



Rhodospiridium toruloides cultivated in NaCl-enriched glucose-based media: adaptation dynamics and lipid production

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1 ***Rhodospiridium toruloides* cultivated in NaCl-enriched glucose-based media:**
2 **adaptation dynamics and lipid production**

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19 **Key-words:** NaCl, microbial lipids, pasteurized conditions, fed-batch culture, *Rhodospiridium*
20 *toruloides*
21

22 **Abbreviations and units:** X – total biomass (dry cell weight, DCW), (g/L); L – total cellular lipids,
23 (g/L); Glc – glucose, (g/L); $Y_{L/X}$ – lipid in DCW (% w/w); $Y_{X/Glc}$ – total biomass yield, g of total
24 biomass produced per g of glucose consumed; $Y_{L/Glc}$ – lipid yield, g of cellular lipids produced per g
25 of glucose consumed; q_{Glc} – specific glucose uptake rate, g of glucose/g of biomass/h; r_{Glc} – glucose
26 uptake rate, g/L/h; μ – specific glucose uptake rate, g of glucose/g of biomass/h.
27

28 **Practical application**

29 The yeast strain *Rhodosporidium toruloides* DSM 4444 was found capable of producing sufficient lipid
30 amounts when grown in media supplemented with NaCl. This particular feature of the strain, combined with its
31 tolerance against relatively high amounts of glucose, could denote the feasibility of microbial oil production without the
32 need of stringent sterile conditions. The utilization of agro-industrial residues such as salty or brackish waste-water
33 streams deriving from fisheries or olives production establishments as fermentation media could reduce the cost of
34 microbial oil production by the particular yeast, whereas waste bio-remediation could offer an additional environmental
35 benefit in the process. Likewise, the accomplishment of microbial lipid fermentation by this strain in substrates
36 supplemented with NaCl and without previous sterilization of the culture medium can further reduce the cost of the
37 bioprocess.

38

39 **Abstract**

40 In the present report and for the first time in the international literature, the impact of the addition of NaCl
41 upon growth and lipid production on the oleaginous yeast *Rhodospiridium toruloides* was studied. Moreover, equally
42 for first time, lipid production by *R. toruloides* was performed under non-aseptic conditions. Therefore, the potentiality
43 of *R. toruloides* DSM 4444 to produce lipid in media containing several initial concentrations of NaCl with glucose
44 employed as carbon source was studied. Preliminary batch-flask trials with increasing amounts of NaCl revealed the
45 tolerance of the strain against NaCl content up to 6.0% (w/v). However, 4.0% (w/v) of NaCl stimulated lipid
46 accumulation for this strain, by enhancing lipid production up to 71.3% (w/w) per dry cell weight. The same amount of
47 NaCl was employed in pasteurized batch-flask cultures in order to investigate the role of the salt as bacterial inhibiting
48 agent. The combination of NaCl and high glucose concentrations was found to satisfactorily suppress bacterial
49 contamination of *R. toruloides* cultures under these conditions. Batch-bioreactor trials of the yeast in the same media
50 with high glucose content (up to 150 g/L) resulted in satisfactory substrate assimilation, with almost linear kinetic
51 profile for lipid production, regardless of the initial glucose concentration imposed. Finally, fed-batch bioreactor
52 cultures led to the production of 37.2 g/L of biomass, accompanied by 64.5% (w/w) of lipid yield. Lipid yield per unit
53 of glucose consumed received the very satisfactory value of 0.21 g/g, a value amongst the highest ones in the literature.
54 The yeast lipid produced contained mainly oleic acid and to lesser extent palmitic and stearic acids, thus constituting a
55 perfect starting material for “second generation” biodiesel.

56

57 **Introduction**

58 Environmental concerns have driven scientific research towards alternative energy
59 resources, as means of disengagement from fossil oil [1, 2]. Biodiesel consists one of the major
60 renewable transportation fuels, deriving by trans-esterification process of long chain fatty acids of
61 plant or animal origin. However, constant rising demand of biodiesel production competes with the
62 availability of existing raw materials and as a result, other non-conventional oil resources are
63 explored, mainly of non-edible nature [2]. In this light, scientific interest on microbial lipids as
64 alternative source of oil has gain momentum the last decades.

65 Microbial oil production can be carried out by a number of heterotrophic (mostly yeast and
66 fungi) or phototrophic (algae) organisms which are found to accumulate oil up to 80% of their dry
67 weight [1, 3]. This lipid, namely single cell oil (SCO), is mainly composed of neutral fractions
68 (principally triacylglycerols-TAGs and to lesser extent steryl-esters) [4], while these lipid-
69 accumulating microorganisms are called “oleaginous” [1-4]. It has been well established that when
70 culture is performed on sugars or similarly metabolized compounds (e.g. polysaccharides, glycerol,
71 etc), the conditions required to trigger lipid production are met in a culture environment with carbon
72 excess and (at least) one essential nutrient depletion (usually nitrogen) [1, 3-5]. As indicated, SCOs
73 could constitute the starting material for the synthesis of the “2nd” or the “3rd generation” biodiesel
74 [2, 6]. Nevertheless, and despite the huge upsurge of interest concerning the production of microbial
75 oils amenable to be converted into biodiesel [2, 4], the first industrial applications related with the
76 utilization of oleaginous microorganisms referred to the production of specialty (and expensive)
77 lipids, rarely found in the plant or animal kingdom, like the cocoa-butter substitutes [1-4, 7, 8]. In
78 any case though, the feasibility of sustainable bioprocess development for SCO production is
79 determined by the cost of both raw materials and (mainly) the fermentation process [1-3, 9-12].
80 Yeast strains belonging to the genera *Cryptococcus* sp., *Lipomyces* sp., *Rhodotorula* sp.,
81 *Rhodospiridium* sp, and *Trichosporon* sp. are among those reported as possible biodiesel producers
82 [13-19]. Among those, *Rhodospiridium toruloides* Y4 has been reported capable of producing

83 106.5 g/L of biomass containing 67.5% (w/w) of oil, during cultivation in a 15-L bioreactor under
84 fed-batch mode [20], designated as the highest oil production from the particular strain so far.

85 One important aspect developed in several industrial fermentations, refers to the potential of
86 the accomplishment of the microbial conversion under non-aseptic conditions, due to obvious
87 process cost reduction [6]. While this is feasible in fermentations in which inhibiting (for any
88 contaminant microorganisms) metabolites are accumulated into the production media (e.g. ethanol
89 during the alcoholic fermentation processes; see Sarris and Papanikolaou [6]), generation of such
90 extra-cellular metabolites inhibiting contaminant cells is not obvious during the fermentation of
91 SCO production. Thus, addition of such a “hurdle” compound into the medium should be
92 considered. Preliminary works have identified the potential of the microorganism *R. toruloides*
93 DSM 4444 to grow on media containing NaCl (an important inhibiting agent towards contaminant
94 microorganisms) quantities, while in some previous studies it has been demonstrated that amongst
95 other yeasts, *Rhodospiridium* sp. strains have been isolated from hyper-saline habitats [13] and,
96 therefore, could potentially grow on media presenting relatively high salinity. The objective of the
97 study, thus, was double: to evaluate the performance of this yeast strain on glucose-based media
98 supplemented with different initial quantities of NaCl and to perform SCO production by this yeast
99 under non-aseptic conditions, due to the addition of salt into the medium.

100

101 **Materials and Methods**

102 i) Microorganism and media. *Rhodospiridium toruloides* DSM 4444, provided by the
103 DSMZ culture collection (Leibniz, Germany), was maintained on yeast peptone dextrose agar
104 (YPDA), supplemented with malt extract, at T=4 °C and sub-cultured every month in order to
105 maintain its viability. Pre-cultures of the strain contained glucose, yeast extract and peptone at 10
106 g/L each. The synthetic medium used had the following salt composition (g/L): KH₂PO₄, 7.0;
107 Na₂HPO₄, 2.5; MgSO₄·7H₂O, 1.5; CaCl₂, 0.15; FeCl₃·6H₂O, 0.15; ZnSO₄·7H₂O, 0.02;
108 MnSO₄·H₂O, 0.06. Peptone and yeast extract were used as nitrogen sources in concentrations of

109 0.75 g/L and 0.5 g/L respectively. Unless otherwise stated, cultures were supplemented with NaCl
110 at concentrations of 0.5, 1.0, 1.5, 2.5, 4.0 and 6.0 % (w/v). Commercial glucose provided by the
111 “Hellenic Industry of Sugar SA” (Thessaloniki, Greece) was used as carbon source in the
112 fermentations performed [purity *c.* 95%, w/w, impurities composed of maltose (2%, w/w), malto-
113 dextrans (0.5%, w/w), water (1.5%, w/w) and salts (1.0%, w/w)]. The initial pH for all media
114 before and after sterilization (121 °C/ 20 min) was 6.0±0.1. Glucose was added at different
115 concentrations into the medium before heat sterilization. In all trials, initial glucose (Glc_i)
116 concentration was measured after the sterilization. Assay of glucose before and after the
117 sterilization demonstrated very small glucose destruction (*c.* 2%) due to the heat sterilization.

118 ii) Culture conditions. Batch-flask cultures were conducted in 250-mL conical flasks,
119 containing 50±1 mL of growth medium, previously sterilized (121 °C/ 20 min) and inoculated with
120 1 mL of a 24-h exponential pre-culture (*c.* 3×10⁷ cells, initial biomass concentration at the flasks at
121 *c.* 0.12±0.02 g/L). Cultures were performed in an orbital shaker (Lab-Line, Illinois-USA) at an
122 agitation rate of 185±5 rpm and incubation temperature T=26±1 °C. It was desirable to maintain a
123 medium pH in a value greater than 5.2, therefore an appropriate volume of KOH (5 M) was
124 periodically and aseptically added into the flasks when needed, in order to maintain the pH value at
125 6.0±0.2 [19].

126 Batch-flask cultures were also realized under previously pasteurized media by subjecting the
127 flasks filled with the fermentation media at 100 °C for 7 min. Then 3 mL of 24-h pre-culture (*c.*
128 9×10⁷ cells, initial biomass concentration at the flasks at ~0.36±0.06 g/L) were used as inoculum. A
129 Jenway 3020 pH-meter was used for pH-measurements of cultures. Dissolved oxygen (DO)
130 concentration was determined using a selective electrode (OXI 96, B-SET, Germany). Before
131 harvesting, the shaker was stopped and the probe was placed into the flask. Then, the shaker was
132 switched on and the measurement was taken after DO equilibration (usually within 10 min).
133 Oxygen saturation was kept above 20% (v/v) during all growth phases.

134 Batch-bioreactor experiments were carried out in a 3.5-L bioreactor (Infors HT, Labfors 5),
135 with a working volume of 2.0 L. A 7.5% (v/v) inoculum was employed using 24-h exponential
136 yeast pre-culture. The stirrer speed was on cascade mode, automatically varying from 200 to 500
137 rpm to maintain a dissolved oxygen (DO) concentration above 20% (v/v) of saturation. Aeration
138 and temperature were maintained at 1.0 vvm and $T=26\text{ }^{\circ}\text{C}$, respectively. The pH was maintained at
139 6.0 ± 0.1 by automatic addition of 5 M KOH. Fed-batch fermentations were initiated in batch mode
140 and when glucose concentration was reduced to 10 g/L, a volume of concentrated glucose solution
141 (60%, w/v) was added in the bioreactor. Samples were taken periodically from the bioreactor
142 throughout fermentation for subsequent analysis as described in the next section.

143 iii) Analytical methods. The whole content of flasks (c. 50 mL) or bioreactor samples (c. 20
144 mL) were periodically collected and cells were harvested by centrifugation ($9000 \times g/15\text{ min}$ at 10
145 $^{\circ}\text{C}$) in a Hettich Universal 320R (Germany) centrifuge and washed twice with distilled water.
146 Biomass (X , g/L) was determined by means of dry cell weight (DCW) ($95\pm 2\text{ }^{\circ}\text{C}/24\text{ h}$). Consumed
147 glucose was determined by 3,5-dinitrosalicylic acid (DNS) assay [21]. Total cellular lipid (L , g/L)
148 was extracted from the dry biomass with a mixture of chloroform/methanol 2/1 (v/v) and was
149 determined gravimetrically. Cellular lipids were converted to their methyl-esters in a two-step
150 reaction [19]. FAMES were analyzed according to Fakas et al. [21] and were identified by reference
151 to standards. In some of the performed trials, in order to investigate whether glycerol (or other
152 polyols) was secreted into the culture medium, HPLC analysis [21] of the supernatant obtained after
153 centrifugation was performed. All experiments were performed in duplicate by using different
154 inocula. All of the experimental points presented in the tables and the figures are the mean value of
155 two independent determinations, with standard error $\leq 10\%$.

156

157 **Results**

158 This study was focused on the evaluation of the *Rhodospiridium toruloides* yeast ability to
159 grow on media containing glucose as carbon source, supplemented with various amounts of NaCl.

160 Special attention was paid to the evolution of lipid production and the potential effect of NaCl on
161 yeast metabolism, in batch-flask trials as well as batch- and fed-batch bioreactor experiments.

162 i) Effect of NaCl concentration on *Rhodospiridium toruloides* cultures. The yeast strain *R.*
163 *toruloides* DSM 4444 was cultivated in batch-flask trials, in media containing 50 g/L glucose
164 supplemented with increasing NaCl concentrations. Cultures were done under nitrogen-limited
165 conditions (carbon-to-nitrogen ratio equal to 106 mol/mol) in order to stimulate lipid accumulation.
166 Moreover, an experiment without NaCl addition was included that served as control. The obtained
167 results with regard to the impact of NaCl upon the physiological behavior of *R. toruloides* are
168 depicted in Table 1. Generally, it should be stressed that only at increased initial NaCl concentration
169 (e.g. NaCl at 60 g/L or higher) some negative effect upon the biomass and lipid produced was
170 observed (Table 1). Likewise, by taking into consideration the specific growth rate for the
171 fermentations in the media with different initial salinity imposed, similar μ_{\max} values ($0.09 \pm 0.01 \text{ h}^{-1}$)
172 were recorder for all trials performed except for the fermentation presenting the highest initial NaCl
173 quantity (=60 g/L), in which the μ_{\max} value slightly declined (see Table 1). Moreover, analysis of
174 the supernatant at the end of all trials was performed in order to demonstrate whether secretion of
175 glycerol or other polyols was performed due to the increasing osmotic pressure into the medium; it
176 has been seen that up to the threshold of 1.5% (w/v) not any polyols have been identified into the
177 medium. Thereafter, very small glycerol accumulation into the medium occurred (the maximum
178 quantity of glycerol, *c.* 1.5 g/L, was obtained at the trial with initial concentration of NaCl imposed
179 of 40 g/L). No other polyol was synthesized as response to the osmotic stress situation by *R.*
180 *toruloides*. On the other hand, for NaCl concentrations varying from 0.5 to 2.5% (w/v), microbial
181 growth as well as lipid production was maintained in similar levels; specifically, 88-97% of initial
182 glucose concentration was consumed within 120-168 h of fermentation, whereas biomass
183 production ranged between 8.2 and 8.9 g/L. In terms of lipid production, cells accumulated 60.6-
184 62.4% (w/w) of oil. However, initial NaCl concentration of 4.0% (w/v) was found to positively
185 affect biomass and, specifically, lipid production, yielding 9.4 and 6.7 g/L respectively.

186 Consequently, lipid quantity per DCW increased to 71.3% (w/w). Higher NaCl additions exerted
187 inhibitory effects on yeast growth, as only 6.3 g/L of biomass were synthesized, with concomitant
188 impact on lipid production and yield (Table 1). On the other hand, for all the above-mentioned
189 trials, the yield of total biomass produced per glucose consumed ($Y_{X/Glc}$) was *c.* 0.20 g/g, ranging
190 between 0.19 and 0.21 g/g.

191 Taking into account the satisfactory performance of the strain at NaCl supplementation of
192 4.0% (w/v), subsequent batch-flasks cultures were carried out with the same NaCl addition and
193 increased glucose concentration ($Glc_i \approx 100$ g/L). In the same manner, a control experiment without
194 NaCl addition was included. In the absence of NaCl, elevated glucose concentrations prolonged the
195 course of the fermentation up to 433 h. At that time, *c.* 88% of initial carbon source concentration
196 (≈ 93 g/L) was finally consumed by the strain, leading to 17.5 g/L of biomass. The μ_{max} in the
197 above-mentioned culture, calculated at the early exponential growth phase by fitting the equation

198 $\ln\left(\frac{X}{X_0}\right) = f(t)$ on the experimental data within this phase, was found to be ≈ 0.08 h⁻¹ (slightly lower

199 than that observed on the respective trial with $Glc_i = 50$ g/L, potentially due to slight inhibition
200 exerted by the increased initial concentration of glucose). However, biomass production per
201 substrate consumption yield ($Y_{X/Glc}$) was ≈ 0.19 g/g, the same value as in trials with $Glc_i = 50$ g/L.
202 Furthermore, the higher Glc_i concentration employed and the increased carbon-to-nitrogen ratio
203 (C/N=211 mol/mol) seemed to enhance lipid production in terms of absolute values, reaching a
204 maximum SCO production of 8.1 g/L. On the other hand, and despite the significant increase of
205 lipid in absolute values compared with the respective trial in which Glc_i was adjusted to *c.* 50 g/L
206 (see Table 1 entry 1), total lipid in DCW ($Y_{L/X}$) value was lower than the one obtained in the trial
207 with $Glc_i = 50$ g/L (*c.* 46.3% against 55.1% w/w). The presence of 4.0% (w/v) NaCl in media with
208 100 g/L of glucose, extended the fermentation duration to more than 500 h, a time point in which *c.*
209 80% of the initial glucose was consumed. Biomass synthesis was slightly reduced to 16.1 g/L
210 compared with the culture with $Glc_i \approx 100$ g/L and no NaCl addition was performed. Nonetheless,
211 $Y_{X/Glc}$ and μ_{max} values were unaffected ($= 0.20$ g/g and 0.08 h⁻¹ respectively) by the addition of salt

212 into the medium. On the other hand, lipid in terms of both absolute (g/L) and relative (% in DCW)
213 values was noticeably higher compared with the equivalent experiment ($\text{Glc}_i \approx 100$ g/L) in which no
214 NaCl addition occurred ($L=9.2$ g/L, $Y_{L/X}=57.1\%$ w/w). Surprisingly enough, *R. toruloides*
215 accumulated oil in an almost linear manner, whereas shortly after virtual exhaustion of the
216 assimilable nitrogen from the culture medium (i.e. *c.* 50 h after inoculation) lipid in DCW almost
217 reached its plateau (see Fig. 1).

218 Table 2 shows the FA profiles of *R. toruloides* cellular lipids, during growth on media with
219 increasing NaCl concentrations. In every case, the predominant fatty acid of the yeast was oleic
220 ($\Delta^9\text{C18:1}$), followed by palmitic (C16:0) stearic (C18:0) and linoleic acid ($\Delta^{9,12}\text{C18:2}$). The
221 implementation of NaCl did not seem to affect the amounts of individual fatty acids in the
222 composition of the accumulated lipids. On the contrary, the unsaturated nature of lipids increased
223 during the course of fermentation, as indicated by the SFA/UFA ratio. This fact could be mainly
224 attributed to the increased amounts of the unsaturated oleic and linoleic acids and the declining
225 percentage of stearic acid that occurred in the late growth phase.

226 ii) Trials of *Rhodospiridium toruloides* on pasteurized media supplemented with NaCl.

227 Based on evidence of the tolerance against noticeable amounts of NaCl (e.g. 4.0% w/v) shown by
228 the employed yeast strain, it was decided to further investigate the stability of microbial growth and
229 lipid production under pasteurized conditions and assess whether the presence of 4.0% (w/v) NaCl
230 could reduce the probability of culture contamination. It is evident that a successful accomplishment
231 of SCO production unsterile media can reduce the cost of the process when a scale-up is envisaged.
232 To this end, batch-flask trials were carried out with *c.* 50 and 100 g/L of glucose as substrate, in
233 media supplemented with 4% (w/w) NaCl. At $\text{Glc}_i \approx 50$ g/L, substrate exhaustion occurred around
234 160 h after inoculation, yielding 11.7 g/L of biomass production (Table 3A). However, lipid
235 accumulation was lower than in the experiment in which the medium had been previously subjected
236 to heat sterilization ($L=5.9$ g/L against 6.7 g/L). Microscopy observations revealed the presence of
237 bacterial contamination (rods), accounting for *c.* 8% of the total microbial population. When higher

238 Glc_i concentrations were employed in pasteurized media, equally some bacterial contamination
239 occurred (c. 5% of the total microbial population). As in the trial with Glc_i≈50 g/L, glucose
240 assimilation in the pasteurized medium was more rapid than in the aseptic fermentation, possibly
241 due to this contamination. Equally, biomass formation was enhanced in the pasteurized medium in
242 comparison with the aseptic culture, reaching a DCW value of 17.9 g/L that contained 9.1 g/L of oil
243 (lipid in DCW of c. 51% w/w) while in the aseptic culture the respective values were for DCW 16.1
244 g/L and for lipid 9.2 g/L (see Table 3A). The kinetics of biomass produced, lipid accumulated and
245 glucose assimilated for the trials with Glc_i≈100 g/L are seen in Fig. 2.

246 Table 4A shows the FA profiles of the produced cellular lipids for the previously
247 pasteurized media, in which Glc_i concentration was adjusted to c. 50 and 100 g/L and constant NaCl
248 quantity added. Despite the fact that the cultures were not axenic (as stated, some contamination by
249 bacilli existed), the FA composition presented significant similarities with the trials in which
250 growth and lipid accumulation occurred in previously sterilized media (see and compare Tables 2
251 and 4A). In any case, lipid produced through the non-aseptic experiments was rich in the FA
252 Δ^9 C18:1, constituting, thus, a perfect fatty material amenable to be converted into biodiesel [2, 22].

253 iii) Batch-bioreactor cultures of *Rhodospiridium toruloides*. The next step in the
254 experimental process involved the realization of batch-bioreactor cultures of the yeast with
255 increasing glucose concentrations and NaCl supplementation, aiming to promote, if possible, lipid
256 production. Previously sterilized fermentation media containing c. 50, 100 and 150 g/L of glucose
257 and 4.0% (w/v) NaCl were used in bench top bioreactor cultures. Table 3B summarizes the
258 quantitative data of *R. toruloides* trials in bioreactor experiments. At 50 g/L of glucose, the strain
259 exhibited rapid substrate assimilation within 72 h. Biomass production was notably enhanced, as
260 opposed to the respective batch-flask experiment, yielding 12.7 g/L with 8.1 g/L of oil. Increasing
261 amounts of carbon source did not seem to drastically negatively affect the microbial metabolism,
262 while in all cases, it is interesting to indicate that glucose was linearly consumed (Fig. 3). On the
263 other hand, the more the Glc_i concentration (and, hence, the initial molar ratio C/N of the medium)

264 increased, the more the glucose consumption rate ($r_{Glc} = -\frac{\Delta Glc}{\Delta t}$) decreased; for Glc_i adjusted at c.
265 50 g/L, r_{Glc} was =0.62 g/L/h decreasing to 0.54 g/L/h for Glc_i≈100 g/L. Finally, at Glc_i≈150 g/L,
266 r_{Glc} value eventually dropped to 0.34 g/L/h (Fig. 3). During these trials, lipid accumulation process
267 demonstrated remarkable stability; in accordance with the trial performed in shake flasks, as
268 depicted in Fig. 3, the evolution of lipids' kinetic profile was almost linear, regardless of the applied
269 initial glucose concentration. Maximum biomass production was achieved at 150 g/L of glucose
270 equal to 34.1 g/L, containing 65.1% (w/w) of oil. It is interesting to indicate that under the present
271 culture conditions, growth was not inhibited by the increment of Glc_i concentration up to the
272 threshold of 150 g/L; this assumption can be justified by the fact that the yields $Y_{X/Glc}$ and $Y_{L/Glc}$
273 presented their higher values at the trial in which the concentration of carbon substrate had been
274 adjusted at c. 150 g/L (=0.33 and 0.21 g/g respectively), being clearly the higher ones obtained in
275 all of the previously performed trials (including fermentations in shake-flasks or bioreactor). In
276 addition, specifically the yield $Y_{L/Glc}$ value obtained in the bioreactor experiment with Glc_i≈150 g/L
277 (=0.21 g/g) was a value very close to the maximum achievable one of 0.22-0.23 g per g of
278 consumed sugar that has been achieved so far in the international literature [1, 3, 11, 21, 23, 24],
279 suggesting, once more, the absence of inhibitory phenomena of increased Glc_i concentrations upon
280 the growth of *R. toruloides* under the present culture conditions.

281 Table 4B shows the FA profiles of *R. toruloides* cellular lipids, during growth on bioreactor
282 cultures in media presenting increasing Glc_i concentrations and constant NaCl quantity added. As in
283 the shake-flask trials, the predominant FA of the yeast was the Δ^9 C18:1, followed by the C16:0,
284 C18:0 and $\Delta^{9,12}$ C18:2. Moreover, as in the case of the trials with the increasing initial NaCl
285 quantities into the medium, the increment of Glc_i concentration did not seem to have serious impact
286 upon the FA composition of the strain, while cellular FAs were slightly more saturated at the
287 beginning of the fermentation (Table 4B).

288 iv) Fed-batch bioreactor culture of *Rhodospiridium toruloides*. In an attempt to further
289 investigate lipid production potential of the yeast *R. toruloides* and to reduce the time of the

290 fermentation (as seen in the previous paragraph, the more the Glc_i concentration increased, the more
291 the time of the fermentation rose) fed-batch cultures were performed in bench top bioreactor, in
292 media containing 4.0% (w/v) NaCl. Trials were initiated batch-wise ($\text{Glc}_i \approx 50$ g/L) and when the
293 glucose level dropped below 10 g/L, a volume of concentrated glucose solution was aseptically
294 introduced to the culture. In every case, it was desirable to maintain the feeding of glucose to
295 concentrations ≤ 50 g/L, in order to increase the uptake rate of glucose. In the first cycle of the fed-
296 batch operation (0-72 h), the r_{Glc} was =0.63 g/L/h and the respective specific glucose consumption
297 rate (q_{Glc} ; $q_{\text{Glc}} = \frac{r_{\text{Glc}}}{X_{\text{average}}}$) was ≈ 0.1 g/g/h. In the second cycle (72-192 h), r_{Glc} was ≈ 0.40 g/L/h and
298 q_{Glc} was ≈ 0.06 g/g/h. In the third cycle (192-272 h) r_{Glc} was =0.51 g/L/h and q_{Glc} was ≈ 0.08 g/g/h.
299 During 272 h of the fermentation, feeding pulses were done twice resulting in the total consumption
300 of c. 127 g/L of glucose (Fig. 4A). Maximum biomass achieved was 37.2 g/L with 64.5% (w/w) of
301 accumulated oil. ($L_{\text{max}} \approx 24$ g/L). Furthermore, overall yields for lipid and biomass production per
302 consumed substrate in fed-batch process were 0.21 and 0.33 g/g, respectively (Fig. 4B). Compared
303 to the batch-bioreactor cultures with high initial glucose concentration ($\text{Glc}_i \approx 150$ g/L), both biomass
304 formation and lipid accumulation were slightly improved during the fed-batch culture mode,
305 whereas the fermentation was accomplished more rapidly in the later case than in the former one,
306 thus the lipid volumetric productivity achieved in the fed-batch experiment was improved compared
307 with the batch process presenting high Glc_i concentration (0.088 against 0.075 g/L/h).

308

309 **Discussion**

310 The oleaginous nature of *Rhodospiridium toruloides* has been a topic of interest for many
311 studies in the international literature. Origin of carbon or nitrogen sources, nutrient limitation and
312 feeding strategy has been assessed for the optimization of SCO production by the particular yeast.
313 Lipid accumulation by *R. toruloides* has been shown to improve in the presence of organic nitrogen
314 sources [25, 26]. Although nitrogen limitation has long been recognized as a determinant factor for
315 *de novo* lipid synthesis in oleaginous microorganisms [1-5], phosphorus- and sulfate-limitation

316 conditions have been also investigated as lipid inducing factors for *R. toruloides* strains [26, 27]. In
317 terms of carbon sources, strains of the particular yeasts are reported to withstand carbon-rich media
318 and under certain conditions, to promote high density cell cultures [20]. On the other hand, in
319 earlier studies, strains of *R. toruloides* have been flask-cultured in media composed of pure stearic
320 acid or blends of pure stearic acid, glucose and glycerol and tailor-made lipids presenting
321 similarities with the cocoa-butter had been synthesized [8, 29]. Equally in early studies, strains of
322 this species had been cultivated on glucose-based media in which $\Delta 9$ and $\Delta 12$ natural or artificial
323 desaturase inhibitors had been added into the culture medium in order to suppress the desaturation
324 reactions inside *R. toruloides* cells, so as finally, again to synthesize lipids presenting compositional
325 similarities with the cocoa-butter [30, 31]. More recently Zhu et al. [32] have carried out a massive
326 study based on genomic sequencing of *R. toruloides*, in an attempt to unravel lipid accumulation
327 process on a genetic level, spent cell mass hydrolysates used as nutrients and spent water from lipid
328 production process were used as substrates by *R. toruloides* for SCO production [33] while other
329 species belonging to the genus *Rhodospiridium* (e.g. *R. kratochvilovae*, *R. babjevae*, *R.*
330 *diobovatum*) have been successfully used as cell factories used for SCO on several waste streams or
331 low-cost materials [34-36].

332 One major objective of the study was to identify the effect of NaCl addition into the culture
333 medium upon the process of lipid accumulation of *R. toruloides* DSM 4444. To this end, batch-
334 flask cultures of the microorganism were performed in media containing increasing amounts of
335 NaCl up to 6.0% (w/v). The particular strain can be categorized as halotolerant, due to its ability to
336 grow sufficient in the presence or absence of salt [13, 37]. Furthermore, NaCl concentrations up to
337 4.0% (w/v) were found to promote optimum growth of the yeast and thus, according to Larsen [38]
338 the strain can be designated as moderate halotolerant. This feature is commonly encountered in
339 microorganisms isolated from marine environments, possessing unique adaptation mechanisms in
340 high salinity conditions [13, 39-42]. A major part of the mechanisms involved in osmotic balance
341 regulation for halotolerant microorganisms represents the production and accumulation of solutes,

342 such as glycerol, trehalose, mannitol and erythritol, etc [43]. In the current investigation and after a
343 threshold NaCl value, small (but not negligible) glycerol quantities were found into the medium,
344 apparently as response to the osmotic stress imposed. Under this optic, the last years there has been
345 a number of reports in which the polymorphic yeast *Y. lipolytica* has been cultivated in media
346 composed of high initial concentrations of (pure or biodiesel-derived) glycerol supplemented with
347 increased initial concentrations of NaCl, and enhancement of production of erythritol, the
348 concentration of which in some cases reached in indeed very high levels (e.g. >45 g/L or even *c.*
349 200 g/L) has been reported when glycerol has been employed as fermentation substrate by wild or
350 mutant *Y. lipolytica* strains [44-47]. Likewise, besides mannitol and erythritol, due to their high
351 osmotic tolerance, halotolerant yeasts have been proposed as candidates for bioethanol production
352 [48], enzyme production [49] as well as xylitol biosynthesis [50]. However, none of these
353 microorganisms has ever been reported as oleaginous. Surprisingly enough, in the current study the
354 addition of NaCl not only did not suppress microbial growth, but on the contrary was found to
355 enhance lipid accumulation, as such was the case in media containing 4.0% (w/v) NaCl. Under
356 these conditions, lipid accumulation increased to *c.* 29% compared to the control experiment (see
357 Table 1). Positive correlation between salt and lipid production has been shown for oleaginous
358 microalgae strains belonging to the genera of *Dunaliella* sp. and *Nannochloropsis* sp. [51, 52].

359 Another aspect of the study was the realization of microbial lipid production in the presence
360 of NaCl under pasteurized conditions. In batch-flask trials with 50 or 100 g/L of initial glucose
361 concentration, signs of bacterial contamination were noted, despite the use of a more concentrated
362 inoculum. However, bacterial presence was less than 5% of the total microbial population and was
363 not found to be detrimental for yeast growth and lipid formation. In the case of microbial
364 conversions, pasteurized grape must has been used for alcoholic fermentation [53], while the effect
365 of pasteurized whey-based medium on propionic acid production has also been evaluated [54].
366 Additionally, the application of completely non-aseptic conditions has been tested for microbial
367 solvent production such as ethanol and 1,3-propanediol [55-58], as means of energy and operation

368 cost reduction. However, in all of the above-mentioned cases (e.g. production of bio-ethanol and
369 1,3-propanediol), it was the main metabolic compound-target (the bio-alcohol) that was synthesized
370 rapidly and in high concentrations that hindered the microbial contamination with undesirable
371 microorganisms, fact that does not happen during the SCO fermentation. As far as the SCO
372 bioprocess is concerned, in order to perform a relatively successful non-aseptic culture, a “hurdle”
373 should be added into the medium; i.e. Moustogianni et al. [59] have successfully produced SCO by
374 using oleaginous Zygomycetes grown on glycerol when essential oils or antibiotics had previously
375 been added into the fermentation medium. In any case, the current study is one of the first in the
376 literature that deals with the production of SCO under non-aseptic conditions.

377 During batch-bioreactor trials, the yeast *R. toruloides* exhibited notable tolerance against
378 high substrate concentrations. The particular strain grew satisfactorily without obvious substrate
379 inhibition being exerted in media containing up to 150 g/L of glucose, while of importance was the
380 almost linear profile of lipid accumulation, regardless of the initial substrate concentration
381 employed into the medium. Tolerance against high substrate (e.g. sugar or glycerol) input is
382 essential, in order to achieve high-density cultures that have been proved as effective cultivation
383 strategy in the case of microbial lipid production. In a similar manner, Li et al. [14] demonstrated
384 that the yeast *R. toruloides* Y4 grew well in media containing glucose up to 150 g/L, a fact directly
385 correlated with the tolerance of the yeast against osmotic stress. On the other hand, for several
386 oleaginous yeasts, substrate (e.g. sugar or glycerol) concentrations at *c.* 100 g/L or even lower (e.g.
387 60-70 g/L) have been reported as threshold, above which microbial cell growth was repressed
388 significantly [60-62]. In contrast, oleaginous fungi seem more resistant in high initial substrate
389 (sugar or glycerol) quantities, since initial concentrations ranging between 80-100 g/L have been
390 considered as ideal in order to enhance the process of lipid accumulation for the species *Mortierella*
391 *isabellina*, *Thamnidium elegans* and *Cunninghamella echinulata* [21, 24, 63-65].

392 *R. toruloides* DSM 4444 presented remarkable cell growth and lipid production in both
393 shake-flask and bioreactor experiments. As indicated in the previous paragraphs, strains of this

394 particular species have been employed already from early studies in order for SCO production to be
395 performed. Initially (mid 80s) these strains had been employed as microbial cell factories in order to
396 produce lipids that mimicked the composition of cocoa-butter [2, 4, 8, 29-31]. The last decade, with
397 the potentiality of the replacement of edible oils by non-conventional fatty substances (e.g. yeast
398 oils) as starting materials in order for biodiesel precursors to be synthesized has been assessed, *R.*
399 *toruloides* has been considered as a microorganism of importance amenable to be used in the
400 conversion of low-cost hydrophilic materials (e.g. lignocellulosic sugars, glucose, sorghum extracts,
401 glycerol, etc) into SCOs. Characteristic results concerning production of lipid in several
402 fermentation configurations are depicted in Table 5.

403 In the current investigation and specifically in the bioreactor experiments, conversion yields
404 of the order of *c.* 0.21 g of lipid produced per g of glucose consumed have been seen. The
405 stoichiometry of glucose (and similar sugars such as lactose, fructose, etc) metabolism indicates that
406 about 1.1 moles of acetyl-CoA are generated from 100 g of glucose [1, 3, 5]. Assuming that all the
407 acetyl-CoA produced is channeled towards lipid synthesis, the maximum theoretical yield of SCO
408 produced per glucose consumed is around 0.32 g/g [3, 5]. This value concerning the fermentation of
409 xylose ranges between 0.31-0.34 g/g, while with reference to glycerol, the maximum theoretical
410 yield of SCO is around 0.30 g/g [1, 3]. However, even under ideal conditions for SCO production
411 (e.g. highly aerated bioreactor cultures) lipid yield on glucose consumed can rarely be higher than
412 0.22-0.23 g/g [5, 11, 23]. It may be assumed therefore that in the current investigation one of the
413 highest conversion yields of SCO produced per sugar consumed has been achieved. As previously
414 indicated [5, 23] cultivation in highly aerated bioreactors was considered as an important
415 prerequisite in order to achieve such high conversion yields. However, in some cases, in shake-flask
416 experiments equally high lipid yields can be achieved; in trials with *T. elegans* grown on sucrose in
417 shake flasks, the conversion yield of lipid produced per sugar consumed was *c.* 0.24 g/g, while
418 utilization of other sugars (glucose or fructose) equally resulted in exceptional conversion yields,
419 i.e. >0.20 g/g [24]. Likewise, maximum conversion yields of the same magnitude compared with

420 growth of *T. elegans* on sucrose (c. 0.23 g/g) have been reported for *Cunninghamella echinulata*
421 cultivated on xylose in shake-flask experiments [21].

422 Fatty acid analysis was carried out in lipids produced during *R. toruloides* batch-flask
423 cultures with increasing NaCl content. The main fatty acids detected in yeast oil composition were
424 oleic (Δ^9 C18:1), palmitic (C16:0), linoleic ($\Delta^{9,12}$ C18:2) as well as stearic acid (C18:0). NaCl
425 presence did not affect the concentration of individual fatty acids, whereas microbial lipid became
426 generally more unsaturated, during lipid accumulation phase. This is attributed to the increase of
427 oleic acid as major constituent of accumulated triglycerides [61]. The distribution of *R. toruloides*
428 fatty acids is similar to oil profiles obtained by other oleaginous yeasts [10, 17-19, 75]. Recent
429 studies have shown the suitability of microbial oil as starting material for biodiesel production,
430 through its direct transformation from microbial biomass [21].

431

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440

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442

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609 fermentation of glucose and xylose by the oleaginous yeast *Lipomyces starkeyi*. *Eur. J. Lipid Sci. Technol.* 2008,
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- 611
- 612

613 Table 1

614 Quantitative data of *Rhodospiridium toruloides* DSM 4444 originated from kinetics in media with six different initial
615 NaCl concentrations with the same initial glucose concentration (50 g/L). Representation consumed glucose (Glc_{cons},
616 g/L), produced biomass (X, g/L), lipid content (L, g/L) and lipid in dry weight (Y_{L/X}, % w/w) when the maximum
617 quantity of lipid (in g/L) was achieved. The maximum specific growth (μ_{\max} , h⁻¹), was calculated for every of the trials
618 performed by fitting the equation $\ln\left(\frac{X}{X_0}\right) = f(t)$ within the early exponential growth phase of the respective culture.
619 Culture conditions: growth in 250-ml flasks at 185 rpm, initial pH=6.0±0.1, DO>20% (v/v), incubation temperature
620 T=26 °C. Two lots of independent cultures were conducted by using different inocula. In all of the determinations,
621 standard error calculated was less that 15%.

622

Entry	NaCl (%, w/v)	Time (h)	μ_{\max} (h ⁻¹)	Glc _{cons} (g/L)	X (g/L)	L (g/L)	Y _{L/X} (%, w/w)
1	0.0	168	0.091	48.8	8.9	4.9	55.1
2	0.5	168	0.088	43.6	8.7	5.4	62.1
3	1.0	144	0.101	48.7	8.2	5.1	62.2
4	1.5	144	0.093	44.5	8.5	5.3	62.4
5	2.5	120	0.084	47.9	8.9	5.4	60.6
6	4.0	192	0.090	48.6	9.4	6.7	71.3
7	6.0	168	0.070	30.0	6.3	2.8	44.4

623

624

625 Table 2

626 Fatty acid composition of cellular lipids of *Rhodospiridium toroloides* DSM 4444 on glucose-based media (Glc_i=50

627 g/L), containing various NaCl concentrations. Very early (VE) growth phase is that in which the incubation time is

628 between 20-30 h. Early (E) growth phase is that in which the incubation time is between 50-70 h. Late growth phase is

629 that in which incubation time is c. 150 h. Culture conditions as in Table 1.

630

Fatty acid composition (% , w/w)											
NaCl (% , w/v)	Growth phase	C14:0	C16:0	Δ^9 C16:1	C18:0	Δ^9 C18:1	$\Delta^{9,12}$ C18:2	$\Delta^{9,12,15}$ C18:3	C20:0	C22:0	*SFA/ UFA
0.0	VE	1.1	28.2	0.8	11.5	48.6	7.7	2.1	-	-	0.69
	E	0.8	24.1	1.5	9.9	51.2	9.1	2.4	-	-	0.54
	L	0.6	22.2	1.4	7.1	54.5	11.5	3.0	-	-	0.43
0.5	VE	1.4	27.5	1.2	10.0	49.0	8.6	2.1	-	-	0.68
	E	1.2	25.5	0.8	12.5	48.8	7.8	2.3	0.3	0.7	0.67
	L	1.1	24.6	1.2	10.0	49.0	10.4	2.8	0.6		0.57
1.0	VE	1.6	23.8	0.9	16.7	45.7	6.6	-	-	4.3	0.87
	E	1.6	25.5	1.1	10.9	49.3	8.1	2.3	0.3	0.2	0.63
	L	1.0	23.3	0.8	8.6	52.2	11.2	2.2	0.2	0.2	0.50
1.5	VE	1.1	26.8	-	10.0	51.3	8.6	1.9	-	-	0.61
	E	1.2	25.8	0.5	11.2	50.7	7.3	2.1	0.3	0.5	0.56
	L	0.9	24.5	1.2	9.8	51.5	8.0	2.6	-	-	0.56
2.5	VE	1.2	26.9	1.2	10.5	51.3	7.0	1.6	-	-	0.63
	E	1.1	25.8	0.7	9.6	50.5	9.0	2.3	0.2	0.3	0.59
	L	0.9	24.0	1.5	9.0	53.0	8.0	2.7	-	-	0.52
4.0	VE	1.4	23.4	0.4	15.5	47.2	9.7	2.6	0.4	0.4	0.68
	E	1.2	26.3	0.4	10.3	50.4	8.2	2.4	-	0.5	0.62
	L	0.8	25.5	1.5	8.5	51.8	8.9	2.8	-	-	0.54

631 *Ratio of saturated to unsaturated fatty acids

632

633

634 Table 3
 635 Quantitative data of *Rhodospiridium toruloides* DSM 4444 originated from kinetics in shake-flask experiments in
 636 sterilized and pasteurized media, supplemented with 4.0% (w/v) NaCl and 50 and 100 g/L initial glucose concentration
 637 (A) and from kinetics in media containing 50, 100 and 150 g/L of glucose and 4% (w/v) NaCl in batch-bioreactor
 638 experiments (B). Representation of initial glucose (Glc_i, g/L), consumed glucose (Glc_{cons}, g/L), produced biomass (X,
 639 g/L), produced lipid (L, g/L), lipid in dry weight (% w/w), lipid yield per consumed substrate (Y_{L/Glc}, g/g) and biomass
 640 yield per consumed substrate (Y_{X/Glc}, g/g). Culture conditions for the shake flasks: growth in 250-mL flasks at 185 rpm,
 641 initial pH=6.0±0.1, DO>20% (v/v), incubation temperature T=26 °C; for the bioreactor: agitation speed 200-500 rpm,
 642 pH=6.0±0.1, DO>20% (v/v), temperature T=26 °C. Two lots of independent cultures were conducted by using different
 643 inocula. In all of the determinations, standard error calculated was less than 10%.

644

645 A)

Culture mode and heat-treatment	Glc _i (g/L)	Time (h)	Glc _{cons} (g/L)	X (g/L)	L (g/L)	Y _{L/X} (% w/w)	Y _{L/Glc} (g/g)	Y _{X/Glc} (g/g)
Flasks, sterilized	≈50	192	48.6	9.4	6.7	71.3	0.14	0.19
Flasks, pasteurized	≈50	160	48.0	11.7	5.9	50.4	0.12	0.24
Flasks, sterilized	≈100	505	93.0	16.1	9.2	57.1	0.10	0.18
Flasks, pasteurized	≈100	311	80.0	17.9	9.1	50.8	0.11	0.22

646 B)

Culture mode	Glc _i (g/L)	Time (h)	Glc _{cons} (g/L)	X (g/L)	L (g/L)	Y _{L/X} (% w/w)	Y _{L/Glc} (g/g)	Y _{X/Glc} (g/g)
Batch-bioreactor	≈50.0	72	44.5	12.7	8.1	63.8	0.18	0.29
	≈100.0	160	90.9	25.2	14.2	56.3	0.16	0.28
	≈150.0	312	110.3	36.2	23.6	65.1	0.21	0.33

647

648

649 Table 4

650 Fatty acid composition of cellular lipids of *Rhodospiridium toroloides* DSM 4444 growing in shake flasks in previously
 651 pasteurized media presenting increasing initial glucose (Glc_i) concentration and constant NaCl concentration (A) and
 652 fatty composition of lipids of the same microorganism growing in batch-bioreactor experiments in media presenting
 653 increasing initial glucose (Glc_i) concentration and constant NaCl concentration (B). In the flask experiments, for
 654 Glc_i≈50 g/L, very early (VE) growth phase is that in which the incubation time is between 10-20 h and late growth
 655 phase is that in which incubation time is c. 150 h. For Glc_i≈100 g/L, very early (VE) growth phase is that in which the
 656 incubation time is between 20-30 h and late growth phase is that in which incubation time is c. 300 h. In the bioreactor
 657 experiments, for Glc_i≈50 g/L, very early (VE) growth phase is that in which the incubation time is between 10-20 h,
 658 early (E) growth phase is that in which the incubation time is between 30-40 h and late growth phase is that in which
 659 incubation time is c. 70 h. For Glc_i≈100 g/L, very early (VE) growth phase is that in which the incubation time is
 660 between 20-30 h, early (E) growth phase is that in which the incubation time is between 50-80 h and late growth phase
 661 is that in which incubation time is c. 150 h.

662

663 A)

Glc _i (g/L)	Growth phase	C14:0	C16:0	Δ ⁹ C16:1	C18:0	Δ ⁹ C18:1	Δ ^{9,12} C18:2	Δ ^{9,12,15} C18:3
≈50	VE	0.6	26.4	0.7	8.4	54.4	5.9	2.3
	L	1.1	23.0	0.5	7.9	57.4	6.9	2.6
≈100	VE	1.2	24.8	-	9.6	52.9	6.5	2.7
	L	1.6	22.0	1.5	8.2	55.0	6.8	2.2

664 B)

Glc _i (g/L)	Growth phase	C14:0	C16:0	Δ ⁹ C16:1	C18:0	Δ ⁹ C18:1	Δ ^{9,12} C18:2	Δ ^{9,12,15} C18:3
≈50	VE	1.5	25.2	-	12.0	50.2	8.4	2.0
	E	1.0	23.8	1.1	10.1	54.7	9.3	-
	L	1.2	22.1	1.5	10.8	55.7	6.5	1.4
≈100	VE	-	23.8	-	12.2	53.7	6.7	1.5
	E	-	22.1	2.5	11.9	55.4	5.8	2.0
	L	-	20.9	3.0	9.1	54.9	8.9	2.5

665

666

667 Table 5
 668 Experimental results of *Rhodospiridium toruloides* strains producing microbial lipid during growth on several carbon
 669 sources under various fermentation configurations and their comparisons with the present study.
 670

Strain	Substrate	Culture mode	X (g/L)	Y _{LX} (% w/w)	Reference
<i>R. toruloides</i> *	Pure stearic acid	Shake flasks	11.7	35.0	Gierhart [8]
<i>R. toruloides</i> AS2.1389	Glycerol	Shake flasks	19.2	47.7	Xu et al. [9]
<i>R. toruloides</i> AS2.1389	Glycerol	Batch bioreactor	26.7	69.5	”
<i>R. toruloides</i> AS2.1389	Glucose	Shake flasks	18.3	76.0	Li et al. [14]
<i>R. toruloides</i> CCT 0783	Sweet sorghum extract	Shake flasks	41.7	33.1	Matsakas et al. [15]
<i>R. toruloides</i> DSM 444	Glycerol/sunflower meal hydrolysate blend	Shake flasks	27.9	29.0	Leiva-Candia et al. [18]
<i>R. toruloides</i> DSM 444	Glycerol/sunflower meal hydrolysate blend	Fed-batch bioreactor	37.4	51.3	”
<i>R. toruloides</i> NRRL Y- 27012	Biodiesel-derived glycerol	Shake flasks	30.1	40.0	Tchakouteu et al. [19]
<i>R. toruloides</i> Y4	Glucose	Fed-batch bioreactor	106.5	67.5	Li et al. [20]
<i>R. toruloides</i> Y4	Glucose (phosphate- limited trial)	Shake flasks	20.6	51.4	Wu et al. [27]
<i>R. toruloides</i> Y4	Glucose (phosphate- limited trial)	Shake flasks	19.4	62.4	”
<i>R. toruloides</i> Y4	Glucose (sulphate- limited trial)	Shake flasks	23.0	20.8	Wu et al. [28]
<i>R. toruloides</i> Y4	Glucose (sulfate-limited trial)	Shake flasks	14.2	55.6	”
<i>R. toruloides</i> Y4	Glucose (sulfate-limited trial)	Shake flasks	17.8	55.7	”
<i>R. toruloides</i> *	Glucose/pure stearic acid	Shake flasks	9.8	46.1	Gierhart [29]
<i>R. toruloides</i> CBS14	Glucose	Shake flasks	12.3	30.8	Moreton [30]
<i>R. toruloides</i> CBS14	Glucose	Shake flasks	8.0	42.5	Moreton [66]
<i>R. toruloides</i> CBS14	Fructose	Shake flasks	7.9	25.3	”
<i>R. toruloides</i> CBS14	Glycerol	Shake flasks	5.8	34.6	”
<i>R. toruloides</i> CBS14	Glucose	Batch bioreactor	12.5	42.9	”
<i>R. toruloides</i> CBS14	Fructose	Batch bioreactor	8.7	39.8	”
<i>R. toruloides</i> CBS14	Xylose	Batch bioreactor	8.3	42.2	”
<i>R. toruloides</i> Y4	Jerusalem artichoke extracts	Shake flasks	25.5	40.0	Zhao et al. [67]
<i>R. toruloides</i> Y4	Jerusalem artichoke hydrolysates	Fed-batch bioreactor	70.1	56.5	”

<i>R. toruloides</i> CBS14	Glucose	Fed-batch bioreactor	35.0	71.4	Wiebe et al. [68]
<i>R. toruloides</i> CBS14	Glucose/xylose/arabinose blend	Fed-batch bioreactor	27.0	55.5	”
<i>R. toruloides</i> Y4	Biodiesel-derived glycerol	Batch bioreactor	35.3	46.0	Uçkun Kiran et al. [69]
<i>R. toruloides</i> 2F5	Inulin	Shake-flasks	15.8	62.1	Wang et al. [70]
<i>R. toruloides</i> 2F5	Inulin	Batch bioreactor	15.6	70.4	”
<i>R. toruloides</i> CCT 0783	Glucose/xylose blend	Batch bioreactor	13.3	42.0	Bonturi et al. [71]
<i>R. toruloides</i> DSM 444	Glucose	Batch bioreactor	~22	~40	Bommareddy et al. [72]
<i>R. toruloides</i> DSM 444	Glycerol	Batch bioreactor	~15	~57	”
<i>R. toruloides</i> AS2.1389	Glucose	Single-stage continuous bioreactor	8.7	61.8	Shen et al. [73]
<i>R. toruloides</i> AS2.1389	Glucose	Single-stage continuous bioreactor	5.7	53.1	”
<i>Rhodospiridium toruloides</i> Y4	Biodiesel-derived glycerol	Shake flasks	24.9	48.9	Yang et al. [74]
<i>R. toruloides</i> DSM 444	Glucose (NaCl-enriched culture)	Fed-batch bioreactor	37.2	64.5	Present study

671 *: No indication of the strain

672

673

674 Fig. 1

675 Kinetics of lipid production (L; g/L) and lipid in dry cell weight (Y_{LX} ; % w/w) during growth of *Rhodospiridium*
676 *toruloides* DSM 4444 on glucose-based media (100 g/L) supplemented with 4% (w/v) NaCl. Culture conditions as in
677 Table 1. Two lots of independent cultures were conducted by using different inocula. In all of the determinations,
678 standard error calculated was less than 10%.

679

680 Fig. 2

681 Kinetics of residual glucose (Glc; g/L), biomass production (X; g/L) and lipid accumulated (L; g/L), during
682 *Rhodospiridium toruloides* DSM 4444 growth in previously sterilized (filled symbols) and pasteurized (open symbols)
683 media with 100 g/L of glucose and 4% (w/v) NaCl. Culture conditions as in Table 2. Two lots of independent cultures
684 were conducted by using different inocula. In all of the determinations, standard error calculated was less than 10%.

685

686 Fig. 3

687 Kinetics of residual glucose (Glc; g/L) and lipid accumulated (L; g/L) during batch-bioreactor cultures of
688 *Rhodospiridium toruloides* DSM 4444 in media containing 50 g/L (open symbols), 100 g/L (grey symbols) and 150 g/L
689 (filled symbols) of glucose, supplemented with 4.0% (w/v) of NaCl. Culture conditions as in Table 4B. Two lots of
690 independent cultures were conducted by using different inocula. In all of the determinations, standard error calculated
691 was less than 10%.

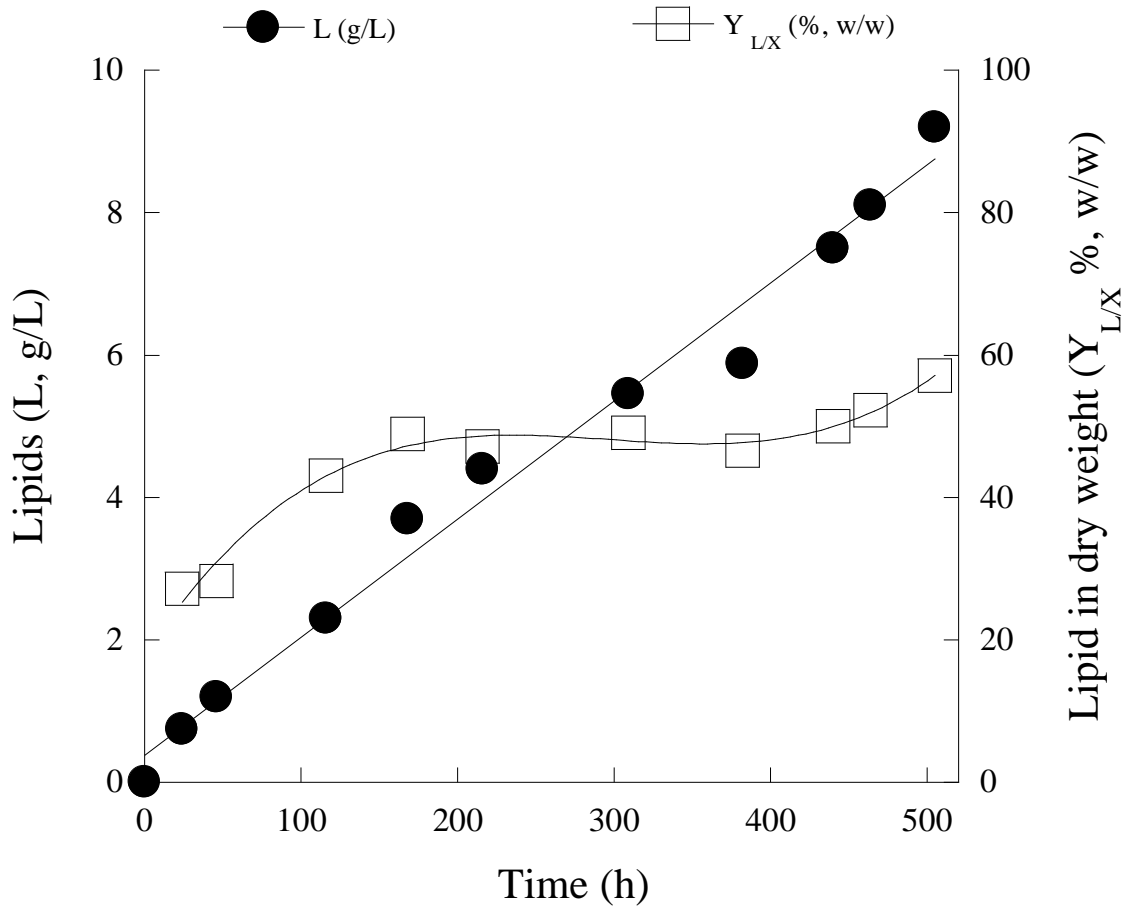
692

693 Fig. 4

694 Kinetics of residual glucose (Glc; g/L), biomass production (X; g/L) and lipid accumulation (L; g/L) (A) and
695 representation of biomass production (X; g/L) and lipid production (L; g/L) per substrate consumed (B) during fed-
696 batch bioreactor cultures of *Rhodospiridium toruloides* DSM 4444, in media supplemented with 4.0% (w/v) NaCl.
697 Culture conditions as in Table 4B. Two lots of independent cultures were conducted by using different inocula. In all of
698 the determinations, standard error calculated was less than 10%.

699

700



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703

704

Fig. 1

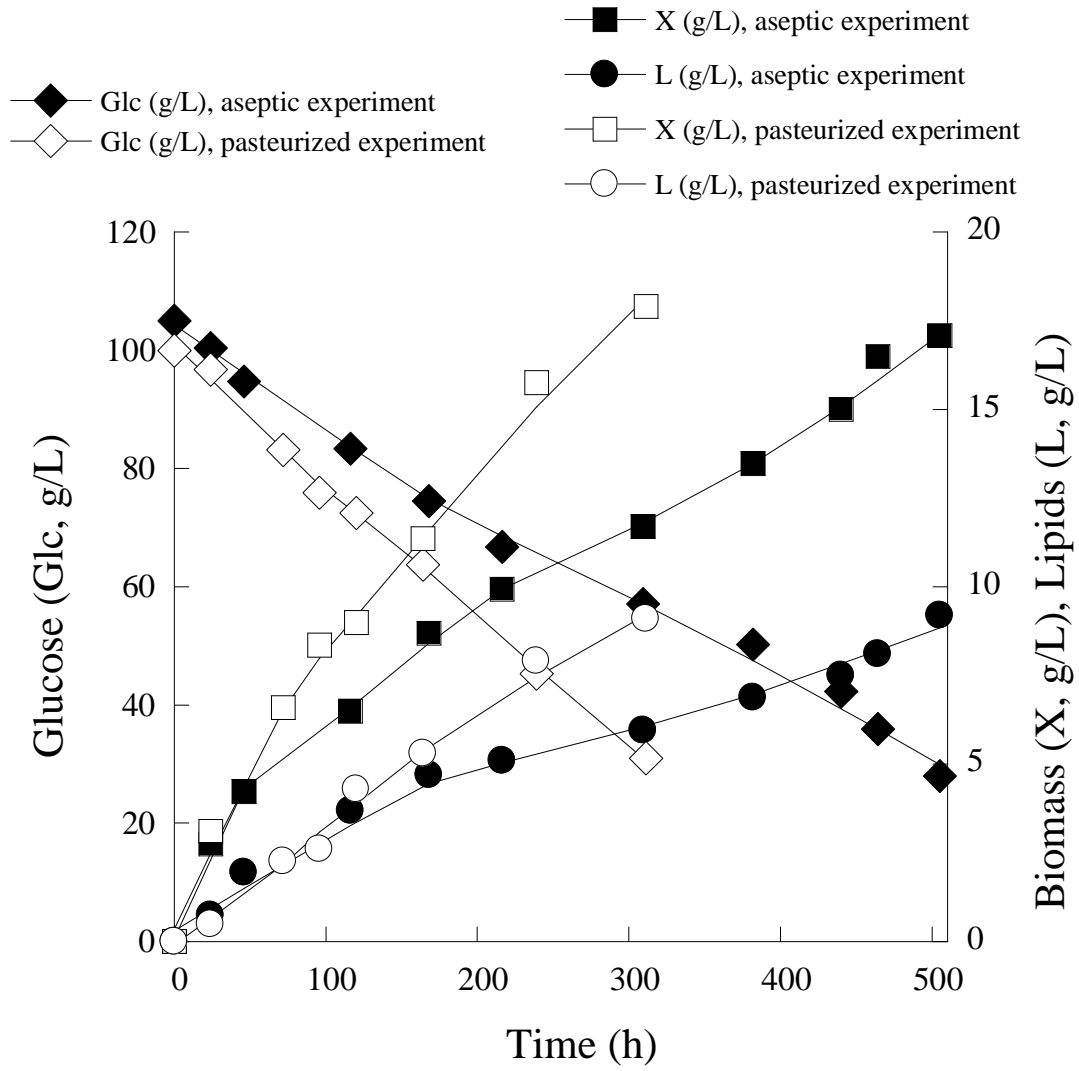


Fig. 2

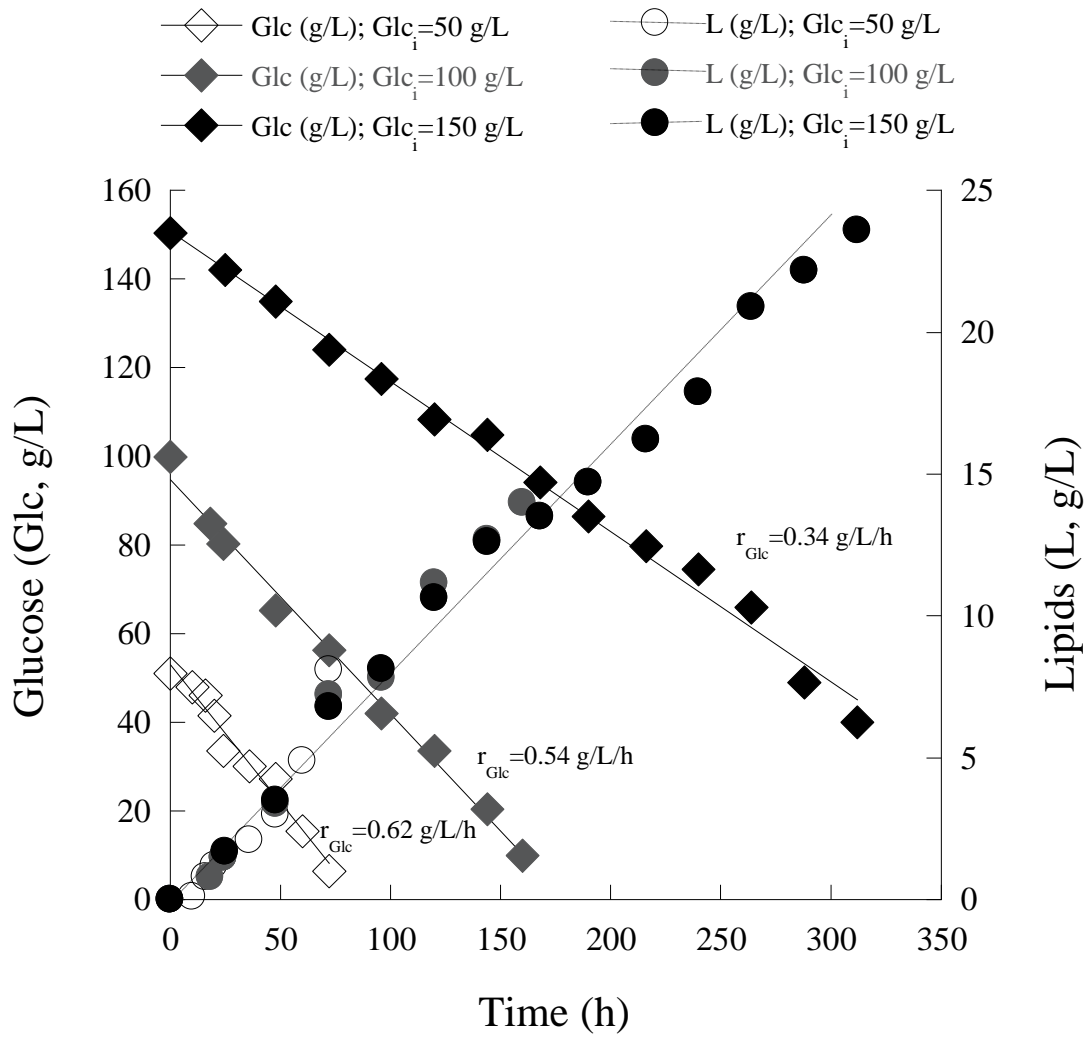


Fig. 3

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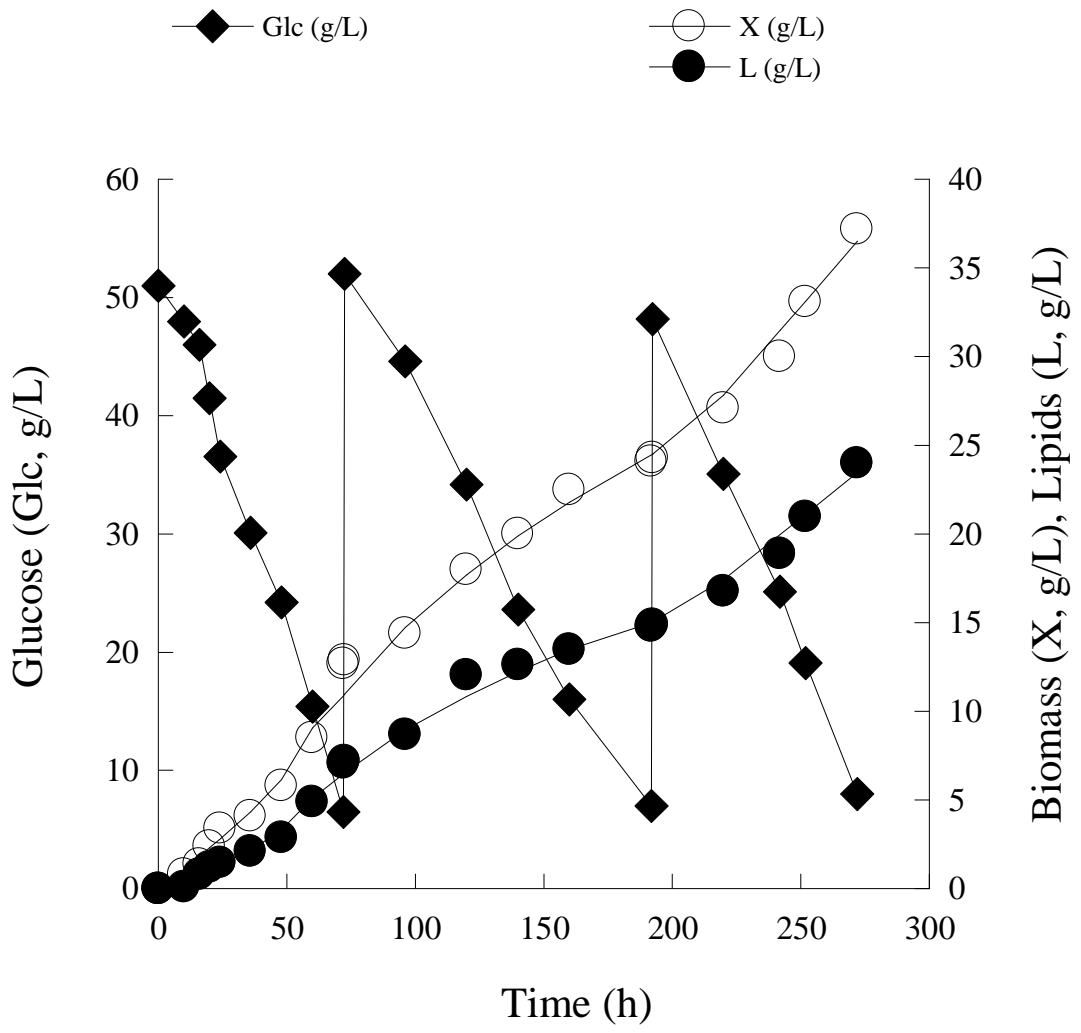


Fig. 4A

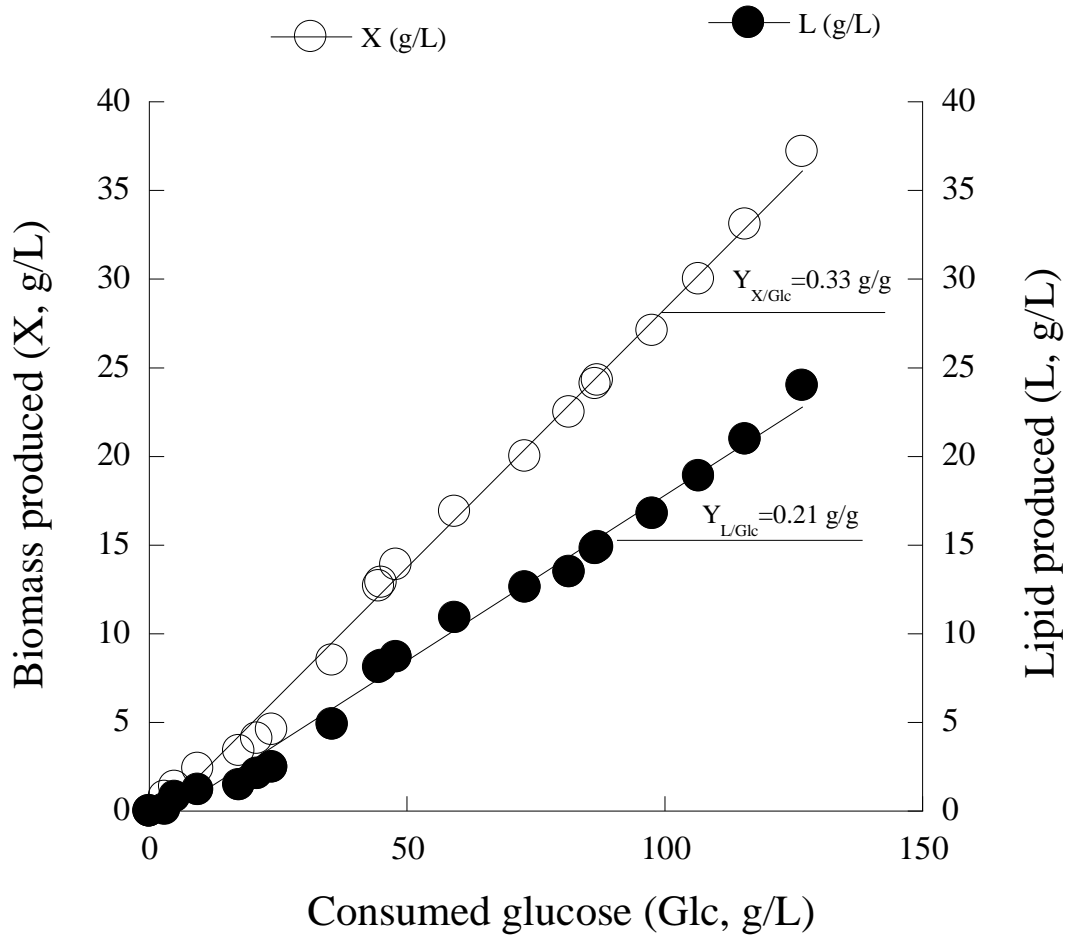


Fig. 4B