
Original paper/research article

Biorefinery-based approach to exploit mixed cultures of *Lipomyces starkeyi* and *Chloroidium saccharophilum* for Single Cell Oil production

Gaetano Zuccaro^{1*}, Angelo del Mondo², Gabriele Pinto³, Antonino Pollio³, Antonino De Natale³

¹ Department of Chemical, Materials and Production Engineering, University of Naples Federico II, 80125 Napoli, Italy

² Stazione Zoologica Anton Dohrn, Istituto Nazionale di Biologia, Ecologia e Biotecnologie marine, Villa Comunale, 80121 Napoli, Italy

³ Dipartimento di Biologia – Università degli Studi di Napoli Federico II – Via Cinthia 26, 80126 Napoli, Italy

* Correspondence: gaetano.zuccaro@unina.it

Abstract: The mutualistic interactions between the oleaginous yeast *Lipomyces starkeyi* and the green microalga *Chloroidium saccharophilum* in mixed cultures were investigated to exploit possible synergistic effects. As a matter of facts, microalga could act as an oxygen generator for the yeast, while the yeast could provide carbon dioxide to microalga. A lignocellulosic hydrolysate from steam exploded *Arundo donax* (Giant reed) was used as low cost feedstock. The overall lipid content and lipid productivity obtained in the mixed culture treating the hydrolysate of *Arundo donax* were equal to 0.081 g_{lipid}.g_{biomass}⁻¹ and 37.2 mg_{lipid}.L⁻¹.d⁻¹, respectively. They represented promising results if compared to the model systems where synthetic media were used. This study provided new input for the integration of Single Cell Oil (SCO) production with agro-industrial feedstock and the fatty acid distribution mainly consisting of stearic (C18:0) and oleic acid (C18:1) allows promising applications in biofuels, cosmetics, food additives and other products of industrial interest.

Keywords: Mixed culture; *Lipomyces starkeyi*; *Chloroidium saccharophilum*; Single Cell Oils (SCOs); *Arundo donax*; biorefinery.

1. Introduction

A sustainable economic growth, devoted to the future generations, requires long-term available resources for industrial production, in terms of raw materials and energy. A biorefinery-based approach, aimed to the conversion of low cost feedstocks into marketable chemicals, fuels and products, has consequently to be preferred [1]. So far, several strategies have been explored to enhance the productivity and competitiveness of microbial-based processes and to improve simultaneously the biorefinery efficiency [2]. Particular attention has been devoted to the Single Cell Oil (SCO) production or microbial oil synthesis used as supplier of functional oils and for biodiesel [3] but the high fermentation costs make this production still undeveloped industrially. Therefore, the identification of substrates able to reduce the costs can be considered a solution that positively affects the related industrial implementation as well as the identification of strategies able to contribute positively in the same direction. Among them, co-culturing oleaginous yeasts and microalgae has been studied in the recent years for enhancing Single Cell Oil productivity by utilizing minimal resources in various fields such as wastewater treatment, biogas

production, enzyme production and bioremediation [4]. While the benefits that can be derived from these systems are clear, the nature of mutualistic interactions between yeast and microalgae in co-culture systems are still largely unexplored [5]. The photoautotroph-heterotroph partnership has been defined as able to overcome the high oxygen accumulation that causes a significant problem for microalgal growth especially in closed systems, since it inhibits photosynthesis. So, the inclusion of a heterotroph partner able to consume the oxygen, mitigates this problem and, at the same time, can contribute to increase microbial biomass and metabolite production [6,7]. In addition, microalgae can convert the dissolved CO₂ in the medium into bicarbonate. When it is consumed, releasing OH⁻ ions, it makes the medium alkaline. Conversely, yeast growth results in acidic medium, which can hinder microalgal growth. The combination of both can be of help to balance this phenomenon [4]. Additionally, the reduction of toxic reactive oxygen species (ROS) by the heterotroph partner has been shown capable to protect the phototroph microorganisms from oxidative stress in these co-cultures systems [7]. To promote the microbial oil synthesis, it is necessary to provide sufficient organic carbon in culture medium or enhance the photosynthesis of microalgae. The research about the microalgae oil production, has been focused mainly on the photoautotrophic growth mode, but there are significant drawbacks associated. As a matter of facts, it is difficult to find operating conditions for the simultaneous achievement of biomass accumulation and lipid synthesis during the microalgae life cycle [8]. In addition, light attenuation is unavoidable for photoautotrophic cultures from lab to pilot scale, leading to significantly reductions of productivity [9]. For these reasons, other cultivation modes in which microalgae are able to use also the source of organic carbon have been explored in order to improve the productivity of microbial oils. On their side, oleaginous yeasts can grow in presence of different carbon sources, for example, hexose and pentose sugars with high growth rates [10]. Generally, they have the ability to accumulate Single Cell Oils (SCOs) more than 20% of their total dry weight [11]. In culture medium with high C/N ratio, oleaginous microorganisms utilize the remaining carbon source for the synthesis of lipids, mainly triacylglycerols (TAGs). So far, yeasts, molds or microalgae have been used for lipid production more frequently than bacteria [12]. *Lipomyces starkeyi* [13] and *Chloroidium saccharophilum* (W. Krüger) [14], were chosen as species and their interactions in mixed cultures were investigated. *L. starkeyi* displays a greater capacity to accumulate lipids and a native ability to assimilate several feedstocks. It also tolerates low pH [15,16] and can metabolize inhibitors present in cellulosic hydrolysates [17]. *C. saccharophilum* has been chosen for its high capacity of CO₂ assimilation and high tolerance to acidic environments [18], as well as for its high capacity of lipid accumulation [19], which represent positive aspects with regard to the use of mixed cultures to increase the lipid yield but also for the potential use of mixed cultures for CO₂ mitigation that would make the process more economically feasible where CO₂-rich flue gases are available, i.e. in the vicinity of power plants. Moreover, *C. saccharophilum* is able to grow under heterotrophic conditions [20] that are not secondary in view of using different feedstocks. It is known, in fact, that the costs of feedstock represent a bottleneck in the successful development of heterotrophic microbial cultures [21]. For this reason, we adopted a growth medium obtained by *Arundo donax* L. (Giant reed), a perennial grass largely diffused in Mediterranean Region. *A. donax* is considered a promising crop for industrial applications, thanks to its high biomass productivity, its adaptability to different climatic and soil conditions (e.g. polluted or salinized soils), and for the efficient protection offered against the erosion of hilly soils [22]. *A. donax* hydrolysate has been successfully employed for growing oleaginous yeast strains [23] but, to the best of our knowledge, there are very few studies about mixed oleaginous yeast-microalgae cultures fed with lignocellulosic hydrolysates [4]. This study was performed in order to verify the presence of synergistic effects comparing the operating conditions and the dynamics of synthetic media inoculated by single and mixed cultures of *L. starkeyi* and *C.*

saccharophilum strains with a real system represented by *Arundo donax* hydrolysate previously subjected to a steam explosion pre-treatment. The incidence of inhibitors and volatile organic acids were also evaluated.

2. Materials and Methods

2.1 Strains

The oleaginous yeast *Lipomyces starkeyi* (DBVPG 6193) was supplied by the Dipartimento di Biologia Vegetale di Perugia, Italy. The strains were maintained at 5 °C on a YPD agar slants and then transferred in YPD medium for the seed culture contained (per liter): yeast extract 10 g, peptone 20 g, D-glucose 20 g and agar 20 g, when required. Both media were sterilized at 121 °C for 20 minutes before use.

The green microalga *Chloroidium saccharophilum* strain 042 was supplied by the ACUF microalgae collection (<http://www.acuf.net>) of the Department of Biology, at the University Federico II of Naples, Italy. Single colonies were picked up from the plates and suspended in BBM medium. Tubes and plates were grown at 25 °C under continuous light supply (100 $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$).

2.2. Culture media and operating conditions

Lipomyces starkeyi and *Chloroidium saccharophilum* were grown in 500 mL Erlenmeyer flasks with an initial volume of 150 mL which contained (g/L): KH_2PO_4 (1.0), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.5), $(\text{NH}_4)_2\text{SO}_4$ (2.0), yeast extract (0.5), glucose (10.0) (YEG). The culture flasks were inoculated, separately and simultaneously, to achieve the initial cell density of about $3.0 \cdot 10^6 \text{ Cells.mL}^{-1}$ for both microorganisms. The pH was adjusted to 6-6.5 and prior to inoculation, the culture medium was sterilized at 121 °C for 20 min. As seed medium was also chosen the Bold Basal Medium (BBM) supplemented with yeast extract and $(\text{NH}_4)_2\text{SO}_4$ to replace NaNO_3 as nitrogen source and glucose as carbon source (BBM+G). The medium was autoclaved for 20 minutes at 121 °C. The enriched Bold Basal Medium (BBM+G) medium contained the following components: $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ($1.70 \cdot 10^{-4} \text{ M}$), KH_2PO_4 ($1.29 \cdot 10^{-3} \text{ M}$), EDTA anhydrous ($1.71 \cdot 10^{-4} \text{ M}$), KOH ($5.52 \cdot 10^{-4} \text{ M}$), K_2HPO_4 ($4.31 \cdot 10^{-4} \text{ M}$), NaCl ($4.28 \cdot 10^{-4} \text{ M}$), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ($3.04 \cdot 10^{-4} \text{ M}$), H_3BO_3 ($1.85 \cdot 10^{-4} \text{ M}$), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ($1.79 \cdot 10^{-5} \text{ M}$), H_2SO_4 ($1.79 \cdot 10^{-5} \text{ M}$), $\text{ZnO}_4 \cdot 7\text{H}_2\text{O}$ ($3.07 \cdot 10^{-5} \text{ M}$), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ($3.07 \cdot 10^{-5} \text{ M}$), MnO_3 ($4.93 \cdot 10^{-6} \text{ M}$), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ($6.29 \cdot 10^{-6} \text{ M}$), $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ($1.68 \cdot 10^{-6} \text{ M}$), yeast extract (0.5 g.L⁻¹), $(\text{NH}_4)_2\text{SO}_4$ (2 g.L⁻¹) and glucose (10 g.L⁻¹). The initial pH was adjusted to 6-6.5. The flasks were incubated at 25 °C and under continuous light intensity equal to 100 $\mu\text{mol photons.m}^{-2}.\text{s}^{-1}$. All chemicals were purchased from Sigma-Aldrich. *Chloroidium saccharophilum* and *Lipomyces starkeyi* were incubated into 500 mL Erlenmeyer flasks containing 150 mL of medium. The culture flasks were inoculated, separately and simultaneously, to achieve the initial cell density of about $3 \cdot 10^6 \text{ Cells.mL}^{-1}$ for both microorganisms. The synthetic media were compared to lignocellulosic hydrolysate from pretreated *Arundo donax*. Steam explosion was applied as pretreatment. *A. donax* (giant reed) was processed in a continuous pilot plant (mod. StakeTech System Digester) located at ENEA-Trisaia Research Centre (Rotondella, Matera, Italy). The biomass was treated processing 150-200 kg.h⁻¹ of dry biomass, to which water was added to raise the intrinsic humidity up to 50%. The pretreatment was carried

out at 210 °C for 4 minutes. The severity factor (SF) was determined to be 3.84 according to the following Equation 1 [24]:

$$SF = \log(t \times e^{\frac{T-100}{14.75}}) \quad (1)$$

where t is the residence time in minutes, T is the temperature of pre-treatment, 100 is the reference temperature and 14.75 is the arbitrary constant.

Pretreated *Arundo donax* was mixed with distilled water (pH 5.2) to obtain a solution with 5% w/v solid content and was then treated with commercial enzymes purchased from Sigma-Aldrich consisting of cellulase from *Trichoderma reesei* ATCC 26921 (15 FPU/g of cellulose) and β -glucosidase from *Aspergillus niger* (30 CBU/g of cellulose). Cellulase activity was measured following the NREL filter paper assay [25] and reported in filter-paper units (FPU) per milliliter of solution. β -glucosidase activity was measured using the method described by Wood & Bhat [26] and reported in cellobiase units (CBU). Enzymatic hydrolysis was carried out at 160 rpm and 50 °C for 48 h (Minitron, Infors HT, Switzerland). The initial pH was adjusted to 6-6.5. At the hydrolysate of *Arundo donax* (ADH), 8 mL of phosphate buffer (0.2 M) were added, then it was inoculated with *Lipomyces starkeyi* and *Chloroidium saccharophilum* alone and mixed to achieve the initial cell density of $3.0 \cdot 10^6$ Cells.mL⁻¹ for both microorganisms.

2.3 Analytical methods

Measurements of pH were made by an inoLab® Multi 740 Multimeters pH-meter (WTW). The biomass concentration was monitored with a Shimadzu UV6100 spectrophotometer (Japan) and by measuring turbidity of liquid samples at 600 and 680 nm. Microbial biomass (g.L⁻¹) was determined by filtering 2-3 mL of culture over pre-weight PES filters (0.45 μ m; Sartorius Biolab, Germany). The retained biomass on filters was washed, dried at 105 °C for 24 hours and then stored in a desiccator before being weighed. The individual cell counts of yeast and microalga were determined with a haemocytometer, using the microscope, due to the different appearance of *L. starkeyi* and *C. saccharophilum* being different under the microscope. After centrifugation and filtration with 0.2 μ m cut-off filters, the liquid samples were analysed for residual substrate content (glucose) and soluble fermentation products (VFA, alcohols). Glucose and VFAs were analyzed by HPLC (LC2010, Shimadzu, Japan), equipped with a refractive index detector (RID-20A, Shimadzu, Japan). The total concentration of phenolic compounds was determined using Folin-Ciocalteu assay [27]. A simple method based on UV spectra was followed for the estimation of total furans (furfural and hydroxymethylfurfural) in the hydrolysates [28]. Lipids were extracted following a method adapted from Bligh and Dyer [29]. The samples were stirred in a CHCl₃/CH₃OH mixture (2:1 w/v) over 24 hours, and the oleaginous biomass was filtered off and washed with additional CHCl₃. This procedure was repeated three times. The solvent was then removed by evaporation under N₂ stream. The total lipid concentration was estimated by gravimetric method. To calculate the lipid concentration of the cells, they were dried to a constant weight in the oven at 80 °C. The lipids extracted were

subjected to transesterification reaction in a stirred container at 60 °C for 10 min, using NaOH (1% w/v) as catalysts and using methanol as reagent. The samples were dried by N₂ stream and subsequently 1 mL of heptane was added for the analysis. The content of fatty acid methyl esters was determined by gas chromatography. The GC (GC-MS 2010, Shimadzu, Japan) was equipped with a flame ionization detector and an Omegawax 250 (Supelco) column (30 m x 0.25 mm I.D., 0.25 µm). Helium was used as carrier gas (flow rate: 30 mL/min). The FAME samples were initially dissolved in 1 mL of heptane and 1 µL of this solution was loaded onto the column. The temperature of the column was kept at 50 °C for 2 min, then heated to 220 °C at a rate of 4 °C/min, and finally kept constant for 2 minutes. Methyl decanoate was used as internal standard. The peak of each methyl ester was identified by comparing the retention time with the peak of the pure standard compound.

2.4 Parameter analysis

The specific growth rate (μ_x) and Yield factor ($Y_{x/s}$) were calculated according to Equations 2, 3 and 4:

$$\frac{dX}{dt} = \mu X \quad (2)$$

$$\mu_x = [\ln (X_t/X_0)]/(t - t_0) \quad (3)$$

$$Y_{x/s} = \frac{X - X_0}{S_0 - S} \quad (4)$$

where X and X₀ are the concentrations of microbial biomass at time t and at initial time, respectively. μ_x is the specific growth rate of microbial biomass. S₀ and S are the values of substrate concentrations at initial time and during the cultivation time, respectively. Lipid content, lipid yield (Y_{lipid}) and lipid productivity were calculated according to Equations 5, 6 and 7, respectively:

$$Lipid\ content\ [g_{lipid}.g_{biomass}^{-1}] = \frac{m_{lipid}}{m_{microbial\ biomass}} \quad (5)$$

$$Y_{lipid}\ [g_{lipid}.L^{-1}] = \frac{m_{lipid}}{V} \quad (6)$$

$$Lipid\ productivity\ [mg_{lipid}.L^{-1}.d^{-1}] = \frac{m_{lipid}}{\Delta t} \quad (7)$$

3. Results and discussion

3.1. Effects of synthetic media on *C. saccharophilum* and *L. starkeyi* growth performances in mono and mixed culture

A preliminary experimental campaign was conducted in order to evaluate the behaviour of the two pure strains selected for this study, *L. starkeyi* and *C. saccharophilum*, in two different synthetic media, BBM+G and YEG, at a C/N ratio equal to 11, that was not very different from the Redfield one equal to about 7 [30], and thus avoiding initial N limitations. Tests were performed by inoculating each batch with single or mixed cultures. In this latter case, the inoculum ratio between the yeast and the microalga was 1:1. Anyway, no evidences were observed in terms of microbial biomass and cell proliferation, even changing this ratio, as already demonstrated in a previous study [31]. In addition, an increase in the microalga:yeast ratio would promote algal metabolic activity by inducing an increase in pH, that could inhibit yeast growth. On the contrary, microalgae growth would be inhibited, due to excessive nutrient consumption caused by a high yeast: microalga ratio [32]. In BBM+G, the growth of *C. saccharophilum* showed an initial trend correlated to the glucose consumption (Fig. 1a). This could be justified by an initial photosynthetic activity lower than the metabolism of organic carbon, that could produce an endogenic source of CO₂ [31] reducing CO₂-limiting effect, explaining the increase in microbial biomass once the predominantly heterotrophic metabolism has stopped in the latter part of the cultivation time (Fig. 1a). In the meantime, the reduced cell proliferation - from $2.7 \cdot 10^7$ to $3.6 \cdot 10^7$ cells.mL⁻¹ - would presuppose an increase in size more than in number, due to a phase of lipid accumulation. This hypothesis seemed to be confirmed by the trend of microbial biomass, which increased from 0.93 to 2.3 g.L⁻¹. The biomass growth rate (μ_x), the related value of biomass productivity and the yield factor ($Y_{x/s}$), equal to 0.381 d⁻¹, 328.6 mg.L⁻¹.d⁻¹, 0.471 g.g⁻¹ respectively (Tab. 1), are comparable with the values obtained from Herrera-Valencia et al. and Tan and Johns [19,20], taking into account the different growth conditions. On the other hand, the single cultures of *L. starkeyi* did not show any growth (Fig. 1b), in spite of the presence of yeast extract and glucose. Probably, the initial glucose consumption led to the production of ethanol and acetic acid due to a reduced activity of TCA-cycle, that, in turn, moved the culture to anaerobiosis, due to oxygen limitation in the medium, essential for glucose transport. The batch culture of *L. starkeyi* was exposed to transient fermentation inhibition (Custers effect), and the absence of an alternative oxygen-independent transport mechanism for glucose absorption, led to the lack of microbial growth [33]. Fig 1c shows the performances of *C. saccharophilum* and *L. starkeyi* grown in mixed cultures. From the trends associated to the cell proliferation it seemed evident the presence of a synergistic effect, as evidenced by the comparison of glucose consumption rate values. The microalga was able to provide the oxygen necessary for *L. starkeyi* to metabolize the glucose, which was partially assimilated by *C. saccharophilum* and this effect led to an increase of glucose consumption rate from 1.47 g.L⁻¹.d⁻¹ for *C. saccharophilum* alone to 2.15 g.L⁻¹.d⁻¹ for mixed culture. The final constant values in terms of cell concentration (Fig. 1c) are justified by the metabolic shift due to an imbalance in the C/N ratio, which induced lipid accumulation phase rather than cell duplication. Since the microbial biomass productivity is a result of gravimetric methodology, the relative value increased from 328.6 mg.L⁻¹.d⁻¹ for *C. saccharophilum* alone, to 366.2 mg.L⁻¹.d⁻¹ for mixed culture (Tab. 1). An increase was also observed in terms of specific growth rate, but not in terms of yield factor ($Y_{x/s}$), which is likely affected by endogenous metabolism and a faster glucose

consumption. In mixotrophic conditions, the microalga should be less dependent on the yeast for CO₂, whereas the latter still benefits from the alga for O₂ production, that is partially consumed by respiration of the alga, negatively affecting yeast growth. However, this aspect did not affect the metabolic activity of the yeast, which, on the contrary, was favored by the presence of alga (Fig. 1b, 1c). On the other hand, if comparing the max values of cells.mL⁻¹ for *C. saccharophilum* alone and in mixed culture (3.6·10⁷ and 5.8·10⁷ cells.mL⁻¹ respectively), it emerges that the trend is similar; therefore, there was no real competition for N or P sources nor an effective reduced production of O₂ by the microalga, necessary to metabolize glucose, which would otherwise have been the reason for a reduced growth of yeast as well. Moreover, the yeast proliferation has undoubtedly induced a progressive photo-inhibition effect which could be another reason for a slowdown of microalgal growth in the final part of the test. The latter effect was associated to a limiting O₂ concentration which in turn did not allow the yeast to grow further and to produce CO₂ available to the microalga. Tests were carried out in an attempt to verify the behavior of *L. starkeyi* alone in a more suitable medium, classified as YEG since it was found to be unable to grow in a BBM+G medium although enriched with yeast extract and glucose because of a Cluster effect above discussed. The individual cultures of *C. saccharophilum* (Fig. 2a) and *L. starkeyi* (Fig. 2b) show similar trends, confirmed also by the corresponding values of specific growth rate (μ_x) equal to 0.94 d⁻¹ and 0.8 d⁻¹, respectively, and by the maximum value of cell concentration (cells.mL⁻¹) which for the alga was equal to 3.3·10⁷ and for the yeast to 3.1·10⁷. Mixotrophic activity of *C. saccharophilum* seemed to be confirmed by glucose consumption profile (Fig. 2a). Instead, *L. starkeyi* alone showed an initial lag phase (Fig. 2b). Therefore, although initially there were no limiting conditions with regard to oxygen concentration, the yeast needed an evident acclimation phase in terms of microbial biomass rather than the profile associated to the number of cells (cells.mL⁻¹), that increase with linear trend from the beginning (Fig. 2b). Once the oxygen became limiting, i.e. after the third day, as observed also by the absence of further substrate consumption able to affect C/N ratio and consequently cell duplication, *L. starkeyi* showed a shift in metabolic activity directed on lipid accumulation. The effect of this metabolic turnover could be explained by the significant increase in microbial biomass, related to the occurrence of lipid bodies within the cell. Concerning *C. saccharophilum* alone, an initial phase of mixotrophic metabolism was followed by an autotrophic final phase, visible from the interruption of glucose assimilation. Also for the alga, therefore, during the final phase of the test a lipid accumulation seems to be present, but it was related to a significant increase in the cell concentration due to the autotrophic metabolism. When *C. saccharophilum* and *L. starkeyi* were grown in mixed culture of YEG medium (Fig. 2c), a synergistic effect was observed, confirming the results obtained with the enriched BBM+G medium. Therefore, the mixotrophic metabolism of the microalga and the yeast fermentation were not in competition for the organic C source, nor for N and P, but rather they benefited from each other's metabolic activity, i.e. the consequent presence of a O₂-CO₂ virtuous loop. This seems evident looking at the initial glucose consumption rate in case of *L. starkeyi* alone (Fig. 2b), and in *L. starkeyi* and *C. saccharophilum* mixed culture (Fig. 2c). The presence of O₂ overproduction by the microalgal metabolism supported the fermentation and

therefore the consumption of the organic substrate. If the specific growth rate (μ_x) (Tab. 1) was positively influenced by the mixed culture, different was the case related to the yield factor ($Y_{x/s}$), since the need of microbial biomass for organic carbon conversion increased as consequence of higher cell concentration in the inoculum. In fact, it changed from a 0.583 g.g⁻¹ for *C. saccharophilum* alone, to 0.596 g.g⁻¹ for *L. starkeyi* alone and finally 0.44 g.g⁻¹ for mixed culture. The choice of a 1:1 ratio of microalga:yeast was made in relation to what already observed in a previous study [31] and according to what already highlighted, for example by Ling et al. [34] and Li et al. [32]. An imbalance in the microalga:yeast inoculum ratio generally motivated by their different growth rates, besides not inducing a net increase in terms of lipid productivity, can sometimes lead to an accentuation for N and P competition, and consequently to an imbalance in pH. Fig. 2c also shows that once the carbon source was depleted, the consequence was, also in this case, the stopping of cell proliferation as shown by the unmodified cell concentration.

3.2. Study of *C. saccharophilum* and *L. starkeyi* single and mixed cultures in *Arundo donax* hydrolysate

The performances in synthetic media described in the previous paragraph were considered necessary before moving to a real system, represented by the hydrolysate, in which the performance of the alga and the yeast was evaluated individually and then in a mixed culture. *Arundo donax* was previously subjected to steam explosion and enzymatic hydrolysis. These two preliminary processes were chosen taking into account evaluations supported by experimental campaigns previously performed and aimed to optimize the operating conditions, to minimize the release of inhibiting compounds and, at the same time, to maximize the concentration in fermentable sugars. The steam explosion represents a pre-treatment able to facilitate the access of the crystalline structure of lignocellulosic biomass to hydrolytic enzymes and to limit the negative effects just discussed. Enzymatic hydrolysis, on the other hand, has been chosen for its operating conditions, which remain less impacting compared to other hydrolytic processes, further reducing the risk to increase the production and the release of inhibitory compounds [35]. As observed in Fig. 3a, *C. saccharophilum* alone did not present any form of microbial growth that could be associated to the presence of inhibitory compounds. The choice of *C. saccharophilum* was made also in view of its high tolerance to the presence, for example, of phenols, recognizing to these latter compounds also a positive effect on the regulation of enzymatic activity, cell membrane structure and macromolecule synthesis [36,37,38]. Furans could be also a reason of inhibition since they are able to cause long lag-phase [39]. Therefore, the permanence of this regime led to the exhaustion of CO₂ and subsequently of O₂ source, making the culture substantially anaerobic and therefore unable to grow up. Further aspect preliminarily taken into account was on the possible inhibition due to the turbidity of the culture medium that already in other studies led to adopt a dilution factor to promote photosynthetic activity [40] involving, at the same time, a loss in organic substrates concentration. On the other hand, the inhibition could be attributed to acetate potentially present in the dissociated form at neutral pH, as in our case [41]. Russel [42] attributed the inhibitory effects of weak acids to two mechanisms: uncoupling and intracellular anion

accumulation. For all these reasons, i.e. associated to the possible occurrence of limiting conditions in terms of CO₂ or O₂, and to the potential inhibiting aspects such as turbidity or to high concentration in acetate, the study of mixed culture was found to be fundamental and potentially able to answer the doubts resulting from the single culture of *C. saccharophilum*. Another not secondary aspect that could have been the cause of algae inhibition is C/N ratio. In fact, the use of low cost feedstock, which is motivated by the need to reduce the costs of the growth medium and at the same time by the positive effect on the increase of lipid accumulation, presents, however, a high C/N ratio that exceeds the optimal values for the algae.

Before arriving at the mixed culture analysis, an additional control culture was monitored considering *L. starkeyi* alone as inoculum. Fig. 3b and 4b show the relative trends in terms of metabolites (substrates and products) and microbial biomass. The lag phase associated to the first two days seemed caused by the presence of furans, which, although not in high concentration, represented a compound able to delay the growth phase. The hypothesis that the yeast inhibition is affected by the C/N ratio has been ruled out, given its ability to tolerate values of even equal to 100 [4]. The lag phase was therefore followed by a growth phase, more visible in terms of microbial biomass than in terms of cells.mL⁻¹, which was the result of the simultaneous assimilation of glucose, xylose and acetate. This aspect confirmed what has already been observed by Anschau et al. [43], Gong et al. [44] and Yang et al. [45] related to the potential use of this microorganism in presence of more complex feedstocks where co-metabolism is of great interest to solve inhibition problems due to volatile organic acids which are formed during the pretreatments and it should be valuable towards conversion of acetate lignocellulosic biomass materials into Single Cell Oils. Moreover, acetate assimilation by *L. starkeyi* represents a positive aspect with respect to pH adjustment, but it leads to an additional demand for dissolved oxygen.

Figs. 3b and 4b show how the consumption patterns of the substrates, i.e. mainly glucose, xylose and acetate, followed a diauxic mechanism. Once the dissolved oxygen was depleted, the respiratory metabolism became fermentative. The concentration and productivity of microbial biomass (Tab. 1) were 3.7 g.L⁻¹ and 411 mg.L⁻¹.d⁻¹, respectively. These values can be considered in line with those already obtained by Pirozzi et al. [46] taking into account that *Arundo donax* was submitted to different pre-treatment processes, but different by one order of magnitude when compared to hydrolysates of wheat corn, corn bran and corn stover [15,47,48].

Finally, *Arundo donax* hydrolysate was inoculated with a mixed culture of *L. starkeyi* and *C. saccharophilum* in an attempt to observe the positive and less positive effects of two microorganisms combination also in relation to what was previously studied with regard to synthetic media in which all the aspects due to inhibitory effects resulting from the complexity of the hydrolysate were missing. Meanwhile, as shown in Fig. 3c, the lag phase lasted for the first 4 days, apart from a slight increase in *L. starkeyi* cell number that passed from 3.3·10⁶ to 5.9·10⁶ cells.mL⁻¹, mainly as a result of glucose consumption rate, equal to 0.28 g.L⁻¹.d⁻¹. At this stage, inhibition due to the presence of inhibiting compounds, such as furans and phenols was confirmed, but once the lag phase was exceeded both microorganisms showed a growth capacity that, in case of *C. saccharophilum*, had not been

observed in single culture. The max value in terms of cells.mL⁻¹ for *L. starkeyi* and *C. saccharophilum* in mixed culture was $1.1 \cdot 10^8$ and $2.1 \cdot 10^7$, respectively. These values were understandably lower than those found for mixed culture in YEG medium, respectively equal to $5.6 \cdot 10^8$ and $6.7 \cdot 10^7$ cells.mL⁻¹, because of faster glucose consumption and absence of inhibition phenomena.

What seemed evident, confirming what has already been observed in previous studies [31, 32,49,50,51], was the presence of a synergistic effect that in our case was attributed to different processes. During the lag phase, *L. starkeyi* showed a latent growth phase (Fig. 3c) that induced a progressive endogenous source of CO₂ able to avoid limiting conditions for the microalga growth. As observed in the single culture (Fig. 3a), the microalga didn't grow neither according to a heterotrophic metabolism, nor mixotrophic because of the various forms of inhibition just discussed. This progressive accumulation of CO₂, not to the point of representing a form of inhibition for the yeast, could be the cause of a partial acidification of the culture medium, but both *C. saccharophilum* and *L. starkeyi* tolerate acid pH. This acidification was therefore counteracted by acetate consumption after the fourth day, since a pH increase is typically observed when a microorganism grows in a salt of an organic acid. The progressive consumption of the organic substrate, and thus the decrease in the C/N ratio that was crucial for the inhibition of single microalgae growth, together with the pH control due to acetate consumption to balance the potential acidification due to yeast respiration, were the positive aspects that favoured the symbiotic growth of *C. saccharophilum* in mixed culture. While in terms of the final microbial biomass, the differences were not evident when passing from 4.72 to 4.14 g.L⁻¹ for mixed cultures in YEG medium and in *Arundo donax* hydrolysate (Fig 2c and 3c), different was what evaluated in terms of microbial biomass productivity measured in the same time-range, and equal respectively to 611.9 and 365.5 mg.L⁻¹.d⁻¹, paying in the latter case the incidence of inhibiting phenomena that slowed down the growth phase. The microbial biomass growth rate (μ_x) and the yield factor ($Y_{x/s}$) in mixed culture were respectively equal to 0.791 d⁻¹ and 0.539 g.g⁻¹ (Tab. 1). Once this synergistic effect stopped as a consequence of limiting conditions achievement in terms of O₂, yeast growth rate changed, switching to a fermentative metabolism and consequently to ethanol accumulation, negatively influencing also the alga, whose growth also suffered a slowdown (Fig. 3c).

Studies about the tolerance of single microbe species to inhibitory compounds are quite limited. Several attempts have been made to promote the microbial growth by reducing toxic compounds before fermentation process with certain detoxification steps. However, the detoxification which directly leads to high cost will decrease the economy of the whole process [52]. The fact that depletions of hydrolysis degradation products were enhanced by the mixed culture mode should be regarded positively because the overall process of microbial lipid production from lignocellulosic hydrolysate can be simplified by omitting the need of detoxification step [49].

3.3. Lipid production and fatty acid distribution

Different parameters were evaluated and reported in Tab. 1, in an attempt to compare the incidence of the culture conditions adopted onto the lipid content, lipid yield and lipid

productivity. The lipid content reached a maximum value of $0.081 \text{ g}_{\text{lipid}}.\text{g}_{\text{biomass}}^{-1}$ for mixed culture in *Arundo donax* hydrolysate, which was higher when compared to the mixed culture in BBM+G or YEG where the same value was equal about to $0.063\text{-}0.064 \text{ g}_{\text{lipid}}.\text{g}_{\text{biomass}}^{-1}$. Therefore, these values were significantly lower to those obtained by Liu et al. [49], where the maximum lipid content was $0.53 \text{ g}_{\text{lipid}}.\text{g}_{\text{biomass}}^{-1}$ for *Chlorella pyrenoidosa* and *Rhodotorula glutinis* consortium. The explanation could be found in the differences in substrate concentration, which in the latter case, amounted from 30 to 60 g.L^{-1} promoting consequently an imbalance in the C/N ratio that could have favoured microbial oil accumulation phase mainly by the yeast, whose metabolic activity is positively conditioned by the increase of this ratio [4]. The lipid yield values of mixed cultures in our study were 0.211 g.L^{-1} for BBM+G, 0.320 g.L^{-1} for YEG and 0.335 g.L^{-1} for *Arundo donax* hydrolysate. These values were very far from those measured by Liu et al. [49], where an average value of 7.73 g.L^{-1} was obtained; but in line with those measured by Wang et al. [53] in presence of *C. pyrenoidosa* and *R. glutinis* consortium, where the lipid yield was 0.75 g.L^{-1} or even much higher if compared with Iasimone et al. [54] where the lipid yield was 0.05 g.L^{-1} in presence of a mixed culture of *L. starkeyi* and algae consortium mainly represented by *Chlorella* sp. and *Scenedesmus* sp.

Cell number normalized lipid contents (Lipid Yield/Cell) were also calculated (Tab. 1), obtaining values for *C. saccharophilum* and *L. starkeyi* mixed culture respectively equal to $16 \text{ e } 3 \text{ } \mu\text{g}$ for 10^{-6} Cells for *Arundo donax* hydrolysate. These values were compared with those by Liu et al. [49] where for *C. pyrenoidosa* and *R. glutinis* were equal about to 16 and $14 \text{ } \mu\text{g}$ for 10^{-6} Cells, respectively, indicating that the C/N ratio of cassava bagasse hydrolysate probably enhanced the lipid accumulation metabolism of yeast rather than alga. Fatty acid distribution was also monitored at the end of each culture as shown in Fig 5a, b, c. Generally, this volumetric distribution in terms of C16:0, C16:1, C18:0, C18:1, C18:2 for yeast monoculture is as follows: 7-20, 0.1-0.8, 3-12, 28-85, 5-20, while for alga monoculture is 12-40, 0.1-1, 2-32, 21-71, 0-10. The effect of co-culture is a progressive increase in the concentration of saturated fatty acids (SFAs) and a reduction in polyunsaturated fatty acids (PUFAs) [4] which was previously observed by Zuccaro et al. [31] and partially confirmed in this study. In fact, the compounds mainly present in our study were C18:0, C18:1 and C14:0, with an increasing concentration of C18:2 and C18:3 for single and mixed culture in *Arundo donax* hydrolysate. For the latter case, specifically, the distribution in terms of C14:0, C16:0, C18:0, C18:1, C18:2, C18:3, C20:0 was the following: 7, 39, 30, 19, 2, 0.1. In general, the lipids produced by microorganisms are converted into biodiesel via a process known as transesterification. The two most important properties of fatty acids that affect the fuel properties as listed above are (a) length of the carbon chain and (b) number of double bonds [55]. The degree of unsaturation in the fatty acids affects the oxidative stability of the biodiesel with SFAs being the most stable followed by MUFAs compared to the least stable PUFAs, respectively [56]. Additionally, C16:0 and C18:1 are used as food additives and cosmetics, indicating as Single Cell Oils from mixed culture using low cost lignocellulosic feedstock as *Arundo donax* could have several potential applications.

3.4. Figures and Tables

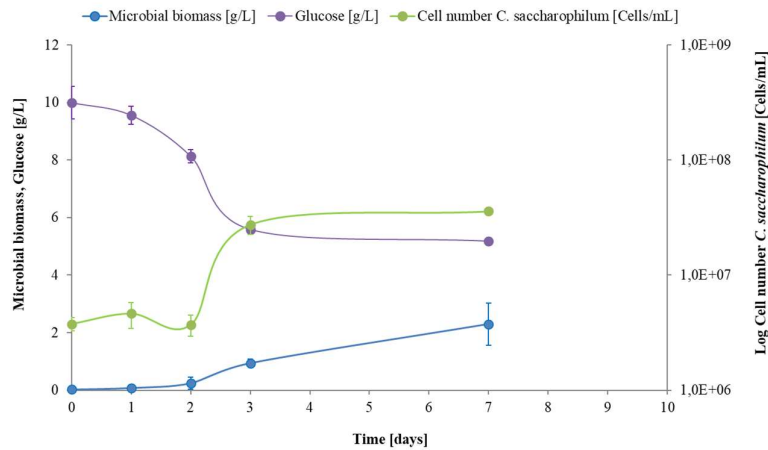


Figure 1a. Profiles of microbial biomass (g/L), glucose consumption (g/L) and cell number (Cells/mL) in BBM+G medium supplemented with yeast extract and glucose inoculated by *C. saccharophilum*

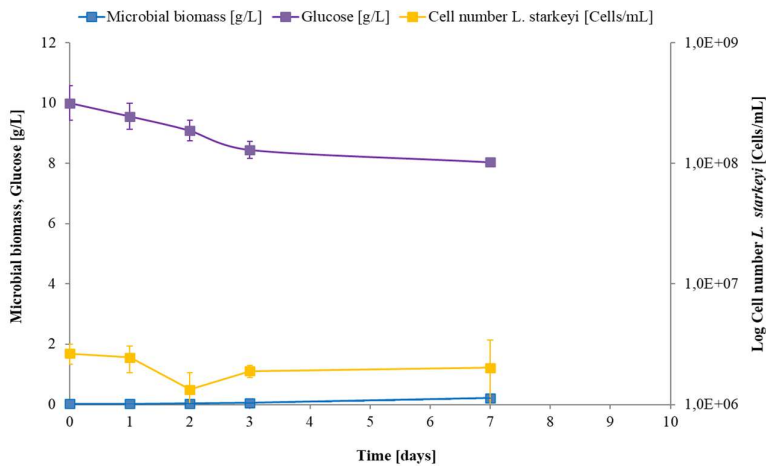


Figure 1b. Profiles of microbial biomass (g/L), glucose consumption (g/L) and cell number (Cells/mL) in BBM+G medium supplemented with yeast extract and glucose inoculated by *L. starkeyi*

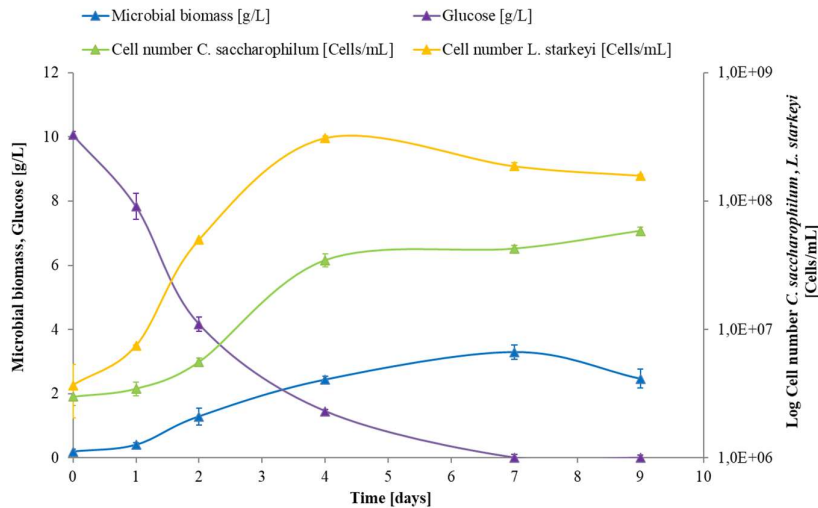


Figure 1c. Profiles of microbial biomass (g/L), glucose consumption (g/L) and cell number (Cells/mL) in BBM+G medium supplemented with yeast extract and glucose inoculated by *C. saccharophilum* and *L. starkeyi*

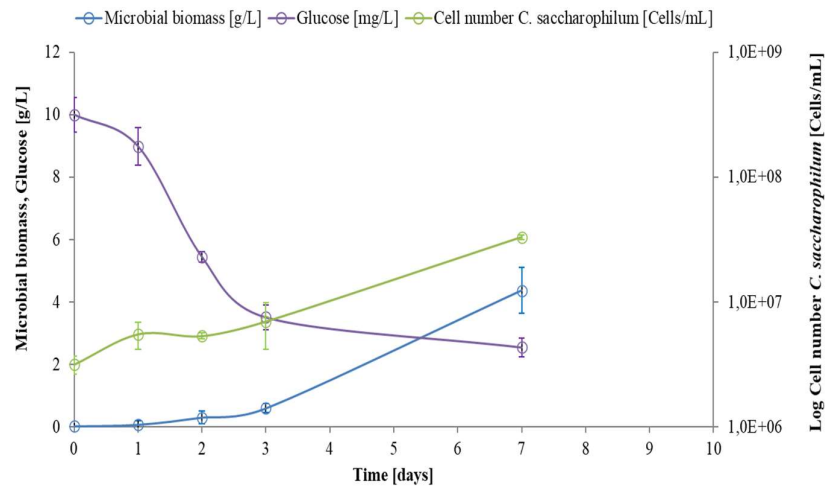


Figure 2a. Profiles of microbial biomass (g/L), glucose consumption (g/L) and cell number (Cells/mL) in YEG medium inoculated by *C. saccharophilum*

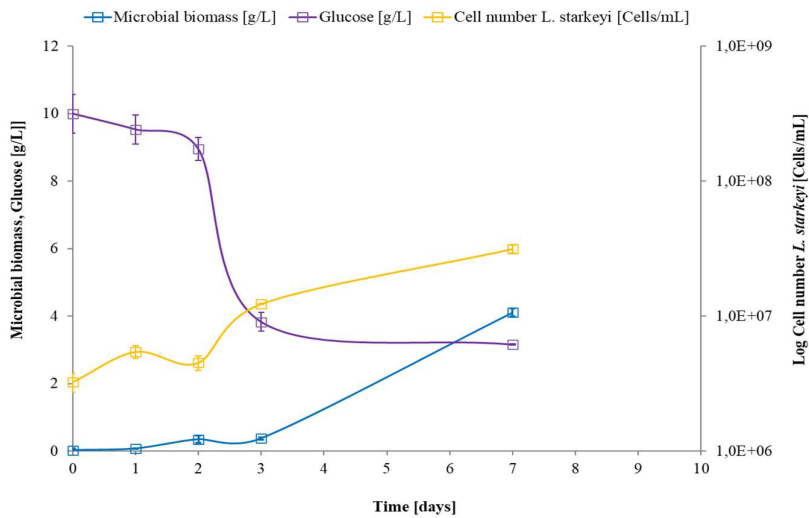


Figure 2b. Profiles of microbial biomass (g/L), glucose consumption (g/L) and cell number (Cells/mL) in YEG medium inoculated by *L. starkeyi*

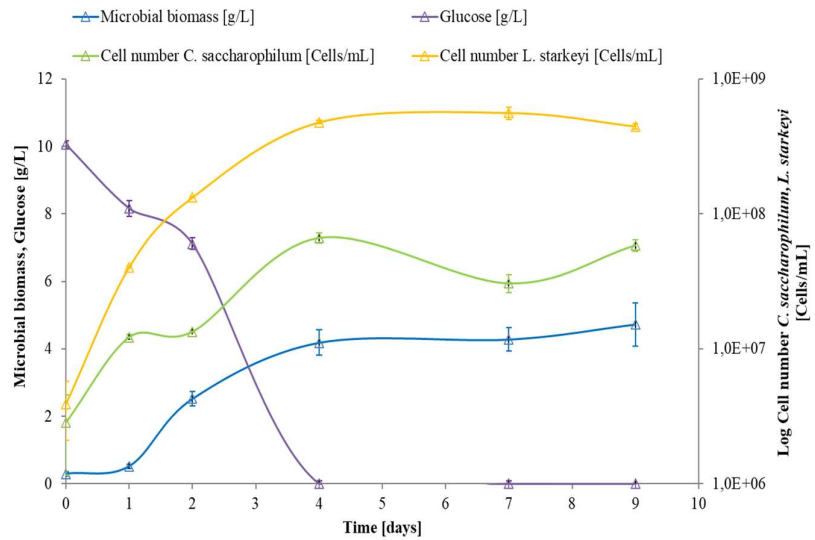


Figure 2c. Profiles of microbial biomass (g/L), glucose consumption (g/L) and cell number (Cells/mL) in YEG medium inoculated by *C. saccharophilum* and *L. starkeyi*

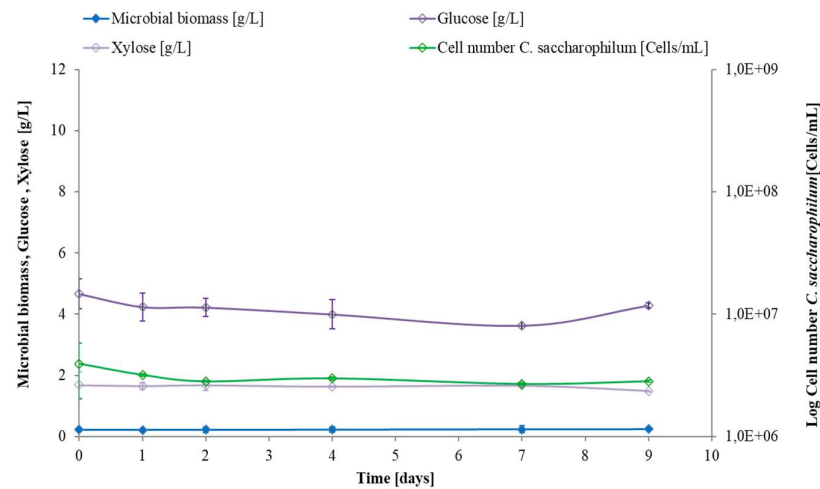


Figure 3a. Profiles of microbial biomass (g/L), glucose consumption (g/L), xylose consumption (g/L) and cell number (Cells/mL) in *Arundo donax* hydrolysate (ADH) inoculated by *C. saccharophilum* and *L. starkeyi*

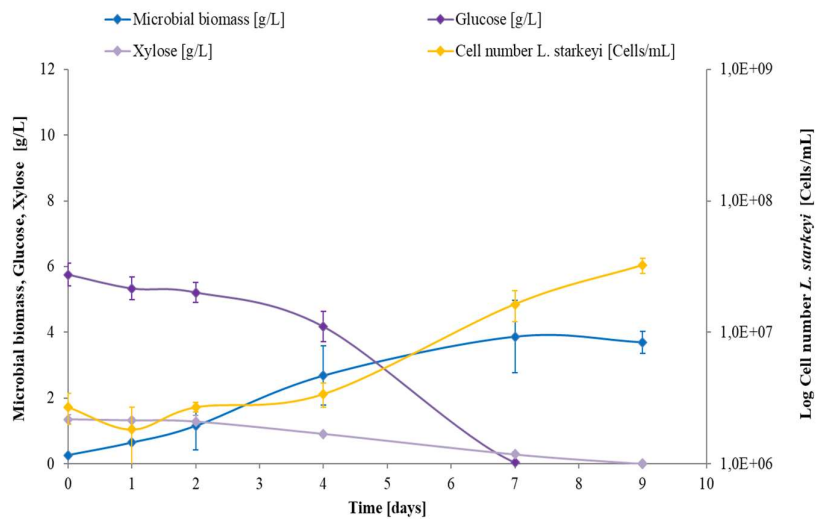


Figure 3b. Profiles of microbial biomass (g/L), glucose consumption (g/L), xylose consumption (g/L) and cell number (Cells/mL) in *Arundo donax* hydrolysate (ADH) inoculated by *L. starkeyi*

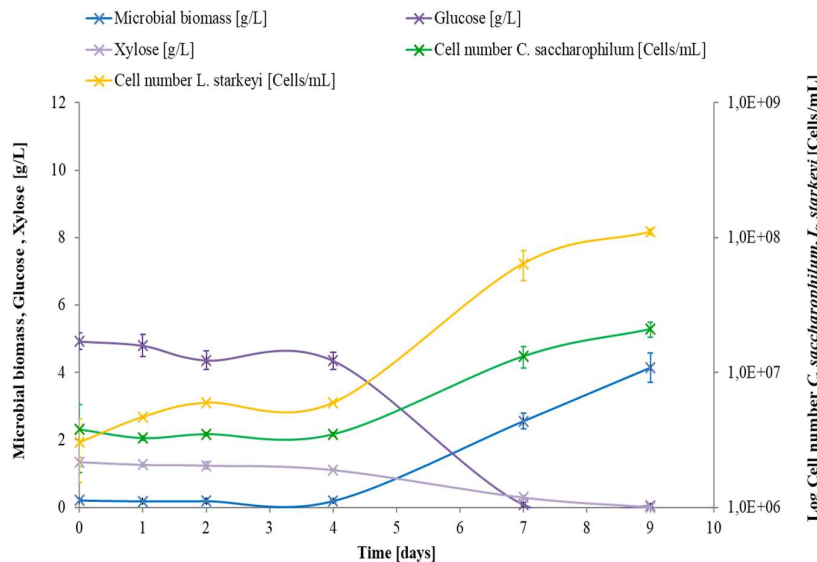


Figure 3c. Profiles of microbial biomass (g/L), glucose consumption (g/L), xylose consumption (g/L) and cell number (Cells/mL) in *Arundo donax* hydrolysate (ADH) inoculated by *C. saccharophilum* and *L. starkeyi*

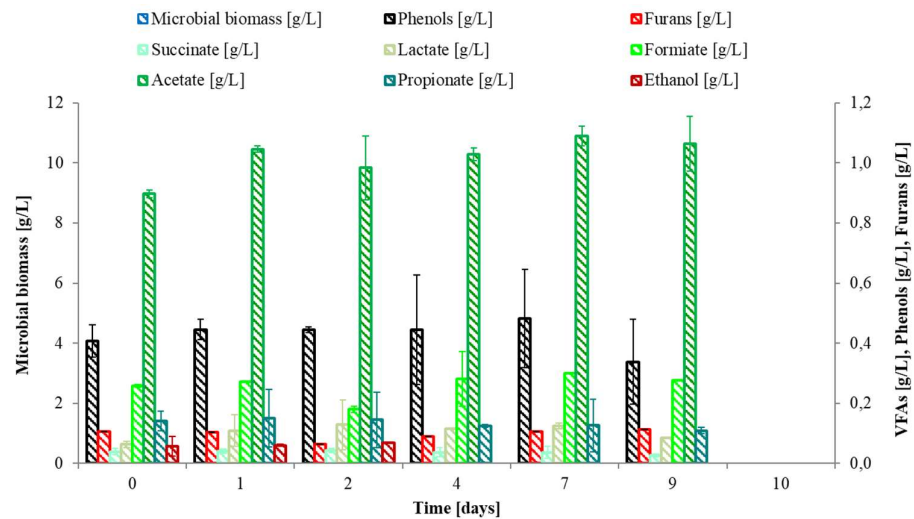


Figure 4a. Profiles of microbial biomass (g/L), VFAs, Phenols and Furans concentration (g/L) in *Arundo donax* hydrolysate (ADH) inoculated by *C. saccharophilum*

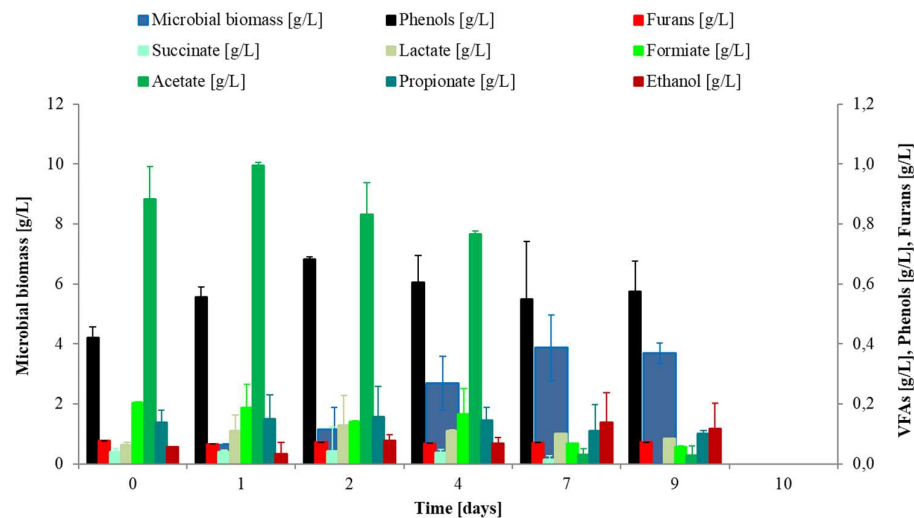


Figure 4b. Profiles of microbial biomass (g/L), VFAs, Phenols and Furans concentration (g/L) in *Arundo donax* hydrolysate (ADH) inoculated by *L. starkeyi*

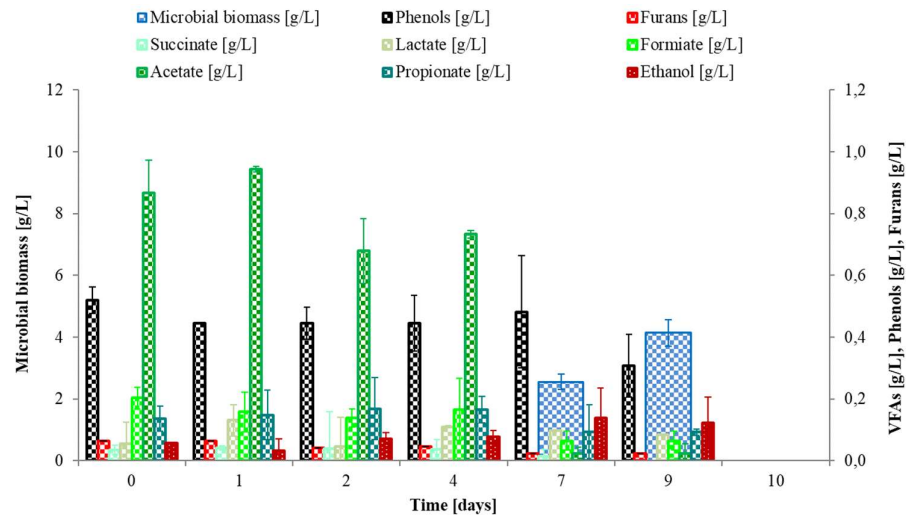


Figure 4b. Profiles of microbial biomass (g/L), VFAs, Phenols and Furans concentration (g/L) in *Arundo donax* hydrolysate (ADH) inoculated by *C. saccharophilum* and *L. starkeyi*

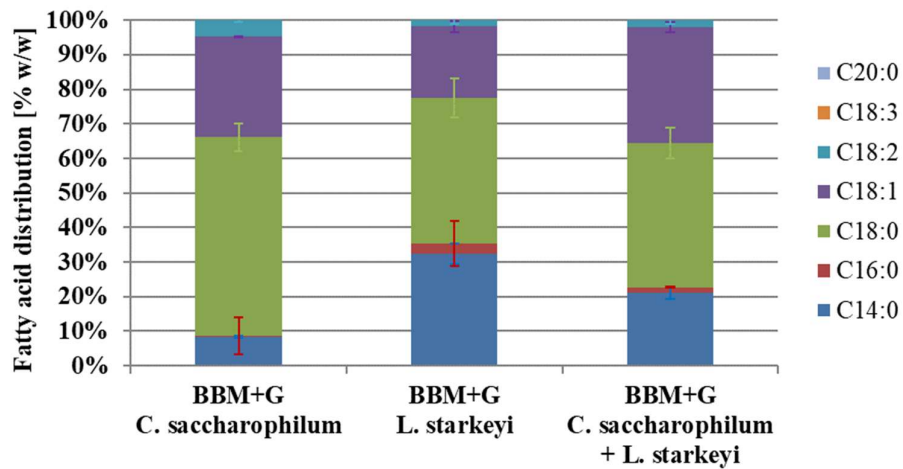


Figure 5a. Fatty acid distribution (% w/w) in microbial biomass (individual and mixed cultures) in BBM+G media

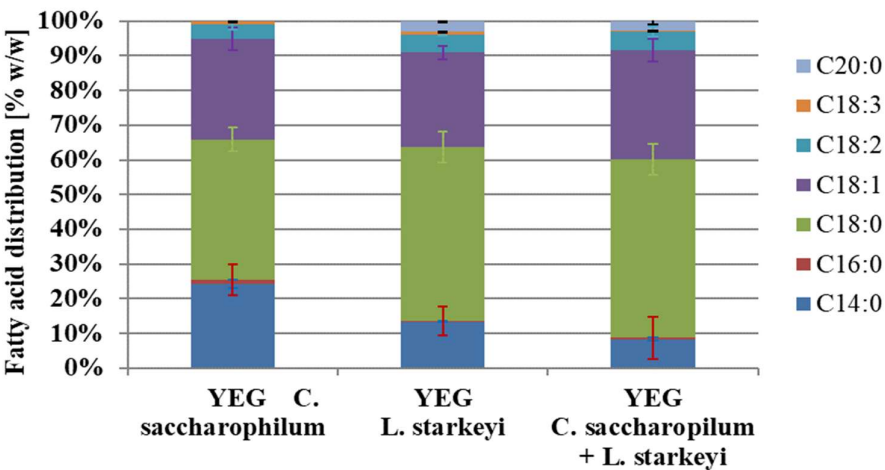


Figure 5b. Fatty acid distribution (% w/w) in microbial biomass (individual and mixed cultures) in YEG media

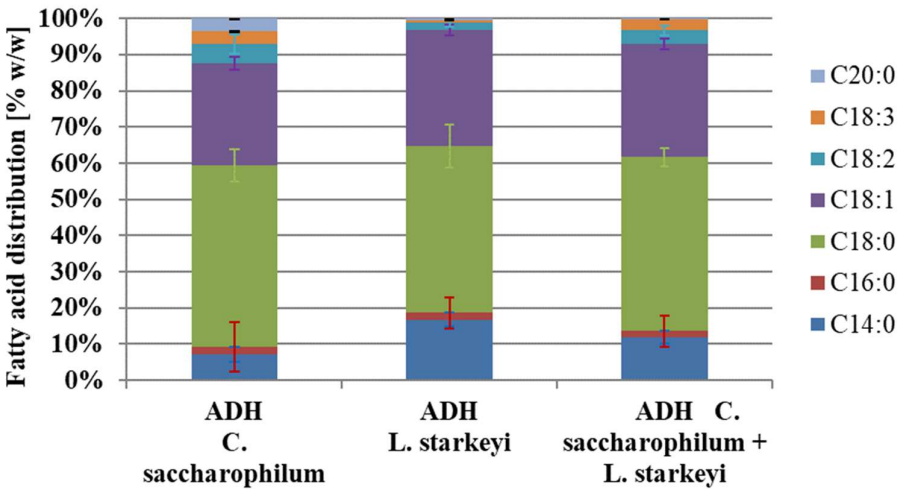


Figure 5c. Fatty acid distribution (% w/w) in microbial biomass (individual and mixed cultures) in ADH hydrolysates

Table 1. Growth characteristics of the mixed cultures and the strains alone in BBM+G, YEG media and *Arundo donax* hydrolysates. Data shown as mean \pm SD, n=3
(*Yield factor, $Y_{x/s}$, was calculated taking in account the contribute of all the monitored substrates)

SAMPLE	μ_x [d ⁻¹]	$Y_{x/s}$ [g.g ⁻¹]	Lipid content [g _{lipid} .g _{biomass} ⁻¹]	Lipid Yield Y_{lipid} [g.L ⁻¹]	Biomass productivity [mg.L ⁻¹ .d ⁻¹]	Lipid productivity [mg.L ⁻¹ .d ⁻¹]	Cells _{max} <i>C. saccharophilum</i> [10 ⁶ Cells.mL ⁻¹]	Cells _{max} <i>L. starkeyi</i> [10 ⁶ Cells.mL ⁻¹]	Lipid Yield /Cell <i>C. saccharophilum</i> [µg*10 ⁶]	Lipid Yield/Cell <i>L. starkeyi</i> [µg*10 ⁶]
BEM+G Chl	0.381	0.471	0.076 ± 0.03	0.175 ± 0.04	328.57	25.00	36.0 ± 1.76		4.9	
BBM+G Lip	0.037	0.095	0.093 ± 0.01	0.020 ± 0.04	30.65	2.87		2.0 ± 0.65		10.0
BBM+G Chl Lip	0.472	0.308	0.064 ± 0.02	0.211 ± 0.17	366.20	30.14	58.7 ± 1.93	309.3 ± 5.91	3.6	0.7
YEG Chl	0.944	0.583	0.051 ± 0.01	0.221 ± 0.24	625.00	31.57	33.0 ± 1.27			
YEG Lip	0.803	0.596	0.052 ± 0.03	0.212 ± 0.07	586.31	30.29		31.5 ± 0.77		6.7
YEG Chl Lip	1.166	0.440	0.068 ± 0.01	0.320 ± 0.28	611.90	35.56	66.7 ± 3.21	561.3 ± 16.64	1.0	0.1
ADH Chl	0.003	0.000*	0.069 ± 0.03	0.016 ± 0.00	26.75	1.86	3.2 ± 0.37		5.0	
ADH Lip	0.614	0.422*	0.074 ± 0.01	0.318 ± 0.05	552.38	35.33		32.45 ± 1.87		9.8
ADH Chl Lip	0.791	0.539*	0.081 ± 0.04	0.335 ± 0.14	365.47	37.22	20.9 ± 1.23	110.0 ± 3.83	16.0	3.0

4. Conclusions

Lignocellulosic extracts have the potential to provide a complex substrate of fermentable sugars and volatile organic acids, mainly acetate, at low cost. In this study, it was shown that *C. saccharophilum* and *L. starkeyi* were able to grow according to a synergistic effect on complex substrate such as *Arundo donax* hydrolysate, while showing that this synergistic effect allowed to overcome the problems associated to inhibitory phenomena due to lignin or sugar degradation products and a non-optimal C/N ratio. The reason was mainly attributed to the virtuous exchange of O₂ and CO₂, but also to phenomena of pH regulation. The promising results in terms of microbial growth and lipid accumulation were correlated with those of cultures in less complex synthetic media. The mixed cultures, in all cases, proved to be the most performing. The operational and economic impacts associated with the introduction of a detoxification phase in an attempt to overcome the inhibition effects of the above mentioned products remain to be clarified and deepened. Single Cell Oil production from lignocellulosic biomass offers a new direction for bio-refinery approach, and it will have a great future if the above mentioned problems will be properly handled. In fact, Single Cell Oils represent intermediates for biodiesel production, polymers, biosurfactants and the control of unsaturation degree in their chain, for example by hydrogenation, could be critical to ensure selectivity and stability. The exploitation of Single Cell Oils (SCOs) related to the possibility of ensuring mono or polyunsaturation could represent an alternative to the emergence of diseases such as atherosclerosis. Therefore, a decisive step in the direction of developing an economically sustainable method for the recovery of high purity SCOs is still awaited.

Author Contributions: Conceptualization, G.Z.; methodology, G.Z. and A.d.M.; validation, G.Z., A.d.M., G.P., A.P. and A.D.N.; formal analysis, G.Z. and A.d.M.; investigation, G.Z. and A.d.M.; resources, A.P. and A.D.N.; data curation, G.Z. and A.d.M.; writing—original draft preparation, G.Z.; writing—review and editing, G.Z., A.d.M., G.P., A.P. and A.D.N.; supervision, G.Z., A.P. and A.D.N.; project administration, A.D.N., funding acquisition, A.P. and A.D.N. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: G.Z. would like to thank ENEA–Trisaia Research Center (Rotondella, Matera, Italy). G.Z. gratefully acknowledges the Department of Chemical, Materials and Production Engineering (DICMaPI) - University of Naples Federico II for the support.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Pérez, A.T.E.; Camargo, M.; Rincón, P.C.N.; Marchant, M.A. Key challenges and requirements for sustainable and industrialized biorefinery supply chain design and management: a bibliographic analysis. *Renewable Sustainable Energy Rev.* **2017**, *69*, 350–359.
2. Abghari, A.; Chen, S. *Yarrowia lipolytica* as an oleaginous cell factory platform for production of fatty acid-based biofuel and bioproducts. *Frontiers in Energy Research* **2014**, *2*, 1–21.
3. Huang, C.; Chen, X.F.; Xiong, L.; Chen, X.D.; Ma, L.L.; Chen, Y. Single cell oil production from low-cost substrates: the possibility and potential of its industrialization. *Biotechnol Adv* **2013**, *31*, 129–139.
4. Arora, N.; Patel, A.; Methani, J.; Pruthi, P.A.; Pruthi, V.; and Poluri, K.M. Co-culturing of oleaginous microalgae and yeast: paradigm shift towards enhanced lipid productivity. *Environmental Science and Pollution Research* **2019**, *26*, 16952–16973.
5. Naidoo, R.K.; Simpson, Z.F.; Oosthuizen, J.R.; and Bauer, F.F. Nutrient exchange of carbon and nitrogen promotes the formation of stable mutualisms between *Chlorella sorokiniana* and *Saccharomyces cerevisiae* under engineered synthetic growth conditions. *Front. Microbiol.* **2019**, *10*, 609.
6. Hays, S.G.; Yan, L.L.W.; Silver, P.A.; and Ducat, D.C. Synthetic photosynthetic consortia define interactions leading to robustness and photoproduction. *J. Biol. Eng* **2017**, *11*, 4.
7. Li, T.; Li, C.T.; Butler, K.; Hays, S.G.; Guarnieri, M.T.; Oyler, G.A.; *et al.* Mimicking lichens: incorporation of yeasts strains together with sucrose secreting cyanobacteria improves survival, growth, ROS removal, and lipid production in a stable mutualistic co-culture production platform. *Biotechnol. Biofuels* **2017**, *10*, 55.
8. Liu, J.; Huang, J.; Sun, Z.; Zhong, Y.; Jiang, Y.; Chen, F. Differential lipid and fatty acid profiles of photoautotrophic and heterotrophic *Chlorella zofingiensis*: Assessment of algal oils for biodiesel production. *Bioresource Technology* **2011**, *102*, 106–110.
9. Wilhelm, C.; Jakob, T. From photons to biomass and biofuels: evaluation of different strategies for the improvement of algal biotechnology based on comparative energy balances. *Appl. Microbiol. Biotechnol.* **2011**, *92*, 909–919.

10. Chi, Z.; Zheng, Y.; Lucker, B.; Chen, S. Integrated System for Production of Biofuel Feedstock, Patents, 2010.
11. Beopoulos, A.; Nicaud, J.M. Yeast: a new oil producer? *OCL* **2012**, *19*, 22-28.
12. Huang, C.; Chen, X.F.; Xiong, L.; Chen, X.D.; Ma, L.L.; Chen Y. Single cell oil production from low-cost substrates: the possibility and potential of its industrialization. *Biotechnol Adv* **2013**, *31*, 129–139.
13. Lodder, J.; Kreger-van Rij, N.J.W. The Yeast: a taxonomic study, 1st ed., North-Holland Publishing Company, Amsterdam, The Netherlands, 1952.
14. Darienko, T.; Gustavs, L.; Mudimu, O.; Menendez, C.R.; Schumann, R.; Karsten, U.; Friedl, T.; Proschold, T. *Chloroidium*, a common terrestrial coccoid green alga previously assigned to *Chlorella* (Trebouxiophyceae, Chlorophyta). *European Journal of Phycology* **2010**, *45*(1), 79-95.
15. Calvey, C.H.; Su, Y.K.; Willis, L.B.; McGee, M.; Jeffries, T.W. Nitrogen limitation, oxygen limitation, and lipid accumulation in *Lipomyces starkeyi*. *Bioresour Technol* **2016**, *200*, 780–788.
16. McNeil, B.A.; Stuart, D.T. Optimization of C16 and C18 fatty alcohol production by an engineered strain of *Lipomyces starkeyi*. *J Ind Microbiol Biotechnol* **2018**, *45*, 1–14.
17. Xavier, M.C.A.; Coradini, A.L.V.; Deckmann, A.C.; Franco, T.T. Lipid production from hemicellulose hydrolysate and acetic acid by *Lipomyces starkeyi* and the ability of yeast to metabolize inhibitors. *Biochem Eng J* **2017**, *118*, 11–19.
18. Beardall, J. CO₂ accumulation by *Chlorella saccharophilum* (Chlorophyceae) at low external pH: evidence for active transport of inorganic carbon at the chloroplast envelope. *J. Phycol.* **1981**, *17*, 371-373.
19. Herrera-Valencia, V.A.; Contreras-Pool, P.Y.; Lopez-Adrian, S.J.; Peraza-Echeverria, S.; Barahona-Perez, L.F. The green microalga *Chlorella saccharophilum* as a suitable source of oil for biodiesel production. *Curr Microbiol* **2011**, *63*, 151–157.
20. Tan, C.K.; and Johns, M.R. Fatty acid production by heterotrophic *Chlorella saccharophilum*. *Hydrobiologia* **1991**, *215*, 13-19.
21. Zhang, T.-Y.; Hu, H.-Y.; Wu, Y.-H.; Zhuang, L.-L.; Xu, X.-Q.; Wang, X.-X.; Dao, G.-H. Promising solutions to solve the bottlenecks in the large-scale cultivation of microalgae for biomass/bioenergy production. *Renew Sustain Energy Rev.* **2016**, *60*, 1602-1614.
22. Fagnano, M.; Impagliazzo, A.; Mori, M.; Fiorentino, N. Agronomic and Environmental Impacts of Giant Reed (*Arundo donax* L.): Results from a Long-Term Field Experiment in Hilly Areas Subject to Soil Erosion. *BioEnergy Research* **2015**, *8*(1), 415-422.
23. Zuccaro, G.; Travaglini, G.; Caputo, G.; Pirozzi, D. Enzymatic Hydrolysis and Oleaginous Fermentation of Steam-Exploded *Arundo donax* and *Lipomyces Starkeyi* in a Single Bioreactor for Microbial Oil Accumulation. *Journal of Multidisciplinary Engineering Science Studies (JMESS)* **2019**, 2577-2586.
24. Garrote, G.; Domínguez, H.; Parajó, J.C. Hydrothermal processing of lignocellulosic materials. *Holz Roh-Werkst* **1999**, *57*, 191–202.
25. Adney, B.; Baker, J. Measurement of Cellulase Activities. NREL Analytical Procedure LAP-006. National Renewable Energy Laboratory: Golden, CO, 1996.
26. Wood, T.M.; Bhat, K.M. Methods for measuring cellulase activities. *Methods in Enzymology* **1988**, *160*, 87-112.
27. Singleton, V.L.; Orhofer, R.; Lamuela-Raventos, R.M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol* **1999**, *299*, 152–178.
28. Martinez, A.; Rodriguez, M.E.; York, S.W.; Preston, J.F.; Ingram, L.O. Use of UV absorbance to monitor furans in dilute acid hydrolysates of biomass. *Biotechnol Prog* **2000**, *16*, 637–641.
29. Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can J Biochem Phys* **1959**, *37*, 911-917.
30. Redfield, A.C. On the Proportions of Organic Derivatives in Sea Water and Their Relation to the Composition of Plankton, in: James Johnstone Memorial Volume, (University Press of Liverpool), 1934, pp. 176–192.
31. Zuccaro, G.; Steyer, J.-P.; van Lis, R. The algal trophic mode affects the interaction and oil production of a synergistic microalga-yeast consortium. *Bioresour Technology* **2019**, *273*, 608–617.
32. Li, H.; Zhong, Y.; Lu, Q.; Zhang, X.; Wang, Q.; Liu, H.; Diao, Z.; Yao, C.; and Liu, H. Co-cultivation of *Rhodotorula glutinis* and *Chlorella pyrenoidosa* to improve nutrient removal and protein content by their synergistic relationship. *RSC Adv.* **2019**, *9*, 14331–14342.
33. Wijsman, M.R.; van Dijken, J.P.; van Kleeff, B.H.A.; and Scheffers, W.A. Inhibition of fermentation and growth in batch cultures of the yeast *Brettanomyces intermedius* upon a shift from aerobic to anaerobic conditions (Custers effect). *Antonie van Leeuwenhoek* **1984**, *50*, 183-192.
34. Ling, J.; Nip, S.; Cheok, W.L.; Alves de Toledo, R.; Shim, H. Lipid production by a mixed culture of oleaginous yeast and microalga from distillery and domestic mixed wastewater. *Bioresour Technology* **2014**, *173*, 132–139.
35. Zuccaro, G.; Pirozzi, D.; Yousuf, A. Lignocellulosic biomass to biodiesel. In *Lignocellulosic Biomass to Liquid Biofuels* (Academic Press, Elsevier), 2020, pp. 127-167.
36. Miazek, K.; Remacle, C.; Richel, A.; and Goffin, D. Effect of lignocellulose related compounds on microalgae growth and product biosynthesis: a review. *Energies* **2014**, *7*, 4446-4481.
37. Bajguz, A.; Czerpak, R.; Piotrowska, A.; Polecka, M. Effect of isomers of hydroxybenzoic acid on the growth and metabolism of *Chlorella vulgaris* Beijerinck (Chlorophyceae). *Acta Soc. Bot. Pol.* **2001**, *70*, 253–259.
38. Larson, L.J. Effect of phenolic acids on growth of *Chlorella pyrenoidosa*. *Hydrobiologia* **1989**, *183*, 217–222.
39. Larsson, S.; Palmqvist, E.; Hahn-Hägerdal, B.; Tengborg, C.; Stenberg, K.; Zacchi, G.; Nilvebrant, N.O. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. *Enz. Microb. Technol.* **1988**, *24*, 151-159.
40. Cea Barcia, G.E.; Imperial Cervantes, R.A.; Torres Zuniga, I.; van Den Hende, S. Converting tequila vinasse diluted with tequila process water into microalgae-yeast flocs and dischargeable effluent. *Bioresour Technology* **2020**, *300*, 122644.

41. Gong, Z.; Zhou, W.; Shen, H.; Yang, Z.; Wang, G.; Zuo, Z.; Huo, Y.; Zhao, Z.K. Co-fermentation of acetate and sugars facilitating microbial lipid production on acetate-rich biomass hydrolysates. *Bioresource Technology* **2016**, *207*, 102–108.
42. Russell, J.B. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. *J. Appl. Bacteriol.* **1992**, *73*, 363–370.
43. Anschau, A.; Xavier, M.C.; Hernalsteens, S.; Franco, T.T. Effect of feeding strategies on lipid production by *Lipomyces starkeyi*. *Bioresour Technol* **2014**, *157*, 214–222.
44. Gong, Z.; Wang, Q.; Shen, H.; Hu, C.; Jin, G.; Zhao, Z.K. Co-fermentation of cellobiose and xylose by *Lipomyces starkeyi* for lipid production. *Bioresour Technol* **2012**, *117*, 20–24.
45. Yang, X.B.; Jin, G.J.; Gong, Z.W.; Shen, H.W.; Song, Y.H.; Bai, F.W.; Zhao, Z.B.K. Simultaneous utilization of glucose and mannose from spent yeast cell mass for lipid production by *Lipomyces starkeyi*. *Bioresour Technol* **2014**, *158*, 383–387.
46. Pirozzi, D.; Ausiello, A.; Yousuf, A.; Zuccaro, G.; Toscano, G. Exploitation of oleaginous yeasts for the production of microbial oils from agricultural biomass. *Chem Eng Trans* **2014**, *37*, 469–474.
47. Probst, K.V.; Vadlani, P.V. Production of single cell oil from *Lipomyces starkeyi* ATCC 56304 using biorefinery by-products. *Bioresour Technol* **2015**, *198*, 268–275.
48. Matsakas, L.; Sterioti, A.A.; Rova, U.; Christakopoulos, P. Use of dried sweet sorghum for the efficient production of lipids by the yeast *Lipomyces starkeyi* CBS 1807. *Ind Crop Prod* **2014**, *62*, 367–372.
49. Liu, L.; Chen, J.; Lim, P.-E.; Wei, D. Enhanced single cell oil production by mixed culture of *Chlorella pyrenoidosa* and *Rhodotorula glutinis* using cassava bagasse hydrolysate as carbon source. *Bioresource Technology* **2018**, *255*, 140–148.
50. Qin, L.; Wei, D.; Wang, Z.; Alam, M.A. Advantage assessment of mixed culture of *Chlorella vulgaris* and *Yarrowia lipolytica* for treatment of liquid digestate of year industry and cogeneration of biofuel feedstock. *Applied Biochemistry and Biotechnology* **2019**, *187*, 856–869.
51. Zhang, Z.; Pang, Z.; Xu, S.; Wei, T.; Song, L.; Wang, G.; Zhang, J.; Yang, X. Improved carotenoid productivity and COD removal efficiency by co-culture of *Rhodotorula glutinis* and *Chlorella vulgaris* using starch wastewaters as raw material. *Applied Biochemistry and Biotechnology* **2019**, *189*, 193–205.
52. Liu, Y.; Wang, Y.; Liu, H.; Zhang, J. Enhanced lipid production with undetoxified corn cob hydrolysate by *Rhodotorula glutinis* using a high cell density culture strategy. *Bioresour. Technol.* **2015**, *180*, 32–39.
53. Wang, S.; Wu, Y.; Wang, X. Heterotrophic cultivation of *Chlorella pyrenoidosa* using sucrose as the sole carbon source by co-culture with *Rhodotorula glutinis*. *Bioresour. Technol.* **2016**, *220*, 615–620.
54. Iasimone, F.; Zuccaro, G.; D'Oriano, V.; Franci, G.; Galdiero, M.; Pirozzi, D. Combined yeast and microalgal cultivation in a pilot-scale raceway pond for urban wastewater treatment and potential biodiesel production. *Water Sci. Technol.* **2018**, *77*, 1062–1071.
55. Stansell, G.R.; Gray, V.M.; Sym, S.D. Microalgal fatty acid composition: implications for biodiesel quality. *J Appl Phycol* **2011**, *24*, 791–801.
56. Asraful, A.M.; Masjuki, H.H.; Kalam, M.A.; Rizwanul Fattah, I.M.; Imtenan, S.; Shahir, S.A.; Mobarak, H.M. Production and comparison of fuel properties, engine performance, and emission characteristics of biodiesel from various non-edible vegetable oils: a review. *Energy Convers Manag* **2014**, *80*, 202–228.