1 **Sync between leucine, biotin and citric acid to improve lipid production**

2 **by *Yarrowia lipolytica* on crude glycerol-based media**

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27 **Abstract**

28 The use of biodiesel-derived glycerol as a carbon source for single cell oil (SCO) production is a
29 biorefinery engineering strategy that aims to valorize the by-product waste and make the microbial
30 lipid production process more cost-effective. This work aimed to improve the capacity of *Yarrowia*
31 *lipolytica* to produce large amounts of lipids and replace genetic engineering by a metabolic
32 approach based on the stimulation of the rate-limiting enzymes and reducing the activation energy
33 thereby increasing the rate of lipids synthesis. The effects of biotin and leucine addition on the lipid
34 content of *Y. lipolytica* have been investigated. The lipid content of *Y. lipolytica* was strongly
35 influenced by the addition of biotin. In fact, an increase in biotin concentration from 25 µg/L to
36 200 µg/L practically increased the lipid concentration up to 15 g/L. Besides, to channel metabolic
37 flux into lipid biosynthesis, the addition of citric acid as lipid precursor led to an increase in total
38 catabolism activation and lipid accumulation to reach around 63% (w/w). The biochemical approach
39 can be a useful target for improving the efficiency of lipid-producing yeast strain rather than genetic
40 engineering.

41

42 **Keywords:** *Yarrowia;* citric acid; glycerol; biorefinery; lipid accumulation.

43

44 ## 1 Introduction

45 Owing to the high productivity, justified by the shorter duplication time and higher lipid content
46 compared to vegetable oils (up to 70% on dry weight) [1-2], it is clear that microorganisms
47 accumulating lipids above 20% of the biomass on a dry basis represent an ideal source of lipids. Thus,
48 concerns about energy supply have driven the development and the production of microbial-derived
49 biodiesel. Currently, microbial lipids provide a unique platform for biodiesel, hydrocarbons
50 production through the thermochemical processes such as pyrolysis. Latter, hydrocarbons are
51 considered a potential source for biofuels [3-4]. In fact, oleaginous microorganisms contributed
52 efficiently to fuel oils and responded sufficiently to global needs [5-6]. Accordingly, increasing lipid
53 accumulation by oleaginous microorganisms has attracted significant attention as single cells oils
54 (SCO) can serve as an important source of petroleum diesel replacement and other oleochemicals
55 [7-10]. However, the current productivities of microorganisms-based processes are still very low,
56 which has restricted their scale-up application. Although the extensive works carried out on lipid
57 accumulation in oleaginous microorganisms, the efficient microbial production of lipids at the
58 commercial stage is still challenging and even though the substantial improvements achieved in lipid
59 production with *Y. lipolytica,* up to now, only high-value lipids are economically feasible and able to
60 reach the market. Consequently, developing a bioprocess with higher lipid production from
61 renewable carbon source with low cost is very promising since these bio-oils microbially produced
62 do not compete with food feedstock production and do not require arable lands [11]. Generally,
63 lipid overproduction has been performed through genetic modifications, however, the genetic
64 engineering and the overexpression of one or few key genes involved in lipid biosynthesis results on

65 a modest increase in the lipid content [12] and engineered strains are usually recognized with their
66 poor stability during scale-up.
67 Magdouli et al. stated that higher lipid production is a balance between metabolic and genetic
68 approaches [2,10]. Unlike genetic modification, the metabolic approach relies on phenotypic
69 screening and does not require specific knowledge of molecular targets in metabolic and catabolic
70 pathways involved in the synthesis of lipid droplets. In addition to various efforts in strain
71 improvement and cultivation optimization, it was proposed that the higher lipid productivity can
72 also be achieved using various biochemical and metabolic engineering strategies to enhance cell
73 growth and lipid accumulation. Thus, various metabolic triggers or enhancers can directly modulate
74 cellular metabolism and can be applied to improve lipid productivity. Moreover, the application of
75 cheap renewable sources in large-scale cultures with low concentrations and lower costs could be a
76 valuable and practical approach for addressing the low productivity issue. Recently, numerous
77 studies have shown that the lipid accumulation in oleaginous microorganisms is an adaptative
78 response to particular environmental factors and the concentration and quality of lipids can vary as
79 a result of changes in growth conditions [temperature and pH, dissolved oxygen or nutrient media
80 characteristics [carbon source concentration, C/N ratio, macro and micro nutrients] [13-16]. Besides,
81 the examination of enzymatic activity and comparative genomics suggests that ATP: citrate lyase
82 [ACL] may play a crucial role in directing excess carbon to be stored as lipids rather than
83 carbohydrates in oleaginous yeasts [17-18]. Moreover, acetyl-CoA carboxylase [ACC] activity is
84 reported to increase during the lipid biosynthesis [19-20]. ACC is a biotin-dependent enzyme that
85 catalyzes the carboxylation of pyruvate to oxaloacetate and acetyl-CoA to malonyl-CoA.
86 Additionally, leucine is a precursor to β-hydroxy-β-methylglutaryl CoA, an intra-mitochondrial

87 intermediate which is reported as a precursor of sterols and helps to the generation of acetoacetate
88 and acetyl CoA. Their mechanism is presented in Fig. 1.

89 The activity of β-hydroxy-β-methylglutaryl CoA is reduced when yeast grows on media lacking or
90 deficient in biotin. The decreased activity of acetyl CoA carboxylase has been reported to lead to
91 reduced lipid content due to biotin deficiency [21-22]. Recently, the highest lipid content of 39% was
92 produced by the culture of *Metschnikowia pulcherrima* without yeast extract but with a
93 supplementation of biotin and nitrogen [23]. Besides, Yu et al. [24] have illustrated the pivotal role
94 of many chemicals to enhance microalgal growth and accumulation of high-value bioproducts and
95 Wahbi et al. [25] have patented many types of bio-modulators to increase biofuel production in
96 microalgae. To the author's knowledge, no study was reported in yeasts to increase lipid through
97 modulators addition as done with microalgae and in crude glycerol based-media in particular.

98 In this regard, this work aimed to increase the lipid content in yeast *Y. lipolytica* through
99 biomodulators addition. Herein, the biomodulators include amino acids (i.e.leucine), vitamin (i.e
100 biotin) and as well as citric acid. Accordingly, citric acid (CA) is defined as a key precursor in lipid
101 accumulation and during the metabolic shift between growth and lipid production, a transient
102 citrate excretion step occurred [26]. More often, CA has been reviewed to activate allosterically ACC
103 enzyme [27]. Moreover, the metabolism of CA and lipid accumulation are competitive and both
104 phenomena required a nitrogen limitation [28]. Despite the current knowledge on both
105 mechanisms, many points arise as to how oleaginous microorganisms could coordinate both steps
106 and metabolic shift from oxidative to CA production over lipid accumulation still need to be
107 elucidated. Hence, the idea was to direct the carbon dissipation from CA production towards lipid
108 accumulation. Blocking the activities of tricarboxylic-acid, glyoxylate cycle enzymes and CA transport

109 through mitochondrial is potential [29]. However, this approach remains complex and costly. Thus,

110 a feed batch strategy is proposed in the current study to block CA production [feed-back inhibition]

111 and strengthen the effect of biomodulators. Moreover, Blazek et al. (2018) reported a link between

112 leucine signalling and lipogenesis in *Y. lipolytica* [30]. Biotin is reported to enhance the production

113 of alkaloids and lipids [21]. Taken all together, the present study aimed to evaluate how low

114 concentrations of bio-modulators supplied at an early stage combining with a dissolved oxygen shift

115 strategy [31] can induce lipid accumulation in *Y. lipolytica*. The current study aims to enhance *de*

116 *novo* lipid accumulation in *Y. lipolytica* through leucine-biotin mediated approach and the addition

117 of CA as lipid precursor for high lipogenesis in a crude glycerol-based media. This work opens a new

118 avenue towards economically viable microbial fuels and chemicals.

119

120 # 2 Materials and methods

121 ## 2.1 Strain

122 The yeast *Y. lipolytica* SM7, isolated from the woody forest and selected as an oleaginous yeast in
123 previous studies [31-33] was maintained in stock cultures on agar slants at 4°C containing [g/L]: 20
124 glucose, 5 yeast extract, 10 malt extract, and 20 agar, pH = 6.5. This strain was used in the current
125 study and its higher potential to accumulate lipids was investigated.

126 ## 2.2 Inoculum preparation

127 The yeast *Y. lipolytica* SM7 stored at 4°C was reactivated on potato dextrose agar Petri dishes for
128 24 h [31]. Subsequently, a sloop of reactivated microbial was transferred to Erlenmeyer flasks
129 containing the growth medium (5% (v/v) inoculum). The culture medium used for inoculum was
130 (GPY) composed of (g/L): Glucose 20, Peptone 20 and Yeast extract 10 named as at pH = 6.5. The
131 inoculum preparation process is presented in Fig. 2. Experiments were conducted in mineral
132 medium composed of: crude glycerol, 0.54 g/L NH_4OH,1 g/L yeast extract, 3 g/L K_2HPO_4, 3 g/L
133 $NaH_2PO_4.H_2O$, 0.5 g/L $MgSO_4.7H_2O$, 0.040 g/L $ZnSO_4.7H_2O$, 0.016 $FeSO_4.7H_2O$, 0.25 µg/L biotin,
134 pH = 6.5, maintained at 28°C in flasks agitated at 180 rpm for 72 h.

135 ## 2.3 Fermentation conditions

136 To evaluate the effect of modulators at large scale, fermentation was conducted in the 5-L fermenter
137 (Biostat B plus, Sartorius) to improve lipid production in crude glycerol-based media. pH-electrode
138 (Mettler Toledo, USA) was calibrated using buffers of pH = 4 and 7 (VWR, Canada). Before the
139 sterilization cycle, the oxygen probe was calibrated to zero (using sodium thiosulfate water) and
140 100% (air-saturated water). Propylene glycol (Sigma-Canada) was used as an anti-foam agent. The

141 fermenter with the medium was then sterilized in situ at 121°C for 20 min. After the fermenter
142 cooled down to 28°C, the DO probe was recalibrated to zero and 100% saturation by sparging N_2 gas
143 and air, respectively, at an agitation rate of 250 rpm. The pH of the fermenter solution was adjusted
144 to 6.5 with 4 N H_2SO_4. Thereafter, sterilized crude glycerol [83% w/v, c koi] and the mineral solution
145 was transferred to the fermenter under aseptic conditions. Agitation at 250 rpm was carried out to
146 mix the solution, after mixing, pre-culture of *Y. lipolytica* was added to the fermenter [31]. During
147 fermentation, the airflow rate was kept constant at 2.5 L/min. The agitation rate was varied during
148 fermentation to keep the DO above 30% saturation. The temperature was around 28 ± 2°C and was
149 kept constant during fermentation.

150 **2.4 Analytical methodology**

151 ***2.4.1 Biomass determination***

152 Biomass was determined gravimetrically [34]. Briefly, the samples were centrifuged at 5,000 x *g* and
153 the cells were washed twice to remove residual glycerol. Remaining pellets were frozen and
154 lyophilized (VirTis Lyophilizer, USA) for 36 h. Cell dry weight was calculated by subtracting the weight
155 of the biomass before and after lyophilization.

156 ***2.4.2 Glycerol consumption***

157 For the measurement of glycerol and others organics acids in the broth, LC/MS/MS technique was
158 employed [35]. The technical details of the LC/MS/MS instrument used for the analysis were: (a) for
159 sugar estimation: Thermo TSQ Quantum model, equipped with an Electrospray Ionization (ESI) in
160 negative ion mode; Zorbax Carbohydrate [4.6 mm x 150 mm; 5 mm, Agilent] analytical column; 75%

161 acetonitrile; 0.1% NH_4OH; 25% water and 0.1% NH_4OH mobile phase and 10 mL injection volume.

162 Glycerol and citric acid [all from Sigma] were used as the internal standards.

163 ***2.4.3 Determination of lipids and the fatty acid profile [FAME]***

164 Lipids from previously lyophilized biomass were extracted according to the method of Bligh and Dyer
165 (1959), with modifications. Resulting biomass was vortexed with methanol: chloroform for
166 extraction of lipids, followed by centrifugation at 785 x *g* for 10 min for phase separation; the lower
167 phase contained the lipids dissolved in chloroform was evaporated and dried at 60ºC to measure
168 the dry lipid mass. The extracted lipid fraction was esterified to obtain the fatty acid methyl esters.
169 For fatty acid, the analysis was performed essentially as described earlier [31]. FAMEs were
170 subsequently analyzed by GC–MS (Perkin Elmer, Clarus 500). The dimensions of the column used
171 were 30 m × 0.25 mm, with a phase thickness of 0.25 µm. A 37-component FAME mix (47885-U, 37
172 Component FAME Mix; Supelco, Bellefonte, PA, USA) was used for qualitative analysis. 1.3-
173 dichlorobenzene was used as an internal standard to quantify the fatty acid methyl esters. The
174 results are expressed as a mean ± standard deviation. The results were compared by variance
175 analysis (ANOVA) at 5% significance using Excel's Analysis ToolPak.

176

177 # 3 Results and discussion

178 ## 3.1 Effect of C/N ratio on cell growth and lipid accumulation in *Y. lipolytica*

179 Recent studies have shown that *Y. lipolytica,* a potentially attractive organism, can grow at higher
180 C/N ratio with higher glycerol concentrations [31-33], this fact seems to be strain-dependent and
181 many strains belonging to *Yarrowia* species can tolerate up to 200 g/L of glycerol and can achieve
182 efficient cell growth ranging from 16.5–26.5 g/L [36]. The selected strain used in this study can
183 accumulate up 52 ± 4% in the presence of crude glycerol of 89 g/L and C/N = 75 at 66 h and a further
184 increase of C/N will not increase the lipid content as well as biomass, Fig. 3.

185 The upper limit of lipid content in *Y. lipolytica* SM7 is 47.7% (w/w) in shake flasks at C/N = 75 and
186 higher C/N ratio will lower the biomass content and the lipid content, for example for higher
187 C/N = 200, lipid content and biomass decreased and reached around 7.37 g/L and 24.4% (w/w).
188 Meanwhile, at C/N = 120, 9.54 g/L of dry biomass and a lipid content of 43.2% (w/w) was obtained.
189 As shown earlier, a higher C/N ratio above 200 will not be suitable to achieve the higher lipid content
190 and the metabolism of the strain seemed to be inhibited by higher glycerol concentration. These
191 observations agree with Karamerou et al. [15], who found that a glycerol concentration of 80 g/L
192 and C/N = 182 resulted in lower biomass 4.62 g/L and a lipid content of 17.4% (w/w) in *Rhodotula*
193 *glutinis.* Consequently, to trigger lipid accumulation, the C/N ratio should be adjusted according to
194 the requirements of the cell. Thus, the amount of carbon in the medium must be higher than that
195 required for growth and maintenance of the yeast so that there is a surplus for lipids synthesis.
196 However, this surplus cannot exceed since high glycerol concentration up to 150 g/L represses the

197 growth [32,37-38] and glycerol at 100 g/L will lead to lower consumption efficiency and more than
198 50% of glycerol will be left unconsumed (Fig. 4).

199 To increase the lipid productivity, metabolic alternatives can be suggested through the optimization
200 of the two phases: growth (biomass) and lipogenesis phases. The variation of physicochemical
201 parameters, such as temperature, pH and nutrient composition of the growth medium is crucial and
202 may impact positively the lipogenesis [15-16]. In the current study, the first phase will be devoted
203 to stimulating the growth of strain and biomass production within a higher oxygen supply (60%),
204 followed by the second phase of lipid accumulation that consists on applying lower oxygenation
205 stress (30%) or/and some modulators. Besides, the application of biological modulators under
206 nitrogen-limited conditions aimed to stimulate the production of lipids. The choice of the type of
207 stress, biomodulators type and quantity and their timing supply seems to be primordial to monitor
208 the efficacy of the process. Besides, a better fundamental knowledge about the requirements of
209 yeast (i.e. molecular and enzymatic systems implicated during the synthesis and the accumulation
210 of lipid) should be well understood to well monitor the process. To mention the fact that the
211 molecular mechanism that regulates lipid accumulation in *Y. lipolytica* remains a debate despite the
212 extensive studies carried out compared to microalgae in which the lipogenesis is well understood in
213 terms of ammonia nitrogen depletion and absorption. For instance, microalgae have both active and
214 passive ammonia nitrogen transport systems that are depending on ammonia concentration and
215 enzymatic (e.g. activity glutamine synthetase). Generally, ammonium nitrogen is directly converted
216 to amino acids by amination or transamination. Thus, free ammonia (NH_3) enters the cell via passive
217 diffusion. With microalgae, the pH is the most critical parameter that impacts the nitrogen
218 adsorption and algal growth [39]. For instance, when pH is alkaline due to CO_2 consumption, the

219 free ammonia can impact on the stability of the biological cell membrane and the enzymatic
220 hydrolysis reaction and inhibit microalgal growth. In contrast, for *Y. lipolytica,* nitrogen adsorption,
221 citric and lipid production are an energy-dependent (ATP utilizing) transport mechanisms. Generally,
222 the lipid accumulation is initiated by a sharp decrease in adenosine monophosphate (AMP)
223 concentrations. AMP is latter deaminized by AMP deaminase to produce inosine monophosphate
224 (IMP) and ammonia, compensating for intracellular nitrogen depletion. Depending on the carbon
225 substrate used in the culture medium, yeasts can perform de novo lipid accumulation process
226 involving the formation of acetyl-CoA resulting from the inhibition of the Krebs cycle in sugar-based
227 media. On the other side, yeast can perform *ex novo* route which is characterized by the
228 incorporation of final products or intermediates of fatty acid β-oxidation into triacylglycerol
229 molecules in hydrophobic carbon source based- media. Collectively, yeast and microalgae are
230 promising candidates for lipid production, however, the production cost of microalgae oils is still too
231 high to be commercialized due to microalgae's slow growth rate, inefficiency in large scale photo-
232 bioreactors, difficulty in contamination control especially for open ponds systems, and finally the
233 high cost in downstream recovery [40-41]. Due to the current challenges, most attention is paid to
234 yeasts-based biodiesel production.

235 **3.2 Effect of modulators to enhance lipid accumulation**

236 The biotin trigger has been tested and optimized on various species, including chlorophytes [42-43]
237 and some of the baker's yeasts [44] and filamentous fungi of *Claviceps* species [21]. In the latter
238 case, the production was principally aimed at the biological production of alkaloids. However, Desai
239 et al. [21] observed a concomitant production of alkaloids and lipids after supplying biotin to the
240 culture. The present study aimed to evaluate how low concentrations of bio-modulators can induce

241 triacylglycerols (TAG) accumulation and their lower concentrations at an early stage of lipid
242 accumulation associated with a dissolved oxygen shift could enhance efficiently the growth and the
243 lipid accumulation scenario in *Y. lipolytica*.
244 The supplementation of cultures with low doses of biotin (50, 100 and 200 µg/L) at an early and late
245 stage of lipid accumulation (16 h and 60 h), was evaluated in the 5-L fermenter. Briefly, batch
246 cultures of *Y. lipolytica* were grown in crude glycerol medium until just before nitrogen depletion at
247 16 h. As expected based on our previous work [31], the cultures that did not receive any biotin had
248 a maximum of growth rate until 12 h of 0.15 h^{-1}, and biomass tends to increase until reaching
249 stationary growth. The original medium supplemented with 25 µg/L of biotin showed a lipid content
250 of 52.6 ± 1.3% (w/w) with maximum lipid concentration of 13.5 ± 0.5 g/L at 66 h and biomass
251 25.1 ± 0.4 g/L. The effect of biotin addition on different growth stages is presented in Table 1. The
252 biotin addition at an early stage showed improvement in lipid quantities. However, the biomass did
253 not increase and remained almost stagnant at 26 g/L. Even though cell dry weight did not increase
254 as much in biotin supplemented media, lipid contents in all biotin supplemented cultures increased.
255 Thus, the addition of 50 µg/L of biotin at an early stage increased the lipid content up to
256 14.0 ± 0.3 g/L, and the increase of biotin concentration led to around 15.5 and 14.9 ± 0.5 g/L in the
257 presence of biotin concentration 100 and 200 µg/L, respectively. This slight decrease can be
258 attributed to yeast requirements; thus, biotin is involved in both amino acid metabolism and lipid
259 synthesis and variations in its level may impact the production of intermediates and fatty acids [45-
260 46]. Previous reports showed that the yeast is more active at lower biotin concentration and the
261 genes involved in biotin uptake and biosynthesis are highly expressed acids [47-48]. In *E. coli*, only a
262 few hundred molecules of biotin per cell are sufficient for growth [49] and some enzymatic pathways

263 are expressed at very low levels (< 350 molecules/cell [50]. Although biotin is widely recognized to
264 be involved in the regulation of several biological processes in both prokaryotic and eukaryotic
265 systems, the molecular details surrounding its exact cellular function in relation with lipid synthesis
266 remain elusive.

267 In the later stage of accumulation at 60 h, the addition of biotin did not improve the lipid
268 concentration and the maximum value was obtained in the case of 100 µg/L, where only
269 13.8 ± 0.4 g/L was reported. Thus, the biotin factor acted preferentially at an early stage and the
270 active phase of lipid accumulation was between 16 h and 60 h. The action of biotin could be also
271 attributed to an effect on intermediary metabolism, correlating with the activity of fatty acid
272 synthases and acetyl-CoA carboxylases, besides, cells require more time for biotin uptake since the
273 entry of biotin into cells is performed by passive diffusion [51]. Likewise, microbial transport systems
274 of biotin are dependent upon, or stimulated by, the presence of an energy carbon source, which is
275 in the present case glycerol, and only 9.2 ± 0.3 g/L are available in the culture medium at 60 h
276 compared to 49.7 ± 0.9 g/L at 16h. The growth seemed to be less affected by biotin addition which
277 was probably due that biotin acts preferentially during the lipogenic phase and if the biotin is
278 required for growth and cell wall synthesis, the strain will use the exogenous biotin supplied in the
279 medium , otherwise, it will synthesize biotin according to its needs.

280 The analysis of glycerol consumption indicated no statistical difference between the control
281 treatments ($Y_{X/S}$ = 0.32 g/g), and those that received biotin addition ($Y'_{X/S}$ = 0.32 g/g). This was due
282 to the delay or arresting of the cellular division, and this phenomenon is assumed to be the result of
283 a fundamental metabolic shift from growth metabolism to lipid accumulation metabolism after
284 nitrogen depletion [31].

285 The addition of up to 200 µg/L biotin did not affect the lipid accumulation activity of *Yarrowia*. Here,
286 the addition of biotin resulted in a single fold increase of lipid yield over the biotin-free control,
287 which suggested that biotin can enhance fatty acid synthesis in agreement with nitrogen depletion
288 and oxygen limitation [concentration of dissolved oxygen DO = 30% of saturation].

289 The results provided evidence that the timely addition of biotin, when coupled with nitrogen
290 depletion, can induce significant lipid accumulation. Therefore, the effect of the biotin on lipid
291 production is explained by the stimulation of genes involved in the lipid production pathway, such
292 as ACC1 genes [52]. Generally, biotin participates in carboxylase reactions and various enzymes are
293 activated in the presence of biotin, such as ACC and pyruvate carboxylase which contained biotin.
294 Their mechanism is clarified by the work of the Lynen group [45-46]. Generally, biotin is reported to
295 function as the prosthetic group of various carboxylases [45-50]. Meanwhile, the only biotin-
296 enzymes functioning in yeast growing in a synthetic medium containing sugar and ammonium salt
297 are pyruvate carboxylase and acetyl-CoA carboxylase catalyzing the carboxylation of pyruvate to
298 oxaloacetate and acetyl-CoA to malonyl-CoA.

299 More often, it was hypothesized that higher lipid productivity might be achieved by adding lower
300 concentrations of leucine near nitrogen depletion. A recent study revealed that leucine auxotrophy
301 caused a 2.5-fold decrease in cell fatty acid content and that *leuA* gene expression restored its level
302 in *M. circinelloides* strain R7B [53]. Moreover, acetyl-CoA generated from the endogenous leucine
303 metabolic pathway was postulated to be another rate-limiting step during fatty acid synthesis in *M.*
304 *circinelloides* [53]. Interestingly, Kamisaka et al. [54] have elucidated a correlation between leucine
305 biosynthesis and oleaginicity. Thus, in that work, the complementation of leucine in *Saccharomyces*
306 *cerevisiae* increased lipid accumulation in comparison with auxotrophic strains.

307 In this regard, in an attempt to increase biomass production prior to inducing lipid accumulation,
308 the culture was also supplemented with leucine [60 µg/mL] at 16 h, just prior to nitrogen depletion,
309 to induce TAG accumulation. The results are shown in Table 2.

310 As indicated earlier, the leucine enhanced the lipid accumulation and lipid content reached around
311 14.7 g/L which is better than the control experiment, besides, biotin and leucine, supplemented
312 together enhanced lipid synthesis and the positive effect of leucine was verified. This suggests that
313 the leucine metabolic pathway is participating in acetyl-coenzyme A [acetyl-CoA] generation which
314 may be critical during fatty acid synthesis [55-57]. The key two-carbon metabolite for lipid
315 biosynthesis, acetyl-CoA, can be produced via degradation of branched-chain amino acids, e.g,
316 leucine, in addition to other metabolic pathways presented in Fig. 1. Interestingly, a recent
317 comparative genome analysis of non-oleaginous and oleaginous species identified a theoretical
318 pathway for leucine degradation that was specific to oleaginous strains and might provide acetyl-
319 CoA for lipid biosynthesis [18]. Besides, a higher concentration of leucine up to 120 µg/mL showed
320 a decrease of the lipid concentration only 12.7 ± 0.3 g/L, and the biomass increased comparatively
321 to control experiment up to 30 g/L, this fact may be due to that higher concentration of leucine
322 concentration can induce leucine biosynthesis and block leucine biodegradation, therefore, the
323 strain will use the leucine for growth and protein synthesis and not synthesizes lipid.

324 This may provide a proof that operating leucine metabolic pathway is required for the accumulation
325 of lipids, suggesting that if the strain is endogenously producing leucine, this latter is degraded for
326 the generation of the corresponding acetyl-CoA, which is incorporated into fatty acid biosynthesis,
327 more often, the strain may present smaller amount of leucine coming mainly from its biosynthesis
328 which is used for lipid synthesis. This fact goes in line with the latter observation in which, the

329 selected strain synthesizes biotin during fatty acid biosynthesis as well as leucine which are both
330 indispensable for lipid synthesis and their overproduction via supplementation may have a crucial
331 role in the present study. As shown in Table 2, leucine and biotin act in synergy and up to 16.8 g/L
332 of lipid was obtained. The essential vitamin biotin is a covalent and tenaciously attached prosthetic
333 group in several carboxylases that play important roles in the regulation of energy metabolism. Thus,
334 the biotin activates the methylcrotonyl-CoA carboxylase (MCCase) enzyme involved in leucine
335 catabolic pathway named as branched-chain catabolism (BCC) which provides more acetyl-CoA as
336 shown in Fig. 1. Blazek et al. (2018) have observed a link between leucine signalling [30] and
337 lipogenesis in *Y. lipolytica* [56]. A comparative study on the effects of leucine and isoleucine
338 supplementation on lipogenesis showed that only leucine promote lipogenesis and *Y.*
339 *lipolytica* possesses degradation pathways for leucine to produce acetyl-CoA fatty-acid precursors
340 [57]. Thus, leucine is found to act as an intracellular signal to favor lipid production. Moreover,
341 MCCase is a biotin-dependant enzyme that enhance the amino acid leucine catabolism. The ultimate
342 product of catabolism is acetyl-CoA which has a wide array of potential fates especially lipogenesis.
343 Consequently, next to a carbon source, the most prerequisite elements for lipid production are
344 biomodulators, which play a pivotal role during lipid synthesis.

345 **3.3 Effect of citric acid production to enhance lipid production in *Y. lipolytica***

346 The addition of citrate is reported to activate allosterically ACC1 enzymes [18]. ACC 1 is allosteric
347 requiring tricarboxylic acid intermediates such as citrate for their activation and are inhibited by
348 long-chain saturated fatty acyl-CoA [58-60]. Herein, the nitrogen depletion occurred at 16 h
349 announcing the lipogenesis phase. The nitrogen concentration of 90 mg/L was kept constant along
350 with the fermentation. In fact, the nitrogen exhaustion activates AMP deaminase which is

351 responsible for the decrease in AMP concentration and increases cellular ammonium concentration
352 [61]. The decrease in AMP concentration inhibits isocitrate dehydrogenase, blocking the citric acid
353 cycle at the isocitrate level. Aconitase mediates the accumulation of citrate in mitochondria, with an
354 exit from the mitochondria-mediated by the citrate/malate cycle [61-62]. This reaction provides
355 large amounts of acetyl-CoA for fatty acid synthesis. Thus, acetyl-CoA is provided by the cleavage of
356 citrate coming from the mitochondria by ATP-citrate lyase [ACL] in the cytosol. Later, ACL cleaves
357 the citrate to give oxaloacetate and acetyl-CoA. Both CA production and lipid accumulation are
358 competitive and they are induced by lower nitrogen concentration [28,63]. Fig. 5 showed the kinetic
359 growth of *Y. lipolytica* in the optimized process [e.g. 89 g/L of crude glycerol and C/N = 75], *Y.*
360 *lipolytica* produced low concentrations of CA 7.8 ± 0.7 g/L and this concentration seemed to be
361 dependent on glycerol concentration. Thus, the variation of glycerol concentration from 75 to
362 100 g/L, increased the CA production around from 2.5 to 14 g/L [32]. The increase of CA
363 concentration in cells at higher glycerol concentration contributed to low levels of lipid accumulation
364 12.8 g/L at 72 h within cells which justify that both phenomena are competitive and more knowledge
365 is required to understand this competitiveness. Likewise, Papanikolaou et al. [2002] assumed that
366 ATP-citrate lyase was inactive in the presence of excess carbon, resulting in low levels of lipid
367 accumulation [64-66].

368 Hence, the idea herein was to inhibit the production of CA. Once CA is available, the metabolic
369 pathway of CA production is blocked and the cells avoided CA production since this latter is available
370 in the growth media. In this manner, the strain maintains nitrogen levels optimal for high lipogenesis
371 and prevented extracellular carbon flux, promoting easier lipid accumulation [30]. For this purpose,
372 the addition of 30 g/L of CA to the culture was investigated, the time was chosen according to the

373 quantities detected in the culture [0.50 g/L] of CA at 36 h. Thus, the feed of the current solution with
374 citric acid at the lipogenic phase was assumed to repress the CA metabolism towards lipid
375 accumulation and provide more acetyl Co during the lipogenic phase as well as reinforcing the biotin
376 effect. Hence, a higher C/N ratio of 75, combined with a buffered pH, lower oxygenation rate at 30%
377 of dissolved oxygen and higher biotin supplement will have a significant effect [$p < 0.05$] on lipid
378 accumulation. The *p*-values of the models were 0.0002 indicating that the models were significant.
379 Usually, a model term is considered to be significant when its value of ''*p*-value'' is less than 0.05.

380 In the control experiment, the final biomass reached around 25.1 g/L. This concentration increased
381 sharply to 28.7 ± 0.5 g/L at 72 h when the culture is supplemented by citric acid and biomodulators.
382 Fig. 6 represents the growth of *Y. lipolytica* on crude glycerol with different biochemical modulators
383 [citric acid (30 g/L) and biotin (100 µg/L)]. As shown in Fig. 6, *Y. lipolytica* simultaneously consumed
384 crude glycerol and CA. Once CA is introduced to the media, its concentration shows a sharp increase
385 and concentration raised up from 2g/L to 33 g/L following the supply of CA at 16h. The exogenous
386 source of CA supplemented to the growth media as well as the endogenous CA produced during
387 growth media and the lipogenesis phase will constitute a potential CA pool and justify the sharp
388 increase. The CA exogenously supplied enhances the production of CA compared to control
389 experiment where the concentration of CA was not exceeding 3g/L during lipogenic phase (Fig. 5).
390 Latter, a slight decrease observed is due to the consumption of CA by *Y. lipolytica.* This phenomenon
391 seemed to be not frequent and the simultaneous utilization of two different substrates is not typical
392 of microorganisms, which first assimilates one of the two available substrates, whereas the
393 assimilation of the other substrate starts only after the first substrate is fully consumed from the
394 medium. Herein, *Yarrowia* was assimilating citric acid [3 g/L], followed by glycerol [0.5 g/L]. This

395 simultaneous consumption can be attributed to metabolic signaling since some sugars can produce
396 signals which modify the conformation of certain proteins that, in turn, directly or through a
397 regulatory cascade affect the expression of the genes subject to catabolite repression. These genes
398 are not at all controlled by a single set of regulatory proteins [66-68]. The glycerol consumption rate
399 was decreased principally because the cells consumed both substrates at low rates. Thus, the
400 strategy of the CA feeding was efficient to create a pool of CA required during metabolism shift
401 towards lipid production. More often, the influence of CA on the production phase of lipid
402 production has not been described until date. Iske et al. [69] have determined the effect of CA on
403 the growth rate of the bioprocess and they found that the addition of initial concentrations of CA at
404 30 g/L into the culture had no remarkable influence on the growth rate. Nevertheless, with
405 increasing concentrations of CA, a significant decline of the growth was observed, which is not the
406 case in the present study, where growth was activated and lipid production was more promoted.
407 Herein, the high-lipid production is due to the continuous production of acetyl-COA, mainly achieved
408 under nitrogen-limited condition leading to a citrate accumulation in the mitochondria.
409 Subsequently, the activity of AMP deaminase responsible for the cleavage of AMP to IMP and
410 ammonia increased considerably. The decrease of AMP inhibits the activity of isocitrate
411 dehydrogenase, which catalyze the conversion of isocitrate into α-ketoglutarate. Therefore,
412 isocitrate is converted back to citrate by the enzyme aconitase, which leads to citrate acid
413 accumulation. The formed citric acid is transported to cytosol, further converted to acetyl-CoA by
414 ACL, and eventually goes to the β-oxidation pathway to produce fatty acids [61-62]. Thus, citric acid
415 can be used as a carbon source for the fatty acid pathway rather than being a major by-product. The

416 addition of CA directs this pool of acetyl-CoA to fatty acid and lipid production under nitrogen-
417 limited conditions.

418 During the lipogenic phase, K_La was kept between 50-60 h^{-1} and OTR varied between 0.2 to 0.3.
419 During fed-batch fermentation, CA was added at 36 h. The supplementation of the medium with CA
420 at 30 g/L led to a decrease of K_La reaching 21.1 h^{-1}, however manual adjustment of agitation was
421 required to keep the DO around 30% [i.e. agitation 420 rpm and air flow rate 2.5 L/min].

422 The combined effect of biomodulators (e.g. leucine) and CA, which were reviewed to work
423 simultaneously [30] increased the lipid concentration to 18.3 g/L and lipid accumulation up to
424 63.7 ± 0.9% (w/w). These results were advantageous compared to the control experiment where
425 only 52.7% (w/w) were obtained. Blazek et al. (2013) confirmed that lipid accumulation phenotypes
426 are dependent on leucine-mediated signalling and that high lipogenesis can be uncoupled from
427 nitrogen starvation and entails a reduction in citric acid cycling [30]. Besides, as biotin is involved in
428 both amino acid metabolism and lipid synthesis (Fig1), the present study, showed that biotin acts in
429 synergy with leucine and citric acid. These results are promising compared to other *Yarrowia* species
430 such as *Y. lipolytica* TISTR 5151 that accumulate around 50.8% (w/w) [70] and *Y. lipolytica* MUCL
431 28849 which accumulate 34.6% (w/w) [71]. The feasibility of this biomodulator-based approach
432 should be assessed using a techno-economic analysis to compare costs associated with biotin
433 addition compared to carbon source addition. Beyond further optimization of modulators supply,
434 additional optimization can perceivably be achieved via metabolic engineering tools.

435 **3.4 Fatty acid composition**

436 The fatty acid profile is essential to assess the biodiesel quality. In the current study, in the presence
437 of CA and biomodulators, C16 and C18 were the mainly fatty acids present (Table 3) and the
438 concentration of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) in *Y. lipolytica*
439 was predominating the total lipid pool. *Y. lipolytica* showed that the major fatty acid detected was
440 oleic acid [C18:1]. This was 33–45% of the fatty acid pool during biomass and lipid production
441 phases. Also, palmitic [C16:0] and linoleic [C18:2] acids have also been detected in high content in
442 the cells [14–20%]. The predominance of palmitic and linoleic acids is propitious for biodiesel
443 synthesis [72-73]. However, in the current study, *Y. lipolytica* produced more stearic acid 12.98%,
444 compared to the control experiment where 8.30% of stearic acid was obtained. These results agree
445 with previous reports [31,74]. Several studies indicated that oleaginous yeasts produce lipids with a
446 similar composition to plant oils with a predominance of SFA and MUFA [20,73]. Fatty acids
447 speciation data exhibited by *Y. lipolytica* agrees with previously reported values of C16:0, C18:1 and
448 C18:2, C18:1 being the main species present in oleaginous yeasts [72-74]. This study presented a
449 significant advantage in fatty acid composition for further potential biodiesel production because
450 polyunsaturated fatty esters have low cetane number, low melting points, and reduced oxidative
451 stability which restricts their use in diesel fuel [75]. In general, the FA composition of lipids produced
452 by *Y. lipolytica* agrees with the FA profile of *Rhodosporidiobolus fluvialis DMKU-RK253, R. glutinis*
453 *R4,* vegetable oils and *Chlorella vulgaris,* [1, 76-79] (Table 3). Therefore, this research provides new
454 insight into fatty acid production by *Y. lipolytica.* Taken together, our results demonstrate that *Y.*
455 *lipolytica* could be a promising feedstock for third-generation biodiesel. From a technological point-
456 of-view, it is much easier to use this strain to produce biodiesel by only supplementing biochemical

targets products. To sum up, SCO production based on crude glycerol with an advanced biochemical approach can yield promising breakthroughs in low cost and effective synthesis of fatty acids.

4 Conclusion

The influence of various modulators concentrations on growth and lipid accumulation in *Y. lipolytica* was explored through the strategic addition of biotin and leucine to enhance growth and lipid accumulation rates as compared to traditional growth regimes which usually supply elevated concentrations of carbon source up to 89 g/L. These data indicated that the type and strategy [e.g, timing, concentration] of modulators had a significant influence [p value < 0.05] lipid production in *Yarrowia* and at an early stage of lipid accumulation, the strain was accumulating up to 15.5 g/L. An optimized two-phase growth with the biochemical approach of biotin and citric acid is an effective strategy to increase its fatty acid biosynthesis and provide pathway precursors for the production of targeted products. This could offer a promising strategy for optimizing productivity and reducing resource costs. Moreover, this strain can be regarded as a valuable tool for generating large amounts of lipids owing to the recent identification of additional putative rate-limiting steps required during the lipid synthesis and regulatory elements involved in lipid accumulation.

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476 ## Figure Captions List

477 **Figure 1** **The diagram of inoculum preparation process under optimal conditions. The crude**
478 **glycerol solution was used without any aseptic techniques**

479 **Figure 2** **Overview of metabolic pathways for fatty acid synthesis in the presence of glycerol**
480 **as carbon source, underlined enzymes are activated in the presence of biotin**
481 **[pyruvate carboxylase, acetyl-CoA carboxylase, and methylcrotonyl-CoA**
482 **carboxylase].**

483 **Figure 3** **Effect of C/N on the growth and lipid production in *Y. lipolytica*, cultured in shake-**
484 **flasks (Culture conditions: T = 28°C, pH = 6.5, 180 rpm, 72 h).**

485 **Figure 4** **Effect of initial glycerol concentration on the growth of *Y. lipolytica* (Culture**
486 **conditions: T = 28°C, pH = 6.5, 180 rpm, 72 h).**

487 **Figure 5** **Batch bioreactor fermentation at C/N 75. Crude glycerol was used as a carbon**
488 **source (89 g/L).**

489 **Figure 6** **Effect of citric acid and biotin on the lipid by *Y. lipolytica* SM7, Conditions: T = 28°C,**
490 **pH = 6.5, DO = 30%, 440-480 rpm, 100 h [duplicates].**

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689

TABLE 1 *Y. lipolytica* culture characteristics during nitrogen limited growth when supplemented with various concentrations of biotin at different stages

	Biotin concentrations (μg/L)					
	50		**100**		**200**	
	Lipid	**DCW**	**Lipid**	**DCW**	**Lipid**	**DCW**
Early stage	14.0 ± 0.3	26.4 ± 0.4	15.5 ± 1.1	25.8 ± 0.5	14.9 ± 0.5	27.1 ± 0.6
Late stage	13.5 ± 0.5	25. 9 ± 0.3	13.8 ± 0.9	27.8 ± 0.5	13.8 ± 0.4	25.5 ± 0.7

DCW: dry cell weight (g/L), Lipid (g/L), The values are means ± standard deviations of three independent experiments.

TABLE 2 Effect of biomodulators on *Y. lipolytica* characteristics

Biomodulators	**DCW (g/L)**	**Lipid concentration (g/L)**	**Lipid content (% w/w)**
With Leucine	26.7 ± 0.6	14.7 ± 0.9	54.9 ± 0.5
With Biotin	25.8 ± 0.6	15.5 ± 1.1	60.1 ± 0.7
With Biotin+leucine	27.4 ± 0.5	16.8 ± 0.5	61.3 ± 0.9

DCW: cell dry weight (g/L), The values are means ± standard deviations of three independent experiments.

Table 3 : Fatty acids composition of lipid produced by different oleaginous microorganisms and vegetable oils

	C16:0	C16:1	C18:0	C18:1	C18:2	
Y. lipolytica SM7 [a]	14.50	0.97	12.98	45.0	20.1	*Current study*
Y. lipolytica SM7[b]	12.50	0.93	8.30	33.0	14.0	*Current study*
R. fluvialis DMKU-RK253	*18.8*	0.00	6.20	35.8	34.2	[1]
R. glutinis R4	16.78	1.81	1.35	61.60	11.64	[76]
Olive Oil	11.60	1.00	3.10	75.00	7.80	[77]
Jatropha Oil	14.90	1.00	6.10	40.40	36.20	[78]
Palm Oil	36.70	0.10	6.60	46.10	8.60	[79]
C. vulgaris	10.66	2.76	5.17	24.42	7.17	[79]

a: Crude glycerol media supplied with 100µg/L of biotin; 60 µg/L of leucine and 30g/L of citric acid at an early stage (16h)

b: Control media without biomodulators supply

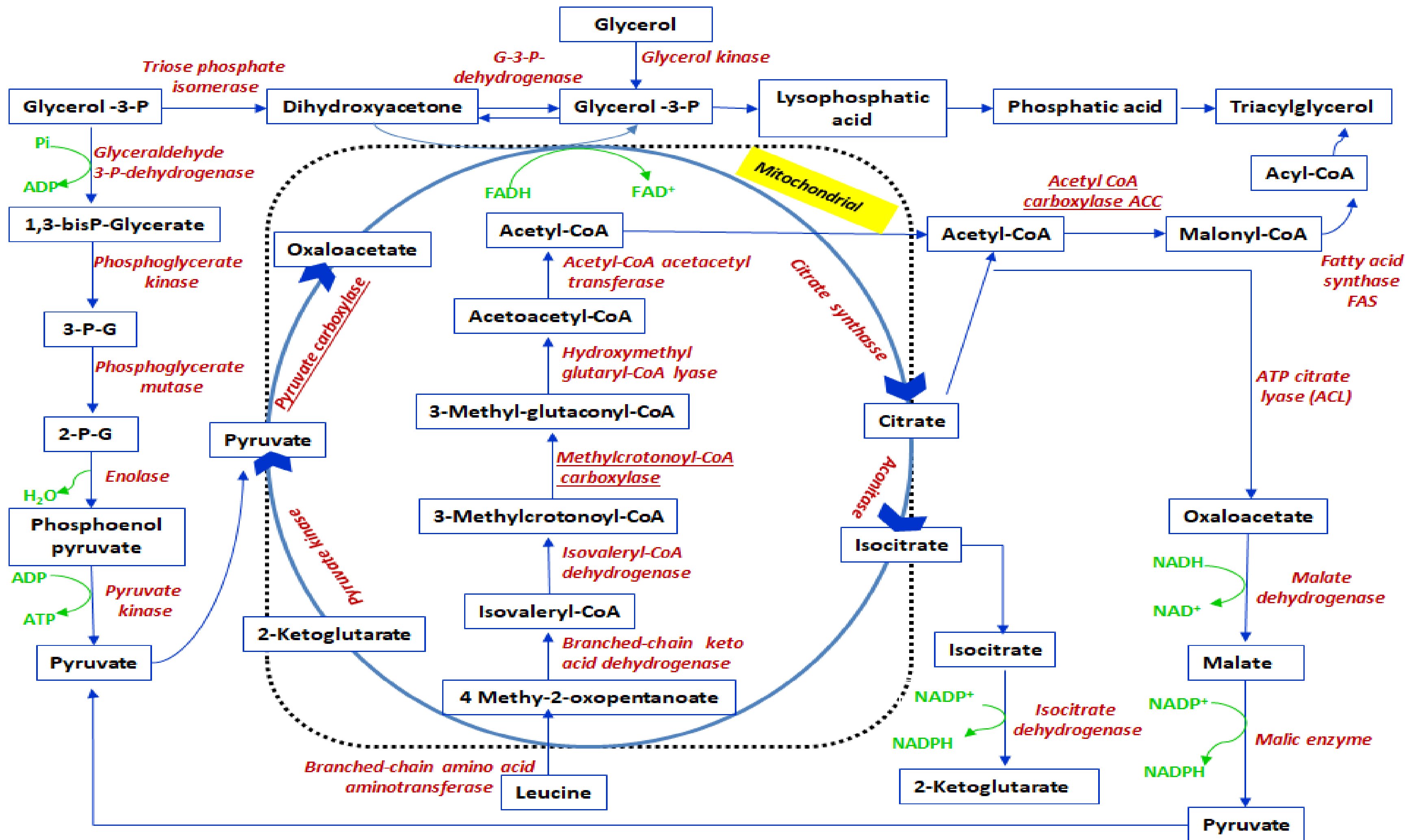

FIG 1

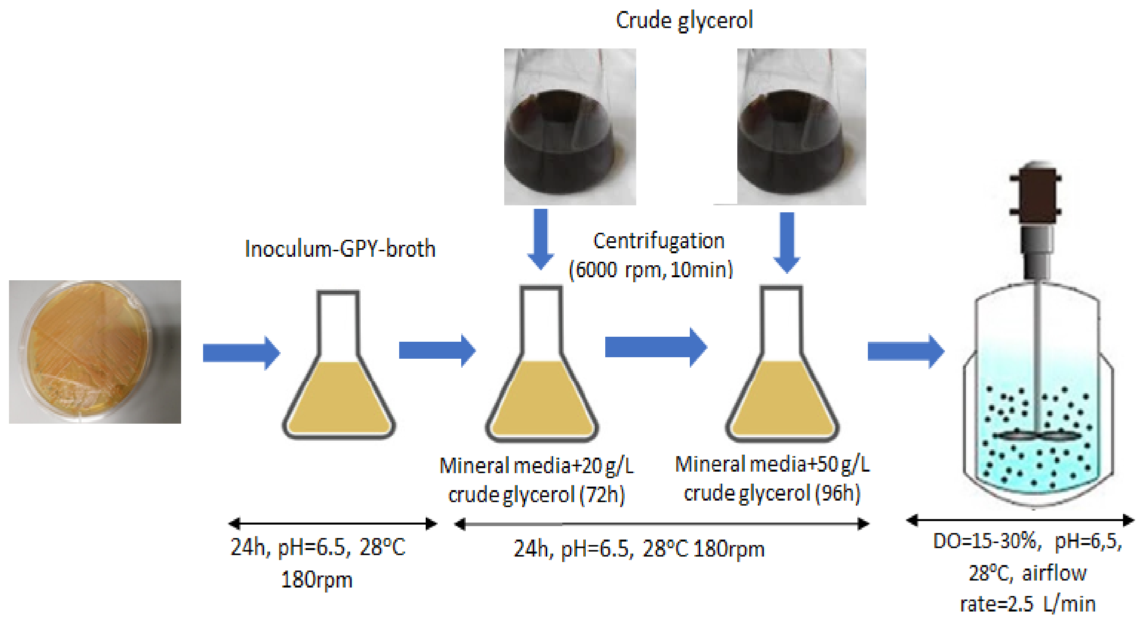

FIG 2

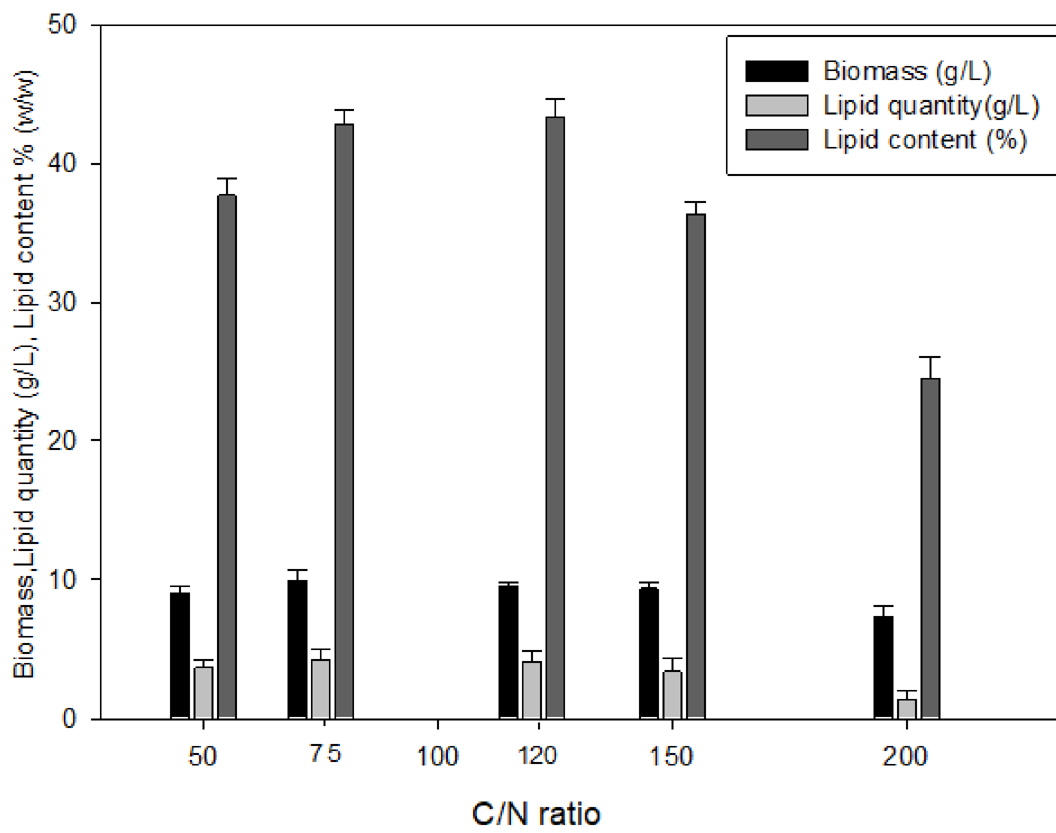

FIG 3

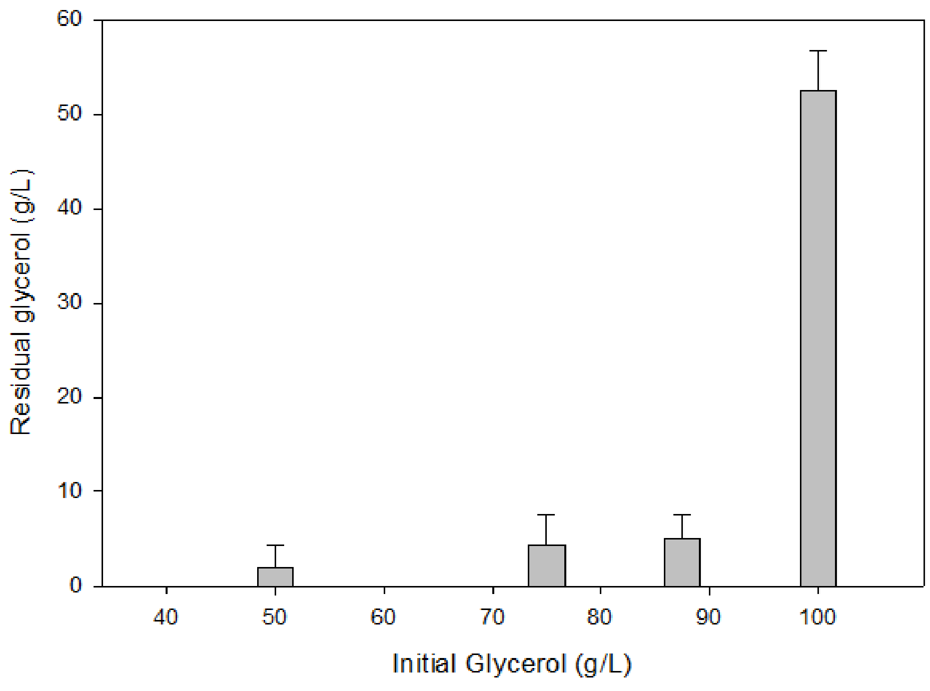

FIG4

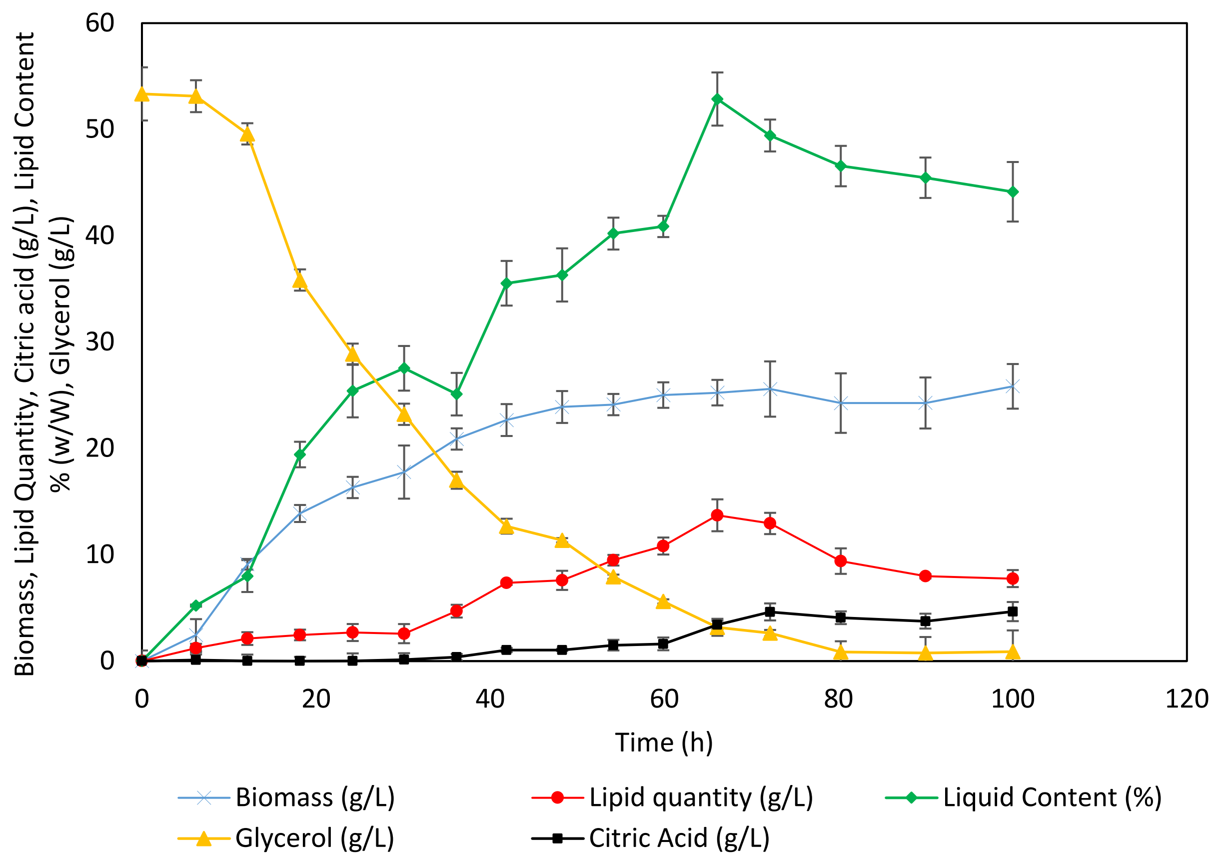

FIG 5

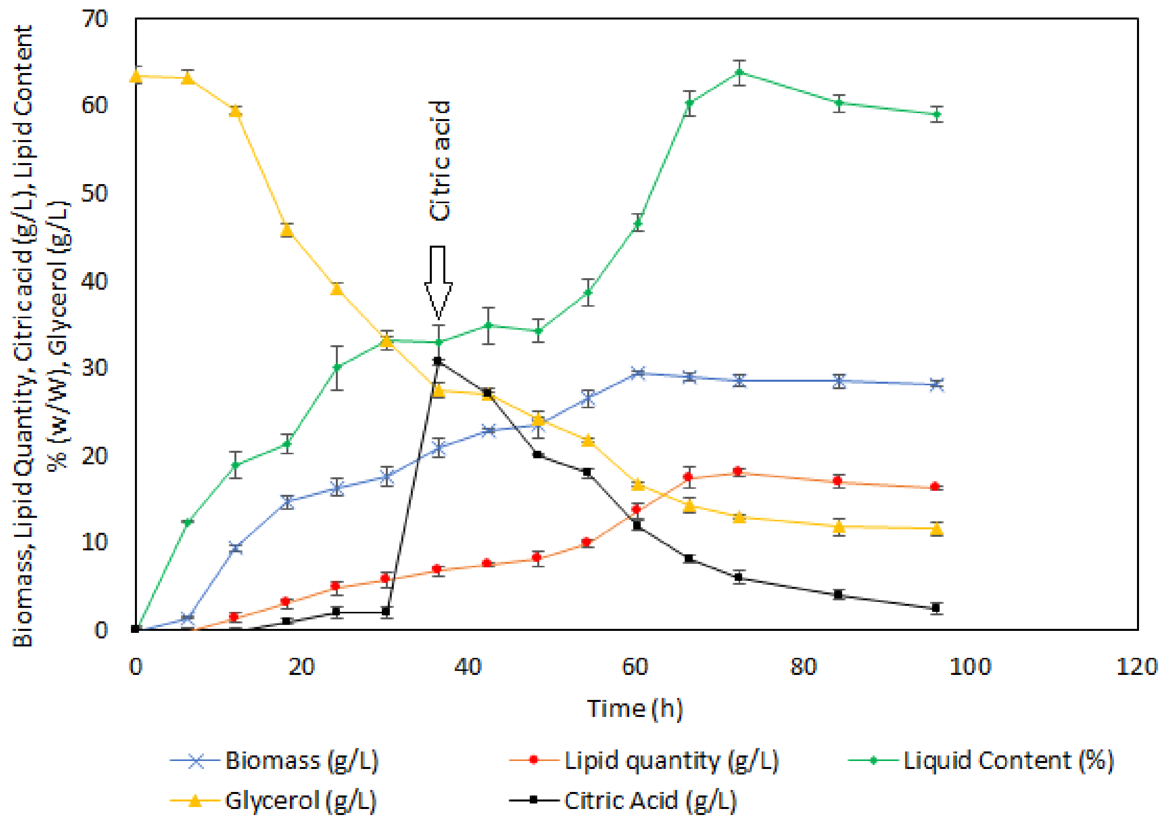

FIG 6