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Valorization of raw glycerol and crustacean waste into value added products by *Yarrowia lipolytica*

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1 **Valorization of raw glycerol and crustacean waste into value added products by**
2 ***Yarrowia lipolytica***
3

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23 **Abstract**
24
25 Crude glycerol has been widely investigated as a renewable carbon source for biodiesel
26 production. In the present study, this feedstock was supplemented by various inducers
27 surfactants and oils to enhance lipid and lipase production by the newly isolated yeast,
28 *Yarrowia lipolytica*. Results have shown that a culture medium composed of olive oil
29 could enhance lipase activity at 25 U/mL and lipid content up to 35% (w/w). The
30 fortification of the medium with crustacean waste increased the lipase activity up to 38
31 U/mL. The hydrolytic activity of the extracellular lipases produced in mentioned medium
32 was satisfactory and opened avenues for other biotechnological processes.

33 **Keywords:** *Yarrowia lipolytica*, Glycerol, Lipid production, Lipase, crustacean waste

34 **1. Introduction**
35
36 *Yarrowia lipolytica* has been known to convert many triglycerides, industrial fats and
37 hydrophobic substrates to single cell biomass or to produce environmentally friendly
38 compounds,such as single cell oils (SCO) (Kamzolova et al., 2005; Papanikolaou et al.,
39 2007) and has gained interest owing to its high secretory activity of various biomolecules,
40 including citric acid, 1,3 propanediol and enzymes such as protease, RNase, phosphatase,
41 esterase and lipase. Besides, *Yarrowia lipolytica* was reported to utilize various aliphatic
42 and hydrophobic substrates (Rywińska et al., 2013, Papanikolaou & Aggelis, 2010,
43 Fickers et al. 2005a). Crude glycerol has been investigated as by-product residual to
44 produce various metabolites (Rywińska et al., 2013, Beopoulos et al., 2009, Kamzolova
45 et al., 2005). However, there is scarce information dealing with the possibility of these
46 microorganisms to produce lipids and lipase. In this regard, *Y. lipolytica* NRRL Y-2178

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47 was reported to synthesize alkaline lipase when glycerol is used as a carbon source (Lee
48 et al., 2007). In a recent study, Fabiszewska et al. (2014) have cultivated wild-type strain
49 *Y. lipolytica* KKP 379 in based glycerol media and have determined its efficiency to
50 synthesize lipolytic enzymes (Fabiszewska et al., 2014). Moreover, the authors have
51 concluded that glycerol utilization in microbiological lipase production is possible, but
52 that this process cannot proceed without the addition of one or more stimulators of lipase
53 synthesis, such as olive oil. Relevant results were obtained while using a medium
54 containing waste cooking olive oil as inducer (Goncalves et al., 2013). More often, some
55 oils were reported to increase the surfactant production, which could act as an inducer to
56 enhance lipid accumulation in yeast (Aksu & Eren, 2005). Therefore, the use of these
57 substrates for fermentation aims to decrease the final cost of these enzymes and make the
58 industrial enzymatic processes cost-competitive with chemical ones. For this purpose,
59 researchers have investigated the role of various inducers to enhance lipid and lipase
60 activity in the presence of oils. To the best knowledge of the authors, this is the first
61 report on complete assessment of lipase production by the yeast using crude glycerol and
62 crustacean wastes. In the first part, a suitable inducer was first screened for enhanced
63 production of lipids and lipase activity. Secondly, crustacean waste was supplemented to
64 the media to enhance lipase activity. The use of CW industrial wastes, in the culture
65 media, as nitrogenous source can be a good replacement and nutrient supplementation
66 instead of using different culturing materials, such as protein hydrolysates (e.g. peptones,
67 yeast and malt extracts) that are very expensive. This alternative may decrease the cost of
68 lipase production and contribute to the valorization of these residues. Thus, a

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69 combination of crude glycerol, crustacean waste and/or other inducers could be a
70 promising strategy to enhance metabolite production in *Y. lipolytica*.

71 **2. Materials and Methods**

72 **2.1. Microorganism**

73 *Y. lipolytica* SM7 was newly isolated (Magdouli et al., 2016). The micro-organism was
74 maintained at 4°C on (yeast extract peptone dextrose agar) (YEPD) slants. Subcultures
75 were made on fresh agar slants every three months to maintain viability.

76 **2.2. Crude glycerol, Crustacean waste, Reagents and Chemicals**
77

78 All chemicals were of reagent grade. Growth media were purchased from Sigma-Aldrich,
79 Fisher scientific (Mississauga, Ontario, Canada).
80 Domestic vegetable oil (purchased from local grocery) is composed of canola oil and/or
81 soybean oil. It was supplemented with some antioxidants and anti-foaming agents as
82 inducers in the present study
83 Olive oil was also obtained from the local grocery and used as an inducer in the current
84 study.
85 Crude glycerol was obtained from Rothsay Biodiesel (Ontario, Canada), resulting from
86 the transesterification of animal fats was used as carbon source, with high purity of
87 83.30% with impurities mainly composed of potassium and sodium salts (1-2%),
88 methanol (1-3%), and water (2-5%).
89 The crustacean waste (CW) was obtained from a seafood processing plant located at
90 Gaspesie, Quebec. The seafood waste (shells of crab, shrimp, prawn, krill and lobster)
91 was oven dried, powdered using a grinder and stored at 4±1 °C.

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92 **2.3. Cultivation Conditions**
93
94 Cultures were grown on a minimal medium containing, 1 g/L yeast extract, 1.5 g/L
95 $(NH4)_2SO_4$, 3 g/L K_2HPO_4, 3 g/L NaH_2PO_4 H_2O, 0.5 g/L $MgSO_4$ $7H_2O$, 0.040 g/L $ZnSO_4$
96 $7H_2O$, 0.016 $FeSO_4$ $7H_2O$, 0.25 µg/L biotin), and 40$\pm$5 g/L of crude glycerol. As
97 methanol in the crude glycerol was not detectable after autoclaving, filtration sterilization
98 was carried out to sterilize the crude glycerol. Methanol concentration varied form 1 to 3
99 % (w/w). Herein, the objective was to provide all components presented in the crude
100 glycerol, especially methanol which can be evaporated after sterilization. The crude
101 glycerol was diluted to obtain 4% glycerol concentration. The final pH of the medium
102 was 6.5. The medium was sterilized by autoclaving at 121°C for 20 min. The inoculum of
103 *Y. lipolytica* SM7 was prepared by transferring cells grown on a slant to 50 mL
104 erlenmeyer flasks containing (YEPD) broth. The seed culture was incubated in an orbital
105 shaker at 28°C, 180 rpm for 24 h. The YEPD broth was used to initiate growth 5% (v/v).
106 Cultivation was carried out in 2L Erlenmeyer flasks containing 500 mL minimal medium
107 at 28°C with shaking at 180 rpm for 150 h. There was no adjustment of pH during
108 cultivation. At regular intervals, samples were withdrawn for analyses. All analyses were
109 performed in triplicates and did not vary more than 5%.
110 To screen a suitable inducer to obtain the maximum lipid and lipase activities, various
111 surfactants and oils were used. After screening step, the effect of inducer on lipase
112 production was investigated. Thus, experiments were carried out in the shake flasks as
113 described earlier and lipase activity was determined periodically. No control of pH during
114 fermentation was performed.

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115 The experiments were performed in two stages. The first stage experiments were carried
116 out to determine the appropriate inducer to obtain best growth performance and higher
117 metabolites production. In the presence of different inducers, lipase activity, biomass and
118 lipids were monitored throughout the fermentation. During the second stage, experiments
119 were carried out to determine the effect of CW supplementation to induce and enhance
120 lipase production.

121 **2.4. Analytical methods**

122 Five mL aliquot of culture was centrifuged at 5000 rpm for 5 min. The cell free
123 supernatant obtained after centrifugation was used for estimation of lipase activity. For
124 the determination of lipase activity, titration method was employed which consists of use
125 of olive oil emulsion as substrate (Lopes et al., 2011). An emulsion containing 25 mL of
126 olive oil in 7% Arabic gum (75 mL) in 0.1 M phosphate buffer (pH 7.0) was used as
127 substrates, respectively for lipase. The reaction mixture which contains substrate, 2 mL of
128 0.1 M phosphate buffer (pH 7.0) and 1 ml of the enzymatic suspension (10 mg /mL) was
129 incubated at 37±1 °C for 30 min using orbital shaking. The emulsion was immediately
130 disrupted by the addition of 15 mL of acetone-ethanol (1:1 v/v) and the liberated free
131 fatty acids were titrated with 0.05 M NaOH. One unit of enzyme activity is defined as the
132 amount of enzyme which liberated 1 μmol of fatty acid per minute.

133 To study the effects of pH on the enzyme stability, buffered sample was incubated at
134 various pH ranging from 5 to 9 for 1 h at 37 °C. To keep the sample pH constant, the
135 following 100 mM buffers systems were used: Na_2HPO_4/ Citrate, pH 3.0–6 and
136 KH_2PO_4/Na_2HPO_4, pH 6.0–7.0. The thermal stability was studied by incubating the
137 enzyme at various temperatures and measuring the residual activity with time under

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138 standard titrimetric assay conditions. For determination of biomass weight (g/L), culture
139 broths were centrifuged at 5000 rpm for 15 min. After rinsing the pellet once with
140 deionized water, the pellet was frozen at −80 °C for 1–2 days, and then dried for 24 h in a
141 lyophilizer for dry cell weight measurement. All experiments were performed in
142 triplicates, and average results were reported with standard deviation less than 5%.

143 Extraction of lipid from lyophilized biomass was performed according to the modified
144 procedure of Folch et al. (1957). Lyophilized biomass with a mixture of chloroform:
145 methanol (2:1, v/v) was placed in contact with zirconium beads (0.7 mm) for cell
146 disruption by bead beater for 5 min (Biospecs, USA). Afterwards, the extracted lipids
147 were centrifuged to obtain a clear supernatant and the resulting biomass was re-
148 suspended in a mixture of chloroform: methanol (1:1, v/v). The same procedure of bead-
149 beating was repeated. After centrifugation, the solvent was removed by evaporation under
150 vacuum.

151 Fatty acid profile of the lipid was determined by methylation for conversion of fatty acids
152 to fatty acid methyl esters (FAMEs). The fatty acid composition in the FAMEs was
153 analyzed using Gas Chromatography- Mass Spectroscopy (GC–MS) (Perkin Elmer,
154 Clarus 500) equipped with a column (dimensions 30 m × 0.25 mm, 0.25 μm film
155 thickness) and flame ionization detector. The calibration curve was prepared with a
156 mixture comprising 37 FAMEs (47885-U, 37 Component FAME Mix; Supelco,
157 Bellefonte, PA, USA). 1,3-dichlorobenzene was used as an internal standard. All
158 experiments were performed in triplicates, and average results were reported with
159 standard deviation less than 5%.

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160 **3. Results and Discussion**

161 **3.1. Dynamics of lipase and lipids biosynthesis in glycerol based medium**

162 **3.1.1. Effect of surfactants on growth kinetics, lipid production and composition and**
163 **lipase activity**
164

165 *Y. lipolytica* was cultivated in different media containing filtered crude glycerol (final
166 product of lipase activity that lacks a fatty acid or carbon chain) at initial concentration of
167 40 g/L and various inducers (e.g. olive oil, vegetable oil and motor oil, Tween 80, Tween
168 20, Triton X 100) of 5.0 % (w/v). These components were investigated for their potential
169 in increasing the metabolites production of SM7 (i.e. biomass, lipid, citric acid (CA), and
170 lipase). Previously, many agents such as Tween 80, Tween 20, and Gum arabic were
171 investigated to enhance biomass and lipid production in oleaginous yeast (Saenge et al.,
172 2011). Still, the mechanism of their action is not well understood, hence, Kruszeka et al.
173 (1990) reported that these agents appear to cause different alterations in membrane
174 fluidity (Kruszewska et al., 1990). It was found that the crude glycerol could serve as a
175 sole carbon source for *Yarrowia* without any additional supplementation of inducers
176 (control experiment). Biomass, lipid concentration and lipid content were around
177 8.71±0.5 g/L, 2.68± 0.5 g/L, 30.76±2.0% (w/w) of dry biomass, respectively. The higher
178 biomass concentration was obtained at 120 h and reached around 12.57±0.8 g/L. Lipid
179 content decreased slightly in the latter stage from 30.76± 2.0 g/L to corresponding 14.95
180 ±1.0 g/L to the exhaustion of glycerol from the medium. Thus, the exhaustion of carbon
181 source induced reserve lipid turnover. This phenomenon is routinely observed in
182 oleaginous yeasts after the transition from carbon excess to carbon starvation.
183 Accumulated fatty acids are degraded owing to the induction of intracellular lipase
184 system that cleaves the crude glycerol into fatty material. Only lipid free biomass is

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185 synthesized. Reserve lipid turnover occurred because the extracellular flow rate of
186 aliphatic chains decreased considerably and it did not meet and saturate the microbial
187 metabolic requirements (Papanikolaou et al., 2002). During lipid turnover, some enzymes
188 are activated, such as isocitrate lyase (ACL) which is reported to be induced during the
189 cultivation on fatty materials (Wynn and Ratledge 2000). Besides, Papanikolaou et al.
190 (2004) have suggested that lipid turnover can be repressed when growth occurred in
191 double- or multiple-limited media or in the presence of several nutrients, such as vitamins
192 or oligo-elements, ferrous and magnesium indispensable to mobilize reserve lipid
193 (Papanikolaou et al. 2004).

194 Further increase in biomass, lipid content and lipid quantity was observed when
195 surfactant was added in different concentrations (Table 1). Among the three investigated
196 surfactants, Tween 80 was found to be most effective in increasing both biomass and
197 lipid content. It gave the highest amount of biomass, lipid content (16.93 g/L, 5.94 g/L,
198 35.06% (w/w), respectively). Thus, the role of different detergents as growth promotors
199 and lipid inducers is well illustrated. Herein, the addition of supplementary hydrophobic
200 carbon source (e.g. Tween 80) brought around 11 g/L of oleic acid which generated more
201 carbon flux and enhanced the lipid production to around 35.06 % (w/w). Meanwhile,
202 Tween 20 and Triton X-100 were added as a supplementary source of lauric acid (C12:0)
203 and hydrocarbon. All these hydrophobic substrates contributed efficiently to enhanced
204 lipid production in *Y. lipolytica.* They are assimilated via ex-novo pathway and further
205 degraded via peroxisomal *β*-oxidation to be stocked later in lipid bodies. The assimilation
206 of these hydrophobic substances is accompanied by the modification of surface
207 hydrphobicity and the formation of protrusions scattered across the cell surface. These

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208 facts imply that morphological and physiological adaptations observed in *Y. lipolytica*
209 were a response of the presence hydrophobic substances (Mlickova et al. 2004; Fickers
210 et al. 2005a; Beopoulos et al. 2009). All these changes are responsible for the uptake
211 and the transport of these substrates to be degraded by β-oxidation or re-integrated in the
212 form of triglycerides and esters in the cell.

213 These findings are in agreement with Boudour et al. (2003) who found that the higher
214 consumption rate could be attributed to the capacity of the surfactant to emulsify
215 hydrocarbon-based compounds, breaking them down into more manageable molecules,
216 so that the microbes can then more efficiently digest them (Bodour et al., 2003). Besides,
217 Saengea et al. (2011) have observed that surfactants acted efficiently as activator of TAG
218 accumulation in *R. glutinis* TISTR (Saenge et al., 2011), which are in concordance with
219 the current study in which higher lipid content up to 35.22$\pm$ 2.5 % (w/w) of dry biomass
220 was obtained compared to control experiment at 30.90$\pm$ 1.5 % (w/w) of dry biomass.

221 Besides, the lipid yield decreased between 24h and 48h in the early stage of growth, as
222 well as in the late growth stage, thus the exploration of metabolites produced was
223 conducted. Higher quantity of CA was observed and maximum of 3.3 g/L was obtained at
224 100 h. The production of CA is also accompanied by lipase secretion at 24h and 72h. As
225 seen in Table 1, lipase production appeared to be critically influenced by the presence of
226 various surfactants used along experiments, in the flask cultures. In the control
227 experiment, crude glycerol alone was able to induce lipase activity, however, this activity
228 was very low (2-4$\pm$ 1.7 U/mL) and U_{max}=4 $\pm$ 1.7 U/mL was observed at 72h. At 24h, a
229 lipase activity of 1.66 U/mL was detected, due to the presence of triacylglycerols and
230 fatty acids in the crude glycerol which is estimated by soap content in the crude glycerol

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231 of around 1.5 g/L. In addition to soap, the present activity can be attributed to the
232 presence of fatty acids, vitamins and trace elements resulting from the transesterification
233 of animal's fats diffusing to the glycerol phase during the biodiesel formation reactions,
234 and thus enriching the crude glycerol (Cüelik et al., 2008). Accordingly, lipase secretion
235 is known to be induced by the presence of triacylglycerols and fatty acids in different
236 microorganisms (Benjamin & Pandey, 1996). During lipogenic phase, no activity was
237 detected between 36h and 60h, which could be attributed to a repressive phenomenon in
238 the presence of glycerol as sole carbon source, the latter is generated as an end product of
239 lipase activity and triglycerides hydrolysis and when it is available at the beginning of
240 cultivation, there is no immediate need for lipase production by cell. Corzo and Revah
241 (1990) also reported that lipase is not produced in medium containing glycerol as the sole
242 carbon source (Corzo & Revah, 1999). Besides, Szczesna-Antczak et al. (2006), showed
243 that carbohydrates could inhibit biosynthesis of extracellular lipases and the addition of
244 an inducer is highly required (Szczesna-Antczak et al., 2006), since the lipase production
245 is not a constitutive phenomenon in *Y. lipolytica* and the presence of inducer is highly
246 required.

247 Maximum cell concentration was obtained in the presence of Tween 80>Tween
248 20>Triton X100 (X_{max}=16.97g/L> 16.55g/L>14.73g/L respectively, the higher biomass
249 was associated with higher lipase activity (U_{max}=15.10$\pm$2.5, U_{max}=14.33$\pm$1.9,
250 U_{max}=12.50$\pm$ 2.5 U/mL) respectively detected at 72h. The pH dropped from an initial
251 value of 6.5 to 4.5 from day 3 onwards, probably due to the action of the produced lipases
252 and the subsequent increase of organic acids concentration in the medium, especially
253 citric acid (CA) (Magdouli et al., 2016). Generally, surfactants have been well reviewed

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254 to enhance extracellular lipase activity by causing changes in cell permeability of the cell
255 (Kruszewska et al., 1990). However, their role in the lipase production is not conclusive
256 since some authors reported that surfactants do not increase the lipase production in some
257 species (Lin et al., 1995). Therefore, the effect seems to be strain dependant. Herein, the
258 presence of surfactants seemed to bring about a slight increase in biomass production:
259 maximum cell concentration was found to be between 15.00-19.00 $\pm$2.5 g/L between 72h
260 and 100h, compared with the control.

261 No activity was observed in the late growth phase (96h) due to the possible degradation
262 of lipase since *Yarrowia* species are known to produce alkaline extracellular protease
263 (AEP) (Davidow et al., 1987), which is responsible for the degradation of extracellular
264 lipase produced by selected strain. Between 120 h and 150 h, the activity fell down to
265 undetectable levels.

266 In the present study, whatever the surfactant used, lipase activity peaked twice at 24h and
267 72h medium, however, it was lower in the early stage (i.e. 24h) and accounts around
268 (9+3.5 U/mL for Tween 80, 5.33+ 2.5 U/mL for Triton X 100 and 6.0+3.0 U/mL for
269 Tween 20). The double peak of lipase activity was correlated with sequential
270 consumption of glycerol and surfactant, indicating that *Yarrowia* consumed methanol,
271 soap (free fatty acids in the early stage were mobilized to consume glycerol) and later, the
272 surfactant employed.

273 Furthermore, the quantification of lipid content in the presence of these stimulators was
274 carried out, and lower content of lipid during the phase of biomass production 36h
275 corresponded to lipid quantity between (0.20$\pm$0.3 and 1.13$\pm$0.25 g/L). Between 48-70h
276 (i.e. lipogenesis phase), a higher lipid content was observed and maximum of 35.06

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277 %(w/w) at 60h, however, during the CA phase between 72h-120h (lipid content was
278 decreased to reach 17.59± 3.5 % (w/w) due to the presence of lipolytic activity, to the
279 exhaustion of carbon source and the induction of lipase owing to the presence of
280 accumulated TAG. Besides, another hypothesis can be evolved corresponding to lipase
281 activity during extraction which remains bound to the cells and subsequently induced
282 lysis of intracellular lipid and lower the content below 17% (w/w). Hence, the rapid
283 extraction is required to avoid such phenomena since in the presence of
284 chloroform/methanol, lipase activity disappeared (Najjar et al., 2011). Moreover, lipid
285 accumulated can be preserved owing to different strategies (Zhang et al., 2015).
286 The analysis of the composition of fatty acids is provided in Table 2. In all cultures, in the
287 presence of different surfactants, lipid composition showed significant amounts of stearic
288 acid, oleic and linoleic acid. As compared to the control, with sole glycerol as carbon
289 source, an improvement in the content of oleic acid C18:1 was observed which increased
290 from 39.81% to 47.5% in the presence of Tween 80. Linoleic acid (C18:2) content was
291 also improved and reached 7.50 %. More unsaturated fatty acids are observed in the
292 presence of different surfactants used in the current study. Therefore, the use of crude
293 glycerol with the cited surfactants as co-substrate, is an interesting possibility to produce
294 a fat with a particular fatty acid composition having a higher content in C18:1 and in
295 C18:2.

296 **3.1.2. Effect of oil inducers on growth kinetics, lipid production and composition**
297 **and lipase activity**
298

299 Addition of oils to flask cultures induced an increase in biomass value as found with
300 surfactants, compared with the medium in which no addition of oil was carried out. As

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301 shown in Table 3, *Y. lipolytica* showed efficient cell growth when fermentation was
302 carried out in the presence of olive oil and glycerol based medium (X=17.12 g/L). During
303 the lipogenic phase, biomass was around 17.0 $\pm$0.5 g/L compared to 8.70$\pm$0.5 g/L. A
304 higher biomass was recorded with olive oil (X_{max}=21.93 $\pm$ 0.8 at 120h).

305 However, in the presence of motor oil, a little improvement of biomass production was
306 observed (X_{max}= 10.25 g/L) and visually, the oil remained on the surface, this oil was not
307 consumed, thus, the composition of engine oil inhibited the uptake of glycerol and the
308 microorganism was unable to degrade and use the oil as carbon source while glycerol was
309 there. Accordingly, engine oil is reported to be composed of synthetic antioxidant and
310 corrosion inhibitor, required to minimize its susceptibility to oxidation (Meira et al.,
311 2014). These components seemed to inhibit the growth in the present case. Conversely,
312 the higher lipid content is obtained in the presence of olive oil at 35.80% (w/w) compared
313 to 33.06 % (w/w) in the presence of vegetable oil.

314 Growth kinetics of *Y. lipolytica* in the presence of crude glycerol and olive oil were
315 presented in Figure (1 A, B). Compared to the control (glycerol sole), a substantial
316 biomass production was observed in the presence of glycerol and olive oil. Besides, the
317 uptake of glycerol (Fig. 1B) was accelerated compared to the control experiment, which
318 could be due to the presence of olive oil as co-substrate. Around 12.90$\pm$1.5 g/L was
319 remained when olive was supplemented to the media. Papanikolaou et al. (2003) have
320 reported that *Y. lipolytica* could consume simultaneously hydrophobic substrates and
321 carbohydrates (glycerol or glucose), and the presence of substrate fat increased the
322 glycerol uptake and the citric acid biosynthesis which is in accordance with the current
323 study. The determination of substrate fat consumption and the release of free fatty acids

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324 upon lipolysis as well as the determination of other lipolysis products (diglycerides and
325 monoglycerides)will be provide a sufficient information on the fate of substrate fat during
326 lipogeneis. In this regard, further analyses and studies have to be conducted.

327 Therefore, olive oil and vegetable oil, composed of fatty acids (mainly oleic acid), are
328 known to promote the novo synthesis without affecting the glycerol uptake. These results
329 suggested the high performance of *Y. lipolytica* during growth in various oil-glycerol
330 based media. In fact, *Yarrowia* is known to produce an emulsifying bio surfactant
331 (Lyposan) in the presence of hydrophobic substrates, which emulsify the medium and
332 enhance the uptake of oleic acid and traicylglycerols present in the olive oil. The higher
333 capacity to accumulate lipids when grown on these substrates is probably related to
334 protrusions formed on cell surfaces, facilitating the uptake of hydrophobic substrates
335 from the medium (Mlickova et al., 2004). Motet et al. (1985) reported that only 2% of
336 total lipids in its dry matter were accumulated in the presence of glucose as carbon
337 source. These results were in accordance with Papanikolaou et al. (2002) who found that
338 when glucose was a carbon source, only 5-9% (w/w) of lipid was accumulated in dry
339 cellular mass. The metabolism in this case was more devoted to the citric acid production
340 so that the ATP-citrate lyase (key enzyme for lipid biogenesis) was not active. The
341 addition of palmitate, oleate or arachidate, 1-eicosenoate improved the lipid content,
342 between 3-5.4 % instead of 2% of total lipids in its dry matter (Motet et al., 1985). Thus,
343 the use of fats as co-substrates was efficient to improve the lipid accumulation. These
344 observations are in accordance with Fickers et al. (2005a) who found that *Y. lipolytica*
345 easily assimilated hydrophobic substrates, such as fats and oils etc via specific metabolic
346 pathways and used them for the production of single cells oils (SCO). Further, often,

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347 Papanikolaou and Aggelis, (2010) have also reviewed that once oils or fats are used as
348 substrates or co-substrates, *Y. lipolytica* follow ex-novo pathway which involves the
349 capture of fatty acids, oils and triglycerides on the growth medium, their hydrolysis,
350 transport, re-assimilation as triglycerides and esters followed by their accumulation
351 within the cell in lipid bodies.

352 In the current investigation, surfactants and oils display an inducer role and efficiently
353 contributed to the improvement of lipid accumulation. These statements are in agreement
354 with Papanikaloau et al. (2003) who noticed that the cultivation of *Y. lipolytica* in the
355 presence of technical glycerol and a mixture of saturated free fatty acids (derivative of
356 animal fat=stearin) resulted in higher lipid synthesis, the lipid yield $Y_{L/S}$=0.41 g/g as
357 compared to the combination of glucose and stearin where the lipid yield $Y_{L/S}$=0.23 g/g.

358 In the current investigation, the lipid content increased rapidly compared to control
359 experiment. Therefore, *Y. lipolytica* is capable of to use triglycerides as carbon source
360 and the first step of this metabolism involved hydrolysis of olive oil by lipases to produce
361 fatty acids and glycerol, and a higher activity was detected at 24h, 15$\pm$ 3.2 U/mL and
362 12.80 $\pm$ 2.5 U/mL for olive oil and vegetable oil, respectively. Once oils are present in the
363 medium, *Y. lipolytica* hydrolyses them by the presence of residual lipase present in the
364 inoculum. This fact explained that higher activity observed at 24h which reached 15$\pm$ 3.2
365 U/mL. Once glycerol is released, the latter is consumed as carbon source during the
366 lipogenic phase without producing lipase. During the exhaustion of glycerol, there is
367 induction of lipase activity. Accordingly, a cascade of enzymes of β- oxidation of fatty
368 acids are activated and specific genes that encode for proteins controlling lipase
369 production are expressed, especially specific for oleic acid (SOA) genes (that control

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370 gene expression LIP-2, responsible for the extracellular lipase by *Y. lipolytica*
371 (Desfougeres et al.,2010).
372 Papanikolaou et al. (2002) have reviewed the *ex-novo* pathway of the fatty acids, oils and
373 triglycerides. The pathway follows their capture and their accumulation within the cell,
374 their hydrolysisand finally their transport to be re-assimilated as triglycerides and esters,
375 followed by their accumulation in lipid bodies (Beaopolus et al. 2009).
376 Besides, a double peak was observed at 72h and lipase activities increased sharply and
377 attained (25.10 U/mL and 22.45 U/mL) for olive and vegetable oils, respectively which
378 corresponded to the initiation of the degradation of TAG accumulated. The higher lipase
379 activity could be attributed to the composition of olive oil as discussed earlier. In this
380 regard, the microorganism starts degrading triglycerides and glycerol present in the
381 medium and produced free fatty acids and glycerol due the action of lipase produced at
382 24h, around 15 U/mL for olive oil and 12.80 U/mL for vegetable oil. Afterwards,
383 resulting glycerol is reverted to biomass without lipase production and represses the
384 uptake of fatty acids. During lipogenic phase, the activity remains low and does not
385 exceed 3 U/mL with is mainly due to the production of organics acids that lower the pH
386 of the medium and inactivate the lipase secretion as well as the catabolic repression
387 exerted owing to the presence of glycerol. It is noteworthy that the activity was slow in
388 the later growth stage, which questioned stability during the culturing or carbon source
389 availability where the substrate was completely consumed at 72h. This observation was in
390 agreement with Kamzolova et al. (2005) and Papanikolaou et al. (2007), who found that
391 lipase activity significantly decreased when fermentation progressed and the quantity of
392 substrate lipid into the medium decreased (Kamzolova et al., 2005; Papanikolaou et al.,

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393 2007). The selected microorganism, thus is a potential candidate for cell growth, lipid and
394 lipase production in the presence of oils.

395 In the present study, both oils (olive and domestic vegetable oil) displayed a crucial role
396 in inducing the lipase activity with a higher activity observed in the presence of olive oil,.
397 This fact could be related to the fact that most of *Y. lipolytica* strains display lipase that
398 acts preferentially on oleyl residues at positions 1 and 3 of the glyceride. Besides, lipase
399 activity is reported to be depending on oleic acid concentration, hence, olive oil has
400 around 55-83% of oleic acid content (Royer et al., 1999), while other vegetable oil
401 contains around 55% of oleic acid. Therefore, more the oleic acid content, higher is the
402 lipase activity. These observations are in agreement with the present work. Moreover,
403 expression system containing the *LIP2* gene is expressed under the control of oleic acid
404 inducible promoter, POX2 (Pignede et al., 2000).

405 Besides, the activity detected in the presence of motor oil was lower as compared to those
406 recorded in the presence of olive oil and vegetable oil (Fig.2 B).
407 Thus, the lipase activity is correlated to biomass growth and both work in synergy. In the
408 presence of motor oil, growth and lipase activity was repressed. Moreover, the lipid
409 quantity seemed to be affected by the components present in motor oil. And even the
410 remarkable lipid quantity was detected at 60h (i.e. 3.33 g/L), possibly coming from the
411 residual motor oil which is not used and bound to cell membrane before extraction. On
412 the other side, the absence of lipase activity in the presence of motor oil may be due to
413 nature and the composition of the oil (Meira et al., 2014). Mafakher et al. (2010) showed
414 that the lipase activity could be inhibited in the presence of hydrocarbons (Mafakher et
415 al., 2010).

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416 These results lead to consider *Y. lipolytica* as an efficient producer of SCO and make the
417 current investigation base for the use of other hydrophobic residues such tallow
418 derivatives (Papanikolaou et al., 2007) and olive mill wastes (Finogenova et al., 2008;
419 Moftah et al., 2013; Papanikolaou et al., 2008) and also other metabolites of industrial
420 significance such as lipases. As described earlier, the evolution of lipase production was
421 different depending on the lipid material used (i.e. surfactants or oils). The highest lipase
422 activity levels were obtained when olive oil was added to the medium U_{max}=25.10$\pm$4.5
423 U/mL. Data are in accordance with data reported by Corzo and Revah (1990) which
424 indicates that Yarrowia strains produce high levels of lipase on medium containing olive
425 oil (Corzo & Revah, 1999). These results could be also justified by the fact that *Y.*
426 *lipolytica* strains display a lipase activity, which acts preferentially on oleyl residues at
427 positions 1 and 3 of the glyceride and the extracellular lipase requires oleic acid as
428 stabilizer/activator (Barth & Gaillardin, 1996). Moreover, in the later stage of growth,
429 biomass continues to gradually increase and cells are using fatty acids as well as the main
430 components of degradation of olive oil to support their growth (Del Río et al., 1990).

431 Herein, due to the presence of motor oil and glycerol, the activity of lipase seemed to be
432 lower compared to the activity recorded in the presence of olive oil and vegetable oil.
433 *Yarrowia* was able to metabolize glycerol which had a repressive phenomenon on motor
434 oil uptake and subsequently lowered the activity to 5 U/mL. Thus, the concentration of
435 glycerol and motor oil should be optimised to avoid such inhibitory effect since the role
436 of glycerol to induce lipolytic activity is not conclusive and authors confirmed that a
437 certain amount of glycerol in the culture medium may not have a repressive nature in
438 relation to the production of lipolytic enzymes. For example, Volpato et al. (2008)

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439 observed that maximum lipolytic activity was achieved in the presence of 30 g/L glycerol
440 and 3 g/L olive oil. Moreover, Corzo and Revah (1999) found that the addition of 0.5–
441 6.0 g/L glycerol to the medium did not significantly inhibit the synthesis of extracellular
442 lipase by *Y. lipolytica* 68*1*(Corzo & Revah, 1999). Similarly, Fabiszewska et al. (2015)
443 have found an activity in the presence of 30 g/L compared to lower activity at higher
444 glycerol concentration (up to 150 g/L). The upper limit is 37.5 g/L (Volpato et al., 2008).
445 These conclusions are in accordance with the present results, where the activity is
446 recorded at 40 g/L in the presence of olive oil and vegetable oil, and a lower activity is
447 obtained at 40 g/L glycerol and 5% (w/v) motor oil. Thus, a hypothesis has to be evolved
448 to understand the possible inhibitory effect of both substrates on the lipase activity.
449 Besides, the microorganism will start using the simple carbon source available, and later
450 on moving to the degradation of hydrophobic substrate which can justify such results.

451 The extension of fermentation time up to 150h decreased the lipid content and the lipid
452 productivity to around 19% (w/w). The main cause of lipid degradation was attributed to
453 the lipase activity recorded at 72h. However, to the best of author knowledge, with an
454 exception of a recent study (Szczena-Antczak et al., 2006), the simultaneous lipid and
455 lipase production is not frequent.

456 As shown in Table 2, the lipid composition is dependent on the nature of oils used as
457 inducers. In the presence of olive oil and glycerol, cells are enriched with saturated fatty
458 acids (C16:0 and C18:0) which reached around 22.0% and 17.05%, respectively in the
459 presence of olive oil. Besides, the levels of unsaturated fatty acids was also important
460 (C18:1, C18:2). The C18:1 content rose from 39.81 % to 48.3 %.

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461 No significant change was observed in the presence of glycerol and vegetable oil and
462 cells are more enriched with saturated fatty acids (16 :0 and C18 :0). A slight decrease is
463 observed in C18:1 of around 42.62%. On the contrary, in the presence of glycerol and
464 motor oil, a slight decrease in C18:1 from 39.81 % to 35.60 % was observed.

465 Thus, the analysis of lipid composition in the present study suggested that cells are
466 enriched with saturated as well unsaturated fatty acids, especially when olive or vegetable
467 oil is used as co-substrate. These features suggest that *Y. lipolytica* possess an active
468 desaturase involved in the conversion of palmitic and stearic acid into unsaturated fatty
469 acids before their incorporation into lipid bodies (Montet et al., 1985) and the fatty acid
470 composition is highly dependent on the carbon source present in the media. When
471 vegetable oil is employed as co-substrate, more unsaturated fatty acids are noticed, which
472 is due to the fact that some fatty acids are incorporated into the cell lipids in the presence
473 of oils in the media.

474 **3.2. Use of crustacean waste to enhance more lipase production**

475 *Y. lipolytica* was also tested for the secretion of lipase and maximum activities were
476 found in the presence of crude glycerol and olive oil, vegetable oil, Tween 80, Tween 20
477 and Triton X-100 in the following order. Recent attempts have been made to increase the
478 lipase production via supplementation of medium with highly nitrogenous source.
479 Literature reports use of organic nitrogen source (i.e. urea, peptone, yeast extract) to
480 enhance lipase activity (Fickers et al., 2005 b ; Sharmaa et al., 2001). Thus, crustacean
481 waste (CW) was added into the medium as nitrogen source to improve the lipase
482 production. Usually, the nitrogen content in CW is assumed to be around 7% by weight.

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483 (Fig. 3A, B) represents the evolution of lipase production in the presence of CW,
484 surfactants and oils.
485 As seen from (Fig. 3 A, B), lipase activity drastically increased during the early stage of
486 fermentation and reached a maximum level at 48h in the presence of different oils and
487 surfactants. A higher lipase activity of 38± 3 U/mL is observed in the case of CW and
488 Tween 80 and CW and olive oil. Surprisingly, a higher activity was recorded in the
489 presence of motor oil and CW compared to previous experiments where only 5.5 U/mL
490 was obtained. Thus, motor oil composed of hydrocarbons stimulated the lipase
491 production and nitrogen rich source (CW) was required to obtain 30 U/mL at 48h. The
492 activity continued to be present in the medium and decreased around 72h. Afterwards,
493 undetectable activities were observed which is in agreement with the previous studies,
494 where no activity was observed after 72h although the biomass still increased. In the
495 presence of CW, around 38 ± 3 U/mL was obtained and this value was higher than the
496 values reported by *Candida rugosa* ATCC which produced around (2-15 U/mL)
497 (Montesinos et al., 2003) and values reported by Pereira-Meirelles et al. (2000) who
498 obtained extracellular lipase around 5- 8.5 U/mL in media enriched with both yeast
499 extract and peptone (Pereira-Meirelles et al., 2000). Besides, these activities were
500 significant compared to the activities reported in the presence of 13 g/L glycerol and
501 10 g/L peptone 1% of olive oil (12.2 U/mL) (Galvagno et al., 2011). Thus, the addition of
502 CW to the culture medium is very promising alternative to enhance the lipase production.
503 In fact, this is the first report to deal with the production of lipase in the presence of CW
504 and motor oil. Most of the reports so far investigated the possibility of using wastes such
505 as palm oil mill effluent (POME) (Louhasakul et al., 2016; Moftah et al., 2013) and other

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506 organic nitrogenous sources (Fickers et al., 2005 b, Sharmaa et al., 2001). This bioprocess
507 avoided possible glycerol inhibition on lipase in the case of motor oil where 30 U/mL
508 were obtained. Therefore, the current study provided alternative ways of valorization of
509 CW, by using it as substrate by *Yarrowia* species, in order to produce lipases enzymes,
510 thus, lipases exhibited hydrolytic activities against hydrocarbons, this fact, opens the door
511 for economical process of bioremediation oil spillage contaminated site by enzymatic
512 tools and the optimization of the lipase production should be carried out.

513 **3.3. Characterization of Lipase Produced by *Y. lipolytica***
514

515 The effect of pH on lipase produced by *Y. lipolytica* was determined at pH values ranging
516 from 6 to 8 in phosphate buffer (Fig. 4). Residual activity is expressed as a percentage of
517 the initial activity. The pH was chosen according to the literature where the lipase activity
518 is recorded in this pH range. The activity of lipase increased by increasing the pH up to
519 7.5 and the optimal showed an activity around 7 which is expected since the optimum pH
520 for growth of *Y. lipolytica* in this study is 6.5. The increase of pH leads to the decrease of
521 the activity of the enzyme by 60%. Besides, the enzyme was stable at pH f6 and 7 and
522 decreased while increasing pH values more than 7. These results are in accordance with
523 Corzo and Revah (1999), who found that the lipase activity of *Y. lipolytica* 681 was
524 higher at pH ranging from 5 to 9 and decreased in basic pH (Corzo & Revah, 1999).
525 Moreover, Brigida, et al. (2014) found that lipases from *Y. lipolytica* are active at pH
526 ranging from 6 to 10 and the optimum varied depending on the strain used (Brigida et al.,
527 2014). The effect of temperature on lipase activity has been also studied and investigated
528 at temperature ranging from 25 to 50 ^{o}C using 50mM phosphate buffer (pH=7.0). Results
529 showed that higher activity was observed at 30 ^{o}C. The maintenance of enzymes in

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530 phosphate buffer at pH 7 for 12 h resulted in high thermal stability at temperatures from 531 25 to 35 ^{o}C. Such increase in temperature to 50 ^{o}C resulted in loss of enzyme activity, 532 which justified the effect of high temperature on enzyme denaturation, responsible for its 533 loss of activity. Yadav et al. (2011) reported that the lipase from *Y. lipolytica* NCIM 3639 534 showed a higher activity at 25 ^{o}C and its activity decreased at higher temperatures 535 starting from 30 ^{o}C. (Yadav et al., 2011). However, Yu et al. (2007) showed an optimum 536 of lipase activity obtained at 40 ^{o}C and decreased at 45 ^{o}C until no activity was observed 537 at 60 ^{o}C (Yu et al., 2007). This characterization demonstrate distinct advantages of the 538 lipase produced by *Y. lipolytica* and its stable properties that permit its use in 539 biotechnologically interesting fields

540 **4. Conclusion**
541

542 The potential of *Y. lipolytica* SM7 for the biotechnological valorisation of crude glycerol 543 (industrial derivative) to single-cell oil 35% (w/w) and lipase 25 U/mL was investigated. 544 It was found that he use of crustacean residue as nitrogen source can contribute to costs 545 reduction in lipase production as the latter permitted to obtain around 38 U/mL. The 546 lipase so produced showed high thermal stability, activity in a broad range of pH values, 547 with the highest stability at a slightly acidic pH of 6. Further studies on the concomitance 548 production of cited metabolites are purposeful and higher lipase activity can be used for 549 degradation of waste lipids, as well as the synthesis of esters of fatty acids via enzymatic 550 transesterification.

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553

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558 **6. Bibilography**

559 Aksu, Z., Eren, A.T. 2005. Carotenoids production by Rhodotorula mucilaginosa: use of
560 agricultural wastes as a carbon source. *Process Biochem* **40**: 2985-91.
561 Barth, G., Gaillardin, C. 1996. Yarrowia lipolytica, In: Wolf K (ed) Nonconventional yeasts in
562 biotechnology. Springer-Verlag, Berlin, Heidelberg, New York, .
563 Benjamin, S., Pandey, A. 1996. Optimization of liquid media for lipase production by Candida
564 rugosa. *Bioresour Technol* **55**: 167-170.
565 Beopoulos, A., Cescut, J., Haddouche, R., Uribelarrea, J.-L., Molina-Jouve, C. Nicaud, J.-M. 2009.
566 *Yarrowia lipolytica* as a model for bio-oil production. *Prog. Lipid Res* 48, 375–387.
567 Bodour, A., Drees, K., Maier, R. 2003. Distribution of biosurfactant-producing bacteria in
568 undisturbed and contaminated arid southwestern soils. *Appl Environ Microbiol* **69**: 3280-
569 3287.
570 Brigida, A.I.S., Amaral, P.F.F., Coelho, M.A.Z., Goncalves, L.R.B. 2014. Lipase from Yarrowia
571 lipolityca: production, characterization and application as an industrial catalyst. *J Mol*
572 *Catal B Enzym* **101**: 148-158.
573 Corzo, G., Revah, S. 1999. Production and characteristics of the lipase from Yarrowia lipolityca
574 681. *Bioresour Technol* **70**: 173-180
575 Cüelik, E., Ozbay, N., Oktar, N., Cüalık, P. 2008. Use of biodiesel byproduct crude glycerol as the
576 carbon source for fermentation processes by recombinant Pichia pastoris. *Ind Eng Chem*
577 *Res* **47**: 2985-90.
578 Davidow, L.S., O'Donnell, M.M., Kaczmarek, F.S., Pereira, D.A., DeZeeuw, J.R, Franke, A.E. 1987.
579 Cloning and sequencing of the alkaline extracellular protease gene of Yarrowia lipolytica.
580 *J Bacteriol* **169**: 4621-4629.
581 Del, Río J.L., Serra, P., Valero, F., Poch, M., Sola, C. 1990. Reaction scheme of lipase production
582 by Candida rugosa growing on olive oil. *Biotechnol Lett* **12**: 835-838.
583 Desfougeres, T., Haddouche, R., Fudalej, F., Neuveglise, C., Nicaud, J.-M. 2010. SOA genes
584 encode proteins controlling lipase expression in response to triacylglycerol utilisation in
585 the yeast Yarrowia lipolytica. *FEMS Yeast Res* **10**, 93–103
586 Fabiszewska, A.U., Stolarzewicz, I., Zamojska, W., Białecka-Florjańczyk, E. 2014. Carbon sources
587 influencing profile of lipases produced by yeast Yarrowia lipolytica. *Appl Biochem*
588 *Microbiol* **50**:404-41.
589 Folch, J., Lees, M., Slane-Stanley, G. H. 1957. Simple method for the isolation and purification of
590 total lipids from animal tissues. *J Biol Chem **226**,* 497–509
591 Fickers, P., Benetti, P.H., Wache, Y., Marty, A., Mauersberger, S., Smit, M.S. 2005a. Hydrophobic
592 substrate utilisation by the yeast Yarrowia lipolytica, and its potential applications. *FEMS*
593 *Yeast Res* **5**, 527–543
594 Fickers, P., Fudalej, F., Nicaud, J.M., Destain, J., Thonart, P. 2005b. Selection of new over-
595 producing derivatives for the improvementof extracellular lipase production by the non-
596 conventional yeast Yarrowia lipolytica. *J Biotechnol,* **115**, 379-386.
597 Finogenova, T.V., Puntus, I.F., Kamzolova, S.V., Lunina, Y.N., Monastyrskaya, S.E., Morgunov, I.G.,
598 Boronin, A.M. 2008. Mutant Yarrowia lipolytica strains producing citric acid from
599 glucose. *Appl Biochem Microbiol* **4**: 197-202.
600 Galvagno, M.A., Iannone, L.J., Bianchi, J., Kronberg, F., Rost, E., Carstens, M.R., Cerrutti, P. 2011.
601 Optimization of biomass production of a mutant of Yarrowia lipolytica with a an
602 increased lipase activity using raw glycerol. *Rev Argent Microbiol* **43**: 218-225.

ACCEPTED MANUSCRIPT

603 Goncalves, F.A.G., Colen, G., Takahashi J.A. 2013. Optimization of cultivation conditions for
604 extracellular lipase production by Yarrowia lipolytica using response surface method. *Afr*
605 *J Biotechnol* **12**: 2270-2278.
606 Kamzolova, S.V. , Morgunov, I.G., Aurich, A., Perevoznikova, O.A., Hishkanova, N.V.S,
607 Stottmeister, U., Finogenova, T.V. 2005. Lipase secretion and citric acid production in
608 Yarrowia lipolytica yeast grown on animal and vegetable fat. *Food Technol Biotechnoll*
609 **43**: 113-122.
610 Kruszewska, J., Palamarczyk, G., Kubicek, C.P. 1990. Stimulation of exoprotein secretion by
611 choline and Tween 80 in Trichoderma reesei QM 9414 correlates with increased
612 activities of dolichol phosphate mannose synthase. *J Gen Microbiol* **136**: 1293-8.
613 Lee, G.H., Bae, J.H., Suh, M.J., Kim, I.H., Hou, C.T., Kim, H.R. 2007. New finding and optimal
614 production of a novel extracellular alkaline lipase from Yarrowia lipolytica NRRL Y-2178.
615 *J Microbiol Biotechnol* **17**: 1054-7.
616 Lin, S.F., Chiou, C.M., Tsai, Y.C. 1995. Effect of Triton X-100 on alkaline lipase production by
617 Pseudomonas pseudoalcaligenes F-111. *Biotechnol Lett* **17**: 959-962.
618 Lopes, D.B., Fraga, L.P., Fleuri, L.F., and Macedo, G.A. 2011. Lipase and esterase: to what extent
619 can this classification be applied accurately? *Braz J Food Technol* **31**, 603–613.
620 Louhasakul, Y., Cheirsilp, B., Prasertsan, P. 2016. Valorization of Palm Oil Mill Effluent into Lipid
621 and Cell-Bound Lipase by Marine Yeast Yarrowia lipolytica and Their Application in
622 Biodiesel Production. *Wast Biomass Valor* **7**: 417-426.
623 Mafakher, L., Mirbagheri, M., Darvishi, F., Nahvi, I., Zarkesh-Esfahani, H., Emtiazi, G. 2010.
624 Isolation of lipase and citric acid producing yeasts from agro-industrial wastewater. *N*
625 *Biotechnol* **27**: 337-340.
626 Magdouli, S., Brar, S.K., Blais, J. F. 2016. Lipid production by Yarrowia lipolytica grown on
627 biodiesel-derived crude glycerol: Optimization of growth parameters and their effects
628 on the fermentation efficiency. *RSC Adv* **6**: 90547-90558
629 Meira, M., Santana, P.M.B., Araújo, A.S., Silva, C. L., Filho, J.R.L.L., Ferreira, H.T. 2014. Oxidative
630 degradation and corrosiveness of biodiesel. *Corros Rev* **32**: 143-161.
631 Mlickova, K., Roux, E., Athenstaedt, K., d'Andrea S., Daum, G., Chardot, T., Nicaud, J.M. 2004.
632 Lipid accumulation, lipid body formation, and acyl coenzyme A oxidases of the yeast
633 Yarrowia lipolytica. *Appl Environ Microbiol* **70**: 3918-3924.
634 Moftah, O., ŽGrbavčić, S., Moftah, W.A.S., Knežević-Jugović, Z. 2013. Lipase production by
635 Yarrowia lipolytica using olive oil processing wastes as substrates. *J Serb Chem Society*
636 **78**: 781-794.
637 Montesinos, J.L., Dalmau, E., Casas, C. 2003 Lipase production in continuous culture of Candida
638 rugosa. *J Chem Technol Biotechnol* **78**: 753-761.
639 Montet, D., Ratomahenina, R., Galzy, P., Pina, M. and Graille, J. (1985) A study of the influence
640 of the growth media on the fatty acid composition in *Candida lipolytica* DIDDENS and
641 LODDER. *Biotechnol Lett* **7**, 733–736
642 Najjar, A., Robert, S., Gu'erin, C., Violet-Asther, M., Carriere, F. 2011. Quantitative study of lipase
643 secretion, extracellular lipolysis, and lipid storage in the yeast Yarrowia lipolytica grown
644 in the presence of olive oil: analogies with lipolysis in humans. *Appl Microbiol Biotechnol*
645 **89**: 1947-1962.
646 Papanikolaou, S., Muniglia, L., Chevalot, I., Aggelis, G. and Marc, I. 2003. Accumulation of a
647 cocoa-butter-like lipid by *Yarrowia lipolytica* cultivated on agro-industrial residues. *Curr*
648 *Microbiol* **46**, 124–130.
649 Papanikolaou, S., Aggelis, G. (2010) *Yarrowia lipolytica*: a model microorganism used for the
650 production of tailor-made lipids. *Eur J Lipid Sci Technol* **112**, 639–654

ACCEPTED MANUSCRIPT

651 Papanikolaou, S., Sarantou, S., Komaitis, M. 2004. Repression of reserve lipid turnover
652 in Cunninghamella echinulata and Mortierella isabellina cultivated in multiple-limited
653 media. *J Appl Microbiol* **97**, 867–875
654 Papanikolaou, S., Muniglia, L., Chevalot, I., Aggelis, G. and Marc, I. 2002. Yarrowia lipolytica as a
655 potential producer of citric acid from raw glycerol. *J Appl Microbiol* **92**, 737–744.
656 Papanikolaou, S., Chevalot, I., Galiotou-Panayotou, M., Komaitis, M., Marc, I., Aggelis, G. 2007.
657 Industrial derivative of tallow: a promising renewable substrate for microbial lipid,
658 single-cell protein and lipase production by Yarrowia lipolytica. *Electron J Biotechn* **10**:
659 425-35.
660 Papanikolaou, S., Galiotou-Panayotou, M., Fakas, S., Komaitis, M., Aggelis, G. 2008. Citric acid
661 production by Yarrowia lipolytica cultivated on olivemill wastewater-based media.
662 *Bioresour Technol* **99**: 2419-2428.
663 Pereira-Meirelles, F.V., Rocha-Leao, M.H.M., Sant'Ana, G.L. Jr. 2000. Lipase location in Yarrowia
664 lipolytica cells. *Biotechnol Lett* **22**: 71-75.
665 Pignede, G., Wang H.J., Fudalej, F., Seman, M., Gaillardin, C., Nicaud, J.M., 2000. Autocloning
666 and amplification of LIP2 in Yarrowia lipolytica," *Appl Environ Microbiol* **66**, 3283–3289.
667 Royer, A., Gerard, C., Naulet, N., Lees, M., and Martin, G. J. 1999. "Stable isotope
668 characterization of olive oils. I. Compositional and carbon-13 profiles of fatty acids," *J*
669 *Am Oil Chem Soc* **76**, 357–363.
670 Rywińska, A., Juszczyk, P., Wojtatowicz, M., Robak, M., Lazar, Z., Tomaszewska, L., Rymowicz, W.
671 2013. Glycerol as a promising substrate for Yarrowia lipolytica biotechnological
672 applications. *Biomass Bioenergy* **48**: 148-166.
673 Saenge, C., Cheirsilp, B., Suksaroge, T., Bourtoom, T. 2011. Potential use of oleaginous red yeast
674 Rhodotorula glutinis for the bioconversion of crude glycerol from biodiesel plant to
675 lipids and carotenoids. *Process Biochem* **46**:210-218.
676 Sharmaa, R., Chistib, Y., Banerjeea, U.C. 2001. Production, purification, characterization, and
677 applications of lipases. *Biotechnol Adv* **19**: 627-62.
678 Szczesna-Antczak, M., Antczak, T., Piotrowicz- Wasiak, M., Rzyska, M., Binkowska, N., Bielecki, S.
679 2006. Relationships between lipases and lipids in mycelia of two Mucor strains. *Enzym*
680 *Microb Technol* **39**: 1214-1222.
681 Volpato, G., Rodrigues, R.C., Heck, J.X., Ayub M.A.Z. 2008. Production of organic solvent tolerant
682 lipase by Staphylococcus caseolyticus EX17 using raw glycerol as substrate. *J Chem*
683 *Technol Biotechnol* **83**: 821-828.
684 Wynn, J.P. and Ratledge, C. 2000. Evidence that the rate-limiting step for the biosynthesis of
685 arachidonic acid in Mortierella alpina is at the level of the 18:3 to 20:3 elongase.
686 *Microbiology* **146**, 2325–2331.
687 Yadav, K.N.S., Adsul, M.G., Bastawde, K.B., Jadhav, D.D., Thulasiram, H.V., Gokhale, D.V. 2011.
688 Differential induction, purifi- cation and characterization of cold active lipase from
689 Yarrowia lipolytica NCIM 3639. *Bioresour Technol* **102**: 10663-10670
690 Yu, M.R., Lange, S., Richter, S., Tan, T.W., Schmid, R.D. 2007. Highlevel expression of
691 extracellular lipase Lip2 from Yarrowia lipolytica in Pichia pastoris and its purification
692 and characterization. *Protein Expr Purif* **53**: 255-263.
693 Zhang, X., Yan, S., Tyagi, R.D. 2015. Strategies of preserving lipids in microorganism after
694 fermentation. *Bioresour Technol* **192**: 718-25.

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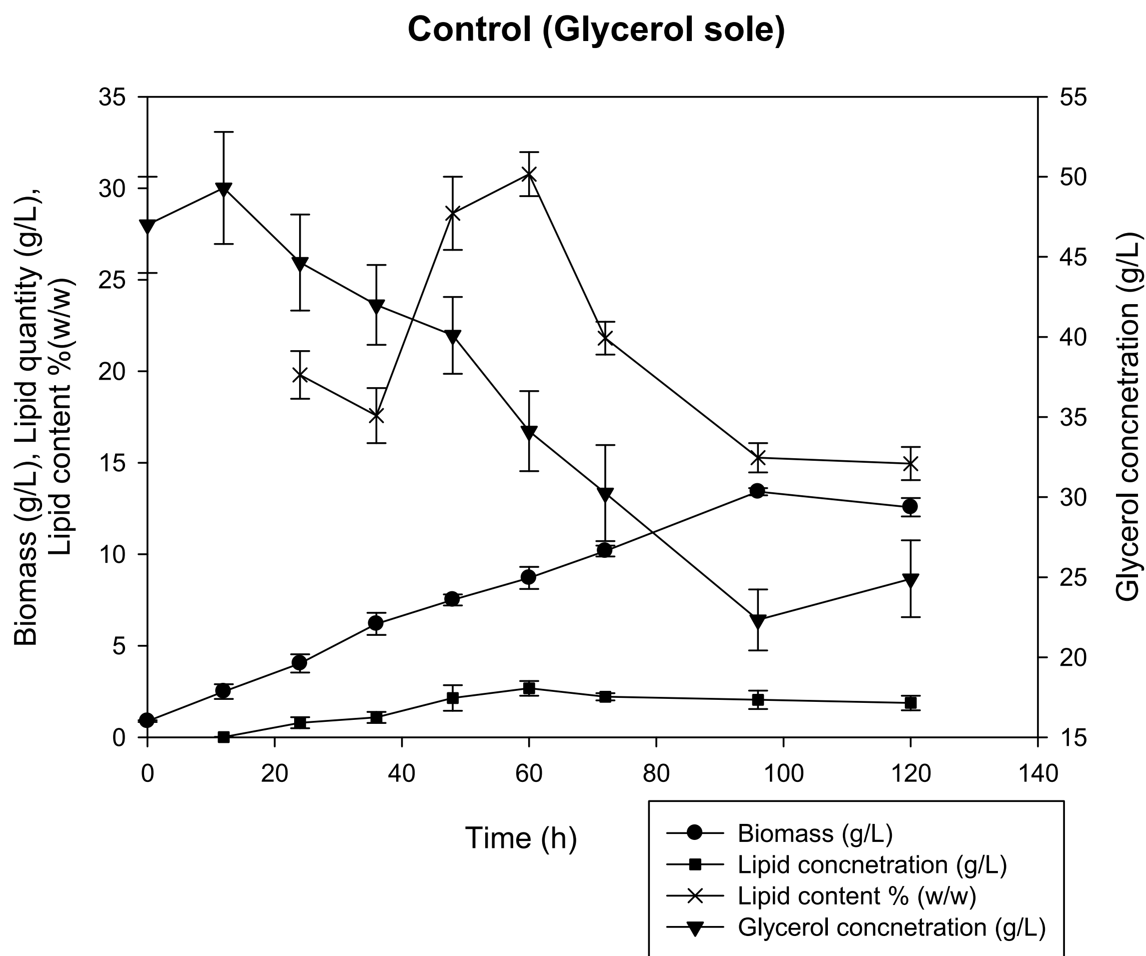

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699 Fig. 1 Growth Kinetics of Yarrowia lipolytica grown at initial pH=6.5 and T 28°C in the presence of
700 glycerol (A) and on a mixture of crude glycerol and olive oil

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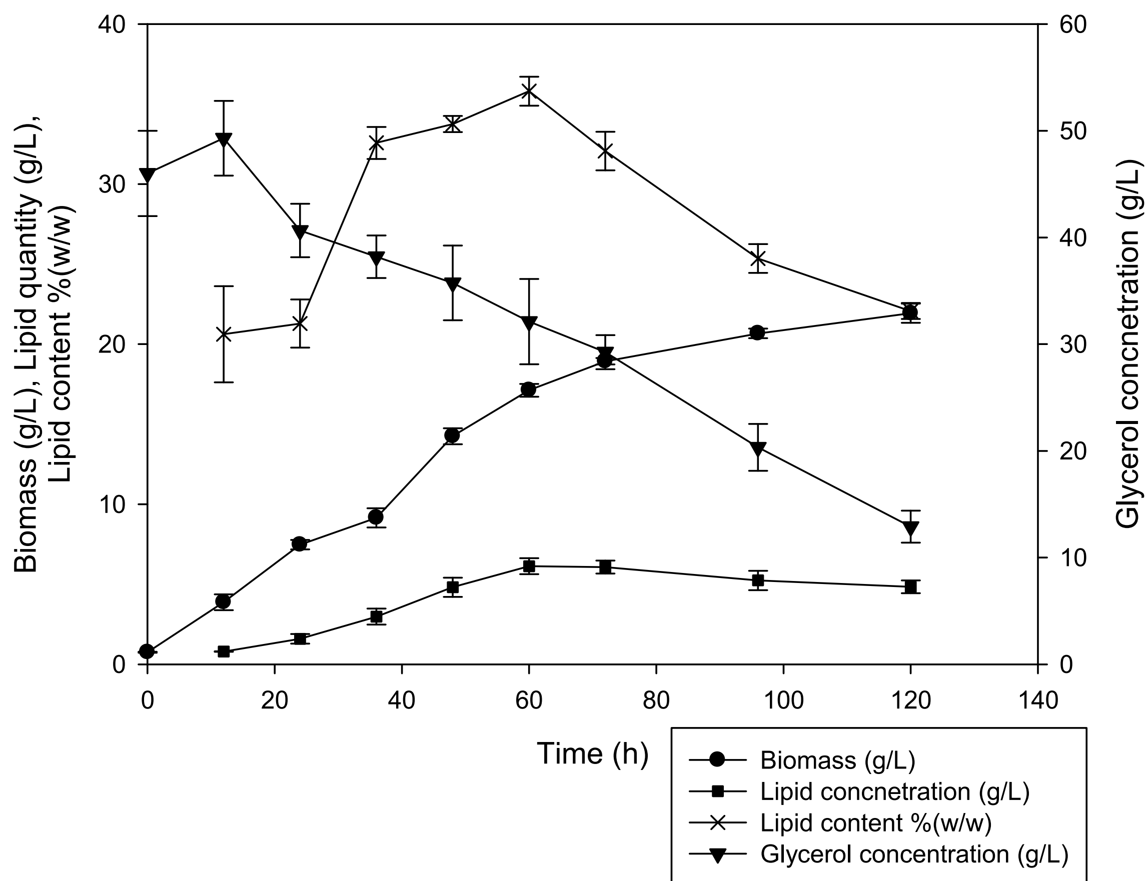

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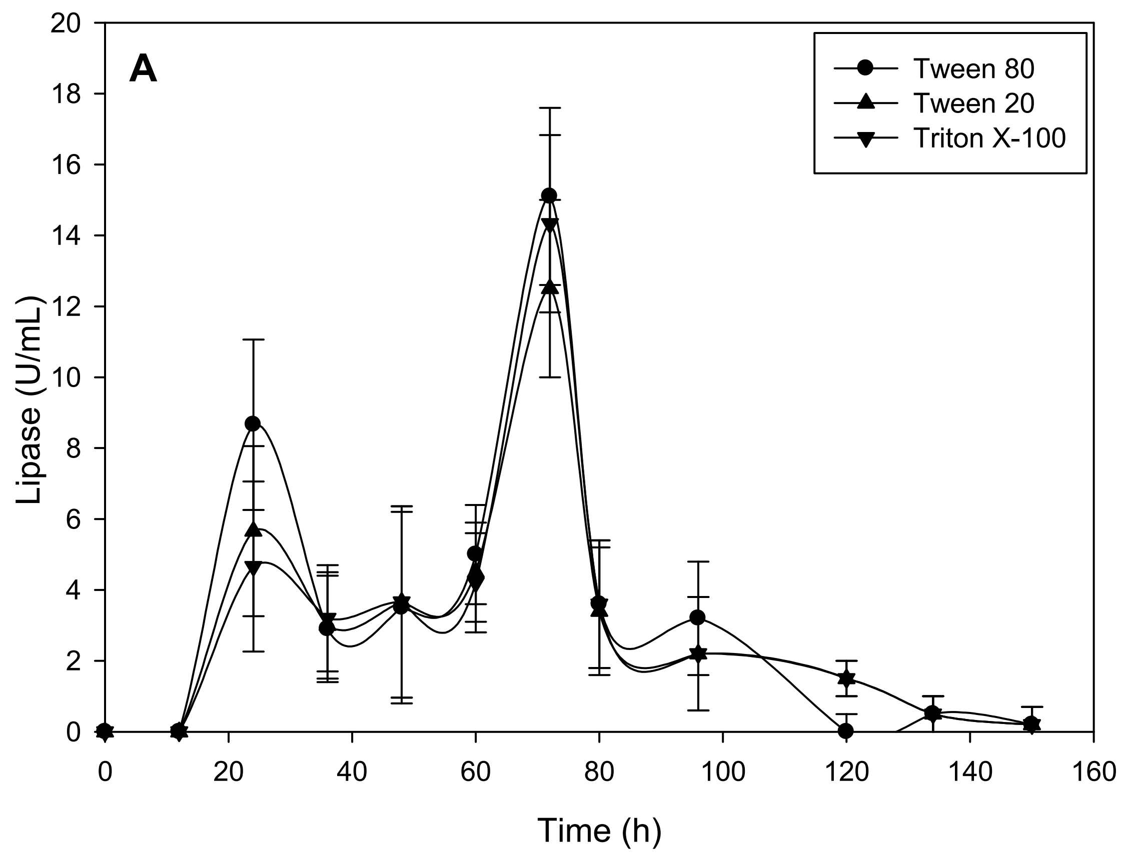

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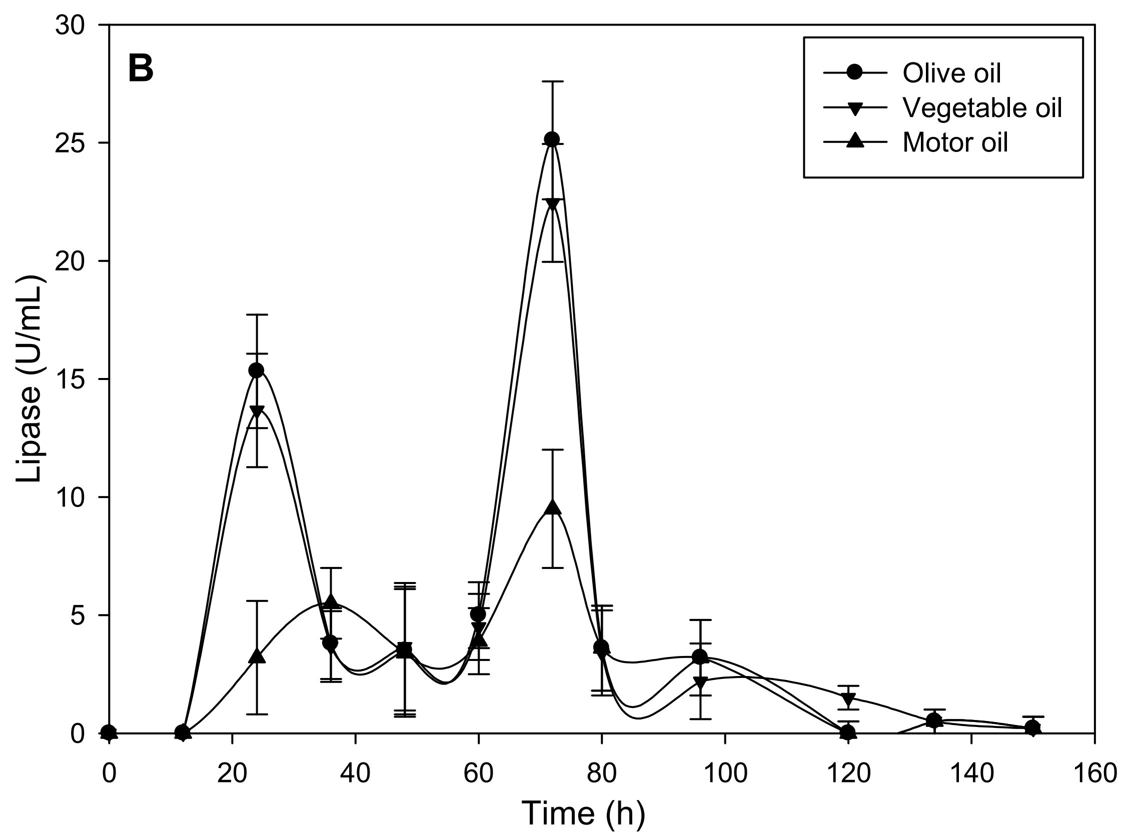

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707 Fig.2. Lipase activity in the presence of surfactants and oils as inducers (A: in the

708 presence of surfactants, B: in the presence of oils)

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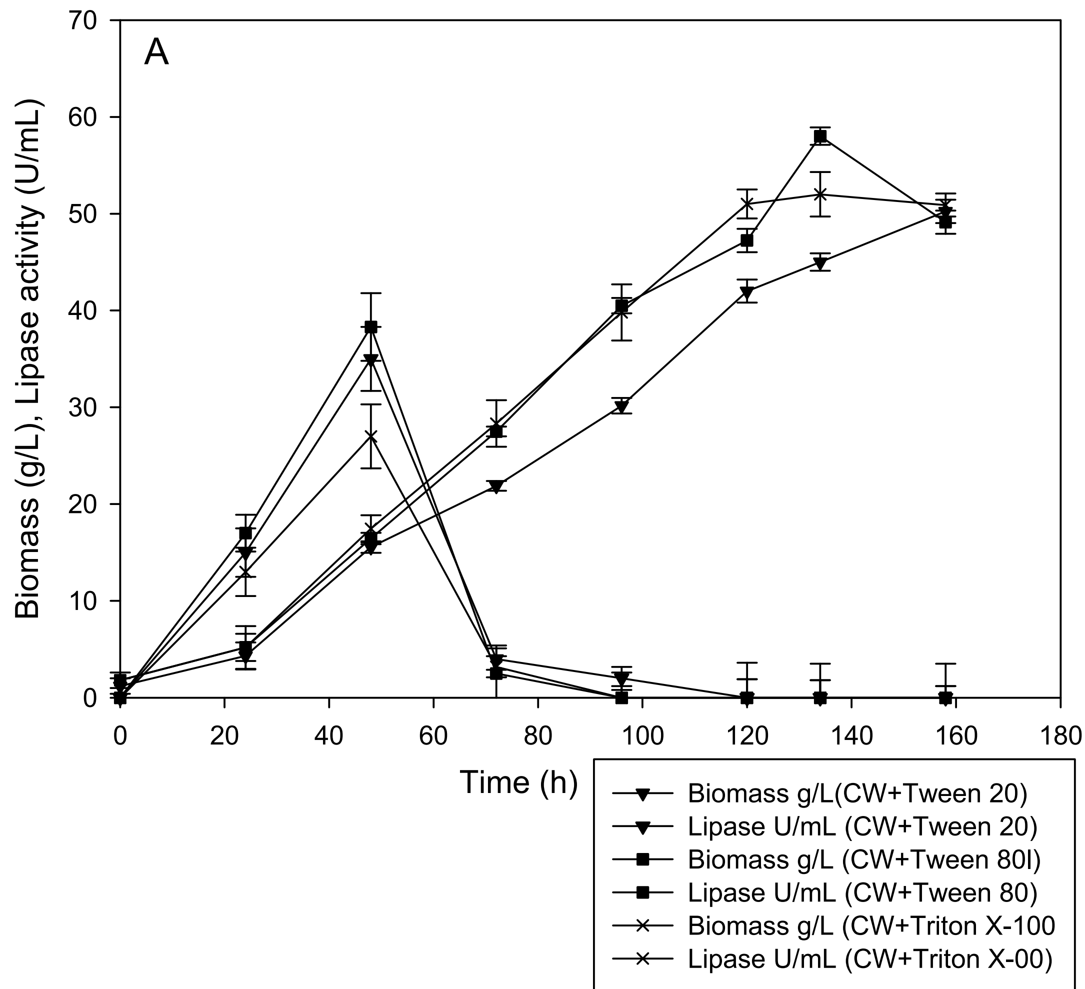

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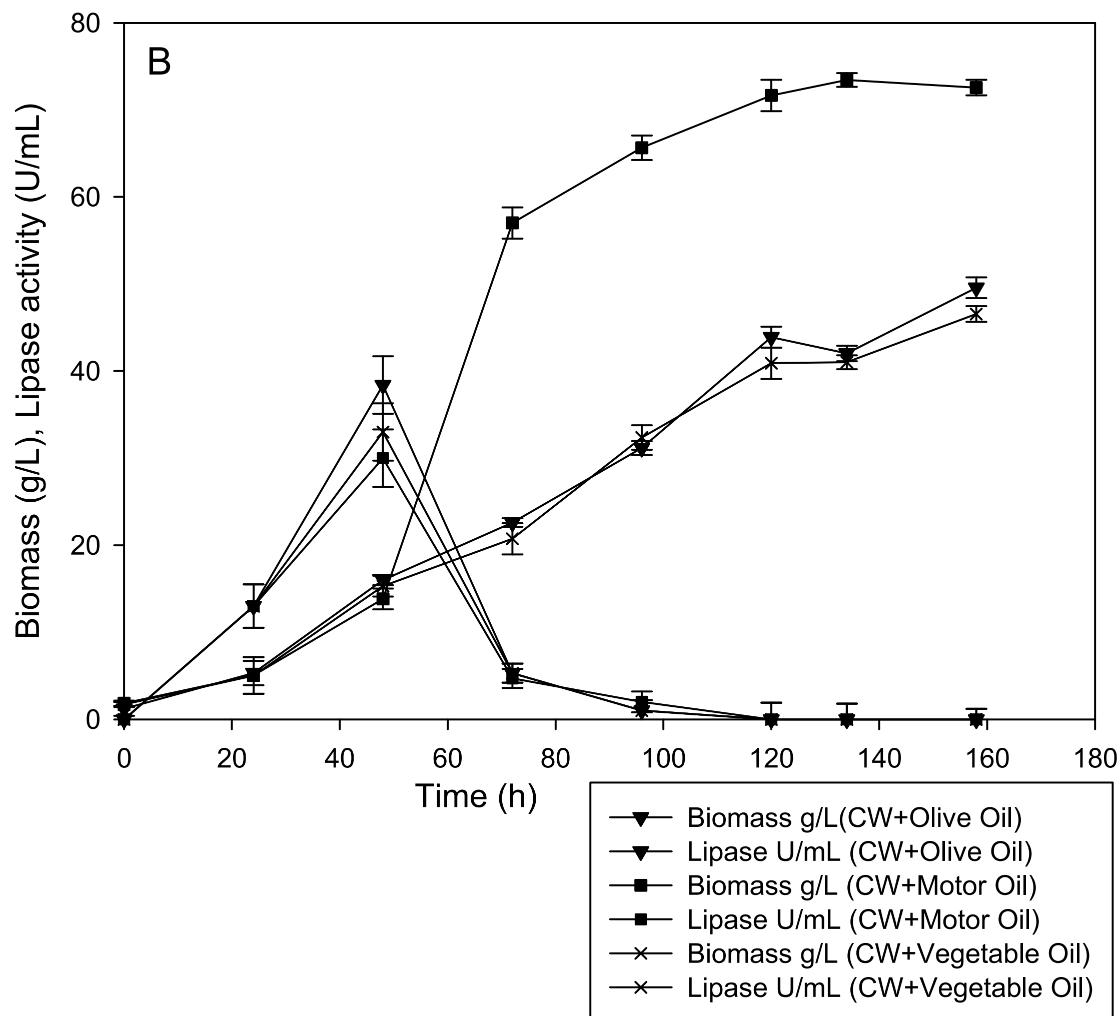

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718 Fig. 3. Evolution of biomass production and lipase activity in the presence of crustacean

719 wastes and different inducers (A: in the presence of surfactants, B: in the presence of

720 oils), crustacean waste (CW),

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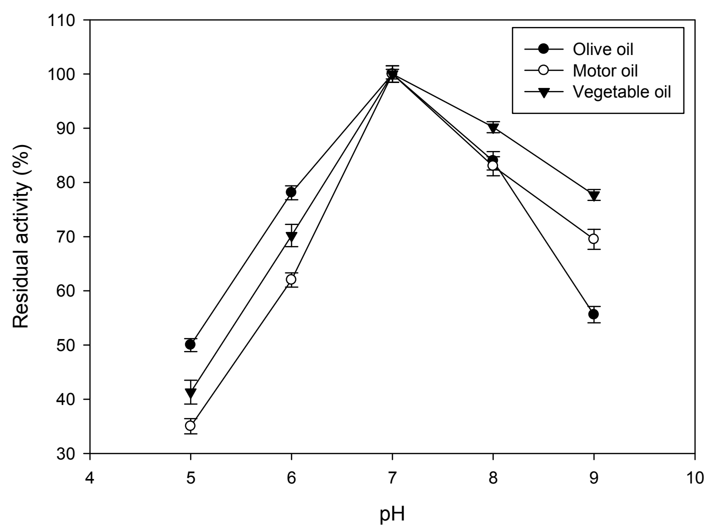

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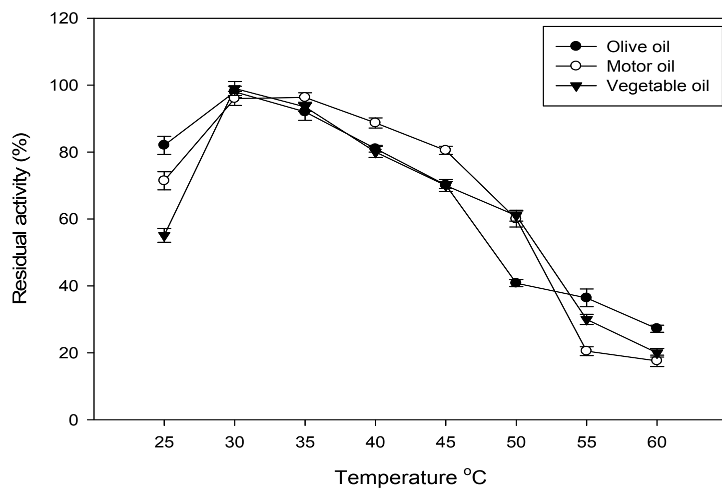

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725 Fig. 4. Residual activity of crude lipase as a function of time.

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729 Table 1 Effect of surfactants on metabolites production of *Y. lipolytica* cultured in crude

730 glycerol

Surfactants	Biomass (g/L)	Lipid (g/L)[a]	Lipid content (%)	Lipase activity (U/mL)[b]	CA (g/l)[c]
Control	8.70	2.68	30.90	2-4	1.2
Tween 20	16.55	5.48	33.12	12.50	2.2
Tween 80	16.97	5.94	35.06	15.10	3.3
Triton X-100	14.73	5.10	34.62	14.33	3.5

731

732 a) the maximum lipid quantity was observed at 60h
733 b) the maximum lipase activity is observed at 72h
734 c) the maximum CA observed at 100h

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737 Table 2 Fatty acid composition of *Y. lipolytica* grown on crude glycerol in the presence of

738 differents inducers. Analyses were performed in duplicates

Media	C14 :0	C16 :0	C18 :0	C18 :1	C18 :2
Glycerol (control)	4.05	14.40	10.80	39.81	5.38
Glycerol+Tween 80	4.10	17.36	16.30	47.5	7.50
Glycerol+Tween 20	4.15	16.20	14.40	42.5	6.41
Glycerol+TritonX-100	3.68	18.12	13.25	45.0	6.50
Glycerol+Olive oil	6.78	22.0	17.05	48.3	8.90
Glycerol+Vegetable oil	5.85	20.0	15.82	42.62	7.66
Glycerol+Motor oil	2.20	9.25	12.0	35.60	5.14

739 Other fatty acids such as linolenate (C18:3n3), arachidic (C20:0), and Lignoceric (C24:0) are

740 detected in minor quantities.

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743 Table 3 Effect of oil on metabolites production of *Y. lipolytica* cultured in crude glycerol
744 media

Surfactants	Biomass (g/L)	Lipid (g/L)[a]	Lipid content (%)	Lipase activity (U/mL)[b]	CA (g/l)[c]
Control	8.70	2.68	30.90	2-4	1.2
Olive oil	17.12	6.13	35.80	25.10	1.7
Vegetable oil	17.06	5.64	33.06	22.45	2.3
Motor oil	10.25	3.33	32.48	5.50	0.5

745

746 a) the maximum lipid yield was observed at 60h
747 b) the maximum lipase activity is observed at 72h
748 c) the maximum CA observed at 100h
749
750

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