*Environmental Engineering and Management Journal* *August 2019, Vol. 18, No. 8, 1693-1701*
*http://www.eemj.icpm.tuiasi.ro/; http://www.eemj.eu*

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# ALKALI PRE-TREATMENT AND ENZYMATIC HYDROLYSIS OF *Arundo donax* FOR SINGLE CELL OIL PRODUCTION

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## Abstract

Microbial oil obtainable from the fermentation of lignocellulose hydrolysates represents a promising and sustainable alternative to first generation biodiesel. Among the lignocellulosic crops, giant reed (*Arundo donax*) is attracting interest due to the impressive biomass yield and the low input requirement. However, a delignification step is needed to facilitate the fermentable sugar release from the lignocellulosic matrix, paying attention to the production of growth inhibitors that represent a bottleneck in the development of microbial oil production. The aim of this study was to evaluate the suitability of an enzymatic hydrolysate of alkali pre-treated *A. donax* biomass, as a substrate for the growth of the oleaginous yeast *Lipomyces starkeyi*. Specific attention was also paid to possible inhibitory effects of compounds generated by the alkaline pre-treatment (10% slurry, NaOH 0-1.5% w/w, 120 °C, 20 min). Increasing NaOH levels enhanced the release of phenolic compounds and increased the fermentable sugar yield after enzymatic hydrolysis of the washed pre-treated fiber. Saccharification yields reached a plateau in correspondence to NaOH 1.2% dose, which gave 407 mg of sugars per g of dry biomass. A medium containing 30 g/L of reducing sugars from the hydrolysate resulted suitable for the growth of *L. starkeyi* and for lipid accumulation, achieving 12.2 g/L of dry cell biomass with 43% w/w of total lipids. The pre-treatment produced soluble inhibitors that affected moderately the yeast growth in an initial phase, followed by a recovery. Thus, extensive washing of the fiber could be avoided, while a thorough filtration after the pre-treatment would be recommended.

*Key words:* fermentable sugars, giant reed, inhibitors, *Lipomyces starkeyi*, microbial oil

*Received: July, 2018; Revised final: January, 2019; Accepted: May, 2019; Published in final edited form: August, 2019*

## 1. Introduction

Biofuel production from renewable sources is widely considered as one of the most sustainable alternatives to petroleum sourced fuels and a viable mean for environmental and economic sustainability, lowering greenhouse gas emissions and meeting rural development goals. At first, biodiesel and bioethanol were obtained from edible vegetable sources, then second generation biofuels have been developed, by processing non-food oils and lignocellulosic materials, to overcome the competition "foods vs energy" (Dragone et al., 2010). The research interests also moved towards microbial oils or single cell oils (SCO) from microalgae, yeasts, filamentous fungi and bacteria, able to convert different carbon sources into lipids. These microbes accumulate intracellular triacylglycerol and free fatty acids, more efficiently than plants, with yields from 20 up to 87% of their dry biomass, and with a shorter production cycle (Bharathiraja et al., 2017; Sitepu et al., 2014). Moreover, microbial oil production does not compete with food production for land use and presents scale-up possibility. SCO production has been mostly carried out using low-cost sugars or grease substrates, like glycerol, cheese whey, food processing wastes or cooking oils. Lignocellulosic materials have been also extensively studied as a source of fermentable sugars for second generation bioethanol industry, but only recently they have been rediscovered as interesting

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low-cost and sustainable substrates for SCO production (Martínez et al., 2015; Sitepu et al., 2014).

Lignocellulose is composed of chains of cellulose, hemicellulose (xylan, mannan, galactan and arabinan polymers) and lignin, closely associated in a complex crystalline structure (Van Dik and Pletschke, 2012). Thus, an opportune pre-treatment is needed to disrupt cell wall structures and expose the holocellulose to enzymatic attack, to release monomeric sugars that can be converted into lipids by the oleaginous microorganisms.

A variety of pre-treatment methods are described in the literature: from biological (Cianchetta et al., 2014) to physical, physico-chemical and chemical (Mosier et al., 2005). Among these, alkaline pre-treatments present the advantage of low production of fermentation inhibitors compared to acid (McIntosh and Vancov, 2011). Alkali enhances the porosity of the lignocellulosic materials by solvation and saponification of intermolecular ester bonds, with the removal of cross-links among xylan, lignin and other hemicelluloses (Chang and Holtzapple, 2000) and by the removal of acetyl groups (Kootstra et al., 2009). Moreover, alkali pre-treated fibers retain most of the hemicellulose, potentially improving the final sugar yield. After the pre-treatment, a hydrolysis step is required with cellulolytic (endo- and exo-cellulase, β-glucosidase) as well as xylanolytic enzymes, which play an important accessory role, acting synergistically with cellulase. Moreover, the hydrolysis step should be performed at the lowest enzymatic load, not only to reduce costs, but also the final nitrogen content in the hydrolysate. In fact, to favor lipid accumulation by the oleaginous microorganisms, a high C/N ratio is desirable in the growth substrate, since nitrogen concentration determines the quantity of the biomass produced, while the carbon source concentration found in excess determines the amount of accumulated lipids (Papanikolaou and Aggelis, 2011).

Giant reed (*Arundo donax* L.) is a perennial grass, widely diffuse in warm-temperate environments under different pedo-climatic conditions. When cultivated under favorable situations, giant reed displays an impressive biomass yield, up to 40 Mg/ha of dry matter per year in the Mediterranean environment (Ceotto et al., 2013). Moreover, its rusticity and low input requirement make its cultivation suitable also for marginal lands. Therefore, giant reed is currently regarded as an emerging energy crop, as supported by a number of studies on its use for heat, bioethanol and biogas production (Corno et al., 2014). Very few information is available on the possibility to exploit giant reed biomass as a substrate for second generation biodiesel production. A recent study regarding the lipid production from giant reed biomass by the oleaginous yeast *Lipomyces starkeyi*, revealed high sensitivity of this microorganism to inhibitors generated during the acid hydrolysis (Pirozzi et al., 2015). Inhibitors represent one of the bottlenecks to the industrial development of microbial lipids from lignocellulosic hydrolysates and a key technological challenge (Rahman et al., 2017).

This work presents the results obtained in a study on the suitability of an enzymatic hydrolysate of alkali pre-treated *A. donax* biomass as a substrate for the growth of the single-cell oil producer *L. starkeyi*.

The alkaline pre-treatment was chosen with the aim of reducing the generation of inhibitors during lignin removal and of retaining most of the hemicellulose so that the enzymatic hydrolysate contained both hexoses and pentoses, which are exploitable by *L. starkeyi*. The effect of increasing doses of sodium hydroxide was evaluated in terms of delignifying ability and digestibility after enzymatic hydrolysis. Specific attention has been dedicated to the effect of inhibitors generated by the alkaline pre-treatment on the yeast growth since no information was yet available.

## 2. Material and methods

### *2.1. Materials*

#### *2.1.1. Plant material*

Representative plants of giant reed were collected in January 2017 from two-year plots at the CREA experimental farm of Anzola dell'Emilia (Bologna, Italy), then they were oven-dried at 60 °C for 48 h, milled at 0.2 mm and sieved to pass a 125 μm screen. Samples were collected and stored at room temperature in plastic bags. The fiber composition was determined on total solids according to Van Soest et al. (1991) and the C/N ratio was calculated after elemental analysis (Leco, CHN Truspec) (Table 1).

**Table 1.** Fiber composition and C/N ratio of giant reed (*Arundo donax* L.) dry biomass collected from two-year plots at the end of the growth cycle (January 2017)

| ***Fiber components*** | ***Mean values (s.d.)*** |
|---|---|
| Total Solids, TS (g/kg) | 937 (15.4) |
| Cellulose (% TS) | 37.3 (2.5) |
| Hemicellulose (% TS) | 25.7 (1.8) |
| Lignin (% TS) | 15.2 (1.6) |
| Ash (% TS) | 5.3 (0.1) |
| C (% TS) | 46.1 |
| N (% TS) | 0.44 |
| C/N (moles/moles) | 122 |

#### *2.1.2. Yeast strain*

The strain DSM 70295 of the yeast *L. starkeyi* used in this study was maintained at 4°C in Petri dishes on Yeast extract Peptone Dextrose Agarized (YPDA) medium (peptone 20 g/L, yeast extract 10 g/L, dextrose 20 g/L, agar 15 g/L). For short term use, the yeast was transferred in Petri dishes on the same fresh medium and kept at room temperature.

### *2.2. Methods*

#### *2.2.1. Alkaline pre-treatment and mass balance*

Slurries of giant reed powder (≤125 m diam., 10% w/w) were treated with increasing concentration

of NaOH (0, 0.17, 0.34, 0.5, 0.67, 0.84, 1.0, 1.17, 1.34, 1.5% w/w, in a final volume of 10 mL), at 120 °C, 20 min, in duplicates. The pre-treated samples were centrifuged (10 min, 2000 rcf, Allegra X-22 Beckman Coulter). Pellets were three-fold washed in sterile water, while the pooled supernatants were dried at 80 °C until constant weight, to perform a mass balance, calculating the dry weight of the insoluble and of the soluble fractions. The pH of the washed pellets was checked and corrected up to 7 with HCl when necessary, then pellets were maintained at 5 °C, until saccharification was performed.

*2.2.2. Spectrophotometric analysis of the liquid fraction*

The liquid fractions obtained by centrifugation of the pre-treated biomass (*par. 2.2.1*) were sampled (20 µl), opportunely diluted with a NaOH solution (10 g/L) and analyzed by a spectrophotometer (Infinite 200 PRO series, Tecan) in 96 well-microplates (EIA/RIA Clear Flat Bottom Polystyrene Not Treated Microplate, 3591, Corning). Absorbance values were recorded between 280 and 420 nm, with 2 nm intervals. A solution of 10 g/L of NaOH was used as blank.

*2.2.3. Saccharification of the pre-treated samples and reducing sugar determination*

The pre-treated samples were saccharified in 15 mL tubes (in duplicate) containing the washed solid fraction (7.0% w/w slurries, in a final volume of 7-11.5 mL), citrate buffer 50 mM, pH 4.8, sodium azide (0.2 mg/mL) and a mix of commercial enzymes at a cellulase load of 25 FPU/g of substrate (d.w.), using (in µL/g biomass): cellulase C2730 (Sigma) 250; β-glucosidase Cellic CTec2 NS22118 (Novozymes, A/S Bagsvaerd, Denmark) 15; xylanase Cellic CTec2 NS22083 (Novozymes) 7.5; enzyme complex Cellic CTec2 NS22119 (Novozymes) 10; hemicellulase Cellic CTec2 NS22002 (Novozymes) 50; amylase Cellic CTec2 NS22119 (Novozymes) 1.25. Tubes were continuously mixed in an orbital shaker (200 rpm, 2 mm radius, 50 °C, 144 h).

Samples of 20 µL were withdrawn at 0, 2, 4, 24, 48, and 144 h, kept in ice during manipulation and stored at -20 °C before analysis for reducing sugars. Reducing sugars were quantified by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959) adapted for 96-well microplates, in duplicates (Cianchetta et al., 2010). A mix of glucose and xylose (1:1) and dilutions of a control enzyme mix, were included, as standards. The assay conditions were: citrate buffer 50 mM, pH 4.8, 20 min, 50 °C. Saccharification yields are reported as mg of reducing sugars per g of dry untreated biomass.

*2.2.4. Evaluation of* L. starkeyi *growth in presence of the hydrolysate derived from pre-treated giant reed biomass*

Fermentation was carried out in 250 mL flasks, containing two different liquid substrates (MM-S and MM-GX) and 2 mL of yeast inoculum, in a final volume of 40 mL, at 28 °C, for 140 h, on an orbital shaker (180 rpm). The yeast inoculum was prepared as follows: fresh cells of *L. starkeyi* collected from YPDA Petri dishes were inoculated into 150 mL flasks containing 20 mL YPD broth. The flasks were incubated at 28 °C, for 24 h on an orbital shaker (180 rpm).

Both substrates MM-S and MM-GX were based on a minimal medium (MM) containing: $MgSO_4$*7 $H_2O$ (1.2 g/L); $CaCl_2$ (0.4 g/L); $(NH_4)_2SO_4$ (2.0 g/L); $KH_2PO_4$ (8 g/L); $Na_2HPO_4$ (7.2 g/L); $CoCl_2$*6 $H_2O$ ($8*10^{-3}$g/L); $MnSO_4$ ($6.4*10^{-3}$ g/L); $ZnSO_4$*7 $H_2O$ ($13.8*10^{-3}$ g/L); $FeSO_4$*7 $H_2O$ ($20*10^{-3}$ g/L).

MM-S (pH 6.2) was prepared with 10 mL of MM and 27 mL of hydrolysate obtained from the enzymatic saccharification of pre-treated giant reed powder (4 g, 10% slurry, NaOH 1.2% w/w), as described before (*par. 2.2.1 and 2.2.3*) using citrate buffer 10 mM instead of 50 mM.

MM-GX (pH 6.0) was prepared as above, substituting the hydrolysate with a solution of glucose and xylose (1:1) (Sigma Aldrich), at a corresponding amount of sugars (30 g/L). A denatured enzymatic mix (boiling water, 5 min) was also added (1.75 mL/L), as well as citrate buffer (6.25 mM).

MM-S and MM-GX contained also yeast extract (1 g/L), Tween 80 (0.63 g/L), and ampicillin (100 µg/mL). Finally, few mL of water were added up to 40 mL of final volume.

Samples of 100 µL were withdrawn from the cultures at 0,18, 24, 33, 41, 48, 55, 65, 89 and 140 h, diluted with 100 µL $H_2O$ and analyzed in duplicates with the spectrophotometer in 96-well microplates (600 nm), to evaluate the growth kinetics. The biomass weight at the final time-point was determined after culture centrifugation at 4500 rcf for 10 min at 5°C and drying at 80°C until constant weight. Two independent experiments were performed.

*2.2.5. Lipid extraction and content determination*

The total lipid content was determined at the final time-point of growth (140 h) from 50 mL cultures grown in MM-S and MM-GX media, in duplicates. Washed cell pellets were oven-dried at 80°C until constant weight and hydrolyzed with 12.5 mL/g d.w. of HCl 2M for 2 h at 80°C. Total lipids were extracted with hexane: isopropanol (3:2) (Hara and Radin, 1978). Briefly, 20 mL of solvent/g of dry cells were used, then the organic phase was recovered by centrifugation. The procedure was repeated 4-folds on the same pellet. The organic phases were pooled, heated at 75°C to remove most of the solvent, then dried under vacuum until constant weight.

*2.2.6. Evaluation of the inhibitory effect on* L. starkeyi *of the liquid fraction*

The liquid fraction obtained after the alkaline pre-treatment of the giant reed biomass was neutralized, centrifuged and added to MM-GX at increasing doses (0, 2.5, 5, 10, 20% v/v) in a final volume of 1500 µL in 24 multiwell plates with cover

(3820-024 Iwaki). Plates were inoculated with 6% v/v of actively growing cells (from 24 hours-old culture of *L. starkeyi* in MM-GX) and incubated at 28°C in a spectrophotometer (Infinite 200 PRO series, Tecan) with cycles of 6 mm orbital agitation (20 sec every 15 min) for 56 h. Every 30 min the optical density was measured at the wavelength of 600 nm (OD). The experiment was repeated with two replicates each time.

The Specific Growth Rate at time t ($SGR_{(t)}$) was calculated according to (Eq. 1):

$$SGR_{(t)} = \frac{\ln \frac{OD_{(t+1.5h)}}{OD_{(t-1.5h)}}}{\Delta t} \quad (1)$$

where: *t* is measured in hours.

The growth inhibition was calculated on the basis of OD measured at 48 hours ($GI_{OD}$) according to (Eq. 2):

$$GI_{OD} = \frac{OD_{control} - OD_{treated}}{OD_{control}} \quad (2)$$

and on the basis of the maximum specific growth rate ($GI_{MSGR}$) according to (Eq. 3):

$$GI_{MSGR} = \frac{MSGR_{control} - MSGR_{treated}}{MSGR_{control}} \quad (3)$$

where MSGR is the maximum specific growth rate, measured in $h^{-1}$.

## 3. Results and discussion

### *3.1. Mass balance after alkaline pre-treatment of the giant reed biomass*

Lignocellulosic substrates need to be pre-treated to remove lignin and facilitate the enzymatic hydrolysis of the holocellulose, with the release of sugars that can be subsequently converted into SCO by oleaginous microorganisms. The effect of a hydrothermal pre-treatment in combination with different level of NaOH on the solubilization of giant reed dry biomass is reported in this paragraph.

The mass balance of the pre-treated samples showed 15% of hot water extractives as reported in Fig. 1 (NaOH 0%, grey dashed bars). This result is consistent with what reported by other authors who found 13.9-18.4% dry matter in the liquor after filtration, depending on the pretreatment severity (hot water, 170-230 °C, 5-15 min) (Jiang et al., 2016).

The total extractives increased up to 44%, along with the NaOH concentration (Fig. 1, grey bars), while the solid insoluble fraction decreased from 85% down to 56% (Fig. 1, black bars). Some authors found lower values of total extractives (23.3-25.1%), for giant reed pre-treated with similar NaOH doses (1.2-2.0 % w/w, at room temperature for 24 h) (Jiang et al., 2016). The higher value found in our study (44%), is probably due to the higher temperature applied during the pre-treatment (120 °C instead of room temperature). The increase in solubilization observed with the increase of NaOH doses is most probably due to the solvation and saponification of intermolecular ester bonds in the biomass, by NaOH (Sun and Cheng, 2002), with partial solubilization of lignin and hemicellulose. These effects are expected to increase the susceptibility to the enzymatic saccharification of the solid fraction of the biomass, unmasking cellulose fibers and facilitating the enzymatic attack.

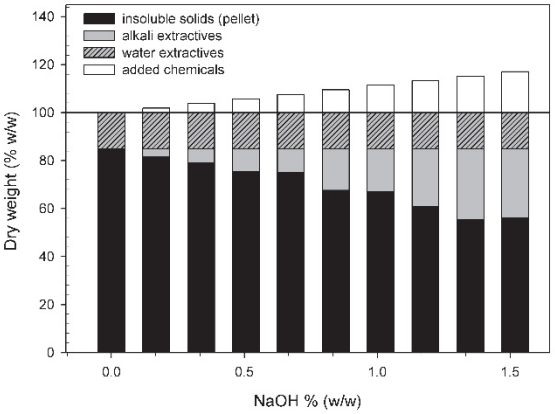


**Fig. 1.** Dry weight (% w/w) of the solid (black bars) and liquid (grey bars) fractions after the pre-treatment of giant reed biomass with increasing alkali doses (10% slurry, NaOH 0-1.5% w/w, 120 °C, 20 min)

### *3.2. Spectrophotometric analysis of the liquid fraction derived from the pre-treatment*

The liquid fraction collected after centrifugation of the alkali pre-treated samples were analyzed by a spectrophotometer in the UV-visible range to evaluate the pre-treatment effectiveness. UV-Vis absorbance spectra showed two overlapped bands with the maximum at 300 and 330 nm (Fig. 2), that can be related to the presence of lignin and other phenolic compounds, solubilized by the alkaline pre-treatment. In fact, lignin shows different absorption maxima between 219 and 365 nm, depending on the type of phenolic structures (Jablonský et al., 2015).

The absorbance increased along with the NaOH concentration (Fig. 2). In particular, a sigmoid increase of the band areas was recorded up to a plateau observed at NaOH doses higher than 1.2%, suggesting that only negligible increases in delignification would be obtained with these doses (not shown). Thus, the optimal NaOH dose for delignifying giant reed at 120 °C appears to be 1.2% w/w, corresponding to 120 mg of NaOH per g of dry biomass.

### *3.3. Saccharification of the pre-treated solid fraction*

Substrates rich in hemicellulose, such as alkali pre-treated substrates, require a varied cocktail of enzymes, among which xylanolytic enzymes play an important role also acting synergistically with cellulase on hydrolyzing pre-treated lignocellulosic materials (Hu et al., 2011; Li et al., 2014). In order to

effectively hydrolyze the complex lignocellulosic matrix of *A. donax*, after the alkaline pre-treatment, a high load (25 FPU/g d.w.) of an enzymatic mix was utilized, containing commercial cellulase, xylanase, endo-xylanase, *β*-glucosidase, but also accessory enzymes, like amylase, polygalacturonase, mannanase, and *β*-glucanase. The relatively high cellulase dose and the rich mix of accessory enzymes were utilized considering the high recalcitrance of giant reed, as highlighted in a comparison with other lignocellulosic feedstocks, like switchgrass or miscanthus (Cianchetta et al., 2017).

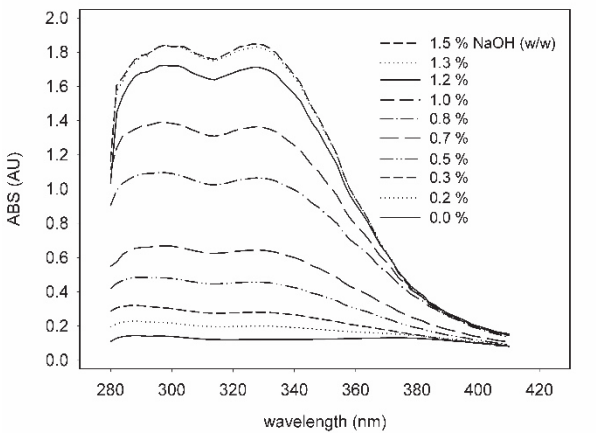


**Fig. 2.** Absorbance spectra of the liquid fractions derived from the pre-treatment of giant reed biomass with increasing alkali doses (10% slurry, NaOH 0-1.5% w/w)

The saccharification was followed at different time-points from 0 up to 144 h in order to study the efficacy of the different levels of pre-treatment and to point out the most convenient NaOH dose, evaluated in terms of the amount of reducing sugars released.

Saccharification time-course graph shows that increasing the pre-treatment severity facilitated the enzymatic hydrolysis, as resulted by the greater and faster release of reducing sugars from the lignocellulosic matrix (Fig. 3). The time-course related to the NaOH 0.8 % dose showed a sudden increase in effectiveness in comparison to the lower doses. This could be attributable to the pH level during the pre-treatment: in fact, with NaOH 0.8 % dose the pH was 11.5 at the end of the pre-treatment, while with the lower doses the pH was below 10.5, decreasing the pre-treatment effectiveness (data not shown).

The extent and speed of the sugar release further increased with NaOH 1% dose, while at higher doses the curves tended to overlap, without any apparent increase in speed (Fig. 3). The final sugar yields obtained at 144 h gradually increased along with the pre-treatment intensity, with a similar trend to that observed for the absorbance in the UV-Vis region of the liquid fraction (Fig. 2). This is consistent with other studies on alkaline pre-treatment of corn stover and giant reed, where a higher enzymatic digestibility resulted correlated with a higher lignin removal (Cui et al., 2012; Jiang et al., 2016). In correspondence with the NaOH 0.8% dose, the saccharification increased by 3.8 folds compared to the lowest pre-treatment level (NaOH 0%), yielding 329 mg of monosaccharides per g of dry biomass. With NaOH 1% the sugar yield further increased up to 393 mg/g, then a plateau was reached at NaOH 1.2%-1.5%, with final yields of 407-432 mg/g of reducing sugars (Fig. 3). Thus, pre-treating giant reed biomass with NaOH doses higher than 1.2% appears not convenient, as already highlighted when discussing the efficacy of the pre-treatment on the lignin removal (section 3.2).

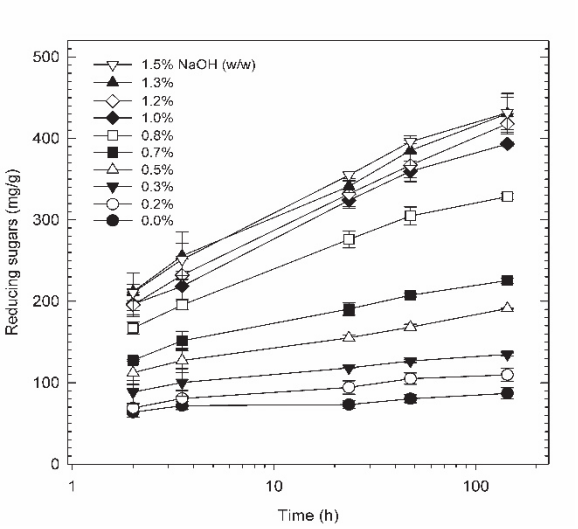


**Fig. 3.** Time courses of the release of the reducing sugars (mg/g of dry untreated biomass) from giant reed biomass after alkaline pre-treatment (10% slurry, NaOH 0-1.5% w/w, 120 °C, 20 min) and enzymatic hydrolysis with a mix of commercial enzymes (25 FPU/g, pH 4.8, 50°C, 144 h). The data points represent the mean ±standard deviation of two independent experiments (N=2)

A plateau for glucose yield after the pre-treatment of giant reed with NaOH 1.2% w/w at room temperature was also reported by other authors (Jiang et al., 2016). Other authors recently reported slightly lower sugar yield (380 mg/g) after alkaline pre-treatment of giant reed (Lemões et al. 2018).

### *3.4. Fermentation of the giant reed hydrolysate with L. starkeyi*

The fermentation experiment with *L. starkeyi* was carried out with an enzymatic hydrolysate of giant reed, pre-treated as described before with NaOH 1.2% w/w. This dose was chosen on the basis of the results reported above, in order to maximize the hydrolysate sugar content. This pre-treated material contained 58 ± 2% of cellulose, 27 ± 1.7% of hemicellulose and 12 ± 1% of lignin, in percent of total solids, according to Van Soest method. With respect to the starting material, the pre-treated biomass showed, as expected, a significant enrichment in the cellulose content and a decrease in lignin.

To promote SCO production by oleaginous microorganisms, a substrate with a high C/N ratio is required since these microorganisms begin to accumulate lipids in limiting condition of nitrogen.

Lipid accumulation is usually optimal at C/N molar ratio exceeding 65 and near 100 (Jin et al., 2015). In particular, for *L. starkeyi,* C/N ratios between 60 and 150 were found optimal (Angerbauer et al., 2008). The giant reed biomass utilized in this study displayed a molar C/N ratio of 122, based on CHN elemental analysis (Table 1), thus it appeared promising to produce low nitrogen hydrolysate. The high C/N ratio was due to the low content of nitrogen in the winter harvested biomass, since nitrogen is gradually translocated from the aboveground biomass towards rhizomes, from the end of the vegetative cycle until the new growth (Nassi o di Nasso et al., 2011).

After the alkaline pre-treatment and washing, C/N molar ratio of the fiber was 600, while that of the hydrolysate was 64, with the nitrogen mainly provided by the added enzymes. The use of enzymes with a higher specific activity (FPU per mg of protein) would allow producing hydrolysates with even higher C/N ratios.

To verify the suitability of the hydrolysate obtained from NaOH 1.2% pre-treated giant reed for the growth of *L. starkeyi*, a medium containing this hydrolysate (MM-S) was utilized in comparison to a semi-synthetic substrate with equivalent sugar content (MM-GX, glucose +xylose = 30 g/L). After an initial 24 h lag phase, the yeast grew fast up to 60 h at the expenses of the sugars contained in the substrates, as demonstrated by the fast increase of the culture turbidity measured at the spectrophotometer (Fig. 4a) and by the corresponding decrease of the sugar content, slightly delayed, determined by the DNS assay (Fig. 4b). After 60 h the growth slowed until the sugars were almost completely consumed (140 h) (Fig. 4b). The yeast grew better in the presence of the hydrolysate of pre-treated giant reed (MM-S) than in the semi-synthetic medium (MM-GX), with final dry cell biomass concentrations of 12.2 and 8.8 g/L, respectively. Since the two substrates contained the same compounds and differed only for the sugar sources, probably the hydrolysate contained other minor nutrients, derived from giant reed, that were limiting or absent in the semi-synthetic substrate.

Cell biomass concentration obtained with MM-GX is consistent with that reported in the literature for the same yeast in a synthetic medium containing 30g/L of glucose, without inhibitors (Rhaman et al. 2017). Differently, cell biomass concentration obtained in presence of the hydrolysate is higher than that reported in the literature for the same yeast in the presence of a giant reed hydrolysate (9.9 g/L) (Pirozzi et al., 2015). Such hydrolysate was obtained after acid hydrolysis and contained an even higher concentration of sugars (40 mg/L), but also some inhibitors.

The final lipid content of *L. starkeyi* cells grown in MM-S medium was higher than that obtained in MM-GX medium, being 43% w/w in comparison to 35% w/w, corresponding to final lipid concentrations of 5.3 and 3.1 g/L, respectively. Thus, a quite good sugar-to-lipid conversion (0.17 g lipid/g sugars) was achieved with the hydrolysate, taking into account that the practical yield after production of biomass is generally considered to be approximately 0.22 g lipid/g glucose (Jin et al., 2015). Further improvements in the conversion can be obtained, by optimizing the culture conditions.

Overall, the relatively high concentrations of cell biomass and lipids obtained with *L. starkeyi* suggest that the enzymatic hydrolysate from alkali pre-treated giant reed is both suitable for the growth of *L. starkeyi* and for lipid accumulation.

### *3.5. Inhibitory effects of by-products derived from the alkaline pre-treatment*

The protocol included a washing step after the pre-treatment to remove most of the lignin-derived soluble compounds. Washing of the pre-treated giant reed fibers before fermentation was effective in removing possible inhibitors.

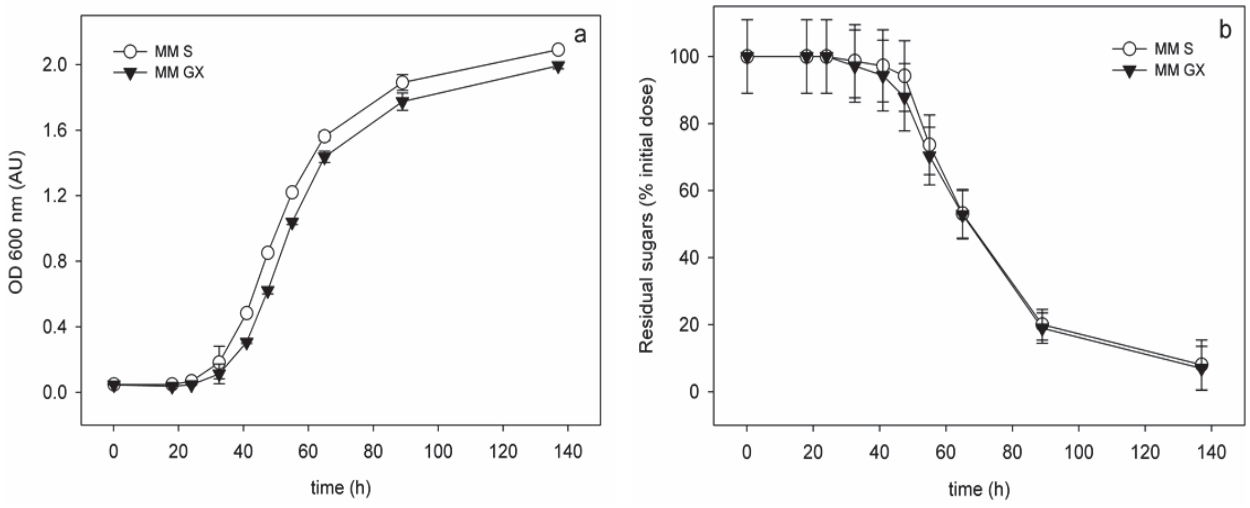


**Fig. 4.** *Lipomyces starkeyi* growth curves (a) and corresponding sugar consumption (b) during fermentation (28 °C, 160 rpm, 250 mL shake flasks) on a medium containing giant reed hydrolysate (MM-S) or an equivalent glucose and xylose mix (30 g/L) (MM-GX). The data points represent the mean ±standard deviation of two independent experiments (N=2)

In general, the need to perform a detoxification step after the pre-treatment, like fiber washing, before fermentation of lignocellulosic hydrolysates, depends on the pre-treatment type and severity, as well as on the strain tolerance to inhibitors (Jin et al., 2015). Some authors highlighted a relatively good tolerance by *L. starkeyi* to phenolic inhibitors at low concentrations, like those derived from acid hydrolysis of lignocellulosic materials (Rhaman et al., 2017). Other authors, who actually carried out acid hydrolysis of giant reed biomass, found, instead, significant inhibitory effects towards the yeast and adopted different strategies to reduced them, like dilution, over-liming and/or treating with activated charcoal, as well as pre-adapting the yeast by a progressive increase of the hydrolysate concentration (Pirozzi et al., 2015).

Currently, nothing is known on the inhibitory effect towards *L. starkeyi* of compounds eventually generated by an alkaline pre-treatment of giant reed. As reported in *par. 3.2*, the liquid fraction derived from the NaOH pre-treatment appeared enriched in phenolic compounds along with the increasing NaOH doses, suggesting a potential inhibitory effect of this fraction on *L. starkeyi*, since phenolic compounds are considered among the most inhibitory compounds for this yeast (Rahman et al, 2017).

To gather information on the possible inhibitory effect of by-products derived from the alkaline pre-treatment of giant reed (NaOH 1.2% w/w, as described before), the phenol rich liquid fraction was neutralized and added at increasing doses to a semi-synthetic medium (MM GX), that was then inoculated with *L. starkeyi* and incubated to follow the yeast growth. The liquid fraction produced an increasing inhibitory effect proportional to the dose, as it can be seen from the representative tracings of the growth curves reported in Fig. 5a, b in terms of optical density and specific growth rate. The greatest differences among the curves were observed around 20 h, while they tended to converge again after this time-point, indicating a sort of recovery by the culture (Fig. 5a). This is confirmed by the trends reported for the specific growth rate. The maxima observed at 10 h were lower in presence of the inhibitors than in the control, while after 20 h the specific growth rate was higher in presence of the inhibitors than in the control (Fig. 5b).

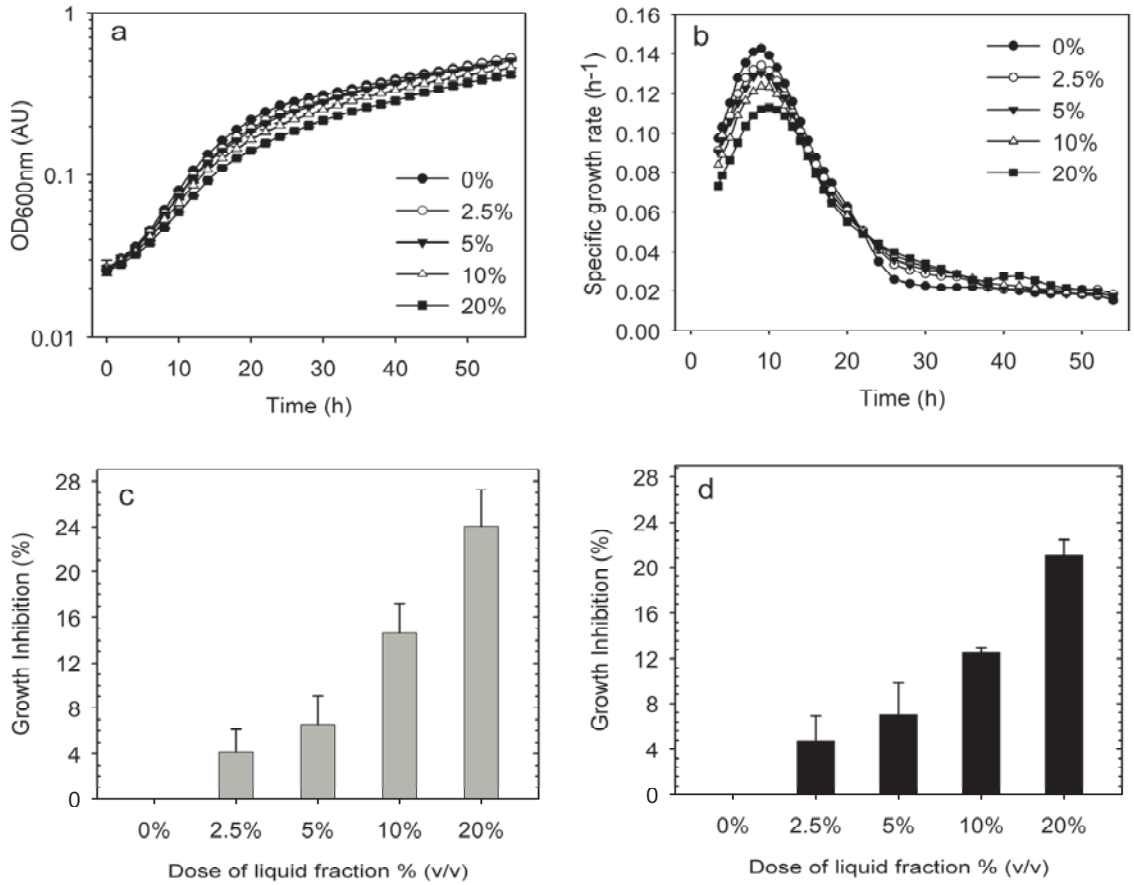


**Fig. 5.** Upper panels: growth curves (a) and specific growth rate (b) of *Lipomyces starkeyi* in a minimal medium containing glucose and xylose, 30 g/L (MM GX) and increasing amounts (0, 2.5, 5, 10, 20%) of the neutralized liquid fraction obtained after the alkaline pre-treatement of giant reed (10% slurry, NaOH 1.2% w/w, 120 °C, 20 min). Lower panels: growth inhibition of *L. starkeyi* (%), calculated on the basis of the optical density at 48 h (c), and on the basis of the maximum specific growth rate (d). Bars represent the mean ± s.d. of two independent experiments with 2 replicates

The growth inhibition towards *L. starkeyi* related to the increasing doses of liquid fraction is reported in Fig. 5c, d. Both panels show the same trend, with an increasing effect of growth inhibition along with the increasing dose of liquid fraction, reaching up to 21-24% inhibition with the maximum dose (20%). These results suggest that in order to reduce inhibitory effects on the yeast, it could be sufficient to remove most of the liquid fraction after the pre-treatment by filtration/centrifugation, without extensively washing the pre-treated fiber. Cycles of adaptation in presence of inhibitory compounds released by the alkaline pre-treatment could be carried out to improve the yeast tolerance, as suggested by other authors who studied giant reed acid hydrolysis (Pirozzi et al., 2015).

Finally, considering the biomass yield obtainable for giant reed under favorable conditions, that can reach 40 Mg/ha dry weight per year (Ceotto et al., 2013), an alkaline pre-treatment with NaOH 1.2%, as described here, followed by enzymatic hydrolysis, would provide up to 16 Mg/ha of fermentable sugars. On the basis of a sugar-to-lipid conversion of 17%, like that obtained in this preliminary study, an estimated microbial oil yield of 2.7 Mg/ha could be obtained. Further improvements could be achieved, optimizing the enzymatic hydrolysis and the culture conditions. As a reference, the actual oil yield obtainable in the case of first-generation biodiesel (sunflower) is 1-2 Mg/ha (Ragaglini et al., 2011).

## 4. Conclusions

The results obtained demonstrated that the enzymatic hydrolysate obtained after the alkaline pre-treatment of giant reed biomass is suitable for the growth of the oleaginous yeast *L. starkeyi*, achieving also a remarkable production of microbial oil (43% w/w).

Increasing the alkali dose of the pre-treatment produced an increase in the delignification efficacy and consequentially also an increase in the enzymatic hydrolysis efficacy, with fermentable sugar yields reaching a plateau in correspondence of NaOH 1.2% w/w dose (on a 10% slurry of biomass), with a sugar yield of 407 mg/g of dry biomass.

Since inhibitors represent an important issue for the development of SCO from lignocellulosic hydrolysate, this study also provided information on the possible inhibitory effects of alkaline pre-treatment by-products which are present in the discarded liquid fraction: *L. starkeyi* appeared rather tolerant up to 20% dose of the liquid fraction. Thus, filtering and discarding the liquid fraction prior to the enzymatic hydrolysis step could be sufficient to reduce inhibitory effects, suggesting that extensive washing of the pre-treated fiber could be avoided.

Further studies are envisaged to optimize the enzymatic load, in order to obtain a hydrolysate with the highest sugar and the lowest N content and maximize SCO production.

**Acknowledgements**

This work was supported by the Italian Ministry of Agricultural, Food and Forestry Policies (MiPAAF) under the AGROENER project (D.D. n. 26329, 1st April 2016) - http://agroener.crea.gov.it. Authors wish to thank Dr. Enrico Ceotto for providing *A. donax* biomass and prof. Alejandro Hochkoeppler for valuable supporting.

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