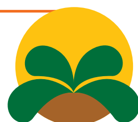
In the Field



Biofpr Biofuels Bioproducts & Biorefining

Scaling up bioethanol production from the farmed brown macroalga *Macrocystis pyrifera* in Chile

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Abstract: Interest in third-generation biomass such as macroalgae has increased due to their high biomass yield, absence of lignin in their tissues, lower competition for land and fresh water, no fertilization requirements, and efficient CO_2 capture in coastal ecosystems. However, several challenges still exist in the development of cost-effective technologies for processing large amounts of macroalgae. Recently, genetically modified micro-organisms able to convert brown macroalgae carbohydrates into bioethanol were developed, but still no attempt to scale up production has been proposed. Based on a giant kelp (*Macrocystis pyrifera*) farming and bioethanol production program carried out in Chile, we were able to test and adapt this technology as a first attempt to scale up this process using a 75 L fermentation of genetically modified *Escherichia coli*. Laboratory fermentation tests results showed that although biomass growth and yield are not greatly affected by the alginate:mannitol ratio, ethanol yield showed a clear maximum around a 5:8 alginate:mannitol ratio. In *M. pyrifera*, a much greater proportion of alginate and lower mannitol abundance is found. In order to make the most of the carbohydrates available for fermentation, we developed a four-stage process model for scaling up, including acid leaching, depolymerization, saccharification, and fermentation steps. Using this process, we obtained 0.213 Kg ethanol Kg^{-1} dry macroalgae, equivalent to 9.6 m^3 of ethanol $hectare^{-1}$ $year^{-1}$, reaching 64% of the maximum theoretical ethanol yield. We propose strategies to increase this yield, including synthetic biology pathway engineering approaches and process optimization targets. © 2016 Society of Chemical Industry and John Wiley & Sons, Ltd

Supporting information may be found in the online version of this article.

Keywords: bioethanol production yield; fermentation process scale up; farmed macroalgae, *Macrocystis pyrifera*, Chile

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Introduction

Methods to convert biomass to competitive biofuels are increasingly attractive as fossil hydrocarbons are likely to become scarce and costly. Interest has now been diverted to third-generation biomass like macroalgae, since first-generation feedstock (edible crops, sugars, and starches) is under serious controversy considering the competition between food and fuel, and second-generation biomass (lignocellulosic biomass) is limited by the high costs of lignin removal.[1] Despite the obvious potential of aquatic biomass production for energy applications (e.g. no lignin content, high biomass yield, rapid growth rates, no competition for land and fresh water, no fertilization need, efficient CO_2 capture),[2–6] challenges now lie in further developing cost-effective transformation technologies for processing large quantities of macroalgae.

Process operations used for algal-derived biofuel production can be grouped in two main areas, (i) cultivation and harvesting and (ii) energy extraction. In the case of brown macroalgae, like the giant kelp *Macrocystis pyrifera*, the first farming technology steps have been already developed.[7–10] This species has shown many advantages over other brown macroalgae in terms of productivity, yield, geographical distribution, and ease of reproduction and farming.[10] By having two production cycles per year and placing seeded lines 4 m apart, cultivation is technically feasible and productions around 41.3 Kg (wet) per meter of culture line per year can be obtained.[11] These values allow establishing commercial farming activities that can become profitable starting at production scales higher than 30 hectares of cultivation and if the value paid by the fresh biomass reaches at least US$ 78 per tonne.[11] By duplicating culture line density and obtaining especially selected kelp strains, the productivity could even be significantly increased and it has been indicated that production values can potentially reach up to 200 tonnes (fresh) per hectare per year.[10] These production values make it possible to think that kelp biomass for industrialization could be available in larger quantities by potentially developing large-scale sea cultivation of *M. pyrifera*.

On the other hand, for the energy extraction step, different technologies have been reported to produce different biofuels from brown macroalgae (e.g. gasification to produce methane,[12] pyrolysis to produce charcoal,[3] and liquefaction to produce oil).[13] Bioethanol can be produced through enzymatic hydrolysis and fermentation.[2,6] Freshly harvested brown macroalgae contain about 40% carbohydrates based on total wet weight.[14] Laminaran and mannitol can be easily extracted and promising results have been obtained with anaerobic fermentation of *Saccharina japonica* (kombu) with *Enterobacter* sp., a mannitol-degrading bacterium.[15] However, the full potential of bioethanol and renewable chemical production from brown macroalgae cannot be realized unless alginate is co-fermented.[16] Alginate (amounting up to 30–40% by dry weight)[14] is harder to release from algal biomass and micro-organisms used so far in fermentation processes to produce ethanol are not able to metabolize alginate. For maximal bioethanol production from brown macroalgae, co-fermentation of mannitol and alginate has been reported by Wargacki *et al.*,[17] using a genetically engineered *Escherichia coli* strain (BAL1611).[17] The authors used 1 L bioreactors with an 8:4:1 mannitol:alginate:glucose carbon source ratio, the carbohydrate proportion present in *S. japonica*, although this is not a representative composition for all commercially relevant brown macroalgae. A final titer of ~4.7% *v/v* was obtained, which corresponds to a bioconversion value of ~0.281 wt ethanol/wt biomass with a yield of ~0.41 wt ethanol/wt total sugars (alginate, mannitol, and glucan). Eighty-three percent of this production was achieved within the first 48 h of fermentation, corresponding to an overall rate of 0.64 g $liter^{-1}$ $hour^{-1}$. Similarly, a *Saccharomyces cerevisiae* strain was also engineered for the same purpose and authors also acknowledged the application of a consolidated bioprocessing strategy for ethanol production directly from brown macroalgae.[16] Although this approach is technically feasible, scaling the complete process for industrial application (including pretreatments and obtainment of co-products from the brown macroalgae biorefinery) implies several challenges. These include, for example, reducing consumption of expensive alginate lyase enzymes and in-house production of oligoalginate lyases, reducing fermentation time, increasing productivity and yield, and adjusting the molar ratio of sugars in order to accommodate different feedstock sources. The latter is extremely important since sugar concentration in brown macroalgae significantly varies between species, regions, and seasons.[18–20]

From a metabolic point of view, alginate and mannitol are very oxidized and have reduced carbon sources, respectively. Their catabolic pathways, and also their energetic and reduced cofactor yields, are radically different between them and when compared to glucose. Moreover, complex regulatory systems in *E. coli* that prioritize the sequential metabolism of sugar mixtures, with preference for glucose, can impede the rapid and complete utilization of sugar mixtures during fermentation.[21] The best understood of these in *E. coli* is carbon catabolite repression (glucose) which involves cyclic AMP, cyclic AMP-binding

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protein (Crp),[22–24] enzymes of the phosphotransferase system,[23,24] the Cra,[22,25,26] and Fis[27,28] global regulators and other components. With this system, glucose effectively blocks the expression of sugar-specific transporters and key enzymes needed for the metabolism of alternative sugars, a mechanism that is known to affect mannitol utilization.[29–33] Therefore, even if the engineered alginate catabolic pathway is constitutively expressed in *E. coli* BAL1611 strain,[17] growth and product yields must be tightly dependent on cytoplasmic redox constraints, oxygen availability, alginate:mannitol ratios, and the presence of other sugars, especially glucose. Most probably, this strain is suboptimal in this sense and its productivity and yield could be further improved by careful metabolic and process engineering.

Therefore, to move into the industrialization of algal bioethanol and co-products, it is relevant to develop a process that allows making the carbohydrate fraction entirely available for fermentation. Importantly, the process should also be flexible, allowing to be adjusted to a variable amount and composition of carbohydrates in biomass. Strain metabolic optimization is also necessary to increase productivity and yield and decrease processing time. Which strategy to use for *E. coli* BAL1611[17] remains obscure due to the lack of experimental exploration of mixed sugar fermentations with this strain.

Considering that *Macrocystis* is a highly productive organism for which amenability for large-scale cultivation has been demonstrated, we report for the first time the scale-up of a bioethanol production process from the brown seaweed *M. pyrifera* using the genetically modified *E. coli* strain BAL1611 of Wargacki *et al.*[17] In a first step, we characterize the growth of *E. coli* BAL1611 on mixed alginate and mannitol media in order to determine the metabolic requirements and yields of the strain to produce ethanol from sugar mixtures with different redox balance. We also propose a biorefinery pre-treatment process to make all major carbohydrates contained in the biomass available for fermentation. This process also generates co-products and allows the possibility of varying the proportion of alginate, mannitol, and glucose in the fermentation medium, independently of the specific ratios contained in macroalgae. Then, we performed fermentations at a larger scale on alginate- and mannitol-containing *M. pyrifera* macroalgae in two stages: first optimizing a fermentation protocol at 1 L scale and then scaling-up the process to a 75 L bioreactor. In this study we clearly demonstrate that although biomass growth and yield is not greatly affected by alginate:mannitol ratio, ethanol yield showed a clear maximum around a 5:8 alginate:mannitol ratio. Unfortunately, this is not the proportion present in most brown macroalgae, including the giant kelp *M. pyrifera*, but we propose strategies based on our results that can be used to optimize bioethanol production from this macroalgae using both process and metabolic engineering approaches.

Materials and methods

Fermentation of pure sugars in Erlenmeyer flasks

Fermentations in Erlenmeyer flasks were carried out using pure sugars as carbon sources to determine optimum sugar ratios for *E. coli* BAL1611 strain[17] growth. Sodium alginate and mannitol (Sigma Aldrich, Saint Louis, MO, USA) were used as the sole carbon sources in M9 minimal medium (Supporting Information). All experiments were carried out at 2.6% *w/v* total carbohydrate concentration (alginate plus mannitol). This concentration was selected based on previous experiments that indicated growth inhibition was observed when larger amounts were used.

We used four different alginate:mannitol ratios in Erlenmeyer flask fermentations (Table 1). We used a 5:8 proportion to represent *S. japonica* composition and a 10:3 ratio to represent carbohydrate abundance in *M. pyrifera*.

Table 1. Carbohydrate ratio used on fermentations performed at different scales: Erlenmeyer flasks, 1-L and 75-L bioreactors.

	Carbohydrate ratio			Alginate fraction [alginate/(alginate + mannitol)]
	Alginate :	Mannitol :	Glucose	
Fermentation in Erlenmeyer flasks	1	0	0	1
	10	3	0	0.77
	8	5	0	0.62
	5	8	0	0.38
	3	10	0	0.23
	0	1	0	0
Fermentation in 1 L bioreactor	1	2	1	
Fermentation in 75 L bioreactor	1	2	1	

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For symmetry considerations, we chose the other two proportions to be 8:5 and 3:10. In this way, the assayed alginate:mannitol ratios represent typical composition ranges of brown macroalgae. Fermentations with pure alginate and pure mannitol were also performed as comparative end points. With this, our experiments covered the entire range of alginate fractions [defined as the alginate/(alginate+mannitol) ratio] (Table 1).

Glycerol stocks of *E. coli* BAL1611[17] stored at −80 °C were used to inoculate 5 mL of LB medium in glass lab tubes and bacteria were grown overnight at 37 °C and 250 rpm. This culture was used to prepare inocula for Erlenmeyer flask fermentations. Each inoculum was grown in M9 minimal medium with 2.6% total sugar concentration at the respective alginate:mannitol ratio of the final fermentation in order to adapt bacteria to the desired growth condition before larger-scale fermentations. Final fermentations were carried out at 37 °C and 250 rpm for 48 h in 500 mL Erlenmeyer flasks containing 100 mL M9 medium supplemented with the respective sugar amounts and inoculated with tube cultures grown in the same medium. Culture optical density was measured at 600 nm every 2 h and ethanol production was quantified using high performance liquid chromatography (HPLC) (Supporting Information). Biomass growth curves were used to calculate exponential growth rates from logarithmic plots during the exponential growth phase. Biomass yields and ethanol yields were obtained for each sugar ratio.

Each experiment was performed in triplicate. Logarithmic plots were used to identify the exponential growth phase and an exponential model was fitted to these points by non-linear parameter fitting. Ninety-five percent confidence errors for the adjusted growth rates were calculated for each experiment. Data were checked for homogeneity of variances and normality and all results were statistically compared using multiple two-tailed t-tests for the difference between two sample means. All calculations were done using the Matlab software package (The Mathworks).

Pre-treatments and fermentation of *M. pyrifera* in a 1 L bioreactor

Acid leaching in a 1L bioreactor

M. pyrifera was collected at Calbuco, Puerto Montt, Chile (41°46´S; 73°08´W) by scuba divers and transported immediately to avoid decomposition. Once in the laboratory, the seaweed was washed with tap water and debris (stones, shells, epiphytes) were manually removed. The tissue was milled in a hammer mill (custom made) down to half-inch pieces. A portion of 250 g of milled seaweed was introduced in a previously sterilized 1 L bioreactor (Biostat® APlus, Sartorius Stedim, Bohemia, NY, USA) with 250 mL of 0.3% HCl. Temperature and agitation were set to 25 °C and 750 rpm, respectively, for 1 h. Once the reaction was completed, solids were separated by centrifugation. Leached seaweed was introduced again in the 1 L bioreactor and the procedure described was repeated twice more. Samples of the complete volume of leached liquid were analyzed through HPLC to determine sugar composition (Supporting Information details carbohydrate determination). The solid-phase weight was determined using a precision balance to calculate process yields. The complete leaching process was performed by triplicate. The results were statistically analyzed using one-way ANOVA followed by a Tukey test for pairwise comparison. Before testing, data were checked for homogeneity of variances and normality. All calculations were done using Minitab v.17 software.

Depolymerization of leached seaweed in a 1 L bioreactor

For depolymerization, 100 g of leached seaweed was added to a previously sterilized 1 L bioreactor (Biostat® APlus, Sartorius Stedim, Bohemia, NY, USA) together with 500 mL of distilled water. The following parameters were set: temperature 50 °C, 200 rpm and pH 5.5, adjusted with KOH if necessary. The bioreactor was inoculated with 0.4 mL of CTec2 and 40 μL of HTec2 (Novozymes, Bagsvaerd, Denmark), and the process was allowed to continue for 20 h. After that time, the temperature was lowered to 25 °C and the pH adjusted to 7.5 with KOH. Once temperature reached a stable level, 2 mL of M9 buffer (M6030, Sigma Aldrich, Saint Louis, MO, USA) with 7.5 mg mL^{-1} of alginate lyase (A1603, Sigma Aldrich, Saint Louis, MO, USA) were added, and the process continued for another 20 h. Once completed the time, separation proceeds through centrifugation at 5500 rpm and 4 °C for 10 min. The complete depolymerization process was repeated three times.

Saccharification of depolymerized liquid in a 1 L bioreactor

Five hundred mL of depolymerized liquid were introduced to a previously sterilized 1 L bioreactor (Biostat® APlus, Sartorius Stedim, Bohemia, NY, USA) and conditions were adjusted to 25 °C, 500 rpm, and pH 7.5. The bioreactor was inoculated with 7.5 mL of crude oligoalginate lyase lysate (OAL lysate) and then stirred for 20 h, maintaining con-

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stant conditions. To prepare the crude OAL lyase extract, the following procedure was followed: *E. coli* BL21 (DE3) strain harboring pETAtu_OAL plasmid[17] was grown in LB medium overnight. Aliquot of this culture was inoculated into fresh LB medium (10 mL) and grown in an orbital shaker at 37 °C at 200 rpm. When the culture reached OD_{600} ~0.6, induction was carried out with isopropyl β-D-l- thiogalactopyranoside (IPTG), and the culture was further grown overnight in an orbital shaker at room temperature. The culture was then centrifuged. The resulting pellet was suspended in a BugBuster cocktail (100 µL BugBuster mix, 20 µL protease inhibitor cocktail, 2 µL lysonase (71230, Novagen, Darmstadt, Germany), 0.15 mM phosphate buffer, 150 µL 2 M NaCl). The solution was chilled on ice for 30 min to lyse the cells. The cell lysate was centrifuged at 4300 rpm for 15 min, which resulted in the crude oligoalginate lyase lysate. The carbohydrate composition of the saccharification product was analyzed through HPLC following the analytical methodology described in Supporting Information. The complete saccharification process was repeated three times.

Fermentation in a 1 L bioreactor

To prepare the inoculum, glycerol stocks of *E. coli* BAL1611 strain[17] were used to inoculate 50 mL of LB medium and bacteria were grown at 37 °C and 250 rpm for 5 h. This culture was used to prepare inocula for fermentations. The fermentations were carried out at 25 °C, 200 rpm for 141 h in the 1 L bioreactors containing mannitol (22.3 g L^{-1}), DEHU (4-deoxy-L-erythro-5-hexoseulose uronate) (22.3 g L^{-1}), and glucose at a ratio of 2:1:1 (Table 1). The fermentation was repeated three times.

DEHU was prepared according to the following protocol: 17.5 g sodium alginate, 14 mL 0.3 M EDTA, and 875 µL 10 mg mL^{-1} alginate lyase solution were added to 44 mL of 10X M9 buffer at pH 5.5. The resulting solution was stirred and incubated in shaker at 200 rpm and 37 °C overnight. Total volume was brought to 50 mL with deionized water. A sample of 10 mL was digested with an oligoalginate lyase degradation buffer (M9 buffer, alginate lyase, OAL lysate, sodium azide, and deionized water), incubated without stirring at 37 °C for 24 h. DEHU was quantified by HPLC using an UV detector (Supporting Information details the analytical methodology).

Samples from the bioreactors were collected every hour for carbohydrate and ethanol determination (Supporting Information details the analytical methodology). The results were statistically analyzed using one-way ANOVA. Before testing, data were checked for homogeneity of variances and normality.

Pre-treatment and fermentation of *M. pyrifera* at pilot scale

Acid leaching

Prior to fermentation, three pre-treatments were performed: acid leaching to remove large quantities of potassium chloride; depolymerization to enzymatically digest alginate; and saccharification to degrade oligoalginate into DEHU, which is the monomeric form of alginate produced by lyase hydrolysis (ß-elimination). For acid leaching, 100 Kg of *M. pyrifera* were collected at Calbuco, Puerto Montt, Chile (41°46´S; 73°08´W) by scuba divers and transported immediately to avoid decomposition. Once in the laboratory, seaweed was washed with tap water and debris (stones, shells, epiphytes) were manually removed. The tissue was milled in a hammer mill (custom made) down to half-inch pieces. 100 kg of milled seaweed and 200 L of 0.3% HCl were mixed in a stirred reactor at 25 °C, 200 rpm for 1 h. Once completed, the liquid was drained. The process was repeated three consecutive times. The total volume of leached liquid was subjected to membrane-based separation and concentration (Labstack M20 unit, Alpha Laval) to recover the mannitol. Leached mannitol was purified by micro-, ultra-, and nanofiltration and then concentrated using reverse osmosis. Since mannitol has a size of 183 Dalton, first it was microfiltered and ultrafiltered using GR95PP-101204 and UFX5pht-522577 membranes, respectively. For nanofiltration a NF-517820 membrane was used and finally the solution was concentrated by reverse osmosis (RO98pht-100457 membrane). Mannitol concentration in solution was determined by HPLC (Supporting Information). The final solution was stored until fermentation stage.

Depolymerization of leached seaweed

For depolymerization, the leached seaweed was mixed with 200 L of tap water inside the stirred reactor and the pH was adjusted to 5.5 with KOH and kept constant throughout the entire procedure. 160 mL of hemicellulase and 16 mL of cellulose commercial preparations (Ctec2 and Htec2, Novozymes, Bagsvaerd, Denmark) were subsequently added and the mixture was stirred at 200 rpm and 50 °C for 20 h. After that period, the mixture was cooled to 25 °C and the pH was adjusted to 7.5 using KOH. 800 mL of M9 buffer (Sigma Aldrich, Saint Louis, MO, USA) containing 7.5 mg mL^{-1} of alginate lyase (Sigma Aldrich, Saint Louis, MO, USA) was added and the mixture was stirred again at 200 rpm for 20 h. After enzymatic digestion, the depolymerized seaweed was separated from the liquid by centrifugation for 10 min at

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5500 rpm and 4 °C in a centrifuge (Sorval RC6+, Thermo, Waltham, MA, USA). Decanted solids were submitted for proximal analysis at an external certified laboratory (ICYTAL UACH, Puerto Montt, Chile), which included estimation of protein concentration with the Kjeldahl method, fat concentration using a Soxhlet extraction method, carbohydrate concentration through a colorimetric method, crude fiber determination using calcination method and humidity and ash using gravimetry.

Saccharification of depolymerized seaweed

Saccharification started with 34 L of depolymerization liquid that were transferred to a previously sterilized 75 L fermentor (Biolafitte, LSL, St. Cloud, MN, USA). pH was adjusted to 7.5, temperature was set at 25 °C and rotation speed at 500 rpm. Finally, 510 mL of crude oligoalginate lyase lysate was added and then stirred for 20 h maintaining constant conditions. At the end of each stage, samples were analyzed for glucans, mannitol and alginate by HPLC using the methodology described in the Supporting Information.

Fermentation of *M. pyrifera* in a 75 L bioreactor

Before starting the fermentation, an *E. coli* BAL1611 strain[17] inoculum was prepared overnight as in the section Fermentation in a 1 L bioreactor. Flasks were maintained at 30 °C, 200 rpm overnight, to allow bacterial growth. This was used to inoculate fresh depolymerized/saccharified medium containing a 2:1:1 mannitol:DEHU:glucose proportion (Table 1). pH was adjusted to 7.0 using KOH and the fermentation was conducted at 25 °C and 200 rpm with no aeration for 48 h. Ethanol production and sugar consumption were monitored by HPLC (Supporting Information) over the entire fermentation period.

Results

Fermentation of pure sugars in Erlenmeyer flasks

Fermentations in Erlenmeyer flasks were carried out using pure sugars as carbon sources, using different alginate:mannitol ratios in order to determine optimum conditions for *E. coli* BAL1611 strain[17] growth and ethanol production (Table 2). Biomass growth rate and sugar to biomass yield have no significant differences for all alginate:mannitol ratios tested as long as both sugars were present in the medium. Although no significant differences were detected, a slight tendency to higher growth rates and lower biomass yields were observed for higher alginate:mannitol ratios. When alginate or mannitol were used as sole carbon sources, both biomass growth and yield were significantly lower. Growth rate and biomass yield on pure mannitol were three times higher than on pure alginate.

Ethanol production was more influenced by sugar ratio than biomass and varied significantly ($p < 0.05$) with fermentation time. Ethanol yields at 12 h were not significantly different for all alginate:mannitol ratios, but dropped to undetectable levels if pure sugars were used. However, at 48 h, a clear productivity optimum was detected at a 5:8 alginate:mannitol ratio. Ethanol yields for the other sugar ratios tested were similar at about 60% that of the maximum and ethanol levels remained undetectable after 48 h for fermentations with pure alginate or mannitol.

Fermentation of *M. pyrifera* in a 1 L bioreactor

For acid leaching, 500 g of wet milled seaweed was produced. After three successive extractions with

Table 2. Biomass growth rates, sugar to biomass yields and sugar to ethanol yields at different alginate:mannitol ratios.

Alginate:mannitol ratio	Growth rate [liter hour^{-1}]	Biomass yield [g biomass g^{-1} substrate]	Ethanol yield after 12 h [g ethanol g^{-1} substrate]	Ethanol yield after 48 h [g ethanol g^{-1} substrate]
Pure alginate	0.099 ± 0.068^{a}	0.008 ± 0.003^{d}	0.000^{g}	0.000^{i}
10:3	0.478 ± 0.035^{b}	0.067 ± 0.008^{e}	0.102 ± 0.022^{h}	0.146 ± 0.043^{j}
8:5	0.527 ± 0.036^{b}	0.061 ± 0.008^{e}	0.086 ± 0.022^{h}	0.173 ± 0.049^{j}
5:8	0.480 ± 0.041^{b}	0.072 ± 0.009^{e}	0.042 ± 0.007^{h}	0.304 ± 0.023^{k}
3:10	0.470 ± 0.033^{b}	0.077 ± 0.006^{e}	0.064 ± 0.024^{h}	0.177 ± 0.012^{j}
Pure mannitol	0.325 ± 0.032^{c}	0.041 ± 0.011^{f}	0.000^{g}	0.000^{i}

Superindex letters denote statistically different groups.

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Table 3. Acid leaching results in 1 L bioreactor. Results are presented for each replicate in terms of mannitol percentage in leachate and yield of leached seaweed (wet weight percentage).

Replicate	Extraction	% mannitol	Total mannitol (%)	Yield leached seaweed (%)
1	1	0.277 ± 0.002	0.341	44.56
	2	0.053 ± 0.002		
	3	0.010 ± 0.001		
2	1	0.205 ± 0.003	0.279	50.12
	2	0.056 ± 0.001		
	3	0.018 ± 0.000		
3	1	0.235 ± 0.000	0.311	49.42
	2	0.060 ± 0.001		
	3	0.016 ± 0.001		

0.3% HCl, a yield of leached seaweed around 50% (Table 3) and 0.3% of mannitol in the leachate were obtained. No significant differences between replicates were detected in mannitol concentrations ($F_{(2,26)} = 0.09$; $P = 0.918$). After depolymerization and saccharification of the leached seaweed, 23.8 ± 1.02 g liter^{-1} of DEHU were quantified for the three replicates. Finally, the fermentation using a ratio of 2:1:1 mannitol:DEHU:glucose achieved a maximum ethanol production of 10.36 g liter^{-1} after 41 h of fermentation, with a productivity of 0.25 g liter^{-1} hour^{-1} (Fig. 1). Glucose was consumed in the first 3 h, followed by mannitol and DEHU. No significant differences between replicates were observed for carbohydrate composition (glucose $F_{(2,8)} = 0.03$, $P = 0.972$; mannitol $F_{(2,24)} = 0.56$, $P = 0.582$; DEHU $F_{(2,15)} = 0.15$, $P = 0.963$) and ethanol production ($F_{(2,26)} = 0.22$, $P = 0.806$).

Fermentation of *M. pyrifera* in a 75 L bioreactor

To start with the fermentation process at the larger scale, the first step began with 100 Kg of wet milled *M. pyrifera* and produced 380 L of leachate and 48 Kg of leached seaweed (48% yield by weight). The leachate contains 0.63% mannitol, which was concentrated using a membrane-based separation and concentration process up to a final concentration of 1.67%. After depolymerization, a solid/liquid separation process was performed and 210 L of solution with 2.6 g liter^{-1} of glucose were obtained. Mannitol and DEHU were undetectable. 2.5 kg of dry depolymerized seaweed were obtained containing 35.77% of proteins (Kjeldahl method), 7.19% fat (Soxhlet method), 27.25% carbohydrates (colorimetric method), 9.85% crude fiber (calcination method), 6.51% humidity (gravimetry), 13.55% ash (gravimetry) and 3.17 Kcal g^{-1}.

Thirty-four and a half liters of saccharified liquid was produced, containing 1.54 g liter^{-1} of glucose and 22.3 g liter^{-1} of DEHU. For the final fermentation stage, 7 L of inoculum was prepared and inoculated in the fermenter with 475 g of mannitol in 22.3 L, 238 g of DEHU in 10.7 L and 268 g of glucose in order to achieve a final volume of 40 L with the desired 2:1:1 proportion. This proportion mimics the sugar concentration ratio of *M. pyrifera*.[18] After 48 h of micro-aerobic fermentation, the maximum ethanol concentration achieved was 8.87 g liter^{-1} (Fig. 2), with a productivity of 0.22 g liter^{-1} hour^{-1}. Assuming a

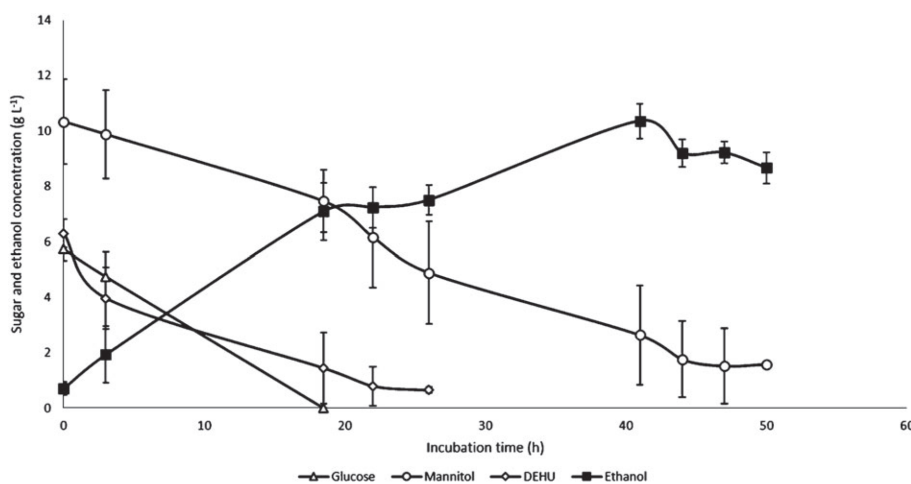

Figure 1. Sugar consumption and ethanol production during fermentation in a 1L bioreactor.

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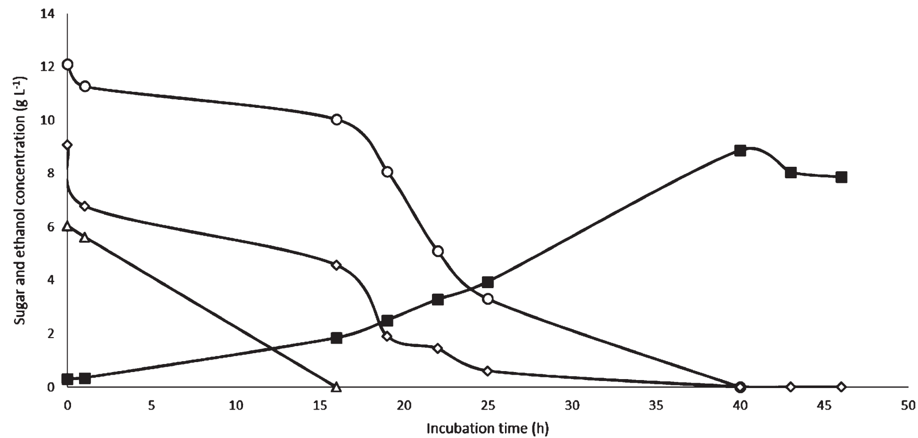

Figure 2. Sugar consumption and ethanol production during fermentation in a 75L bioreactor.

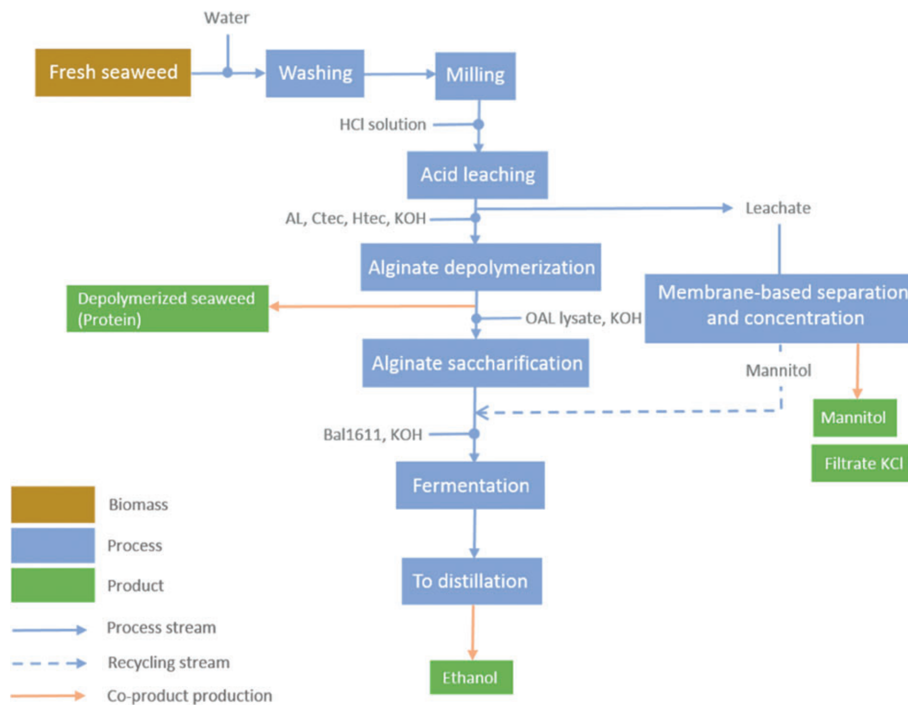

Figure 3. Process flow diagram for ethanol production from *Macrocystis pyrifera*, showing the biomass input (yellow), the different process steps (blue), and products (green). Arrows of different colors show the main process streams (blue), the mannitol recycle stream (blue dotted line), and co-products production (orange) as indicated in the legend. Mannitol can be used as feed stream for the fermentation step or as a process co-product.

maximum theoretical yield of 0.51 g ethanol g^{-1} sugar,[29] we obtained a yield of 64% of this maximum. The overall process, showing also possible co-products that can be obtained from the main ethanol production process, is summarized in Fig. 3.

Discussion

Wargacki *et al.*[17] reported their results using a 5:8:1 alginate:mannitol:glucose ratio to simulate brown macroalgae composition. However, these are not typical

concentrations for all macroalgae and in fact *M. pyrifera* composition differs significantly from this sugar ratio. On the other hand, *E. coli* can naturally ferment glucose, and mannitol to some extent, but not alginate. The extensive genetic engineering performed on *E. coli* BAL1611 makes it possible to metabolize these sugars. Alginate and mannitol, producing less and more reduction equivalents than glucose, respectively, mutually balance their influence on the intracellular redox state of the cell, although it is not easy to predict how cell growth and ethanol production will vary with different sugar ratios.

Our Erlenmeyer flask fermentation results clearly show that although biomass growth and yield are not greatly affected by the alginate:mannitol ratio, ethanol yield showed a clear maximum around a 5:8 alginate:mannitol ratio. Unfortunately, this is not the proportion present in most brown macroalgae, including the giant kelp *M. pyrifera*. In this species, a much greater amount of alginate and much smaller amount of mannitol are encountered. For the perspective of an industrial process for ethanol production from cultivated *M. pyrifera*, 2:1 alginate:mannitol is a much more representative proportion for the raw material. Given our flask results, we expected ~40% less ethanol yield for this condition than that reported by Wargacki *et al.*[17] using the optimum ratio. Considering that kelp also contains some glucose that can be metabolized by *E. coli* BAL1611, ethanol yields can be higher than these expected values based only on alginate and mannitol, which was in fact what we actually observed.

In flask fermentations (data not shown), 1-L and pilot scale fermentations (Figs 1 and 2), alginate and mannitol consumption rates and ethanol production rates are lower at the beginning of the process. This is coincident with the presence of glucose in the medium, which is totally consumed in the first 15 h in 1 L and 75 L fermentations. Alginate and mannitol consumption rates are greatly increased after glucose depletion, which suggests a glucose catabolite repression mechanism.

In *E. coli*, mannitol catabolism is mediated by the *mtlADR* operon, which codifies for a mannitol phosphotransferase transport system (mtlA) and a mannitol-1-phosphate 5-dehydrogenase (mtlD). This is the original metabolic route used by *E. coli* BAL 1611 to incorporate mannitol into the glycolytic pathways.[17] The *mtlADR* operon is repressed by the pleiotropic catabolite repressor/activator (Cra) protein, the factor for inversion stimulation (Fis) and the intrinsic operon self-repressor mtlR, and is activated by the cAMP receptor protein-cAMP complex (Crp-cAMP).[22,30–33] In the presence of glucose, binding of the abundant glycolytic intermediate fructose 1,6-bisphosphate (FBP) release Cra from the operator site of the *mtlADR* regulatory region and derepresses mtlA and mtlD expression, thus allowing mannitol catabolism during glucose consumption.[25–27] Fis is abundant during early exponential growth in rich medium, when glucose is present, but decreases afterwards,[23,28] which represses and derepresses the *mtlADR* operon in the exponential phase and during later growth stages, respectively.

In an additional mechanism, when glucose is actively transported and phosphorylated by the phosphotransferase system (PTS), the glucose-specific EIIA ($EIIA^{Glc}$) is mainly dephosphorylated, whereas the phosphorylated $P\sim EIIA^{Glc}$ form is the main enzyme form present when glucose is not available for transport. $P\sim EIIA^{Glc}$ directly associates and stimulates adenylate cyclase (CyaA) to generate cyclic AMP (cAMP), which can in turn bind Crp[24,34] to form a cAMP-Crp complex that activates the transcription of the *mtlADR* mannitol catabolism operon.[25]

According to the former mechanisms, glucose probably represses mannitol utilization during the exponential stage through a combination of Fis repression and lack of cAMP-Crp activation in the exponential phase. Cra repression is deemed inexistent in this culture conditions due to the permanent abundance of FBP caused by the influx of glucose or mannitol through the glycolytic pathways. A direct inducer exclusion mechanism similar to that described for glucose-lactose diauxie[35] cannot be ruled out, although an analogous inhibitory effect of $EIIA^{Glc}$ on mannitol transport has not been reported to the best of our knowledge.

Our results showed that growth of *E. coli* BAL1611[17] on pure mannitol is slower and on pure alginate is much slower when compared to growth on mannitol:alginate mixtures (Table 2), probably because mixtures of oxidized and reduced carbohydrates are needed to balance the intracellular NAD^+:NADH ratio. In the presence of glucose, mannitol utilization is repressed, but the alginate degrading pathways introduced in this strain are not subject to glucose repression. Since mannitol catabolism is not operative when glucose is present in the culture medium, utilization of alginate in the presence of glucose must be at least as slow and inefficient as it is when *E. coli* BAL1611[17] grows on pure alginate. Therefore, these results indicate that alginate utilization is indirectly repressed by glucose in the medium and growth must be ascribed largely to glucose catabolism in this condition. This observation can be used to develop a first strategy to metabolically optimize ethanol production in *E. coli* BAL1611[17] by removing glucose repression through engineering the mannitol utilization pathway to be independent of Fis- and Crp-mediated

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effects. Other additional strategies, such as that used by Yomano *et al.*[21] can also be predicted to be useful to enhance ethanol production in this strain.

To analyze our fermentation yields, it is interesting to take into account that maximum yields of bioethanol production from mannitol and glucan have been reported to be approximately 0.152 and 0.196 Kg ethanol Kg^{-1} dry brown algae respectively. These values are low compared to yields on glucose from sugar crops and lignocellulosic biomass (0.212 to 0.341 Kg ethanol Kg^{-1} dry feedstocks).[36] When considering production from mannitol, glucose and alginate or DEHU, the yields increase significantly to 0.281 and 0.362 Kg ethanol Kg^{-1} dry brown algae, respectively using a modified *E. coli*[17] and a modified *S. cerevisiae*[16] for fermentation. These values agree with the expected ethanol production from seaweeds according to the calculations of Kraan,[2] which assumed a carbohydrate content of 60% of dry weight and a 90% conversion ratio to ethanol. Through fermentation, 1 g of sugar can yield 0.4 g of ethanol and this will yield 0.22 Kg or 0.27 L ethanol from 1 Kg dry weight seaweed biomass, corresponding to approximately 0.05 L ethanol per Kg wet weight. However, our fermentation in a 75-L pilot-scale vessel achieved a maximum ethanol concentration of 8.87 g $liter^{-1}$ using DEHU, mannitol and glucose from *M. pyrifera*, or 0.213 kg ethanol from 1 kg dry weight. These experimental values are higher than fermentation using only mannitol and glucans and close to the yields achieved in the lignocellulosic industry.[36]

Wargacki *et al.*[17] report the production of ethanol directly by fermentation of dry *S. japonica*. From a cost perspective, it is important to avoid drying algal biomass. However, in this study it was necessary to add a three-step pre-treatment (leaching, depolymerization and saccharification) in order to obtain the monomer DEHU, which was fermented together with mannitol and glucose, thus making the process more complex and expensive. Certainly, there are critical aspects that need to be addressed and optimized in order to reduce processing times and cost and increase bacterial efficiency, to finally develop the technology for commercial success. For example, (i) avoiding the separation of depolimerization and saccharification steps by adding the complete enzyme cocktail (cellulase complex, alginate lyase and oligoalginate lyase) to leached seaweed and modifying associated process parameters, (ii) reducing the pre-treatment processing time, (iii) producing a less expensive alginate lyase to significantly reduce the cost of the process, and (iv) evaluating methods for recovery and re-utilization of enzymes. In the same line, increasing the concentration of ethanol seems to be a requisite for distillation, since otherwise an additional processing step should be added (e.g. pervaporation) to produce the final product.

At this point, it is important to emphasize that not all kelps are equal. *S. japonica* used by Wargacki *et al.*[17] have a higher mannitol:alginate ratio that coincides with the maximal ethanol production capacity of the strain. This means that the process requires to be adjusted for each different kelp. Furthermore, kelp sugars vary very significantly in time and space[18–20] and therefore any industrialization process will require to be highly dynamic to be efficient. The process presented in this study allows separating the carbohydrates from other components of the biomass. Then it allows separating the mannitol from the other carbohydrates, and this can be used to control the redox balance to control the fermentation efficiency.

As demonstrated at a pilot scale, kelp production can potentially reach ca. 200 fresh tonnes $hectare^{-1}$ $year^{-1}$.[10] With the ethanol production values obtained in this study in a 75 L fermenter, a production of 9.6 m^{-3} of ethanol $hectare^{-1}$ $year^{-1}$ can potentially be obtained. This value is lower than that for lignocellulosic biomass, corn or sugarcane,[36] due to the higher water contents of algal biomass and lower yields from dry seaweed mass. Considering a maximum calculated theoretical yield of ~51% of the polysaccharides transformed into bioethanol, ethanol production in the 75 L fermenter reached a conversion of 64% of the maximum theoretical yield. This value is still high considering the scale and complexity of the described process, but lower than the previous report at a laboratory scale of 83%.[17]

Considering the additional complexity that is needed in a more industrialized process to obtain bioethanol from kelp, the transformation of *M. pyrifera* in bioethanol is far from a reality. Nevertheless, the previously developed breakthrough technology[16,17] and the process described in this paper make the high levels of polysaccharides that can be found in kelps to become directly available for biorefining and not just solely for alginate extraction as takes place today.[37] In addition to the polysaccharides, this paper describes (Fig. 3) several additional co-products. In a first approach, salts (KCl) and protein concentrates can be obtained. These co-products, in addition to polysaccharides, require to be considered in a biorefinery concept. The challenge now is to process these co-products and develop applications. Very recently, there has been a call to move toward searching for more valuable products that can be extracted from seaweed in general,[38] such as phlorotannins.[39] This paper comes with an alternative to that view, a process that can make the main different polysaccharides and several additional co-products available. The

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industrialization of the described process will demand a joint private-university research collaboration.

Conclusions

It is technically possible to produce bioethanol from macroalgae at a larger scale with the technology described. Also several other co-products (i.e., minerals, proteins, phlorotannins) can become available and this study describes for the first time a process that is focusing on how to move into industrialization using a biorefinery approach. However, there are still several technical issues to be addressed in order to optimize productivity and yield. In particular, we have demonstrated that both heterologous alginate and native mannitol utilization pathways in *E. coli* BAL1611[17] are still largely underused and suboptimal due to a combination of mannitol operon self-repression, catabolite repression/activation, and redox balance-related mechanisms. In this sense, fermentation can be greatly enhanced by using synthetic biology tools to engineer more efficient pathways and metabolic regulation strategies in this strain.

From a processing point of view, upstream pre-treatment processes, still require further optimization, and down-processing steps, such as distillation, have not yet been developed. The production model also makes some co-products available, which will need to be considered in the next future in order to improve the global process profitability. Considering that *Macrocystis* is a highly productive organism and space for cultivation is available, it is interesting to further explore the development of a more comprehensive exploitation of this biomass. The extraction of alginate is today a reality,[37] but a more efficient use of macroalgae as carbohydrate sources seems especially important from an industrial and economic perspective.

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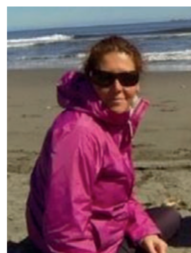

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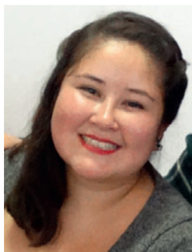

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