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

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ABSTRACT

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

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

1. INTRODUCTION

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

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

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

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

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

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

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

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

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

2. MATERIAL AND METHODS

2.1 Microorganisms and inoculum

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

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

For the inoculum preparation, the yeasts were transferred to 250 mL Erlenmeyer flasks containing 25 mL of culture medium of the same composition as the culture medium without agar. Before inoculation, it was sterilized in an autoclave, at 121°C, for 20 min, namely the standard procedure to remove even heat-resistant spores.

The inoculated flasks were incubated in a rotating shaker (New Brunswick, INNOVA 40, NJ, USA) at 20 rad sec^{-1}, 180 rpm ±1 rpm, 28°C ± 0.1°C, the optimal temperature for yeast growth, for 18 h in order to obtain high cell density. At the end of the incubation period, cells were centrifuged aseptically (3000 rpm, 4°C and 5 min), resuspended in 25 mL KCl (150 mM) and then centrifuged again in similar conditions. The suspension obtained after harvesting cells and re-suspending in 10 mL of KCl 150 mM was used for inoculation.

2.2 Fermentation medium

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

2.3 Fermentation experiments

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

2.4 Analytical methods

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

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

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

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

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

2.5 Ethanol$_{o/t}$ ratio

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*.

Table 1

Sugars consumed and ethanol production by the four yeast strains selected, after 144h 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

Concerning xylose, wild types of *S. cerervisiae* were shown to be not able to assimilate pentose [40]; While *P. stipitis* is known to be the most efficient for xylose fermentation [41]. However, no xylose consumption was observed. Moreover, this strain showed the lowest ethanol production. This should be related to the sensibility of this strain to the aeration conditions and it need for a microoxygenation of the medium, essentially given by a high agitation [33]. From this, the aeration conditions applied in this work did not seem adequate for ethanol production by *P. stipitis*.

Comparing cell growth displayed in Figure 1, *C. guilliermondii* led to the highest cell growth rate and final biomass amount, followed by *S. cerevisiae*, *K. marxianus* and *P. stipitis*. However *C. guilliermondii* consumed only 75.9% of the glucose present in the culture medium. That lets suppose that *C. guilliermondii* used more glucose for cell formation than *S. cerevisiae*, instead of producing ethanol. Based on ethanol yields, which represents the ethanol produced over the glucose consumed (expressed in carbon/carbon (mol/mol), the results were as follows: *S. cerevisiae* (68.0% C/C) > *C. guilliermondii* (61.9% C/C) > *K. marxianus* (48.4% C/C) > *P. stipitis* (30.2% C/C) (Figure 2). $Ethanol_{o/t}$ ratio (ethanol observed over ethanol theoretically produced) was also found to be the lowest for *P. stipitis*, 44.8%; while *S. cerevisiae* led to the highest value, 100.0%. So, *S.cerevisiae* appeared to be the most promising candidate for the valorization of glucose and galactose contained in *Ulva sp* hydrolysates.

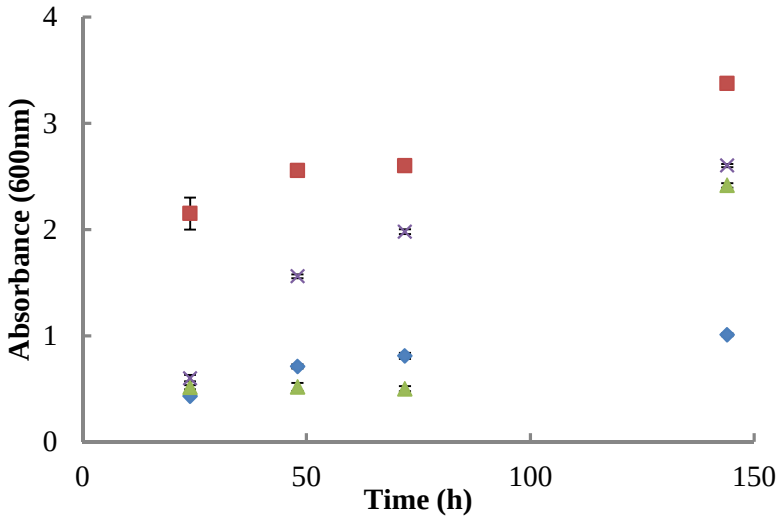


Figure 1. Absorbance at 600nm measured during 144 h of fermentation by (▲) *K. marxianus*, (◆) *P. stipitis*, (■) *C. guilliermondii* and (×) *S. cerevisiae*

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).

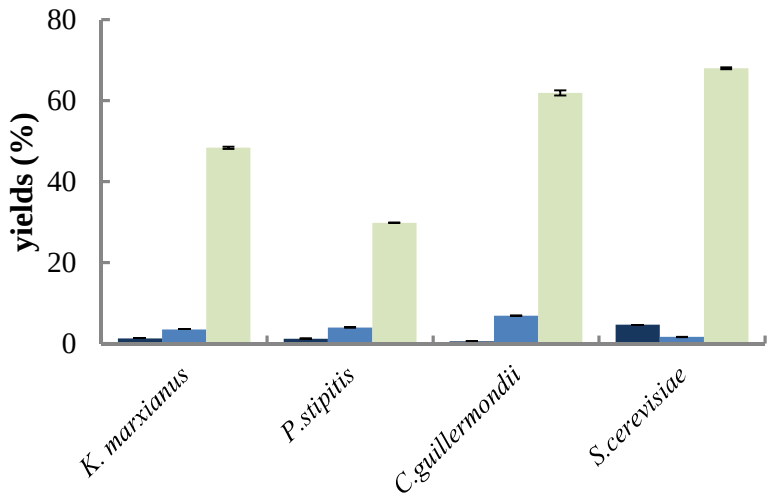


Figure 2. Glycerol (■), acetic acid (■) and ethanol (■) yields (% C/C) obtained after 144 h of fermentation with the four yeast strains

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

3.1.2 Influence of the inoculum size

Inoculum size could influence sugar consumption and ethanol production. An optimization of this parameter could improve ethanol production and production rate. A variation of inoculum size 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 investigated. As presented in Table 2, in the case of 11.8 $mg.L^{-1}$ inoculation, glucose was not totally consumed (95.1%) and no galactose consumption was observed within 72 h of fermentation; while

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

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

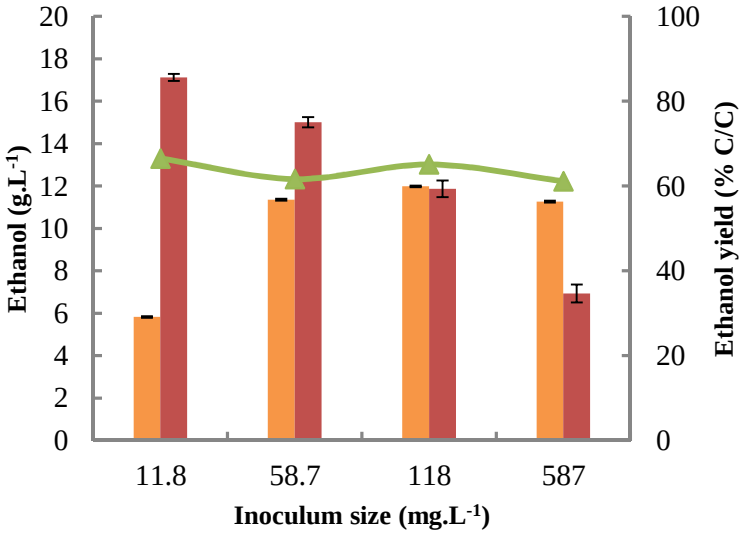


Figure 3. Influence of inoculum size on ethanol production ( ), ethanol/ biomass ratio ( ) and ethanol yield ( )

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

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

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 600nm)	2.05	10.48
Ethanol yield (% C/C)	60.65	52.47
Glycerol yield (% C/C)	4.24	2.14

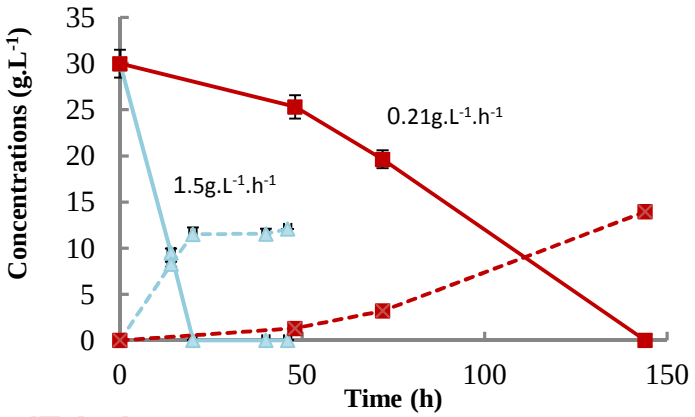


Figure 4. Kinetics of glucose consumption (continuous line) and ethanol production (dashed line) with peptone (▲) and NH_4Cl (■) as nitrogen source

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

The use of peptone led also to a decrease of the glycerol yield, 2.1 instead of 4.2% C/C in the presence of ammonium (Table 3). This should be related to the nitrogen source. In fact, with a better assimilation of nitrogen, cell growth is favored, leading to a higher glucose consumption and also a decrease of the oxygen content, both having a direct impact on glycerol and ethanol yields, lowering the former and increasing the latter [51].

However, even though ethanol production rate and cell growth was faster, $ethanol_{o/t}$ ratio and ethanol yield were not improved using peptone instead of NH_4Cl (Table 3) showing that *S. cerevisiae* growth by an anabolic pathway is favored over fermentation in the presence of peptone.

These results highlight the importance of the nitrogen source. For *S. cerevisiae*, a mineral source allows to obtain a high ethanol yield with a low biomass yield; while an organic source, such as peptone, allows to improve ethanol production rate in spite of a loss of carbon substrate for biomass formation.

According to the supplier (Biokar Diagnostics, A1702AH), peptone is mostly composed of glutamic acid (17.4%), proline (8.4%), leucine, lysine and aspartic acid (between 6.4 and 7.2%). Hou et al [15], who studied *Laminaria digitata* as nitrogen source, found that amino-acids contained in peptone were also abundant in this macroalgae. Therefore, the use of peptone as source of nitrogen can lead to approach algal hydrolysate conditions.

Moreover, algae could be used as nitrogen source to enhance ethanol production from corn stover [57] or high gravity sweet potato medium [58]. It is proven that yeast growth and ethanol production are enhanced by this supplementary source of nitrogen. Rich in proteins, their composition in amino-acids are close to those of yeast extract and peptone, confirming that macroalgae could be used as substituent in yeast culture or as fermentation media.

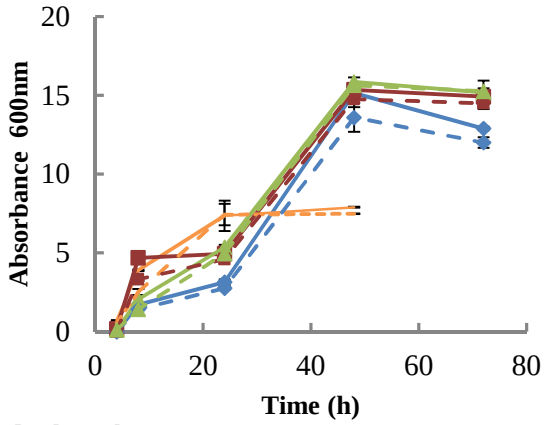
3.3 Influence of salts

Due to the presence of salts in algal hydrolysate, yeast strains could suffer from their impact on the osmotic pressure. The supplementation of synthetic medium with sodium chloride and sulfate at similar concentrations found in *Ulva sp* hydrolysates (0.25 and 0.21 M respectively; data not shown) should allow to study the behavior of the four yeast strains selected facing this change of osmolarity.

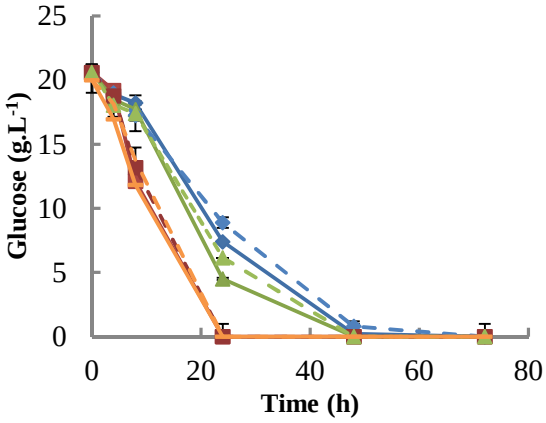
Referring to Figure 5.a, a slight impact of the presence of salts was only really noticeable for *P. stipitis*. Due to the presence of salts, yeasts need to adapt to a higher osmotic pressure. During osmoregulation, biomass development is slowed in favor of the production of neutral solutes, like

glycerol (Blomberg, 2000). From this, *S. cerevisiae*, *K. marxianus* and *C. guilliermondii*, whose biomass growth was less affected, could better adapt and resist to osmotic pressure than *P. stipitis*.

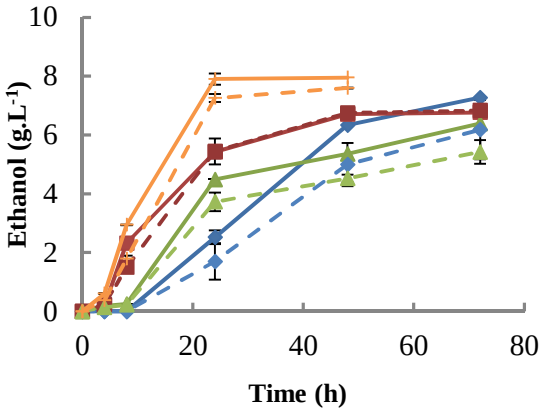
Growth rates differed from one strain to another. After 22 h of fermentation and contrarily to the other strains, *S. cerevisiae* growth reached a stationary state at a lower absorbance value than those observed for the other strains, for which growth was observed until 48 h. For *S. cerevisiae* and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



(a)



(b)



(c)

Figure 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)

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

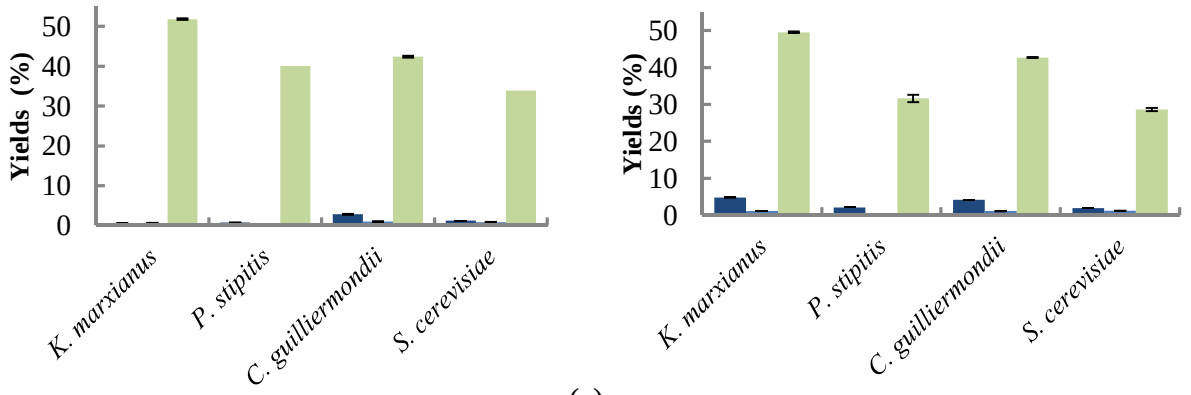
Table 4

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

S. cerevisiae	0.83	0.83	0.33	0.30	77.8	74.3	5.55	5.43

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



(a) (b)

Figure 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)

CONCLUSIONS

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

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

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REFERENCES

[1] S. Fernandes, N. M. Trautmann, D. G. Streets, C. A. Roden, and T. C. Bond, "Global biofuel use, 1850–2000," *Glob. Biogeochem. Cycles*, vol. 21, no. 2, pp. 1–15, 2007.

[2] N. Trivedi, V. Gupta, C. R. Reddy, and B. Jha, "Enzymatic hydrolysis and production of bioethanol from common macrophytic green alga Ulva fasciata Delile," *Bioresour Technol*, vol. 150, pp. 106–112, 2013.

[3] S. Chniti, H. Djelal, M. Hassouna, and A. Amrane, "Residue of dates from the food industry as a new cheap feedstock for ethanol production," *Biomass Bioenergy*, vol. 69, pp. 66–70, 2014.

[4] C.-M. Liu and S.-Y. Wu, "From biomass waste to biofuels and biomaterial building blocks," *Renew. Energy*, vol. 96, no. B, pp. 1056–1062, 2016.

[5] J. Milano *et al.*, "Microalgae biofuels as an alternative to fossil fuel for power generation," *Renew. Sustain. Energy Rev.*, vol. 58, pp. 180–197, 2016.

[6] J. Milledge, B. Smirh, P. W. Dyer, and P. Harvey, "Macroalgae-Derived Biofuel: A Review of Methods of Energy Extraction from Seaweed Biomass," *Energies*, vol. 7, no. 11, pp. 7194–7222, 2014.

[7] T. Suganya, M. Varman, H. H. Masjuki, and S. Renganathan, "Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: A biorefinery approach," *Renew. Sustain. Energy Rev.*, vol. 55, pp. 909–941, 2016.

[8] X. Briand and P. Morand, "Anaerobic digestion of Ulva sp. 1. Relationship between Ulva composition and methanisation," *J. Appl. Phycol.*, vol. 9, pp. 511–524, 1997.

[9] A. Holzinger, K. Herburger, F. Kaplan, and L. A. Lewis, "Desiccation tolerance in the chlorophyte green alga Ulva compressa: does cell wall architecture contribute to ecological success?," *Planta*, vol. 242, no. 2, pp. 477–492, 2015.

[10] S. Shimada, M. Hiraoka, S. Nabata, and M. Masuda, "Molecular phylogenetic analyses of the Japanese Ulva and Enteromorpha (Ulvales, Ulvophyceae), with special reference to the free-floating Ulva," *Physiol. Res.*, vol. 51, no. 2, pp. 99–108, 2003.

[11] C. Wang, R. Yu, and M. Zhou, "Acute toxicity of live and decomposing green alga Ulva (Enteromorpha) prolifera to abalone Haliotis discus hannai," *Chin. J. Oceanol. Limnol.*, vol. 29, no. 3, pp. 541–546, 2011.

[12] M. Yanagisawa, T. Ojima, and K. Nakasaki, "Bioethanol from sea lettuce with the use of crude enzymes derived from waste," *J. Mater. Cycles Waste Manag.*, vol. 13, no. 4, pp. 321–326, 2011.

[13] E. T. Kostas, D. A. White, C. Du, and D. J. Cook, "Selection of yeast strains for bioethanol production from UK seaweeds," *J Appl Phycol*, vol. 28, no. 2, pp. 1427–1441, 2016.

[14] N. Suriyachai, K. Weerasaia, N. Laosiripojana, V. Champreda, and P. Unrean, "Optimized simultaneous saccharification and co-fermentation of rice straw for ethanol production by Saccharomyces cerevisiae and Scheffersomyces stipitis co-culture using design of experiments," *Bioresour. Technol.*, vol. 142, pp. 171–178, 2013.

[15] X. Hou, J. H. Hansen, and A.-B. Bjerre, "Integrated bioethanol and protein production from brown seaweed Laminaria digitata," *Bioresour. Technol.*, vol. 197, pp. 310–317, 2015.

[16] A. Fathima, M. Sanitha, T. Kumar, S. Iyappan, and M. Ramya, "Direct utilization of waste water algal biomass for ethanol production by cellulolytic Clostridium phytofermentans DSM1183," *Bioresour. Technol.*, vol. 202, pp. 253–256, 2016.

[17] N. Kim, H. Li, K. Jung, H. Chang, and P. Lee, "Ethanol production from marine algal hydrolysates using Escherichia coli KO11," *Bioresour Technol*, vol. 102, no. 16, pp. 7466–7469, 2011.

[18] R. C. Kuhad, G. Mehta, R. Gupta, and K. K. Sharma, "Fed batch enzymatic saccharification of newspaper cellulosics improves the sugar content in the hydrolysates and eventually the ethanol fermentation by Saccharomyces cerevisiae," *Biomass Bioenergy*, vol. 34, no. 8, pp. 1189–1194, 2010.

[19] A. Mishra *et al.*, "Lignocellulosic ethanol production employing immobilized Saccharomyces cerevisiae in packed bed reactor," *Renew. Energy*, pp. 1–7, 2016.
[20] H. van Hurk, E. Postma, W. A. Scheffers, and J. P. van Djiken, "Glucose transport in crabtree-positive and crabtree-negative yeasts," *J Gen Microbiol*, vol. 135, no. 9, pp. 2399–2406, 1989.
[21] M. G. Borines, R. L. de Leon, and J. L. Cuello, "Bioethanol production from the macroalgae Sargassum spp.," *Bioresour. Technol.*, vol. 138, pp. 22–29, 2013.
[22] H. Kim, S. Wi, S. Jung, and H. Bae, "Efficient approach for bioethanol production from red seaweed Gelidium amansii," *Bioresour. Technol.*, vol. 175, no. C, pp. 128–134, 2014.
[23] P. Kötter and M. Ciriacy, "Xylose fermentation by Saccharomyces cerevisiae," *Appl. Microb. Cell Physiol.*, vol. 38, no. 6, pp. 776–783, 1993.
[24] I. De Bari, P. De Canio, D. Cuna, F. Liuzzi, A. Capece, and P. Romano, "Bioethanol production from mixed sugars by Scheffersomyces stipitis free and immobilized cells, and co-cultures with Saccharomyces cerevisiae," *New Biotechnol.*, vol. 30, no. 6, pp. 591–597, 2013.
[25] T. Hanly and M. Henson, "Dynamic metabolic modeling of a microaerobic yeast co-culture: predicting and optimizing ethanol production from glucose/xylose mixtures," *Biotechnol. Biofuels*, vol. 6, no. 44, p. 16, 2013.
[26] Y. Chen, "Development and application of co-culture for ethanol production by co-fermentation of glucose and xylose: a systematic review," *J.Ind.Microbiol.Biotechnol*, vol. 38, pp. 581–597, 2011.
[27] D. R. J. Grootjen, L. H. H. M. Meijlink, R. G. J. M. van der Lans, and K. C. A. M. Luyben, "Cofermentation of glucose and xylose with immobilized Pichia stipitis and Saccharomyces cerevisiae," *Enzyme Microb. Technol.*, vol. 12, no. 11, pp. 860–864, 1990.
[28] H. Rouhollah, N. Iraj, E. Giti, and A. Sorah, "Mixed sugar fermentation by Pichia stipitis, Sacharomyces cerevisiaea, and an isolated xylosefermenting Kluyveromyces marxianus and their cocultures," *Afr. J. Biotechnol.*, vol. 6, no. 9, 2007.
[29] S. I. Mussatto, E. M. S. Machado, L. M. Carneido, and J. A. Teixeira, "Sugars metabolism and ethanol production by different yeast strains from coffee industry wastes hydrolysates," *Appl. Energy*, vol. 92, pp. 763–768, 2012.
[30] N. Rodrussamee, N. Lertwattanasakul, K. Hirata, S. Limtong, T. Kosaka, and M. Yamada, "Growth and ethanol fermentation ability on hexose and pentose sugars and glucose effect under various conditions in thermotolerant yeast Kluyveromyces marxianus," *Appl.Microbiol.Biotechnol*, vol. 90, pp. 1573–1586, 2011.
[31] W. Wu, W. Hung, K. Lo, Y. Chen, H. Wan, and K. Cheng, "Bioethanol production from taro waste using thermo-tolerant yeast Kluyveromyces marxianus K21," *Bioresour Technol*, vol. 201, pp. 27–32, 2016.
[32] A. Acourene and A. Ammouche, "Optimization of ethanol, citric acid, and α-amylase production from date wastes by strains of Saccharomyces cerevisiae, Aspergillus niger, and Candida guilliermondii," *J Ind Microbiol Biotechnol*, vol. 39, pp. 759–766, 2012.
[33] V. F. Silva, P. V. Arruda, M. G. Felipe, A. R. Gonçalves, and G. J. Rocha, "Fermentation of cellulosic hydrolysates obtained by enzymatic saccharification of sugarcane bagasse pretreated by hydrothermal processing," *J Ind Microbiol Biotechnol*, vol. 38, no. 7, pp. 809–817, 2011.
[34] H. Djelal, A. Amrane, F. Lahrer, and G. Martin, "Effect of medium osmolarity on the bioproduction of glycerol and ethanol by Hansenula anomala growing on glucose and ammonium," *Appl. Microbiol. Biotechnol.*, vol. 69, no. 3, pp. 341–349, Dec. 2005.
[35] H. Djelal, F. Lahrer, G. Martin, and A. Amrane, "Effect of the dissolved oxygen on the bioproduction of glycerol and ethanol by Hansenula anomala growing under salt stress conditions," *J. Biotechnol.*, vol. 125, no. 1, pp. 95–103, 2006.
[36] S. I. Martins, W. M. Jongen, and M. A. Van Boekel, "A review of Maillard reaction in food and implications to kinetic modelling," *Trends Food Sci. Technol.*, vol. 11, no. 9, pp. 364–373, 2000.

[37] H. Djelal, F. Larher, G. Martin, and A. Amrane, "Continuous culture for the bioproduction of glycerol and ethanol by Hansenula anomala growing under salt stress conditions," *Ann Microbiol*, vol. 62, no. 1, pp. 49–54, 2012.
[38] P. Karagöz and M. Özkan, "Ethanol production from wheat straw by Saccharomyces cerevisiae and Scheffersomyces stipitis co-culture in batch and continuous system," *Bioresour. Technol.*, vol. 158, pp. 286–293, 2014.
[39] S. Mateo, J. G. Puentes, I. C. Roberto, S. Sanchez, and A. J. Moya, "Optimization of acid hydrolysis of olive tree pruning residue. Fermentation with Candida guilliermondii," *Biomass Bioenergy*, vol. 69, pp. 39–46, 2014.
[40] A. Tesfaw and F. Assefa, "Current Trends in Bioethanol Production by Saccharomyces cerevisiae: Substrate, Inhibitor Reduction, Growth Variables, Coculture, and Immobilization," *Int. Sch. Res. Not.*, vol. 2014, p. 11, 2014.
[41] F. K. Agbogbo, G. Coward-Kelly, M. Torry-Smith, and K. S. Wenger, "Fermentation of glucose/xylose mixtures using Pichia stipitis," *Process Biochem.*, vol. 41, no. 11, pp. 2333–2336, 2006.
[42] J. Pagliardini, G. Hubmann, C. Bideaux, S. Alfenore, E. Nevoigt, and S. E. Guillouet, "Quantitative evaluation of yeast's requirement for glycerol formation in very high ethanol performance fed-batch process," *Microb Cell Fact*, vol. 9, no. 1, p. 36, 2010.
[43] S. Galafassi, M. Toscano, I. Vigentini, J. Piškur, and C. Compagno, "Osmotic stress response in the wine yeast Dekkera bruxellensis," *Food Microbiol.*, vol. 36, no. 2, pp. 316–319, 2013.
[44] Y.-H. Lin, W.-S. Chien, K.-J. Duan, and P. R. Chang, "Effect of aeration timing and interval during very-high-gravity ethanol fermentation," *Process Biochem.*, vol. 46, no. 4, pp. 1025–1028, 2011.
[45] A. Blomberg, "Metabolic surprises in Saccharomyces cerevisiae during adaptation to saline conditions: questions, some answers and a model," *FEMS Microbiol. Lett.*, 2000.
[46] O. Deesuth, P. Laopaiboon, P. Klanrit, and L. Laopaiboon, "Improvement of ethanol production from sweet sorghum juice under high gravity and very high gravity conditions: Effects of nutrient supplementation and aeration," *Ind. Crops Prod.*, vol. 74, pp. 95–102, 2015.
[47] A. Blomberg and L. Adler, "Roles of glycerol and glycerol-3-phosphate dehydrogenase (NAD+) in acquired osmotolerance of Saccharomyces cerevisiae.," *J. Bacteriol.*, pp. 1087–1092, 1989.
[48] A. Blomberg and L. Adler, "Physiology of osmotolerance in fungi," *Adv Microb Physiol*, vol. 33, pp. 145–212, 1992.
[49] M. Bely, P. Stoeckle, I. Masneuf-Pomarède, and D. Dubourdieu, "Impact of mixed Torulaspora delbrueckii–Saccharomyces cerevisiae culture on high-sugar fermentation," *Int. J. Food Microbiol.*, vol. 122, no. 3, pp. 312–320, 2008.
[50] A. Tahir, M. Aftab, and T. Farasat, "Effect of cultura conditions on ethano production by locally isolated Saccharomyces cerevisiae bio07," *J App Pharm*, vol. 3, no. 2, pp. 72–78, 2010.
[51] P. Vijaikishore and N. G. Karanth, "Glycerol production by fermentation," *Appl. Biochem. Biotechnol.*, vol. 9, no. 3, pp. 243–253, 1984.
[52] S. Chniti *et al.*, "Effect of the Nitrogen Source on Bioethanol Production from Syrup Dates by Saccharomyces cerevisiae," *Int. J. Agric. Innov. Res.*, vol. 4, no. 3, 2015.
[53] S. H. da Cruz, E. Maffud Cilli, and J. R. Ernandes, "Structural Complexity of the Nitrogen Source and Influence on Yeast Growth and Fermentation," *J. Inst. Brew.*, vol. 108, no. 1, pp. 54–61, 2002.
[54] E. Albers, C. Larsson, G. Lidén, C. Niklasson, and L. Gustafsson, "Influence of the nitrogen source on Saccharomyces cerevisiae anaerobic growth and product formation.," *Appl. Environ. Microbiol.*, vol. 62, no. 9, pp. 3187–3195, 1996.
[55] B. Bach, F. X. Sauvage, S. Dequin, and C. Camarasa, "Role of γ-Aminobutyric Acid as a Source of Nitrogen and Succinate in Wine," *Am J Enol Viticult*, vol. 60, pp. 508–516, 2009.

[56] D. D. da Silva, J. Cândido Ede, P. V. de Arruda, S. S. da Silva, and M. Felipe, "New cultive medium for bioconversion of C5 fraction from sugarcane bagasse using rice bran extract," *Braz J Microbiol*, vol. 45, no. 4, pp. 1469–1475, 2015.

[57] Z. Yue, D. Ma, S. Peng, X. Zhao, T. Chen, and J. Wang, "Integrated utilization of algal biomass and corn stover for biofuel production," *Fuel*, vol. 168, pp. 1–6, 2016.

[58] Y. Shen, Y. P. Guo, H. D. Zhang, X. X. Zheng, X. M. Zhang, and F. W. Bai, "Application of low-cost algal nitrogen source feeding in fuel ethanol production using high gravity sweet potato medium," *J. Biotechnol.*, vol. 160, no. 3–4, pp. 229–235, 2012.

[59] J. Moreno-Garcia, T. Garcia-Martinez, M. Carmen Millan, J. Mauricio, and J. Moreno, "Proteins involved in wine aroma compounds metabolism by a Saccharomyces cerevisiae flor-velum yeast strain grown in two conditions," *Food Microbiol.*, vol. 51, pp. 1–9, 2015.

[60] S. Swinnen, P.-W. Ho, M. Klein, and E. Nevoigt, "Genetic determinants for enhanced glycerol growth of Saccharomyces cerevisiae," *Metab. Eng.*, vol. 36, pp. 68–79, 2016.

[61] X.-C. Hu, L.-J. Ren, S.-L. Chen, L. Zhang, X.-J. Ji, and H. Huang, "The roles of different salts and a novel osmotic pressure control strategy for improvement of DHA production by Schizochytrium sp.," *Bioprocess Biosyst. Eng.*, vol. 38, no. 11, pp. 2129–2136, 2015.

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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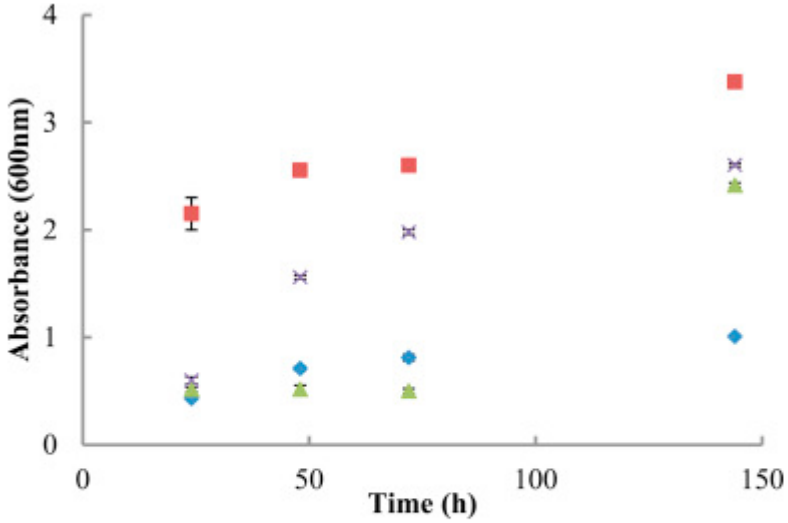

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Fig. 1. Absorbance at 600 nm measured during 144 h of fermentation by (▲) *K. marxianus* (◆), *P. stipitis* (■), *C. guilliermondii* and (×) *S. cerevisiae*.

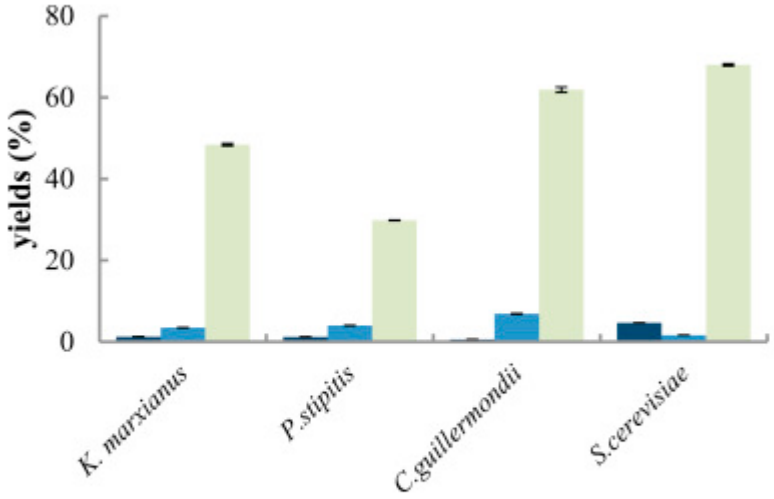

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Fig. 2. Glycerol (■), acetic acid (■) and ethanol (■) yields (% C/C) obtained after 144 h of fermentation with the four yeast strains.

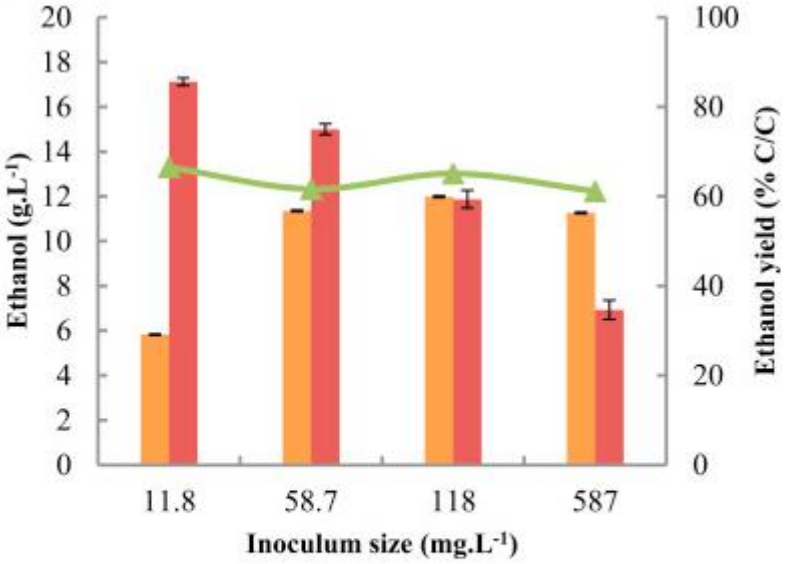

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Fig. 3. Influence of inoculum size on ethanol production ( ), ethanol/biomass ratio ( ) and ethanol yield ( ).

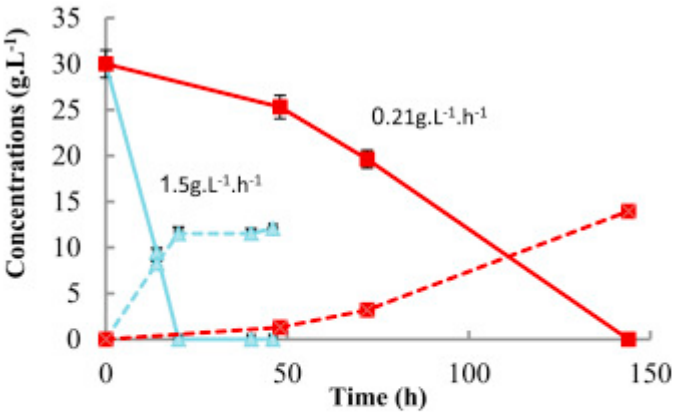

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Fig. 4. Kinetics of glucose consumption (continuous line) and ethanol production (dashed line) with peptone ( ) and NH_4Cl ( ) as nitrogen source.

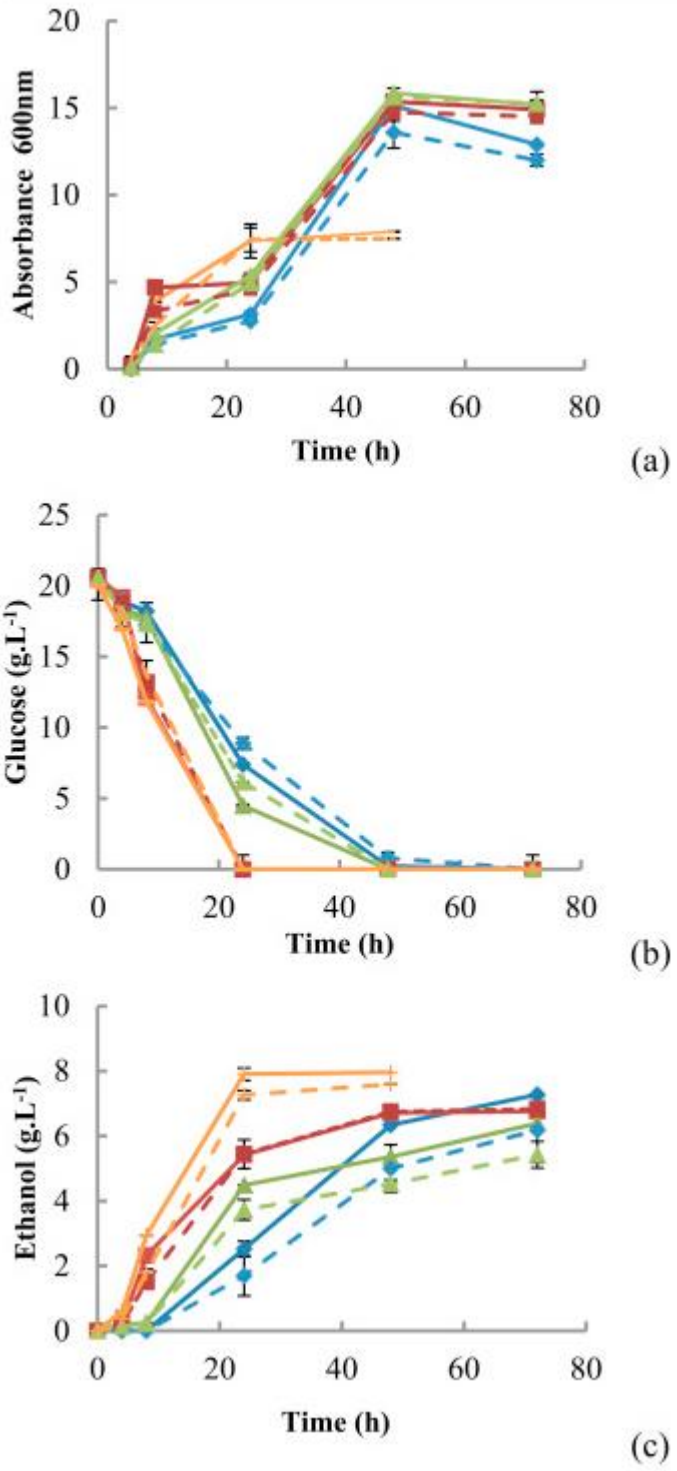

Fig. 5. Growth rate (a), glucose consumption (b) and ethanol production (c) for *K. marxianus* (■), *P. stipitis* (◆), *C. guilliermondii* (▲) and *S. cerevisiae* (+), during fermentation in absence (continuous line) and presence of salts (dashed line).

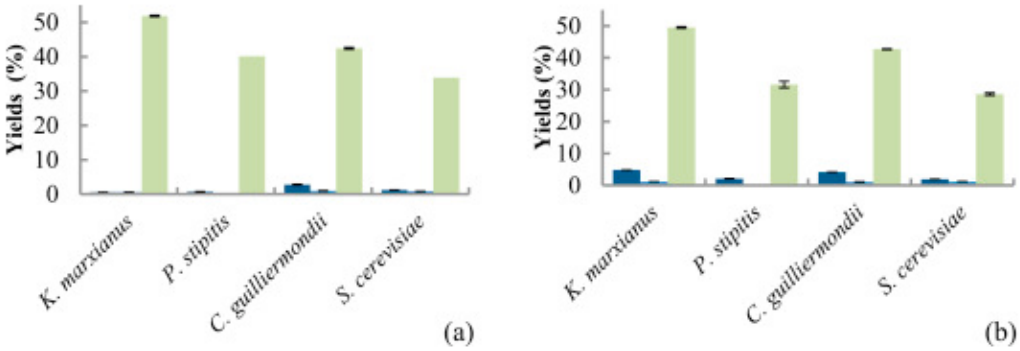

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Fig. 6. Glycerol (■), acetic acid (■) and ethanol (■) yields obtained after 72 h of fermentation with the four strains, in absence (a) and presence of salts (b).

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