *et al.*, and used to monitor the yeast carotenoid content throughout the culturing process [34], making FC a powerful tool in the monitoring of *R. toruloides* cultures.

The present work studies an integrated process that combines the fed-batch cultivation of the oleaginous yeast *R. toruloides*, for the production of SCO and carotenoids, the co-extraction of these products, and the production of biogas by AD of the organic residues generated in these steps, aiming to valorize all biomass fractions and process organic residues.

2. Materials and methods

2.1. Fermentations

The fed-batch culture of the oleaginous yeast *R. toruloides* was performed using glucose as the carbon source. A liquid semi-defined culture medium was used in the pre-inoculum, inoculum and fermentation broths, containing, as described by Pan *et al.*, (g L⁻¹): KH₂PO₄, 12.5; Na₂HPO₄, 1.0; (NH₄)₂SO₄, 5.0; MgSO₄·7 H₂O, 2.5; CaCl₂·2 H₂O, 0.25; yeast extract, 1.9; and 0.25 mL L⁻¹ of a trace minerals solution prepared in 5 N-HCl, containing (g L⁻¹): FeSO₄·7 H₂O, 40.0; CaCl₂·2 H₂O, 40.0; MgSO₄·7 H₂O, 10.0; AlCl₃·6 H₂O, 10.0; CoCl₂, 4.0; ZnSO₄·7 H₂O, 2.0; Na₂MoO₄·2 H₂O, 2.0; CuCl₂·2 H₂O, 1.0; H₃BO₄, 0.5. [27].

Unless stated otherwise, sterilization of materials and solutions was achieved through autoclaving (Uniclave 88, A.J. Costa (Irmãos) LDA, Portugal). To avoid glucose caramelization, glucose was autoclaved as a separate solution.

The microorganism used in the fermentation assays was *Rhodospiridium toruloides* NCYC 921, supplied by the National Collection of Yeast Cultures (Norwich, United Kingdom). The yeast was stored in malt extract agar slants at 4 °C and, prior to the preparation of the inoculums, transferred to fresh malt extract agar slants and incubated (NIR 252 Incubator, SANYO Electric Co., Ltd., Japan) at 30 °C for 72 h.

Three fermentation assays were performed. Fermentation I was carried out in a 7 L benchtop borosilicate glass bioreactor (5 L working volume) coupled to a dedicated controller for the measurement and control of agitation rate, dissolved oxygen (DO), temperature, pH and foaming (FerMac 310/60 Bioreactor, Electrolab Biotech, United Kingdom). pH was measured with a pH probe (Mettler Toledo, Switzerland) and adjusted through the automatic addition 5 M NaOH or HCl solutions. A few drops of pure polypropylene glycol (PPG) were

added at the beginning of the cultivation process to prevent excessive foaming. The foam levels were monitored throughout the process and managed through the addition of a sterilized PPG 1:10 aqueous solution. Agitation relied on two Rushton turbines and mixing/aeration was enhanced by four baffles inside the vessel. The temperature of the medium was controlled with an external heating blanket and an internal cold finger. An oxygen probe (Mettler Toledo, Switzerland) was used to monitor DO levels. In order to avoid oxygen limitation, DO level were maintained above 40% of the medium air saturation by coupling the agitation rate to the DO readings. Fermentations II and III were performed in a 50 L stainless steel bioreactor (35 L working volume) coupled to a dedicated control module (C 809, Biolafitte, France) to monitor and control the stirring rate and reactor temperature. An external module (MOD 7F, Setric Genie Industriel, France) was used to monitor and control the DO and pH, measured with, respectively, DO (Mettler Toledo, Switzerland) and pH (Ingold InFit 764-50, Mettler Toledo, Switzerland) probes. The pH was adjusted through the addition of 10 M NaOH or 5 M HCl solutions. DO was adjusted through changes in the agitation and aeration rates and in the vessel's internal pressure. Agitation was controlled manually and relied on three Rushton turbines. Mixing and aeration were enhanced by the reactor's four internal baffles. The temperature was controlled by the an integrated heating/cooling external jacket. Excessive foaming was managed through the manual addition of PPG.

Inocula were prepared in baffled Erlenmeyer flasks containing the previously described medium supplemented with an initial concentration of 35 g L⁻¹ of glucose as the carbon source. Inoculation of the 7 L bioreactor was achieved through the addition of 150 mL of inoculum, prepared by inoculating 150 mL of medium with yeast cells transferred from two slants and incubating at 30 °C under agitation (150 rpm), for 24 h (Unitrom Infors incubator, Switzerland). For the inoculation of the 50 L bioreactor, two 150 mL pre-inoculums were prepared as described above and used to inoculate two 5 L baffled Erlenmeyer flasks, each containing 1350 mL of culture medium, resulting in two 1500 mL inoculums that were incubated at 30 °C under agitation (150 rpm), for 24 h (Unitrom Infors, Switzerland). These two inoculums were used to inoculate the 50 L bioreactor.

The cultivations were performed at 30 °C, using a two-step pH control fed-batch strategy described by Dias *et al.* [35]. The fermentations were initiated in batch mode, at a fixed pH of 4.0, with a 35 g L⁻¹ initial glucose concentration. Once the carbon source was exhausted, feeding at a variable

rate was initiated using a glucose (600 g L⁻¹) solution supplemented with yeast extract (20 g L⁻¹) and MgSO₄·7H₂O (9 g L⁻¹), and maintained until the end of the growth phase. These conditions were optimized by Dias *et al.* to promote yeast biomass production and lipid accumulation. Once the culture reached the stationary phase, the medium pH was altered to 5.0 and the feed changed to a concentrated glucose solution (600 g L⁻¹), at a variable rate, aiming to promote carotenoid accumulation during the stationary phase [35].

Samples (40 to 100 mL) were typically collected three times a day. Aliquots were used for immediate analysis and the remainder was centrifuged (Sigma 2-16K centrifuge, Sartorius, Germany) for 10 min, at 9000 rpm and 5 °C, to allow the sedimentation of the biomass. The supernatant and the pellet were then separated and frozen at -18 °C for later determination of the glucose and FA content, respectively.

Biomass dry cell weight (DCW) concentration (g L⁻¹) was calculated using a correlation previously established by Parreira [36], between the optical density (OD) of the samples measured at 600 nm (OD₆₀₀) and the biomass DCW concentration. The samples' OD₆₀₀ were measured in duplicate (in a ThermoSpectronic Genesys 20 spectrophotometer, Thermo Scientific, USA).

The residual glucose concentration (g L⁻¹) in the medium was determined by the 3,5-dinitrosalicylic acid (DNS) method [37], using the supernatant obtained from each sample.

The yeast cellular FA content was quantified and its composition determined by gas chromatography (GC) following derivatization of the FA into their respective FAME. The extraction and conversion of the FA to FAMES was done according to the protocol described by Freitas, *et al.* [34], itself an adaptation from earlier work by Lepage and Roy [38]. The samples were analyzed with a gas chromatographer (SCION GC 436, Bruker, Germany), equipped with a flame ionization detector (FID). Separation was achieved in a fused silica capillary (0.32 mm × 30 m) column (Supelcowax 10, Supelco, USA) with a film thickness of 0.25 µm, using helium, at a flow rate of 1.6 mL min⁻¹, as the carrier gas. A split ratio of 1:20 was used for the initial 5 min, and changed to 1:10 for the remaining time. Injector and detector temperatures were 250 and 280 °C, respectively. The column was programmed for an initial temperature of 200 °C, increasing to 220 °C at a 2 °C min⁻¹ rate. The column pressure was 13.5 psi. Peak identification and response factor calculations were carried out using heptadecanoic acid (17:0) as the 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_i is the mass of a specific FAME i , A_i is its respective peak area, $A_{17:0}$ is the area of the peak corresponding to the internal standard and RF_i is the FAME's response factor (considered to be equal to 1).

$$m_i = \frac{A_i}{A_{17:0}} \cdot RF_i \quad (1)$$

Total carotenoids content was determined by FC using a FACScalibur benchtop flow cytometer (BD Biosciences, USA) equipped with blue argon (488 nm) and red diode (635 nm) lasers, FSC and SSC detectors detecting in the same wavelength as the blue argon laser (488 ± 10 nm), and four fluorescence detectors: FL1 (530 ± 30 nm), FL2 (585 ± 42 nm), FL3 (> 670 nm) and FL4 (661 ± 16 nm). All buffers were filtered prior to use with a 0.22 µm filtration membrane (TPP Syringe-Filter 0.22 µm, Techno Plastic Products AG, Switzerland). The gathered data was analyzed with the FCS Express 4 Flow Research Edition software (De Novo Software, USA). The total carotenoids cellular content was determined through a correlation established by Freitas *et al.*, relating this parameter to the AF levels measured in the FL2 detector [34].

2.2. Biomass processing

At the end of the fermentations, the bioreactors were emptied and a fraction of broth was centrifuged (Sigma 2-16K centrifuge, Sartorius, Germany) at 9000 rpm, 5 °C, for 10 min. The wet biomass and the supernatant were separated and stored at -18 °C for further analysis. This biomass was processed for co-extraction of the FA and carotenoid content, using a protocol described by González *et al.* [39], involving the direct saponification of wet biomass, extraction of the unsaponifiable fraction, acidification of the soaps and extraction of the FA. 17 g of wet biomass (5 g DCW) were treated, in a 1 L flask, with 380 mL of ethanol (96%, v/v), containing 8 g of KOH (85% purity). Saponification was carried out in a N₂ atmosphere, at room temperature, overnight, with constant agitation. The suspension was then transferred to a (light protected) separatory funnel where 100 mL of water were added (to allow a better phase separation), and the unsaponifiables extracted with 200mL of hexane, under minimal light and at room temperature. The two phases were decanted and the extraction repeated (two to three times) until a colorless hexane phase was obtained. Aliquots of the unsaponifiables were analysed by high performance liquid chromatography (HPLC) for carotenoid quantification and determination.

To recover the saponified fraction the wa-

ter/alcoholic solution was adjusted to pH 1.0 using HCl 1:1 (v/v) and the FA and torularhodin were extracted with hexane under minimal light at room temperature by repeating the process described above. The total carotenoids content and composition of the extracted saponifiables were determined by HPLC and the lipid profile of the extracted FA was determined by GC, following their conversion into the respective FAME.

The total carotenoids content and composition of the extracted fractions were determined by HPLC using a chromatograph (HP 1100 series, Hewlett-Packard, Germany) equipped with a UV detector set to 450 nm, a reverse phase column (μ Bondapak C18, 4.6 x 250 mm, Waters Corporation, USA) and an injection loop of 20 μ L. The mobile phase was a (75:15:10) mixture of acetonitrile, methanol (with 0.1% trimethylamine, TEA) and ethyl acetate, at a flow rate of 0,8 mL min⁻¹. After the sample elution (12 min), the column was flushed for 2 min with methanol (100%) and then with the eluent mixture for 5 min before the next analysis cycle. β -carotene (97%, Sigma-Aldrich, UK) solutions with concentrations ranging from 0.05 to 15.00 mg mL⁻¹ were prepared in the same solvent as the as samples and used as external standards.

2.3. Anaerobic digestion

The energetic valorization of several residues generated in the yeast cultivation and product extraction stages was attempted through an anaerobic co-digestion process. The glucose supernatant (GS) obtained from the centrifugation of the culture broth of a fermentation where glucose was used as the carbon source, the yeast residue (YR) generated in the products co-extraction process, the carob supernatant (CS) produced in previous works, where carob pulp syrup (CPS) was used as a low cost carbon source and the carob residue (CR) resulting from the CPS preparation steps [22, 40], were used as substrates in the anaerobic digestion (AD) process. These residues were co-digested as mixtures of YR and GS (mixture I), YR and CS (mixture II) or YR, CS and CR (mixture III) where the relative quantity of each residue was the same as the estimated proportion in which they are generated in the yeast cultivation and product extraction steps. These mixtures were characterized in regard to their total solids (TS), volatile solids (VS), COD and total sulfates content.

Biological solids collected from the anaerobic digester from SIMLIS in Leiria, a unit that processes municipal aerobic sludge and piggery effluents, were used as the inoculum in the AD assay. The inoculum was characterized in terms of its pH and TS, VS, total suspended solids (TSS) and volatile suspended solids (VSS) contents.

The AD process was performed in triplicate, using batch vials with a 71.5 mL working volume containing 40 mL of liquid phase, and a 31.5 mL headspace. The liquid phase was comprised of 60% (v/v) inoculum and 40% (v/v) of a suspension of the substrates in a sodium bicarbonate buffer (3 g L⁻¹ NaHCO₃) adjusted to pH 7.0. The initial substrate concentration was chosen as to achieve an initial substrate VS to inoculum VS ratio equal to 0.75. The control assay consisted of a liquid phase containing 60% (v/v) inoculum and 40% (v/v) sodium bicarbonate buffer. 240 mL of each of the liquid phases were prepared and aliquots of 40 mL were distributed in the respective vials. The vials were then flushed with a N₂ stream for oxygen removal and encapsulated with airtight stoppers and aluminum caps. The remainder of each mixture (about 120 mL) was stored at 4 °C for further analysis of the initial pH value and COD, TS, VS, total Kjeldhal nitrogen (total-N), ammonia nitrogen (NH₃-N) and volatile fatty acids (VFA) contents. The vials were placed in a thermostatic bath maintained at 37 \pm 1 °C (mesophilic range).

The biogas production was monitored daily by measuring the pressure inside the sealed vials, using a previously calibrated pressure transducer (Centrepoint Electronics). The pressure readings were used to calculate the biogas produced volumes at standard temperature and pressure (STP) conditions (1 atm, 0 °C). The biogas composition in the vials was determined weekly by GC. 0.5 mL gas samples were extracted from the vials with an airtight syringe, depressurized to atmospheric pressure and injected in a gas chromatograph (Varian CP 38000, Varian Inc., USA) equipped with a thermal conductivity detector (TCD) and a fused silica column (Agilent Select Permanent Gases/CO₂ HR set, with CP-Molsieve 5Å and CP-Porabond Q columns in tandem). The injector, column, detector and filament temperatures were 80 °C, 50 °C, 120 °C and 170 °C, respectively. An isobaric column pressure of 14 psi and injection split ratio of 5 were used, with helium as the carrier gas (63.5 mL min⁻¹ flow rate). The percentages of CH₄, CO₂, H₂S, H₂, O₂ and N₂ were calculated by using a calibration curve (R² > 0.99) obtained with external standard gases.

The TS, VS, TSS, VSS, COD, total-N, NH₃-N and total sulfates contents were determined according to the Standard Methods for the Examination of Water and Wastewater [41]. All determinations were performed in triplicate.

The VFA content was determined through GC. The samples were centrifuged (15000 rpm, 2 min) and filtered (0.22 μ m cellulose nitrate membrane) before injection in a gas chromatograph (HP 5890 GC, Hewlett Packard, USA) equipped with a FID

Table 1: Summarized results from fermentations I, II e III.

Parameter	Fermentation I	Fermentation II	Fermentation III
Working volume	5 L	35 L	35 L
Max. biomass concentration (g L ⁻¹)	118.9 (t = 186.0 h)	38.3 (t = 241.6 h)	198.2 (t = 406.5 h)
Max. biomass productivity (g L ⁻¹ h ⁻¹)	1.33 (t = 22.0 h)	0.33 (t = 23.3 h)	0.52 (t = 307.4 h)
Max. carotenoid content (mg g ⁻¹)	0.20 (t = 48.5 h)	0.44 (t = 217.3 h)	0.24 (t = 167.8 h)
Max. carotenoid productivity (mg L ⁻¹ h ⁻¹)	0.26 (t = 48.5 h)	0.07 (t = 217.3 h)	0.08 (t = 378.2 h)
Max. FA content (% w/w)	24.25 (t = 66.0 h)	30.24 (t = 241.6 h)	40.89 (t = 162.4 h)
Max. FA productivity (g L ⁻¹ h ⁻¹)	0.29 (t = 72.0 h)	0.05 (t = 241.6 h)	0.09 (t = 426.2 h)

and a (2 mm x 2 m) packed column (Carbopack B-DA/4% Carbowax 20 M 80/120 packing, Sigma-Aldrich, USA). The column, injector and detector temperatures were 170, 175 and 250 °C, respectively. Helium was used as carrier gas (30 mL min⁻¹). Calibration was done with an external standard VFA mixture (Supelco WSFA-2, Sigma-Aldrich, USA) and pivalic acid was used as the internal standard. VFA determinations were made in duplicate and expressed as acetic acid equivalents.

3. Results and discussion

3.1. Fermentations

The summarized results obtained in the fermentation assays are shown in table 1.

Fermentation I resulted in the highest biomass (1.33 g L⁻¹ h⁻¹), total carotenoids (0.26 mg L⁻¹ h⁻¹) and FA (0.29 g L⁻¹ h⁻¹) productivities of the three assays. This is due to the fact that the operational conditions for *R. toruloides* cultivation at this scale had already been optimized in previous works [35]. The productivities in fermentations II and III were also limited by some technical difficulties with the equipment used. Dias *et al.*, using the same culture strategy achieved a similar FA content (24.42%) but a higher biomass concentration (126.84 g L⁻¹), carotenoid content (0.29 mg g⁻¹) and biomass (2.35 g L⁻¹ h⁻¹), carotenoid (0.29 mg L⁻¹ h⁻¹) and FA (0.40 g L⁻¹ h⁻¹) productivities [35].

In fermentation II, problems with DO monitoring led to the culture being subjected to oxygen-deficient conditions throughout most of the process duration. This led to impaired culture growth, resulting in the lowest biomass concentration of the three assays (38.8 g L⁻¹) and low overall pro-

ductivities. Nonetheless, higher FA (30.24%) and carotenoid (0.44 mg g⁻¹) contents were obtained in this assay. These results highlight the importance of oxygen availability for *R. toruloides* growth and product synthesis.

Fermentation III led to the highest biomass concentration (198.2 g L⁻¹) and FA content (40.89%). This biomass concentration is higher than any described in the literature for *R. toruloides* or *R. glutinis*, including the one reported by Pan *et al.*, using the same medium and oxygen enriched air (185 g L⁻¹) [27]. However, problems in the beginning of assay III led to an extended fermentation time that resulted in overall low productivities. Nonetheless, the biomass concentration obtained in assay III is near the limit at which the increase in the viscosity of the medium, the reduction in the oxygen transfer rate and the cultures high oxygen demand, makes it impractical to sustain the cultivation process [42].

Overall, the FA contents obtained in this work were lower than the values reported in the literature (>60% w/w) for other strains of *R. toruloides* [43–45]. The carotenoid contents obtained were also significantly lower than the 35.5 mg g⁻¹ content described by Aksu and Eren in shake flask cultures of *R. glutinis* [46].

Table 2 shows the average FA composition (% w/w) obtained in fermentations I-III, in terms of the major FA identified and of the saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) FA contents. A FA composition high in palmitic (16:0), oleic (18:1 ω 9) and linoleic (18:2 ω 6) acids, typical of *R. toruloides* [19], was obtained, with 18:1 ω 9 being the major FA in all three fermentations. Palmitoleic acid (16:1 ω 9) was

Table 2: Average fatty acid composition (% w/w) in fermentations I, II and III.

Assay	Composition (% w/w)								
	16:0	16:1 ω 9	18:0	18:1 ω 9	18:2 ω 6	18:3 ω 3	SFA	MUFA	PUFA
I	26.36%	0.13%	6.93 %	35.35%	22.79%	7.22%	34.41%	35.58%	30.01%
II	17.84%	0.02%	23.30%	53.23%	4.81%	0.22%	41.67%	53.30%	5.03%
III	18.32%	7.01%	4.57%	50.66%	15.55%	3.52%	23.27%	57.67%	19.06%

one of the major FA components only in fermentation III. The FA composition in fermentation II was distinctive, with a higher stearic acid (18:0) content and the lowest average PUFA content, possibly due to the oxygen limitations, as synthesis of unsaturated fatty acids is an oxygen dependent process [47].

Fermentations II and III exhibited a FA composition low in PUFA and rich in MUFA, favorable characteristics for obtaining a good quality biodiesel [48]. The European standard for biodiesel (FAME) quality (EN 14214) specifies that its content in linolenic acid (18:3 ω 3) derived methyl esters must not exceed 12% (w/w). Despite the 18:3 ω 3 content having sometimes exceeded this value at the beginning of the fermentation assays, the value stayed well below the 12% limit during the remaining duration of the assays. This standard also specifies that the content in methyl esters derived from PUFA with four or more double bonds must not exceed 1% (w/w), none of which were detected in any of the assays.

3.2. Biomass processing

Two extraction assays were performed, in duplicate, using biomass derived from two different fermentations. Table 3 shows the carotenoid and FA extraction yields (CEY and FAEY, respectively) relative to their initial contents in the processed biomass. In extraction I 77.7% of the carotenoid content of the processed biomass was recovered, with 71.7% and 6.0% extracted from the unsaponifiable and saponifiable fractions, respectively. In extraction II a global CEY of 96.6% was achieved, with 82.1% of the biomass carotenoid content being extracted from the unsaponifiables and 14.5% from the saponifiable fraction. The carotenoids extracted from the unsaponifiable fractions were mainly β -carotene and torulene. Most of the torularhodin was extracted from the saponifiable fractions, because, unlike β -carotene and torulene, torularhodin has a carboxylic acid functional group that renders it, like the FA, susceptible to saponification. The global FAEY were 76.4% and 93.2% in extractions I and II, respectively.

The CEY from the unsaponifiable fractions obtained in this work were higher than those reported by González *et al.* (60.1%), but the FAEY from the

saponifiable fractions were lower than the >90% yield reported by González *et al.*. This may be due to an excess of water in the mixtures, resulting in the formation of emulsions that hampered the FA extraction [39].

3.3. Anaerobic digestion

Table 4 shows the biogas production (mL) and composition (% v/v) and the CH₄ productivities (mL CH₄ g⁻¹ VS) at the end of the AD process. The digestion of all mixtures resulted in biogas productions significantly higher than the control assay, with final CH₄ contents above 60%. Relative to control, mixture I resulted in the highest biogas (55.4 mL) and CH₄ (31.6 mL) productions and highest CH₄ productivity (236.4 mL CH₄ g⁻¹ VS). The digestion of the substrate mixtures containing carob derivatives resulted in lower productions and productivities, perhaps due to a high content in phenolic compounds, known to inhibit microorganisms. Nonetheless, mixture III yielded the biogas with the highest quality, with the highest CH₄ content (68.0%) and lowest H₂S (a toxic and corrosive gas) concentration (771 ppm). Mixture III is particularly interesting, as it contains all the digestible residues generated in a *R. toruloides*-based biorefinery using CPS as a low cost carbon source. The CH₄ productivities obtained in this assay were, in general, lower than the values reported in the literature for the AD of oleaginous microalgae-derived biomass [49]. Batista *et al.* reports a 346 mL CH₄ g⁻¹ SV yield from the AD of oleaginous microalga *Scenedesmus obliquus* after extraction of its FA content. Ehimen *et al.* reports a 308 mL CH₄ g⁻¹ SV yield from the AD from *Chlorella* biomass following its direct transesterification.

4. Conclusions

This work aimed to study a process integrating the fed-batch culturing of the oleaginous yeast *R. toruloides* for the production of FA (intended for biodiesel production) and carotenoids, the extraction and separation of these products and the valorization of the energy content of the residues generated in the substrate preparation (CR), yeast cultivation (GS and CS) and product extraction (YR) steps.

The fermentation assays show that it is possi-

Table 3: Carotenoid and fatty acids extraction yields relative to their respective contents in the processed biomass.

Assay	Component	Extraction yield (% w/w)		
		Unsaponifiables	Saponifiables	Global
Extraction I	Carotenoids	71.7% ± 5.4%	6.0% ± 4.2%	77.7% ± 15.9%
	Fatty acids	19.9% ± 2.4%	56.5% ± 4.8%	76.4% ± 5.3%
Extraction II	Carotenoids	82.1% ± 14.5%	14.5% ± 0.6%	96.6% ± 14.5%
	Fatty acids	17.3% ± 0.8%	75.9% ± 14.8%	93.2% ± 14.8%

Table 4: Biogas production (mL) and final composition (% v/v) and CH₄ productivities obtained in the AD assay.

Parameter	Control	Mixture I (YR + GS)	Mixture II (YR + CS)	Mixture III (YR + CS + CR)
Biogas production (mL)				
Assay	13.9 ± 0.5	69.3 ± 1.3	45.1 ± 2.4	43.2 ± 3.2
Assay – control	-	55.4 ± 1.4	31.2 ± 2.4	29.3 ± 3.2
CH ₄ production (mL)				
Assay	10.7 ± 0.4	42.2 ± 1.0	30.8 ± 1.7	29.4 ± 1.6
Assay – control	-	31.6 ± 1.1	20.1 ± 1.7	18.8 ± 1.6
CH ₄ yield				
Volumetric (mL CH ₄ g ⁻¹ SV)	-	236.4 ± 16.7	179.0 ± 22.2	145.4 ± 9.7
Energy (kWh kg ⁻¹ SV)	-	2.3 ± 0.2	1.8 ± 0.5	1.4 ± 0.5
Biogas final composition				
CH ₄ (% v/v)	76.8 ± 0.4	60.9 ± 0.4	68.2 ± 0.4	68.3 ± 2.3
CO ₂ (% v/v)	24.1 ± 0.5	39.1 ± 0.4	32.0 ± 0.4	32.1 ± 2.2
H ₂ S (ppm)	1160 ± 123	3779 ± 648	3278 ± 1798	757 ± 840

ble to achieve significant biomass concentrations and to make the scale-up of the culture strategy described by Dias *et al* [35]. However the process can only be economically sustainable if the *R. toruloides* cultivation process scale-up can be achieved using low cost carbon sources. In this regard, CPS, in particular, is a potential candidate as it has shown to be a promising low cost substrate in the cultivation of *R. toruloides* in lab-scale reactors [22, 40]. A better understanding of the factors affecting carotenoid and FA accumulation is also needed. The results highlight the importance of oxygen availability for yeast growth and the need for a tighter control over the carbon source concentration to increase biomass, FA and carotenoid productivities.

In the biomass processing assays carotenoids and FA were extracted and separated with good yields, albeit with a significant degree of variability between assays, the causes of which need to be better understood to further optimize the process.

The energetic valorization of the residue mixtures was successfully achieved in the AD assays, showing that the integration of an AD process is an important step to achieve the economic and environmental sustainability of a *R. toruloides*-based

biorefinery. Since significant quantities of organic residues are generated in the CPS preparation, yeast cultivation and product extraction steps, the AD process scale-up should be investigated.

Acknowledgements

The present work was carried out under the project FCOMP-01-0124-FEDER-019317 (previously PTDC/AAC-AMB/116594/2010) entitled “CAROFUEL – New process for a sustainable microbial biodiesel production: The yeast *Rhodotorula glutinis* biorefinery as a source of biodiesel, biogas and carotenoids” co-funded by FCT (Fundação para a Ciência e a Tecnologia) and by the European Regional Development Fund (FEDER) through COMPETE – Programa Operacional Factores de Competitividade).

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