Biological treatment of effluents from the brewing industry with simultaneous production of intracellular lipids using a symbiotic system with yeasts and microalgae

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Abstract: Microalgae, besides being very robust and being involved in the mitigation of greenhouse gases, have the capacity to grow under adverse environmental conditions, making them a very attractive hypothesis for the biological effluents treatment. Oleaginous yeast are potential producers of carotenoids and oils for the production of biofuels. The objective of this dissertation was the biological wastewater treatment from the brewing industry, using mixed cultures of the microalgae *Scenedesmus obliquus* and the yeasts *Rhodosporidium toruloides* taking advantage of the symbiosis resulting from their complementary nutritional modes and the potential production of valuable added products. The effluent was supplemented with sugarcane molasses and the different treatments were tested at various total sugars concentrations. The best treatment was achieved with the growth of *S. obliquus* only in efluente (with the following final concentrations: 0,20 g/L of CQO; 17,2 g/L of $N-NH_3$; 32,0 g/L of ${PO_4}^{3-}$; 10,0 g/L of $P-{PO_4}^{3-}$ and 24,0 g/L of P_2O_5), followed by the treatment with mixed culture of *S. obliquus* and *R. toruloides* in 10 g/L of total sugars (with the following final concentrations: 9,50 g/L of CQO; 74,1 g/L of $N-NH_3$; 20,5 g/L of ${PO_4}^{3-}$; 6,5 g/L of $P-{PO_4}^{3-}$ and 15,0 g/L of P_2O_5) and, finally, with *R. toruloides* in 20 g/L of total sugars (with the following final concentrations: 21,63 g/L of CQO; 43,2 g/L of $N-NH_3$; 43,0 g/L of ${PO_4}^{3-}$; 14,0 g/L of $P-{PO_4}^{3-}$ and 12,8 g/L of P_2O_5). On the other hand, the treatment with mixed culture achieved the highest production of intracelular lipids (19,6% (w/w)), which greatly values the process economically. The treatment of the effluent with mixed culture in non-sterile conditions was tested, resulting in the most efficient of all treatments, however, the lipid production was very low (1,6% (w/w)).

1. Introduction

1.1. Industrial Effluents

Effluent is any water containing contaminants, which can be very harmful to man and to the environment (AL-Rajhia et al., 2012). Effluents originating from industrial processes, particularly from the agro-food industry, are rich in carbon and nitrogen (AL-Rajhia et al., 2012; Raposo et al., 2010), in the form of organic and inorganic compounds, reason why the treatment of these is a necessary condition for their discharge in nature or reuse in the processes, in order to avoid any environmental impact (Raposo et al., 2010).

1.2. Treatment of Industrial Effluents

The quality of a water is assessed by quantifying some of its physico-chemical and microbiological properties, followed by comparison with legally required limit values (Alves, 2007).

When the destination of the treated effluent is the reutilization in industrial processes or the discharge in nature, special emphasis should be given to: ammoniacal nitrogen ($N-NH_3$), Kjeldahl total nitrogen (KTN), total organic carbon (TOC) and total phosphorus (P). These parameters must be within the legally required specific limit values (Table 1.1).

Table 1.1: Emission limit values (ELV) in waste water discharge, where: BOD5 is the biochemical oxygen demand at day 5, COD is the chemical oxygen demand, TSS is the total suspended solids, N-NH3 is the ammoniacal nitrogen and KTN is the Kjeldahl total nitrogen ("Decreto-Lei nº 236/98 , de 1 de agosto de 1998," 1998).

Emission limit values (ELV) in waste water discharge		
Parameters	Expression of Results	ELV
pH	Sorensen Scale	6,0-9,0
BOD_5, 20℃	mgO_2/L	40
COD	mgO_2/L	150
TSS	mg/L	60
$Total\ P$	mgP/L	0,5-10
$N-NH_3$	$mgNH_4/L$	10
KTN	mgN/L	15

1.2.1. Conventional Treatment/Biological Treatment of Industrial Effluents

The steps involved in conventional effluent treatment are: (i) pretreatment, (ii) primary treatment, (iii) secondary treatment, and (iv) tertiary treatment or disinfection (Mihelcic & Zimmerman, 2010). The effluent that arrives at the secondary treatment (clarification) already underwent significant removal of the suspended solids (SST), however, still presents high load in dissolved organic matter, in N and P. Secondary treatment is a type of biological treatment and, as such, uses microorganisms (Mihelcic & Zimmerman, 2010).

In addition to conventional treatment, there are many techniques to be developed for the treatment of effluents, mainly by biological processes (Arbib et al. , 2014). In part, in response to the serious global environmental problems that have been felt and, above all, with the objective of reducing greenhouse gases emissions, such as CO_2. Photosynthesis has been recognized as a form of anthropogenic CO_2 fixation, and algae have been identified as photosynthetic agents with high fixation rates of C. Since the biological treatment of effluents with microalgae simultaneously allows the removal of organic C and inorganic nutrients, both N and total P (Arbib et al., 2014).

The microalgae, in addition to being very robust and once in proper conditions can grow in a practically unlimited, have the capacity to accumulate intracellularly more than 70% of their dry weight in oil, for the production of biofuel (AL-Rajhia et al., 2012). The major difficulties in biological treatment with microalgae are the low cellular densities, typically between $0,5-5\ g/L$ and the reduced cellular dimensions of some algae, typically $2-40\ \mu m$ (Brennan & Owende, 2010).

Oleaginous yeast are efficient producers of added value products, being able to accumulate cellular lipids corresponding to more than 70% by weight of their biomass, being able to be extracted and transformed into biodiesel, which can favor economically the treatment process (Freitas et al., 2014; Papone et al., 2015). Thus, a mixed culture of microalgae and yeasts, allows to take advantage of the symbiosis resulting from their

complementary nutritional modes (Papone et al., 2015) and the potential production of value added products.

In the specific case of this symbiosis applied to the treatment of effluents, which organic load is low, the yeasts may not have sufficient carbon source to their growth. In these cases, it is necessary to consider supplementing the effluent with an organic carbon source. However, in these cases it is essential to take into account the economic viability of the process and, as such, to consider the choice of cheap organic carbon sources (Yan et al., 2011). An economically viable and sustainable option is the use of industrial by-products of residual commercial value, such as sugar cane molasses, which is a by-product of the sugar industry (Papone et al., 2015).

1.3. Brewery Industry

Water consumption varies between $4-10\ hL/hL$ of beer produced, since besides constitutes $90-95\%$ (by mass) of the beer and is used in almost all stages of the production process, being a very important consumable in this industry, not only in terms of water consumption, but also of effluent production (Olajire, 2012). Generally, the effluents of the brewing industry have a high chemical oxygen demand (COD) due to the presence of organic compounds, however, they are non-toxic and as a rule do not contain appreciable quantities of heavy metals (Olajire, 2012).

The treatment and the discharge of effluents from a brewing industry have very high costs. Therefore, a few aspects should be taken into account in the operation of a brewery.

1.4. Intracellular Products

It is increasingly common knowledge that fossil fuels are not sustainable at environmental and market level, since their extraction costs are increasing and their sources are exhausting (Freitas et al., 2014). An alternative to fossil fuels is biofuels, and fatty acids are the most attractive raw materials for their development, since the methyl esters of fatty acids are the constituents of biodiesel, which is a biofuel. (Zhu et al., 2012). Fatty acids are synthesized by microbial cells and accumulated intracellularly as storage materials, in the form of triglycerides. Biodisel results from the transesterification of a triglyceride with a molecule of methanol or ethanol, thus being constituted by a mixture of methyl or ethyl esters, respectively (Defanti et al., 2010). Currently, the production of biofuel from microorganisms is not economically viable (Freitas et al., 2014a). Therefore, their production as by-products of the processes is a favorable alternative to the economic valuation of the processes.

Generally, the production of lipids from yeast has an advantage over the production from bacteria, fungi or algae, since the specific rate is higher in yeast and therefore is more easy to obtain appreciable amounts of biomass and higher lipid productivities (Freitas et al., 2014a).

1.5. *Scenedesmus obliquus*

S. obliquus is a green microalga that belongs to the kingdom Chloroplastida and to the phylum Chlorophyta. It has microscopic dimensions, of diameter between $5-10\ \mu m$, is a unicellular circular eukaryotic organism (Becker, 2007), presents phenotypic plasticity (Lürling, 2003), and it is common to form aggregates, usually with two or four cells, although under adverse conditions it can form colonies with eight cells (Lürling, 2003; Singh & Singh, 2014). It presents high productivities of biomass and lipids (Singh & Singh, 2014), accumulating high value intracellular co-products (Ferreira et al., 2018), and it is the microalga that presents a profile of fatty acids more suitable for the production of biodiesel (Gouveia & Oliveira, 2009). The *S. obliquus* microalgae has been used to treat effluents from the brewing industry.

1.6. *Rhodosporidium toruloides*

R. toruloides is a yeast belonging to the Fungi kingdom, from the phylum *Basidiomycota*, from the class *Microbotryomycetes*, from the order *Sporidiobolales* and from the genus *Rhodosporidium*. The oleaginous yeast *R. toruloides* NCYC 921, known as "pink yeast", has been described as a potential producer of oils for the production of biofuels, and as a promising producer of carotenoids with commercial interest (Freitas et al., 2014a; Zhu et al., 2012).

1.7. Flow Cytometry

Flow cytometry allows the detection of cells as well as obtaining detailed information about their characteristics, structure, behaviors and functions, namely about its enzymatic activity and membrane integrity (Silva et al., 2004). If the cell synthesizes fluorescent compounds (chlorophylls, carotenoids, NAD(P)H, etc.) or compounds capable of binding to fluorescent dyes, they may be detected (Silva et al., 2004). In addition, the signal obtained by the FSC photodiode gives information on the cell size, and the signal obtained by the SSC photomultiplier gives information about the internal complexity of the cell (Cutzu et al., 2013; Freitas et al., 2014).

2. Materials and methods

2.1. Microorganisms and Pre-Inoculums

The microorganisms used on this work were the oleaginous yeast *R. toruloides* NCYC 921, supplied by the National Collection of Yeast Cultures (Norwich, United Kingdom), and the microalgae *S. obliquus* ACOI 204/07, from the Coimbra University Algotec, Portugal.

2.1.1. *R. toruloides*

Malt Extract Agar (MEA) ramps were made from a pure culture of *R. toruloides*, which were incubated at 30℃ for 72 h and stored at 4℃ (Freitas et al., 2014a). For preparation of the solid culture medium: dissolve $25\ g$ of MEA in $500\ ml$ of distilled water and sterilize for 10 min at 115℃.

Preparation of the liquid culture medium of the inoculum (Yoon & Rhee, 1983) (g/L): $CaCl_2 \cdot 2H_2O$, 0,134; $MgSO_4$, 0,73; Na_2HPO_4, 2; KH_2PO_4, 7; $(NH_4)_2SO_4$, 1; yeast extract, 0,5; glucose, 35 (Freitas et al., 2014a). The pH was adjusted to 4,0 and sterilized for 20 min at 121℃. The pre-inocula were prepared in Erlenmeyers with $1\ L$ bulkheads, in duplicate, and each containing $150\ ml$ of culture medium and the *R. toruloides* biomass corresponding to 4 growth ramps. It was incubated with light (LED bulbs, 12 W), at 30℃ and $150\ rpm$ for 24 h (Freitas et al., 2014a).

2.1.2. *S. obliquus*

The *S. obliquus* microalgae (ACOI 204/07) was maintained on a seaweed bank, in Erlenmeyer flasks, in Bristol medium (g/L): $NaNO_3$, 0,250; KH_2PO_4, 0,175; K_2HPO_4, 0,075; $MgSO_4 \cdot 7H_2O$, 0,075; Fe-EDTA, 0,060; $CaCl_2 \cdot 2H_2O$, 0,033; $NaCl_2$, 0,025; $1\ ml/l$ of Chu's trace elements solution ((g/L): H_3BO_3, 0,286; $MnSO_4 \cdot 4H_2O$, 0,203; $ZnSO_4$, 0,022; $CoSO_4 \cdot 7H_2O$, 0,009; $Na_2MoO_4 \cdot 2H_2O$, 0,006; $CuSO_4$, 0,005) (Ferreira et al., 2017). The pre-inocula were prepared in $500\ mL$ Erlenmeyers and incubated at room temperature, at 110 rpm and under artificial light conditions (4 Philips 36W lamps) for 5 days.

2.2. Inoculums

The tests were carried out in baffled $1\,L$ Erlenmeyer flasks containing $180\,ml$ of liquid medium and $20\,ml$ of inoculum (10% v/v) (Freitas et al., 2014a). The culture medium used was secondary effluent from the brewing industry supplemented with sugarcane molasses.

Tests were carried out with pure culture of *S. obliquus*, with initial pH 7,0 (Ferreira et al., 2017) and incubated at 30℃ and at 110 rpm (in order to test the growth only in effluent and in $5\,g/L$, $10\,g/L$, $20\,g/L$, $40\,g/L$ and $60\,g/L$ of total sugars); with pure culture of *R. toruloides*, with initial pH 4,0 (Dias t al., 2015) and incubated at 30℃ and at 150 rpm; and with mixed culture of *S. obliquus* e *R. toruloides*, with initial pH 6,0 and incubated at 30℃ and at 150 rpm. After optimization of the concentration in total sugars ($10\,g/L$), two tests were performed, one in sterile condition and another in non-sterile condition.

The growths were monitored by measuring biomass, lipid content, contaminants concentrations (COD, $N-NH_3$, KTN and P) and cell viability (membrane integrity and enzymatic activity analysed by flow cytometry).

2.3. Growth Monitoring

The cultures were monitored by optical microscopy (Olympus Bx60, Japão) and the biomass was measured by optical density (OD) at: 540 nm for the pure cultures of *S. obliquus* and 600 nm for the pure cultures of *R. toruloides* and mixed cultures, in duplicate and with a spectrophotometer Genesys 20 Visible. On the last day of the test, the correlation between OD and dry weight was established.

2.4. Flow Cytometry

Membrane integrity and enzymatic activity were evaluated by flow cytometry (FACSCalibur Flow Cytometer, Becton-Dickinson biosciences, CA, USA). The flow cytometer has four more photomultipliers: FL1, which detects green fluorescence ($530 \pm 30\,nm$); FL2, which detects yellow fluorescence ($585 \pm 42\,nm$); FL3, which detects orange-red fluorescence ($> 670\,nm$); FL4, which detects red fluorescence ($660 \pm 16\,nm$) (Cutzu et al., 2013; Freitas et al., 2014).

2.4.1. Cell membrane integrity

The integrity of the membrane was evaluated using the SytoxGreen dye. SytoxGreen is a fluorescent dye, which penetrates membranes with compromised integrity and once inside the cell binds to the DNA strands, increasing its fluorescence which is detected in FL1 when excited by radiation at $488\,nm$. The samples were diluted with phosphate buffered saline (PBS) in order to analyze $1000\,events \cdot s^{-1}$ (Freitas et al., 2014), and incubated in the dark with $5\,\mu l$ of $30\,\mu M$ SytoxGreen (obtained by diluting the $5\,mM$ SytoxGreen in dimethylsulfoxide (DMSO)), for 25 min (the working SytoxGreen incubation time and concentration were previously optimised).

2.4.2. Enzymatic activity

The enzymatic activity was analyzed using carboxyfluorescein diacetate, CFDA. CFDA is a non-fluorescent compound, which penetrates all cells by passive diffusion and if there are intracellular esterases, they hydrolyze CFDA, resulting in a fluorescent compound that is detected in FL1 (when excited at $488\,nm$). The samples were diluted with McIlvaine buffer solution (citric acid $100\,mM$ and Na_2HPO_4 $200\,mM$, at pH 4,0 (Freitas et al., 2014)), in order to analyze $1000\,events \cdot s^{-1}$ (Freitas et al., 2014), and incubated in the dark with $20\,\mu L$ of CFDA $10\,mg/mL$, for 50 min (the working CFDA incubation time and concentration were previously optimised).

2.5. COD analysis

It was placed in each tube of the digester (HACH COD REACTOR): $1{,}5\,mL$ of sample, $1\,mL$ of solution A ($10{,}216\,g$ of dry $K_2Cr_2O_7$ at 103℃ for 2 h; $167\,mL$ of H_2SO_4 concentrated; $33{,}3\,g$ of $MgSO_4$; give $1\,L$ with distilled water) and $2\,mL$ of solution B ($10{,}12\,g$ of $AgSO_4$ in H_2SO_4 concentrated); was carried to the digester at 150 ± 2℃ for 2h; transferred to Erlenmeyers of $50\,mL$; 1 to 2 drops of ferroin was added and titrated with $0.0125\,M$ D solution ($49{,}01\,g$ of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in $500\,mL$ of distilled water; $20\,mL$ of concentrated H_2SO_4 was added; $1\,L$ with distilled water was preferred; Dilute $1{:}10$ (v/v) in distilled water). The analyzes were done in duplicate and for each digestion a blank was made ($1{,}5\,mL$ of distilled water rather than of sample was placed). The determination of COD concentration was done by the following equation:

$$mgO_2/L = (A - B) \times \frac{M}{V_S} \times 8000$$

Where A and B are the volumes of solution D used in titration of the blank and the sample, respectively; M is the molarity of solution D and V_S is the sample volume.

2.6. Nitrogen analysis

2.6.1. Ammoniacal Nitrogen analysis

$10\,mL$ of sample and $1\,mL$ of ISA solution ($MgSO_4$ $1M$) were placed in a $50\,mL$ plastic beaker; was read with an ammonia selective electrode (CRISON-Multimeter MM 41) under stirring.

2.6.2. Total Kjeldahl Nitrogen analysis

For the determination of KTN, a procedure collected from the Solvay Group was used: $25\,mL$ of sample, $3\,ml$ of concentrated H_2SO_4 and $3\,mL$ of $CuSO_4$ ($10\%\,w/v$) were placed in a Kjeldahl flask; it was heated to $120-130$℃, for 3 h; $100\,ml$ of distilled water was added and the contents of the Kjeldahl flask transferred to a $200\,mL$ volumetric flask; the $200\,mL$ is perfected; $10\,mL$ of the solution, $15\,mL$ of distilled water and $2\,mL$ of $NaOH$ solution ($40\%\,w/v$) were placed in a beaker; was stirred for 8-10 min and was read with the specific ammonium electrode (CRISON-Multimeter MM 41) under stirring.

2.7. Phosphorus analysis

To determine phosphorus, a commercial kit (PhosVer 3 Phosphate Reagent, HACH) was used to quantify (mg/L) PO_4^{3-} (phosphates), P -PO_4^{3-} (phosphorus present in the phosphates) and P_2O_2 (phosphorus pentoxide): $25\,mL$ of sample was transferred into an appropriate flask; was placed on the spectrophotometer as white; a kit bag was added; stirred vigorously and allowed to stand for 2 min; was read.

2.8. Fatty acid analysis

Into the reaction tubes previously filled with inert gas (N) and in duplicate, about $100\,mg$ of lyophilized biomass were weighed; $2\,mL$ of methanol/acetyl chloride solution ($95{:}5$, v/v) and $0{,}2\,mL$ of heptadecanoic acid ($5\,mg/mL$, Nu-Check-Prep, Elysian, USA) were added as an internal standard; the tubes were coated with aluminum foil and heated at 80°C for 1 h to effect the transesterification reaction; cooled; $1\,mL$ of water and $2\,mL$ of n-hexane were added to each tube; the organic phase, with the methyl esters, was finally extracted, filtered through an anhydrous sodium sulfate bed, and analyzed by gas-liquid chromatography.

The methyl esters were analyzed on a GC BRUCKER SCION 436-SC (Billerica, Massachusetts, USA), equipped with a flame

ionization detector. Separation of the compounds was done on a silica capillary column with $0{,}32\ mm \times 30\ mm$ ($0{,}32\ \mu m$ film) Supelcowax 10 (Supelco, Bellafonte, Palo Alto, CA, USA), which operates with helium as the carrier gas , with a flow rate of $3{,}5\ ml/min$, and a pressure of $13{,}5\ psi$. The column is programmed for an initial temperature of 200℃ for 8 min, and then increased $4℃/min$ to 240℃, which temperature it operated for 16 min. The injector and detector are respectively at 250℃ e 280℃, and at a ratio of 1:20 for 5 min and 1:10 for the remaining run time. Peak identification was done by comparison with methyl ester blend standards (Nu-Chek-Prep, Minesota, USA).

2.9. Quantification of Sugars by HPLC

The quantification of sugars was done by high performance liquid chromatography (HPLC) (LaChrom Merck/Hitachi, Germany), using a differential refractive index (RI) detector. The analysis was performed using a Waters SugarPak 1 column (Bio-Rad Laboratories, CA, USA), column characteristics and operating conditions being in Table 2.1.

Prior to HPLC analysis, the samples were centrifuged (BECKMAN COULTER TM 18 Microfuge), the supernatant was recovered, placed in vials and analyzed; the chromatograms were analyzed with the software ChromeleonTM SP6 build 783 (1994-2003, Dionex, Thermo Fischer Scientific Inc., USA).

Table 2.1: Key features of Waters SugarPak 1 column and operating conditions.

Characteristics	SugarPak Column
Dimensions	$6{,}5 \times 300\ mm$
Mobile Phase	Ca-EDTA, $50\ mg/L$
Flow Phase Mobile	$0{,}5\ ml/min$
Column Temperature	75℃
Temperature Detector RI	30℃
Running Time	20 in

2.10. Removal Efficiency

From the values obtained from the analysis of COD, N, P and sugars, the removal efficiencies (RE) of the effluent nutrients were determined. The determination of RE was made by the equation below, where C_0 and C_f are the initial and final concentrations, respectively.

$$RE\ (\%) = \frac{C_0 - C_f}{C_0} \times 100$$

3. Results and Discussion

In an attempt to achieve a biological treatment for effluents from the brewing industry with *S. obliquus* and *R. toruloides*, several conditions were tested. In an initial phase, pure cultures of each microorganism, conducted under asseptic conditions, were developed on the brewery efluente, in order to verify if they grew on the effluent, if sugar cane molasses supplementation was necessary, and if so, what was the optimal sugar concentration for growth. Eventually the mixed culture was developed on efluente supplemented with sugarcane molasses in order to confirm that the two microorganisms grew in the presence of each other, and again what was the optimum sugar concentration to growth. At last, after selecting the optimal total sugar concentration for the mixed culture growth was conducted under non-asseptic conditions.

3.1. Treatment with *S. obliquus*

The growth of *S. obliquus* only in effluent and in $5\ g/L$, $10\ g/L$ and $20\ g/L$ of sugar was studied.

The maximum biomass concentration, $X_{max}(OD_{540\ nm})$ (Figure 3.1(a)), was achieved with growth in $5\ g/L$ of sugar, although it was also significant for growth only in effluent and in $10\ g/l$. Increasing the sugar concentration in the medium increases the latency phase period and reduces the exponential phase period. Growth in $5\ g/L$ was characterized by a higher maximum specific growth rate ($\mu = 0{,}73\ d^{-1}$) and a shorter duplication time ($t_d = 0{,}95\ d$).

The highest cell densities are obtained with the growths only in effluent and in $20\ g/L$ of sugar (Figure 3.1(b)). $5\ g/L$ is the concentration with the highest values of $X_{máx}(OD_{540\ nm})$ and μ, but one of the concentrations with lower cell density, which is justified by the greater cellular sizes and complexities verified for this concentration. In $20\ g/L$ of total sugars the cells were smaller and formed large cellular aggregates, indicative that this concentration represents adverse conditions to the microalga (Lürling, 2003).

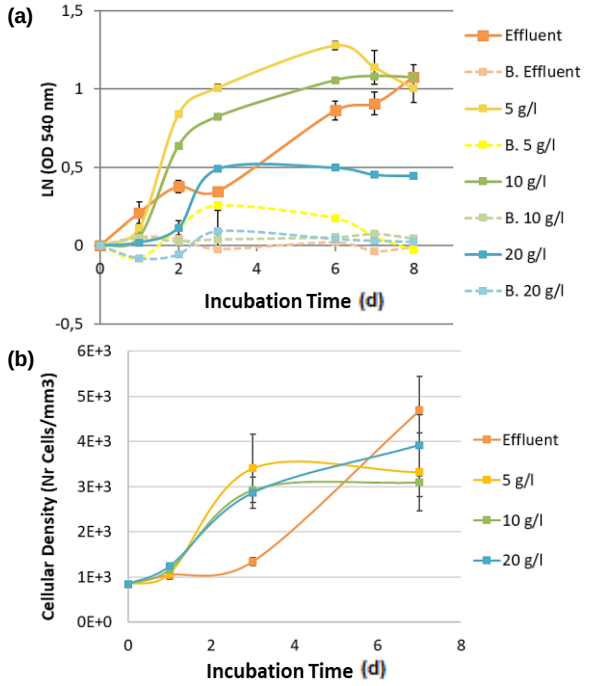

Figure 3.1: (a) Normalized growth curves in order to eliminate molasses interference. (b) Curves of cell density. B. corresponds to the blank assay. The cell count were made by optical microscopy.

The highest production of fatty acids was verified for the growth in $20\ g/L$ of sugar $2{,}6\%\ (w/w)$. However, the production of fatty acids was very low for all studied concentrations ($1{,}8 - 2{,}6\%\ (w/w)$), because according to Becker (2007), *S. obliquus* microalgae is capable to accumulate $12 - 14\%\ (w/w)$ of lipids.

The higher percentage of stained cells with CFDA (Figure 3.2(a)), representative of the higher enzymatic activities, are verified for growth at $5\ g/L$, most probably as a result of higher metabolic performances and higher cell growth.

The growth in $20\ g/L$ of sugar is the one that display greater percentage of cells stained with SytoxGreen (Figure 3.2(b)), revealing greater membranar fragility. On the other hand, the growth only in effluent and in $5\ g/L$, are the ones that display smaller percentage of cells stained, revealing the presence of cells with greater cellular integrity.

It is concluded that $5\ g/L$ is the total sugars concentration in the medium that most favors the growth of *S. obliquus*, which ensures higher enzymatic activities and greater integrity of the membrane.

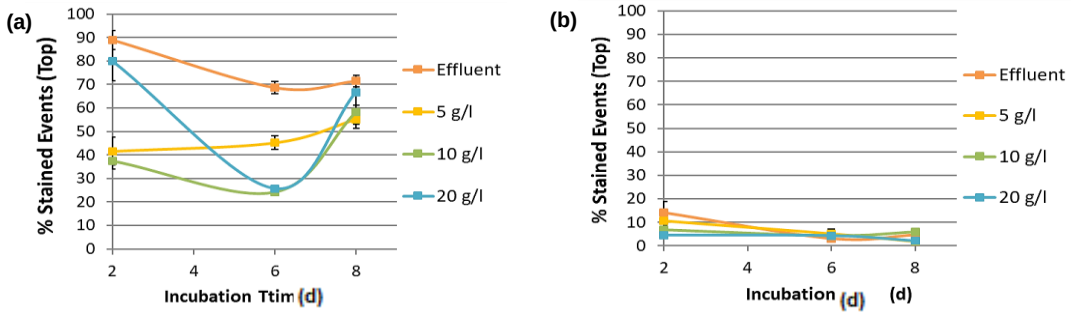

Figura 3.2: Curves based on the information obtained by flow cytometry (a) were diluted with McIlvain buffer and incubated in the dark for 50 min, with 20 μL CFDA 10 mg/mL dye; (b) were diluted with PBS buffer and incubated in the dark for 20 min with 5 μL of 30 μM SytoxGreen dye.

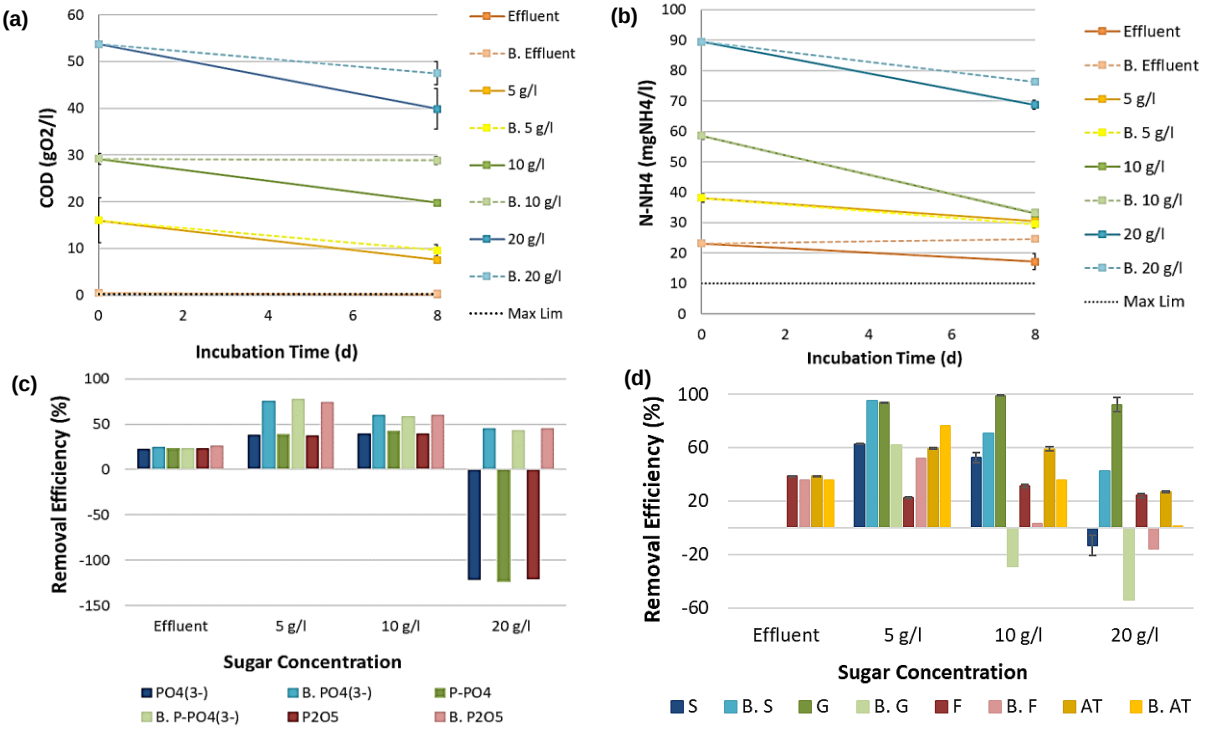

Figura 3.3: (a) Chemical oxygen demand (COD) and (b) Concentration of ammoniacal nitrogen (mgNH4/L), corresponding to initial and final test times. (c) and (d) removal efficiencies of the three phosphorus species and sugars (S-sucrose, G-glucose, F-fructose and AT-total sugars), respectively. B. corresponds to the blank assay.

COD removal is more efficient for growth only in effluents ($RE = 54{,}0\%$) and in 5 g/l ($RE = 52{,}9\%$) (Figure 3.3(a)). Being these the growths for which the final COD concentrations (0,20 gO_2/L and 7,54 gO_2/L, respectively) are closer to the legally required maximum level for discharge of treated effluents (0,15 gO_2/L).

The highest RE of $N - NH_3$ was achieved for growth in 10 g/L of total sugars ($RE = 43{,}7\%$), followed by growth only in effluent ($RE = 25{,}9\%$) (Figure 3.3(b)). For these concentrations and at 5 g/L, the final load of $N\text{-}NH_3$ (33,0 $mgNH_4/L$, 17,2 $mgNH_4/L$ and 30,4 $mgNH_4/L$, respectively) is very close to the legal maximum limit for the treated effluent to be discharged (10,0 $mgNH_4/L$).

Removal of P is more efficient at 5 g/L and 10 g/L of total sugars (Figure 3.3(c)). However, the removal in whites is even more significant, revealing that it is not made by microalgae. Contrarily, the microalgae contribute to this not being so efficient, probably due to cell lysis phenomena, which is confirmed by the heavy increase of the concentrations of P in the growth in 20 g/L, concentration for which there is a greater cellular death.

When only in effluent the microalga does not require sugars to its growth nor activity, typical of autotrophy. The same was observed for growth at 5 g/L, for which the removal of sugars was even higher in white than in the duplicates. On the other hand, for the growths in 10 g/L and 20 g/L, the high sugar removal reveals the predominance by a mixotrophic nutritional mode.

Another assay was performed for higher concentrations of total sugars in the medium: 40 g/L and 60 g/L. It was observed that the cellular growth and viability of *S. obliquus* are affected when subjected to conditions of total sugars concentration in the medium greater than 20 g/L, resulting in low cellular densities, reduced cellular activities, and high cellular death, besides the infeasible process of treatment as a result of the inefficient removal of the contaminants. Probably due to the presence of inhibitory compounds present in molasses (Freitas et al., 2014b).

3.2. Treatment with *R. toruloides*

The growth of *R. toruloides* was studied only in effluent and in 20 g/L, 40 g/L, 60 g/L, 80 g/L and 100 g/L of total sugars.

From Figure 3.4(a), it can be concluded that *R. toruloides* yeast does not grow only in effluent and it is with the growth in 20 g/L of total sugars in the medium that it is observed the highest $X_{max}(OD_{600\,nm})$, the highest μ (0,42 d^{-1}) and the smallest t_d (1,67 d).

The cell count is concordant with the $OD_{600\,nm}$, with the highest cell density verified for growth at 20 g/L of total sugars (Figure 3.4(b)). From 20 g/L, the cell density of the cultures decreases with increasing total sugars concentration in the medium.

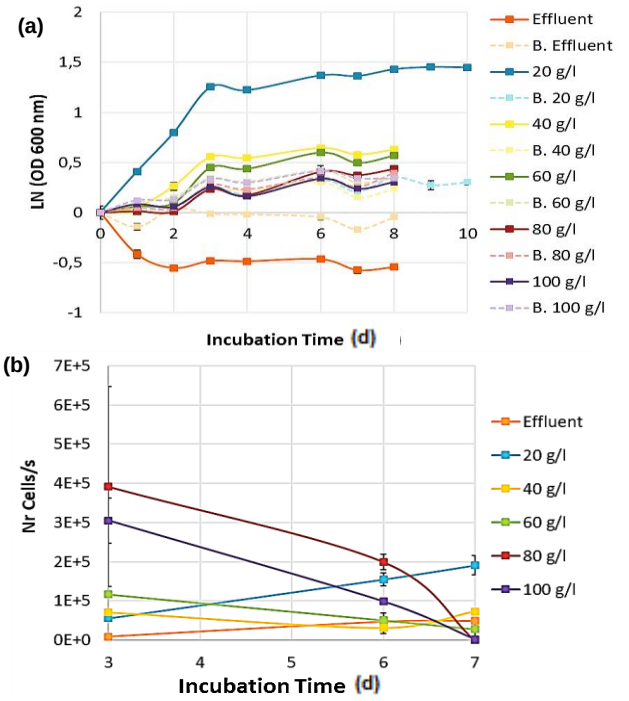

Figure 3.4: (a) Normalized growth curves, to eliminate interference of sugarcane molasses, variation of $DO_{600\,nm}$ with incubation time. (b) Cell count by flow cytometry. *B.* corresponds to the blank assay.

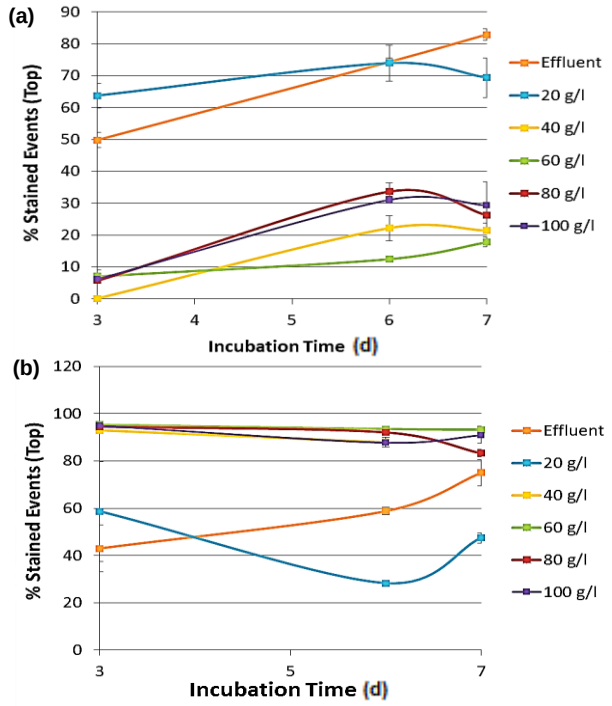

Figure 3.5: Curves based on the information obtained by flow cytometry, having been diluted with buffer (a) McIlvain and incubated in the dark for 50 min with 20 μL of CFDA 10 mg/mL dye; (b) PBS and incubation in the dark for 20 min with 5 μL of 30 μM SytoxGreen dye.

The highest production of fatty acids was also observed for growth in 20 g/l of total sugars 16,5% (w/w). However, from the information obtained by flow cytometry, increasing the concentration of total sugars in the medium increases the size and complexity of *R. toruloides* cells, which is justified by the presence of larger cell aggregates at these concentrations.

By CFDA analysis, it is observed that the growths with higher percentages of cells with enzymatic activity (Figure 3.5(a)), are: at 20 g/l sugar, which is consistent with the higher production of biomass and fatty acids registered in this culture, because they are cells in constant cell division and lipid synthesis; and only in effluent, probably because the lack of C and N in the effluent induced damage to the cell membrane, however the cells preserved their enzymatic activity.

By the analysis with SytoxGreen (Figure 3.5(b)), it is perceived that the concentration for which the cell death is lower (with lower percentage of stained cells), is 20 g/l, and that for the growths in higher concentrations of total sugars, there are much higher percentages of cells stained with SytoxGreen, revealing the superior cellular fragility and, consequently, the higher rates of cell death.

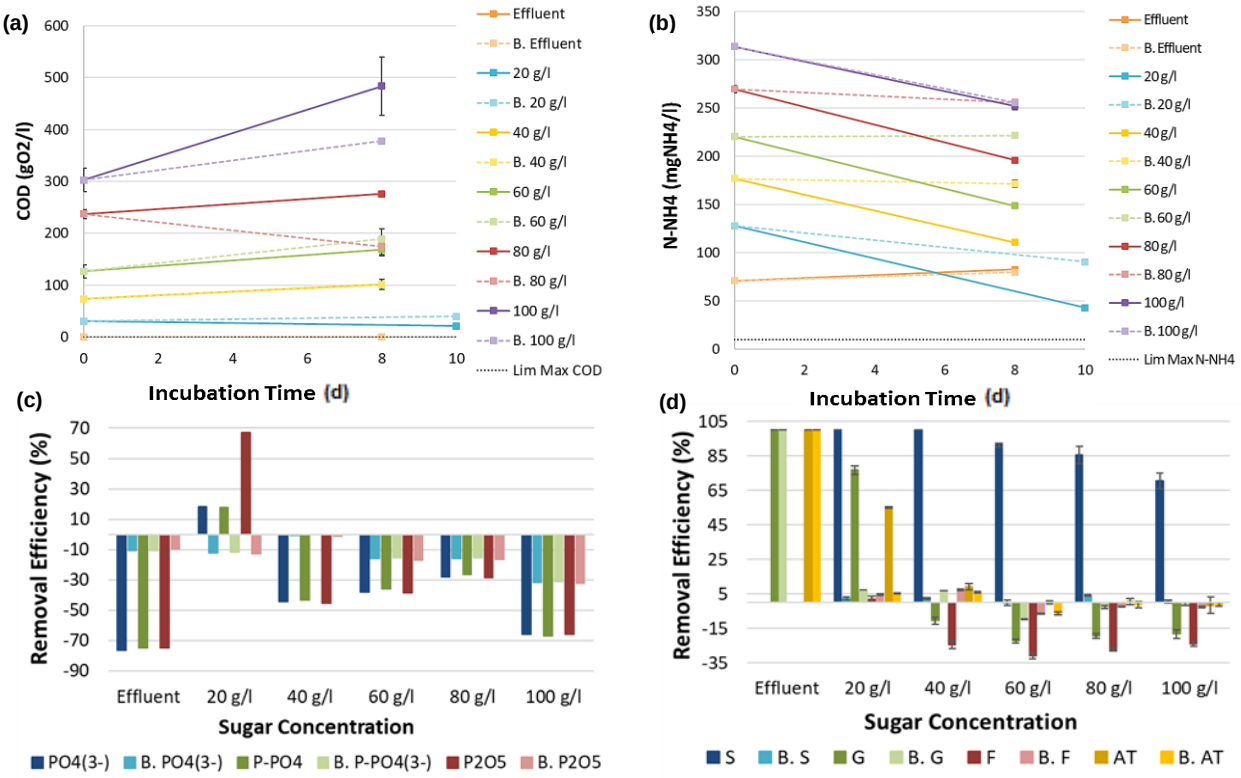

Figure 3.6: (a) Chemical oxygen demand (COD) and (b) Concentration of ammoniacal nitrogen (N-NH4), corresponding to initial and final test times. (c) and (d) removal efficiencies of the three phosphorus species and sugars (S-sucrose, G-glucose, F-fructose and AT-total sugars), respectively. B. corresponds to the blank assay.

Only with growth at $20\ g/L$ did COD removal occur (RE=30,2%) (Figure 3.6(a)). The COD increase observed for the remaining growths is a result of reduced cell metabolism (due to inhibition by growth inhibitors present in molasses (Freitas et al., 2014b) and/or limitation of N or O_2 or other nutrient other than C), high rates of cell lysis, and evaporation phenomena.

It is also for the growth in $20\ g/L$ that the highest RE of $N-NH_3$ ($RE = 66{,}2\%$) and of P ($RE_{PO4^{3-}} = 18{,}1\%$, $RE_{P-PO4^{3-}} = 17{,}6\%$, $RE_{P_2O_5} = 67{,}2\%$) are observed, and for which the final concentrations of these contaminants are closer to the maximum limits legally required to discharge the treated efluente (Figure 3.6(b) and Figure 3.6(c)).

From Figure 3.6(d) it can be seen that the only growth for which the three sugars were removed and, consequently, for which the RE of the TS was positive and significant, was $20\ g/L$ ($RE(S) = 56{,}4\%$; $RE(G) = 0{,}8\%$; $RE(F) = 1{,}9\%$; $RE(TS) = 140{,}8\%$). The consumption of sugars at higher concentrations was practically nil, and what happened was the decomposition of sucrose (dimer) into glucose and fructose (Freitas et al., 2014b).

Thus, it was concluded that among the total sugars concentrations in the medium studied, which allowed a greater cellular adaptation, a more efficient treatment of the effluent, and a superior production of fatty acids, was $20\ g/L$.

3.3. Treatment with Mixed Culture

The growth of the mixed culture was studied only in effluent and in the following concentrations of total sugars: $5\ g/L$, $10\ g/L$, $20\ g/L$, $40\ g/L$ and $60\ g/L$.

From the cell count by citometry, it was verified that: concentrations of $40 g/L$ and $60 g/L$ did not assure the growth of *S. obliquus* microalgae; for $20\ g/L$ of total sugars there is dominion of the population of *R. toruloides*; unsupplemented medium (effluent only) is not conducive to yeast growth; $5\ g/L$ compromises the growth of *R. toruloides*; and the concentration that best ensured the symbiosis between the two populations was $10\ g/L$. All of this, coupled with the fact that $10\ g/L$ was the concentration of total sugars in the medium which ensured higher values of $X_{max}(OD_{600\ nm})$ and μ ($1{,}20\ d^{-1}$), and lower t_d ($0{,}58\ d$) (Figure 3.7).

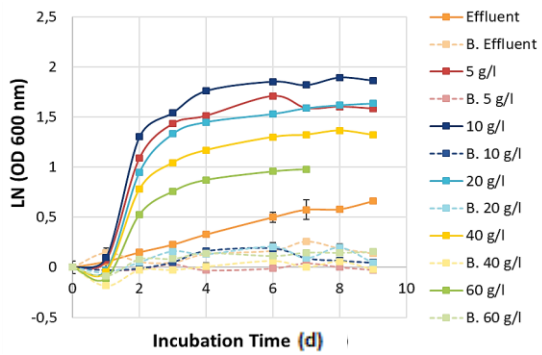

Figure 3.7: Normalized growth curves, to eliminate interference from molasses, variation of OD with incubation time. B. corresponds to the blank test.

Thus, $10\ g/L$ seems to be the best hypothesis, based on the biomass production and the ratio of cell densities achieved between the two populations.

From the analysis to the fatty acids, it was also verified that this is superior to $10\ g/L$ of total sugars in the médium $19{,}6\%\ (w/w)$.

From Figure 3.8(a), it can be seen that: at $40\ g/L$ and $60\ g/L$ the organic load at the end of the assay is higher than the initial one, probably due to the high rate of cell death; to $20\ g/L$ the organic load is also not removed ($RE \approx 0\%$); and therefore, the only growth that allowed COD removal was those that occurred only in effluent and in $5\ g/L$ and $10\ g/L$ of total sugars in the medium ($RE_{Efl.} \approx 50{,}0\%$, $RE_{5\ g/l} \approx ER_{10\ g/l} \approx 37{,}7\%$).

The growths that ensured higher RE and allowed the final concentration of $N-NH_3$ to approach more to the legally required maximum limit for discharging the treated efluente ($10{,}0\ mgNH_4/L$), were those that occurred only in effluent and in $5\ g/L$ and $10\ g/L$ (with the following final concentrations, in $mgNH_4/L$: 8,1; 13,7 and 22,2, respectively) (Figure 3.8(b)).

The concentration for which higher RE of the three P species are achieved is $10\ g/L$ (Figure 3.8(c)). By comparing these results with those obtained for the pure cultures of *S. obliquus* and *R. toruloides* under the same conditions, the advantage that exists in the removal of P by the mixed culture is clear.

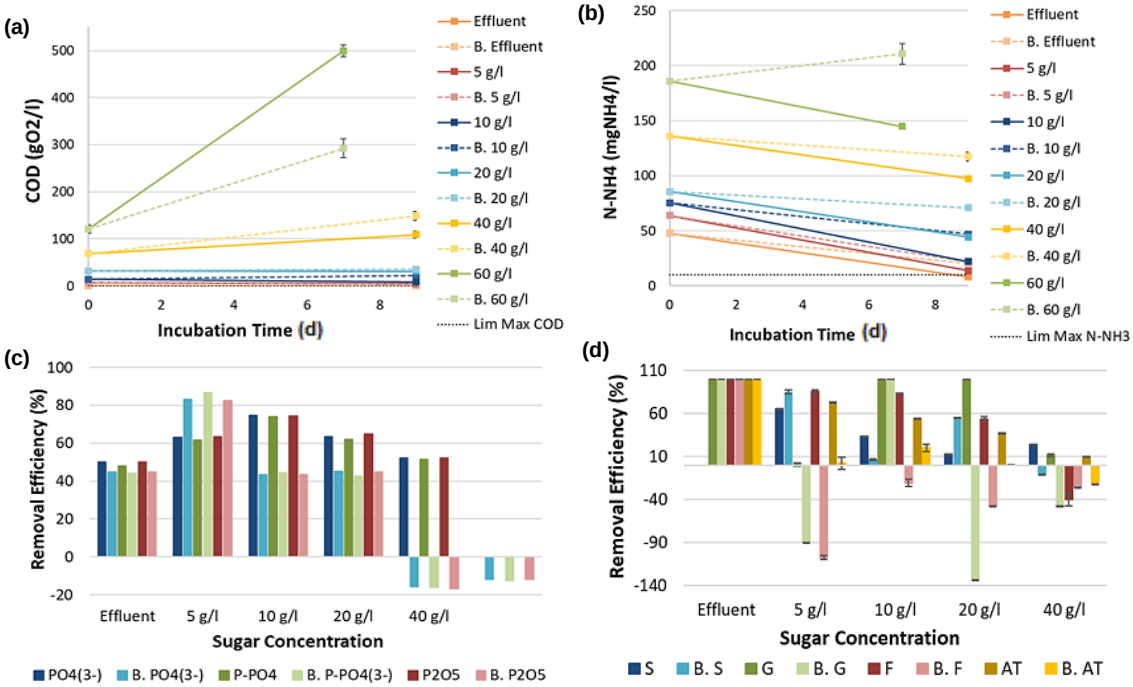

Figure 3.8: (a) Chemical oxygen demand (COD) and (b) Concentration of ammoniacal nitrogen (mgNH4/L), corresponding to initial and final test times. (c) and (d) removal efficiencies of the three phosphorus species and sugars (S-sucrose, G-glucose, F-fructose and AT-total sugars), respectively. B. corresponds to the blank assay.

With the exception of the growth only in effluent, for which the total sugars RE is 100% but the initial concentration is practically zero, the growth in $5\ g/L$ was the one that ensured a greater removal of total sugars, reaching a value of RE of $72{,}9\%$. Even so, the removal of sugars in $10\ g/L$ is also satisfactory ($54{,}8\%$ of RE). The low RE of total sugars found in the $40\ g/L$ assay suggests inhibition by growth inhibitors present in molasses (Freitas et al., 2014b), or by limitation of a nutrient other than C, for example N (Taskin et al., 2016) or O_2, and some evaporation may have occurred simultaneously.

Thus, taking into account the biomass production, the cell viability of *S. obliquus* and *R. toruloides*, the symbiosis between them, the production of intracellular lipids and the treatment of the effluent, we conclude that $10\ g/L$ is the concentration of total sugars in the medium most favorable. The non-sterility condition was then tested for treatment with growth at $10\ g/L$.

3.4. Treatment with Mixed Culture in $10\ g/l$

Under sterile conditions, higher productivities are obtained (Figure 3.9(a)). However, for non-sterility conditions greater μ ($0{,}52\ d^{-1}$ and $0{,}79\ d^{-1}$, respectively for sterility and non-sterility) and lower t_d ($1{,}33\ d$ and $0{,}88\ d$, respectively for sterility and non-sterility) are obtained. It is also observed that for non- sterile conditions the stationary phase is reached much earlier than under sterile conditions, which was to be expected since, in the case of non-sterile medium, the microbial population is much more dense and varied, consisting of *S. oliquus* and *R. toruloides*, but also by all the microorganisms present in the effluent, including microorganisms of several genera, such as bacteria that divide much faster than yeasts and microalgae, and therefore, the consumption of substrates occurs more rapidly.

From fatty acids analysis, it is confirmed that the production of Intracellular lipids is much superior in conditions of sterility than in non-sterility ($17{,}6\%\ (w/w)$ and $1{,}6\%\ (w/w)$, respectively).

Removal of COD is much more efficient under non-sterile conditions (the RE in the sterile and non-sterile assay were, respectively, $35{,}1\%$ and $90{,}7\%$), which is to be expected, since the number of microorganisms in heterotrophy consuming the organic substrates is much higher (Figure 3.10(a)).

Under sterile conditions there is no removal of $N-NH_4$ or NTK loading, whereas, for non-sterility conditions, the loads of these two contaminants are removed, although not very significant: $RE_{N-NH4} = 71{,}0\%$ e $RE_{KTN} = 8{,}1\%$ (Figure 3.10(b) and Figure 3.10(c)). However, it has been observed that sterilization of the medium reduces the $N-NH_4$ and NTK loading, so with this step, the RE is even superior in sterility, however, due to the sterilization step and not the biological treatment, so it has the disadvantage of having associated costs of sterilization. The low N removal may be the result of the nitrogen compounds present in molasses not ensuring the growth of yeasts.

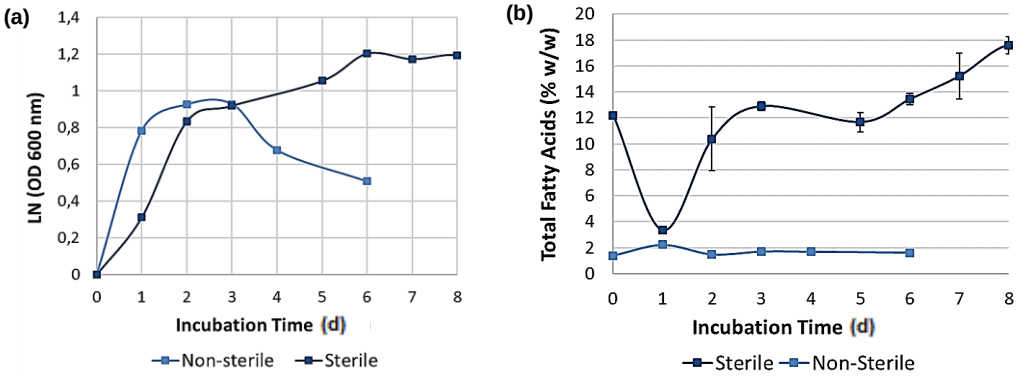

Figura 3.9: (a) Normalized growth curves, to eliminate interference from molasses, variation of OD with incubation time. (b) Result of fatty acid analysis for the tests under sterile conditions and under non-sterile conditions.

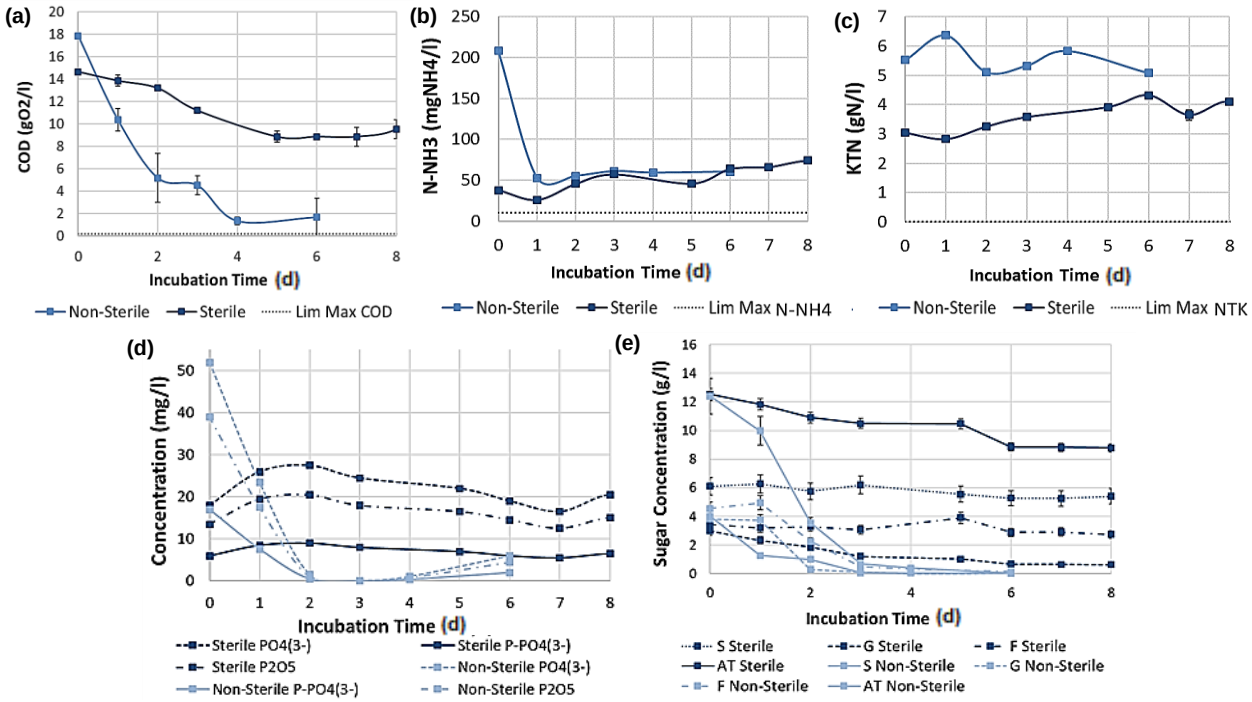

Figura 3.10: (a), (b) and (c): COD, $N-NH_4$ e KTN, variation curves, respectively, from the beginning of the cultures until the stationary phases have been reached, for sterility and non-sterility conditions. (d) and (e): removal efficiencies of the three phosphorus species and sugars (S-sucrose, G-glucose, F-fructose and AT-total sugars), respectively. B. corresponds to the blank assay.

It is concluded, from Figure 3.10(d), that the removal of P is more effective under non-sterility conditions, and that the sterilization step also reduces the loads of the 3 P species. In non-sterile conditions, the P concentrations in the medium become zero, and on day 3, when the culture reaches the death phase, they begin to increase. This may mean that the death phase began as a result of the depletion of the P in the medium. Even so, the fact that at the 2nd day the P has already been removed, is a great advantage compared to the treatment under sterile conditions.

From Figure 3.10(e), it is concluded that the removal of sugars is much more efficient under non-sterility conditions, with complete consumption of sugars available in the medium. For the sterility assay, sucrose is the sugar with the highest RE (79,0% on the 8th day), but probably does not correspond to consumption by the microorganisms, but to their decomposition into glucose and fructose.

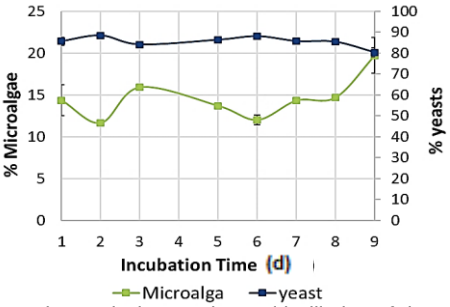

Figure 3.11: The analysis was done with dilution of the samples in PBS and according to the sets of the two microorganisms. The results presented are the average of those obtained with each set.

It can be seen that the ratio between the two populations remains approximately constant throughout the assay and therefore the symbiosis in 10 g/L of sugar in the medium is possible.

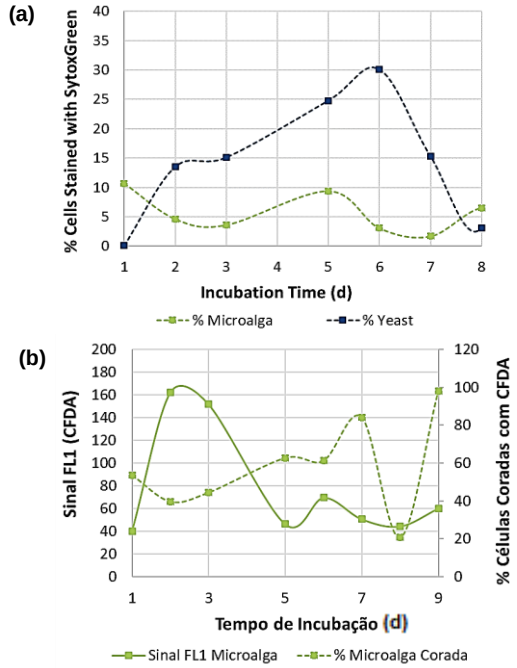

Figure 3.12: (a) Analysis of membrane integrity by dilution in PBS buffer and incubation with 5µL of 30 µM SytoxGreen in the dark for 20 min. (b) Analysis of the enzymatic activity of *S. obliquus* by dilution in McIlvain buffer and incubation with 20µl of 10 mg/ml CFDA in the dark for 50 min.

Figure 3.12(a) shows the existence of the symbiosis relationship between *S. obliquus* and *R. toruloides*, because when the cellular viability of one of the populations is affected, the other is also affected.

It was not possible to evaluate the enzymatic activity of *R. toruloides* by flow cytometry. The enzymatic activity of *S. obliquus* increases until the end of the period of the exponential phase with greater μ, and from there tends to decrease. The percentage of stained cells increases from the 6th to the 7th day of growth, which corresponds to the entry of the culture in the stationary phase and is probably related to the cellular response to the cause of growth inhibition.

In conclusion, it is only possible under sterile conditions to maintain *S. obliquus* and *R. toruloides* cultures, and even symbiosis between them. However, effluent treatment is much more effective under non-sterile conditions, as opposed to the production of intracellular lipids, which was neglegible under non-sterility and quite significant under asseptic conditions.

3.5. Staged Treatment

Once in the presence of organic compounds, *S. obliquus* microalgae opts to follow the myxotrophic pathway, undermining the initial goal of removing compounds such as $N-NH_3$, NTK and P, by autotrophy. Thus, it was thought to make a treatment by stages, in which at an early stage *S. obliquus* and *R. toruloides* contribute to the removal of the organic load from the medium and, in a second phase, when there is substrate limitation, *R. toruloides*, an obligate heterotroph microorganism, enters into the death stage and *S. obliquus* follows the autotrophic pathway, through which it will remove the $N-NH_3$, NTK and P charges. Thus, the assay under sterile conditions in 10 g/L of total sugars in the medium was prolonged until the 14th day of incubation.

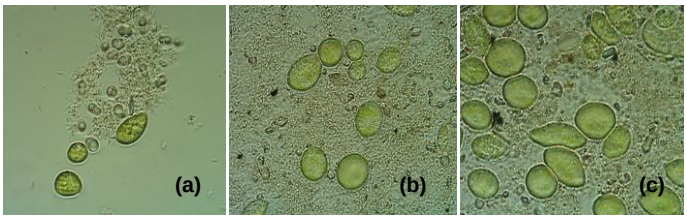

Figure 3.13: Images obtained by optical microscopy, with magnification 100x, for (a) 8th, (b) 9th and (c) 13rd days of growth.

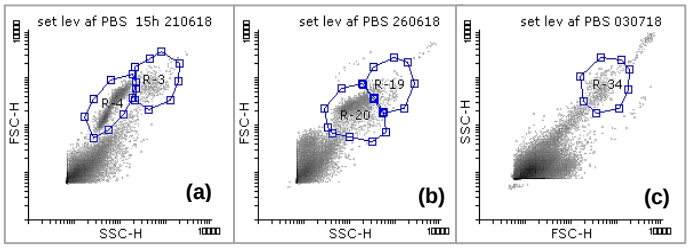

Figure 3.14: Cytograms corresponding to: (a) 1st, (b) 6th and (d) 13th days of growth, for which the regions corresponding to *R. toruloides* are, respectively, R4, R20 and absent for the 13th day; the region corresponding to *S. obliquus* are, respectively, R3, R19 and R34.

When the stationary phase was reached, the number of yeasts was significantly reduced due to a shortage of the C source. However, microalgae become greener and larger (Figure 3.13) revealing that they chose to follow the autotrophic pathway when they do not have available C. From the flow cytometric analysis, it can be seen that *R. toruloides* FSC and SSC signals values were progressively reduced, revealing that yeast population was disappearing due to carbon depletion in the medium. At 13th day the yeast population couldn't be detected (Figure 3.14).

A reduction in the $N-NH_3$ and KTN loading was found, however, not enough for the treatment to be satisfactory. It is necessary that the final concentrations of these contaminants (were 40,6 $mgNH_4/L$ and 2,74 gN/L, respectively) are closer to the corresponding legally required maximum limits (10,0 $mgNH_4/L$ and 15,0 mgN/L, respectively). COD loading increased (the final COD concentration at 8th and 14th days were, respectively, 9,50 gO_2/L and 10,17 gO_2/L), most probably as a consequence of increased cell debris, as a result of the *R.*

toruloides death phase. As for the media load on the three P and sugar types, the removal was not very significant. As for sugars, it is expected, since the microalga follows the autotrophic route. Regarding the removal of P, it was expected that it would have been superior, and the fact that this had not happened could be the result of several causes, namely: the fact that *R. toruloides* was in the death phase, with consequent liberation of P to the medium, which is consistent with the observed COD increase; the fact that *S. obliquus* is not efficient in the removal of P, as was also seen in this work; phenomena of evaporation, more significant at the end of the assay, as a consequence of the smaller volume of medium in the flask; etc.

4. Conclusions

It is concluded that the effluent treatment with mixed culture presents advantages over the treatment with pure cultures of *R. toruloides*, however, it does not present advantages over the treatment with pure cultures of *S. obliquus*. The supplementation of the effluent with sugarcane molasses and the consequent increase of the contaminant load, means that the final treatment result with pure culture of *R. toruloides* and with mixed culture is much less satisfactory than that obtained with pure culture of *S. obliquus* without supplementation. However, the lipid production by *S. obliquus* and *R. toruloides* pure cultures under the most favorable conditions, is inferior to that obtained by the mixed culture, also for the more favorable condition, which is a great advantage of the processing as a mixed culture compared to treatments as pure cultures.

During the work, some limitations were observed, namely: high evaporation rates of the medium; nutritional limitations, the most significant of which is probably the N limitation, since the N effluent load is very low and the nitrogen compounds of sugar cane molasses do not guarantee the growth of yeasts; and the presence of inhibitory compounds in sugarcane molasses. It is true that these factors conditioned the growth and prevented greater biomass production, as well as more effective treatments.

In conclusion, the main objective was not achieved, since in none of the conditions studied was it possible to obtain, after biological treatment, a treated efluente, below the maximum limits legally required for its discharge in nature or for reuse in processes industries. However, high yields of intracellular lipids have been achieved, especially with treatment with mixed culture in medium supplemented with $10\ g/L$ of total sugars, which economically may favor the process.

It should be borne in mind that sugarcane molasses have organic compounds, including organic nitrogen compounds, which are not assimilable by the microorganisms in this work. Therefore, supplementing the effluent with sugarcane molasses conditions the treatment of the effluent, at least in terms of the removal of organic carbon and nitrogen.

From the assay in which the sequential treatment was tested, it was noticed that there were improvements in the results, especially in terms of ***N*** removal, so it is concluded that the biological treatment of the effluent with *S. obliquus* and *R. toruloides* has a future**,** just having to be optimized. A hypothesis is to develop the treatment in sequential mode, instead of mixed culture, in which in a first step the supplemented effluent ($20\ g/L$) is treated by the yeast *R. toruloides*, in order to remove the organic load (COD) and P, and there may be recovery of the process by production of lipid products; in a second step, when the organic load is trace, the yeasts are removed from the medium by separation processes and the *S. obliquus* microalgae population is added to the already partially treated effluent for removal of the $N - NH_3$ and NTK charges.

It would also be interesting to carry out the process developed in this work, under the optimum conditions selected, using a reactor (initially bench scale, in view of the scale increase) with a controlled aeration and agitation system, in order to avoid low mass transfers which are often observed in cultures grown in shaken flasks, and which are responsible for nutritional limitations that reduce the yield and productivity of bioprocesses.

It would be still interesting to develop this work using primary effluent, since the secondary effluent presents a degree of contamination already quite low. Future work aimed at the growth of yeast in secondary effluents of the brewing industry with supplementation of sugarcane molasses, it is essential to supplement the medium with a source of nitrogen.

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