From paper mill waste to single cell oil: enzymatic hydrolysis to sugars and their fermentation into microbial oil by the yeast *Lipomyces starkeyi*

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ABSTRACT

Single cell oil (SCO) represents an outstanding alternative to both fossil sources and vegetable oils from food crops waste. In this work, an innovative two-step process for the conversion of cellulosic paper mill waste into SCO was proposed and optimised. Hydrolysates containing glucose and xylose were produced by enzymatic hydrolysis of the untreated waste. Under the optimised reaction conditions (Cellic® CTec2 25 FPU/g glucan, 48 h, biomass loading 20 g/L), glucose and xylose yields of 95 mol% were reached. The undetoxified hydrolysate was adopted as substrate for a batch-mode fermentation by the oleaginous yeast *Lipomyces starkeyi*. Lipids yield of 20.2 wt%, lipid content for single cell of 37 wt%, lipids production of 3.7 g/L, and maximum oil productivity of 2.0 g/L/d were achieved. This new generation oil, obtained from a negative value industrial waste, represents a promising platform chemical for the production of biodiesel, biosurfactants, animal feed and biobased plastics.

Keywords: Paper mill waste; Enzymatic hydrolysis; *Lipomyces starkeyi*; Single cell oil; Biodiesel.

1. Introduction

The main global energy concerns are related to the depletion of fossil and natural resources, the exponential growth of the world's population and inhomogeneous supplies of food (Ulucak & Khan, 2020). World petroleum reserves are limited and their exploitation rate will be increased if sustainable and renewable energy sources cannot be implemented. Global oil consumption has risen from 89.8 to 98.5 million barrels per day since 2011 to the end of 2017 (Sutanto et al., 2018) and it is estimated to reach 112

49 million barrels per day by 2035 (Sutanto et al., 2018). Among biofuels produced
50 starting from biomass, biodiesel is the most sustainable and renewable substitute for
51 fossil diesel fuel (Mahlia et al., 2020). In fact, biodiesel can replace diesel fuel without
52 any engines modifications (d'Espaux et al., 2015). World biodiesel is currently
53 produced from edible vegetable oils, which belong to the so-called first-generation
54 feedstocks. The use of food oil for energy purpose involves the ethical "food versus
55 fuel" debate which can cause the increase of food price, the decrease of land availability
56 for edible crops, and deficit in food oils (Sutanto et al., 2018). For these reasons,
57 second-generation feedstocks, such as waste cooking oils, animal fats, and inedible oils,
58 represent promising renewable resources to replace edible vegetable oils (Patel et al.,
59 2020; Patel et al., 2019). Finally, the biodiesel production based on the so-called third-
60 generation feedstocks, such as oleaginous yeasts, microalgae and bacteria, is currently
61 under development (Vasconcelos et al., 2019). An "oleaginous" microorganism can
62 accumulate oil over 20 wt% of its dry cell weight and in some species the intracellular
63 lipid content can reach up to 60-70 wt%, yielding what is called "single cell oil" (SCO).
64 Oleaginous yeasts are the most promising microorganisms for the production of oils
65 similar to vegetable ones, due to their rapid growth, the requirement of smaller areas for
66 their cultivation, and their lower sensitivity to climatic conditions than other production
67 systems (Vasconcelos et al., 2019). More than other yeast species, oleaginous yeasts
68 show a high metabolic flexibility as the majority of them are indeed capable of
69 converting both pentoses, such as xylose and arabinose, and hexoses, such as glucose,
70 sugars into lipids (Sitepu et al., 2014). Moreover, SCO from oleaginous yeasts may
71 serve as a source of platform chemicals for several products, such as surfactants,
72 lubricants, food additives, plastics, paints, and detergents (Probst et al., 2016). In

particular, *Lipomyces starkeyi* can provide high lipid yields from both hexoses and pentoses, re-utilising small amounts of its intracellular lipids, and can grow in simple media (e.g. without vitamin supplementation), being able to perform extracellular polysaccharide degradation. Moreover, it shows good tolerance to inhibitory compounds, such as organic acids, aldehydes and alcohols (Sitepu et al., 2014; Sutanto et al., 2018; Wang et al., 2014).

Over 70% of the total cost of biodiesel production is related to the raw material (Go et al., 2016). Thus, the main strategy to reduce the process cost is the use of waste materials and biomass residues which are abundantly available and characterised by a low or even negative value. In this regard, the utilisation of agro-industrial lignocellulosic wastes significantly reduces the cost of feedstock for SCO production (Brummer et al., 2014). In this perspective, industrial wastepaper is a very attractive raw material for biofuels production as it is more readily available compared with other substrates. Indeed, despite the encouraging legal framework and the promotion of the economic model of recycling, a huge amount of wastepaper still ends up in landfills (Guerfali et al., 2015).

In the present investigation, the paper mill waste derived from the converting process for the production of tissue paper products. This industrial process uses pure cellulose as starting feedstock for the production of toilet paper and handkerchiefs. In particular, the waste cellulosic powder is produced in the converting section, where the paper coil is unrolled and the sheet is subjected to mechanical operations (stripping, embossing, cutting, etc.) to give the final commercial product (Licursi et al., 2018). This cellulosic waste is not suitable to be recycled within the same papermaking process. For this reason, it is typically recovered by aspiration and sent to the landfill. It is

remarkable that this kind of waste tissue paper is mainly composed of short-fibers of pure cellulose (Licursi et al., 2018) and, since it has been already mechanically treated, it resulted more easily hydrolysable to monosaccharides, thus resulting an ideal feedstock for second-generation sugars production. Previous studies have demonstrated the potential for obtaining reducing sugars solutions from wastepaper using a variety of process designs, achieving in the best case the glucose yield of around 65 mol% (Brummer et al., 2014; Guerfali et al., 2015). The hydrolysis of lignocellulosic feedstocks to fermentable monosaccharides typically requires dealing with rigid lignocellulosic structures making necessary various pretreatments, different in their mode of action and severity. On this basis, the direct employment of enzymes appears an advantageous strategy for the hydrolysis of wastepaper feedstock, offering some advantages than chemical conversion approaches, such as high sugars yield, high selectivity, high energy saving, mild reaction conditions and low environmental impact processing (Guerfali et al., 2015).

Up to now, wastepaper hydrolysis has been extensively investigated for bioethanol production, while it has been rarely studied for SCO production (Zhou et al., 2017) and no investigation applying *L. starkeyi* is reported, so far, in the literature. The present work aimed to assess the conversion of the tissue paper mill waste into SCO in the perspective of an innovative and sustainable biorefinery model. In particular, the enzymatic hydrolysis of this unpretreated lignocellulosic material was optimised in the presence of the commercial enzymatic mixture Cellic® CTec2. Then, glucose and xylose in the obtained undetoxified hydrolysate were converted into microbial oil by the oleaginous strain *L. starkeyi* DSM 70296.

2. Materials and methods

2.1. Feedstock and materials

Paper mill wastepaper was collected from different local paper companies (Sofidel®, ICT® and Eurovast®, all in Lucca, Italy) and provided by the Center of Paper Quality Lucense® (Lucca, Italy). The starting solid raw material was employed as received without any pretreatment. It was just oven-dried at 105 °C up to reach a constant weight, cooled and stored in a desiccator.

All chemicals of analytical reagent grade were provided by Sigma-Aldrich (USA). The enzymatic preparation Cellic® CTec2 was kindly supplied by Novozymes (Denmark) and adopted as received.

2.2. Characterisation analyses of wastepaper feedstock

Chemical composition of wastepaper feedstock was evaluated through the standard NREL protocols (Sluiter et al., 2008a; Sluiter et al., 2008b; Sluiter et al., 2008c; Sluiter et al., 2008d). The Bruker D2 Phaser diffractometer (30 kV, 10 mA) operating in Bragg-Brentano geometry (θ – θ scan mode) was employed for the XRD analysis of the cellulosic powder. A 1-dimensional Lynxeye detector and a Ni-filtered Cu Kα radiation were employed. The experimental analysis and the evaluation of the crystallinity index (CrI) were performed according to the procedure described in our previous study (Di Fidio et al., 2020).

The Perkin-Elmer Spectrum Two spectrophotometer was employed for the FT-IR analysis of wastepaper in the Attenuated Total Reflectance (ATR) mode. The adopted operating conditions were equal to those described in our previous work (Di Fidio et al., 2019a).

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146 *2.3. Enzymatic hydrolysis of waste tissue paper*

147 Cellic® CTec2 is a commercial cellulase mixture, obtained from the fungus
148 *Trichoderma reesei*, that have proven effective on a wide variety of lignocellulosic
149 materials for the conversion of the carbohydrates into monosaccharides. It consisted of a
150 mixture of endo- and exocellulase, β-glucosidases and hemicellulase. For the enzymatic
151 activity evaluation, the NREL protocol (Adney & Baker, 1996) was employed. The
152 adopted Cellic® CTec2 was characterised by 134.5 FPU/mL.

153 The enzymatic hydrolysis of wastepaper was performed in a 150 mL flask with the
154 biomass loading of 2 wt%. The values of pH, temperature and agitation speed were set
155 at 4.8, 50 °C and 160 rpm, respectively. The 0.05 M citrate buffer solution was used as
156 a solvent. Samples of 2 mL were withdrawn every day, cooled in ice, centrifuged at
157 8,000 ×g for 10 min, and analysed by HPLC for glucose and xylose quantification. Both
158 enzymatic hydrolysis and HPLC analysis were carried out in triplicate and the
159 associated error resulted within 5%.

160

161 *2.4. Oleaginous yeast strain and cultivation*

162 The yeast strain *Lipomyces starkeyi* DSM 70296, provided by DSMZ (Germany),
163 was stored at 4 °C and propagated every 4 weeks on solid medium (glucose 20 g/L,
164 peptone 10 g/L, yeast extract 10 g/L, agar 20 g/L, pH 6.0). The cultivation protocol was
165 already described in our previous work (Di Fidio et al., 2019b). In order to obtain the
166 starting dry cell weight (DCW) of 5 g/L, a proper volume of preculture was inoculated
167 in the fermentation medium.

168

169 *2.5. Batch-mode fermentations*

170 Batch cultures (50 mL) were performed in 250 mL Erlenmeyer flasks at 30 °C.
171 Fermentations were extended until the complete depletion of sugars in the medium.
172 Each test was replicated three times. The initial pH was adjusted to 5.5 with 1 M NaOH
173 solution. The agitation was guaranteed by a rotary shaker set at 180 rpm. Wastepaper
174 hydrolysate and synthetic model medium were supplemented with nutrients selected
175 according to the study of Zhao et al. (Zhao et al., 2008) and then sterilised by
176 microfiltration (0.22 μm). The chemical composition of fermentation media was the
177 following one: glucose 17.0 g/L, xylose 3.0 g/L, $MgSO_4 \cdot 7H_2O$ 1.5 g/L, phosphate
178 buffer (KH_2PO_4 7 g/L, $Na_2HPO_4 \cdot 2H_2O$ 5 g/L), $FeSO_4 \cdot 7H_2O$ 0.08 g/L, yeast extract 1.5
179 g/L. The concentration of micronutrients was selected conforming to the work of
180 Juanssilfero et al. (Juanssilfero et al., 2018). The C/N weight ratio was set at 40 based
181 on the range 30-50 reported in previous studies on the *Lipomyces starkeyi* (Sutanto et
182 al., 2018; Zhao et al., 2008). The yeast extract was selected as the best nitrogen source
183 according to previous studies (Di Fidio et al., 2019b; Zhao et al., 2008) and its
184 concentration was set in line with the carbon content in order to obtain the desired C/N
185 ratio.

186 During batch cultivation, yeast growth, sugars concentration and intracellular lipid
187 content were monitored by DCW quantification, HPLC analysis and lipids extraction,
188 respectively. Two samples of 1 mL were withdrawn every 24 h and centrifuged for
189 separating cells from the culture medium. Cells were washed three times with deionized
190 water and oven-dried at 70 °C in order to obtain the DCW concentration. The liquid
191 fraction was used for quantifying the sugars concentration by HPLC.

192

193 *2.6. Triglycerides extraction*

194 During and after fermentation processes, yeast cells were harvested by
195 centrifugation, washed twice with distilled water, lyophilised and stored in a desiccator
196 until the triglycerides extraction. Lipids extraction was performed by modification of a
197 reported extraction method (Tasselli et al., 2018). Lyophilised cells (200-400 mg) were
198 suspended in 10 mL of 4 M HCl solution for 1 h at 60 °C in the microwave reactor
199 CEM Discover S-class System in order to promote the cell membrane lysis. Then, 15
200 mL of a chloroform/methanol 2:1 v/v mixture was added to the acid-hydrolysed cells
201 and the obtained suspension was incubated at room temperature for 1 h under magnetic
202 stirring. After incubation, the separation of different phases was obtained by
203 centrifuging the sample at 5,000 ×g for 10 min. The organic one containing the
204 triacylglycerols was transferred in a glass vial and the drying of lipids was performed by
205 fluxing gas nitrogen in the absence of light. Subsequently, the sealed vial was weighed
206 to gravimetrically quantify the total amount of triacylglycerols and stored at -20 °C until
207 GC analysis.

208 The amount of yeast biomass produced at the end of batch cultures, the amount of
209 extracted lipids, the glucose and xylose consumption and the incubation time required
210 for obtaining the complete depletion of sugars were used to calculate the intracellular
211 lipid content (wt%), the lipid production (g/L), the lipids yield (wt%) and the process
212 productivity (g/L/d) according to the following equations:

213 $$\text{Lipid content (wt\%)} = (m_L / m_{cells}) \times 100 \quad (1)$$

214 $$\text{Lipid production (g/L)} = (m_L / m_{cells}) \times c_{cells} \quad (2)$$

215 $$\text{Lipid yield (wt\%)} = (c_L / c_s) \times 100 \quad (3)$$

216 $$\text{Productivity (g/L/d)} = (c_L / t) \quad (4)$$

where m_L is the amount of the lipids in g, m_{cells} is the lyophilised yeast biomass in g, c_{cells} is the yeast biomass concentration (g/L) obtained at the end of the batch culture, c_L is the production of lipids in g/L, c_s is the concentration (g/L) of consumed sugars (glucose and xylose), and t is the fermentation time in days.

2.7. Analytical methods

Sugars (glucose, xylose), organic acids (acetic, formic and levulinic acids) and furan-derivatives (5-hydroxymethylfurfural, furfural) were qualitatively and quantitatively analysed by High Performance Liquid Chromatography (HPLC) PerkinElmer Flexar Isocratic Platform equipped with a Benson 2000-0 BP-OA column (7.8 mm × 300 mm × 10 µm) and a differential refractive index detector. The adopted operating conditions were already described in our previous study (Di Fidio et al., 2019a). Both standards concentration and samples were analysed three times and the error resulted within 3%.

The amount m_i of the different compounds was obtained as follows:

$$m_i = c_i \times V \quad (5)$$

where c_i is the concentration in g/L and V is the volume in L.

The glucose and xylose yield respect to the glucan and xylan moles of the biomass (m_b), respectively, was calculated according to the following equations:

$$\text{Glucose yield (mol\%)} = \left\lfloor (m_g \times 0.90)/(m_b \times G_f/100) \right\rfloor \times 100 \quad (6)$$

$$\text{Xylose yield (mol\%)} = \left\lfloor (m_x \times 0.88)/(m_b \times X_f/100) \right\rfloor \times 100 \quad (7)$$

where m_g is the glucose amount in g, 0.90 is the ratio between the molecular weight values of the glucan monomer and the glucose, G_f is the percentage of glucan in the biomass (wt%), m_x is the xylose mass in g, 0.88 is the ratio between the molecular

240 weight values of the xylan monomer and the xylose, X_f is the percentage of xylan in the
241 biomass (wt%).
242 The microbial triglycerides were directly transmethylated as already reported in our
243 previous work (Di Fidio et al., 2019b). Briefly, the dry lipids were added with 2 mL of
244 12% v/v BCl_3/MeOH and 1 mL of 2,2-dimethoxypropane, and placed in a water bath at
245 60 °C for 30 min. In order to stop the reaction, distilled water (1 mL) was added. The
246 extraction of the fatty acid methyl esters (FAMEs) was carried out by adding 2 mL of
247 hexane. The FAMEs profile was determined by GC analysis as described in our
248 previous study (Di Fido et al., 2019b).
249
250 **3. Results and discussion**
251 *3.1. Chemical characterisation of the waste tissue paper*
252 The industrial wastepaper adopted as starting raw material was characterised by the
253 following chemical composition (wt% on the dry matter): glucan 75.0 ± 0.5, xylan 12.1
254 ± 0.1, ash 6.8 ± 0.2, extractives 2.2 ± 0.5, Klason lignin (acid-insoluble residue) 3.9 ±
255 0.3. All the values represent the mean of three replicates together with the standard
256 deviation. As expected, glucan is by far the main component, while xylan represents a
257 lower percentage.
258 The XRD analysis was performed on the cellulosic biomass. The calculated
259 Crystallinity Index (CrI) resulted 56.9%. This value is similar to the CrIs values (66 and
260 50%) reported in the literature for office wastepaper (Danial et al., 2015; Mohkami &
261 Talaeipour, 2011). The starting raw material was also characterized by ATR-FTIR
262 spectroscopy. The absorption band at 2925 cm^{-1} corresponds to C-H stretching in
263 methyl and methylene groups, whereas the band at 2858 cm^{-1} is assigned to the C-H

stretching of the O-CH_3 groups (Chen et al., 2019). The peak at 1660 cm^{-1} is related to conjugated C=O and aromatic rings in lignin (Chen et al., 2019), while the band at 1455 cm^{-1} was due to the C=C stretching of benzene rings in lignin (Licursi et al., 2015). The peaks at 1377 and 1322 cm^{-1} were assigned to O-CH_3 and C-H symmetric deformation, and aryl ring breathing with C-O stretching in lignin, respectively (Licursi et al., 2015). The peak at 1164 cm^{-1} corresponds to aromatic C-H in plane deformation (Licursi et al., 2015), whereas that at 1109 cm^{-1} to the C-H deformation in the aromatic skeleton of lignin (Licursi et al., 2015). Furthermore, the absorption peaks at 1056 and 1033 cm^{-1} were assigned to C–O–C stretching of the pyranose ring in cellulose and the C–O stretching of hydroxyl and ether groups of cellulose, respectively (Di Fidio et al., 2020; Mattonai et al., 2018). Finally, the peak at 902 cm^{-1} was attributed to C-O-C, C-C-O, C-C-H stretching (Mattonai et al., 2018).

3.2. Enzymatic hydrolysis of paper mill waste

The wastepaper used as feedstock in the present study derived from the paper mill converting process for the production of tissue products (Licursi et al., 2018). It represents a promising raw material because it is characterised by a negative value. Furthermore, waste tissue paper represents an ideal substrate for the production of high-quality sugars due to its high carbohydrates content and low lignin content.

The composition of lignocellulosic hydrolysates is very important as it affects the performance of the fermentation processes. Enzymatic hydrolysis is a mild and highly selective process: as a consequence, the concentration of by-products, such as furanic compounds and short-chain organic acids, is typically low. Furanic compounds, such as 5-hydroxymethylfurfural (5-HMF) and furfural, are strong inhibitors of most

288 microorganisms growth (Di Fidio et al., 2019b; Van Dyk & Pletschke, 2012). The
289 efficiency of the enzymatic process is strongly related to several parameters, such as the
290 enzyme activity, the chemical composition and the structure of the substrate, the
291 crystallinity of the cellulose fraction and the porosity/size of fibers (Zhang et al., 2014).
292 In particular, the presence of lignin significantly affects the reaction yield since it
293 reduces the accessibility of enzymes to the polysaccharides (Van Dyk & Pletschke,
294 2012). Moreover, lignin could block the specific adsorption of enzymes onto the
295 polysaccharides and inactivate enzymes by forming lignin-enzyme complexes, thus
296 reducing the catalyst efficacy (Berlin et al., 2006).

297 In the present investigation, the enzymatic conversion of the polysaccharide fractions
298 present in the unpretreated wastepaper into glucose and xylose was performed by using
299 the commercial cellulolytic preparation Cellic® CTec2. The kinetics of the enzymatic
300 hydrolysis is reported in Figure 1 where the effect of the enzyme dosage on the
301 cellulose digestibility was displayed at two dosages, namely 15 and 25 FPU/g glucan,
302 according to the typical range reported in the literature for the hydrolysis of
303 lignocellulosic material (Cotana et al., 2015; Wang et al., 2012).

304 (Figure 1, near here)

305 As reported in Figures 1A and 1B, in all the reactions the glucose/xylose
306 concentration and yield exponentially increased during the first 24 h, while during the
307 following 72 h (up to 96 h) only a slight increase was observed. In the presence of 15
308 FPU/g glucan, the glucose and xylose plateau concentrations were 15.2 g/L and 2.6 g/L
309 respectively, corresponding to the yields of 92.2 and 98.6 mol%. However, just after 72
310 h, the glucose and xylose concentrations resulted 15.2 and 2.4 g/L, respectively,
311 corresponding to the yield values of 92.2 and 91.0 mol%. In the presence of 25 FPU/g

glucan, the same trends were observed. After 96 h, the glucose and xylose concentrations were 16.1 and 2.6 g/L, respectively, corresponding to the yields of 97.6 and 99.3 mol%. However, similarly to the previous case, just after 48 h, the glucose and xylose concentrations achieved the values of 15.6 and 2.5 g/L, reaching the yields of 94.4 and 94.7 mol%, respectively. The use of the enzyme dosage of 25 FPU/g glucan ensured higher glucose and xylose yields, shorter reaction time to reach sugars yields over 90 mol% (48 h against 72 h), and higher glucose and xylose concentrations. For these reasons, the optimal reaction conditions for enzymatic hydrolysis of waste tissue paper were Cellic® CTec2 25 FPU/g glucan and reaction time of 48 h. As reported by Brummer and co-workers (Brummer et al., 2014), for industrial applications, it is necessary to achieve extensively high yields and concentrations. In the present work, 90.5 g of total reducing sugars were produced from 100 g of wastepaper, with the whole (glucose + xylose) sugar concentration of 18.1 g/L. Moreover, the absence of any pretreatment step in the biorefinery scheme implemented in the present study potentially increases the economic sustainability of the process. Additional biomass pretreatments were not required to reduce the wastepaper recalcitrance (Elliston et al., 2013) because the feedstock had already undergone a pretreatment during the pulping process, making the direct enzymatic hydrolysis to produce second-generation sugars at high yields possible.

The obtained glucose yield of 95 mol% was around 3-folds higher than those reported by Brummer et al. for different kinds of office wastepaper (Brummer et al., 2014). The authors reported the yield of 25.4 mol% for offset paper, 38.5 mol% for recycled paper, 35.9 mol% for recycled paper - printed, 37.2 mol% for offset paper MY sol matte, 37.7 mol% for cardboard, 10.5 mol% for filter paper and 20.6 mol% for

cellulose pulp. Moreover, differently from the present study, the authors did not use unpretreated wastepaper, but they investigated three different acid pretreatments: (i) 0.25 wt% H_3PO_4, (ii) 0.25 wt% H_3PO_4 + 2 wt% HNO_3, (iii) 0.25 wt% H_3PO_4 + 2 wt% NaOH. Under the best reaction conditions reported in their work (8wt% cardboard pretreated with 0.25 wt% H_3PO4 and 2 wt% NaOH, 10 wt% enzyme loading), the maximum glucose yield was 65.2 mol%, which was significantly lower than that reached in the present study. Similar results with glucose yields in the range of 90-95 mol% were obtained by Kojima and Yoon in the enzymatic hydrolysis of ozone-pretreated office wastepaper (Kojima & Yoon, 2008). In the study of Guerfali et al. (Guerfali et al., 2015) the enzymatic hydrolysis of the cellulose fraction of two types of wastepaper materials, namely newspaper and office paper, was investigated in order to produce fermentable monosaccharides from this renewable feedstock. Cellulolytic enzymes produced locally by *Trichoderma reesei* Rut-C30 and *Aspergillus niger* F38 were employed and the hydrolysis reaction was carried out for 48 h. The effect of the surfactant pretreatment on wastepaper digestibility was investigated and Triton X-100 at 0.5 wt% significantly improved the reaction efficiency. Under the optimal reaction conditions reported in their work, the maximum glucose yields obtained from newspaper and office paper were 67 and 92 mol%, respectively.

The results achieved in the present study confirmed the high efficiency of the enzymatic hydrolysis for the selective production of second-generation sugars from industrial wastepaper. The obtained sugars represent important and versatile platform chemicals for the synthesis of added-value bioproducts by chemical or biological routes.

3.3. Biological conversion of sugars into single cell oil

Sustainable biorefinery schemes fully exploit the feedstock and include the conversion of side streams to additional products. Considering the high quality of the paper mill side stream under investigation, the microbial conversion of sugars represents a versatile process to produce added-value bioproducts. The cultivation of oleaginous yeasts on various substrates as fermentation medium, such as industrial or agricultural wastes, has been extensively studied (Di Fidio et al., 2019b; Wang et al., 2014). In the present paper, the reducing sugars obtained from industrial waste tissue paper were converted into new generation oil, represented by single cell oil or microbial oil. *L. starkeyi* was cultured in batch mode on the undetoxified wastepaper hydrolysate. The reducing sugars profile, the DCW, the lipid content and the production of lipids were monitored during the fermentation. Moreover, in order to investigate the effect of inhibitors eventually present in the wastepaper hydrolysate, a synthetic model medium containing the same sugars concentration and the identical C/N ratio was used as a reference substrate. Figure 2 shows the results of fermentation tests on the undetoxified wastepaper hydrolysate.

(Figure 2, near here)

As reported in Fig. 2, yeast growth, expressed as DCW, was very rapid already in the early hours of the process and reached the maximum value of around 10 g/L at 48 h. From 48 to 72 h the stationary phase was observed. The DCW net production of 5.3 g/L in 72 h was in agreement with the biomass production of around 6 g/L reported by Rahman and co-workers, by working at the same C/N ratio (Rahman et al., 2017). Glucose and xylose were completely consumed after 72 h. Glucose was consumed first, and this result is in accordance with data reported in the literature for *L. starkeyi* (Zhao et al., 2008). The xylose consumption started 24 h later in the presence of glucose

concentration of around 10 g/L and in correspondence of an internal glucose-to-xylose weight ratio of around 4, suggesting a sequential utilisation pattern, which is generally observed in most microorganisms. Differently from yeast strains belonging to the *Saccharomyces cerevisiae* species, *L. starkeyi*, as well as other oleaginous yeast species, is able to use also xylose as carbon source (Xavier et al., 2017). This ability ensured the complete biological conversion of the second-generation sugars of wastepaper hydrolysate into microbial oil, increasing the profitability of the proposed biorefinery scheme. As reported in Figure 2, the intracellular lipid content increased from 10.0 to 37.0 wt%. The same initial lipid content was reported by Wang et al. (Wang et al., 2014). During the first 48 h, the lipid content increased up to 35.4 wt%, according to the sugars consumption and the biomass increase. During the stationary phase observed from 48 to 72 h, a slight increase of the lipid content was observed, ranging from 35.4 to 37.0 wt%. The final lipid content resulted higher than the values reported in the literature for the same yeast strain DSM 70296 (Sutanto et al., 2018). In particular, Xavier et al. reported the maximum lipid content of 17.3 wt% in the batch fermentation of sugarcane bagasse hydrolysate (Xavier et al., 2017). Pirozzi et al. reported the maximum values of 20.3 and 17.6 wt% in the fermentation of *Arundo donax* L. and *Sorghum bicolor* L. hydrolysates, respectively (Pirozzi et al., 2013; Pirozzi et al., 2014). Leiva-Candia et al. reported the maximum lipid content of 30.3 wt% in the fermentation of sunflower meal hydrolysate (Leiva-Candia et al., 2015). Moreover, the maximum oil productivity, calculated in the exponential growth phase (24-48 h), resulted 2.0 g/L/d.

In order to investigate the effect of undetected inhibitors eventually present in the wastepaper hydrolysate, an analogous fermentation was performed on a synthetic

medium by implementing the same process conditions. Figure 3 shows the results obtained from the control test.

(Figure 3, near here)

In the synthetic medium, *L. starkeyi* completely consumed glucose and xylose in 48 h, with a consumption rate slightly higher than that reached in the fermentation of real wastepaper hydrolysate. Glucose was consumed first, confirming the sequential utilisation pattern observed in the previous case. The DCW in Figure 5 reached the maximum value of around 11 g/L at 48 h, remaining constant during the stationary phase until 72 h. This DCW concentration was very similar to that of 10 g/L achieved in undetoxified hydrolysate (Fig. 2). The lipid content profile was very similar too. In the model synthetic medium, it ranged from 9.5 to 40.0 wt%, confirming the maximum performance of the yeast in the implemented process conditions adopting the wastepaper hydrolysate as substrate. The maximum oil productivity, calculated in the exponential growth phase (24-48 h), was 2.8 g/L/d, which resulted slightly higher than the value of 2.0 g/L/d obtained in the wastepaper hydrolysate fermentation. The consumption of 20.0 g/L of total reducing sugars allowed the production of 4.4 g/L of lipids, reaching the lipid yield of 22.0 wt%, slightly higher than that obtained on the hydrolysate (20.2 wt%). The lipid yield obtained by fermenting the model solution of pure glucose and pure xylose agreed with the values reported in the literature for *L. starkeyi*, ranging from 19 to 24 wt% (Sutanto et al., 2018). In fact, Lin and co-workers reported the lipid yield of 24 wt% starting from glucose (Lin et al., 2011), Gong et al. achieved the lipid yield of 20 wt% adopting both glucose and cellobiose/xylose mixture (Gong et al., 2012), whilst Tapia et al. obtained the value of 19 wt% employing glucose/xylose mixture (Tapia et al., 2012).

In the present work, in the wastepaper hydrolysate fermentation, 3.7 g/L of lipids were produced by consuming 18.5 g/L of total reducing sugars, achieving a lipid yield of 20.2 wt%. According to the stoichiometry of biochemical conversion of glucose into triglycerides, the maximum theoretical yield is around 33 wt% (Papanikolaou & Aggelis, 2011). On this basis, the obtained yield of 20.2 wt% represented 61.2% of the maximum theoretical yield for oleaginous yeasts. However, in experimental tests reported in the literature, the real process yield ranges between 10 and 24 wt% for *L. starkeyi* (Sutanto et al., 2018). Thus, the lipid yield obtained in the present work was very close to the upper limit of the yields range found in the literature for most yeast species. Up to now, studies on the fermentation of wastepaper hydrolysates by *L. starkeyi* are not reported in the literature. Considering the fermentation of other kinds of lignocellulosic hydrolysates by the same yeast, Xavier et al. reported the yield of 14 wt% employing sugarcane bagasse hydrolysate (Xavier et al., 2017). Azad et al. claimed the yield of 18.0 wt% using rice straw hydrolysate (Azad et al., 2014), whilst Calvey et al. obtained the yield of 14.0 wt% adopting corn stover hydrolysate (Calvey et al., 2016). The findings of the present investigation confirmed the high quality of the paper mill waste and further emphasise the opportunity of using it in microbial processes.

The mass balance flow diagram of the implemented two-step process is reported in Figure 4.

(Figure 4, near here)

It shows the complete conversion of cellulose and hemicellulose of the paper mill waste into glucose and xylose and the following efficient conversion of all reducing sugars into SCO by the yeast *L. starkeyi*. Starting from 100 g of waste tissue paper, the final oil production of 18.3 g was achieved.

Under the optimised process conditions, the single cell oil produced by *L. starkeyi* was mainly composed of long-chain fatty acids with 16 and 18 carbon atoms. The main fatty acids were palmitic acid (C16:0, 36.7 wt%), palmitoleic acid (C16:1, 4.5 wt%), stearic acid (C18:0, 6.1 wt%), oleic acid (C18:1, 47.5 wt%) and linoleic acid (C18:2, 5.2 wt%). The lipid profile of the microbial oil resulted very similar to those of palm and rapeseed oils (Anschau et al., 2014; Sutanto et al., 2018) and to those obtained by *L. starkeyi* on other kinds of lignocellulosic hydrolysate (Sutanto et al., 2018), confirming the suitability of waste tissue paper as a renewable feedstock for biofuels and bioproducts production.

4. Conclusions

In the perspective of the circular economy, this work reported for the first time the cascade bioconversion of unpretreated tissue paper mill waste into single cell oil. The enzymatic hydrolysis yields of the considered negative-value biomass to glucose and xylose were both 95 mol%, resulting higher than the values reported in the literature for wastepaper hydrolysis. *Lipomyces starkeyi* converted sugars into triglycerides achieving a lipid yield of 20.2 wt% with a productivity of 2.0 g/L/d. The proposed biorefinery scheme allows the efficient conversion of industrial waste into platform chemicals (sugars and lipids) for the production of bio-based fuels and chemicals.

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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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656 **Captions for Figures**

657

658 **Fig. 1.** Kinetics of enzymatic hydrolysis of wastepaper: A) glucose concentration (g/L)
659 and yield (mol%); B) xylose concentration (g/L) and yield (mol%).
660 **Fig. 2.** Glucose concentration (g/L), xylose concentration (g/L), dry cell weight (DCW,
661 g/L), lipids concentration (g/L) and lipid content (wt%) in function of reaction time
662 during the wastepaper hydrolysate fermentation by *Lipomyces starkeyi*.

Fig. 3. Glucose concentration (g/L), xylose concentration (g/L), dry cell weight (DCW, g/L), lipids concentration (g/L) and lipid content (wt%) in function of reaction time during the synthetic medium fermentation by *Lipomyces starkeyi*.

Fig. 4. Mass balance flow diagram of the implemented two-step process based on the enzymatic hydrolysis of paper mill waste and the following fermentation of the hydrolysate to single cell oil.

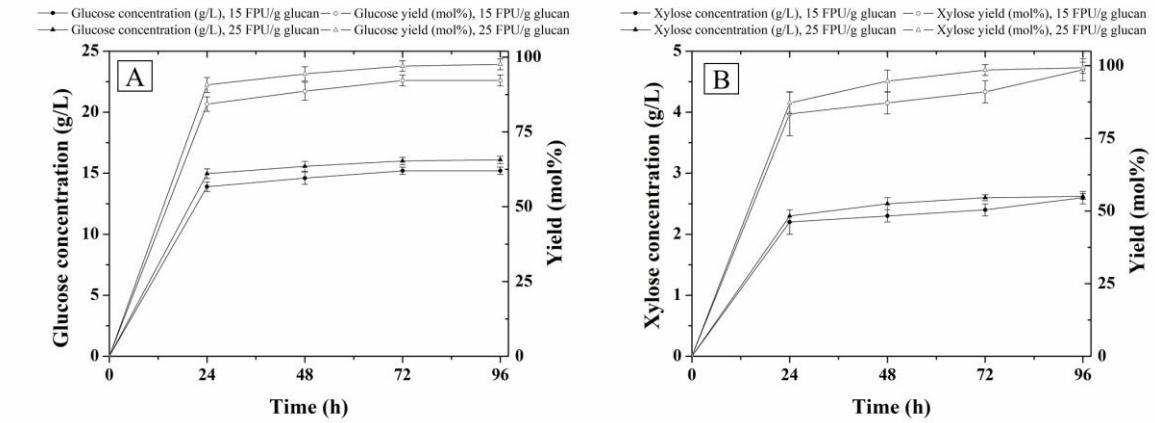


Figure 1

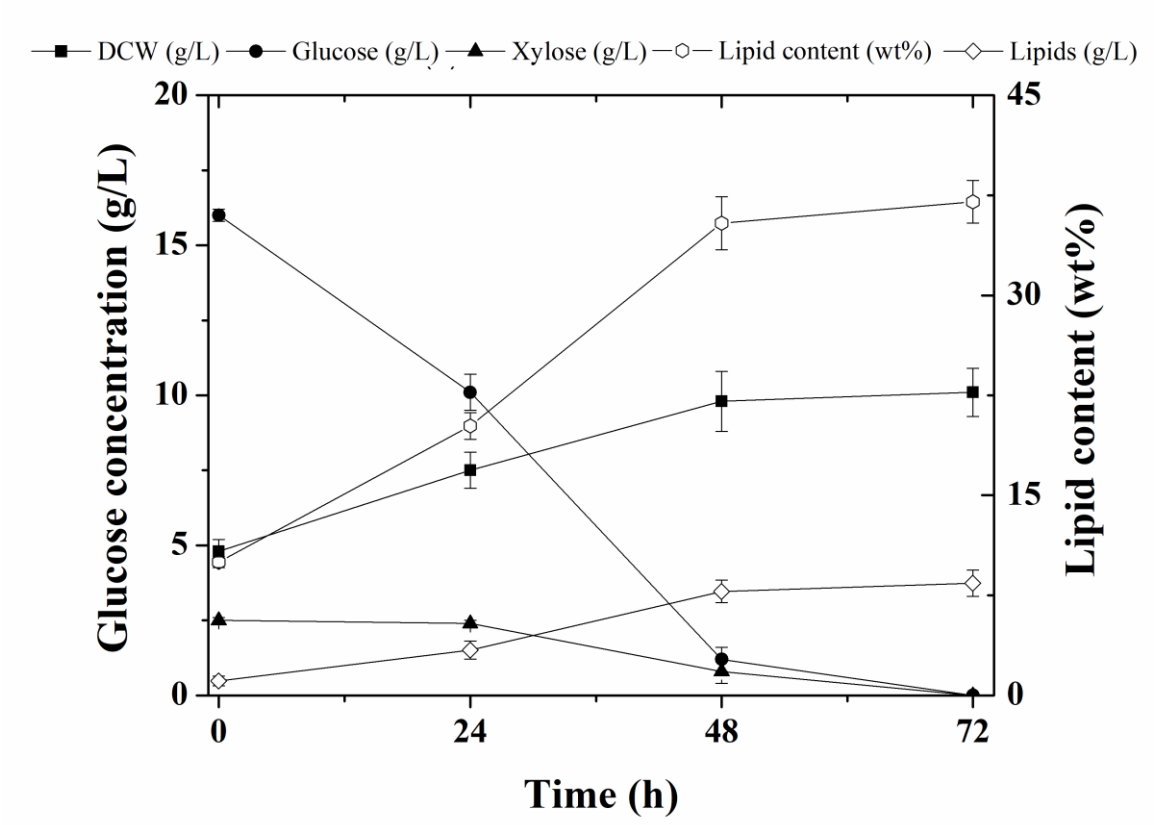

DCW (g/L)
Glucose (g/L)
Xylose (g/L)
Lipid content (wt%)
Lipids (g/L)
Glucose concentration (g/L)
Lipid content (wt%)
Time (h)
0
5
10
15
20
0
15
30
45
0
24
48
72

Figure 2

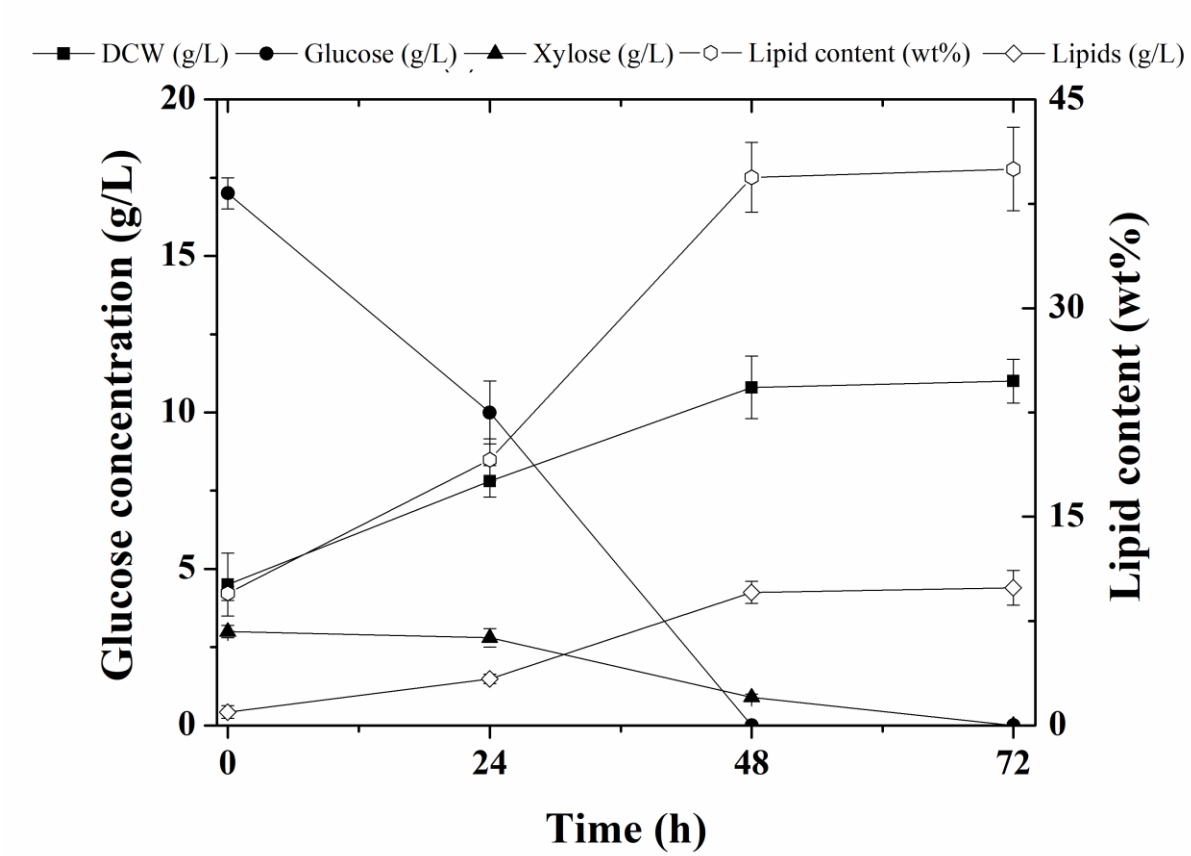
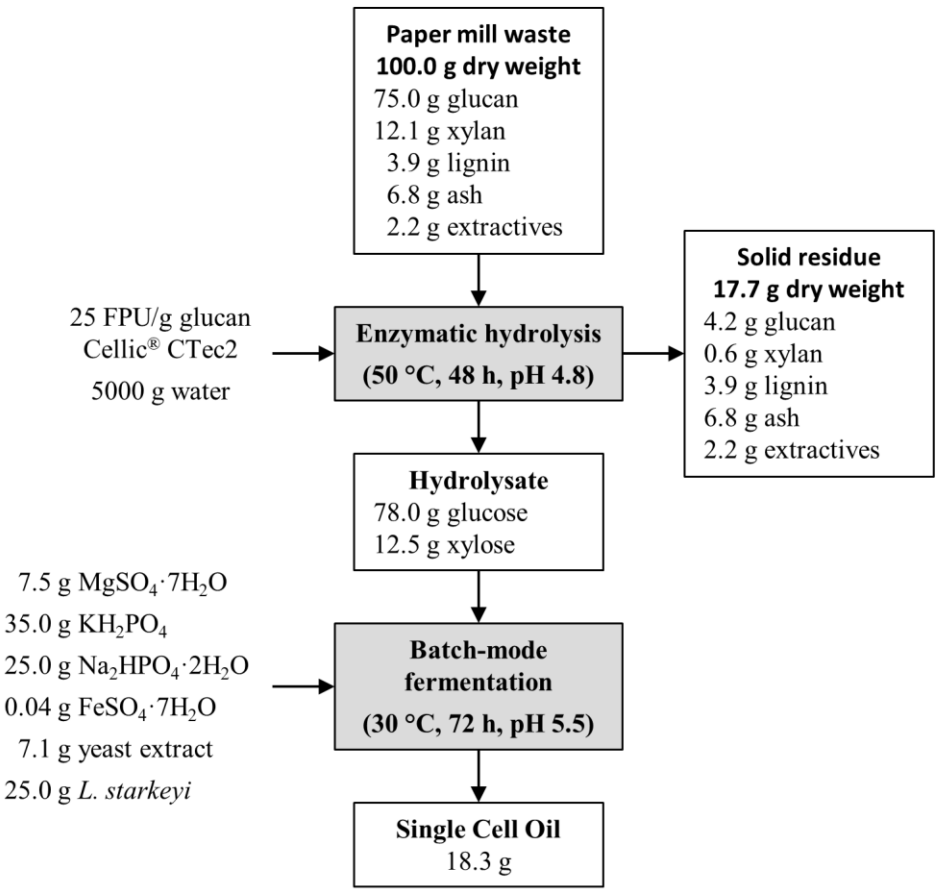

DCW (g/L)
Glucose (g/L)
Xylose (g/L)
Lipid content (wt%)
Lipids (g/L)
Glucose concentration (g/L)
Lipid content (wt%)
Time (h)
0
5
10
15
20
0
15
30
45
0
24
48
72

Figure 3



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708 **Figure 4**