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Enzymatic Hydrolysis and Oleaginous Fermentation of Steam-Exploded Arundo Donax and Lipomyces Starkeyi in a Single Bioreactor for Microbial Oil Accumulation

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***Abstract* — So far, the processes in single bioreactor were mainly developed to produce bioethanol. In this study, this process was developed to produce microbial oils from steam exploded *Arundo donax*, a flexible energy crop that can be cultivated in partially fertile lands. *Lipomyces starkeyi* was used as oleaginous microorganism. The microbial oils can be used as substrate for the synthesis of biodiesel or for the production of high-value biochemicals. Enzymatic hydrolysis and oleaginous fermentation in single bioreactor (SBHF) was performed to decrease enzyme loading and yeast cell concentration, in order to reduce total costs, to maximize final lipid yield (Y_{lipid}) and lipid productivity (q_{lipid}). A pH control was carried out during experimental tests to favor the growth of desired strains under non-sterile conditions. The lipid yield (Y_{lipid}) of samples without pH control (SBHF without pH control), using buffer solution (SBHF Buffer pH 5.2) and NaOH or HCl solution for pH control (SBHF pH 5.2) were equal to 0.05, 0.21 and 0.16 g g^{-1} total glucose, respectively. Distribution of fatty acids showed very high values of oleic acid. Specific glucose consumption rates (μ_s) were 0.00, 0.066 and 0.138 h^{-1}, respectively. A further advantage is the direct consumption of enzymatic hydrolysis products by oleaginous microorganism in the same reactor, which reduces substrate inhibition phenomena. The experimental data showed encouraging results, demonstrating that obtained fatty acids were suitable for production of biodiesel, bioplastics and other products of industrial interest.**

Keywords—Enzymatic hydrolysis, single bioreactor, Arundo donax, steam explosion, microbial oil, Lipomyces starkeyi

I. INTRODUCTION

Lignocellulosic materials are considered a very promising source for biofuel production [1,2,3]. Traditionally, the hydrolysis of lignocellulosic biomasses and the subsequent fermentation of hydrolysates are carried out in separate stages of the process. The integration of cellulose hydrolysis and lipid production into a single reactor reduces capital costs, improving efficiency for cellulose hydrolysis and lipid production [4,5,6,7]. A further advantage of this process is that the sugars produced by hydrolysis are immediately consumed by microorganisms, and possible substrate inhibition of the enzymes during the hydrolysis can be limited [8]. A possible limitation to the applicability stems from the different cultivation requirements of hydrolytic enzymes and oleaginous yeasts. As a matter of facts, the growth rate of *Lipomyces starkeyi* is considerably limited when the temperature exceeds 37°C [9, 7] whereas the activity of the hydrolytic enzymes is maximum in the temperature range (40-50°C) [10, 6, 7]. Furthermore, a major drawback of single reactor process is that the microorganisms cannot be recirculated since they are mixed with the solid particles derived from feedstock hydrolysis [11,12]. As a matter of facts, the enzymatic hydrolysis of the solid portion of pretreated lignocellulosic biomass generates a liquid slurry containing fermentable sugars and insoluble lignin/ashes. Only few papers have been devoted to the enzymatic hydrolysis coupled with the growth of oleaginous yeasts. Liu *et al.* [13] have studied integration of lipid production and enzymatic hydrolysis of corn stover, using diluted acid pretreated. The lipid concentration obtained were 3.03 g L^{-1} and 3.23 g L^{-1} using *Trichosporon cutaneum* yeast cultivated in 5 L and 50 L stirred tank reactor respectively. Gong *et al.* [7] have described simultaneous saccharification and enhanced lipid production of glucose and cellulose by *Criptococcus curvatus*. When cellulose was loaded at 32.3 g L^{-1}, lipid yield reached 0.20 g g^{-1} of cellulose. Gong et al. [7] have demonstrated that *Criptococcus curvatus* can be utilize either oligocelluloses or oligoxyloses as the sole carbon source for microbial oil production. The lipid concentration has been evaluated 2.7 and 1.6 g L^{-1}, while the lipid yield 0.20 and 0.17 g g^{-1} consumed sugar respectively. For commercial production of microbial lipids, low cost raw materials should be explored such as glycerol, sewage sludge, molasses and hydrolysates of lignocellulosic biomasses [14,15,16,17,18]. The present paper describes enzymatic hydrolysis and oleaginous

fermentation in single bioreactor of steam exploded *Arundo donax* for lipid production using *Lipomyces starkeyi* as oleaginous yeast. In this work, it has been investigated the influences of environmental factors as initial pH but also inhibitory effects of organic acids, furans from hexoses decomposition and phenols from lignin decomposition, released during hydrolysis and oleaginous fermentation. Thus, this new process has two features. In one aspect, it provides an example of simultaneous utilization of carbon source which may be of general interests for microbial conversion of some organic wastes and special resources. In the other hand, it offers an opportunity to develop integrated process to reduce time and costs for microbial oil production from lignocellulosic biomasses.

II. MATERIALS AND METHODS

Pretreatment of the lignocellulosic material

Arundo donax (giant reed) is a perennial, rhizomatous grass, classified as energy crop [19], with different characteristics such as high dry biomass yield (30-40 ton ha^{-1} $year^{-1}$) [20,21], the ability to grow in marginal lands [22], with reduced input of water [23], a high content in cellulose and hemicellulose (about 60%) [24,25], that make this interesting crop for bioethanol, biodiesel and bio-polymer production [26,27]. It was pretreated in a continuous pilot plant for the steam explosion (mod. StakeTech System Digester) located at ENEA–Trisaia Research Centre (Rotondella, Matera, Italy). Steam explosion of biomass is a pretreatment process that opens up the fibers, and makes the biomass polymers more accessible for subsequent processes, i.e. fermentation and hydrolysis. It is considered to be one of the most important. Its attractive features, in comparison to autohydrolysis, pulping, and other methods, include the potential to significantly reduce the environmental impact, the investment costs, and the energy consumption [28]. The biomass was steam exploded, processing 150-200 Kg h^{-1} of dry biomass. 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 2.1 [29]:

$$SF = \log(R) = \log\left(t \times e^{\frac{T-100}{14.75}}\right) \quad (2.1)$$

where t is pretreatment time and T is pretreatment temperature.

Enzymes and yeast strain

Cellulase from *Trichoderma reseei* ATCC 26921 and β-glucosidase from *Aspergillus niger* were purchased from Sigma. Enzymatic complexes used for the biomass pre-treatment usually consist of 1,4-β-D-glucanohydrolases (endoglucanases), 1,4-β-D-glucan cellobiohydrolases (exoglucanases), and β-D-glucoside glucohydrolase (β-glucosidase or cellobiase). In this study, only two enzyme complexes with cellulolytic activity were used, due to their high cost: cellulase that hydrolyses cellulose to cellobiose through the combined action of endo and exoglucanases, and β-glucosidase that hydrolyses cellobiose to glucose. Cellulase activity was measured following the NREL filter paper assay [30] and reported in filter-paper units (FPU) per milliliter of solution. β-glucosidase activity was measured using the method described by Wood & Bhat [31] and reported in cellobiase units (CBU). The enzymatic hydrolysis was performed in 500 mL flask with 200 mL of 5.0% w v^{-1} of steam exploded *Arundo donax* at pH 5.2 using a 0.05 M citrate buffer 50°C, 160 rpm for 48 hours. Cellulase and β-glucosidase were loaded at 15 FPU and 30 CBU per gram of cellulose, respectively. *Lipomyces starkeyi* DBVPG 6193 was used as oleaginous yeast, purchased from the Culture Collection of the Dipartimento di Biologia Vegetale of the Perugia University, Italy. The yeast was maintained at 5°C on YPD agar plate containing agar (20 g L^{-1}), D-Glucose (20 g L^{-1}), yeast extact (10 g L^{-1}) and peptone (20 g L^{-1}), that was sterilized by autoclaving for 20 min at 121°C. For seed preparation, *Lipomyces starkeyi* was cultivated in a 100 mL Erlenmeyer flask with an initial volume of 50 mL which contained D-Glucose (20 g L^{-1}), yeast extact (10 g L^{-1}) and peptone (20 g L^{-1}) at 160 rpm and 30°C for 24 h, previously autoclaved for 20 min at 121°C (Minitron, Infors HT, Switzerland). Cell density was measured at 600 nm (1 cm light path) using a UV-vis spectrometer (Shimadzu UV1700, Japan).

Separate hydrolysis and fermentation (SHF) and enzymatic hydrolysis and oleaginous fermentation in single bioreactor (SBHF)

The SBHF experiments were performed under non-sterile conditions. The media, containing substrates, were not autoclaved in an effort to preserve protein and carbohydrate integrity, preventing the Maillard reaction [32] as well to retain the most likely parameters for industrial applications. A 5% w v^{-1} of steam exploded *Arundo donax* was used in 500 mL Erlenmeyer flask, each containing 200 mL of culture suspension. A commercial mixture containing 15 FPU g^{-1} cellulose of Cellulase from *Trichoderma reesei* ATCC 26921 and 30 CBU g^{-1} cellulose of β-glucosidase from *Aspergillus niger* (Sigma-Aldrich) were used. After 48 h, 5% v v^{-1} of yeast cell suspension (*Lipomyces starkeyi* DBVPG 6193) was added to the same flask. A pH control was carried out during the experimental tests to limit the pH fluctuations. The enzymatic hydrolysis of lignocellulosic materials usually leads to a pH reduction, whereas the oleaginous fermentation causes a pH increase. Consequently, the experimental tests in enzymatic hydrolysis and oleaginous fermentation in single bioreactor (SBHF) were performed following three different protocols as regards pH control: (a) without pH control (SBHF

without pH control), (b) keeping a pH value of 5.2 using a citrate buffer solution (SBHF Buffer pH 5.2), (c) adding suitable amounts of NaOH 1 M or HCl 0.1 M to maintain a pH value equal to 5.2 under discontinuous conditions (SBHF pH 5.2).
Tests in Separate Hydrolysis and Fermentation (SHF) were performed simultaneously as control, using the same culture conditions observed for the tests just described, except the separation of hydrolysis and fermentation steps. The highest values of pH (between 5 and 6) were observed when a semi-continuous control was adopted (method c). In the presence of a citrate buffer solution (method b), the measured pH was in the range 4.5-5. When no pH control was adopted (method a), lowest values of pH were observed, exactly below 4. In a typical SBHF test, all the enzymes were added at the beginning and the biomass was hydrolyzed at 40°C and 160 rpm (Minitron, Infors HT, Switzerland). After 48 hours, temperature was changed from 40°C to 30°C and 5% v v^{-1} of yeast cells (*Lipomyces starkeyi*) were used as inoculum. All the tests were carried out, at least, in duplicate.

Analytical methods

Measurements of pH were made by a inoLab® Multi 740 Multimeters pH-meter (WTW). Glucose was measured using an enzymatic kit (Sigma Aldrich). Reducing sugars were measured by Nelson-Somogyi assay [33]. The total concentration of phenolic compounds was determined by the Folin-Ciocalteu reagent method, using cathecol as standard [34]. A simple method based on UV spectra was followed for the estimation of total furans (furfural and hydroxymethylfurfural) in the hydrolysates [35]. UV spectra were recorded on a SHIMADZU-UV1700 spectrophotometer using 1 cm cells. Volatile organic acids and ethanol were determined by GC analysis, using a Shimadzu GC-17A equipped with a flame ionisation detector (FID) and a capillary column with a PEG stationary phase (BP20, 30 m by 0.32 mm i.d., 0.25 µm film thickness, from SGE). Samples of 1 µL were injected with a split-ratio of 1:10. Helium was fed as carrier gas with a flow rate of 6.5 mL min^{-1}. Injector and detector temperatures were set to 320°C and 250°C, respectively. Initial column temperature was set to 30°C, kept constant for 3 min, followed by a ramp of 10°C min^{-1}, then kept constant for 1 min. When the microorganism was cultivated in SBHF, due to the solid-state nature of the biomass/feedstock, absorbance value using turbidometry cannot be utilized to monitor cell growth as a function of time. Consequently, the total count of microorganisms was carried out by sequential dilution in Petri dish containing YPD agar medium (Sigma-Aldrich). The cell proliferation was measured as Colony Forming Unit/mL (CFU mL^{-1}). Lipids were extracted by a method adapted from Bligh and Dyer [36]. The samples were stirred in a $CHCl_3/CH_3OH$ mixture (2:1 w v^{-1}) over 24 hours, and the oleaginous biomass was filtered off and washed with additional $CHCl_3$. This procedure was repeated three times. Then, the solvent was removed by evaporation under N_2 stream. The total lipid concentration ([Lipid]) was estimated by gravimetric method. To calculate the lipid concentration, the cells were dried to a constant weight in an oven at 105°C. The lipids extracted were subjected to transesterification reaction in a stirred container at 60°C for 10 min, using NaOH (1% w v^{-1}). The samples were dried by N_2 stream and subsequently 1 mL of heptane was added for the analysis. Fatty acid distribution was determined by gas chromatography. The GC (GC-MS 2010, Shimadzu, Japan) was equipped with a flame ionization detector on 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^{-1}). The 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^{-1}, and finally kept constant for 2 minutes. Methyl decanoate was used as internal standard. The peaks of each methyl ester were identified by comparing the retention time with the peak of the pure standard compound. All data in this study were the average of three independent experiments.

Equations

The ethanol yield ($Y_{ethanol}$) and lipid yield (Y_{lipid}) were calculated according to Equations 2.3 and 2.3:

$$Y_{ethanol}\ [g\ g^{-1}] = \frac{m_{ethanol}}{m_{total\ glucose}} \quad (2.2)$$

$$Y_{lipid}\ [g\ g^{-1}] = \frac{m_{lipid}}{m_{total\ glucose}} \quad (2.3)$$

The profile of glucose concentration s(t) can be determined following Equation 2.4:

$$s = s_0\, e^{-\mu t} \quad (2.4)$$

where s0 is glucose concentration released at the end of enzymatic hydrolysis (after 48 h).

III. RESULTS

Effects of pH control on enzymatic hydrolysis and oleaginous fermentation in single bioreactor
Figures 1, 2 and 3 show the profiles of reducing sugars, glucose concentrations and other byproducts (ethanol, volatile organic acids, phenols and furans) during enzymatic hydrolysis and oleaginous fermentation in single bioreactor (SBHF). Reducing sugars and glucose conversion was observed in a medium with buffer at initial pH 5.2 (SBHF Buffer pH 5.2) or using NaOH or HCl solution to adjust initial pH at 5.2 (SBHF pH 5.2) significantly higher than without pH control (SBHF without pH control), justified by the variability of pH value considered an inhibitory factor for oleaginous yeast fermentation, that reached 3.0 in the sample without pH control (data not shown), while it was unaffected from significant fluctuations in culture

medium with buffer solution at initial pH 5.2 (SBHF Buffer pH 5.2), further minimized in SBHF pH 5.2. As shown in Figure 1, 2 and 3, during the enzymatic hydrolysis from 0 h to 48 h, reducing sugars and glucose concentrations increased in all the samples. In fact, an increase of reducing sugars (until 15.16 g L^{-1}) and glucose (until 6.25 g L^{-1}) concentration for SBHF Buffer pH 5.2 was observed. Something to SBHF pH 5.2, an increase of reducing sugars (until 19.67 g L^{-1}) and glucose (until 6.96 g L^{-1}) was observed.
Figures 2 and 3 show a complete reducing sugars and glucose conversion during oleaginous fermentation (48-144 h), Specific glucose consumption rate (μ_s), as shown in Table 1, was calculated according to Equation 2.4, demonstrating that the value related to the process with pH control using NaOH or HCl solution (SBHF pH 5.2) was comparable to separate hydrolysis and fermentation (SHF), for the accuracy 0.138 and 0.159 h^{-1}, respectively.

TABLE 1. KINETIC PARAMETERS IN SHF AND SBHF PROCESSES

	μ_s [h^{-1}]
SHF	0.159
SBHF without pH control	0.000
SBHF Buffer pH 5.2	0.066
SBHF pH 5.2	0.138

This result suggests positive potential applications of such process that still remain roughly unexplored for microbial oil production. It could not be measured microbial growth using turbidometry but using CFU mL^{-1} counting. A maximum value of $1.84x10^8$ CFU mL^{-1} for SBHF pH 5.2 sample was evaluated, significantly higher if compared to the sample without pH control (SBHF without pH control) equal to $1.00x10^6$ CFU mL^{-1}. Figures 1, 2 and 3 show also acetic acid production. The inhibitory effects of weak acids were attributed to intercellular acidification and intracellular anions accumulation, more firmly in SBHF without pH control, where acetic acid concentration increased, instead of SBHF buffer pH 5.2 and SBHF pH 5.2 for which a decrease was observed. Acetic acid production is usually associated to the deacetylation of hemicellulose chains. It occurs significantly during the hydrolysis of xylan-rich lignocellulosic raw materials such as hardwoods, and in herbaceous energy crops such as *Arundo donax*. Hovewer, at high concentrations, negative effects are observed on the specific growth rate, increasing also the timing of lag phase [37,38]. Oleaginous yeasts can utilize acetic acid as a nutrient. According to Hu *et al.* [39] also a 7 g L^{-1} of acetate concentration can result an improvement of the lipid content of 68% using *Rhodosporidium Toruloides*. Phenols are a heterogeneous group of compounds originated from a partial lignin degradation [40]. Phenol concentration was about 0.6 g L^{-1}released during first 24 hours. After about 100 hours, a slight decrease in phenol concentration was observed (Figures 1, 2, 3), but it was not able to produce inhibitory effects on microbial growth. Furan concentration profiles showed a variable pattern that didn't appear to produce inhibitory effects on the performance of microbial growth. SBHF process was performed without sterilization and in absence of auxiliary nutrients. Some inhibitory compounds such as furfural and hydroxymethylfurfural in suspension might be related to steam explosion pretreatment. Ethanol production was also monitored during SBHF process in order to evaluate the ability of oleaginous yeasts to metabolize part of fermentable sugars not only for lipid accumulation, but also for alcoholic fermentation. It is known that the ethanol synthesis in the presence of lignocellulosic hydrolysates follows quite complex pathways. As a matter of facts, in addition to direct use of glucose, ethanol production from cellobiose has been demonstrated [41,42]. Ethanol production was affected by pH and dissolved oxygen shift. In fact, as shown in Figures 1, 2, 3 a remarkable ethanol production in SBHF pH 5.2 was observed, due to a higher fermentable sugars concentration used for ethanol production and the depletion of dissolved oxygen concentration that could induce this phenomenon. Ethanol yield was 0.19 g g^{-1} total glucose in SBHF pH 5.2, which was lower than reported for alcoholic fermentations where the yield is instead of 20 g g^{-1} dry biomass [43] or about 0.6 g g^{-1} fermentable sugars [44]. Ask *et al.* [44] obtained a final ethanol concentration 14.1 g L^{-1}. In the experimental activity, already described, a maximum value of 3 g L^{-1} obtained.

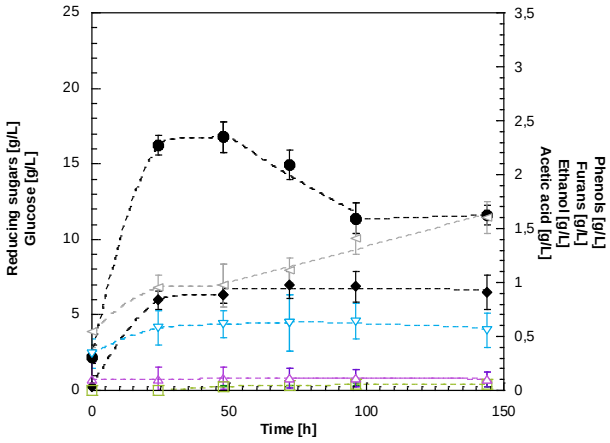

Fig.1. *Concentration profiles SBHF without pH control. Reducing sugars (black full circles), glucose (black full diamond), phenols (blu empty triangles), furans (violet empty triangles), ethanol (green empty squares), acetic acid (grey empty triangles). (T=40÷30 °C, 160 rpm, 144 h)*

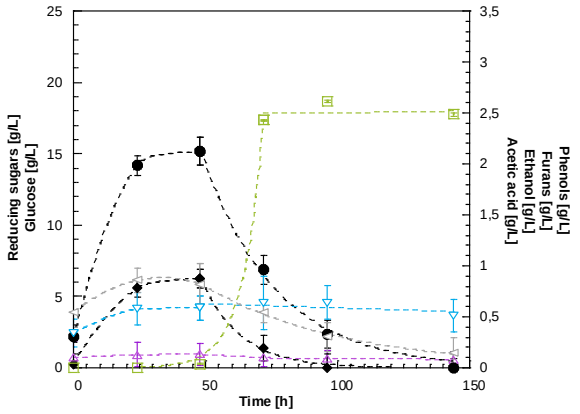

Fig. 2. *Concentration profiles SBHF Buffer pH 5.2. Reducing sugars (black full circles), glucose (black full diamond), phenols (blu empty triangles), furans (violet empty triangles), ethanol (green empty squares), acetic acid (grey empty triangles). (T=40÷30 °C, 160 rpm, 144 h)*

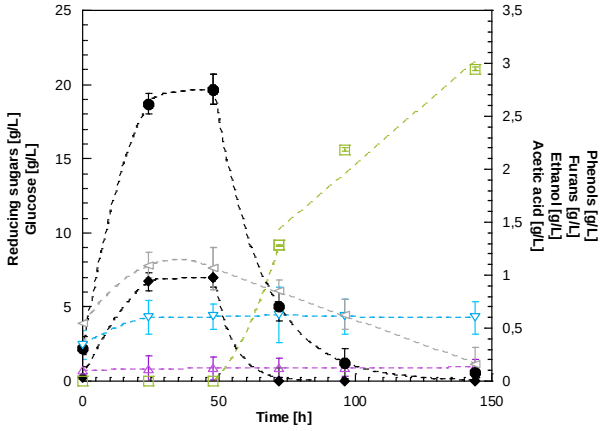

Fig. 3. *Concentration profiles SBHF pH 5.2. Reducing sugars (black full circles), glucose (black full diamond), phenols (blu empty triangles), furans (violet empty triangles), ethanol (green empty squares), acetic acid (grey empty triangles). (T=40÷30 °C, 160 rpm, 144 h)*

Morikawa *et al.* [45] studied biological co-production of ethanol and biodiesel from wheat straw using Simultaneous Saccharification and Fermentation for ethanol production and hemicellulose hydrolysate to produce microbial lipids in two separated processes. Mass balance showed that 0.9 g lipids and 10.1 g ethanol can be produced from 100 g of wheat straw. The experimental data obtained according to Equation 2.3, for lipid yield, and Figures 2 and 3, for ethanol concentration, showed almost 2.4 g lipids and 6 g ethanol for both SBHF Buffer pH 5.2 and SBHF pH 5.2, respectively. The exploitation of ethanol and Single Cell Oil co-production was feasible, though more optimization should be performed to improve economical and mass balance of the process. As a matter of facts, negative effects on specific growth rate and lag phase have been observed at acetic acid concentrations higher than 3 g L^{-1} [37,38], though at lower concentrations acetic acid could increase ethanol yield instead of biomass yield due to the uncoupling of ATP utilization from growth [46].
However, oleaginous yeasts are particularly tolerant to acetic acid inhibition [46]. Furfural and HMF directly inhibit alcohol dehydrogenase (ADH), pyruvate dehydrogenase (PDH), and aldehyde dehydrogenase (ALDH) [47]. Both furfural and HMF are known to inhibit the alcoholic fermentation also in oleaginous yeasts [48].

Lipid, FAME yields and distribution of fatty acids
Lipid yield (Ylipid), according to Equations 2.3, and lipid productivity (qlipid) were calculated. As shown in Table 2, highest lipid yield in SBHF cultures was obtained using buffer solution at pH 5.2 (SBHF Buffer pH 5.2) and this result was comparable to lipid yield in SHF, equal to 0.21 and 0.23 g g-1, respectively.

TABLE 2. LIPID YIELD, LIPID PRODUCTIVITY AND LIPID CONCENTRATION AT THE END OF SHF AND SBHF PROCESSES

	Y_{lipid} [g g^{-1}]	q_{lipid} [g L^{-1} day^{-1}]	[Lipid] [g L^{-1}]
SHF	0.26	0.38	1.48
SBHF without pH control	0.05	0.10	---
SBHF Buffer pH 5.2	0.21	0.34	1.36
SBHF pH 5.2	0.16	0.26	1.08

For this reason, SBHF processes by using *Lipomyces starkeyi*, as oleaginous yeast, and *Arundo donax*, as lignocellulosic biomass, can be considered feasible. Table 2 also shows lipid productivity (q_{lipid}). Under pH control using a buffer solution (SBHF Buffer pH 5.2), the value of productivity was promising (0.34 g L^{-1} day^{-1}) in comparison to lipid productivity calculated by Matsakas *et al.* [49] under optimum conditions but not in single bioreactor process. It was calculated also lipid concentration ([Lipid]) equal to 1.36 g L^{-1} for SBHF Buffer at pH 5.2 and 1.08 g L^{-1} for SBHF pH 5.2. Microbial lipids produced were subjected to alcoholysis. Figure 4 shows the distribution of fatty acids obtained by alcoholysis. The experimental data indicated a prevalence of long-chain fatty acids with 16 and 18 carbon atoms. The most abundant in SBHF Buffer pH 5.2 were oleic acid (C18:1) and palmitic acid (C16:0), equal to 43.92% and 34.59% w w^{-1}, respectively. Smaller amounts of stearic (C18:0) and arachidic (C20:0) were also present [Figure 4]. Similar fatty acid composition of lipids obtained from *Lipomyces starkeyi* also found when the yeast was cultivated on starch [50], mixtures of glucose and xylose [51] and various mixtures of glucose, cellobiose and xylose [18].

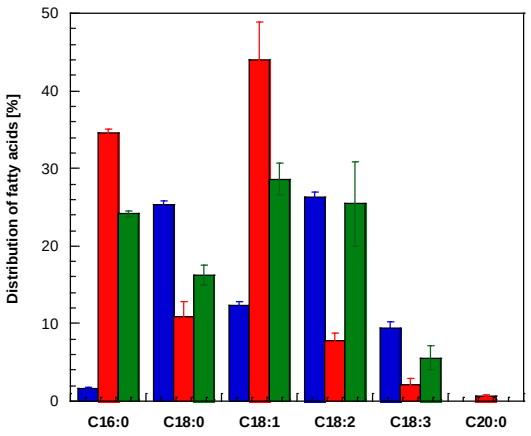

Fig. 4. *Fatty acid distribution (% w/w) with alkali-catalysis in SBHF. Fatty acid distribution for SBHF without pH control (blu columns), SBHF Buffer pH 5.2 (red columns), SBHF pH 5.2 (green columns)*

IV. DISCUSSION

Enzymatic hydrolysis and oleaginous fermentation in single bioreactor (SBHF) process was considered as preferably process because of reduced all operation costs, lower enzyme requirement and increased productivity [52, 14]. However, lignin residues mixed with yeast cells, in a single bioreactor, makes yeast recirculation very difficult. In addition, the optimal temperature for the yeast and the enzymes differs, which means that the conditions used in SBHF cannot be optimal for both enzymes and yeast. Figure 1 shows how the lack of pH control (SBHF without pH control) induces acetic acid production during the test that is usually associated to the deacetylation of hemicellulose chains in energy crops such as Arundo donax and produces an inhibitory effect on microorganisms that has been partially ascribed to the “uncoupling” mechanism [53]. Accordingly, low pH leads weak acids increase in undissociated form, being more hydrophobic and prone to crossing the membrane by simple passive diffusion. The anion of weak acids showed to be key factors in acid toxicity [54, 55] and cause of viable-cell low value (1.0 x 106 CFU/mL) approximately equal to the initial value. It is likely that a higher number of viable cells in high initial cell density culture could increase the likelihood of heterogeneity, and therefore, increase the likelihood that the cells resume growth asynchronously during the acid-adaptation phase. In general, yeast cells exposed to an increase of acid concentration exhibit significant decrease in specific glucose consumption rate, ethanol production [56] (Table 1) and also in terms of lipid content (Table 2). Figures 1, 2 and 3 show that, using a pH control, oleaginous cells converted acetic acid and completely glucose with a high consumption rate (Table 2). The pH of oleaginous yeast cultures is an important key factor, since lipid production significantly decreases at low pH values in some species [57]. Lipid accumulation in *Lipomyces starkeyi* was greatly inhibited at acidic pH below pH 4.0. However, from pH 5.0 to 5.5 (data not shown), *Lipomyces starkeyi* showed good lipid accumulation ability (Table 2) related also to acetic acid conversion into acetyl-CoA, a central intermediate in lipid synthesis, by acetyl-coenzyme A synthetase; this acetyl-CoA is then used for biosynthesis of polyunsaturated fatty acids and lipid accumulation in oleaginous yeast cells [58]. This metabolic pathway is very important to identify alternative organic substrates able to increase lipid yields. In addition to weak acids, lignocellulosic biomass pretreatment could generate a broad range of compounds that can inhibit the following steps as furans and phenolic compounds. The level of furans varies according to the type of raw material and to the pretreatment, strictly related to sugar dehydration while a wide range of phenolic compounds can be generated from lignin breakdown and also from carbohydrate degradation during acid hydrolysis [47]. The concentration of furans and phenols, monitored during the cultivation tests, was too low to determine any significant difference and inhibition effects in terms of microbial growth attested equal to $1.84x10^{8}$ CFU mL^{-1}. Ethanol production was correlated to the oxygen limitation. As noted in *Yarrowia lipolytica* by Workman *et al.* [59], oxygen limiting conditions create cofactor imbalances, particularly an excess of NADH, which are restored by the reduction of sugars into polyols or by the reduction of pyruvate into ethanol or butandiol.

In SBHF Buffer pH 5.2, lipid productivity was equal to 0.34 g L^{-1} day^{-1}, promising value in comparison to lipid productivity calculated by Huang *et al.* [60] for corncob hydrolysate treated with dilute sulfuric acid (0.74 g L^{-1} day-1) in separated reactors. Gong *et al.* [61] studied simultaneous saccharification and enhanced lipid production using *Cryptococcus curvatus*. The productivity was 4.69 g L^{-1} day^{-1} and the lipid content was 9.8 g/100 g raw corn stover. Gong *et al.* [18] calculated lipid coefficient as gram lipid produced per gram substrate consumed. The value for Co-fermentation of cellobiose and xylose by *Lipomyces starkeyi* for lipid production was almost 0.2 g g^{-1}. Lipid concentration ([Lipid]) equal to 1.36 g L^{-1} for SBHF Buffer at pH 5.2 and 1.08 g L^{-1} for SBHF pH 5.2 was lower than observed by Huang et al. [60] and Matsakas *et al.* [49], 8.1 and 6 g L^{-1}, who used corncob hydrolysate treated with dilute sulfuric acid and dried sweet sorghum in separated reactors, respectively. Gong *et al.* [61] obtained up to 11 g L^{-1} in simultaneous saccharification and lipid production in presence of extra nutrients. It can be explained by variability of culture conditions [62] and it dipends upon the carbon and nitrogen sources present in the media during the test and also from the oxygen limited conditions.

In Figure 4, the experimental data indicated a prevalence of mid-chain fatty acids with 16 and 18 carbon atoms in in agreement with Gong *et al.* [7].

V. CONCLUSIONS

The main advantage associated to the present study in single bioreactor mode is that end-product inhibition of the hydrolytic enzymes can be minimized, since produced fermentable sugars can be directly consumed by the microorganisms as soon as released into the fermentation broth [11]. Another advantage over SHF (Separate Hydrolysis and Fermentation) is the lower capital costs connected to SBHF, since the same bioreactor is used for both enzymatic hydrolysis and oleaginous fermentation. However, a compromise between the optimal process conditions of the cellulolytic enzymes and microorganisms has to be made. Moreover, this process doesn't offer opportunity for microorganism recirculation, because the solid particles in the lignocellulosic feedstock interfere with the separation. Another challenge of this study is related to decrease of economic impact using non-sterile fermentation. Non-sterile fermentation can reduce energy consumption and lower the cost associated with operation and maintenance [63].

This work demonstrated the possibility to obtain significant values in term of lipid yield, 0.21 g g^{-1} and 0.16 g g^{-1} total glucose released for SBHF Buffer pH 5.2 and SBHF pH 5.2, respectively; in comparison to 0.16 g g^{-1} obtained from Huang *et al.* [64]. The high tolerance of *Lipomyces starkeyi* to furan and phenol inhibition also suggested that the pH control is necessary for optimal cell growth. The cell viability $1.84x10^{8}$ Cell mL^{-1} in SBHF pH 5.2 demonstrated that semi-continuous conditions improve the growth condition of *Lipomyces starkeyi.* However, a balanced proportion between saturated and unsaturated fatty acids was observed, that encourages the use as automotive fuel [65]. For instance palmitic and oleic acids have been established as main biodiesel components and provide highest cetane response [66], corresponding with those of soybean oil and jatropha oil, which are used as feedstock for biodiesel production. Therefore, the exploitation of Single Cell Oils for other purposes was studied by Simopoulos [67] and Bharathiraja *et al.* [68] and related to the possibility of ensuring mono or polyunsaturation, known as EFAs, essential fatty acids of ω-6 and ω-9 families; chemical mediators that, for example, allow to fight the emergence of diseases such as atherosclerosis [67] and PUFAs as components of thrombocytes, neuronal and muscle cells, cerebral cortex as well as the immunocompetent cells [68].

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