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Integrated cascade biorefinery processes for the production of single cell oil by *Lipomyces starkeyi* from *Arundo donax* L. hydrolysates

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ABSTRACT

Giant reed (*Arundo donax* L.) is a promising source of carbohydrates that can be converted into single cell oil (SCO) by oleaginous yeasts. Microbial conversion of both hemicellulose and cellulose fractions represents the key step for increasing the economic sustainability for SCO production. *Lipomyces starkeyi* DSM 70296 was cultivated in two xylose-rich hydrolysates, obtained by the microwave-assisted hydrolysis of hemicellulose catalysed by $FeCl_3$ or Amberlyst-70, and in two glucose-rich hydrolysates obtained by the enzymatic hydrolysis of cellulose. *L. starkeyi* grew on both undetoxified and partially-detoxified hydrolysates, achieving the lipid content of 30 wt% and yield values in the range 15-24 wt%. For both integrated cascade processes the final production of about 8 g SCO from 100 g biomass was achieved. SCO production through integrated hydrolysis cascade processes represents a promising solution for the effective exploitation of lignocellulosic feedstock from perennial grasses towards new generation biodiesel and other valuable bio-based products.

Keywords: Glucose- and xylose-rich hydrolysates; Yeast fermentation; Biodiesel; Lignocellulosic feedstock; Perennial grasses.

1. Introduction

The transition from a fossil-based economy to a bio-based one is a current global goal in order to contrast some important issues such as climate change and environmental pollution and to reduce the dependency on fossil sources as well. Thus, the replacement of fossil fuels and materials with biofuels and bioproducts represents the key solution. Among biofuels, biodiesel is one of the most promising renewable

energy sources since it does not require new technology and engines for its use (d'Espaux et al., 2015). Conventional biodiesel is produced on an industrial scale starting from vegetable oils obtained from oleaginous crops. However, most of the high productivity oleaginous plant species are food crops, thus determining the ethical debate on the right use of these renewable resources due to the competition between the energy industry and food chain (Mahlia et al., 2020). An innovative and promising solution is represented by new generation biodiesel, produced from microbial oil or single cell oil (SCO) (Patel et al., 2020). Some oleaginous yeasts can accumulate lipids over 20% of their dry cell weight and the typical lipids profile of SCOs is very similar to that of the main vegetable oils such as palm oil, rapeseed oil and sunflower oil (Patel et al., 2020). Moreover, SCO from oleaginous yeasts represents a source of platform chemicals for several biobased products, such as surfactants, lubricants, food additives, plastics, paints and detergents (Probst et al., 2016). Among oleaginous yeasts, *Lipomyces starkeyi* can afford high lipid yields from both hexoses and pentoses, re-utilising small amounts of its intracellular lipids, showing the capability to grow in simple media (e.g. without vitamin supplementation) and to carry out extracellular polysaccharide degradation, as well as good tolerance to inhibitory compounds such as aldehydes, alcohols and organic acids (Sutanto et al., 2018; Wang et al., 2014). For these reasons, *L. starkeyi* is a well-known, promising oleaginous yeast for industrial-scale production of SCO.
Nevertheless, the current high prices of most conventional carbon sources (e.g. sugars) strongly limit the economic competitiveness of SCO respect to crude oil (Javaid et al., 2017). Conversely, lignocellulosic feedstocks obtained from crop residues or high-yielding and resource-efficient perennial grasses, such as giant reed (*Arundo donax* L.),

can be less expensive and reduce the competition with food crops, potentially leading to more sustainable pathways for SCO production.

As a biomass crop, giant reed is characterised by several positive traits: it is a perennial species, thus avoiding annual soil tillage; it has shown high yielding potential under low input management systems, without irrigation, even on marginal, contaminated or underutilised lands; it effectively removes nitrates from the soil, helping to mitigate nitrate pollution risk (e.g. in riparian buffer strips); it does not suffer from any major pathogen or pest (Bosco et al., 2016; Ceotto et al., 2018; Scordia & Cosentino, 2019). Moreover, giant reed biomass can be collected from spontaneous riparian stands, in order to maintain riverbanks and mitigate the flooding risk, or to control populations where this species is considered invasive (e.g. North America) (Pilu et al., 2014). Giant reed is typically rich in both cellulose (about 40% of dry matter) and hemicellulose (about 25% of dry matter) (Nassi o Di Nasso et al., 2011) and presents high reactivity in the hydrothermal conversion thus offering outstanding perspectives for the conversion into chemicals and biofuels. The hemicellulose fraction (the second most abundant polysaccharide in lignocellulosic biomass) is usually wasted in traditional biorefinery plants, where it is generally removed during the pretreatment of the biomass in order to reduce structural constraints on the enzymatic hydrolysis of cellulose (Xavier et al., 2017). In particular, during conventional pretreatments based on organic and inorganic acids, alkali, hot water, steam and ammonia explosion, and ionic liquids, hemicellulose is decomposed in by-products that represent strong inhibitors in the hydrolysate conversion through the fermentation route. Thus, the complete recovery by selective fractionation of the hemicellulose component into sugars to be fermented would significantly improve the economic balance of biofuels and bioproducts

production. Alternative pathways of selective microwave-assisted hydrolysis have been already demonstrated effective in providing xylose-rich hydrolysates from the hemicellulose fraction of giant reed biomass (Di Fidio et al., 2019; Di Fidio et al., 2020c), while the cellulose-rich residues can be source of glucose-rich hydrolysates by means of enzymatic hydrolysis (Di Fidio et al., 2020b; Di Fidio et al., 2020c).

The present study evaluates a novel integrated cascade biorefinery scheme for the production of SCO by *L. starkeyi* DSM 70296 in batch-mode fermentation of both xylose- and glucose-rich hydrolysates. In order to verify the effectiveness of this approach on different types of xylose- and glucose-rich hydrolysates, these last were obtained adopting two different cascade processes both for hemicellulose and for cellulose fractions hydrolysis. As widely reported in the literature, the pretreatment step is expensive, energy-intensive and, often adopts chemicals which require special disposal, handling, or production methods. All these aspects often compromise the economic sustainability of a biorefinery process on an industrial scale (Yang et al., 2018). Therefore, in the present study, the different hydrolysates were obtained from unpretreated giant reed biomass in order to assess their conversion to give SCO, by evaluating: 1) the efficiency of conversion by *L. starkeyi* DSM 70296 of both xylose- and glucose-rich hydrolysates obtained by catalytic and enzymatic conversion respectively; 2) the mass balance of the investigated cascade processes; 3) the fatty acids composition of the obtained SCO.

2. Materials and methods

2.1. Feedstock and materials

Giant reed (*Arundo donax* L.) biomass was collected from a mature, 4-year old plantation, routinely managed by yearly harvests, at the Centre for Agri-environmental Research "Enrico Avanzi" of the University of Pisa in San Piero a Grado (Pisa, latitude 43° 68' N, longitude 10° 35' E). In December 2018, giant reed biomass was harvested and then treated and characterised as described in our previous work (Di Fidio et al., 2019). After the harvesting, whole culms and leaves were ground in 1.0 mm average size particles, dried at 105 °C in an oven until a constant weight, and then stored in a desiccator up to their use.

The feedstock contained 36.3±0.4 wt% glucan, 17.3±0.2 wt% xylan, 1.9±0.1 wt% arabinan, 0.6±0.0 wt% mannan, 3.6±0.1 wt% acetyl groups, 2.0±0.1 wt% ash, 15.4±0.8 wt% extractives, 22.0±0.3 wt% acid-insoluble lignin, 0.9±0.1 wt% acid-soluble lignin. Values represent the mean, n = 3, ± standard deviation.

Chemicals of analytical purity grade were provided by Sigma-Aldrich (USA). Novozymes (Denmark) kindly provided the enzymatic mixture Cellic® CTec2.

2.2 Hemicellulose and cellulose hydrolysis

SCO production by *L. starkeyi* DSM 70296 was evaluated in batch-mode fermentation of xylose- and glucose-rich hydrolysates obtained from two different cascade processes based on two consecutive steps for hemicellulose and cellulose hydrolysis, respectively.

Under the first process, a xylose-rich hydrolysate (X1) was obtained by the selective microwave-assisted hydrolysis of hemicellulose performed in the monomodal microwave reactor CEM Discover S-class System by employing the homogeneous catalyst $FeCl_3$, adopting the following reaction conditions: 9 wt% biomass loading, 150

°C, 2.5 min and $FeCl_3$/giant reed weight ratio 0.17 wt/wt (Di Fidio et al., 2019). In particular, 1 g of raw biomass was charged in the glass vessel (35 mL) containing 10 mL H_2O and about 0.28 g $FeCl_3$. The microwave reactor was heated at 150 °C and the reaction was carried out for the required time under magnetic stirring. At the end of the reaction, the vessel was rapidly cooled at room temperature through an external airflow.

According to the second approach, xylose-rich hydrolysate (X2) was produced by the selective microwave-assisted hydrolysis of hemicellulose performed in the monomodal microwave reactor CEM Discover S-class System by employing the heterogeneous catalyst Amberlyst-70, adopting the following reaction conditions: 17 wt% biomass loading, 160 °C, 20 min, Amberlyst-70/giant reed weight ratio 0.20 wt/wt (Di Fidio et al., 2020c). In particular, 4.1 g of raw biomass was charged in the glass vessel (35 mL) containing 20 mL H_2O and about 0.4 g Amberlyst-70. The microwave reactor was heated at 150 °C and the reaction was carried out for the required time under magnetic stirring. At the end of the reaction, the vessel was rapidly cooled at room temperature through an external airflow. At the end of the reaction, the liquid fraction, containing a high concentration of xylose and a low concentration of glucose, was recovered by filtration under vacuum on a Gooch filter and analysed by HPLC. In order to adapt X2 to the yeast growth condition, the selective removal of furfural (1.0 g/L) and acetic acid (4.7 g/L) was performed by vacuum evaporation (Sutanto et al., 2018). The solution was firstly concentrated in a laboratory rotavapor vacuum system at 8 kPa, 80 rpm and 50 °C for 1 h and then diluted up to the starting concentration values of sugars by adding deionised water. The final composition of X2 used as fermentation medium was the following one (g/L): glucose 8.0, xylose 38.0, 5-HMF 1.3, formic acid 1.0, levulinic acid 0.5.

The separated solid residues obtained by the first step, namely the cellulose-rich residues (CRR1 and CRR2), were washed with deionized water, oven-dried at 105 °C and then used as the substrate for the following enzymatic hydrolysis of the cellulose fraction. The enzymatic activity of the commercial preparation Cellic® CTec2 (a mixture of endo- and exocellulase, β-glucosidases and hemicellulase) was equal to 134.5 FPU/mL. The enzymatic hydrolysis was carried out in a 150 mL flask adopting the following reaction conditions: pH 4.8, 50 °C, 50 mL of the 0.05 M citrate buffer solution, 25 FPU/g glucan Cellic® CTec2, shaking at 160 rpm. At the end of the reaction, the glucose-rich hydrolysate was recovered by filtration under vacuum on a Gooch filter and analysed by HPLC (Di Fidio et al., 2020b; Di Fidio et al., 2020c). Therefore, two different glucose-rich hydrolysates (Table 1) were obtained: the first one by the enzymatic hydrolysis of the CRR recovered after the $FeCl_3$-catalysed hydrolysis of hemicellulose (G1) and the second one after the Amberlyst-70-catalysed hydrolysis of hemicellulose (G2), adopting in both cases the following reaction conditions: 9 wt% biomass loading, 50 °C, 96 h and 25 FPU/g glucan Cellic® CTec2.

The quantification of glucose, xylose, acetic acid, formic acid, levulinic acid, 5-hydroxymethylfurfural and furfural was performed by High Performance Liquid Chromatography (HPLC) PerkinElmer Flexar Isocratic Platform equipped with a differential refractive index detector (Di Fidio et al., 2019).

2.3. Yeast strain and cultivation

The oleaginous yeast strain *Lipomyces starkeyi* DSM 70296 was provided by DSMZ (Germany). Preparation, sterilisation and inoculation of preculture media were performed as previously reported (Di Fidio et al., 2020a). A calculated volume of the

preculture was added to the fermentation medium in order to reach the inoculum concentration of 5.0 g/L dry cell weight (DCW).

2.4. Bioconversion of sugars into single cell oil

Batch-mode fermentations were carried out according to the process conditions previously described (Di Fidio et al., 2020a). Briefly, 50 mL of undetoxified lignocellulosic hydrolysate was set as the working volume in 250 mL Erlenmeyer flasks at 30 °C, pH 5.5 and speed of 180 rpm. The C/N weight ratio was 40 and yeast extract was selected as nitrogen and vitamins source, according to the literature on *L. starkeyi* (Sutanto et al., 2018; Zhao et al., 2008). The selected C/N weight ratio was obtained by adding a proper amount of yeast extract as a function of the carbon content of each hydrolysate. Both xylose- and glucose-rich hydrolysates were supplemented with nutrients and all fermentation media were sterilised by microfiltration (0.22 μm). *L. starkeyi* cultivation was stopped when the complete depletion of sugars in the media was reached, in order to avoid the use of accumulated lipids as a carbon source by the yeast. Each test was replicated three times.

In order to monitor the yeast growth, sugars concentration and intracellular lipid content during batch fermentations, every 24 h two samples of 1 mL were withdrawn and centrifuged in order to perform DCW determination, HPLC analysis and lipids extraction and quantification.

2.5. Single cell oil extraction and FAMEs determination

During and at the end of fermentations, yeast cells were harvested by centrifugation (8,000 ×g for 10 min), washed twice with distilled water, lyophilised, and stored in a

desiccator until the SCO extraction was carried out (Di Fidio et al., 2020a). The lipid yield was calculated by means of the following equation:

$Y_L = (C_L/C_s) \cdot 100$

where Y_L is the lipid yield (wt%), C_L is the final lipids concentration (g/L) and C_s is the concentration (g/L) of total consumed sugars at the end of the fermentation.

The lipid content (wt%, C_L) was calculated by means of the following equation:

$C_L = (m_L/m_{cells}) \cdot 100$

where m_L is the amount of the lipids (g) and m_{cells} is the amount of lyophilised yeast biomass (g).

The chemical characterisation of SCO was performed by GC-FID analysis after the direct transmethylation of microbial triglycerides into fatty acids methyl esters (FAMEs) (Di Fidio et al., 2020a).

2.6. Statistical analysis

Statistical analysis of the results obtained from the batch-mode fermentation of X1, X2, G1 and G2 hydrolysates was performed by a two-way analysis of variance (ANOVA) according to a completely randomised design. The type of hydrolysate and the process time were set as factors, while sugars concentrations, DCW concentration, lipid content, lipids production and lipids production rate were considered as dependent variables. Moreover, a one-way ANOVA was performed in order to compare the final DCW concentration, lipid content, lipids production and the maximum lipids production rate among the four hydrolysates. The Tukey's HSD test was used in order to compare the means of considered treatments. Statistics was performed by R 4.0.2.

3. Results and discussion

3.1. Fermentation of xylose-rich hydrolysates

In the present study, two xylose-rich hydrolysates (X1 and X2), obtained from the hydrolysis of the unpretreated giant reed adopting two different catalytic systems, one homogeneous and the other heterogeneous, were tested as the carbon source for the production of new generation oil. Figure 1 shows the general flow chart of the adopted cascade processes, while the composition of the starting hydrolysates is reported in Table 1.

(Figure 1, near here)

(Table 1, near here)

X1 was produced from the hydrolysis reaction catalysed by the homogeneous catalyst $FeCl_3$, which presents important advantages compared with traditional strong homogeneous inorganic acids, such as less corrosion of reactor, low cost, simple recovery by precipitation, good efficacy at mild reaction conditions, energy-saving, and high selectivity (Loow et al., 2015). On the other hand, X2 was obtained from the hydrolysis reaction catalysed by the heterogeneous catalyst Amberlyst-70, a promising styrene-based sulfonic acid resin for biomass hydrolysis (Qi et al., 2019), in order to evaluate the potentiality of the products obtained by hydrolysis with different catalytic approaches. In fact, the adoption of this heterogeneous system allows easy and safe operations with minor corrosion problems, simple recovery of the catalyst with less waste disposal, catalyst recycle with the maintenance of the catalytic performance (Meena et al., 2015). Nonetheless, a previous optimisation study evidenced that the heterogeneous catalyst requires higher reaction temperature and longer times than

$FeCl_3$, thus causing the presence in the X2 hydrolysate of potential inhibitors, as furanic derivatives and acetic acid (Di Fidio et al., 2020c).

The results of the batch fermentation of the undetoxified lignocellulosic hydrolysate X1 by *L. starkeyi* are reported in Figure 2A.

(Figure 2, near here)

In this case, in the first 24 h, the growth phase was not observed, while from 24 to 72 h the yeast growth significantly increased. The net production of DCW was 11 g/L. It ranged from the starting value of 5 g/L to the final concentration of 16 g/L. The sugars content decreased from 24 to 72 h as the increasing DCW. In particular, the consumption of glucose and xylose was simultaneous, due to the relatively low initial concentration of glucose (5.6 g/L) in the fermentation medium. These results confirmed the ability of *L. starkeyi* to convert xylose into SCO. This important feature is crucial for the complete biological conversion of the second-generation sugars obtainable from giant reed into microbial oil, increasing the profitability of the proposed biorefinery scheme. At 72 h, the complete consumption of both glucose and xylose was observed, thus indicating the end of fermentation. The intracellular lipid content ranged from 10 to about 30 wt% during the fermentation. It was 10% at 0 h and around 30% at 72 h. Moreover, the maximum oil productivity, 2.8 g/L/d, was observed in the last stage between 48 and 72 h. In the X1 fermentation, 25 g/L of total reducing sugars were converted into 4.6 g/L of lipids, achieving the lipid yield of 18.6 wt%.

According to the stoichiometry of biochemical conversion of glucose and xylose into triacylglycerols in most oleaginous yeasts, the maximum theoretical yield is around 33 wt% (Papanikolaou & Aggelis, 2011). However, in the lipogenesis pathway of *L. starkeyi* cytosolic malic enzyme takes NAD^+ as coenzyme rather than $NADP^+$,

determining the lower availability of NADPH in the cytosol, which is one of the key factors for the synthesis of the lipids. As a consequence, in *L. starkeyi* the maximum theoretical yield is 27.6 wt% (Sutanto et al., 2018). On this basis, the obtained yield of 18.6 wt% represented 67.4% of the maximum theoretical yield for this species. Moreover, in the literature, the experimental lipid yields obtained both in synthetic and biomass-derived media ranged from 10 and 24 wt%, thus attesting the achieved value as around 80% of the maximum experimental yield obtained up to now from *L. starkeyi* (Sutanto et al., 2018).

On the other hand, after a preliminary test, *L. starkeyi* showed to be not suited for growing on the xylose-rich hydrolysate X2 as obtained from the selective microwave-assisted Amberlyst-70-catalysed hydrolysis, due to the presence of a relatively high concentration of furfural, HMF and acetic acid (Table 1). The presence of undesired by-products was due to the adoption of the high-gravity approach in the hemicellulose hydrolysis catalysed by Amberlyst-70 which allowed us to use the biomass loading of 200 g/L (17 wt%). This high-gravity approach increases the economic sustainability of the sugars production, despite a slight increase in by-products synthesis. For this reason, furfural and acetic acid were removed by vacuum evaporation and the obtained concentrated hydrolysate, containing 16.0 g/L glucose, 76.0g/L xylose, 2.6 g/L 5-HMF, 1.0 g/L levulinic acid, was tested. Also in this case the yeast was not able to grow, almost surely due to the increased concentration of 5-HMF and other unidentified by-products. After the dilution of the hydrolysate in order to restore the initial concentration of both reducing sugars and other by-products (8.0 g/L glucose, 38.0g/L xylose, 1.3 g/L 5-HMF, 0.5 g/L levulinic acid), the fermentation was successfully

carried out. Figure 2B shows the results of the batch-mode fermentation of the processed X2 furfural-free hydrolysate by *L. starkeyi*.

Similarly to the fermentation of X1, the yeast growth did not occur in the first 24 h. From 24 to 48 h a slight yeast growth was observed, while the major growth phase evolved from 48 to 72 h. The bioconversion of X2 required a longer time than X1 (96 vs 72 h), due to the higher concentration of total reducing sugars (46 vs 25 g/L). The net production of DCW was 19.1 g/L, which resulted higher respect to the value (11 g/L) of the previous case. It ranged from the starting value of 4.9 g/L to the final concentration of 24 g/L. The sugars consumption evolved according to the DCW increase, being slower between 24 and 48 h and 72 and 96 h and faster during the growth phase observed from 48 to 72 h. Compared to X1, the consumption of glucose and xylose was asynchronous. Indeed, glucose depletion started earlier, already during the lag phase of DCW growth, and its complete consumption required almost half the time of xylose. Despite the lower initial content, the consumption of glucose evolved faster, being the favoured carbon source for oleaginous yeasts (Zhao et al., 2008). Instead, the consumption of xylose started after 24 h. Therefore, the main phase of xylose decrease was also observed 24 h later and its complete consumption was delayed at 96 h. The final lipid content was 28 wt%, while the maximum oil productivity, calculated in the major growth phase (48–72 h), resulted 4.1 g/L/d. This value was higher than the maximum productivity reached in the previous case because of the higher cell biomass production (DCW). In X2 fermentation, 46 g/L of total reducing sugars were converted into 6.7 g/L of lipids, achieving the lipid yield of 14.6 wt%. The obtained yield represented 52.9% of the maximum theoretical yield and 60.8% of the maximum experimental yield for *L. starkeyi* (Sutanto et al., 2018). These values resulted lower

than those reached in X1 despite the consumption of a double amount of reducing sugars. This result can be related to the presence of a higher concentration of by-products, such as 5-HMF and organic acids, in X2 than in X1, generating stressful growing conditions for the yeast which hampered the lipogenesis. Moreover, the presence of Fe^{3+} in X1, due to the use of $FeCl_3$ as a homogeneous acid catalyst for the hydrolysis of the giant reed hemicellulose into xylose, is reported to favour the lipid production in oleaginous yeasts (Gong et al., 2014; Zhao et al., 2008).

The cell biomass productions (DCW) ascertained in the X1 and X2 fermentations agreed with the concentrations of 12.3 g/L reported by Tapia et al. (Tapia et al., 2012) and 13.6 g/L reported by Leiva et al. (Leiva-Candia et al., 2015) for the same yeast strain DSM 70296 cultivated in a flask on pure xylose and glucose, respectively. Moreover, the DCW concentration achieved in the present investigation on the xylose-rich giant reed hydrolysate was higher respect to the maximum value of 10 g/L reported by Pirozzi et al. (Pirozzi et al., 2015) on diluted giant reed hydrolysate obtained by H_2SO_4-catalysed hydrolysis.

In both X1 and X2 fermentations, the starting lipid content (10 wt%) agreed with those reported in the literature for the *L. starkeyi* (Wang et al., 2014), while the final values (around 30 wt%) resulted higher respect to the literature data reported in all the other fermentation experiments on giant reed hydrolysates, obtained by different pretreatments and catalytic approaches, by using the same yeast strain (Pirozzi et al., 2014a; Pirozzi et al., 2015; Pirozzi et al., 2014b).

Considering the fermentation of other kinds of lignocellulosic hydrolysates by the same yeast, Xavier et al. reported the lipid yield of 14.0 wt% employing sugarcane bagasse hydrolysate (Xavier et al., 2017). Azad et al. claimed the lipid yield of 18.0 wt% using

rice straw hydrolysate (Azad et al., 2014), whilst Calvey et al. obtained the lipid yield of 14.0 wt% adopting corn stover hydrolysate (Calvey et al., 2016). Moreover, considering the batch-mode fermentation of pure xylose or a combination of xylose and glucose, Tapia et al. reported the lipid yield of 14 wt% in the presence of 30 g/L xylose adopting the C/N weight ratio of 50 (Tapia et al., 2012), while Anschau et al. obtained the lipid yield of 10 wt% in the presence of 42 g/L xylose and 18 g/L glucose with the C/N weight ratio of 50 (Anschau et al., 2014).

All the obtained results confirmed the feasibility of the SCO production from the hemicellulose fraction of giant reed after its selective hydrolysis by means of both homogeneous and heterogeneous acid catalysts. Moreover, the ascertained SCO yields make this approach surely competitive respect to the up to now reported literature ones where synthetic model solutions or hydrolysates obtained from pretreated lignocellulosic biomasses are adopted as substrates for fermentation.

3.2. Fermentation of glucose-rich hydrolysates

In the perspective of the complete valorisation of the carbohydrates present in the starting biomass, the glucose-rich hydrolysates G1 and G2 (Table 1), obtained from the hydrolysis of the giant reed cellulose-rich residues CCR1 and CCR2 remaining after the two different catalytic hemicellulose dissolutions were employed as a carbon source for the production of SCO (Figure 1). For the production of G1 and G2 from the cellulose-rich solids, the enzymatic hydrolysis was preferred because ensured the selective depolymerisation of the cellulose into glucose avoiding the formation of toxic inhibitors for the *L. starkeyi* growth. Figure 2C shows the results of the batch-mode fermentation of G1 by *L. starkeyi*.

The absence of inhibitors in the hydrolysate allowed the prompt yeast growth in the first 24 h. From 24 to 48 h the major growth was observed, while from 48 to 72 h *L. starkeyi* reached the stationary phase. With respect to the previous fermentations, the absence of a long lag phase (at least 24 h) decreased the productive processing time from 72 and 96 h of X1 and X2, respectively, to 48 h observed in G1. In the latter, the DCW concentration moved from the starting value of 4.8 g/L to the final concentration of 17.1 g/L. The net production of DCW was 12.3 g/L, similar to the value (11 g/L) obtained in the X1 fermentation, due to the comparable total reducing sugars concentration. The glucose consumption was consistent with the cell biomass growth. It started during the first 24 h and ended after 72 h, even if just after 48 h the glucose concentration in the G1 was only 1.6 g/L. The lipid content varied from about 10 to 31 wt%. Moreover, the maximum oil productivity, calculated in the major growth phase (24–48 h), resulted 3.5 g/L/d which represented an intermediate result with respect to the values obtained by fermenting X1 and X2. In the G1 fermentation, 21.8 g/L of total reducing sugars were converted into 5.3 g/L of lipids, achieving the lipid yield of 24.3 wt%. The obtained yield represented 88.0% of the maximum theoretical yield and 100% of the maximum experimental yield for *L. starkeyi* on synthetic and biomass-derived media (Sutanto et al., 2018). These values resulted higher than those reached in X1 and X2 fermentations due to the presence of glucose as sole carbon source and the absence of the hydrolysis reaction by-products due to the adoption of the enzymatic catalytic approach. In fact, these process conditions favoured the lipogenesis, increasing the lipid yield in the *L. starkeyi* DSM 70296.

Figure 2D shows the results of the batch-mode fermentation of G2 by *L. starkeyi*. Similarly to G1 fermentation, the yeast growth started already during the first 24 h

without a significant lag phase due to the high quality of the hydrolysate related to the improved catalytic strategy based on the highly selective enzymatic hydrolysis. The major growth phase took place from 24 to 48 h while during the last 24 h of the process a slight yeast growth evolved towards the stationary phase. DCW concentration ranged from the starting value of 5.1 to 22.0 g/L at process end. The net increase of DCW was 16.9 g/L, which resulted the maximum value obtained in the present investigation. The glucose consumption was consistent with the DCW growth. It started within 24 h and ended after 72 h. The lipid content ranged from about 10 to 34 wt%. Moreover, the maximum oil productivity, observed during the major growth phase (24–48 h), 4.4 g/L/d, was higher than the value obtained from G1 and similar to that achieved from X2. In G2 fermentation, 32.8 g/L of total reducing sugars were converted into 7.5 g/L of lipids, achieving the lipid yield of 22.9 wt%. The obtained yield represented the 83.0% of the maximum theoretical yield and the 95.4% of the maximum experimental yield for *L. starkeyi* on synthetic and biomass-derived media (Sutanto et al., 2018), in agreement with the results achieved in the previous fermentation of the glucose-rich hydrolysate G1.

In G1 fermentation, the final cell biomass production was similar to the value of 12.3 g/L achieved on the sole pure glucose in flask by Leiva-Candia et al. (Leiva-Candia et al., 2015) for the same yeast strain with the C/N equal to 50. On the contrary, it resulted higher than the value of 9.4 g/L obtained on the sole pure glucose by Angerbauer et al. (Angerbauer et al., 2008) working with a C/N equal to 150, which favoured the lipogenesis and limited the yeast growth. In G2 fermentation, the final cell biomass production was similar to the value of 21.2 g/L obtained by Bonturi et al. on pure glucose (Bonturi et al., 2015). Moreover, in both G1 and G2 fermentation, the biomass

production was significantly higher than the maximum value of 10 g/L reached on the glucose-rich giant reed hydrolysate obtained by H_2SO_4-catalysed hydrolysis (Pirozzi et al., 2015). Considering the fermentation of other lignocellulosic hydrolysates, the DCW production was similar to those obtained on sugarcane bagasse (Anschau et al., 2014; Xavier & Franco, 2014), Brazilian molasses (Vieira et al., 2014) and sunflower meal (Leiva-Candia et al., 2015).

As previously observed for X1 and X2 fermentation, also in both G1 and G2 fermentations, the lipid content resulted higher respect to the range of 17-21 wt% achieved by fermenting giant reed hydrolysates obtained by different catalytic strategies (Pirozzi et al., 2014a; Pirozzi et al., 2015; Pirozzi et al., 2014b).

The two-way ANOVA showed that glucose, xylose, DCW, lipids concentration and content varied significantly according to the hydrolysate, the process time and their interaction as well.

Figure 3 shows the results of the statistical analysis (one-way ANOVA) of the findings obtained from the fermentation of X1, X2, G1 and G2. In particular, the final DCW and lipids concentration, lipid content and the maximum lipids production rate were compared as a function of the type of the hydrolysate.

(Figure 3, near here)

Regarding the DCW production, X1 and G1 were not significantly different from each other, as well as X2 and G2, but these last two values were significantly higher than those obtained for X1 and G1. Regarding the lipids concentration, the values obtained for X1 resulted significantly lower than those obtained for X2 and G2. The lipids concentration obtained from the fermentation of G1 was not significantly different with respect to the values reached for X1 and X2, while it was significantly lower than G2.

Considering the lipid content, not statistically differences were observed between X1 and X2, as well as between G1 and G2. The only statistically difference was observed between G2 and X1/X2. Considering the maximum lipids production rate, the values achieved for X2 and G2 were not statistically different from each other, but they were significantly higher than X1. The value reached in the case of G1 was not significantly different respect to all the other hydrolysates.

Both the homogeneous and the heterogeneous catalytic approaches for the first process step allowed the production of cellulose-rich residues suitable for the subsequent enzymatic hydrolysis, which provided good-quality glucose-rich hydrolysates. The good performances of *L. starkeyi* in their successive fermentation confirmed the profitability of the SCO production from the cellulose fraction of giant reed following the cascade biorefinery scheme presented in this work.

3.3. Fatty acids composition of single cell oils

The fatty acids composition of microbial oils is strongly affected by the main process parameters, such as the nature of the carbon and nitrogen source, the carbon to nitrogen weight ratio, the sugars concentration, the fermentation mode, the nature and the concentration of growth inhibitors and the yeast species and strain (Pirozzi et al., 2015; Sutanto et al., 2018; Takaku et al., 2020; Wild et al., 2010). In the present investigation, the lipid profiles of the SCO produced by *L. starkeyi* DSM 70296 were listed in Table 2.

(Table 2, near here)

They were characterised by a good percentage (~55 wt%) of unsaturated long-chain fatty acids according to the literature (Gong et al., 2012; Pirozzi et al., 2015; Xavier & Franco, 2014). In particular, the SCO profiles achieved by the fermentation of X1 and

X2 agreed with those obtained by other studies on the xylose-rich hydrolysates or synthetic media (Gong et al., 2012; Wang et al., 2014; Xavier et al., 2017). The oils obtained by the bioconversion of G1 and G2 resulted in agreement with those derived from the fermentation of glucose-rich hydrolysates or synthetic media (Gong et al., 2012; Pirozzi et al., 2014a; Wang et al., 2014). Comparing the lipid profiles obtained in each process configuration of this study, the chemical composition of the SCO produced by *L. starkeyi* on the various xylose- or glucose-rich hydrolysates is quite constant. This result can be explained by considering the use of the same yeast strain, C/N ratio, nitrogen source (yeast extract) and fermentation-mode, together with the low concentration of growth inhibitors such as 5-HMF and furfural, the limited range of sugars concentrations (22-46 g/L) and the similar carbon source (glucose and xylose). Moreover, in vegetable and microbial oils, the presence of unsaturated fatty acids favour the cold properties, such as the cloud point, the pour point and the cold filter plugging point. At the same time, these components reduce the oxidation stability (Pirozzi et al., 2015; Serrano et al., 2014). However, the presence of mostly monounsaturated fatty acids (C16:1, C18:1) and the low relative amount of polyunsaturated fatty acids (C18:2, C18:3) could balance both these antagonistic requirements (Knothe, 2008). In this view, the SCO produced by *L. starkeyi* in the present investigation, containing about 55% of unsaturated fatty acids, appears a valid candidate for the production of new generation biodiesel with good oxidative stability and cold flow properties. Moreover, in support of this perspective, the SCO resulted very similar to palm and rapeseed oils (Anschau et al., 2014; Sutanto et al., 2018), usually employed as a renewable source for the production of traditional biodiesel (Table 2).

3.4 Mass balance of the processes

Chemical and biological catalytic strategies implemented in this study allowed the evaluation of a novel integrated biorefinery scheme for the valorisation of the unpretreated lignocellulosic giant reed. The absence of the pretreatment step and the optimisation of a cascade biomass exploitation would increase the economic sustainability and profitability of this biorefinery model. Moreover, the optimisation of tailored catalytic approaches for the production of second-generation sugars from both hemicellulose and cellulose fractions and of new generation oil offered an important versatility in terms of technology and final high added-value biobased products.

For the first process step, namely the hemicellulose hydrolysis, the use of 27.6 g $FeCl_3 \cdot 6H_2O$, corresponding to 16.5 g $FeCl_3$, and 20 g Amberlyst-70 for 100 g of unpretreated giant reed (Figure 1), is justified by the absence of any chemical or hydrothermal pretreatment of the raw biomass. The adopted catalyst/biomass weight ratio of 0.17 and 0.20 wt/wt, for $FeCl_3$ and Amberlyst-70 respectively, are in agreement with those already reported in the literature for similar hydrolysis reactions. In particular, for the hemicellulose hydrolysis Kamireddy et al. (Kamireddy et al., 2013) adopted 20.3 g $FeCl_3$ for 105 g unpretreated corn stover, corresponding to the catalyst/biomass weight ratio of 0.19 wt/wt; Lòpez-Linares et al. (López-Linares et al., 2013) used 42.2 g $FeCl_3$ for 120 g unpretreated olive tree biomass, corresponding to the catalyst/biomass weight ratio of 0.35 wt/wt; Marcotullio et al. (Marcotullio et al., 2011) adopted 16.2 g $FeCl_3$ for 100 g unpretreated wheat straw, corresponding to the catalyst/biomass weight ratio of 0.16 wt/wt. Similar considerations can be performed for the hemicellulose hydrolysis catalysed by Amberlyst. You et al. (You et al., 2016) used 20 g Amberlyst35 DRY for 100 g of pretreated giant reed (110 °C, 1-n-butyl-3-

methylimidazolium chloride, for 3 h), corresponding to the catalyst/biomass weight ratio of 0.20 wt/wt. In both cases, for $FeCl_3$ and Amberlyst-70, the employed catalyst/biomass weight ratios are comparable or even lower than literature reported values. Moreover, both catalysts can be recycled. In fact, an important advantage of metal chlorides is represented by the possibility of being recovered as metal hydroxides by ultrafiltration. Metal hydroxides can be successively converted back to metal chlorides, when treated with conjugate acids (e.g. HCl), thus allowing the easy catalyst recycle and reuse in the process (Kamireddy et al., 2013). Regarding the heterogeneous catalyst adopted in the cascade process 2, previous research demonstrated that the embedded Amberlyst-70 can be separated by sieving and efficiently recycled by performing a simple washing with acetone, which represents a green and cheap solvent (Di Fidio et al., 2020c).

These aspects, together with the improvement of the SCO yield with respect to other processes involving the same yeast *L. starkeyi* DSM 70296, represent a promising improvement and innovation. Figure 1 shows the mass balance flow diagram of the two adopted cascade processes based on different catalytic strategies. Process 1, based on the combination of MW-assisted $FeCl_3$-catalysed hydrolysis of hemicellulose, enzymatic hydrolysis of the obtained cellulosic residue and yeast fermentation of X1 and G1, allowed the total production of 7.8 g of SCO from 100 g dry matter of raw biomass from giant reed cultivation, 4.7 g lipids deriving from the hemicellulose exploitation while 3.1 g from the cellulose valorisation. Process 2, based on the combination of MW-assisted Amberlyst-70-catalysed hydrolysis of hemicellulose, enzymatic hydrolysis of the obtained cellulosic residue and yeast fermentation of X2 and G2, allowed the total production of 7.7 g of SCO from the same amount and kind of

biomass: 3.4 g lipids deriving from the hemicellulose exploitation, while 4.3 g from the cellulose valorisation. Thus, only considering the final SCO yield with respect to the starting unpretreated lignocellulosic biomass, the two proposed multi-step approaches resulted equivalent. The first one favoured the hemicellulose exploitation and was less performing in the cellulose valorisation, while the second one favoured the cellulose exploitation and was less efficient in the hemicellulose valorisation. A key aspect of this multi-step approach consists in almost doubling the SCO production from the lignocellulosic biomass due to the production of sugars from both hemicellulose and cellulose and their biological conversion into lipids (Figure 1). This process strategy, up to now never investigated, ensured a higher SCO production respect to those schemes which performed a pretreatment step followed by the enzymatic hydrolysis of the biomass and the hydrolysate fermentation (Azad et al., 2014; Pirozzi et al., 2015). Regarding the SCO productivity, the values obtained in the present study, 2.6 g/day for both processes, were similar to those reported in the literature for the same yeast species. In particular, the work of Sutanto et al. (Sutanto et al., 2018) on *L. starkeyi* reported the productivity values in the range 0.04-4.00 g/day. Moreover, the same review reported for the yeast strain DSM 70296 values in the range 0.65-1.44 g/day. Figure 4 shows the kinetics of the lipids production rate as a function of type of hydrolysate (X1, X2, G1 and G2) and the statistical analysis of the results through the two-way ANOVA.

(Figure 4, near here)

No statistically significant difference was observed after 24 h among the four hydrolysates from fermentation start. On the contrary, the lipids production rate observed at 48 h for G1 (around 3.5 g/L/day) and G2 (around 4.5 g/L/day) was

significantly higher than those reached for X1 and X2 (around 1.0 g/L/day). At the same time, the values achieved from G1 and G2 were not statistically different from each other, as well as those obtained from X1 and X2. At 72 h, the lipids production rate for X2 was significantly higher than the values obtained for the other hydrolysates. Moreover, the production rates of X1 (around 2.5 g/L/day) and G2 (around 2.0 g/L/day) were not statistically different from each other. The production rate from G1 (around 0.5 g/L/day) was the lowest one after 3 days. Considering the kinetics within the same hydrolysate, for the two xylose-rich hydrolysates the maximum lipids production rate was reached at 72 h with an increase that resulted statistically different than the values achieved at 24 and 48 h. Differently, for the two glucose-rich hydrolysates, the maximum lipids production rate was reached earlier, 48 h, having values significantly different from 24 and 72 h. Finally, in the case of X2, the difference in the production rate between 72 and 96 h was not statistically different.

On this basis, this integrated cascade process can offer an outstanding opportunity for the exploitation of all the biomasses characterised by a relevant amount of hemicellulose, of which giant reed is a representative example, thus allowing case-specific selection of suitable feedstocks among a variety of similar lignocellulosic crops and residues for this type of biorefinery processes. Considering that giant reed under suited pedoclimatic conditions is able to produce 37.7 tons dry matter ha^{-1} yr^{-1}, as 12-year average under low input and rainfed conditions (Nassi o Di Nasso et al., 2011), the potential SCO production could equal 2.9 tons ha^{-1} yr^{-1}. Under similar conditions, an annual oleaginous crop, such as sunflower, can deliver no more than 2 tons ha^{-1} yr^{-1} of high oleic oil. These results indicate that such process, based on unconventional renewable biomass resources, mild reaction conditions, water as the solvent, safe

catalysts, microwaves as an efficient energy system, has the potential to achieve yields similar to conventional oil crops respecting the principles of Green Chemistry.
The integral use of biomass is essential to ensure sustainability in such supply chains. At this regard, both the adopted approaches can lead to the production of lignin-rich solid residues after the cellulose hydrolysis, which can further be exploited to give valuable aromatic compounds. Analogously, both approaches may lead to the production of a huge amount of spent cell biomass after the oil extraction, similar to the yeast extract produced from spent brewer's yeast (Tanguler & Erten, 2008) and spent baker's yeast (Vukašinović-Milić et al., 2007). This spent biomass could suit for nitrogen recovery in the fermentation process or for biomethane production by anaerobic digestion (Moeller et al., 2018; Sosa-Hernández et al., 2016). Moreover, process 2 could also provide furfural, which represents one of the most promising platform chemicals directly derived from biomass (Wang et al., 2019).

4. Conclusions

This study reported for the first time two alternative multi-step conversions of unpretreated giant reed to SCO. The conversion of hemicellulose and cellulose fractions into xylose- and glucose-rich hydrolysates with the low production of by-products enabled the fermentation of produced undetoxified hydrolysates by *L. starkeyi*. Sugars exhaustion was reached for all the hydrolysates, providing good lipid yields, 15-24 wt%, and oil content, about 30 wt%. The two cascade processes enabled us to achieve about 8 g SCO from 100 g raw biomass. This SCO represents an outstanding alternative to fossil and food oils for the production of biofuels and bioproducts.

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Appendix A. Supplementary data

E-supplementary data of this work can be found in the online version of the paper.

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Captions for Figures

Fig. 1. Flow diagram and mass balance of the different chemical and biological catalytic steps adopted for the production of single cell oil from giant reed.

Fig. 2. Glucose, xylose, dry cell weight (DCW) and lipids concentrations (g/L), and lipid content (wt%) during the fermentation of X1 (A), X2 (B), G1 (C) and G2 (D) by *L. starkeyi* DSM 70296.

Fig. 3. Dry cell weight concentration (g/L), lipids concentration (g/L), lipid contents (wt%) and maximum lipids production (g/L/day) at the end of the fermentation of X1, X2, G1 and G2 hydrolysates. Different letters on the bars indicate significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Fig. 4. Kinetics of the maximum lipids production (g/L/day) as a function of the type of the fermented hydrolysate (X1, X2, G1 and G2). Different letters on the values indicate significant differences (* $p < 0.05$) among process times in the same kinetics (lowercase letters) or among the hydrolysates at the same process time (uppercase letters).

Table 1

Chemical composition of giant reed hydrolysates obtained by different catalytic approaches and used as fermentation substrates.

Giant reed hydrolysate	Glucose (g/L)	Xylose (g/L)	Furfural (g/L)	5-HMF (g/L)	Acetic acid (g/L)	Formic acid (g/L)	Levulinic acid (g/L)
X1[a]	5.6±0.2	19.4±0.3	0.3±0.0	0.4±0.0	2.2±0.2	0.5±0.0	n.d.
X2[b]	8.0±0.3	38.0±0.5	1.0±0.1	1.3±0.1	4.7±0.4	1.0±0.1	0.5±0.0
X2[b*]	8.0±0.2	38.0±0.4	n.d.	1.3±0.2	n.d.	1.0±0.2	0.5±0.0
G1c	21.8±0.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
G2[d]	32.8±0.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a] Hydrolysate obtained by microwave-assisted $FeCl_3$-catalysed hemicellulose hydrolysis; [b] hydrolysate obtained by microwave-assisted Amberlyst-70-catalysed hemicellulose hydrolysis; [c] hydrolysate obtained by enzymatic hydrolysis of the cellulose-rich residue after the microwave-assisted $FeCl_3$-catalysed hemicellulose hydrolysis; [d] hydrolysate obtained by enzymatic hydrolysis of the cellulose-rich residue after the microwave-assisted Amberlyst-70-catalysed hemicellulose hydrolysis; n.d. = not detected.

*X2 after the selective removal of furfural and acetic acid by vacuum evaporation.

Table 2

Chemical composition (wt%) of single cell oils obtained by fermentation of different xylose- and glucose-rich hydrolysates and their comparison with traditional food oils used for the biodiesel production.

Oil	C16:0	C16:1	C18:0	C18:1	C18:2
SCO from X1	37.6	3.5	7.2	47.5	4.2
SCO from X2	34.6	3.1	7.6	49.5	5.2
SCO from G1	35.8	2.5	8.1	50.5	3.1
SCO from G2	31.8	8.0	5.1	48.3	6.8
Palm oil[a]	36.7	0.1	6.6	46.1	8.6
Rapeseed oil[a]	40.1	0.1	4.1	43.0	11.0

[a] FAMEs profiles referred to the work of Sutanto et al. (Sutanto et al., 2018).

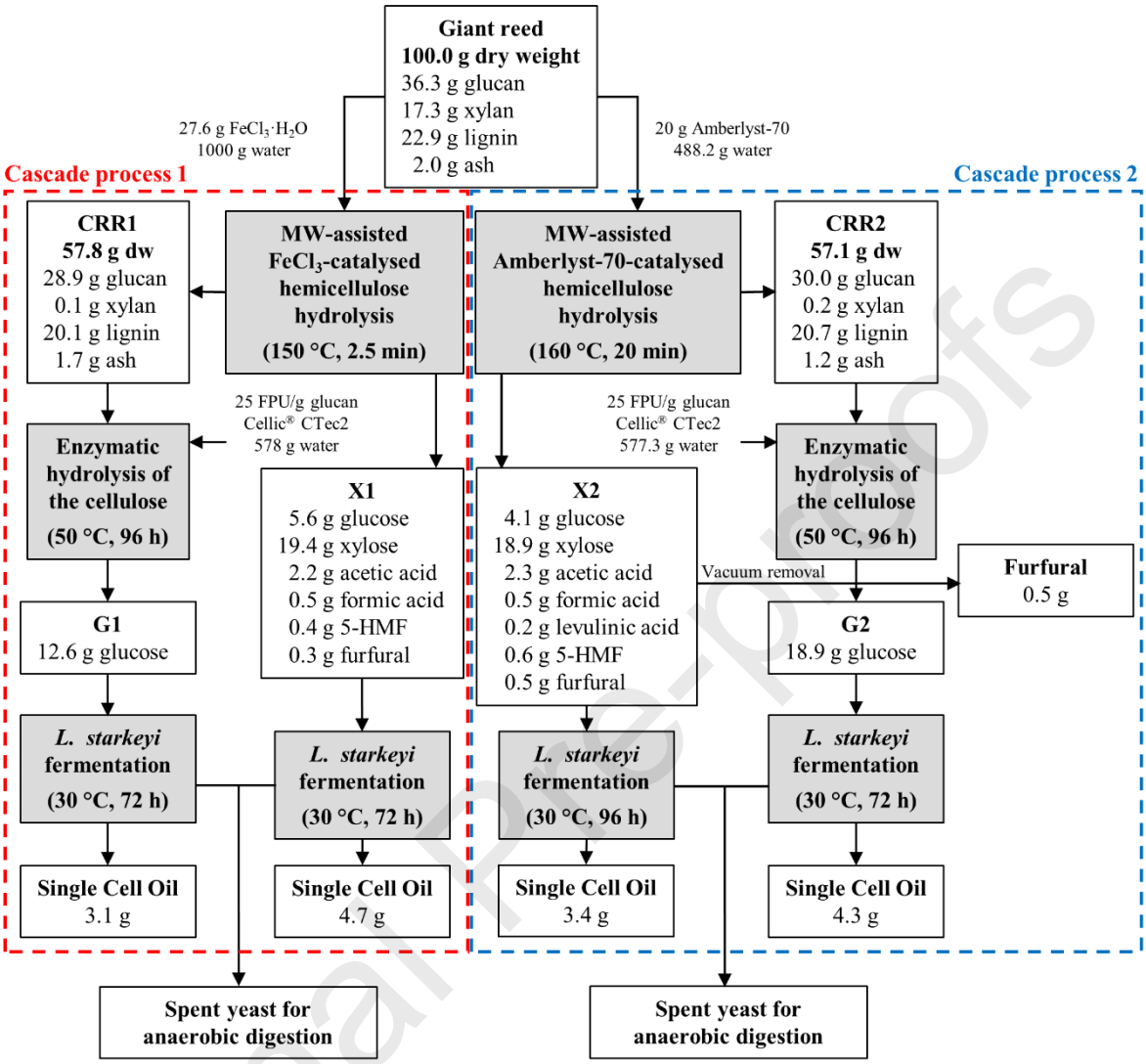


Figure 1

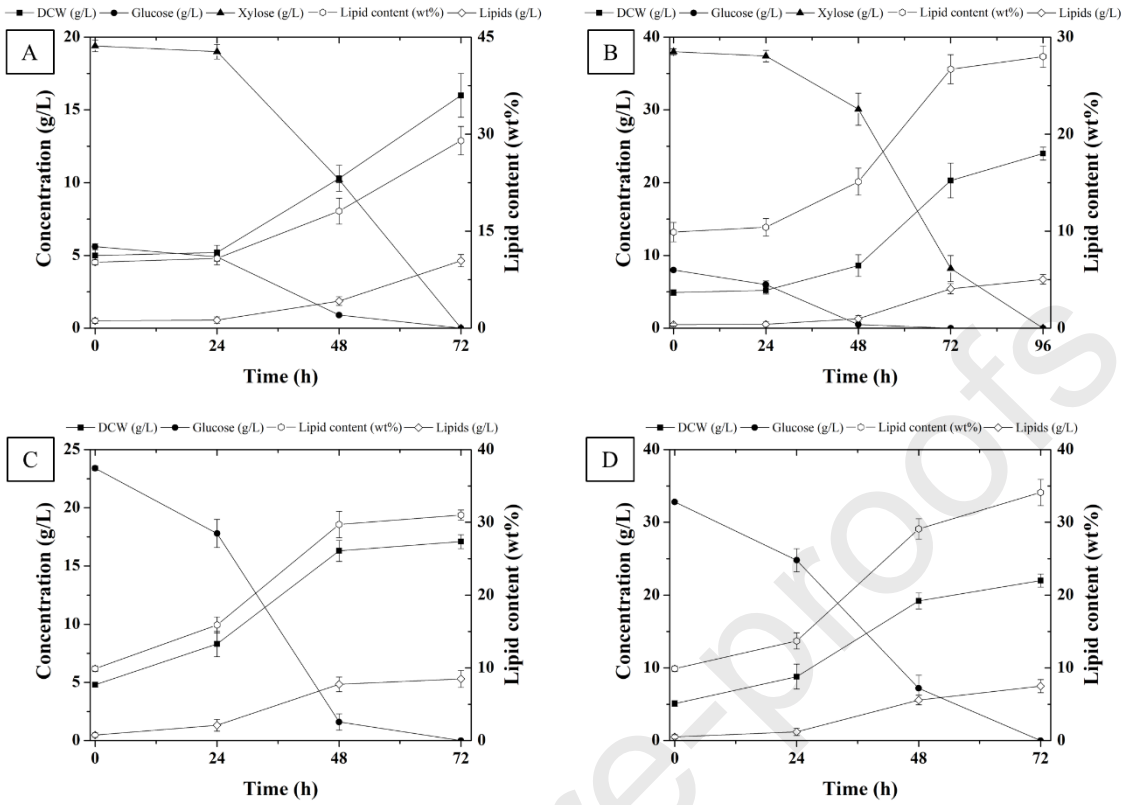


Figure 2

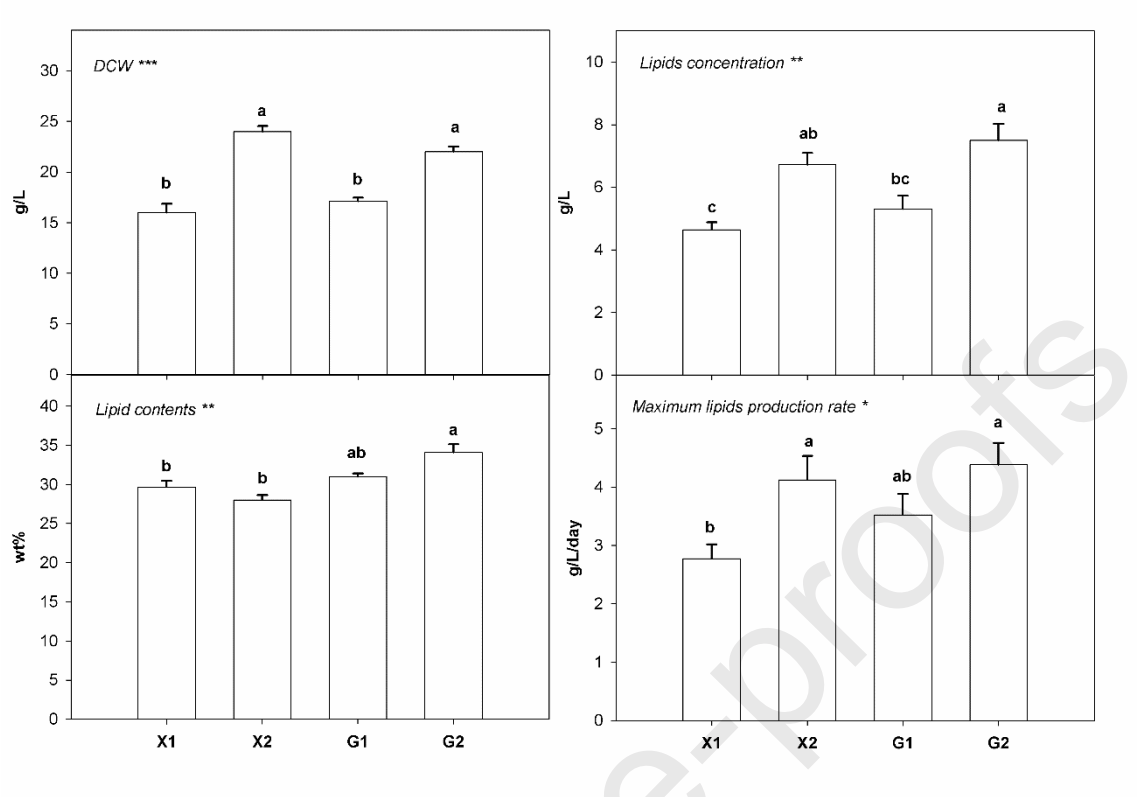


Figure 3

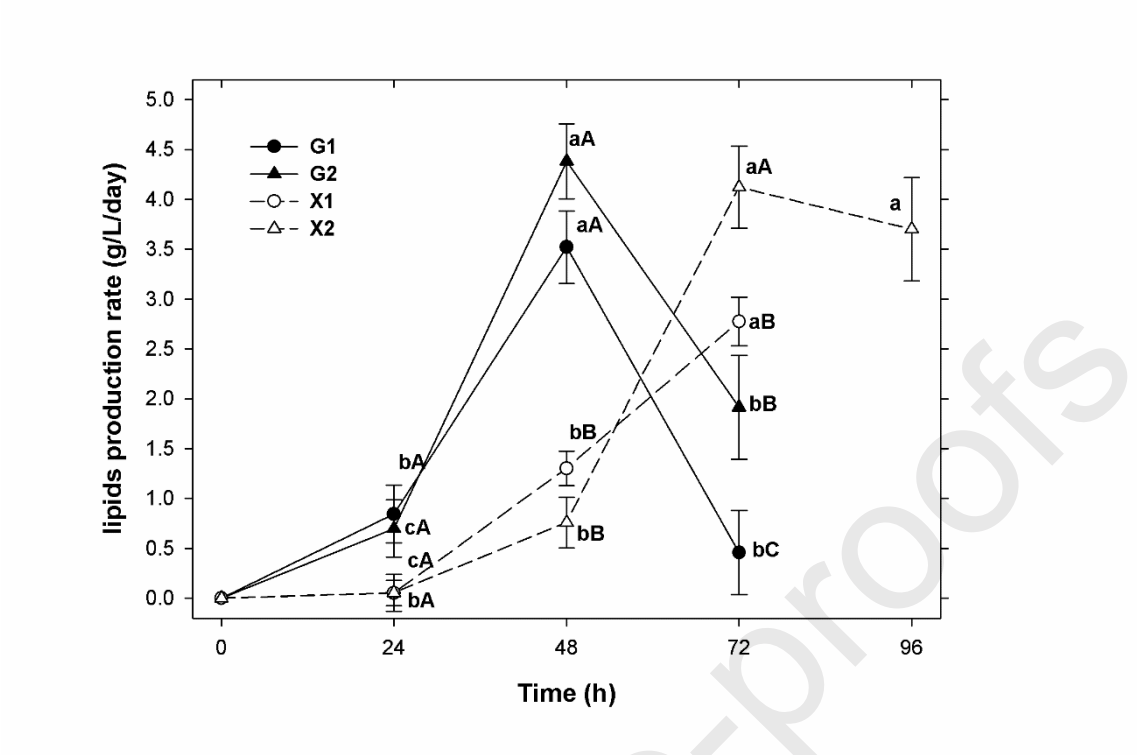


Figure 4

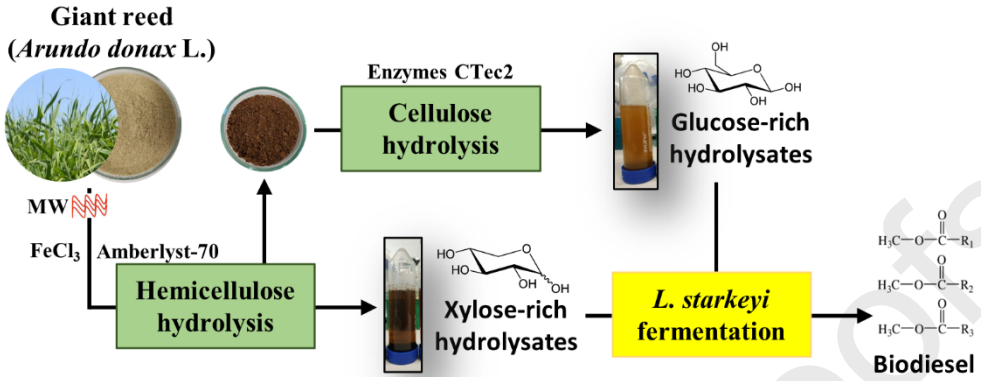
Declaration of interests

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

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

CRediT author statement

Nicola Di Fidio: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft.
Giorgio Ragaglini: Writing - review & editing, Supervision, Resources.
Federico Dragoni: Methodology, Formal analysis, Writing - original draft.
Claudia Antonetti: Writing - review & editing, Formal analysis.
Anna Maria Raspolli Galletti: Writing - review & editing, Supervision, Funding acquisition.



Highlights

- The multi-step exploitation of unpretreated giant reed was assessed.
- Two undetoxified xylose-rich hydrolysates were fermented into microbial oil.
- Two undetoxified glucose-rich hydrolysates were converted into single cell oil.
- The lipid yields of *L. starkeyi* DSM 70296 were in the range 15-24 wt%.
    - The single cell oil composition was similar to those of typical vegetable oils.