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Converting tequila vinasse diluted with tequila process water into microalgae-yeast flocs and dischargeable effluent

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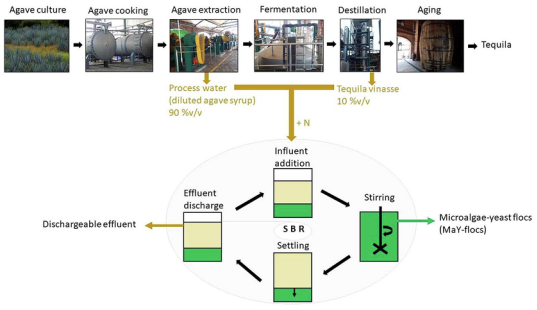
GRAPHICAL ABSTRACT



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ABSTRACT

During tequila production from agave, wastewaters are produced, such as dark-colored vinasse. To add value to this vinasse, microalgae-yeast biomass was produced on vinasse diluted with tequila process water (first rinsing water of agave syrup production). In batch experiments, a vinasse concentration of 10 %v/v resulted in the highest biomass productivity, pH and microalgae growth compared to 20 and 30 %v/v. To ease harvesting, microalgae-yeast flocs (MaY-flocs) were developed in a sequencing batch reactor (SBR). A MaY-floc SBR was run with diluted vinasse (10 %v/v) enriched to 76 mg N-TA L^{-1}, resulting in a doubled biomass productivity (49.5 ± 8.3 mg VSS L^{-1} day^{-1}) of MaY-flocs compared to the best batch reactor performance. Based on

Abbreviations: BOD, biochemical oxygen demand; COD, chemical oxygen demand; CODs, soluble COD; HRT, hydraulic retention time; MaB-flocs, microalgal bacterial flocs; MaY-flocs, microalgae-yeast flocs; $N\text{-}NO_2^-$, nitrite concentration expressed as nitrogen; $N\text{-}NO_3^-$, nitrate concentration expressed as nitrogen; N-TA, total ammonia nitrogen concentration ($N\text{-}NH_4^+$ + $N\text{-}NH_3$) expressed as nitrogen; TN, total nitrogen; OD, optical density; $P\text{-}PO_4^{3-}$, phosphate concentration expressed as phosphorus; TP, total phosphorus; SBR, sequencing batch reactor; SRT, sludge retention time; TSS, total suspended solids; VSS, volatile suspended solids; VDS, volatile dissolved solids; dSVI, diluted sludge volume índex

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response surface experiments, enrichment to 150 mg N-TA L^{-1} and 5.9 %v/v vinasse are recommended. The MaY-floc SBR system is a promising, novel technology to treat tequila wastewaters while producing settleable MaY-floc biomass of interest to aquaculture.

1. Introduction

Tequila is a Mexican regional alcoholic beverage (55 %v/v alcohol) obtained from the fermentation of sugars from the cooked stems of blue agave (*Agave tequilana* Weber var. azul). Tequila production generates tequila vinasses, i.e. liquid residues that remains in the bottom of the still after the distillation of the must of fermented agave. Often these vinasses are discharged in water bodies causing harm to the environment due to their low pH of 3.4–4.5, and high content in phosphates (100–700 mg L^{-1}) and BOD (35,000–60,000 mg L^{-1}) (López-López et al., 2010). For every liter of tequila 10–12 L of vinasse is produced (López-López et al., 2010). Tequila production in Mexico was 309 million liters in 2018, consequently 3090–3708 million liters of tequila vinasses were generated (Regulatory Council of Tequila, 2019), which without proper treatment is equivalent to the annual BOD pollution produced by 8.4 million people (López-López et al., 2010). Approximately 80% of the tequila vinasses are discharged directly into water bodies (rivers, streams, lakes, reservoirs) and municipal sewer systems or directly onto the soil without receiving adequate treatment (López-López et al., 2010). Next to vinasses, tequila distilleries produce also effluents, i.e. process waters resulting from rinsing of tanks of agave extraction (syrup production) and fermentation. Especially micro, small and medium-sized tequila distilleries do not have systems or treatment plants for effectively treating their vinasse and effluents before discharge in waterbodies, mainly because they are faced with limited financial resources and technological challenges. To address these challenges, a new approach for treatment of vinasse and process waters from tequila distilleries should be developed which not only results in a dischargeable effluent, but also recovers wastewater-borne resources into valuable products. The latter provides twin benefits of: (1) offsetting the cost of the treatment and (2) lowering the need to produce fresh supplies of precious resources which consume high amounts of energy and also adversely impact the environment.

Different types of vinasses have been used to grow valuable microalgal biomass. For example, diluted sugarbeet vinasses have been used to grow *Spirulina platensis* (Coca et al., 2015), *Spirulina maxima* (Barrocal et al., 2010), digested tequila vinasse has been used to grow *Chlorella* and *Scenedesmus* sp. (Choix et al., 2018), and diluted sugarcane vinasses have been used to grow *Micractinium* sp. (Santana et al., 2017), *Chlorella vulgaris* (Marques et al., 2013), *Scenedesmus* sp. (Ramirez et al., 2014), *Botryococcus braunnii* (Yeesang and Cheirsilp, 2014), and *Spirulina maxima* (dos Santos et al., 2016). In these previous studies, axenic vinasses were used to avoid contamination of microalgae monocultures with yeast cells or spores. Nevertheless, heterotrophic yeast growth is complementary to autotrophic microalgal growth, in terms of carbon dioxide and oxygen production and uptake. Indeed, provided enough light is available, the high CO_2 levels produced by the yeast should allow a faster autotrophic growth of the microalgae, which in turn can provide high levels of O_2 for an efficient growth of the yeast (Zuccaro et al., 2019). Only few studies exist on the culture of yeast-microalgae biomass on sugar-containing industrial waste effluents, i.e. *Rhodotorula glutinis* with *Chlorella vulgaris* (Cheirsilp et al., 2011) and *Torula maleeae* or *Torula globosa* with *Chlorella* sp. KKU-S2 (Papone et al., 2015), and therefore their true potential is not yet well known (Zuccaro et al., 2019). Certain microalgae could also grow heterotrophically or mixotrophically on sugars present in vinasses, but the assimilation of organic substrates can reduce the photosynthetic CO_2 fixation and O_2 production activity of microalgae (Heifetz et al., 2000). Furthermore, obligate autotrophic microalgae combined with heterotrophic yeast results in a higher biomass production compared to mixotrophic microalgae and heterotrophic yeast (Zuccaro et al., 2019).

To get higher yields in the cultivation of microalgae in vinasse, often a pre-digestion of the vinasse is necessary to lower the content of phenolic compound toxic for certain microalgae species and dark color limiting photosynthesis, but this additional step increases the cost (Candido and Lombardi, 2017). An alternative is diluting the vinasse.

In this study, non-axenic filtered tequila vinasse was diluted with tequila production process water (effluent of extraction process) to grow microalgae-yeast biomass. In a first experiment, a microalgae-yeast consortium was selected in which obligate autotrophic microalgae grow, i.e. microalgae which do not grow mixotrophically at dark on the diluted vinasse but do grow in light. Furthermore, the optimal vinasse dilution was determined.

To lower harvesting costs and to ease reactor operation by decoupling hydraulic retention time (HRT) from sludge retention time (SRT), the growth of microalgae and yeast cells together in a sequencing batch reactor (SBR) to obtain microalgae-yeast flocs (MaY-flocs) which settle fast and efficiently, similar to microalgal bacterial (MaB-flocs) (Van Den Hende et al., 2016a). Therefore, in a second experiment, MaY-flocs were produced in an SBR. To the best of the knowledge of the authors, this is the first report of the growth of MaY-flocs on diluted non-axenic tequila vinasse, and nutrient optimization is still needed. Tequila vinasse has a N:P ratio of 0.09–1.53:1 (López-López et al., 2010). This is very low compared to the general N:P ratio of microalgal biomass of around 16:1 (Redfield, 1934). Therefore, ammonia nitrogen was added to the MaY-floc SBR influent and the biomass and effluent quality were evaluated. In a third experiment, to improve the nutrient removal and the properties of MaY-flocs (productivity and content of chlorophyll and lipids), the nitrogen and vinasse concentrations were optimized by means of response surface methodology.

2. Materials and methods

2.1. Inoculum, vinasse and tequila production process water

Samples of mixed microalgae was collected from an aerated activated sludge reactor from a wastewater treatment plant (Complejo 2, División de Ciencias de la Vida, Universidad de Guanajuato, Irapuato, Guanajuato, Mexico, 20°44′33.9″N 101°20′00.2″W) and grown in Bold's basal medium. In a first period, the inoculum was cultivated in a clear glass media bottle (1 L total volume; 0.5 L effective volume) with magnetic stirring at 20–25 °C for three weeks. This reactor was illuminated with 12:12 h light: dark photoperiods supplied by a 18 W daylight led lamp (Geopower, Mexico) with an intensity of 96 μmol PAR photons m^{-2} s^{-1} (measured at the top of the reactor). In a second period, the culture was aerated at a flow rate of 1 L min^{-1} under the same culture conditions. The biomass growth was monitored by determining total suspended solids (TSS) and volatile suspended solids VSS). For three months, every 15 days, the microalgae inoculum was settled and 90 %v/v was discarded where after fresh culture medium was added.

Tequila vinasse (*Agave tequilana*) was obtained from a local tequila distillery (Tequilera Corralejo S.A. de C.V, Pénjamo, Guanajuato, Mexico, 20°26′59.0.″N 101°37′30.5″W and was directly stored at 4 °C. To remove the remaining suspended solids, agave vinasse was settled and the supernatant was filtered at 1.2 μm with glass fiber filters (Whatman, USA) prior to its dilution and use in experiments. The filtered, undiluted vinasse had a pH of 3.9 and contained 69,560 mg COD_s L^{-1}, 28.0 mg N-TA L^{-1}, < 0.1 mg N-NO_3^- L^{-1}, < 0.1 mg N-

NO_2^- L^{-1}, and 43.6 mg $P\text{-}PO_3^{4-}$ L^{-1}. Agave sugar extraction to produce agave syrup is a step of tequila production in the distillery. To dilute the vinasse, the first rinsing water of agave syrup production was used, which is actually diluted agave syrup. Synthetic process water was prepared using tap water and 0.1 %v/v of concentrated agave syrup (Agave Sweet, Mexico) and further in the text referred to as tequila production process water. This process water had pH of 7.8 and contained 1340 mg COD L^{-1}, < 0.1 mg N-TA L^{-1}, < 0.1 mg N-NO_3^- L^{-1}, and < 0.1 mg $P\text{-}PO_3^{4-}$ L^{-1}. Yeast cells were present in agave vinasses and served as yeast inoculum.

2.2. Microalgae-yeast biomass in batch reactors: vinasse and light

To optimize the growth conditions for microalgae in tequila vinasse, three dilutions of vinasse and two light conditions were screened (Table 1). All three dilution conditions and two light conditions were carried out in triplicate (in total 18 reactors). Dilutions of tequila vinasse (10%, 20% and 30 %v/v) were made by adding tequila production process water. All reactors were inoculated with a 10 %v/v of mixed microalgae inoculum resulting in batch reactors containing (323 ± 12 mg TSS L^{-1} and 250 ± 14 mg VSS L^{-1}). Yeast cells were inoculated by adding yeast-containing vinasse. The initial yeasts/microalgae cells ratio was determined based on the cell count by means of microscopy. Two light conditions were tested: (1) light (mixotrophic growth) similar to the growth conditions as inoculum production and dark by covering reactors with aluminum foil (heterotrophic growth).

Reactors were glass bottles with a volume of 500 mL and effective volume of 250 mL. All reactors were incubated at room temperature (20–25 °C) under continuous stirring at 130 rpm by an orbital shaker (Heathrow Scientific, USA). The pH of the medium was initially set to 7.0 in all reactors (10%, 20%, and 30 %v/v tequila vinasse) by adding 0.5 M NaOH and was measured daily until the end of the experiments. To evaluate the biomass growth, TSS and VSS were measured at the start and the end of the experiment after 15 days.

Statistical analyses were performed using the software R Studio® v1.1.463 and R v3.6.0 (R Foundation for Statistical Computing, Austria). Normality of data was analyzed by a quantile-quantile plot. Homogeneity of variances was analyzed by a Bartlett test. Since all analyzed data showed a normal distribution and homogeneity of variances, differences in means were analyzed with one-way ANOVA and a Tukey posthoc test ($p < 0.05$).

2.3. MaY-flocs in an SBR: nitrogen addition

In the previous experimental stage, microalgae-yeast biomass was obtained. The biomass obtained from the reactor with 10 %v/v of tequila vinasse was used as inoculum to obtain MaY-flocs in an SBR. The yeasts/microalgae cells ratio was determined based on the cell count by means of microscopy. The SBRs had a volume of 1000 mL and an effective volume of 500 mL. It was operated with a hydraulic retention time (HRT) of 10 days. Each cycle lasted 24 h with the following phases: (1) addition of 50 mL of influent (20 min), (2) reaction while stirring (1200 min), (3) settling (180 min), (4) discharge of 50 mL of effluent (20 min), and (5) buffer time to avoid the addition of influent during effluent discharge (20 min). The SBR conditions were: room temperature (20–25 °C); initial pH 7.0; 12 dark: 12 h light at 96 μmol PAR photons m^{-2} s^{-1} (measured at the surface of reactor liquor); stirring at 130 rpm by an orbital shaker (Heathrow Scientific, U.S). Tequila wastewater with 10 %v/v of vinasse and 90 %v/v tequila production process waster was used as influent. To increase the microalgae biomass productivity of the MaY-floc SBRs, from day 28 on, its influent was supplemented with $(NH_4)_2SO_4$ to reach a measured 76.1 ± 1.3 mg N-TA L^{-1}.

The reactor liquor of the SBR was monitored every two days by measuring $chlorophyll_{a+b}$ concentration, pH and OD at 730 nm as an indicator for the total biomass concentration. From day 20 on, once a week, samples were collected from the influent, effluent and MaY-floc biomass. Influent and effluent samples were filtered at 1.2 μm with glass fiber filters (Whatmann, USA), and analyzed for CODs, N-TA, and $P\text{-}PO_3^{4-}$. Biomass samples were analyzed for TSS, VSS, $chlorophyll_{a+b}$ content and dominant microalgae/yeast species. The final MaY-floc biomass was analyzed for lipids.

Means and standard deviations are given of data after adaptation to new reactor conditions and stabilization of reactor performance: of days 8–24 for period 1 without nitrogen addition, and days 36–72 for period 2 with nitrogen addition. Normal distribution of data was screened with a Shapiro-Wilk test and homogeneity of variances with a Levene's test. Data of the first. When normal data distribution and homogeneity of variances were observed, significant differences between means of parameter values of period 1 and 2 were analyzed with a parametric *t*-test; otherwise, a non-parametric Mann-Whitney *U* test was used ($p < 0.05$).

2.4. MaY-flocs in batch reactors: vinasse and nitrogen

To optimize the conditions for the culture of MaY-flocs, response surface methodology was applied. A central composite setup was designed using the software Statgraphics centurion XVI.I ®. Ten experimental conditions were evaluated, using vinasse content and N-TA concentration of the influent as independent variables (Table 2). The experimental conditions were carried out in duplicate in batch reactors, consisting of glass bottles with a volume of 500 mL and an effective volume of 350 mL. MaY-flocs of the SBRs were settled and used as inoculum of all batch reactors of this second experiment, resulting in an initial concentration of 730 ± 28 mg TSS L^{-1} and 676 ± 30 mg VSS L^{-1}. Light was provided by four fluorescent lamps (18 W, Geopower, Mexico) resulting in 96 μmol PAR photons $m^{-2}s^{-1}$. Reactors were stirred at 130 rpm (Heathrow Scientific, USA) at room temperature (21–25 °C).

Batch reactors were monitored by measuring $chlorophyll_{a+b}$

Table 1
Initial (day 0) and final (day 15) pH, TSS, VSS, and biomass productivity of microalgae-yeast batch reactors at two light conditions and fed with tequila vinasse at three different concentrations diluted with tequila production process water.

Light conditions	Vinasse concentration (%v/v)	pH		TSS (mg L^{-1})		VSS (mg L^{-1})		VSS/TSS (%)		Biomass productivity (mg L^{-1} d^{-1})	
		Initial	Final	Initial	Final	Initial	Final	Initial	Final	TSS	VSS
Light	10	7.0 ± 0.1 ^{a}	8.5 ± 0.1 ^{a}	317 ± 10 ^{a}	760 ± 37 ^{a}	244 ± 11 ^{a}	680 ± 39 ^{a}	77.1 ± 2.1^{a}	89.4 ± 1.1 ^{a}	30 ± 2 ^{a}	29 ± 2 ^{a}
Light	20	7.0 ± 0.1 ^{a}	7.9 ± 0.1^{b}	331 ± 14 ^{a}	725 ± 30 ^{a}	257 ± 17 ^{a}	645 ± 23 ^{a}	77.7 ± 2.2 ^{a}	89.1 ± 1.3 ^{a}	26 ± 3 ^{a}	26 ± 2 ^{a}
Light	30	7.1 ± 0.1 ^{a}	6.3 ± 0.1^{c}	323 ± 11 ^{a}	465 ± 15^{c}	248 ± 14 ^{a}	381 ± 19^{c}	76.9 ± 1.9 ^{a}	81.9 ± 1.8 ^{a}	9 ± 0^{c}	9 ± 0^{b}
Dark	10	7.0 ± 0.1 ^{a}	6.1 ± 0.3 ^{cd}	350 ± 39 ^{a}	400 ± 29 ^{d}	231 ± 21 ^{a}	257 ± 19 ^{d}	66.4 ± 4.7 ^{a}	64.4 ± 5.6^{b}	3 ± 1^{d}	2 ± 0^{c}
Dark	20	7.0 ± 0.2 ^{a}	5.8 ± 0.2 ^{de}	365 ± 25 ^{a}	481 ± 15^{c}	262 ± 13 ^{a}	319 ± 37 ^{cd}	71.9 ± 5.5 ^{a}	66.1 ± 6.3^{b}	8 ± 1^{c}	4 ± 2^{c}
Dark	30	7.0 ± 0.1 ^{a}	5.5 ± 0.1 ^{e}	371 ± 35 ^{a}	628 ± 24^{b}	249 ± 31 ^{a}	452 ± 37^{b}	67.0 ± 3.9 ^{a}	71.9 ± 3.4^{b}	17 ± 4^{b}	14 ± 4^{b}

Different letters indicate significant differences between means of the same parameter according to one-way ANOVA and a Tukey posthoc test ($p < 0.05$).

Table 2
Biomass characteristics of MaY-flocs grown on 10 %v/v tequila vinasse diluted with process water and enriched with nitrogen, compared to MaB-flocs grown on effluent of the manure treatment, aquaculture, chemistry and food production industry (Van Den Hende et al., 2016b).

Parameter	Unit	MaY-flocs	MaB-flocs			
		Tequila	Manure	Aquaculture	Chemistry	Food
Biomass productivity	mg TSS $L_{reactor}^{-1}$ day^{-1}	56.1 ± 10.3	136 ± 100	236 ± 73	236 ± 294	257 ± 116
Biomass productivity	mg VSS $L_{reactor}^{-1}$ day^{-1}	49.5 ± 8.3	68 ± 67	109 ± 30	122 ± 141	223 ± 90
TSS of reactor liquor	mg TSS L^{-1}	1008 ± 56	1109 ± 343	1830 ± 333	980 ± 331	1407 ± 410
VSS of reactor liquor	mg VSS L^{-1}	896 ± 67	544 ± 143	782 ± 127	612 ± 145	1208 ± 295
VSS:TSS	%m/m	88.8 ± 1.9	50.0 ± 5.0	43.3 ± 6.1	64.9 ± 10.5	87.0 ± 7.8
Settled floc density	mg TSS L^{-1}	2949 ± 143	6700–1100	5200–3700	7000–24000	12000–15000
Settled floc density	mg VSS L^{-1}	2617 ± 174	3350–5500	2252–1602	4543–15576	10440–13050

concentration and pH until the stationary stage was reached at day 20. At the start and end of the experiment (day 20), reactor liquor samples were taken and analyzed for TSS, VSS and chlorophyll$_{a+b}$ concentration, lipid content and carbohydrate content. Reactor supernatant (after settling the reactor liquor for 3 h) were taken, filtered at 1.2 μm with glass fibre filters (Whatmann, USA) and analyzed for CODs, VDS, N-TA, and P-PO_3^{4-}.

Response surface modeling was used to determine the best influent conditions. The response variables were VSS productivity, total chlorophyll$_{a+b}$ concentration, total lipid content of the biomass, and removals of CODs, VDS, N-TA and P-PO_4^{3-}.

To generate the response surfaces, first and second degree polynomial-based models were first considered. Nevertheless, these polynomial-based models did not fit the experimental data correctly. Natural Neighbor interpolation was then considered both, to model, visualize and better understand the experimental data, and to estimate the value of the response variables at unsampled conditions (Park et al., 2006). Since the interpolated function is exact, the residual between the experimental data and the estimated one is zero, assuring the correct fit. In this work the Natural Neighbor interpolation method was implemented by using the Matlab® function *scatteredInterpolant*. Since the interpolated function is C^1 continuous, optimal conditions were computed by the gradient-based line search optimization method (Nocedal and Wright, 2006). The response surfaces were plotted in Matlab® 2019. A mesh considering the independent variables was first generated using the function *meshGrid*. The response surfaces were then plotted by the function *surf*, while the experimental data were plotted by the function *plot3*.

2.5. Analytical procedures

The TSS, VSS, VDS and dSVI concentrations were analyzed according to standard methods (APHA et al., 2012). For biochemical biomass characterization, 25 mL of biomass sample was centrifuged at 6000 rpm for 10 min. Then the supernatant was removed and 25 mL of NaCl 0.9 %w/v was added to wash the biomass. This procedure was repeated three times in order to avoid interferences of the supernatant on the biomass characterization. The total carbohydrate concentration was determined by the phenol sulfuric acid method, using glucose as a standard according to Dubois et al. (1956). The total lipid content was determined using the Bligh and Dyer (1959) method which has been previously reported for analysis of microalgae biomass.

The total chlorophyll$_{a+b}$ concentrations of reactor liquor were determined on untreated biomass according to the method and the equations reported by Pruvost et al. (2009). Chlorophyll was extracted with methanol (99.9%) during 30 min in the dark at 45 °C where after samples were centrifuged centrifuged (13000 rpm, 5 min). OD were measured at 652, 665 and 750 nm by spectrophotometer (UV/visible; LAMBDA XLS PerkinElmer, USA). OD at 652 and 665 nm were corrected for turbidity by subtracting the OD values at 750 nm (Pruvost et al, 2009). All chlorophyll analyses were performed in triplicate. The PAR photon flux densities (PPFD) in the reactors were measured using a lux meter (Steren, Mexico) and converting the value in lux to μmol photons m^{-2} s^{-1}. For the taxonomic identification of yeast and microalgae based on morphology, a microscope (LEICA DM500, Germany) with the image acquisition system (LEICA ICC50 HD, Germany), identification guides were used (Prescott, 1984; Prescott et al., 2013).

The pH was measured *in situ* using a pH probe (HANNA® instruments, Italy). The CODs, N-TA, P-PO_3^{4-}, N-NO_2^- and N-NO_3^- were analyzed using colometry test kits of Hach (10237, 10206, 8000 and 10127, respectively; Germany). In this work, N-TA represents the total ammonia including both NH_4^+ and NH_3 in nitrogen equivalent.

3. Results and discussion

3.1. Microalgae-yeast biomass in batch reactors: vinasse and light

Tequila vinasse has a low pH of e.g. 3.4–4.5 (López-López et al., 2010) which not all microalgae tolerate (Van Den Hende et al., 2012). It contains certain phenolic compounds (López-López et al., 2010), which at high concentrations are toxic for microalgae (Olguín et al., 2015). Furthermore, it has a dark color which limits photosynthesis. Diluting vinasse addresses these problems, but the dilution which is optimal for microalgae growth needs to be determined. Therefore, in this study tequila vinasse concentrations of 10, 20 and 30 %v/v were diluted with tequila process water, the pH of the medium was set to 7.0 and used to grow microalgae yeast biomass on. Initially, all batch reactors presented an initial biomass ratio of less than 1 yeast cells per 100 cells of microalgae, and after 15 days of reaction, all reactors showed an increase on yeast cells, reaching ratios above of 9 yeast cells for each microalgae cell.

After 15 days in light conditions, vinasse concentrations of 10 and 20 %v/v showed a significant higher TSS and VSS concentration, and biomass productivity compared to 30 %v/v (Table 1). Furthermore, with a vinasse concentration of 10 %v/v a significant higher pH was obtained compared to all other dilutions (Table 1). A higher pH suggests a stronger photosynthetic activity as photosynthesis by microalgae increases the pH due to uptake of CO_2 (Van Den Hende et al., 2012). In contrast, yeast growth produces CO_2 which decreases the pH (Coote and Kirsop, 1976). The reactor with 10 %v/v vinasse also showed the highest abundance of microalgae, i.e. between 6 and 15% of the microorganism biomass (based on the cell count of microalgae and yeast cells), this means a yeast/microalgae ratio of 9.5 ± 4 yeast cells for each microalgae cell, whereas in the reactor with 30 %v/v vinasse less than 1% of the biomass cells consisted of microalgae.

Some microalgae species can grow heterotrophically on organic carbon present in diluted vinasse at dark, e.g. *Micractinium* sp. grew on 2–20 %v/v sugarcane vinasse (Kose-Engin et al., 2018). However, the assimilation of organic carbon by heterotrophic microalgae can reduce the photosynthetic CO_2 fixation and O_2 production of the algae, which may affect the efficacy of the intended system in terms of microalgae-yeast biomass (Zuccaro et al., 2019). Therefore, obligate autotrophic

microalgae species are preferred in microalgae-yeast production systems to avoid any overlap in trophic modes (Zuccaro et al., 2019). To verify if the microalgae consortia selected in light conditions in this study were growing obligate autotrophically on tequila vinasse, batch reactors with 10, 20 and 30 %v/v vinasse were run in parallel without light. After 15 days of reactor operation, no microalgae growth was observed in any of the latter reactors in dark (microscopic observation). The final pH of all these reactors was significantly lower than the initial pH (Table 1), which can be attributed to yeast and/or bacterial growth observed in the reactors at dark. This data demonstrates that the microalgae consortia screened in this study can only grow obligate autotrophically on the tequila wastewaters used, alike as aimed at. Further study is needed to determine the species of these consortia, but chlorophyte microalgae and diatoms were present in the consortia, while no cyanobacteria were observed (determined based on their morphology observed by microscopy).

In conclusion, this experiment shows that to maintain a neutral pH in the reactor in order to obtain a dischargeable effluent, it is important to provide light so that microalgae can grow autotrophically to damp the drastic pH reduction by yeast growth. Furthermore, medium consisting of 10 %v/v tequila vinasse diluted with tequila production process water is recommended above 20 or 30 %v/v. However, the produced biomass was still dominated by yeast cells (identified as *Saccharomyces* sp.). This could be due to the too low levels of nitrogen present in the tequila vinasse (2.8–8.4 mg N-TA L^{-1} in contrast to, for example, Basald Bold medium which contains 40 mg $N\text{-}NO_3^-$ L^{-1}).

3.2. MaY-flocs in an SBR: nitrogen addition

To ease the biomass harvesting, microalgae-yeast (MaY-flocs) flocs were developed, similar to microalgal bacterial flocs (MaB-flocs) (Van Den Hende et al., 2016a) by operating in an SBR instead of a batch reactor. MaY-flocs settle by gravity. Based on the results of experiment 3.1, 10 %v/v agave vinasse diluted with tequila production process water was used as influent for the MaY-floc SBR. During the first 27 days of SBR operation (period 1), the $chlorophyll_{a+b}$ content of reactor liquor remained stable with an average of 2.76 ± 0.60 mg L^{-1} (Fig. 1a). During this period, the SBR contained 640 ± 57 mg TSS L^{-1} and 525 ± 46 mg VSS L^{-1}. This means that the SBR biomass contained around 0.526 mg of $chlorophyll_{a+b}$ 100^{-1} mg^{-1} VSS. Photosynthetic microorganisms have a chlorophyll content of around 1–2 mg 100^{-1} mg^{-1} VSS (Imberger, 1998). These low levels could be due to the low level of available nitrogen present in diluted vinasse (2.8 mg N-TA L^{-1} at 10 %v/v of vinasse). The pH increased, but after the first week (Fig. 1.b), but hereafter stabilized around 8.12 ± 0.23. This increase is due to photosynthesis. To be able to discharge treated wastewater in Mexico, the pH must be within the current norm range of 5–10 (SEMARNAT, 1996). The effluent of this study was within this range.

The molar N:P ratio of vinasse is within the range of 0.09–1.53:1 (López-López et al., 2010). In this study, the N:P ratio of the influent during period 1 was 0.48:1. This is very low compared to the molar N:P ratio of microalgal biomass of around 16:1 (Redfield, 1934). Therefore, from day 28 till day 72 of the SBR operation (period 2), nitrogen was added to the influent to investigate whether this addition could increase the chlorophyll content of MaY-flocs without increasing the effluent pH too much to still enable it discharge. During period 2, the $chlorophyl_{la+b}$ content of the reactor liquor steeply increased (Fig. 1.a), where after it stabilized at 20.45 ± 3.65 mg L^{-1}. The latter is a significant increase of seven times in period 2 compared to period 1, whereas the biomass content of the reactor liquor only doubled to 1008 ± 56 mg TSS L^{-1} and 896 ± 67 mg VSS L^{-1}. The

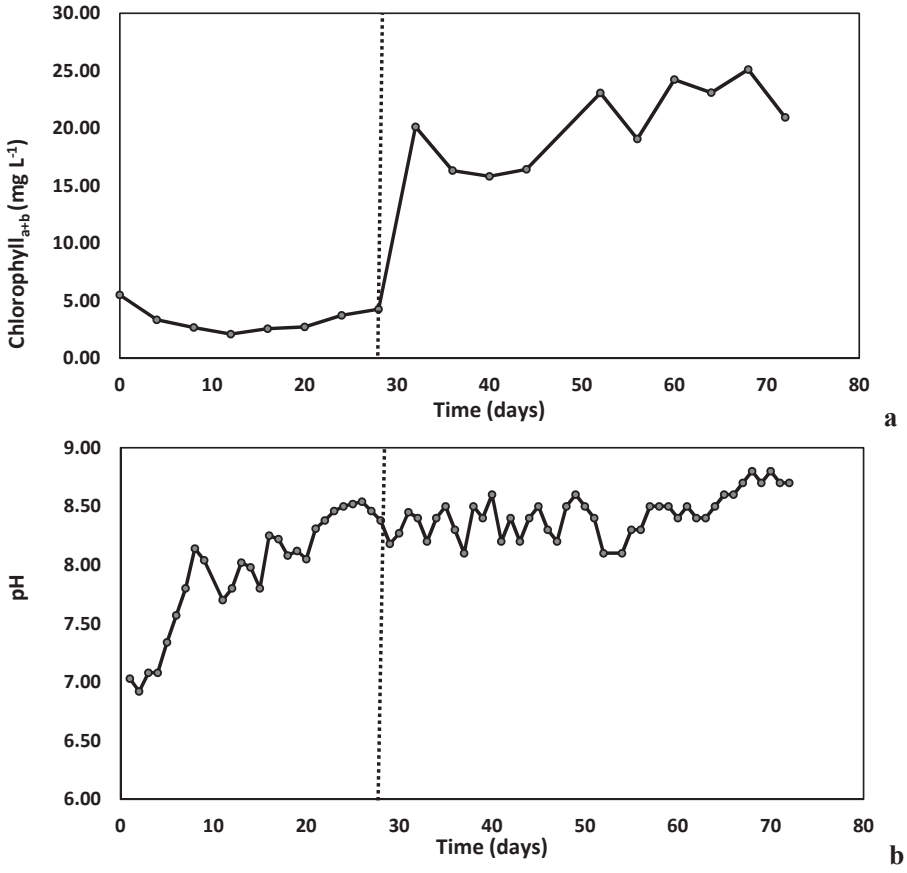

Fig. 1. Performance of the MaY-floc SBR fed with 10 %v/v vinasse diluted with tequila process water during period 1 without nitrogen addition (days 0–27) and during period 2 with nitrogen addition (days 28–72): (a) $chlorophyll_{a+b}$ and (b) pH.

chlorophyll$_{a+b}$ content of the biomass increased four times to 2.82 mg of chlorophyll$_{a+b}$ 100^{-1} mg^{-1} VSS. The dominant microorganisms present in MaY-flocs were microalgae and yeast cells. The pH of the reactor liquor stabilized at 8.45 ± 0.19. Tolerance of yeast genera as *Saccharomyces* sp. to an alkaline pH has been reported (Peña et al., 2015; Zheng et al., 2012). It did not significantly increase during period 2, still allowing effluent discharge with respect to the pH.

Reactor operation as SBR and addition of nitrogen doubled the MaY-floc VSS productivity (Table 2) compared to batch reactor without nitrogen addition (Table 1). VSS productivity of MaY-flocs grown in an SBR fed with diluted tequila vinasse enriched with nitrogen at laboratory scale was similar to MaB-flocs grown on manure-processing effluent, but 2, 3 and 5 times lower compared to MaB-flocs on wastewater grown of the aquaculture, chemistry and food-processing industry, respectively (Table 2). The VSS:TSS of MaY-flocs is 2–1.5 times higher than MaB-flocs grown on effluent from manure treatment, chemistry and aquaculture industry (Table 2). A high VSS:TSS ratio is of interest for inclusion of the biomass in feed. MaY-flocs settled by gravity. The Diluted Sludge Volume Index (dSVI) in mL g TSS^{-1} (after 30 min of settling) was also determined. It was of 95 ± 3 mL g TSS^{-1}, and it was in line with the values obtained for MaB-floc biomass from food-industry effluents (113 ± 18 mL g TSS^{-1}) reported by Van Den Hende et al. (2016a).

Another important parameter is the density of the settled flocs, as dewatering is an important cost of production of dried microalgae biomass (Vulsteke et al., 2017). This value was in the same ranges as MaB-flocs treated on aquaculture wastewater, but 2–4 times lower than the other MaB-flocs (Table 2). The MaY-flocs in period two (weekly samples of day 52 to 72) contained 25 ± 3 mg lipids 100^{-1} mg^{-1} VSS. This is double compared MaB-flocs grown on aquaculture wastewater-grown MaB-flocs which contained 9 mg lipids 100^{-1} mg^{-1} VSS (Van Den Hende et al., 2016b). In future research it should be verified whether decreasing the HRT of the MaY-floc SBR combined with operation outdoors with an increased light intensity increases the settling density of MaY-flocs while maintaining the relatively high lipid content.

Next to biomass characteristics, also the nutrient removal and effluent quality is of importance. Therefore, during period 2 from day 41 to 72, the COD, nitrogen and phosphorus of influent and effluent were measured. The influent contained 9267 ± 7 mg COD$_s$ L^{-1}, while the effluent only 1415 ± 48 mg COD$_s$ L^{-1}. This means a removal efficiency of 84.7 ± 0.5%. Future experiments should be performed to verify whether this remaining COD in the effluent is recalcitrant or/and if the high COD values in the effluent were due to N or P limitation for the biomass. Furthermore, biological oxygen demand in 5 days (BOD$_5$) should be measured, as currently there are only Mexican norms for BOD$_5$ (< 30–200 mg L^{-1}) and not for COD. Tequila wastewater is mainly composed of complex carbohydrates and, in a minor proportion, by simple carbohydrates, such as glucose and fructose (España-Gamboa et al., 2011). The latter can be uptaken by yeast and certain species of microalgae as carbon source. For example, *Scenedesmus* sp. can grow under mixotrophic and heterotrophic conditions, using the organic carbon presents in wastewater and anaerobic digestate (Mai-Linh et al., 2019).

As for phosphorus, the influent contained 16.3 ± 0.3 mg P-PO_3^{4-} L^{-1} and the effluent contained 2.6 ± 0.2 mg P-PO_3^{4-} L^{-1}. This means a TP removal efficiency of 84.0 ± 1.6%. The Mexican norms for discharge of treated wastewater range from 5 to 30 mg TP L^{-1} The effluent of the MaY-floc SBR complied with the norm. As for nitrogen, the influent contained 76.1 ± 1.3 mg N-TA L^{-1} and the effluent contained 0.4 ± 0.3 mg N-TA L^{-1}. This means a N-TA removal efficiency of 84.0 ± 1.6%. The Mexican norms for discharge of treated wastewater range from 15 to 60 mg TN L^{-1}. In this study only N-TA was measured. Removal of N-TA could be via volatilization of N-TA, uptake by microorganisms and/or conversion via nitrification. Different authors have reported that both microalgae and yeast are capable to use ammonia as nitrogen source (Mai-Linh et al., 2019; Huang et al., 2018;

Table 3
Experimental conditions and responses of MaY-flocs grown on different tequila vinasse and nitrogen concentrations.

Experimental conditions			Experimental results							
Reactor name	Tequila vinasse concentration (%v/v)	N-TA (mg N-TA L^{-1})	Biomass productivity (mg VSS L^{-1} d^{-1})	Final chlorophyll$_{a+b}$ content of the reactor liquor (mg chlorophyll$_{a+b}$ g^{-1} VSS)	Final pH of the reactor liquor	Lipids (mg Lip. g^{-1} VSS)	COD removal (%)	VDS removal (%)	N-TA removal (%)	Total P-PO_4^{3-} removal (%)
V5.9_N150	5.9	150	47.3 ± 7.4	15.0 ± 0.5	8.5 ± 0.1	215 ± 38.0	85 ± 7.0	84 ± 2.6	99 ± 0.1	82 ± 2.9
V34_N150	34	150	72.0 ± 9.3	2.4 ± 0.2	8.2 ± 0.1	89 ± 16.4	66 ± 3.3	66 ± 9.0	99 ± 0.5	79 ± 0.7
V20_N100	20	100	32.3 ± 13.6	4.1 ± 0.5	8.6 ± 0.0	44 ± 7.1	76 ± 2.5	49 ± 3.4	98 ± 0.8	91 ± 3.3
V10_N100	10	100	55.6 ± 2.1	4.0 ± 0.3	8.9 ± 0.07	32 ± 13.6	77 ± 0.0	73 ± 5.9	98 ± 2.6	89 ± 0.2
V10_N200	10	200	37.8 ± 3.8	6.9 ± 0.3	8.7 ± 0.07	186 ± 64.4	79 ± 5.7	81 ± 2.6	98 ± 0.3	98 ± 0.1
V20_N100	20	100	32.8 ± 1.7	4.9 ± 0.3	8.3 ± 0.3	119 ± 13.6	62 ± 11.3	68 ± 5.8	96 ± 1.1	89 ± 0.7
V20_N220	20	221	75.3 ± 24.3	3.8 ± 0.1	8.4 ± 0.5	192 ± 72.3	55 ± 12.7	71 ± 7.8	98 ± 1.0	87 ± 5.3
V30_N100	30	100	26.9 ± 20.0	2.8 ± 0.4	8.1 ± 0.07	127 ± 33.7	69 ± 6.8	58 ± 5.4	97 ± 0.5	81 ± 0.2
V30_N200	30	200	65.7 ± 10.5	3.3 ± 1.2	8.1 ± 0.0	272 ± 83.4	68 ± 6.3	83 ± 6.1	99 ± 0.4	77 ± 1.2
V20_N079	20	79	52.4 ± 7.8	3.3 ± 0.4	8.2 ± 0.0	162 ± 23.6	58 ± 15.5	74 ± 3.4	91 ± 1.3	83 ± 0.1

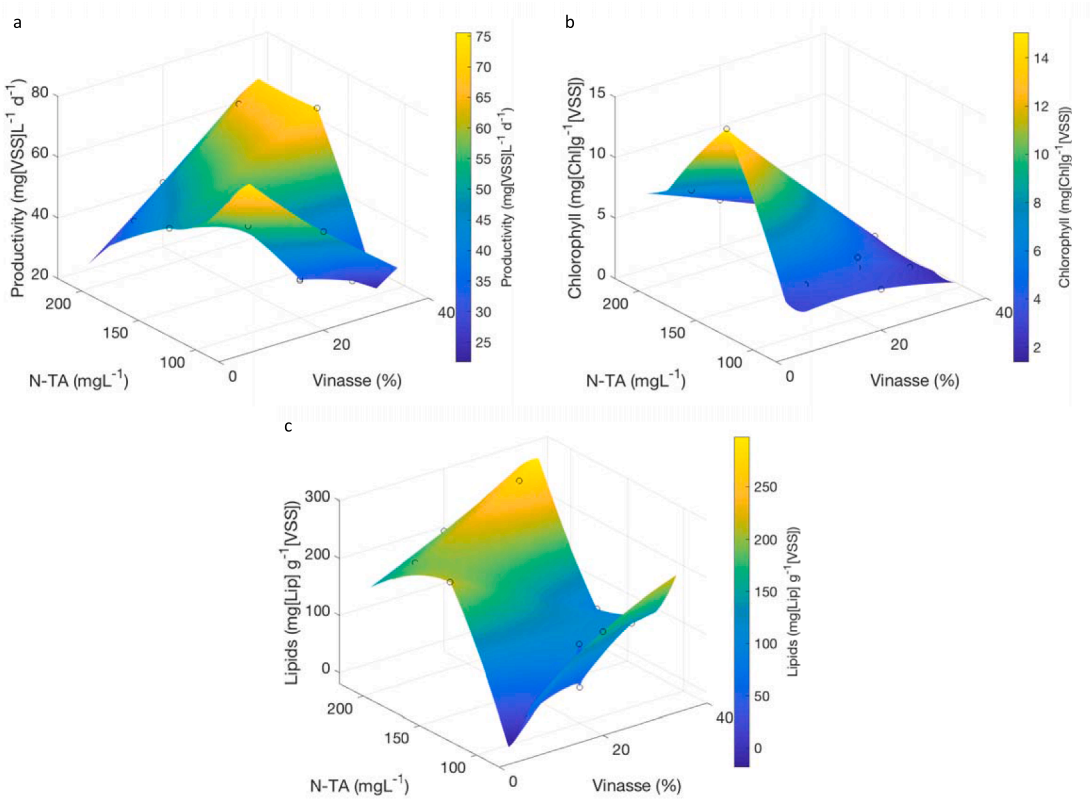

Fig. 2. Response surface models for VSS productivity (a), total chlorophyll$_{a+b}$ (b) and content of lipid (c) of the MaY-flocs with N-TA and vinasse concentration as independent variables.

Zheng et al., 2012). The N-TA concentration for the effluent is very low.

In conclusion, MaY-flocs were successfully developed and grown in an SBR on tequila wastewaters enriched with nitrogen. Nevertheless, the MaY-floc SBR operation needs to be optimized, especially with respect with nitrogen enrichment and vinasse dilution, to increase biomass productivity while reaching a good effluent quality.

3.3. MaY-flocs in batch reactors: vinasse and nitrogen

In the response surface experimental design (Table 2), vinasse and ammonia nitrogen concentrations were selected as independent variables to know their effect on the VSS productivity of MaY-flocs, the chlorophyll$_{a+b}$ concentration of the reactor liquor, the lipids content of the total biomass and the removal of CODs, VDS, N-TA and P-PO_3^{4-}.

The biomass productivity of the MaY-flocs reached the highest value of 75.6 mg VSS L^{-1} d^{-1} at 79.3 mg N-TA L^{-1} in the area corresponding to the lowest vinasse concentrations (5.9 %v/v) (Table 3). However, the highest chlorophyll content of MaY-flocs and the highest N-TA removal were obtained with 5.9 %v/v vinasse and 150 mg N-TA L^{-1}.

In contrast, the highest values of the chlorophyll$_{a+b}$ content of reactor liquor were reached in the area corresponding 100–150 mg N-TA L^{-1} and the lowest vinasse concentrations of 5–15 %v/v (Table 4; Fig. 2b). In the range of 25–35 %v/v vinasse concentration, no microalgae were observed in the MaY-flocs (light microscopy). These results confirm the findings of experiment one that a tequila vinasse concentration of around 30 %v/v it too high if microalgal growth is aimed at. The peak of chlorophyll$_{a+b}$ (15 mg g^{-1} VSS or 1.5 mg 100^{-1} mg^{-1} VSS) was achieved at 5.9 %v/v of vinasse and 150 mg N-TA L^{-1}. Above 180 mg N-TA L^{-1}, the chlorophyll$_{a+b}$ concentration content of the reactor liquor decreased (Fig. 2b). This could be explained by the inhibition on microalgae growth due to too high ammonia concentrations. The highest pH increase was reached in the batch reactor with 10 %v/v vinasse and 100 mg N-TA L^{-1} (Table 4). This stronger pH increase could be explained by an increased photosynthetic activity.

If the N-TA tolerance of the selected microalgae and yeast species are compared, the latter showed a higher level of tolerance, since, it reached its highest VSS productivity values at 200 mg N-TA L^{-1} (Fig. 2a). In fact, the surface response model shows that in the concentrations range of N-TA studied, the maximum VSS productivity value was not yet achieved, suggesting that the inhibitory ammonia concentrations for yeast growth are in a higher range. The tolerance of certain yeast species at high concentrations of ammonia has already

Table 4
Optimal operating conditions obtained from response surface models.

Response variable	Maximum value	Optimum vinasse (%v/v)	Optimum N-TA (mg L^{-1})
Productivity (mg VSS L^{-1} d^{-1})	76	5.9	79
N-TA removal (%)	99	5.9	150
Chlorophyll (mg chlorophyll$_{a+b}$ g^{-1} VSS)	15	5.9	150
Total P-PO_4^{3-} removal (%)	100	8.7	219
COD removal (%)	85	5.9	150
Lipids (mg lipids g^{-1} VSS)	298	34	202
VDS removal (%)	85	5.9	79

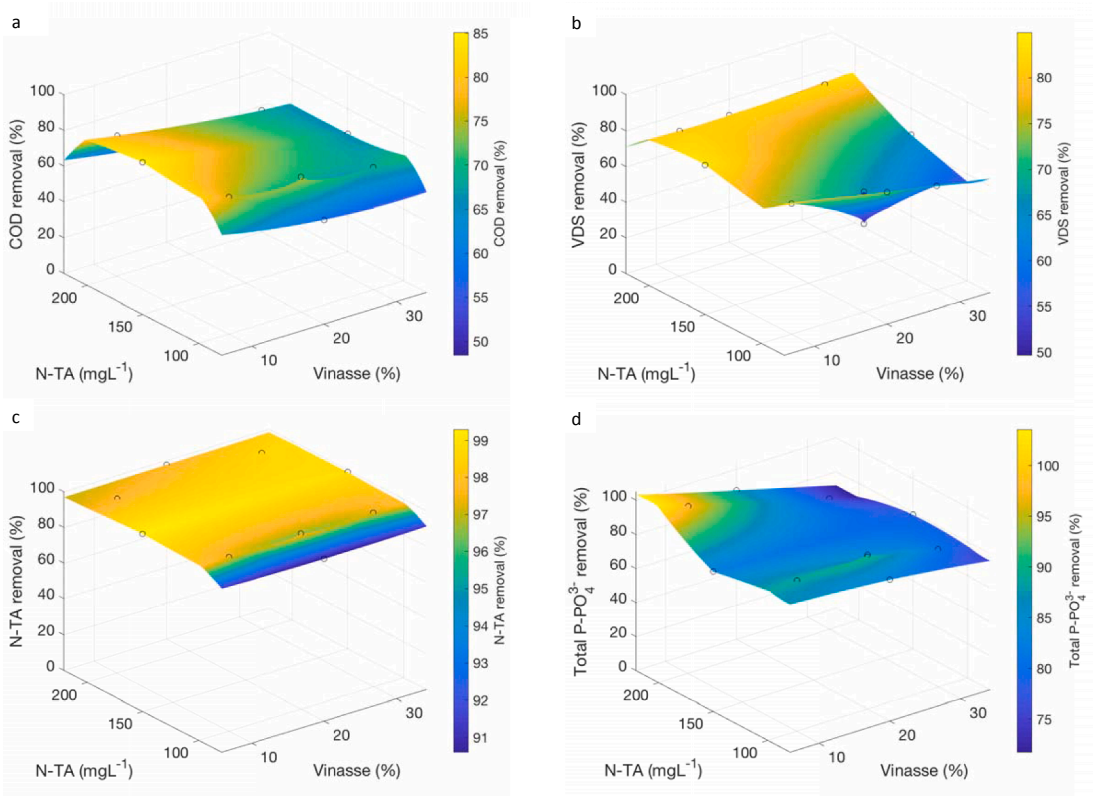

Fig. 3. Response surface models for removal of CODs (a), VDS (b), N-TA (c) and P-PO_3^{4-} (d) of the MaY-flocs with N-TA and vinasse concentration as independent variables.

been reported in the literature. For example, the oleaginous yeast *Cryptococcus curvatus* was capable to grow in up to 785 mg N-TA L^{-1} at a pH of 7.5 and temperature of 30 °C (49.2 mg N-NH_3 L^{-1}) using glucose and acetate as carbon source (Zheng et al., 2012). Likewise, *Cryptococcus curvatus* grew on acetate and 790 mg N-TA L^{-1} at a pH of 6.0 and temperature of 30 °C (24 mg N-NH_3 L^{-1}) (Huang et al., 2018).

With respect to the organic matter, COD and VDS removals show similar profiles (Table 4; Fig. 3a; b). In both cases, removal efficiencies lower than 65% were obtained in the area of a vinasse concentration of above 20 %v/v and a N-TA concentration of below 140 mg N-TA L^{-1}. The N-TA removal efficiencies were similar in all the batch experiments varying between 94 and 99% (Fig. 3c). In contrast, the P-PO_3^{4-} removal efficiency peaked (100%) when the nitrogen concentration was above 200 mg N-TA L^{-1} and with the lowest vinasse concentrations (Table 4; Fig. 3d). Overall, these are very high removal efficiencies which is of importance for discharge of the treated wastewater.

Not only the chlorophyll content and nutrient removal of MaY-flocs, but also their proximal composition is of importance. This study demonstrates that it is feasible to obtain a considerable lipid content of around 25 mg lipids 100^{-1} mg^{-1} VSS (250 mg lipids g^{-1} VSS) in MaY-flocs with a tequila wastewater with a nitrogen concentration of 125–200 mg N-TA L^{-1} (Table 4; Fig. 2c). This result is similar to MaY-flocs in SBR. In the oleaginous yeast *Cryptococcus curvatus*, ammonia nitrogen inhibits (from 26 to 67% of inhibition) the lipid production in the presence of different carbon sources compared to nitrate (Zheng et al., 2012). It is of interest to verify whether nitrate addition and/or addition of nitrifying bacteria would increase the lipid content of MaY-flocs. In addition, unlike the studies found in literature (Cheirsilp et al., 2011; Papone et al., 2015; Zuccaro et al., 2019), the results of the present study were obtained with a non-oleaginous yeast, which suggests that the yield lipid production could be improved if an oleaginous yeast is used. In this study, the highest lipid contents were obtained regardless the vinasse concentration at the screened ranges (Fig. 2c) and the obtained chlorophyll contents (Fig. 2b).

Based on the above findings, a nitrogen content of 150 mg N-TA L^{-1} combined with a vinasse concentration of 5.9 %v/v can be recommended for the culture of microalgae-rich MaY-flocs in tequila wastewater, with COD and N-TA removal efficiencies above 84% (Table 4).

A balance between microalgae and yeast cells in MaY-flocs is wanted, as microalgae and yeast cells are both of interest to the commercial aquaculture sector. Yeast are rich in beta-glucan. The latter compound is already being used as an immunostimulatory additive in commercial aquaculture (Vetvicka et al., 2013), and is quite commonly used in Ecuador, for example during transport of shrimp post-larvae from hatcheries to shrimp ponds. Furthermore, the techno-economic potential of MaY-flocs as supplement in shrimp feed to increase shrimp coloration should be investigated, similar to MaB-flocs (Van Den Hende et al., 2016b). On the other side, optimization of the MaY-floc culture conditions should be part of future research to steer the biochemical biomass composition of these innovative flocs.

4. Conclusions

In batch experiments, a vinasse concentration of 10 %v/v resulted in the highest biomass productivity and microalgae growth compared to 20 and 30 %v/v. To ease harvesting, MaY-flocs were developed in a SBR with diluted vinasse (10 %v/v) enriched to 76 mg N-TA L^{-1}. This resulting in a doubled biomass productivity (49.5 ± 8.3 mg VSS L^{-1} day^{-1}) of MaY-flocs compared to the best batch reactor performance. Based on response surface experiments, enrichment to 150 mg N-TA L^{-1} and 5.9 %v/v vinasse are recommended. The MaY-floc SBR system is a novel technology to treat tequila wastewaters while producing MaY-floc biomass of interest to aquaculture.

CRediT authorship contribution statement

Glenda Edith Cea Barcia: Formal analysis, Data curation, Investigation, Resources, Funding acquisition, Project administration, Writing - original draft, Writing - review & editing, Visualization, Conceptualization, Methodology. **Rocio Alejandra Imperial Cervantes:** Investigation. **Ixbalank Torres Zuniga:** Software, Formal analysis. **Sofie Van Den Hende:** Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Visualization, Conceptualization, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2019.122644.

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