POLITECNICO DI TORINO
Repository ISTITUZIONALE

Control of bacterial contamination in microalgae cultures integrated with wastewater treatment by applying feast and famine conditions

Original
Control of bacterial contamination in microalgae cultures integrated with wastewater treatment by applying feast and famine conditions / Di Caprio, F., Proietti Tocca, G., Stoller, M., Pagnanelli, F., Altimari, P.. - In: JOURNAL OF ENVIRONMENTAL CHEMICAL ENGINEERING. - ISSN 2213-3437. - STAMPA. - Volume 10:(2022), p. 108262. [10.1016/j.jece.2022.108262]

Availability:
This version is available at: 11583/2970858 since: 2022-09-02T09:09:42Z

Publisher:
Elsevier

Published
DOI:10.1016/j.jece.2022.108262

Terms of use:

This article is made available under terms and conditions as specified in the corresponding bibliographic description in the repository

Publisher copyright
Elsevier postprint/Author's Accepted Manuscript

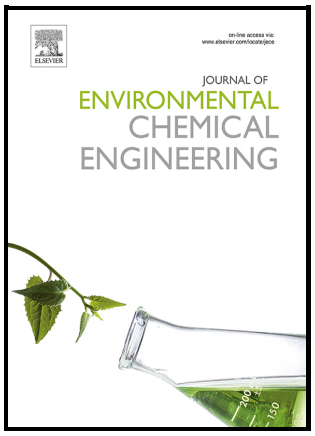
© 2022. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/.The final authenticated version is available online at:
http://dx.doi.org/10.1016/j.jece.2022.108262

(Article begins on next page)

27 November 2026

Journal Pre-proof

Control of bacterial contamination in microalgae cultures integrated with wastewater treatment by applying feast and famine conditions



Fabrizio Di Caprio, Giacomo Proietti Tocca, Marco Stoller, Francesca Pagnanelli, Pietro Altimari

PII: S2213-3437(22)01135-6

DOI: https://doi.org/10.1016/j.jece.2022.108262

Reference: JECE108262

To appear in: *Journal of Environmental Chemical Engineering*

Received date: 28 April 2022
Revised date: 17 June 2022
Accepted date: 10 July 2022

Please cite this article as: Fabrizio Di Caprio, Giacomo Proietti Tocca, Marco Stoller, Francesca Pagnanelli and Pietro Altimari, Control of bacterial contamination in microalgae cultures integrated with wastewater treatment by applying feast and famine conditions, *Journal of Environmental Chemical Engineering,* (2022) doi:https://doi.org/10.1016/j.jece.2022.108262

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2022 Published by Elsevier.

Journal Pre-proof

Control of bacterial contamination in microalgae cultures integrated with wastewater treatment by applying feast and famine conditions

Fabrizio Di Caprio[a,*], Giacomo Proietti Tocca[a,b], Marco Stoller[c], Francesca Pagnanelli[a], Pietro Altimari[a].

[a]Dipartimento di Chimica, Università Sapienza di Roma, Piazzale Aldo Moro 5, 00185, Rome, Italy.

[b]Dipartimento di Scienza Applicata e Tecnologia, Politecnico di Torino, Corso Duca degli Abruzzi 24, 10129, Turin, Italy.

[c]Dipartimento di Ingegneria Chimica, Sapienza Università di Roma, via Eudossiana 18, 00184, Rome, Italy.

*Corresponding author: fabrizio.dicaprio@uniroma1.it

Journal Pre-proof

Abstract

The integration of microalgae production with wastewater treatment can significantly increase economic and environmental sustainability of the treatment process and of the microalgal biomass production. However, a major bottleneck of this strategy is the control of contamination by heterotrophic bacteria, which compete with microalgae for the organic substrate and can negatively affect the quality of the produced biomass. Here, a strategy to control bacterial contamination is proposed, whereby a first batch phase with the medium replete in the organic substrate (feast) is followed by a second batch phase without the organic substrate (famine). Permeates from microfiltration and ultrafiltration of cheese whey were used as sources of organic substrates, with different C/N. Biomass production and pollutant removal were analyzed, and the growth kinetics of

microalgae and bacteria were characterized and modelled to determine the specific growth rate (μ_{max}) during the feast phase and the decay rate (k_D) during the famine phase. Bacteria had μ_{max} (0.16 h^{-1}) about two folds higher than microalgae (0.07 h^{-1}), however, bacteria lysed in larger fraction during the famine phase, allowing to reduce contamination. A remarkable finding was that, for both microalgae and bacteria, only a cell subpopulation experienced lysis during the famine phase. The fraction of resistant cells was higher for microalgae and decreased with increasing the C/N ratio in the initial medium, indicating that cell-to-cell heterogeneity for carbon storage is crucial in determining cell resistance. Guidelines are derived to maximize microalgae biomass productivity and pollutant removal while maintaining a prescribed bacterial contamination.

Keywords

Dairy wastewater; bacteria contamination; feast and famine; control strategy; mixotrophic growth; heterotrophic growth

1. Introduction

Microalgae are photosynthetic microorganisms that are emerging in recent years as next-generation biotechnological systems for the production of food, feed and bio-based products. They can grow using different metabolisms: photoautotrophic, using light as energy source and CO_2 as carbon source, heterotrophic, whereby organic substrates are used as source of carbon and energy, and mixotrophic, where both are implemented. Microalgae have a tunable biochemical profile, in what the fractions of proteins, lipids, starch and other components vary depending on the physiological condition.

Industrial cultivations are currently carried out in fully photoautotrophic or fully heterotrophic [1]. The main limit of fully photoautotrophic processes is represented by the high operative and

investment costs and energy demand required to maintain adequate light supply rate (reactors with high S/V ratio), mixing, O_2 degassing, etc. [1]. Microalgae produced by phototrophic processes have a cost typically > 5 €/kg [2] and an energy demand from 6 to 16,000 kWh/kg [3,4], depending on plant design and its geographical region. These numbers are mainly determined by the light-limited growth regime which limits biomass concentration (typically < 5 g/L) and productivity (typically < 1 g/L per day). Heterotrophic cultivations use fermenters fed with organic substrate (typically pure glucose) attaining biomass concentrations up to 286 g/L, thanks to the light-independent growth [5]. The organic substrate is currently the main factor affecting economic and environmental sustainability of heterotrophic cultures [5,6]. The application of wastewater as source of organic substrate in heterotrophic cultures can enhance the economic profitability and reduce, at the same time, the environmental footprint. Nevertheless, if heterotrophic or mixotrophic microalgae cultures are not operated under axenic conditions, heterotrophic bacteria contaminants can outgrow microalgae, becoming rapidly the predominant biomass [7]. However, wastewater sterilization is usually unfeasible due to the significant increase of operating costs. Despite there are many previous studies reporting microalgae cultivation with wastewater as substrate source for mixotrophic or heterotrophic metabolism [8–11], these were usually conducted without quantifying bacterial contamination and/or without adopting control strategies, other than sterilization, to reduce it.

The need of sterilization can be excluded by developing strategies for nutrient feeding that allow to select microalgae over contaminants. Our research group recently developed a control strategy allowing to cultivate microalgae in heterotrophic “open reactors” (non-axenic condition) without significant contamination [7]. This strategy entails to replenish at different times the N and the organic substrate in way that allows to cyclically replace a N-starvation/Energy-replete medium by a N-replete/Energy-starvation medium. The strategy was initially demonstrated by using only synthetic substrates in a fed-batch reactor and then implemented in a process integrated with olive mill wastewater in a sequencing batch reactor (SBR) [12]. However, this strategy is not applicable

for wastewaters in which there is, along with organic C, a relevant concentration of N, because this would make it impossible the uncoupling of C and N feeding.

In this work, an innovative strategy is proposed to control bacterial contamination in microalgae cultures fed with wastewater containing both N and organic C. The process studied includes a two-stage strategy with a first phototrophic cultivation stage, followed by a batch heterotrophic fermentation with wastewater used as C source. The phototrophic stage allows to obtain a culture inoculum characterized by a low initial contamination (high microalgae/bacteria ratio). The heterotrophic fermentation then includes a phase performed under nutrient replete conditions (feast) followed by a phase without organic substrate (energy starvation, or famine). The objective of this heterotrophic cultivation strategy is to capitalize the competitive advantage of microalgae, as compared to competing heterotrophic bacteria, to store C/energy in the form of carbohydrates and lipids, and then using these energy reserves to sustain maintenance under the famine phase. Such feast and famine approach is similar to that used to select bacteria storing polyhydroxyalkanoates (PHA) from activated sludge [13]. However, to our knowledge, such an approach was never adopted before for the selection of microalgae cultivated with wastewater.

Cheese whey was used in this study as source of N and organic C. About 9 L of whey are produced for each kg of cheese, yielding an estimated annual worldwide production of $40.7 \cdot 10^6$ tons [14]. Whey typically contains 50-80 g/L COD and relevant amount of N and P, which need to be removed to prevent environmental pollution [15]. Its high sugar load can be valorized as substrate source for microalgae, as proved in previous studies [16–18]. However, in these studies, bacterial contamination was not assessed, or it was prevented by adopting conventional sterilization procedures.

Here, along with the quantification of biomass production and pollutant removal, bacteria and microalgae growth kinetics attained during feast and famine phases upon the proposed heterotrophic cultivation process are experimentally analyzed and mathematically modelled. This allowed us to estimate separately microalgae and bacteria specific growth rates (under feast phase) and decay rate

(under famine phase). Based on this analysis, indications were derived on the optimal operative conditions to operate the batch cultivation with wastewater.

2. Materials and Methods

2.1 Cheese whey collection and pre-treatment

Cheese whey was obtained after the production of ricotta cheese (obtained by the precipitation of whey proteins at 85-90 °C). It was collected from Campo Felice cheese factory, in Collimento di Lucoli (AQ, Italy). In the production site, milk was pretreated with lactase to split lactose to D-glucose and D-galactose. Microfiltration was carried out, after centrifuging cheese whey at 2,265 *g* for 5 min, by filtering the supernatant through 0.7 µm glass fiber filters. The resulting permeate (P_{MF}) was stored at -18 °C till its use. For ultrafiltration, 20 L of cheese whey was fed (at 25 L/h) from a feedstock tank to an ultrafiltration membrane (model GM2540F, mean pore size: 2 nm, 2.51 m^2 active area), at an operating pressure of 5 bar. Two pumps were used to drive the feed-stream to the membrane module; two regulation valves were used to set the desired operating pressure and crossflow over the membrane independently; two plate heat exchangers maintained the temperature of the streams stable at ambient temperature. More details about the filtration system can be found in Figure S1 and in a previous work [12]. Ultrafiltration was performed till obtaining about 16 L of permeate (P_{UF}) and 4 L of concentrated whey, which were stored at -18 °C. All the filtrations were performed on whey within 2 d from its collection from the production plant.

2.2 Microalgal strain and cultivation set up.

A strain of *Tetradesmus obliquus* (generally known as *Scenedesmus obliquus*), isolated as previously described [19], was cultivated in 500 mL glass column photobioreactors (PBRs) (h = 35 cm, d = 5 cm), in non-axenic conditions, as previously described [20]. Microalgae were inoculated inside two PBRs at 0.1 g/L and cultivated for 4 d, then they were diluted in fresh medium at 0.3 g/L initial concentration. After 24 h, when the concentration was about 1 g/L, 160

mL were collected from the PBRs, centrifuged (2,265 g, 10 min) and the pellet washed with distilled water. This pellet was used as inoculum to start the heterotrophic cultivation by suspension in 160 mL medium obtained by mixing 80 mL saline medium (C and N free, Table S1) with 80 mL of whey permeate (P_{MF} or P_{UF}, Table 1). Heterotrophic cultivation was carried out inside 300 mL conical flasks, covered with sterile stoppers (Silicosen S35), posed in dark under orbital shaking at 150 rpm and 25 ± 2 °C. The speed of the shaking was set to ensure enough O_2 supply rate [21]. The pH was daily monitored and eventually adjusted to 7.5 ± 0.2 by adding NaOH 1M or HCl 1M. Samples were collected at different times to analyze cell concentration, biomass concentration and nutrient concentration.

2.3 Analysis of biomass and cell concentration.

Overall biomass concentration (i.e., microalgae + bacteria biomass) was determined by filtering a known volume through 0.2 µm cellulose acetate filter, dried at 105 °C. Microalgal cell concentration was measured by optical counting with a microscope (Leitz, Labor Lux 12) using a Thoma chamber. Bacterial cell concentration was measured by flow cytometry (Attune™ NxT Flow Cytometer, ThermoFisher Scientific). Before analysis, the reproducibility of the instrument was verified by using Attune™ Performance Tracking Beads. Before analysis, samples were diluted to 5-10·10^6 microalgal cells/mL and fixed in TE Buffer (10 mM Trizma® base, 1 mM disodium EDTA, pH 8) with 1 % glutaraldehyde, at 4 °C for 1 h. Then, 2 µL of 300x SYBR green I (in dimethyl sulfoxide) was added to 600 µL of fixed sample and incubated for 15 min at 25 °C. Samples were then further diluted in TE buffer till obtaining a total cells concentration < 10^6/mL. Analyses were performed acquiring 50 µL sample at 100 µL/min. Bacteria population was separately gated from noise and microalgae by plotting the signal from DNA green fluorescence emission, BL1 (BP 530/30), vs the signal from chlorophyll red fluorescence emission, BL3 (BP 695/40), obtained after laser excitation at 488 nm. More details about the protocol can be found in a previous work [22].

2.4 Chemical and biochemical analysis

Journal Pre-proof

Total sugars concentration was determined by the phenol-sulfuric acid method [23]. Glucose concentration was measured with the GOPOD reagent (glucose oxidase/peroxidase) [24], from the n°3 bottle of the Megazyme kit (K-TSTA-50A). Total N (TN) and total phosphorus P (TP) were analyzed by the official spectrophotometric method for water analysis reported by IRSA-CNR 4060 [12]. Chemical oxygen demand (COD) was measured by means of the colorimetric kit Lovibond® Vario vials (2420721). To quantify total carbohydrates inside biomass, acid hydrolysis was performed and followed by the spectrophotometric method using the MBTH (3-metil-2-benzothiazolinone hydrazone hydrochloride) reagent, as previously described (NREL/TP-5100-60957) [25]. Biomass nitrogen quota (q) was determined from a mass balance from produced (ΔX) and consumed nitrogen (ΔTN), as described in detail in supplementary material.

2.5 Calculation of process parameters

Process parameters related to biomass production were measured as follows. Biomass productivity was determined by Eq. 1:

$$r_X = \frac{(X(t)-X_0)}{t} \quad (1)$$

with X_0 denoting the biomass concentration at the beginning of the batch, and *t* the time of sampling during the cultivation. $r_{X,max}$ was determined as the maximum value of r_X attained during the batch and $r_{X,f}$ as the value at the end of the batch.

Biomass to substrate yield (g/g) was determined by Eq. 2:

$$Y_{X/S} = \frac{(X(t)-X_0)}{(S_0-S(t))} \quad (2)$$

where S_0 is the initial concentration of total sugars. $Y_{X/S_{max}}$ was determined as the maximum value of $Y_{X/S}$ attained during the batch and Y_{X/S_f} as the value at the end of the batch. X_{max} was the maximum biomass concentration attained during the batch and $\Delta X_{max}=X_{max}-X_0$. X_f was the final

biomass concentration and $\Delta X_f = X_f - X_0$. For each pollutant, C_i, C_f and C_{min} are the initial, final and minimum concentration attained, while Δ_f (mg/L)$= C_i - C_f$, Δ_{max} (mg/L)$= C_i - C_{min}$, Δ_f (%)$= (C_i - C_f)/C_i \times 100$, Δ_{max} (%)$= (C_i - C_{min})/C_i \times 100$.

2.6 Modelling of microalgae and bacteria growth

The variation of microalgal and bacteria cell concentration as function of time was modelled by adopting two different models: a first model was used to describe the kinetics of growth in presence of substrate (feast or energy replete), and a second model was employed to describe the kinetics of the cell lysis taking place during the following nutrient depleted phase (famine or energy starvation). The same models were separately applied for microalgae and bacteria. Microalgae and bacteria growth in presence of substrate was described by means of the following logistic model (Eq. (3)):

$$\frac{dn}{dt} = \mu_{max}\left(1 - \frac{n}{n_{max}}\right)n \quad (3)$$

where n denotes the cell concentration. Which was integrated between n_0 (t=0) and n (t) obtaining the integrated form in Eq. 4.

$$n = \frac{n_0 n_{max} e^{\mu max t}}{n_{max} + n_0 (e^{\mu max t} - 1)} \quad (4)$$

Such equation was fitted to cell concentrations measured during the initial phase of growth (feast) to determine the maximum specific growth rate (μ_{max}) and the maximum cell concentration (n_{max}) attained by microalgae and bacteria cultivated with P_{MF} and P_{UF}. The doubling time (t_d) was calculated as $t_d = \ln(2)/\mu_{max}$.

To describe the evolution of cell concentration during the famine phase (n(t)), the populations of microalgae and bacteria were supposed to be heterogeneous, both of them being composed of a subpopulation with cell concentration n_1, which survived till the end of the famine phase (i.e., n_1(t)

= $n_{1,0}$ at any t during the famine phase), and a subpopulation with cell concentration n_2 (Eq. 5), which lysed by following first order kinetics with constant decay rate k_D (Eq. 6).

$$n\,(t) = n_1(t) + n_2(t) \quad (5)$$

$$-\frac{dn_2}{dt} = k_D t \quad (6)$$

The application of Eqs. (5)-(6) gives:

$$n\,(t) = n_{1,0} + n_{2,0}e^{-k_D t} \quad (7)$$

where $n_{1,0}$ and $n_{2,0}$ are the concentrations of the two subpopulations at the beginning of the famine phase. Since $n_0 = n_{1,0} + n_{2,0}$, Eq. (7) can be re-written in the form of Eq. 8, which was applied to fit experimental data of microalgal and bacteria cell concentration measured during the famine phase.

$$n\,(t) = n_{1,0} + \left(n_0 - n_{1,0}\right)e^{-k_D t} \quad (8)$$

The parameters $n_{1,0}$ and k_D were determined by fitting, while n_0 was maintained constant during fitting and equal the cell concentration measured at the beginning of the famine phase.

2.7 Contamination assessment

Contamination was quantified in terms of cell concentration (Eq. 9) and in terms of biomass concentration (Eq. 10).

$$f_B = \frac{n_B}{n_A} \quad (9)$$

$$\chi_B = \frac{n_B m_B}{n_A m_A + n_B m_B} \quad (10)$$

where n_A and n_B are the microalgal and bacterial cell concentration, respectively, and m_A and m_B the microalgal and bacteria cell mass, respectively, with m_A fixed at 30 pg/cell and m_B at 1 pg/cell.

2.8 Statistical analysis

Batch cultivation tests were performed testing 4 different biological replicates for each condition (P_{MF} and P_{UF}). Here a biological replicate is considered as a physically separated culture, inoculated with an algal suspension coming from a different PBR, operated at the same conditions. However, since half of the replicates were sampled at different times (to cover better the whole dynamic behavior), statistical tests that compared results at the same time point were performed considering a number of replicates (n_r) = 2. Significant differences (α=0.05) were determined by performing analysis of variance (ANOVA) or Student's t-test. Error bars are reported as standard deviation (SD). Nonlinear fitting was performed by the MATLAB function 'nlinfit', which performs the minimization of the sum of the squared residuals. The uncertainty in the model parameters was indicated with 95% confidence intervals ($CI_{95\%}$), calculated with the MATLAB function 'nlparci'.

Table 1. Chemical composition of cheese whey permeates from microfiltration (P_{MF}) and ultrafiltration (P_{UF}) and concentrate from ultrafiltration (C_{UF}). Data are reported as average ± SD (n_r = 2).

Parameter	P_{MF}	P_{UF}	C_{UF}
COD (g/L)	35 ± 1	23.2 ± 0.2	n.d.
Total sugars (g/L)	35 ± 2	28 ± 8	52 ± 5
Glucose (g/L)	13 ± 1	9.6 ± 0.2	14.1 ± 0.1
TN (mg/L)	590 ± 50	180 ± 30	1100 ± 30
TP (mg/L)	81 ± 3	49.4 ± 0.3	152 ± 2
TS (g/L)	30 ± 1	27 ± 1	42 ± 1
C/N	24 ± 2	51 ± 9	n.d.
pH	8.02 ± 0.01	5.4 ± 0.4	4.81 ± 0.07

Results and discussion

3.1 Cheese whey characterization after microfiltration and ultrafiltration

The permeates from ultra- and microfiltration had a COD almost entirely composed of sugars, which was the predominant organic component (Table 1). The concentration of total sugars in the permeates was 20-37% lower than that of lactose usually reported for raw whey (~45 g/L) [26]. This was likely due to a degradation of sugars by microbial flora taking place during storage and

filtration (~9 h for UF and ~2 h for MF). This hypothesis is corroborated by the observation that the sugar concentration decreased with increasing the duration of permeate storage and filtration: P_{MF}, whose storage and filtration lasted about 2 h, had a sugar concentration of 35 g/L, which is significantly larger than that found in the P_{UF} (23 g/L), for which about 9 h were necessary. Biodegradation during filtration and storage was also confirmed by the analysis of pH, which varied from the initial 8.0 (P_{MF}) to the value of 5.4 after ultrafiltration and storage, thus evidencing the occurrence of microbial fermentation.

Since the measured glucose was derived by the hydrolysis of lactose, the difference between total sugars and glucose can be imputed to galactose and residual lactose. A comparison between the concentrations of glucose and total sugars indicated indeed an incomplete lactose hydrolysis: glucose was about 1/3 of total sugars, instead of the 1/2 expected in case of complete lactose hydrolysis. The concentration of N in P_{MF} was in line with previous data [26]. Ultrafiltration was performed to remove soluble whey proteins, which are a high-value product already produced at commercial scale, and to reduce N concentration, thus increasing C/N ratio. It should be remarked that the interest into this ultrafiltration is that the increased C/N ratio of the P_{UF} can promote the selection of microorganisms with increased ability to store energy reserve materials and can thus increase the competitiveness of microalgae compared to bacteria [13]. Indeed, at higher C/N ratio, the production of biomass is mostly due to C accumulation, rather than balanced growth, and in this condition microalgae can be more competitive versus heterotrophic bacteria, due to their high accumulation ability [7].

Ultrafiltration reduced the N concentration from 590 mg/L to 180 mg/L, increasing the C/N from 24 to 51 (Table 1). As a result, in the C_{UF}, the N content increased up to 1100 mg/L. TP was also partially decreased in the P_{UF} and increased in the C_{UF}.

3.2 Biomass production

Total biomass concentration was measured during the cultivation by membrane filtration (0.2 μm), which provides the sum of microalgae and contaminants (bacteria) biomass. With both P_{MF} and P_{UF},

Journal Pre-proof

a continuous increase in the biomass was observed in the feast (substrate replete) phase, until 72 h with P_{MF} and 48 h with P_{UF}, followed, during the famine phase, by a decrease determined by the consumption of endogenous biomass as energy source and/or by cell lysis (Figure 1). The energy starvation condition (famine) was reached earlier with P_{UF}, due to the lower initial concentration of total sugars (S_0). The reduced S_0 contributed to give a maximum biomass concentration (X_{max}) significantly lower with P_{UF} (3.37 ± 0.05 g/L) compared to the experiment with P_{MF} (8.5 ± 0.5 g/L), (p=0.04). However, the difference between the S_0 values in the two experiments was not sufficient to explain the difference between the attained X_{max} values. In fact, this difference was mainly due to a biomass to substrate yield $Y_{X/S_{max}}$ with P_{UF} (0.27 g/g) lower than that observed with P_{MF} (0.54 g/g) in the initial feast phase (Table 2). The $Y_{X/S_{max}}$ obtained with P_{MF} is in the range of values (0.40–0.60 g/g) previously obtained for *Scenedesmus* with synthetic media containing glucose as substrate [5,12], while the reduced $Y_{X/S_{max}}$ for P_{UF} was unexpected, and the exact reason remains not completely clear. Different factors suggest that P_{UF} contained a higher number of fermentative bacteria converting sugars to extracellular organic acids, thus determining a lower biomass yield. These factors were: the longer pre-treatment time of P_{UF} (~9 h) as compared to P_{MF} (~2 h), the higher concentration (about 10 folds) of bacteria in P_{UF} as compared to P_{MF}, the higher fraction of COD that cannot be imputed to sugars at the end of the batch with P_{UF} (87%) as compared to that with P_{MF} (28%), and the lower sugar concentration (S_0) and lower initial pH of P_{UF} (Table 1). Such hypothesis was also confirmed by the measurement of OH^- added to control the pH. In the feast phase, when P_{MF} was used, 0.7 ± 0.6 mmol OH^-/L were added to maintain the pH at 7.5 ± 0.1, while for the test with P_{UF}, 36 ± 8 mmol OH^-/L were added (Figure 2c-d), confirming a much higher concentration of acids in the medium.

Table 2. Process parameters related to biomass production and productivity, as measured in the batch tests with 50% P_{MF} or 50% P_{UF}. X_0 = 1.5 ± 0.1 for P_{MF}. X_0 = 1.2 ± 0.1 for P_{UF}.

	X_{max} (g/L)	ΔX_{max} (g/L)	$r_{X,max}$ (g/L d)	X_f (g/L)	ΔX_f (g/L)	$r_{X,f}$ (g/L d)	$Y_{X/S_{max}}$ (g_X/g_S)	Y_{X/S_f} (g_X/g_S)
P_{MF}	8.5 ± 0.5	6.9 ± 0.5	2.9 ± 0.6	4.3 ± 0.2	2.7 ± 0.3	0.31 ± 0.03	0.54 ± 0.06	0.17 ± 0.02
P_{UF}	3.4 ± 0.1	2.1 ± 0.1	1.7 ± 0.1	2.6 ± 0.3	1.4 ± 0.3	0.16 ± 0.04	0.27 ± 0.02	0.13 ± 0.03

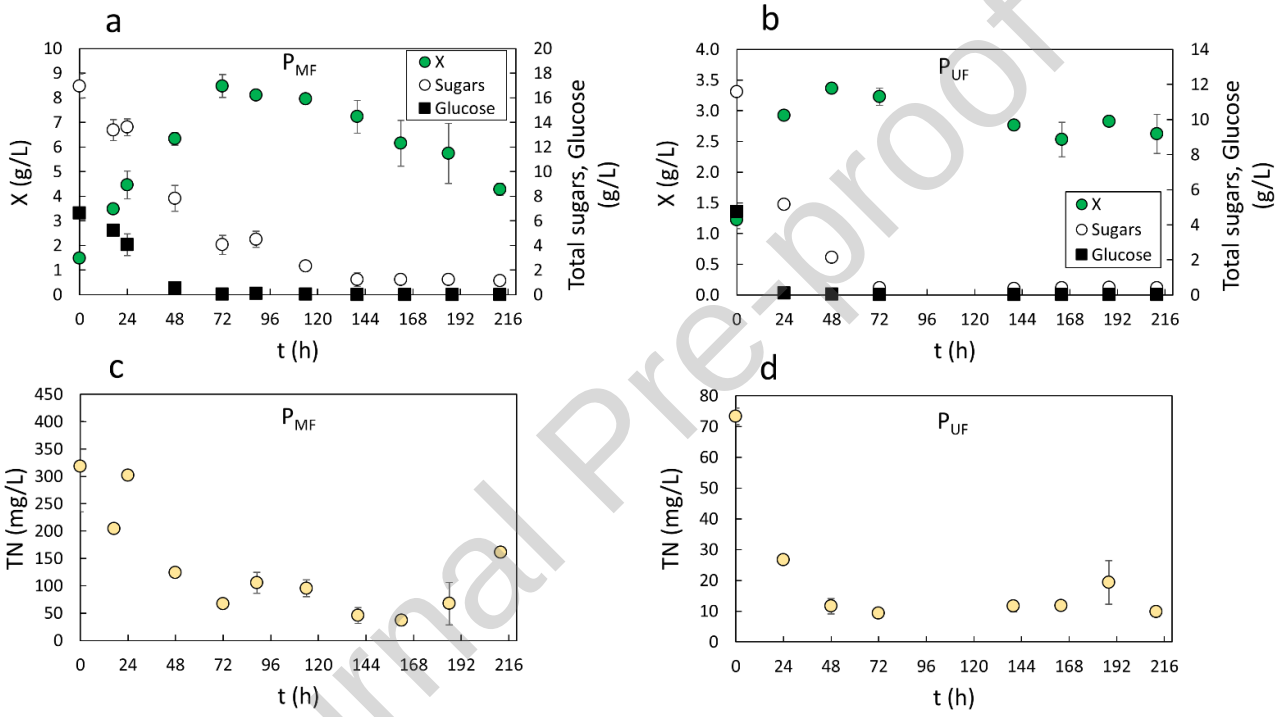


Figure 1. Biomass production, total sugars and glucose consumption throughout batch tests conducted with P_{MF} (a) and P_{UF} (b). Total nitrogen throughout batch tests conducted with P_{MF} (c) and P_{UF} (d). Data are reported as average ± SD (n_r = 2).

Glucose was completely depleted at 48 h for P_{MF} and at 24 h for P_{UF}, thus more rapidly than the other sugars (galactose and some lactose), indicating that biomass grew following a diauxic behavior (Figure 1). A similar diauxic growth has been previously observed also for *Chlorella sorokiniana* growing on acetate and butyrate [27]. Sugars were not completely removed, but a

Journal Pre-proof

minor residual fraction of 380 ± 20 mg/L (6% S_0) for P_{UF} and 1000 ± 80 mg/L (14% S_0) for P_{MF} remained in the residual medium at the end of the batch.

As expected, biomass concentration decreased during the famine phase with both P_{UF} and P_{MF} (Figure 1). This decrement was more significant with P_{MF} (-50% with respect to X_{max}, ΔX: -4.4 g/L) than with P_{UF} (-36% with respect to X_{max}, ΔX: -0.77 g/L). The larger biomass yield during the famine phase of the experiment with P_{UF} could be induced by a higher accumulation of reserve material during the previous feast phase. Indeed, this increased accumulation of reserve material with P_{UF}, which is mainly determined by the higher C/N of P_{UF}, could be used to adapt better cells to starvation. The decrement of biomass during the famine phase progressively reduced the $Y_{X/S}$, which eventually reached a value almost identical with P_{UF} (0.13) and P_{MF} (0.17), attained after 140 h with P_{UF} and 192 h with P_{MF} (Figure 2). The maximum productivity ($r_{X,max}$) was attained after 24 h with both P_{UF} and P_{MF}, and it is mainly related to the availability of glucose.

$r_{X,max}$ and $r_{X,f}$ were 1.7 and 2 folds higher, respectively, in the experiment with P_{MF} compared to the experiment with P_{UF} (Table 2). This difference can be explained by the same mechanisms previously illustrated to explain the different X_{max} values attained with P_{MF} and P_{UF}. The process indicators quantifying biomass production and productivity (Table 2, except $Y_{X/S}$) almost proportionally increased with S_0. Therefore, all these indicators could be further increased by increasing S_0. Since in this study P_{MF} and P_{UF} were diluted two folds, it is foreseen that the indicators related to biomass production and productivity could be increased up to two times by using undiluted P_{UF} or P_{MF} as culture medium.

It should be underlined that the $r_{x,max}$ (Table 2) obtained with heterotrophic cultivation with P_{MF} and P_{UF} are significantly higher than those typically attained with the only phototrophic cultivations (<1 g/L d) [28]. This improvement is due to the light-independent regime of heterotrophic growth, that can overcome the limitation induced by the self-shading effect.

Journal Pre-proof

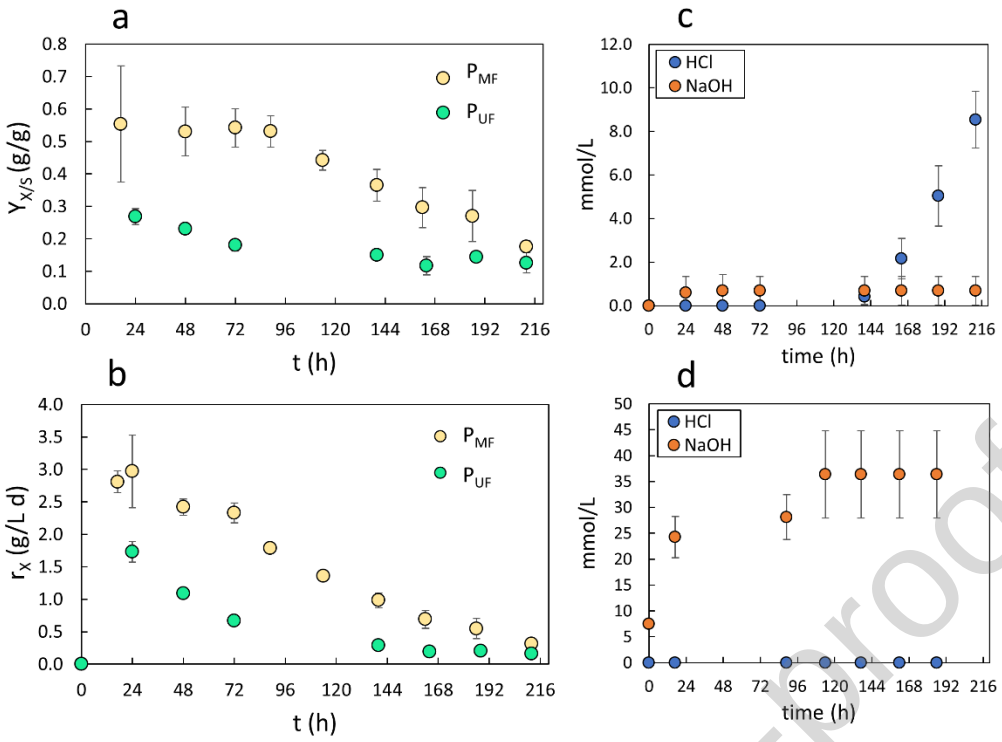


Figure 2. Biomass to substrate yield factor (a) and biomass productivity (b) determined at different time points for biomass produced from P_{UF} and P_{MF}. Amount of HCl and NaOH added throughout the cultivation of microalgae with P_{MF} (c) and P_{UF} (d) to maintain the pH stable at 7.5 ± 0.1. Data are reported as average ± SD (n_r = 2).

3.3 Pollutant removal

Biomass production was made possible by the consumption of organic carbon, N and P, which are the main pollutants present in P_{UF} and P_{MF}. The maximum removal attained for TN, TP and COD were 90%, 87% and 91%, respectively (Table 3). Cultures grown with P_{MF} removed more COD (91%, -15.9 g/L) than with P_{UF} (73%, -8.4 g/L), attaining a final COD lower (1550 mg/L) than with P_{UF} (3200 mg/L). As discussed in the previous section about biomass production, this difference could be induced by a higher fraction of S_0 converted to fermentative extracellular products when P_{UF} was used. Indeed, sugars were only 13% of the final COD for P_{UF} (while 77% for P_{MF}). Since lactic acid is the main fermentative product of microbial flora in cheese whey [29], we can assume that the not-sugar COD fraction was almost entirely composed of lactic acid. This would

correspond to have 2.6 ± 0.2 g/L of lactic acid. An equal mass of sugar would be directed towards fermentative products ($C_6H_{12}O_6$ + 2 ADP + P_i → 2 $C_3H_6O_3$ + 2 ATP) rather than biomass, which is sufficient to explain the lower $Y_{X/S}$ observed with P_{UF}. The molar concentration of the calculated lactic acid is 29 ± 2 mmol/L, in agreement with OH^- added to correct the pH (36 ± 8 mmol/L), according to the reaction stoichiometry.

The removal of TP was higher for P_{MF} (87%, -77 mg/L) than for P_{UF} (71%, -51 mg/L), mainly due to the higher biomass production attained with P_{MF} (Table 3). A relevant part of this P was removed after 1 h (36% for P_{UF} and 47% for P_{MF}) due to chemical precipitation. By assuming that all the residual P was removed by the uptake into biomass, comparable P uptake yields can be estimated with P_{MF} and P_{UF}, corresponding to 13 ± 1 mg_P/g_X and 18 ± 5 mg_P/g_X for P_{MF} and P_{UF}, respectively. These values are consistent with the typical P content reported for microalgal biomass (1-3%). It should be underlined that, in this study, to prevent any P depletion, P was also added to the culture in the form of HPO_4^-. The motivation for this addition is that, by using only permeates, P could have been completely removed, becoming an additional limiting nutrient, making it impossible to clearly distinguish the effect of C/N ratio.

Table 3. COD, TN and TP removal achieved during microalgae cultivation with P_{MF} and P_{UF}. Data are reported as average ± SD (n_r = 2).

		C_i (mg/L)	C_{min} (mg/L)	C_f (mg/L)	Δ_f (%)	Δ_f (mg/L)	Δ_{max} (%)	Δ_{max} (mg/L)
P_{MF}	COD	17400 ± 500	1550 ± 50	1550 ± 50	91 ± 4	15900 ± 500	91 ± 4	15900 ± 500
	TP	88 ± 3	11.7 ± 0.1	11.7 ± 0.1	87 ± 3	77 ± 3	87 ± 3	77 ± 3
	TN	320 ± 80	37 ± 3	161 ± 5	50 ± 10	160 ± 80	90 ± 20	280 ± 80
P_{UF}	COD	11610 ± 80	3200 ± 200	3200 ± 200	73 ± 2	8400 ± 200	73 ± 2	8400 ± 200
	TP	72.7 ± 0.3	21 ± 2	21 ± 2	71 ± 7	51 ± 2	71 ± 7	51 ± 2
	TN	73 ± 3	9.3 ± 0.4	10 ± 1	87 ± 5	63 ± 3	87 ± 5	64 ± 3

Journal Pre-proof

Comparable maximum TN removal yields (~90%) were achieved with P_{MF} and P_{UF} (Table 3). The maximum TN removal yield was attained in correspondence of the end of the batch with P_{UF}, while, in the experiment with P_{MF}, it was attained after 162 h. After this time, with P_{MF}, the TN removal decreased, reaching 50% of the maximum value at the end of the batch (212 h) (Figure 1c). Such anomalous non-monotone evolution of the TN removal in the experiment with P_{MF} can be explained by a significant release of N from the biomass during the late famine phase of the batch (186-212 h). This hypothesis is consistent with the higher drop in biomass concentration observed with P_{MF} during the famine phase (Figure 1a). This biomass concentration drop was determined by more significant cell lysis taking place during the famine phase in the culture fed with P_{MF}, which released N in form of proteins and peptides. This behavior was also confirmed by the pH control: with P_{MF}, a relevant amount of HCl (8.5 ± 1.3 mmol/L) was needed during the last part of the batch to maintain stable the pH (Figure 2c). Such amount of H^+ was equimolar with respect to the released N (8.8 ± 0.4 mmol/L), suggesting that it was mainly used to react with amino groups.

The incomplete N consumption for both P_{MF} and P_{UF} could suggest that N was never a factor limiting the growth. To verify this, the nitrogen quota (q_N) of the produced biomass was determined (Figure S2) by assuming that all the removed N was used to produce new biomass. By this approximation, it was found that q_N was 0.047 after 72 h with P_{MF} and 0.035 after 48 h with P_{UF}. These q_N values are lower than the maximum N quota for *T. obliquus* (~0.095 g_N/g_X [12,30]), despite N was still present in the culture medium. This indicates that the residual N remaining in the culture medium was not used by microalgae likely because it was in form of proteins exhibiting major resistance to proteolytic activity and thus not easily biodegradable, as shown in a previous study [29].

3.3 Evolution of the biomass composition

Since P_{UF} had a higher C/N ratio than P_{MF}, a higher accumulation of storage compounds was expected for microalgae grown on P_{UF}. Results confirm this because the biomass grown on P_{UF} reached a carbohydrate fraction equal to 30.0 ± 0.5% after 24 h and 27.6 ± 0.8% after 72 h, thus

Journal Pre-proof

significantly higher (p=0.004) than the values attained with P_{MF}, which were 18 ± 4% at 24 h and 20 ± 2% at 72 h (Figure 3a).

The carbohydrate content of the biomass cultivated with P_{MF} increased during the feast phase, reaching a maximum at 72-120 h, in correspondence of the depletion of the organic substrate, and then monotonically decreased during the following famine phase (Figure 3a). After the depletion of the organic substrate, the carbohydrate contents of the biomasses cultivated with P_{MF} and P_{UF} progressively decreased to comparable values (p>0.05), corresponding to 14 ± 2% at the end of the batch. The biomass nitrogen quota q_N, which should be proportional to the protein content, followed an opposite behavior: it decreased during the feast phase, attaining a minimum in correspondence of the maximum carbohydrate content (at 24 h with P_{UF} and around 100 h with P_{MF} (Figure S2) and then increased during the famine phase (absence of substrate) owing to consumption of stored carbohydrates [13].

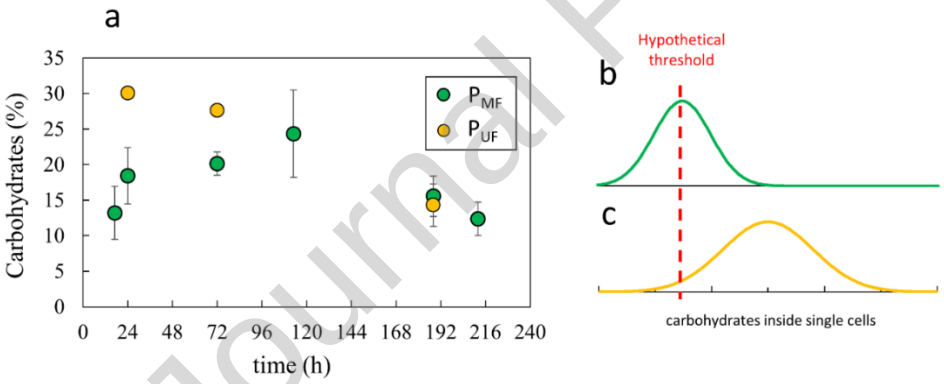


Figure 3. Carbohydrate content inside biomass at different time of the cultivation with P_{UF} and P_{MF} (a). Data are reported as average ± SD (n = 2). Qualitative supposed distribution of single-cell content of carbohydrates inside microalgal cells at the end of the feast (energy replete) phase when cultivated on P_{MF} (b) and P_{UF} (c). Threshold dotted line indicates a hypothetical minimum amount required to resist under the famine phase (energy starvation).

3.4 Dynamic behavior of microalgae and bacteria cell populations

To assess if the alternation of feast and famine conditions induced during the batch cultivation could be effective in controlling bacterial contamination, microalgae and bacteria cells were

separately measured by optical counting and flow cytometry, respectively. The end of the feast condition was defined as the time after which carbohydrates present in the medium were no longer consumed (depletion of bioavailable sugars) and at which X_{max} was attained. This condition was reached at 72 h with P_{MF} and 48 h with P_{UF} (Figure 1). During the feast phase, both microalgae and bacteria cells increased significantly in number by following an initial exponential growth. For both microalgae and bacteria, the growth of cell concentration gradually slowed as the substrate concentration was reduced, till attaining a maximum of cell concentration at substrate depletion. This evolution of the cell concentration was fairly described by the logistic model (Figure 4).

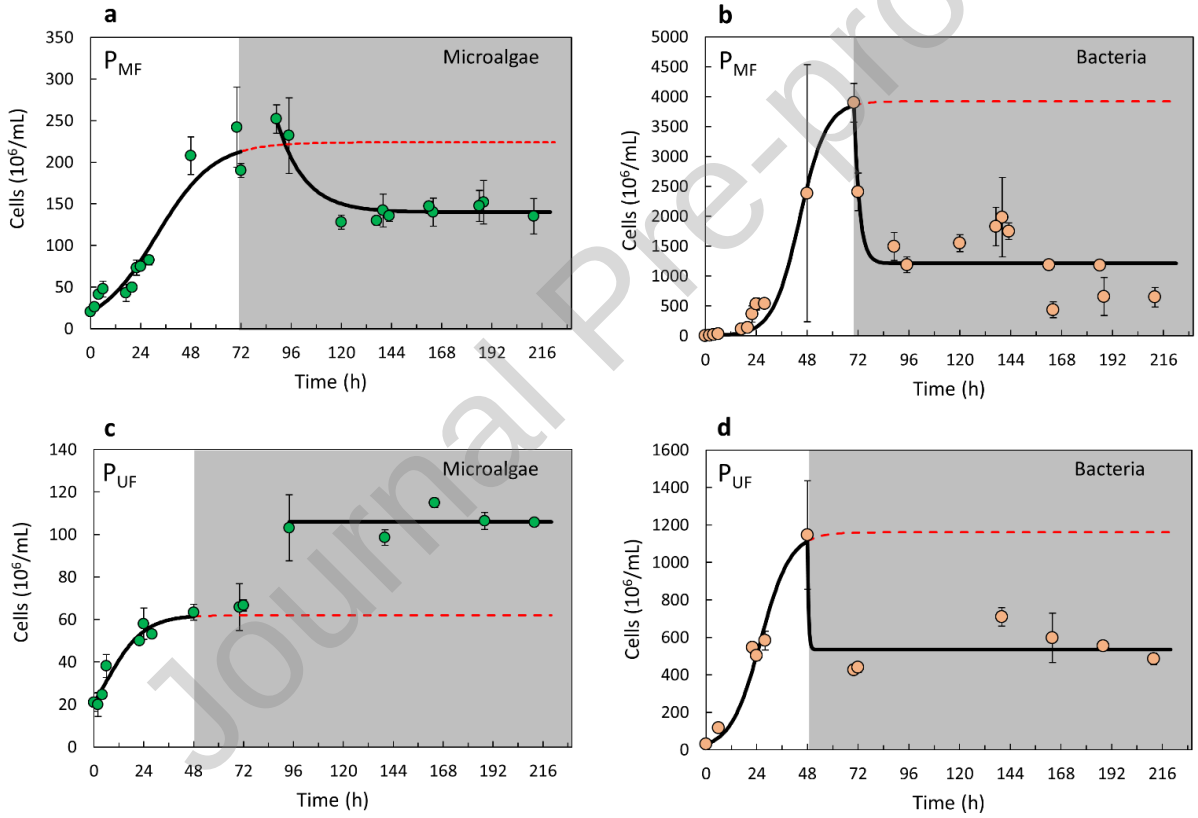


Figure 4. Microalgae (a-c) and bacteria (b-d) cell concentration throughout batch cultivation with P_{MF} (a-b) and P_{UF} (c-d). Solid line indicates the model values obtained after the fitting for the feast (energy replete) phase (white background) and famine, energy starvation, phase (grey background). Dashed line indicates the prediction of the logistic model for times longer than the beginning of the famine phase. Data are reported as average ± SD (n_r = 2).

For both microalgae and bacteria, the use of P_{MF} or P_{UF} did not affect significantly μ_{max} (Table 4). This can be explained by observing that the same substrates in P_{MF} or P_{UF} are used. As expected, bacteria grew faster than microalgae, with a duplication time (t_d) of 4.4-4.9 h, while microalgae had a growth rate about two times lower (t_d=6-9 h). The μ_{max} determined in this study for *T. obliquus* microalgae is higher than values previously reported for the same species under phototrophic conditions, which were between 0.042–0.065 h^{-1} (t_d=16–11 h) [31,32] and comparable to that of *Chlorella sorokiniana* determined on heterotrophic growth on glucose at 25 °C (t_d=8-9 h) [33]. These data indicate that no known microalgal strain could win the competition with heterotrophic bacteria when cultivated heterotrophically in whey under comparable substrate (energy) replete condition.

Table 4. Model parameters estimated for microalgae and bacteria grown with P_{MF} and P_{UF}. Errors are shown as ± $CI_{95\%}$. The parameters n_0, n_1 and n_2 indicate total cells at the beginning of the famine phase (energy starvation), cells resistant to the famine phase and cells lysed during the famine phase, respectively. Data are reported as average ± SD (n_r = 2).

*These parameters were not reported because of the too high uncertainty in their determination.

		μ_{max} (h^{-1})	t_d (h)	n_{max} cell/mL	k_D (h^{-1})	$t_{1/2}$ (h)	n_1 (cell/mL)	n_2 (cell/mL)	n_2/n_0 (%)
microalgae	P_{MF}	0.07 ± 0.01	9 ± 1	$22 \cdot 10^7 \pm 2 \cdot 10^7$	0.08 ± 0.06	9 ± 7	$14 \cdot 10^7 \pm 1 \cdot 10^7$	$11 \cdot 10^7 \pm 1 \cdot 10^7$	44
	P_{UF}	0.11 ± 0.04	6 ± 3	$62 \cdot 10^6 \pm 8 \cdot 10^6$	0	-	$106 \cdot 10^6 \pm 6 \cdot 10^6$	0	0
bacteria	P_{MF}	0.16 ± 0.02	4.4 ± 0.5	$39 \cdot 10^8 \pm 7 \cdot 10^8$	0.4 ± 0.3	1.7 ± 1.3	$12 \cdot 10^8 \pm 2 \cdot 10^8$	$27 \cdot 10^8 \pm 2 \cdot 10^8$	69
	P_{UF}	0.14 ± 0.01	4.9 ± 0.5	$12 \cdot 10^8 \pm 2 \cdot 10^8$	n.d.*	n.d.*	$5 \cdot 10^8 \pm 3 \cdot 10^8$	$6 \cdot 10^8 \pm 3 \cdot 10^8$	54

Journal Pre-proof

The knowledge of the μ_{max} of bacteria and microalgae allows to quantify the difference between microalgae and bacteria competitiveness for substrate uptake, and to predict the optimal operating conditions that should be selected to minimize bacterial contamination. To our knowledge, no previous study quantified the growth rate of bacteria flora in competition with microalgae, neither in real wastewater nor in synthetic consortia.

An issue in the assessment of bacteria growth is given by the heterogeneity of this microbial flora, composed of both flora coming from whey and flora coming from non-axenic microalgal phototrophic culture used as inoculum. However, the native flora from whey is expected to be the most relevant because adapted to the specific substrates that are found in the whey. Previous studies indicated that main native bacteria responsible for whey fermentation are *Lactobacillus delbrueckii* and *Streptococcus salivarius* subsp. *thermophilus* [29,34]. Pure *L. delbruecki* showed μ_{max} between 0.12 – 0.25 h^{-1} (t_d=5.8–2.8 h) [35–38], while *S. thermophilus* between 0.1-0.9 h^{-1} (t_d=0.8–7 h) [38,39], under cultivation conditions similar to this study. The μ_{max} found in this study (Table 4) for bacteria are in the same range reported for *S. thermophilus* and *L. delbruecki*.

The logistic model was inadequate to describe the evolution of cell concentration measured during the famine phase. In fact, this model predicts a constant final cell concentration, which was not attained in all experiments. Bacteria showed a relevant and quick decrease in cell concentration within 24 h after the beginning of the famine phase (Figure 4b,c). Microalgae showed an initial increase in cell concentration at the beginning of the famine phase. This increase was statistically relevant only for microalgae cultivated with P_{UF} (Figure 4c), while for microalgae cultivated with P_{MF}, it was in the same order of the experimental error (Figure 4a). This behavior can be explained by a progression of the cell cycle for the cells that completed the G1 phase before the substrate was depleted [40]. This transient phase, in which cell duplication occurred in absence of substrate, could not be predicted by the used models. After this transient phase, microalgae grown with P_{MF} showed a relevant decrease in cell concentration, while the concentration of microalgae cells grown with P_{UF} remained almost constant. Bacteria concentration showed a relevant decrease with both P_{MF} and P_{UF}.

When cells lysed, however, the cell concentration did not go to 0, but it reached a plateau, evidencing the existence of two different subpopulations: a subpopulation resistant to the famine phase (energy starvation) and a subpopulation undergoing cell lysis. This is a known phenomenon already observed for bacteria [13,41]. The reasons can be different and are mainly related to cell-to-cell variability of the biochemical composition [13]. A possible reason is the presence of a subpopulation with a larger fraction of stored carbohydrates and/or lipids. This variability was observed for lipid accumulation in *T. obliquus* [42] and it is also common in mixed populations of bacteria in wastewater. Such heterogeneity can give a different cell-to cell ability of adaptation, resulting in the lysis of only those cells with insufficient energy storage for adapting to the new environment.

In accordance with this analysis, the model used to describe the evolution of cell concentration during the famine phase considered two different subpopulations: the first one, with cell concentration equal to $n_1(t)$, able to survive till the end of the batch (because of the higher accumulation), and the second one, with cell concentration equal to n_2, unable to survive under the famine phase, and entirely lysing (i.e., $n_2=0$ at the end of the batch) by following first order kinetics (Eqs. (5)-(8)). Therefore, the residual concentration at the plateau attained at the end of the famine phase should correspond to n_1. This approach allowed describing satisfactorily the decreasing of cell concentration during the famine phase (Figure 4). However, for bacteria grown on P_{MF}, in the late phase of the batch, it appeared that cell concentration started again to decrease with a decay rate lower than that observed at the beginning of the famine phase. This latter trend could not be captured by the model.

Remarkably, for microalgae grown with P_{UF}, no relevant decrease in cell concentration was observed, which corresponds in the implemented model to have n_2 always equal to 0. This increased resistance to the famine phase in the experiment with P_{UF}, as compared to P_{MF}, supports the hypothesis that the cell-to-cell heterogeneity in stored carbohydrates plays a relevant role on determining the different cell-to-cell resistance to energy starvation. Indeed, it can be supposed that,

Journal Pre-proof

to survive under the famine phase, a minimum (threshold) amount of accumulated carbon is required. Then, considering that a population with higher average content of accumulated carbohydrates should have a higher fraction of cells over a certain threshold level (Figure 3b,c) [43], it results that a higher average carbohydrate content should induce a higher survival rate under the famine phase. P_{UF} had indeed a higher C/N ratio than P_{MF}, which is known to increase storage compound accumulation [40]. The analysis of carbohydrates in the biomass confirmed that microalgae grown with P_{UF} had higher average carbohydrate content than those grown on P_{MF} before entering the famine phase (Figure 3a).

Specific decay kinetic constants (k_D) and half-life times ($t_{1/2}$) were of the same order of magnitude of μ_{max} and t_d for both microalgae and bacteria (Table 4). These k_D values are similar to the values previously found for *Escherichia coli* [41], and about 10-100 folds higher than those found for activated sludge, which are usually around 0.004 h^{-1} [44,45]. The fraction of resistant cells (n_1/n_0) was 56% and 100% (no lysis) for microalgae grown on P_{MF} and P_{UF}, respectively, and of 31% and 46% for bacteria grown on P_{MF} and P_{UF}, respectively. These fractions indicate that microalgae resist better than bacteria during the famine phase, and that the higher C/N of P_{UF} can increase resistance making all microalgal cells resistant to the famine phase. Therefore, the famine phase can be effective to reduce bacterial contamination. However, because in this phase microalgae consume storage compounds, as carbohydrates (Figure 3a), the application of a famine phase seems more feasible if the harvested biomass is used to produce proteins or as inoculum for further cultivations. The microalga *T. obliquus* can accumulate up to 40-60% carbohydrates as dry weight [46], corresponding to accumulating about 2-3 folds its initial weight as carbohydrates [40]. The accumulation ability of bacteria is more difficult to be predicted because there are different unknown bacteria species in the wastewater and because, also for those known, there are few information about the ability to accumulate carbohydrates. However, it was shown in previous studies that mixed bacteria consortia in wastewater typically show very low accumulation ability, as compared to microalgae, since they increase their weight by accumulation only by a factor lower

than 1.25 (corresponding to reach carbon reserves covering about 20% of dry weight) [47]. In our study we observed a higher survival rate of microalgae in the condition in which more carbohydrates were accumulated (P_{UF}). This suggests that an increased accumulation of carbohydrates may be the reason for the higher survival rate that we observed for microalgae as compared to bacteria in the famine phase. This is in agreement with previous results obtained by uncoupling glucose and N to control bacteria contamination [7].

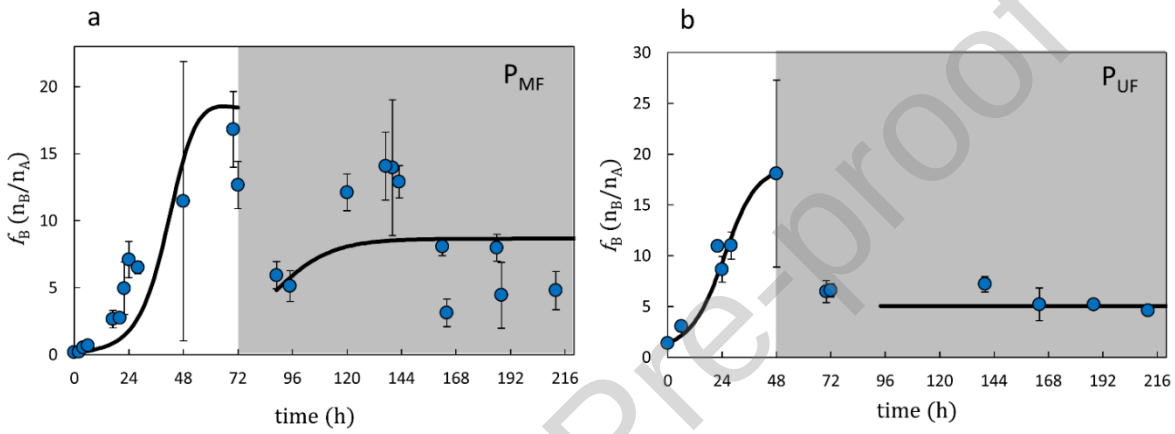


Figure 5. Dynamic evolution of the bacterial contamination (f_B) as measured for P_{MF} (a) and P_{UF} (b) and as predicted by the models. Energy starvation (famine) phase is indicated in grey. Data are reported as average ± SD (n_r = 2).

3.5 Assessment of bacterial contamination and strategies to control it

Bacteria contamination throughout the batch was quantified by the ratio f_B between the concentration of bacteria (n_A) and microalgal cells (n_B). The evolution of f_B predicted by the model with the estimated parameters (Table 4) is compared with the f_B values recorded during the experiment in Figure 5.

In the initial phase of active growth under feast conditions, both bacteria and microalgae grew exponentially:

$$n_A = n_{A,0} e^{\mu_{max,A} t} \quad (10)$$

$$n_B = n_{B,0} e^{\mu_{max,B} t}$$

(11)

where $\mu_{max,A}$ and $\mu_{max,B}$ are the maximum specific growth rates of microalgae and bacteria, respectively. It then follows:

$$f_B = \frac{n_B}{n_A} = \frac{n_{B,0}}{n_{A,0}} e^{(\mu_{max,B} - \mu_{max,A})t}$$

(12)

Since bacteria have a higher μ_{max}, bacteria contamination (Eq. 12) increases exponentially with a net growth rate $\mu_{max,C} = \mu_{max,B} - \mu_{max,A}$. The $\mu_{max,C}$ was equal to 0.09 ± 0.02 h^{-1} for P_{MF} (t_d=7.7 h), which means that bacteria contamination doubled every 7.7 h when substrates are all available (for P_{UF} $\mu_{max,C}$ was not statistically different). To control bacterial contamination, the duration of this phase, which can be varied by modifying the substrate load, could be adequately tailored accordingly to $\mu_{max,C}$, $Y_{X/S}$ and initial $f_{B,0}$. In the case of utilization of P_{UF}, the initial bacterial concentration was higher (due to the longer pre-treatment), indeed $f_{B,0}$ was 0.17 ± 0.05 for P_{MF} and 1.4 ± 0.3 for P_{UF}, thus reducing the widest time window that allows maintaining contamination below a certain threshold.

During the famine phase, some cells lysed due to the shortage of energy. Bacteria had a higher drop in cell concentration than microalgae, thus the contamination could be reduced (Figure 5). For both P_{UF} and P_{MF}, the contamination reduction was the highest around 24 h, immediately after the beginning of the famine phase, and then there was not further relevant improvement. Considering that the famine phase should be minimized to maintain r_X as high as possible, the analysis of bacterial contamination indicates that the batch should takes no more than 24 h after substrate depletion. This strategy can also allow avoiding the releasee of N observed for longer times (Figure 1b), which would reduce N removal yield.

It is here worth to remark that, to select PHA-accumulating bacteria from activated sludge, in analogy with the strategy implemented in the present study, the alternation of energy-replete (feast)

and energy deplete (famine) phases is often exploited [13]. However, in these latter studies, the durations of the phases have been chosen mainly empirically, based on the analysis of the mean PHA content obtained by varying the phase durations [48,49], rather than based on species-specific differences in growth kinetic parameters. The optimal feast to famine ratio found in the present study is 0.66-0.75, significantly higher than the ratio usually reported for selecting PHA-accumulating bacteria (0.1-0.3) [48–50]. However, the length of the famine phase here used was 24 h, higher than that usually employed for selecting PHA-accumulating bacteria (5-11 h) [48,50].

Cell concentration alone may not be enough to quantify the contamination of the produced biomass. It should be taken in consideration that bacterial cells have a mass usually below 1 pg, while *T. obliquus* cells mass is between 20–60 pg (depending on growth condition) [42]. By assuming bacteria and microalgae masses per cell equal to 1 pg and 30 pg, respectively, the fraction of bacterial mass on total biomass (χ_B) was estimated by Eq. 10. The initial biomass was composed for 99.4 ± 0.3% (P_{MF}) and 95.6 ± 0.3% (P_{UF}) of microalgae at t = 0, attaining a minimum in the purity (62-64%) at the end of the feast phase. Then, after 24 h of famine phase, the purity increased again to 82-85% (Figure S3). Despite the famine phase was effective in reducing contamination, the purity of microalgae biomass was not high as in the phototrophic condition (namely, the starter inoculum at t = 0). This latter problem could be overcome by further optimizing the feast phase, in a way that substrate depletion is attained before attaining an exceedingly extended bacterial contamination. To this aim, a prediction on the time-evolution of χ_B under feast conditions was carried out by using the Eq. 12, for different values of microalgal $\mu_{max,A}$ and a fixed value of bacterial $\mu_{max,B}$ = 0.0158 h^{-1} (t_d=4.4 h) (Table 4). This way, it was possible to evaluate the time window for cultivating different known microalgae strains with whey, at 25 °C, preventing excessive contamination (Figure 6). The $\mu_{max,A}$ used were: 0.077 h^{-1} (t_d=9 h), for *T. obliquus* of this study; 0.087 h^{-1} (t_d=8 h), for *Chlorella sorokiniana* [33]; 0.0117 h^{-1} (t_d=59 h), for the high value but slow growing *Hamaetococcus pluvialis* [51] and 0.137 h^{-1} (t_d=5 h), for *Chlorella sorokiniana* under mixotrophic condition [33].

When nutrients are replete, since microalgae have $\mu_{max,A} < \mu_{max,B}$, they have a limited time window before contamination attains a threshold value. Particularly, if the threshold is fixed at 10%, the time window in full heterotrophy is between 21 h (*H. pluvialis*) and 41 h (*C. sorokiniana*) when $\chi_{B,0}$ = 0.0056, as the case of P_{MF} (Figure 6a), and between 6 h (*H. pluvialis*) and 12 h (*C. sorokiniana*) when $\chi_{B,0}$ = 0.044, as the case of P_{UF} (Figure 6b). The threshold should be set case by case depending on the end-use od the biomass. If mixotrophy is exploited, it can allow higher growth rate to microalgae, due to the sum of phototrophic and mixotrophic metabolism. When mixotrophy is considered, *C. sorokiana* can be cultivated for more than 36 h before attaining the threshold (Figure 6). This is a promising prediction worth to be validated with experimental data, since it could allow a more stable non-axenic cultivation of such microalgal species on whey permeate.

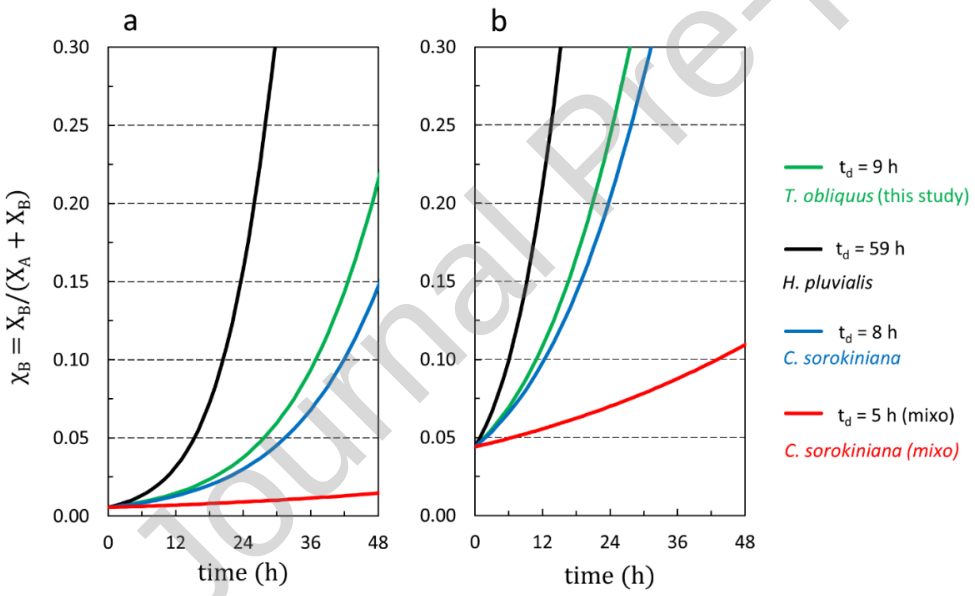


Figure 6. Prediction of bacterial contamination (χ_B, as biomass) evolution in a batch reactor fed with whey permeate under feast (energy replete) condition, for different microalgal specific growth rate. Bacterial specific growth rate: 0.158 h^{-1} (t_d = 4.4 h). In (a), $\chi_{B,0}$ was set 0.0056 (g/g) as measured in P_{MF}, while in (b) $\chi_{B,0}$ was set 0.044 (g/g) as measured in P_{UF}.

The control approach described in this study allowed to minimize bacterial contamination during cultivation in presence of organic substrate, but not to increase the purity of microalgae biomass. In the described configuration, the system can be therefore scaled industrially only in a two-phase

phototrophic-heterotrophic or phototrophic-mixotrophic process. Following the same approach, future studies could evaluate the possibility to find operative conditions to run a sequencing batch reactor (SBR) to select microalgae in fully mixotrophic or heterotrophic conditions. Our results provide general guidelines to be followed that can be used in future studies to define more in detail the operative conditions for specific microalgal strains and specific wastewater employed.

Conclusions

The microalga *T. obliquus* could grow by using the sugars contained in the permeates from whey ultrafiltration or microfiltration as source of C and energy. However, under these conditions, the microalgae growth rate (0.07 h^{-1}) was about two times lower than that of the heterotrophic bacteria flora found in the employed medium (0.16 h^{-1}). Consequently, when sugars were available (feast phase), bacterial contamination increased exponentially with a net specific growth rate of 0.09 h^{-1}. Once substrate was depleted (famine phase), cell lysis took place determining a reduction in cell concentration for bacteria significantly larger than for microalgae. This difference, which may be explained by the higher accumulation of carbohydrates by microalgae during the feast phase, allowed to mitigate bacterial contamination.

The optimal operative conditions to maximize biomass productivity, pollutant removal and maintain bacterial contamination below a prescribed threshold should include a feast phase (substrate replete condition), whose length should be appropriately selected based on the difference between microalgae and bacterial specific growth rates and initial bacteria load, and a 24 h famine phase (energy starvation). The results reported in this study describe how a batch bioreactor can be operated to effectively boost microalgal biomass productivity by the biodegradation of a wastewater containing both nitrogen and organic carbon substrates. It is expected that the proposed process can be industrially scalable in a two-phase phototrophic-heterotrophic or phototrophic-mixotrophic process.

Acknowledgements

This work was supported by the European Union in the framework of the LIFE program (MEWLIFE project - LIFE17 ENV IT 000180).

References

[1] J. Ruiz, R.H. Wijffels, M. Dominguez, M.J. Barbosa, Heterotrophic vs autotrophic production of microalgae: Bringing some light into the everlasting cost controversy, Algal Res. 64 (2022) 102698. doi:10.1016/j.algal.2022.102698.

[2] J. Ruiz, G. Olivieri, J. de Vree, R. Bosma, P. Willems, J.H. Reith, M.H.M. Eppink, D.M.M. Kleinegris, R.H. Wijffels, M.J. Barbosa, Towards industrial products from microalgae, Energy Environ. Sci. 9 (2016) 3036–3043. doi:10.1039/C6EE01493C.

[3] M.R. Tredici, L. Rodolfi, N. Biondi, N. Bassi, G. Sampietro, Techno-economic analysis of microalgal biomass production in a 1-ha Green Wall Panel (GWP®) plant, Algal Res. 19 (2016) 253–263. doi:10.1016/j.algal.2016.09.005.

[4] S. Schade, T. Meier, A comparative analysis of the environmental impacts of cultivating microalgae in different production systems and climatic zones: A systematic review and meta-analysis, Algal Res. 40 (2019) 101485. doi:10.1016/j.algal.2019.101485.

[5] H. Jin, H. Zhang, Z. Zhou, K. Li, G. Hou, Q. Xu, W. Chuai, C. Zhang, D. Han, Q. Hu, Ultrahigh-cell-density heterotrophic cultivation of the unicellular green microalga Scenedesmus acuminatus and application of the cells to photoautotrophic culture enhance biomass and lipid production, Biotechnol. Bioeng. 117 (2020) 96–108. doi:10.1002/BIT.27190.

[6] S. Smetana, M. Sandmann, S. Rohn, D. Pleissner, V. Heinz, Autotrophic and heterotrophic microalgae and cyanobacteria cultivation for food and feed: life cycle assessment, Bioresour. Technol. 245 (2017) 162–170. doi:10.1016/j.biortech.2017.08.113.

[7] F. Di Caprio, P. Altimari, G. Iaquaniello, L. Toro, F. Pagnanelli, Heterotrophic cultivation of T. obliquus under non-axenic conditions by uncoupled supply of nitrogen and glucose, Biochem. Eng. J. 145 (2019) 127–136. doi:10.1016/j.bej.2019.02.020.

[8] N. Poddar, R. Sen, G.J.O. Martin, Glycerol and nitrate utilisation by marine microalgae

Nannochloropsis salina and Chlorella sp . and associated bacteria during mixotrophic and heterotrophic growth, Algal Res. 33 (2018) 298–309. doi:10.1016/j.algal.2018.06.002.

[9] J. Lowrey, M.S. Brooks, P.J. Mcginn, Heterotrophic and mixotrophic cultivation of microalgae for biodiesel production in agricultural wastewaters and associated challenges-a critical review, (n.d.). doi:10.1007/s10811-014-0459-3.

[10] M. Gélinas, T.T.H. Pham, B. Boëns, K. Adjallé, S. Barnabé, Residual corn crop hydrolysate and silage juice as alternative carbon sources in microalgae production, Algal Res. 12 (2015) 33–42. doi:10.1016/j.algal.2015.08.001.

[11] S. Li, P.L. Show, H.H. Ngo, S.H. Ho, Algae-mediated antibiotic wastewater treatment: A critical review, Environ. Sci. Ecotechnology. 9 (2022) 100145. doi:10.1016/J.ESE.2022.100145.

[12] F. Di Caprio, L. Tayou Nguemna, M. Stoller, M. Giona, F. Pagnanelli, Microalgae cultivation by uncoupled nutrient supply in sequencing batch reactor (SBR) integrated with olive mill wastewater treatment, Chem. Eng. J. 410 (2021) 128417. doi:10.1016/j.cej.2021.128417.

[13] F. Di Caprio, Cultivation processes to select microorganisms with high accumulation ability, Biotechnol. Adv. 49 (2021) 107740. doi:10.1016/J.BIOTECHADV.2021.107740.

[14] A.R. Prazeres, F. Carvalho, J. Rivas, Cheese whey management: a review, J. Environ. Manage. 110 (2012) 48–68. doi:10.1016/j.jenvman.2012.05.018.

[15] F. Carvalho, A.R. Prazeres, J. Rivas, Cheese whey wastewater: Characterization and treatment, Sci. Total Environ. 445–446 (2013) 385–396. doi:10.1016/j.scitotenv.2012.12.038.

[16] G. Gramegna, A. Scortica, V. Scafati, F. Ferella, L. Gurrieri, M. Giovannoni, R. Bassi, F. Sparla, B. Mattei, M. Benedetti, Exploring the potential of microalgae in the recycling of dairy wastes, Bioresour. Technol. Reports. 12 (2020) 100604. doi:10.1016/J.BITEB.2020.100604.

[17] D. Zapata, C. Arroyave, L. Cardona, A. Aristizábal, C. Poschenrieder, M. Llugany, Phytohormone production and morphology of Spirulina platensis grown in dairy wastewaters, Algal Res. 59 (2021) 102469. doi:10.1016/J.ALGAL.2021.102469.

[18] J.M. Girard, R. Tremblay, N. Faucheux, M. Heitz, J.S. Deschênes, Phycoremediation of cheese whey permeate using directed commensalism between Scenedesmus obliquus and Chlorella protothecoides, Algal Res. 22 (2017) 122–126. doi:10.1016/j.algal.2016.12.013.

[19] F. Di Caprio, P. Altimari, G. Iaquaniello, L. Toro, F. Pagnanelli, T. obliquus mixotrophic cultivation in treated and untreated olive mill wastewater, Chem. Eng. Trans. 64 (2018) 625–630. doi:10.3303/CET1864105.

[20] F. Di Caprio, R. Chelucci, I. Francolini, P. Altimari, F. Pagnanelli, Extraction of microalgal starch and pigments by using different cell disruption methods and aqueous two-phase system, J. Chem. Technol. Biotechnol. (2021). doi:10.1002/jctb.6910.

[21] F. Garcia-Ochoa, E. Gomez, Bioreactor scale-up and oxygen transfer rate in microbial processes: An overview, Biotechnol. Adv. 27 (2009) 153–176. doi:10.1016/j.biotechadv.2008.10.006.

[22] F. Di Caprio, S. Posani, P. Altimari, A. Concas, F. Pagnanelli, Single Cell Analysis of Microalgae and Associated Bacteria Flora by Using Flow Cytometry, Biotechnol. Bioprocess Eng. 26 (2021) 898–909. doi:10.1007/s12257-021-0054-9.

[23] M. DuBois, K. a. Gilles, J.K. Hamilton, P. a. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356. doi:10.1021/ac60111a017.

[24] A.L. Galant, R.C. Kaufman, J.D. Wilson, Glucose: Detection and analysis, Food Chem. 188 (2015) 149–160. doi:10.1016/J.FOODCHEM.2015.04.071.

[25] S. Van Wychen, W. Long, S.K. Black, L.M.L. Laurens, MBTH: A novel approach to rapid, spectrophotometric quantitation of total algal carbohydrates, Anal. Biochem. 518 (2017) 90–93. doi:10.1016/j.ab.2016.11.014.

[26] A.K. Slavov, General Characteristics and Treatment Possibilities of Dairy Wastewater – A Review, Food Technol. Biotechnol. 55 (2017) 14–28.

[27] V. Turon, C. Baroukh, E. Trably, E. Latrille, E. Fouilland, J.P. Steyer, Use of fermentative metabolites for heterotrophic microalgae growth: Yields and kinetics, Bioresour. Technol. 175 (2015) 342–349. doi:10.1016/J.BIORTECH.2014.10.114.

[28] R. Bosma, J.H. de Vree, P.M. Slegers, M. Janssen, R.H. Wijffels, M.J. Barbosa, Design and construction of the microalgal pilot facility AlgaePARC, Algal Res. 6 (2014) 160–169. doi:10.1016/j.algal.2014.10.006.

[29] M.A. Mazorra-Manzano, G.R. Robles-Porchas, D.A. González-Velázquez, M.J. Torres-Llanez, M.

Journal Pre-proof

Martínez-Porchas, C.O. García-Sifuentes, A.F. González-Córdova, B. Vallejo-Córdoba, Cheese Whey Fermentation by Its Native Microbiota: Proteolysis and Bioactive Peptides Release with ACE-Inhibitory Activity, Food Microbiol. 44 (2014) 136–141. doi:10.3390/fermentation6010019.

[30] G. Breuer, P.P. Lamers, D.E. Martens, R.B. Draaisma, R.H. Wijffels, The impact of nitrogen starvation on the dynamics of triacylglycerol accumulation in nine microalgae strains, Bioresour. Technol. 124 (2012) 217–226. doi:10.1016/j.biortech.2012.08.003.

[31] G.M. León-Saiki, T. Cabrero Martí, D. van der Veen, R.H. Wijffels, D.E. Martens, The impact of day length on cell division and efficiency of light use in a starchless mutant of Tetradesmus obliquus, Algal Res. 31 (2018) 387–394. doi:10.1016/j.algal.2018.02.027.

[32] Y. Yang, K. Gao, Effects of CO 2 concentrations on the freshwater microalgae, Chlamydomonas reinhardtii, Chlorella pyrenoidosa and Scenedesmus obliquus (Chlorophyta), J. Appl. Phycol. 15 (2003) 379–389.

[33] T. Li, Y. Zheng, L. Yu, S. Chen, Mixotrophic cultivation of a Chlorella sorokiniana strain for enhanced biomass and lipid production, Biomass and Bioenergy. 66 (2014) 204–213. doi:10.1016/J.BIOMBIOE.2014.04.010.

[34] A. Aldrete-Tapia, M.C. Escobar-Ramírez, M.L. Tamplin, M. Hernández-Iturriaga, High-throughput sequencing of microbial communities in Poro cheese, an artisanal Mexican cheese, Food Microbiol. 44 (2014) 136–141. doi:10.1016/j.fm.2014.05.022.

[35] F. Rezvani, F. Ardestani, G. Najafpour, Growth kinetic models of five species of Lactobacilli and lactose consumption in batch submerged culture, Brazilian J. Microbiol. 48 (2017) 251–258. doi:10.1016/j.bjm.2016.12.007.

[36] Z. Ai, X. Lv, S. Huang, G. Liu, X. Sun, H. Chen, J. Sun, Z. Feng, The effect of controlled and uncontrolled pH cultures on the growth of Lactobacillus delbrueckii subsp. bulgaricus, LWT - Food Sci. Technol. 77 (2017) 269–275. doi:10.1016/j.lwt.2016.11.057.

[37] Y. Zhang, P. V. Vadlani, D-lactic acid biosynthesis from biomass-derived sugars via lactobacillus delbrueckii fermentation, Bioprocess Biosyst. Eng. 36 (2013) 1897–1904. doi:10.1007/s00449-013-0965-8.

[38] K. Adamberg, S. Kask, T.M. Laht, T. Paalme, The effect of temperature and pH on the growth of

Journal Pre-proof

lactic acid bacteria: a pH-auxostat study, Int. J. Food Microbiol. 85 (2003) 171–183. doi:10.1016/S0168-1605(02)00537-8.

[39] M.A.R. Nor-Khaizura, S.H. Flint, O.J. McCarthy, J.S. Palmer, M. Golding, Modelling the effect of fermentation temperature and time on starter culture growth, acidification and firmness in made-in-transit yoghurt, LWT. 106 (2019) 113–121. doi:10.1016/J.LWT.2019.02.027.

[40] F. Di Caprio, A fattening factor to quantify the accumulation ability of microorganisms under N-starvation, N. Biotechnol. 66 (2022) 70–78. doi:10.1016/J.NBT.2021.04.001.

[41] F.L. Hellweger, V. Bucci, M.R. Litman, A.Z. Gu, A. Onnis-Hayden, Biphasic Decay Kinetics of Fecal Bacteria in Surface Water Not a Density Effect, J. Environ. Eng. 135 (2009) 372–376. doi:10.1061/(ASCE)0733-9372(2009)135:5(372).

[42] F. Di Caprio, F. Pagnanelli, R.H. Wijffels, D. Van der Veen, Quantification of Tetradesmus obliquus (Chlorophyceae) cell size and lipid content heterogeneity at single-cell level, J. Phycol. 54 (2018) 187–197. doi:10.1111/jpy.12610.

[43] A. Garz, M. Sandmann, M. Rading, S. Ramm, R. Menzel, M. Steup, Cell-to-cell diversity in a synchronized chlamydomonas culture as revealed by single-cell analyses, Biophys. J. 103 (2012) 1078–1086. doi:10.1016/j.bpj.2012.07.026.

[44] G. Ziglio, G. Andreottola, S. Barbesti, G. Boschetti, L. Bruni, P. Foladori, R. Villa, Assessment of activated sludge viability with flow cytometry, Water Res. 36 (2002) 460–468. doi:10.1016/S0043-1354(01)00228-7.

[45] M. Friedrich, I. Takács, J. Tränckner, Physiological adaptation of growth kinetics in activated sludge, Water Res. 85 (2015) 22–30. doi:10.1016/J.WATRES.2015.08.010.

[46] L. de Jaeger, R.E. Verbeek, R.B. Draaisma, D.E. Martens, J. Springer, G. Eggink, R.H. Wijffels, Superior triacylglycerol (TAG) accumulation in starchless mutants of Scenedesmus obliquus: (I) mutant generation and characterization, Biotechnol. Biofuels. 7 (2014) 69. doi:10.1186/1754-6834-7-69.

[47] K. Sakai, S. Miyake, K. Iwama, D. Inoue, S. Soda, M. Ike, Polyhydroxyalkanoate (PHA) accumulation potential and PHA-accumulating microbial communities in various activated sludge processes of municipal wastewater treatment plants, J. Appl. Microbiol. 118 (2015) 255–266.

Journal Pre-proof

doi:10.1111/JAM.12683.

[48] C.S.S. Oliveira, C.E. Silva, G. Carvalho, M.A. Reis, Strategies for efficiently selecting PHA producing mixed microbial cultures using complex feedstocks: Feast and famine regime and uncoupled carbon and nitrogen availabilities, N. Biotechnol. 37 (2017) 69–79. doi:10.1016/j.nbt.2016.10.008.

[49] D. Dionisi, M. Majone, G. Vallini, S. Di Gregorio, M. Beccari, Effect of the Length of the Cycle on Biodegradable Polymer Production and Microbial Community Selection in a Sequencing Batch Reactor, Biotechnol. Prog. 23 (2007) 1064–1073. doi:10.1021/BP060370C.

[50] J. Tamis, L. Marang, Y. Jiang, M.C.M. van Loosdrecht, R. Kleerebezem, Modeling PHA-producing microbial enrichment cultures-towards a generalized model with predictive power, N. Biotechnol. 31 (2014) 324–334. doi:10.1016/j.nbt.2013.11.007.

[51] M.J. Moya, M.L. Sa • Nchez-Guardamino, A. Vilavella, E. Barberà, Growth of Haematococcus lacustris: A Contribution to Kinetic Modelling, J. Chem. T Ech. Biotechnol. 68 (1997) 303–309. doi:10.1002/(SICI)1097-4660(199703)68:3.

Journal Pre-proof

CRediT authorship contribution statement

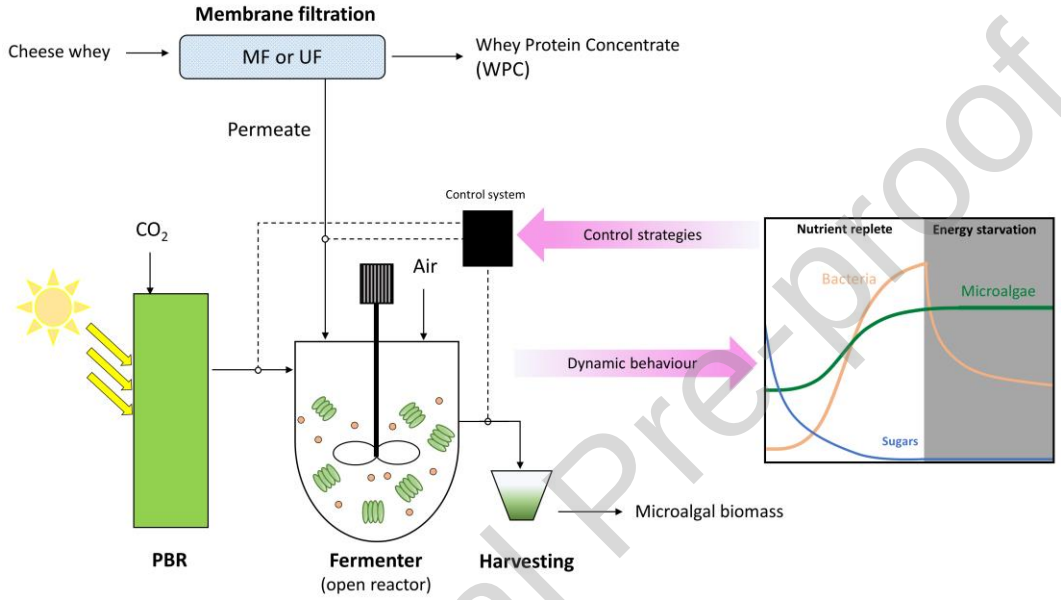
F. Di Caprio: Investigation, Conceptualization, Methodology, Resources, Data curation, Formal analysis, Visualization, Writing – original draft, review & editing, Project administration. **G.P. Tocca:** Investigation, Data curation, Writing – original draft. **M. Stoller:** Investigation, Methodology. **F. Pagnanelli:** Supervision, Project administration, Funding acquisition. **P. Altimari:** Supervision, Writing – review & editing.

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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical abstract



Highlights

- Microalgae were cultivated in UF and MF whey permeates in a feast and famine regime
- Up to 90% TN, 87% TP and 91% COD were removed from whey permeates
- During feast phase bacteria had a μ_{max} (0.16 h^{-1}) two folds higher than microalgae
- Bacteria contamination was remarkably reduced after 24 h energy starvation (famine)
- Only a subpopulation of cells lysed during famine phase; it was lower at higher C/N

Journal Pre-proof