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1 ENHANCEMENT OF ETHANOL PRODUCTION FROM SYNTHETIC MEDIUM MODEL OF
2 HYDROLYSATE OF MACROALGAE

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35 ABSTRACT

36 Among biomass materials available, macroalgae is a promising alternative to traditional energy 37 crops. The absence of lignin, a high growth rate and a richness of fermentable sugars and nitrogen, 38 are real gains for a competitive ethanol production. But the presence of salts can be an obstacle to 39 obtain relevant performances. Experiments were carried out with a synthetic medium adjusted on 40 algal hydrolysate composition in order to reduce resource limitations and variations of composition. 41 The behavior of four yeast strains for ethanol production was investigated: *Candida guilliermondii*, 42 *Scheffersomyces stipitis*, *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*. Glucose, which 43 is the most abundant sugar in the targeted algal hydrolysate (*Ulva* spp), was completely assimilated 44 by all of the considered strains, even in the presence of salts at levels found in macroalgal 45 hydrolysates (0.25 M of sodium chloride and 0.21 M of sulfate). The use of peptone as nitrogen 46 source enhanced kinetics of consumption and production. For instance, the rate of ethanol 47 production by *S. cerevisiae* in the presence of peptone was six times higher than that obtained using 48 ammonium, 0.6 and 0.1 $g.L^{-1}.h^{-1}$ respectively. In the presence of salts, the rates of glucose 49 consumption and ethanol production were lowered for the considered strains, except for *K.* 50 *marxianus*. Nevertheless, *S. cerevisiae* could be the most promising strain to valorize *Ulva* spp 51 hydrolysate in bioethanol, in terms of ethanol produced (7.5- 7.9 $g.L^{-1}$) whether in the presence or 52 in absence of salts.

53

54 *Keywords: macroalgae, yeasts, valorization, ethanol, osmotic pressure, sugar mix*

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69 **1. INTRODUCTION**

70 Nowadays, 88% of energy consumption is mainly derived from fossil fuels, such as petrol, coal or
71 natural gas [1]. The growing concern on depletion of fossil fuels and their environmental effects,
72 particularly greenhouse gas emissions, have led to search for viable renewable fuel alternatives [2].
73 One of these alternative solutions is to produce biofuels like bioethanol. This renewable fuel is
74 made from vegetal wastes, like agricultural residues (rice straw, corn stover, wet birch pulp), agro-
75 industrial wastes (mushroom wastes, cotton cellulose, coffee, date syrup) [3]-[4] and microalgae
76 [5]. Macroalgae are also considered as a potential source for third generation biofuel production [6]-
77 [7]

78 Furthermore, in France, a proliferation of green algae and deposit on the beaches of Brittany can
79 be observed. Up to 98.000 m^3 algal biomass, principally *Ulva*, is gathered during summer along the
80 Brittany coastline [8]. This proliferation is the result of many factors. Among them, years of use of
81 nitrates and phosphates, especially in agriculture. *Ulva* has a negative impact on costal ecosystem
82 and causes problems, such as emission of an offensive odor, killing of shellfish [9,10] or killing of
83 abalone *Haliotis discus hannai* [11]. It was found that both fresh medium and decomposing algal
84 effluent have toxic effects and the decomposed form is more toxic than fresh culture medium,
85 provoking hypoxia due to the release of ammonia and sulfides.

86 But this biomass regarded as a pollutant can be converted into high-value product, such as
87 ethanol via fermentation. Besides having a fast growth rate and a high biomass yield, macroalgae
88 contain high carbohydrates levels (20 to 40% dry weight) but no lignin [12] which is difficult to
89 degrade. So, its valuation offers a double benefit: solving a problem of green tide and help to
90 produce bio-energy and high-value substances without using available food resources. After
91 hydrolysis, this type of carbon source can provide a wide range of simple sugars, such as glucose,
92 galactose, xylose, arabinose, fucose, mannitol and rhamnose [13]. Also rich in protein (almost 20%
93 of dry weight) and free amino-acids, macroalgae are also a potential source of nitrogen. For this
94 reason, they are used as a complement for the fermentation of rice straw [14].

95 Nevertheless, sugar composition and quantities vary from a macroalgae to another and for a
96 given algae, environmental and seasonal variations are also observed [15]. Macroalgae also contain
97 salts, like sodium chloride and sulfates, from sulfated polymer like ulvan [9]. These components
98 could play a role in the osmotic pressure of the culture medium and so on ethanol production
99 performance.

100 In view of the valorization of this bioresource, there is therefore a need to select adequate
101 microorganisms. For this purpose, several studies focused on the selection of natural or genetically

102 modified microorganisms. Bioconversion of algal biomass to ethanol can be operated by bacteria,
103 like *Clostridium phytofermentans* [16], or the recombinant *Escherichia coli KO11* [17]. However,
104 yeast strains are the most used. Among all, *Saccharomyces cerevisiae* is the most commonly
105 studied for ethanol conversion of cellulosic and lignocellulosic biomass [18,19]. This strain has a
106 high ethanol tolerance, but also high yields and rates of fermentation. Moreover, because this yeast
107 is Crabtree-positive, fermentative pathway is favored in the presence of high sugar levels [20]. For
108 these reasons, it is already used for ethanol conversion of macroalgae, such as *Laminaria digitata*,
109 *Chondrus crispus* or *Ulva lactuca* [13], *Sargassum* spp, with on average 89% of ethanol conversion
110 [21], as well as with *Gelidium amansii* [22]. However, due to an insufficient capacity of the non-
111 oxidative pentose phosphate pathway, *S. cerevisiae* is unable to ferment xylose [23].

112 Among the wild type of yeasts capable of fermenting xylose in ethanol, at relatively high yield
113 (0.404 g/g), *Pichia stipitis* is considered as the most interesting [24,25]. This strain is often utilized
114 in co-culture with *S. cerevisiae* for ethanol production from glucose and xylose [25–27]. But it has a
115 low ethanol tolerance (inhibition beyond 30 $g.L^{-1}$ of ethanol) [28]. This strain is already used for
116 biomass conversion in ethanol from coffee industry wastes hydrolysates, which contain xylose,
117 glucose, arabinose, galactose and mannose [29].

118 *Kluyveromyces marxianus* is able to ferment mixed sugars comprising glucose, galactose,
119 xylose, arabinose and mannose from green macroalgae [13]. The advantages of this strain are a fast
120 cell growth rate and a higher ethanol tolerance than *P. stipitis* [28,30]. From 100 $g.L^{-1}$ of glucose, *K.*
121 *marxianus* is capable of producing 49 $g.L^{-1}$ of ethanol in only 22 hours [31]. Due to its broad
122 substrate spectrum (glucose, galactose, xylose, mannitol and rhamnose), *Candida guilliermondii* is
123 also an interesting strain for waste valorization in ethanol [13]. It is already used for sugarcane
124 bagasse, date wastes or macroalgae valorization in ethanol [32]-[33].

125 The objective of this work was to improve ethanol production from a model medium simulating
126 algal hydrolysate to assess for possible subsequent implementation on the hydrolysate. Working
127 with synthetic medium led not only to reduce resource limitations but also to control the
128 composition, avoiding seasonal variations of its composition. This can give insights on the impacts
129 of variable compositions from algal hydrolysates on yeast fermentation and ethanol yields.
130 Adjustment of the synthetic medium was focused on carbon and nitrogen substrates and the
131 presence of salts. According to the above literature review, the choice of the strain is of major
132 importance. Because *S. cerevisiae*, *C. guilliermondii*, *P. stipitis* and *K. marxianus* have already
133 proven their relevance for ethanol fermentation from various wastes, they were selected for this
134 study. Behavior of these four yeast strains was therefore investigated using synthetic medium
135 mimicking green algal hydrolysates.

136

137 ## 2. MATERIAL AND METHODS

138 ### 2.1 Microorganisms and inoculum

139 *Saccharomyces cerevisiae* baker's yeast CLIB 95 (CIRM French), *Pichia stipitis* 3651, *Candida*
140 *guilliermondii* 11947 and *Kluyveromyces marxianus* 11954, obtained from DSMZ (Germany) were
141 used in this study.

142 Culture of *S. cerevisiae* was maintained at 4°C on a Petri plates and agar slant whose
143 composition consisted in ($g.L^{-1}$): glucose (20), peptone (10), yeast extract (10), and agar (20).
144 Cultures of the three other strains were maintained at 4°C on agar plate containing in ($g.L^{-1}$):
145 glucose (10), peptone (5), yeast extract (3), malt extract (3), and agar (15), according to the
146 supplier. Medium components were weighed on a precision scale; the accuracy of the scale was 0.1
147 mg.

148 For the inoculum preparation, the yeasts were transferred to 250 mL Erlenmeyer flasks
149 containing 25 mL of culture medium of the same composition as the culture medium without agar.
150 Before inoculation, it was sterilized in an autoclave, at 121°C, for 20 min, namely the standard
151 procedure to remove even heat-resistant spores.
152 The inoculated flasks were incubated in a rotating shaker (New Brunswick, INNOVA 40, NJ, USA)
153 at 20 rad sec^{-1}, 180 rpm ±1 rpm, 28°C ± 0.1°C, the optimal temperature for yeast growth, for 18 h
154 in order to obtain high cell density. At the end of the incubation period, cells were centrifuged
155 aseptically (3000 rpm, 4°C and 5 min), resuspended in 25 mL KCl (150 mM) and then centrifuged
156 again in similar conditions. The suspension obtained after harvesting cells and re-suspending in 10
157 mL of KCl 150 mM was used for inoculation.

158 ### 2.2 Fermentation medium

159 Synthetic media were prepared following the composition of green algae *Ulva sp*. They were
160 constituted by simple sugars (glucose, galactose, xylose, rhamnose and arabinose) and salts at levels
161 close to those of hydrolysates. The medium was enriched with mineral supplementation, whose
162 composition was (in $mg.L^{-1}$): KH_2PO_4 5200; $MgSO_4$, $7H_2O$ 1200; $CaCl_2$, $6H_2O$ 150; $FeSO_4$, $7H_2O$
163 100; $ZnSO_4$, $7H_2O$ 30; $CuSO_4$, $5H_2O$ 0.79; H_3BO_3 15; KI 2; Na_2MoO_4, $2H_2O$ 5; $MnSO_4$, H_2O 32;
164 $CoCl_2$, $6H_2O$ 5.2; EDTA 100. The medium was enriched with NH_4Cl (1 $g.L^{-1}$) or peptone (5 $g.L^{-1}$)
165 (sources of nitrogen). The pH was adjusted at 6 ± 0.01 (pH meter WTW pH 315i) by addition of
166 sterile KOH 2 mM. Finally the medium was sterilized by filtration through 0.2 µm (Sartorius) filter
167 under aseptic conditions [34]-[35] in order to avoid any modification of the composition which
168 could take place with autoclave, such as Maillard's reaction [36].

169 **2.3 Fermentation experiments**

170 Fermentations were carried out in 250 mL hermetically closed bottles with a working volume of
171 100 mL. The sterilized fermentation medium was inoculated with the yeast suspension under
172 aseptic conditions. The ethanol fermentation was subjected to 180 rpm ±1 rpm, at 28 ± 0.1 °C, via a
173 shaking incubator (INNOVA 40). All experiments were performed in duplicates and samples were
174 withdrawn and centrifuged at 3000 rpm ±1 rpm, 4 ± 0.1°C and 5 min ± 5 s. The cell free
175 supernatant was evaluated for ethanol and sugar concentrations.

176 **2.4 Analytical methods**

177 The various metabolites produced by the yeasts and the sugar concentrations were analyzed
178 using high performance liquid chromatography (HPLC) [34], equipped with an ions exclusion
179 column HPX-87H (300 × 7.8 mm, Bio-Rad, Hercules, CA, USA). The temperature was 45°C (Oven
180 CrocoCil™; Cluzeau-Info-labo, Ste Foy LaGrande, France). Sulfuric acid (0.01 M) was used as the
181 mobile phase at 45°C, and at a flow rate of 0.7 $mL.min^{-1}$. A Shimadzu RIO-6A Refractive index
182 index Detector (Japan) was used for the detection of the various compounds [37]. The various
183 metabolites and sugars were quantified by comparing their peak areas with those of standard of
184 known concentrations. The Nessler method (NF T 90-015) was used to determine the ammonium
185 concentration.

186 Cell growth was monitored by analysis of absorbance at 600 nm, with a spectrophotometer
187 SECOMAM Prim 500, after calibration using a non-inoculated medium. Biomass growth was also
188 measured in terms of dry matter ($g.L^{-1}$). 30 mL of medium was disposed in a previously weighed
189 porcelain cup (P1) and placed in an oven at 105ºC during 24h. Dry medium was then weighed (P2)
190 and the dry matter could be calculated as follows:

191

192
$$Dry\ matter = \frac{(P2 - P1)}{30.10^{-3}} \quad (1)$$

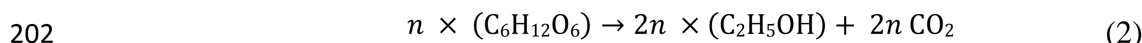
193 Inoculum size of each yeast strain was also controlled by measuring dry matter. After
194 centrifugation of 10 mL of yeast culture, the pellet was deposited in a previously weighed porcelain
195 cup and treated as samples above.

196 One- way analysis of variance (ANOVA) test was performed using R project 2.15.0 software to
197 check for the significance of the data and to discuss their interpretation.

198 **2.5 Ethanol$_{o/t}$ ratio**

199 Fermentation efficiency corresponded to the ratio of the the ethanol produced over the ethanol

theoretically produced ratio (ethanol$_{o/t}$). During ethanolic fermentation, sugars were converted in ethanol and CO_2, by the action of microorganisms. For example with glucose:



$$n \times (C_6H_{12}O_6) \rightarrow 2n \times (C_2H_5OH) + 2n\, CO_2 \quad (2)$$

For a total conversion, one mole of glucose was converted by yeasts in two moles of ethanol. According to equation (2), the ethanol theoretically produced could be calculated as follows:

$$[\text{ethanol}]_{\text{theor}}\ (g.L^{-1}) = 2 \times [\text{glucose}] \times \left(\frac{M_{\text{ethanol}}}{M_{\text{glucose}}}\right) \quad (3)$$

But, this ethanolic fermentation could be in competition with other metabolic pathway, like glycerol production for cell maintenance [34]. So, the ratio of ethanol observed over theoretically produced could give information on the carbon substrate consumption for cell maintenance:

$$\text{Ethanol}_{o/t}\,(\%) = \frac{[\text{ethanol}]_{\text{observed}}}{[\text{ethanol}]_{\text{theor}}} \quad (5)$$

3. RESULTS AND DISCUSSION

3.1 Selection of the carbon substrate

3.1.1 Study of different yeast strains

The synthetic medium was enriched with minerals, ammonium chloride (1 g.L^{-1}) as nitrogen source and the five considered sugars (12 g.L^{-1}), which are the most encountered in *Ulva sp* hydrolysates. Sugar fermentation of each yeast strain, inoculated at 11.8 mg.L^{-1} (0.1% v/v), was studied. Table 1 shows the main results obtained after 144 h of fermentation. For each case, glucose was the first sugar consumed. The level of consumption differed from one strain to another, following this decreasing order: *S. cerevisiae* (100.0%)> *C. guilliermondii* (75.9%)> *K. marxianus* (64.4%)> *P. stipitis* (30.2%) (Table 1). Compared to the other strains, *S. cerevisiae* showed a complete assimilation of the glucose present in the medium within 144 h of fermentation. It was also the only strain which consumed galactose (100% of the feedstock). No yeast strain consumed xylose, rhamnose or arabinose. All considered strains showed a preference for glucose, according with the available related literature. Indeed, glucose is a carbon substrate of choice for yeasts [38]-[39]-[30]. When utilizing this sugar, strains display a high metabolic output [13]. So, the other sugars would not be assimilated until total glucose removal from the medium. This could account for the absence of galactose, xylose, rhamnose and arabinose consumption by *K. marxianus, P. stipitis* and *C. guilliermondii*.

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230 **Table 1**

231 Sugars consumed and ethanol production by the four yeast strains selected, after 144h of
232 fermentation (11.8 mg.L^{-1} inoculation)

Yeast strains	Glucose consumed (%)	Galactose consumed (%)	[ethanol]o/t ratio (%)
K. marxianus	64.4	0.0	72.6 ± 0.25
P. stipitis	30.2	0.0	44.8 ± 0.05
C. guillermondii	75.6	0.0	92.9 ± 0.63
S. cerevisiae	100.0	100.0	100.0 ± 0.25

233
234 Concerning xylose, wild types of *S. cerervisiae* were shown to be not able to assimilate pentose
235 [40]; While *P. stipitis* is known to be the most efficient for xylose fermentation [41]. However, no
236 xylose consumption was observed. Moreover, this strain showed the lowest ethanol production.
237 This should be related to the sensibility of this strain to the aeration conditions and it need for a
238 microoxygenation of the medium, essentially given by a high agitation [33]. From this, the aeration
239 conditions applied in this work did not seem adequate for ethanol production by *P. stipitis*.
240 Comparing cell growth displayed in Figure 1, *C. guilliermondii* led to the highest cell growth
241 rate and final biomass amount, followed by *S. cerevisiae*, *K. marxianus* and *P. stipitis*. However *C.*
242 *guilliermondii* consumed only 75.9% of the glucose present in the culture medium. That lets
243 suppose that *C. guilliermondii* used more glucose for cell formation than *S. cerevisiae*, instead of
244 producing ethanol. Based on ethanol yields, which represents the ethanol produced over the glucose
245 consumed (expressed in carbon/carbon (mol/mol), the results were as follows: *S. cerevisiae* (68.0%
246 C/C) > *C. guilliermondii* (61.9% C/C) > *K. marxianus* (48.4% C/C) > *P. stipitis* (30.2% C/C)
247 (Figure 2). Ethanol$_{o/t}$ ratio (ethanol observed over ethanol theoretically produced) was also found to
248 be the lowest for *P. stipitis*, 44.8%; while *S. cerevisiae* led to the highest value, 100.0%. So,
249 *S.cerevisiae* appeared to be the most promising candidate for the valorization of glucose and
250 galactose contained in *Ulva sp* hydrolysates.

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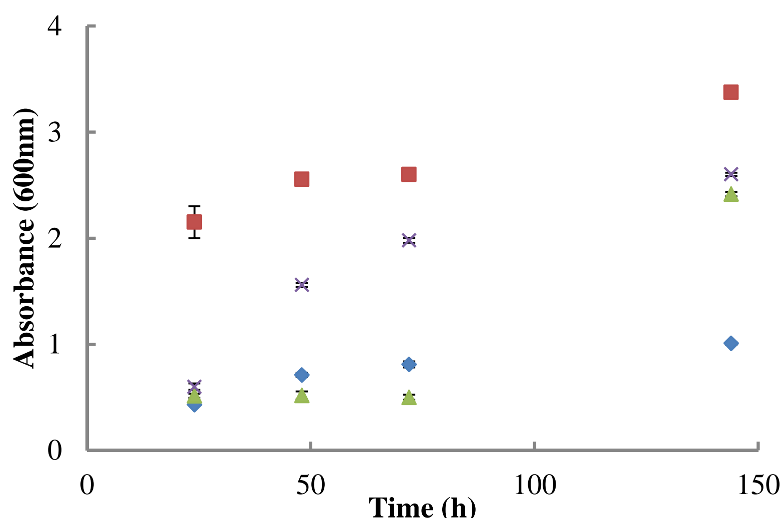

Figure 1. Absorbance at 600nm measured during 144 h of fermentation by (▲) *K. marxianus*, (◆) *P. stipitis*, (■) *C. guilliermondii* and (×) *S. cerevisiae*

The culture medium contained a total of 60 g.L^{-1} of sugars, which can play a role in a possible Crabtree effect [20]. For Crabtree-positive yeast, consumption rate was enhanced by facilitated diffusion of glucose in the cell and alcoholic fermentation is privileged in the presence of a high glucose concentration. Cell growth is also lowered in favor of the co-production of glycerol, acetate and ethanol [42]. *S. cerevisiae* is known to be a Crabtree-positive strain. So, facing high sugar content, this strain should privilege the formation of co-products of fermentation instead of biomass production.

K. marxianus, *P. stipitis* and *C. guilliermondii*, which are Crabtree negative strains, possess a regulated H^+ symport system, which leads to regulate glucose transport in the cell. In the case of high sugar content, Crabtree-negative yeasts restrict the entry of glucose by their high-affinity system and give a weak fermentative response [20]. This could explain *S. cerevisiae* predominance over the other strains, referring to glucose consumption and ethanol production.

Yeast strains also secreted acetic acid and glycerol during fermentation (Figure 2). *S. cerevisiae* was the highest producer of glycerol, with 4.68% C/C, namely 3.5 to 8 times higher than the amounts obtained for the other strains, *K. marxianus* (1.3% C/C) > *P. stipitis* (1.2% C/C) > *C. guilliermondii* (0.6% C/C). The reverse was observed for acetic acid yields, since *S. cerevisiae* was the lowest producer (1.6% C/C) compared to the other strains, and the highest production was found for *C. guilliermondii* (6.9% C/C).

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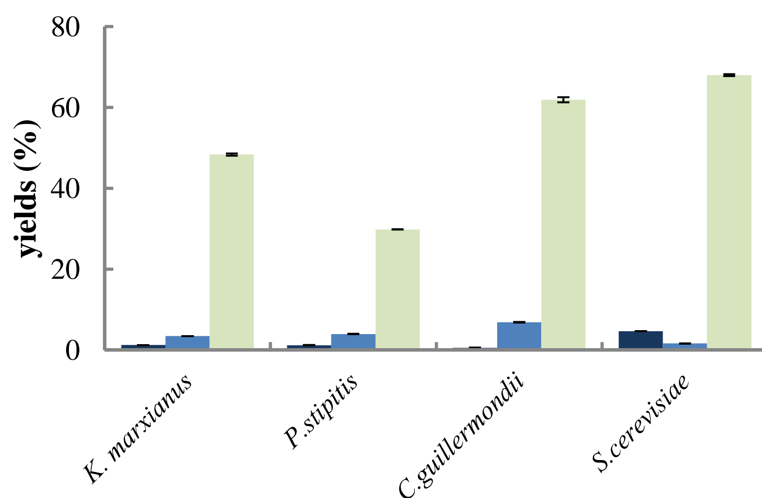


274

275 **Figure 2.** Glycerol (■), acetic acid (■) and ethanol (■) yields (% C/C) obtained after 144 h of
276 fermentation with the four yeast strains

277

278 A total of 60 g.L^{-1} of cumulated sugars in the medium could also cause hyperosmotic conditions
279 [43]. This osmotic pressure to which the yeasts were exposed could significantly impact on yeast
280 viability and on fermentation performances [44]. In fact, that could drive to a dehydration of cells
281 and hence to an inhibition of growth. Then, this loss of yeasts viability drove to a decline of ethanol
282 production [45]-[46]. So, to counteract this loss of water, yeasts produced and accumulated neutral
283 solutes in their cytoplasm, like glycerol, which led to restore thermodynamic equilibrium [34].
284 Osmotolerance induced by glycerol production was due to glycerol-3-phosphate deshydrogenase
285 activity and the control of alcohol dehydrogenase and aldehyde dehydrogenase [47]. One of the co-
286 products generated with glycerol in the case of osmotic pressure was acetic acid [48]. Strains had
287 different strategies for osmo-adaptation and consequently proportions of glycerol and acetic acid
288 produced differed from one strain to another [49]. Figure 2 showed that *S. cerevisiae* excreted more
289 glycerol than acetic acid contrary to *C. guilliermondii*. These two strains could produce more co-
290 products than *K. marxianus* and *P. stipitis*, which might be a reason for a higher resistance to
291 osmotic pressure and then a faster cell growth.

292

293 3.1.2 Influence of the inoculum size

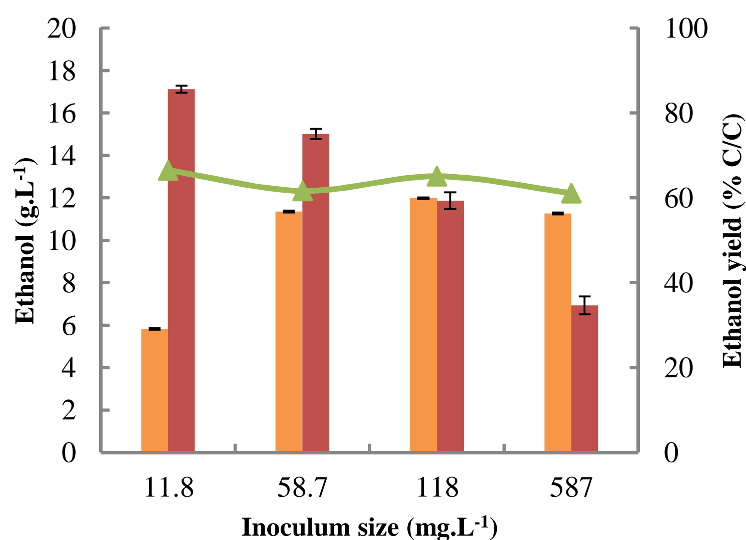
294 Inoculum size could influence sugar consumption and ethanol production. An optimization of
295 this parameter could improve ethanol production and production rate. A variation of inoculum size
296 from 11.8 to 587 mg.L^{-1} (0.1 to 5% v/v) with *S. cerevisiae*, in a mix of five sugars (12 g.L^{-1}) was
297 investigated. As presented in Table 2, in the case of 11.8 mg.L^{-1} inoculation, glucose was not totally
298 consumed (95.1%) and no galactose consumption was observed within 72 h of fermentation; while

299 total glucose and galactose consumption were shown for the other inoculum levels. However, no
300 consumption of arabinose, rhamnose and xylose was observed irrespective of the inoculation level.
301 From 58.7 to 587 mg.L^{-1} (0.5 to 5% v/v), 11-12 g.L^{-1} of ethanol was produced versus only 6 g.L^{-1}
302 for 11.8 mg.L^{-1} inoculum (Figure 3). But inoculum size did not significantly impact ethanol yield
303 (confirmed by ANOVA test, p-value= 0.162), which remained in a short range, between 61 and
304 65% C/C irrespective of the inoculum size. Ethanol to biomass ratio decreased for increasing
305 inoculum size; while a weak peak was observed for the ethanol production, 12.0 g.L^{-1} for 118 mg.L^{-1}
306 (1% v/v) inoculum (Figure 3), as well as for the Ethanol$_{o/t}$ ratio, the ratio of the experimental to
307 the theoretically ethanol produced, found also to be optimal for 118 mg.L^{-1} inoculum (97.7%).
308 From this, 118 mg.L^{-1} seemed to be the optimal inoculum size in terms of ethanol productivity.
309

310 **Table 2**

311 Inoculum size effect on sugar consumption and ethanol production by *S.cerevisiae*, over 72 h of
312 fermentation

Inoculum size (mg.L^{-1})	Glucose consumed (%)	Galactose consumed (%)	[ethanol]o/t ratio (%)
11.8	95.1	-	99.8 ± 0.16
58.7	100.0	100.0	92.5 ± 0.24
118	100.0	100.0	97.7 ± 0.39
587	100.0	100.0	91.8 ± 0.42



313
314 **Figure 3**. Influence of inoculum size on ethanol production ( ), ethanol/ biomass ratio ( ) and
315 ethanol yield ( )

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Tesfaw and Assefa [40] also investigated the influence of the inoculum size on ethanol production by *S. cerevisiae*. They found that lowering the inoculum size reduces costs of production in ethanol fermentation. But the ethanol production raised from 1.29 to 2.35 $g.L^{-1}.h^{-1}$ when the yeast load increased from 0.5 to 5 $g.L^{-1}$, in agreement with the trend also observed in Figure 3, from 11.8 to 118 $mg.L^{-1}$ of inoculation. In their studies, Tahir et al. [50] tested different sizes of inoculum, from 1 to 5% (v/v) for ethanol production by *S. cerevisiae*. The amount of ethanol produced gradually increased with the rise of the inoculum. However, a maximum ethanol production (65.0 $g.L^{-1}$) was achieved at 3.0% inoculum; while a further increase in the inoculum size did not result in a considerable enhancement of ethanol production. This finding is in accordance with the results displayed in Figure 3. Indeed, beyond an optimum, increasing the size of inoculum did not improve ethanol production.

In terms of ethanol to biomass ratio, 11.8 $mg.L^{-1}$ inoculation was the most interesting, but led to the lowest ethanol production. The quantity of the inoculated cells influences the time of fermentation as well as the product yields [51]. The shortening of the fermentation time linked to the increase in the size of the inoculum was due to a fast cell growth; most of the substrate was immediately converted to ethanol. However excessive inoculum volume would largely influences fermentation efficiencies. So, a compromise has to be done between ethanol productivity and costs of production. According to the results obtained, 118 $mg.L^{-1}$ inoculation seemed to be a good compromise between ethanol productivity and ethanol to biomass ratio, with a mix of sugars as carbon sources. These results highlighted therefore the importance of the size of the inoculum regarding ethanol fermentation.

3.2 Effect of the nitrogen source

Two sources of nitrogen were tested, one mineral (NH_4Cl, 1 $g.L^{-1}$) and another one, organic (peptone, 5 $g.L^{-1}$). The behavior of *S. cerevisiae* (11.8 $mg.L^{-1}$ inoculum) with regard to these two nitrogen sources was studied for glucose fermentation (30 $g.L^{-1}$), the main sugar consumed.

S. cerevisiae needed 144 h to totally consume glucose using NH_4Cl as nitrogen source, while only 20 h in the presence of peptone (Table 3), leading to consumption rates of 0.21 and 1.5 $g.L^{-1}.h^{-1}$ with NH_4Cl and peptone, respectively (Figure 4). Ethanol production rate followed the same trend, 0.10 and 0.58 $g.L^{-1}.h^{-1}$ with NH_4Cl and peptone, respectively. Analysis of NH_4^+ at the end of culture showed that the nitrogen content was not limiting, since 75% of the nitrogen source remained in the medium. This confirmed that an organic nitrogen source improves growth and glucose consumption

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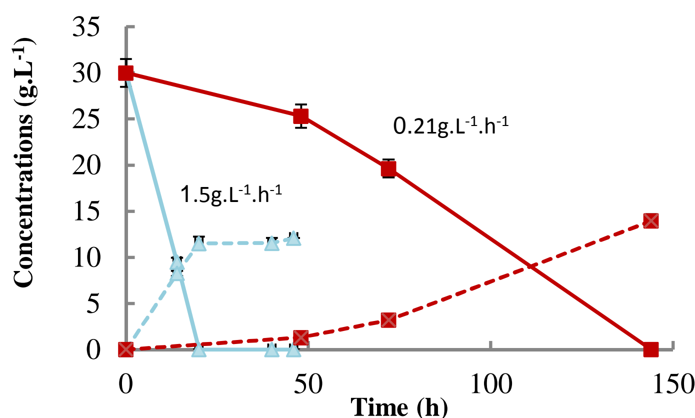
348 and hence ethanol production rate. Chniti et al [52] observed the same trend by studying syrup dates
349 enrichment with either NH_4Cl or yeast extract.

350

351 **Table 3**

352 Influence of the nitrogen source on glucose fermentation

	NH_4Cl	Peptone
Time to totally consume glucose (h)	144	20
Ethanol production rate ($g.L^{-1}.h^{-1}$)	0.10	0.58
[ethanol]o/t ratio (%)	91.06	78.78
Cell growth (Absorbance at 600nm)	2.05	10.48
Ethanol yield (% C/C)	60.65	52.47
Glycerol yield (% C/C)	4.24	2.14



353

354 **Figure 4.** Kinetics of glucose consumption (continuous line) and ethanol production (dashed line)
355 with peptone (▲) and NH_4Cl (■) as nitrogen source

356

357 This preference for peptone over ammonium by *S. cerevisiae* has also been reported in the
358 literature [53]. It reveals that most free and peptide amino acids (particularly glutamic acid) are
359 utilized by the yeast, inducing higher cell growth, an increase of ethanol production rate and a
360 diminution of glycerol production [54]. Another study with γ–aminobutyric acid as nitrogen source
361 reports the preference of *S. cerevisiae* for amino-acids as nitrogen source [55]. This preference is
362 not exclusive to *S. cerevisiae*; *P. stipitis* and *C. guilliermondii* also showed a preference for an
363 organic source like peptone or yeast extract instead of a mineral source ($(NH_4)_2SO_4$) [56].

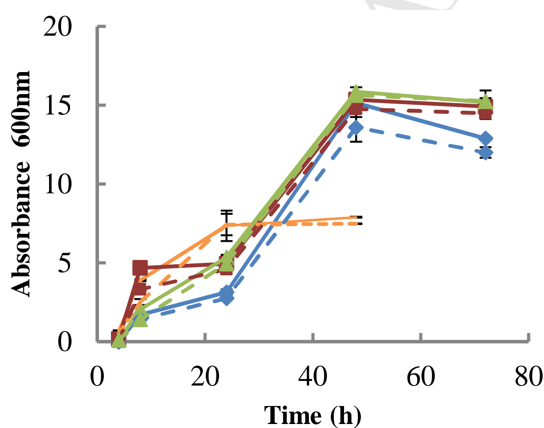
364 The use of peptone led also to a decrease of the glycerol yield, 2.1 instead of 4.2% C/C in the
365 presence of ammonium (Table 3). This should be related to the nitrogen source. In fact, with a
366 better assimilation of nitrogen, cell growth is favored, leading to a higher glucose consumption and
367 also a decrease of the oxygen content, both having a direct impact on glycerol and ethanol yields,
368 lowering the former and increasing the latter [51].
369 However, even though ethanol production rate and cell growth was faster, $ethanol_{o/t}$ ratio and
370 ethanol yield were not improved using peptone instead of NH_4Cl (Table 3) showing that *S.*
371 *cerevisiae* growth by an anabolic pathway is favored over fermentation in the presence of peptone.
372 These results highlight the importance of the nitrogen source. For *S. cerevisiae*, a mineral source
373 allows to obtain a high ethanol yield with a low biomass yield; while an organic source, such as
374 peptone, allows to improve ethanol production rate in spite of a loss of carbon substrate for biomass
375 formation.
376 According to the supplier (Biokar Diagnostics, A1702AH), peptone is mostly composed of
377 glutamic acid (17.4%), proline (8.4%), leucine, lysine and aspartic acid (between 6.4 and 7.2%).
378 Hou et al [15], who studied *Laminaria digitata* as nitrogen source, found that amino-acids
379 contained in peptone were also abundant in this macroalgae. Therefore, the use of peptone as source
380 of nitrogen can lead to approach algal hydrolysate conditions.
381 Moreover, algae could be used as nitrogen source to enhance ethanol production from corn
382 stover [57] or high gravity sweet potato medium [58]. It is proven that yeast growth and ethanol
383 production are enhanced by this supplementary source of nitrogen. Rich in proteins, their
384 composition in amino-acids are close to those of yeast extract and peptone, confirming that
385 macroalgae could be used as substituent in yeast culture or as fermentation media.

386

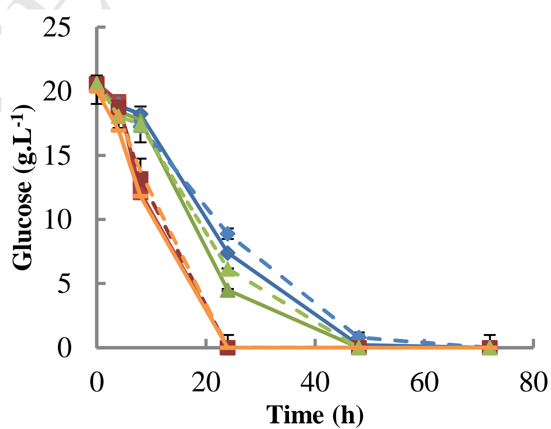
387 **3.3 Influence of salts**

388 Due to the presence of salts in algal hydrolysate, yeast strains could suffer from their impact on the
389 osmotic pressure. The supplementation of synthetic medium with sodium chloride and sulfate at
390 similar concentrations found in *Ulva sp* hydrolysates (0.25 and 0.21 M respectively; data not
391 shown) should allow to study the behavior of the four yeast strains selected facing this change of
392 osmolarity.
393 Referring to Figure 5.a, a slight impact of the presence of salts was only really noticeable for *P.*
394 *stipitis*. Due to the presence of salts, yeasts need to adapt to a higher osmotic pressure. During
395 osmoregulation, biomass development is slowed in favor of the production of neutral solutes, like

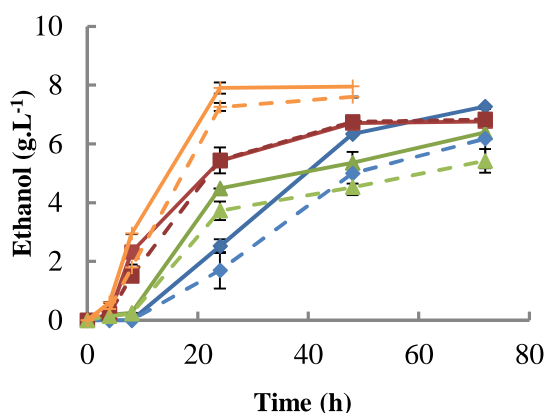
396 glycerol (Blomberg, 2000). From this, *S. cerevisiae*, *K. marxianus* and *C. guilliermondii*, whose
397 biomass growth was less affected, could better adapt and resist to osmotic pressure than *P. stipitis*.
398 Growth rates differed from one strain to another. After 22 h of fermentation and contrarily to the
399 other strains, *S. cerevisiae* growth reached a stationary state at a lower absorbance value than those
400 observed for the other strains, for which growth was observed until 48 h. For *S. cerevisiae* and *K.*
401 *marxianus*, glucose depletion was observed within 22h of culture (Figure 5.b). Following growth
402 and substrate consumption, ethanol production also ceased after 22 h of culture for *S. cerevisiae*;
403 while ethanol continued to be produced during *K. marxianus* culture (Figure 5.c) until the end of
404 growth at 48 h (Figure 5.a). For this latter species, a second carbon substrate was assimilated, most
405 likely peptone. As already seen above, macroalgae are rich in protein. So, such diauxic growth may
406 be also encountered when utilizing algal hydrolysates as fermentation medium. While *S. cerevisiae*
407 could assimilate peptone as nitrogen source, it would not be able to assimilate its carbon content.
408 But it is possible for this strain to consume the glycerol produced as carbon source to maintain cell
409 viability. Glycerol growth in *S. cerevisiae* has been reported in previous studies, in the presence of
410 complex supplements such as yeast extract, peptone or amino acids in the medium[59], [60].



411 (a)



412 (b)



413 (c)

414 **Figure 5**. Growth rate (a), glucose consumption (b) and ethanol production (c) for *K. marxianus* (■
415), *P. stipitis* (◆), *C. guilliermondii* (▲) and *S. cerevisiae* (+), during fermentation in absence
416 (continuous line) and presence of salts (dashed line)

417

418 Glucose consumption (20 g.L^{-1} initially) was not significantly affected by a higher osmotic
419 pressure (Figure 5.b); irrespective of the presence of salts, all the glucose was consumed at the end
420 of culture, after 72 h. However, regarding ethanol production differences can be seen depending on
421 the species considered (Figure 5.c). The most significant impact was observed for *P. stipitis*, in
422 close connection with cell growth. Ethanol$_{o/t}$ ratio for *P. stipitis* was also impacted and decreased
423 from 71.2 to 60.5% (Table 4). *C. guilliermondii* and *S. cerevisiae* also showed a lower ethanol$_{o/t}$
424 ratio at a higher osmotic pressure (53.1 and 74.4% instead of 62.5 and 77.8%) and a slower ethanol
425 production rate (only 0.09 g.L^{-1}.h^{-1} for *C. guilliermondii*) (Table 4). Only ethanol production of *K.*
426 *marxianus* was not impacted, as well as its ethanol$_{o/t}$ ratio. Nevertheless, *S. cerevisiae* still gave the
427 best results in terms of ethanol produced (7.5-7.9 g.L^{-1}), production rates (0.30-0.33 g.L^{-1}.h^{-1}) and
428 ethanol to biomass ratio (24.7-18.0), whether in the presence or in the absence of salts.

429

430 **Table 4**

431 Influence of salts on glucose fermentation, with the four selected strains

	Glucose consumption rate at 24h (g.L^{-1}.h^{-1})		Ethanol production rate (g.L^{-1}.h^{-1})		[ethanol]o/t ratio (%)		Ethanol/ Biomass ratio	
		with salts		with salts		with salts		with salts
K. marxianus	0.86	0.86	0.14	0.14	66.2	66.3	2.64	2.74
P. stipitis	0.55	0.49	0.13	0.10	71.2	60.5	3.27	2.98
C. guilliermondii	0.67	0.60	0.11	0.09	62.5	53.1	2.45	2.07

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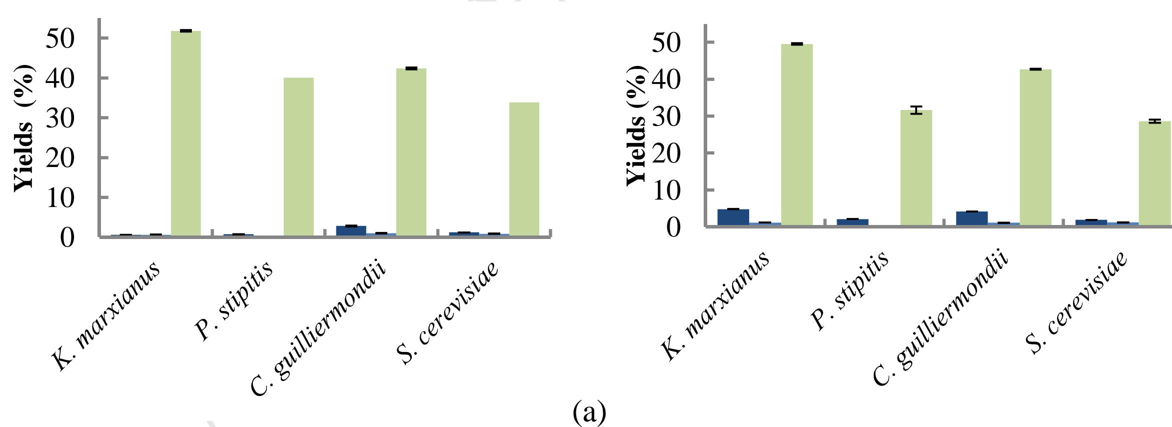
S. cerevisiae	0.83	0.83	0.33	0.30	77.8	74.3	5.55	5.43

432

433 In the case of salts supplementation of the medium, glycerol and acetic acid yields rose for all the
434 considered strains (Figure 6). However, the increase differed from one strain to another and was the
435 most important for *S. cerevisiae*, in agreement with its higher ethanol production. In the literature,
436 glucose consumption is lowered and so fermentation completion time increases in the presence of a
437 higher amount of salts. That also impacts cell growth and ethanol production and promotes glycerol
438 production [51]. Similar behavior was previously recorded with *Hansenula anomala* [37] or with
439 *Dekkera bruxellensis* [43]. Effects of sulfates, like Na_2SO_4, $MgSO_4$ and $(NH_4)_2SO_4$ was also studied
440 in the control of osmotic pressure of culture medium. These electrolytes play a role in osmotic
441 pressure [61]. But the salts added in the media at level encountered in algal hydrolysates did not
442 significantly affect kinetics of consumption, growth and production, except for *P. stipitis* which
443 suffered from a slowdown of metabolism. This means that *S. cerevisiae*, *C. guilliermondii* and *K.*
444 *marxianus* are able to adapt their metabolism to salinity brought by algae and so to survive and
445 grow in these conditions. This is confirmed by Kostas et al [13], who reported that *S. cerevisiae*
446 YPS128 was able to produce 7 $g.L^{-1}$ of ethanol by fermentation of a mix of sugars (12 $g.L^{-1}$) from
447 *Ulva lactuca* hydrolysate. Furthermore, Borines et al [21] recorded higher levels of ethanol with the
448 fermentation of *Sargassum* spp. hydrolysate by a wild *S. cerevisiae* than based on glucose as a
449 substrate.



450

451 Figure 6 Glycerol (■), acetic acid (■) and ethanol (■) yields obtained after 72 h of fermentation
452 with the four strains, in absence (a) and presence of salts (b)

453

454 **CONCLUSIONS**

455 The green seaweed is proposed as a promising biomass material that can be easily converted to

456 ethanol. Synthetic media adjusted on *Ulva sp* hydrolysate composition gave the opportunity to
457 control nitrogen, carbon and salt contents and consequently to understand the importance of these
458 factors on ethanol production. Glucose, which is the most abundant sugar in *Ulva sp* hydrolysate,
459 was the most assimilated by the four studied yeast strains. The use of peptone, a nitrogen source
460 close to macroalgal proteins, confirmed that algae can be used as fermentation medium. Finally,
461 synthetic media supplemented with salts led to study the impact of the latter on the fermentation
462 process. Salts brought by macroalgae did not significantly impede the production, except for *P.*
463 *stipitis*. Among the strains studied, *K. marxianus* seemed to be the most resistant to osmotic
464 pressure and hence appeared promising for the fermentation of *Ulva sp* hydrolysates. But *S.*
465 *cerevisiae* remained the most interesting in terms of ethanol production. This work argues that *Ulva*
466 *sp* hydrolysate can be an adequate biomass resource for ethanol fermentation by yeast strains. To
467 confirm these results, work is in progress in the laboratory on *Ulva* spp hydrolysate.

468

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642 **Figure captions**

643 Figure 1. Absorbance at 600nm measured during 144 h of fermentation by ( ) *K. marxianus*, ( )
644 *P. stipitis*, ( ) *C. guilliermondii and* ( ) *S. cerevisiae*

645 Figure 2: Glycerol ( ), acetic acid ( ) and ethanol ( ) yields (% C/C) obtained after 144 h of
646 fermentation with the four yeast strains

647 Figure 3 Influence of inoculum size on ethanol production ( ), ethanol/ biomass ratio ( ) and
648 ethanol yield ( )

649 Figure 4. Kinetics of glucose consumption (continuous line) and ethanol production (dashed line)
650 with peptone ( ) and NH_4Cl ( ) as nitrogen source

651 Figure 5 Growth rate (a), glucose consumption (b) and ethanol production (c) for *K. marxianus* (
652), *P. stipitis* ( ), *C. guilliermondii* ( ) and *S. cerevisiae* ( ), during fermentation in absence
653 (continuous line) and presence of salts (dashed line)

654 Figure 6 Glycerol ( ), acetic acid ( ) and ethanol ( ) yields obtained after 72 h of fermentation
655 with the four strains, in absence (a) and presence of salts (b)

656

657 **Table captions**

658 Table 1. Sugars consumed and ethanol production by the four yeast strains selected, after 144h of
659 fermentation (11.8 $mg.L^{-1}$ inoculation level)

660 Table 2. Inoculum size effect on sugar consumption and ethanol production by *S.cerevisiae*, over 72
661 h of fermentation

662 Table 3. Influence of the nitrogen source on glucose fermentation

663 Table 4. Influence of salts on glucose fermentation, with the four selected strains

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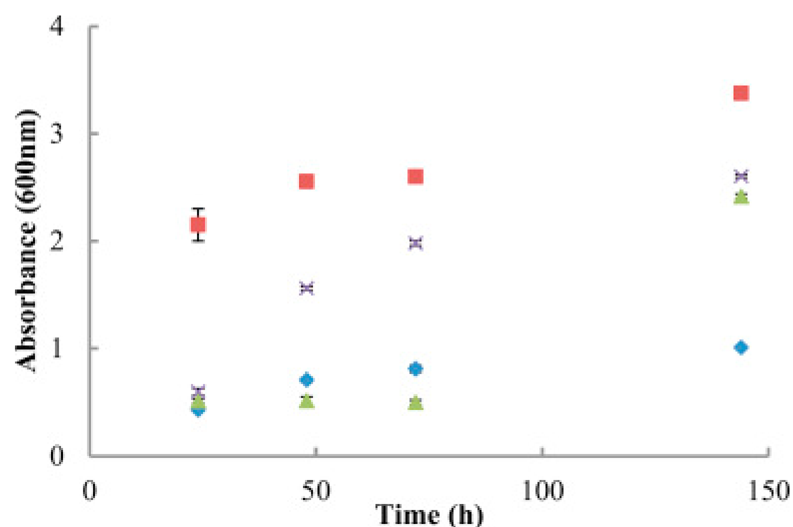

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Fig. 1. Absorbance at 600 nm measured during 144 h of fermentation by (▲) *K. marxianus* (◆), *P. stipitis* (■), *C. guilliermondii* and (×) *S. cerevisiae*.

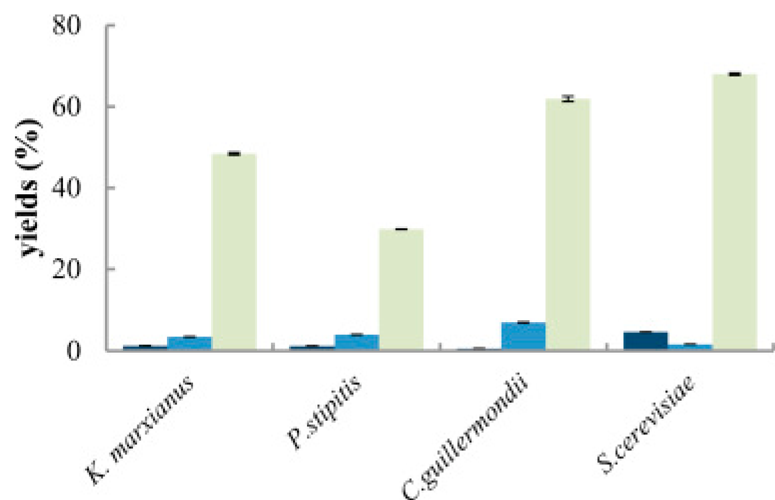

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Fig. 2. Glycerol (■), acetic acid (■) and ethanol (■) yields (% C/C) obtained after 144 h of fermentation with the four yeast strains.

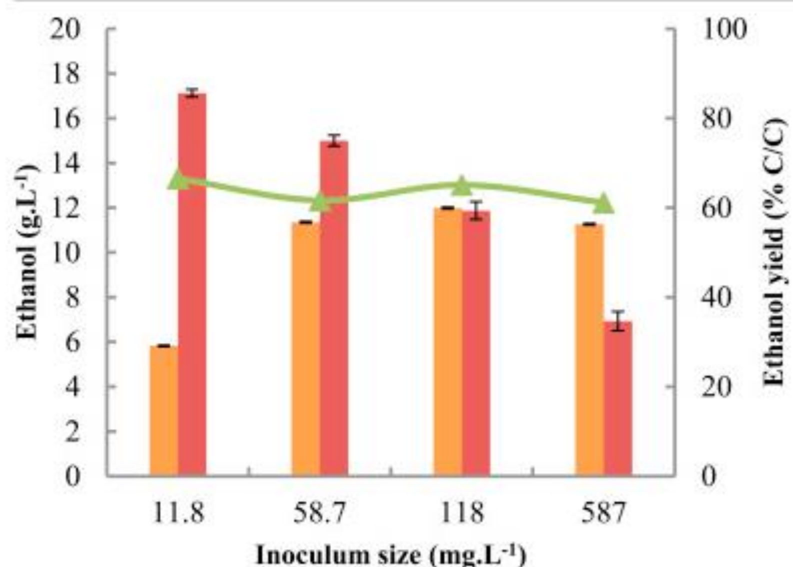

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Fig. 3. Influence of inoculum size on ethanol production (▬), ethanol/biomass ratio (▬) and ethanol yield (▬▲▬).

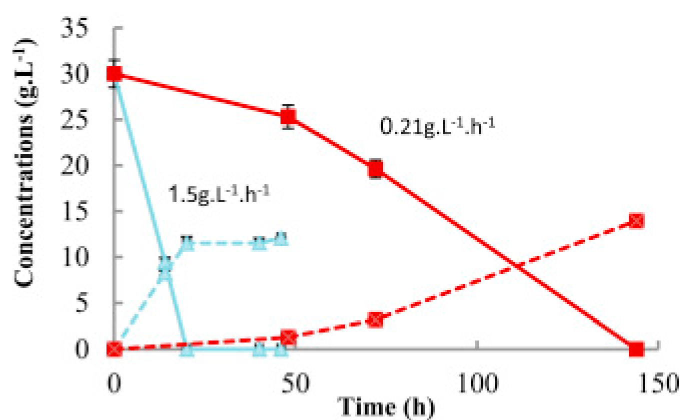

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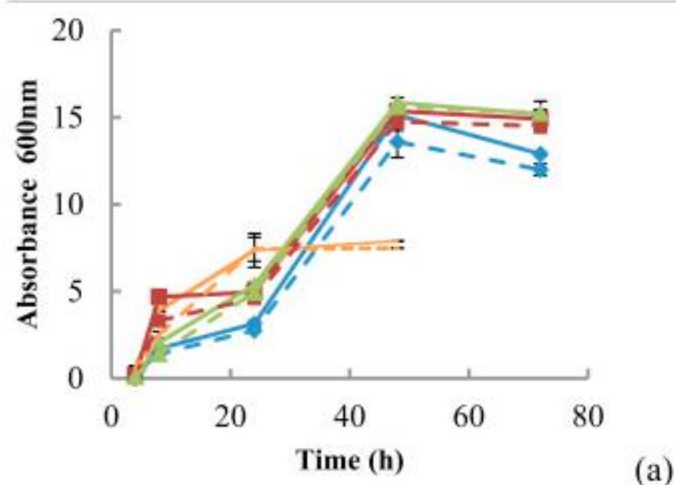
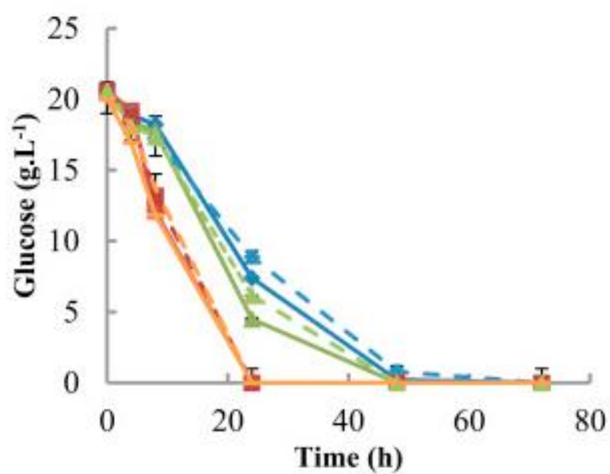
Fig. 4. Kinetics of glucose consumption (continuous line) and ethanol production (dashed line) with peptone (▲) and NH_4Cl (■) as nitrogen source.





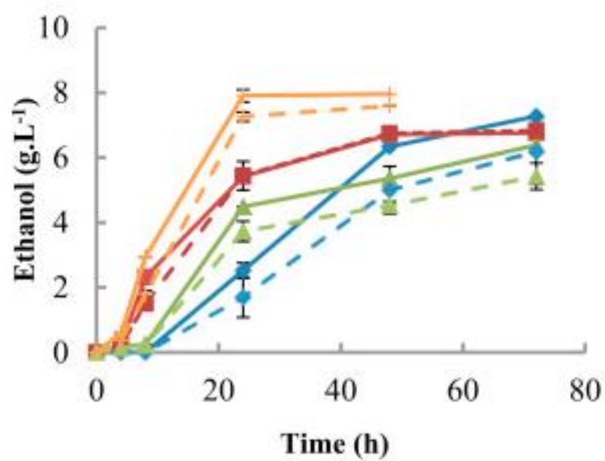


Fig. 5. Growth rate (a), glucose consumption (b) and ethanol production (c) for *K. marxianus* (■), *P. stipitis* (◆), *C. guilliermondii* (▲) and *S. cerevisiae* (+), during fermentation in absence (continuous line) and presence of salts (dashed line).

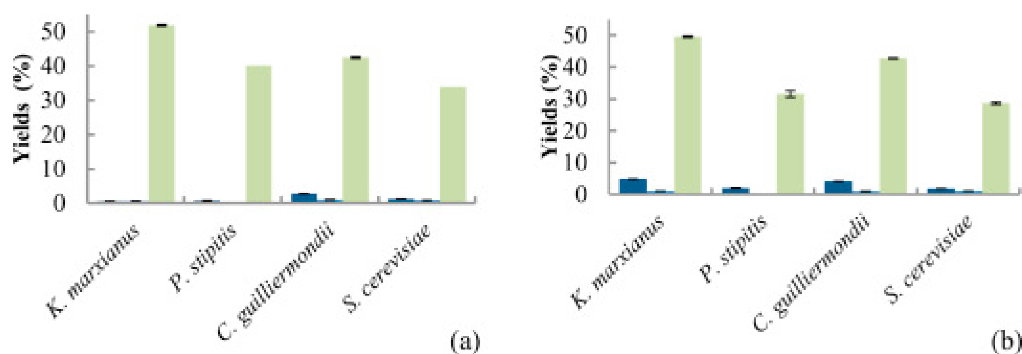


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Fig. 6. Glycerol (■), acetic acid (■) and ethanol (■) yields obtained after 72 h of fermentation with the four strains, in absence (a) and presence of salts (b).

Table 1. Sugars consumed and ethanol production by the four yeast strains selected, after 144 h of fermentation (11.8 mg L^{-1} inoculation).

Yeast strains	Glucose consumed (%)	Galactose consumed (%)	[ethanol]o/t ratio (%)
K. marxianus	64.4	0.0	72.6 ± 0.25
P. stipitis	30.2	0.0	44.8 ± 0.05
C. guillermondii	75.6	0.0	92.9 ± 0.63
S. cerevisiae	100.0	100.0	100.0 ± 0.25

Table 2. Inoculum size effect on sugar consumption and ethanol production by *S.cerevisiae*, over 72 h of fermentation.

Inoculum size (mg.L^{-1})	Glucose consumed (%)	Galactose consumed (%)	[ethanol]o/t ratio (%)
11.8	95.1	–	99.8 ± 0.16
58.7	100.0	100.0	92.5 ± 0.24
118	100.0	100.0	97.7 ± 0.39
587	100.0	100.0	91.8 ± 0.42

Table 3. Influence of the nitrogen source on glucose fermentation.

	NH_4Cl	Peptone
Time to totally consume glucose (h)	144	20
Ethanol production rate ($g.L^{-1}.h^{-1}$)	0.10	0.58
[ethanol]o/t ratio (%)	91.06	78.78
Cell growth (Absorbance at 600 nm)	2.05	10.48
Ethanol yield (% C/C)	60.65	52.47
Glycerol yield (% C/C)	4.24	2.14

Table 4. Influence of salts on glucose fermentation, with the four selected strains.

	Glucose consumption rate at 24 h ($g.L^{-1}.h^{-1}$)		**Ethanol production rate ($g.L^{-1}.h^{-1}$)**		**[ethanol]o/t ratio (%)**		**Ethanol/Biomass ratio**	
		with salts		**with salts**		**with salts**		**with salts**
K. marxianus	0.86	0.86	0.14	0.14	66.2	66.3	2.64	2.74
P. stipitis	0.55	0.49	0.13	0.10	71.2	60.5	3.27	2.98
C. guilliermondii	0.67	0.60	0.11	0.09	62.5	53.1	2.45	2.07
S. cerevisiae	0.83	0.83	0.33	0.30	77.8	74.3	5.55	5.43