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Ethanol production from brown seaweed using non-conventional yeasts

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Abstract: The use of macroalgae (seaweed) as a potential source of biofuels has attracted considerable worldwide interest. Since brown algae, especially the giant kelp, grow very rapidly and contain considerable amounts of polysaccharides, coupled with low lignin content, they represent attractive candidates for bioconversion to ethanol through yeast fermentation processes. In the current study, powdered dried seaweeds (*Ascophylum nodosum* and *Laminaria digitata*) were pre-treated with dilute sulphuric acid and hydrolysed with commercially available enzymes to liberate fermentable sugars. Higher sugar concentrations were obtained from *L. digitata* compared with *A. nodosum* with glucose and rhamnose being the predominant sugars, respectively, liberated from these seaweeds. Fermentation of the resultant seaweed sugars was performed using two non-conventional yeast strains: *Scheffersomyces (Pichia) stipitis* and *Kluyveromyces marxianus* based on their abilities to utilise a wide range of sugars. Although the yields of ethanol were quite low (at around 6 g/L), macroalgal ethanol production was slightly higher using *K. marxianus* compared with *S. stipitis.* The results obtained demonstrate the feasibility of obtaining ethanol from brown algae using relatively straightforward bioprocess technology, together with non-conventional yeasts. Conversion efficiency of these non-conventional yeasts could be maximised by operating the fermentation process based on the physiological requirements of the yeasts.

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1 Introduction

There is currently renewed interest in the conversion of macroalgae (seaweeds) to bioenergy [1–3]. Since brown seaweeds, particularly the giant kelp, grow very rapidly and widely in coastal marine and estuarine environments they can potentially be exploited by different countries to produce bioethanol (fuel alcohol) and thereby reduce dependency on oil imports and increase energy security [4]. Macroalgal biomass contains storage polysaccharides which represent good substrates for microbial degradation [2], although composition does vary with the seasons [5,6]. Brown seaweeds lack lignin and contain low amounts of cellulose making it simpler, compared with terrestrial plants, to microbiologically convert them to biofuels [5-7]. Furthermore, with higher growth rates than most terrestrial biomass, coupled with concerns over feedstock supply and of security issues, seaweeds present distinct advantages [8–11]. According to Ross *et al.* [12] and Aresta *et al.* [13] marine biomass represents the most readily available resource that could be utilised for energy production on a large scale with minimal environmental impact.

Bioethanol is the most important alternative to petroleum as a transport fuel and can contribute in a positive way to reducing CO_2 emissions [4,13-15], particularly if it is produced from sustainable resources. Bioethanol production from sugar and starch faces ethical food security issues whilst the use of lignocellulosic materials such as energy crops raises the concerns over agricultural land availability together with technological challenges in pre-treatment and hydrolysis [15–18]. Marine biomass is fast growing, easily biodegradable with no lignin; and does not compete with land crops for human and animal food [7,17–19]. Therefore, brown seaweeds rich in polysaccharides such as laminarin, mannitol, alginate,

and cellulose represent sustainable bioenergy resource. Laminarin consists primarily of linear β-1,3-linked glucose residues with small amounts of β-1,6-linkages and is the main storage carbohydrate of brown algae. It can be hydrolysed by laminarinase (endo-1,3(4)-β-glucanase) to release glucose monomers. Mannitol on the other hand is a sugar alcohol, which can be converted to fructose by mannitol dehydrogenase. Laminarin and mannitol make up to 55% of dry weight of *Laminaria* spp [19–22]. Alginate and cellulose form the main structural polysaccharides in the cell walls of brown seaweeds, providing mechanical strength. Alginate is made up of linear 1,4-linked β –D mannuronic acid and α-L-guluronic acid of varying length, whilst cellulose is made up of a linear chains of several hundreds to thousands of β-1,4- linked D-glucose units [6,8]. Many macroalgal components are recalcitrant to bioconversion and pose microbiological challenges due to diversity in carbohydrate composition [9, 20]. Therefore, organisms able to utilise the different carbohydrate components are required.

The yeast, *Saccharomyces cerevisiae* is the most exploited organism for industrial ethanol production, but it cannot ferment a wide range of sugars, including pentose sugars [23]. To address this challenge, different microorganisms have been investigated such as pentose-fermenting yeasts *Pichia (Scheffersomyces) stipitis* [20,24] and *Kluyveromyces marxianus* [25].

Scheffersomyces stipitis (also known as *Pichia stipitis*) is a homothallic yeast with unique abilities to ferment pentose sugars, such as xylose. Unlike *S. cerevisiae*, which exhibits a Crabtree Effect, the onset of fermentation in *S. stipitis* is not dependent on sugar concentration but is regulated by a decrease in oxygen availability [24,26]. *S. stipitis* has a greater respiratory capacity than *S. cerevisiae* due to the presence of an alternative respiration system, which donate electrons directly to O_2 from ubiquinone branching out before the cytochrome C complex. It also possess a unique enzyme; dihydroorotate dehydrogenase, which confers on it the ability to grow anaerobically [24].

K. marxianus is a thermotolerant yeast with intrinsic abilities to ferment a wider variety of substrates than *S. cerevisiae*. *K. marxianus* has number of advantages over *S. cerevisiae*, including fermentation of various sugars at high temperatures, weak glucose repression and fermentability of inulin. However, its fermentation activity from xylose by *K. marxianus* is low compared to that of glucose [27,28].

A study by Lee *et al.* [15] using various yeast strains on *Laminaria japonica* fermentation reported that *S. stipitis* produced six times more ethanol than *S. cerevisiae*. *K. marxianus* can ferment sugars at temperatures up to 47°C and has a strong affinity for xylose, which is a component of reducing sugars in seaweed hydrolysates [16,20,28].

Studies [20–22] have shown that the process of releasing sugars from algal biomass can be enhanced by the combination of acid hydrolysis and treatment with a cocktail of different enzymes. To address the heterogeneous nature of algal carbohydrates, multi-enzyme preparations containing mainly cellulases and cellobiases have been advocated [20,22,29,30].

In this study, we aimed to determine the abilities of non-conventional yeasts to ferment brown seaweed sugars obtained following acid hydrolysis and enzymolysis.

2 Materials and methods

2.1 Seaweed substrates

The brown seaweeds, *Ascophyllum nodosum* and *Laminaria digitata*, were harvested from the Broughty Ferry beach, Dundee, Scotland 56° 28′ 1.85″N, 2°52′ 11.68″W, in March, 2010 after collection, the seaweeds were placed on tin foil covered trays and dried at 80°C for 48 h. The dried seaweeds were powdered using a hammer mill (ZM-100, Retsch GmbH, Germany), to pass through 0.5 mm mesh sieve before use. Seaweed powder was stored in sealed containers at room temperature until used.

2.2 Analysis of algal biomass composition

Research has shown that algal biomass composition varies depending on the time and season of harvest [3,12,31,32] and this in turn determines the productivity of any bioconversion processes utilised for energy production. For instance, the amounts of laminarin and mannitol present in *L. digitata* were very low around March and reach its peak between June/July [6]. Similar trends were found in *A. nodosum* [33]. The protein content was analysed using the Coomassie (Bradford) protein assay. Proteins were extracted from seaweed powder using 2M NaOH in a proportion of 10% seaweed powder and 90% NaOH, incubated at 65°C at 150 RPM for 60 minutes. Samples were centrifuged and the supernatant used for the protein assay. The amount of protein was expressed as per cent of dry mass of the seaweeds. Carbohydrate content was determined following hydrolysis using the method described the NREL Chemical and Testing procedure [34]. Ash content, total and volatile solids were determined according to the standardised methods by oven-drying at 105°C and incinerating at 550°C as previously described [34].

2.2.1 Combined acid hydrolysis and enzyme digestion

After the raw seaweeds were dried and ground into powder, they were subjected to acid and enzyme hydrolysis to release sugars prior to fermentation. Seaweed powder (10%(w/v)) was hydrolysed with 0.2M H_2SO_4 by autoclaving at 121°C for 20 minutes. Subsequently, pH was adjusted to 5.5 using 10M NaOH. The solid stem was sieved and discarded before a cocktail of different commercial enzymes (cellulase, β-glucosidase (cellobiase), enzyme complex, xylanase and hemicellulase) present in the Novozymes Biomass Kit (Novozymes, Denmark) were added according to manufacturer's instructions (Table 1). β-Glucosidase hydrolyses cellobiose which is an inhibitor of cellulase activity [17]. Enzymatic digestion was performed by incubating acid-hydrolysed seaweed samples at 50°C with agitation at 150 rpm for 18 h [35].

2.2.2 Chemical analyses

Total reducing sugar concentration was determined by a dinitrosalicylic acid (DNS) method while the concentration of glucose and other monomeric sugars were analysed by high performance liquid chromatography (HPLC) following saccharification with acid and enzymes [14,15]

2.2.3 Preparation of yeast seed cultures and fermentation

Malt extract (20 g/l) broth was inoculated with two yeasts *Schefferomyces (Pichia) stipitis* (NCYC 1542) and *Kluyveromyces marxianus* (NCYC 1424) obtained from the National Collection of Yeast Cultures (NCYC), Norwich, UK and generously donated by Prof Graeme Walker (Abertay University, Dundee, UK) and incubated at 30°C with shaking at 150 rpm for 18 h to produce seed cultures for fermentation. Fermentation was conducted in a modification of the method previously described [11], fermenting samples in 150 ml Pyrex bottles at 30°C with shaking at 100 rpm. Samples (6 ml) were collected after 3, 24, 30, 96, 102, 120, 126, 144 and 150 h of fermentation. Culture supernatants were analysed to determine the reducing sugar, specific sugar and ethanol concentrations. Yeast cell counts were done with a haemocytometer (Neubauer) to monitor yeast cell growth during the fermentation process [35]. Specific sugars were analysed by HPLC using a BioRad Aminex HPX-87P column with a refractive index detector. The amount of sugar was quantified by comparing peaks produced with those of standard sugars with known concentrations. Ethanol content was determined using a gas chromatography mass spectrometer (GCMS-QP2010 Shimadzu fitted with a ZB-23 column) and was expressed in percent (v/v) using 1% propanol as internal standard [35].

2.2.4 Statistical analysis

Experimental error was determined for replicate assays and expressed in standard deviation. The significance of differences in reducing sugar yields was determined by one-way analysis of variance (ANOVA). Statistical significant interactions were further analysed using post hoc test (Tukey) at 95% confidence interval. Differences between species and across treatments were also determined. All statistical analyses were performed using Minitab Statistical Software version 17.0.

3 Results and discussion

3.1 Compositional analysis of experimental seaweeds

One of the most important parameters affecting productivity of seaweeds is the composition of the biomass which exhibits significant seasonal variations [3,12,36]. Analysis of seaweed harvested in Scotland in March has

Table 1. Enzyme parameters used in this study (information from Novozymes A/S).

Enzyme	Activity	pH	Temperature (°C)	Dose (%w/w seaweed)*
Cellulase complex	700EGU[a] g^{-1}	4.5-6.5	45-60	6.0
β-Glucosidase	250CbU[b] g^{-1}	2.5-6.5	45-70	0.6
Multi-complex	500FXU[c] g^{-1}	4.0-6.0	40-65	0.4
Xylanase	500FXU[c] g^{-1}	4.5-6.0	35-55	0.5
Hemicellulose	750FXU g^{-1}	5.0-8.0	45-70	0.4

*Dose values were calculated based on 10% seaweeds substrate. [a]Endoglucanase units [b] β-Glucanase units [c]Fungal xynalase units. 1 unit of enzyme activity was defined as the amount of the respective enzyme required for the release of 1 µmol of glucose/min.

shown that carbohydrates and protein contents are low while inhibitory materials such as polyphenols, ash and metals are high [2]. This will therefore impact significantly on the overall ethanol productivity following fermentation of seaweed sugars.

One-way ANOVA and Tukey's pairwise analysis of the compositional analysis of brown seaweeds shows that total carbohydrates in *L. digitata* (64.5%) was significantly higher than *A. nodosum* (57.8%) ($p<0.003$). These findings are lower than total carbohydrate levels, which have been reported as high as 84% [3]. Statistical comparisons of brown seaweed protein contents revealed that although generally low, the protein content of *L. digitata* was significantly higher than *A. nodosum* ($p<0.032$). The finding that both carbohydrate and protein contents of were higher in *L. digitata* suggests that this species would yield a much more favourable hydrolysate than *A. nodosum*. There were no significant differences in ash ($p>0.5$) and volatile solids ($p>0.682$) composition between the two seaweeds tested (Table 2).

3.2 Combined acid and enzymatic hydrolysis of seaweed polysaccharides

Seaweeds are rich sources of carbohydrates which can be converted to energy during biological processes [4], including fermentation for the production of bioethanol. Glucose, the main feedstock for ethanol production, is limited in seaweeds. It is therefore important that other hexose and pentose sugars present in the biomass are efficiently utilised to achieve high ethanol production [22]. Our main objective was to determine the specific ability of non-conventional yeasts to ferment a range of sugars derived from brown seaweeds in other to achieve efficient fermentation and ethanol production.

The concentrations of both total reducing sugars and specific sugars produced after combined acid and enzyme hydrolysis was determined to assess sugar utilisation by the yeasts *S. stipitis* and *K. marxianus*. One-way ANOVA highlighted that sugar liberation from the two seaweeds was significantly different ($p<0.015$). Enzymolysis of acid-treated *L. digitata* resulted in a significant ($p<0.0001$) 190% increase in reducing sugar production (to 29.3 g/l). A significant ($p<0.002$) increase (25%) in reducing sugar (to 15.6 g/l) was also recorded in *A. nodosum* hydrolysates (Fig. 1).

Tukey's post hoc comparison of sugars obtained from the two seaweeds hydrolysates shows that significantly higher levels ($p<0.0001$) were produced from *L. digitata* compared with *A. nodosum* (81% more sugars). Lower sugar levels obtained from *A. nodosum* may be due to enzymatic inhibition by the high concentration of polyphenols in this seaweed species [3,33]. According to Meon *et al.* [37], this is the most important limiting factor for the biological conversion of seaweeds. Although polyphenols are present in all brown algae, they are more prevalent in *A. nodosum* (up to 14%) than in *L. digitata* at around 2% [33]. Choi *et al.* [38] used a combination of seaweeds (*Laminaria japonica* and *Undaria pinnatifida*) in a 1:1 ratio and found 27.2 g/l of reducing sugar after enzymatic hydrolysis, representing a saccharification yield of over 80%. This is comparable with the 29.3 g/l of reducing sugar produced from *L. digitata* in the current study with around 87% saccharification yield, while only 46% was achieved for *A. nodosum*. Using a high temperature liquefying system combined with high pressure, Yeon *et al.* [39] efficiently hydrolysed the seaweed *Sargassum sagamianum* until a reducing sugar concentration of 30 g/l was achieved. This is similar to the sugar yields from *L. digitata* in the current study.

Fig 2 shows the specific sugars obtained following enzyme hydrolysis of acid-treated seaweeds included: glucose, MGX, (mannose, galactose and xylose measured together), rhamnose and fucose. Fig 2b shows that glucose was the predominant sugar released from *L. digitata* (63% of total sugars), while rhamnose was the main sugar obtained from *A. nodosum* (55% of total sugars). These

Table 2: Compositional analysis of seaweeds used in this study.

Component	*A. nodosum*	*L. digitata*
Total Carbohydrate (alginate, laminarin, mannitol and cellulose) (%)	57.84	64.47
Protein (%)	2.12	2.64
Ash (%)	19.51	19.63
Others[a] (%)	20.52	13.12
VS (%)	80.49	80.33
TS (% wet solid)[b]	24.7	26.4

[a]Other components of algae such as lipid were determined by the difference in 100% determined components. [b]Total solids in seaweeds were determined by drying wet seaweeds at 105°C for 24 h.

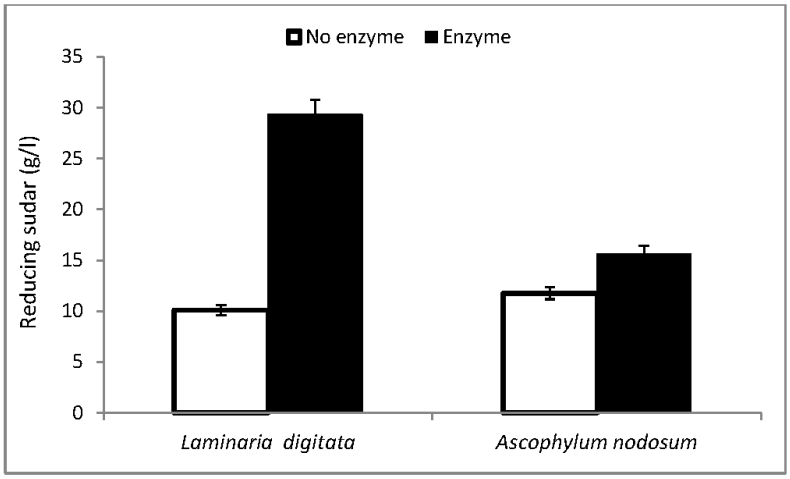

Fig.1. Reducing sugar concentrations before and after enzymatic hydrolysis of seaweeds. Seaweed samples were subjected to acid (0.2 M H_2SO_4) and heat (121°C for 1 h) treatment and the subsequent hydrolysates were subsequently subjected to enzymatic hydrolysis using a cocktail of commercial enzymes to release fermentable sugars. Error bars represent standard error from the mean values of the duplicate samples.

results indicate the greater suitability of *L. digitata* for bioconversion. In *A. nodosum*, the enzymatic liberation of sugars, and glucose in particular, may be deleteriously affected by high levels of polyphenolic compounds in this seaweed [8,14].

3.3 Ethanol fermentation of *L. digitata* and A. nodosum using non-conventional yeasts: *S. stipitis* and *K. marxianus*

Sugars other than glucose are produced from acid and enzymatic hydrolysis of seaweeds. Efficient production of ethanol from seaweed will require complete fermentation of all available sugars, so we tested the abilities of two non-conventional yeast strains to convert hydrolysates obtained from *Laminaria* and *Ascophyllum* brown algae. We conducted 150 h fermentations of seaweed hydrolysates with *S. stipitis* and *K. marxianus* with shaking at 30°C.

Results obtained from the fermentation of *L. digitata* hydrolysates using *S. stipitis* showed a steady increase in both yeast growth and ethanol production over the course of the 150 h fermentation. Fig. 3a shows that peak ethanol formation (5.8g/L) occurring after 144 h fermentation, which is lower than 9-10g/l ethanol produced from *Sargassum sagamianum* after 200 h of repeated batch fermentation by Yeon *et al.* [39]. It is, however, higher than 3.58 g/l (0.45 %v/v) ethanol produced from *Saccharina latissima* which was fermented with *S. cerevisiae* [19].

Increased ethanol production was commensurate with sugar consumption by the yeasts resulting in their rapid growth (Fig. 3b). *S. stipitis* completely utilised all the available mannose, galactose, xylose and fucose, whilst 84% and 87% consumption of glucose and rhamnose, respectively, occurring. Fermentation of *L. digitata* hydrolysates show extensive utilisation of the sugars by *S. stipitis*, especially pentose sugars (Fig. 3b), consistent with previous reports [35,39] on the ability of this yeast to utilise pentoses. Fermentations of *L. digitata* hydrolysates using *K. marxianus* resulted in rapid yeast growth and ethanol production within the first 24 h with maximum ethanol production of 6 g/L being observed after 30 h (Fig. 4a), and this corresponded with almost 80% of glucose consumption (Fig. 4b). The percentage reduction for both fucose and MGX was 100%, glucose 80% and rhamnose 44%, at the end of the 150-hour fermentation process (Fig. 4b).

Although relatively high sugar conversion efficiencies in relation to available sugar utilisation were observed in both yeasts from *L. digitata* hydrolysate, the two yeasts behaved differently during the fermentation process. While *K. marxianus* produced its maximum amount of ethanol in the first 30 h of fermentation, *S. stipitis* took 144 h to produce about the same amount of ethanol. *K. marxianus* therefore metabolises seaweed sugars faster than *S. stipitis*, especially in glucose-rich hydrolysates. Both yeasts completely utilised all the MGX (mannose, galactose and xylose) present in the hydrolysate (Fig. 3b and 4b), but fucose was metabolised more fully with *S. stipitis*. Although *K. marxianus* possesses higher growth rates during fermentation of *L. digitata* (Fig. 4a), it was only able to extensively utilise few of the seaweed-derived sugars [30]. For example, *K. marxianus* consistently

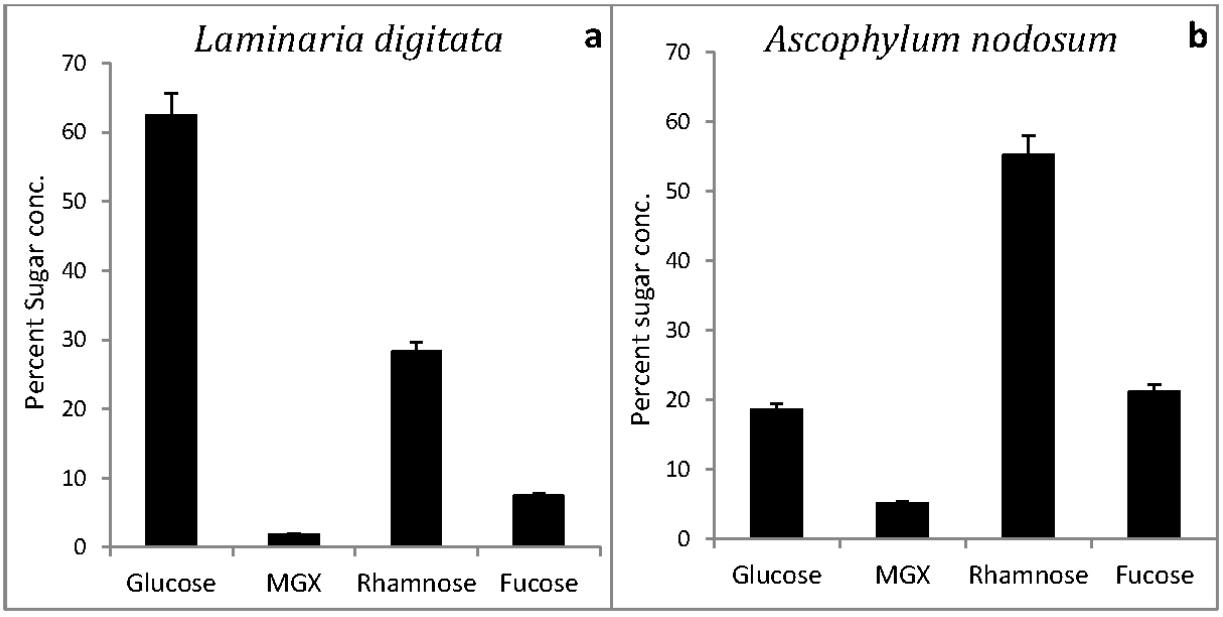

Fig. 2. Seaweed sugar concentrations obtained by enzymatic hydrolysis (a) *L. digitata* (b) *A. nodosum* analysed with HPLC. MGX (mannose, galactose and xylose measured together). Error bars represent standard error from the mean values of the duplicate samples.

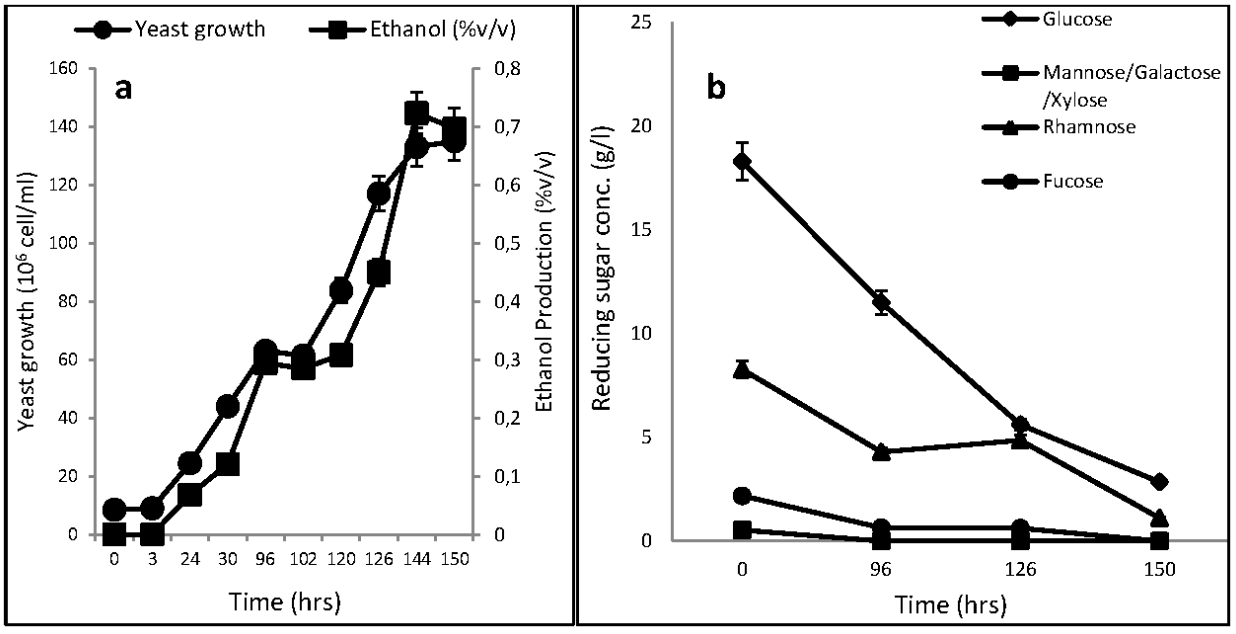

Fig. 3 Fermentation characteristics of *L. digitata* hydroysates with *S. stipitis*(a) Yeast growth and ethanol production (b) Specific sugar fermentation and utilisation in cultures containing *L. digitata* inoculated with *S. stipitis*. Viable yeast cell counting was performed with a haemocytometer. Ethanol concentration was measured by HPLC. MGX= mannose, galactose and xylose measured together. Error bars represent standard error from the mean values of the duplicate samples.

showed a high affinity for glucose and MGX but poor utilisation of rhamnose and fucose [29]. Table 3 shows a summary of yeasts preferences and affinity towards *L. digitata* sugars.

One-way ANOVA and Tukey's pairwise comparison of ethanol produced from *L. digitata* using *P. stipitis* and *K. marxianus* shows there was no significant difference in the concentration of ethanol produced by the 2 yeast

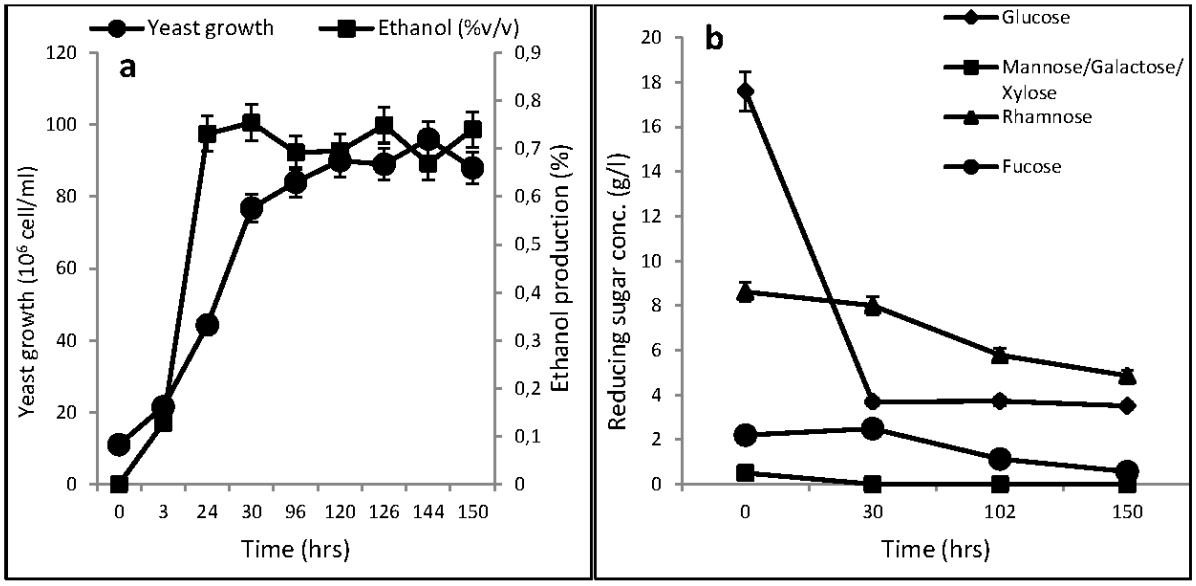

Fig. 4. Fermentation characteristics of *L. digitata* hydrolysates with *K.marxianus* (a) Yeast growth and ethanol production, (b) Specific sugar fermentation and utilisation in the cultures containing *L. digitata* inoculated with *K.marxianus*. Viable yeast cell count was performed with haemocytometer. Ethanol concentration was measured by HPLC. Error bars represent standard error from the mean values of the duplicate samples.

strains ($p > 0.05$). This confirms the suitability of both yeasts for the fermentation of pentose and hexose sugars from seaweeds (Table 4).

Regarding fermentation of *A. nodosum* hydrolysates, these were carried out in a similar manner to the *L. digitata* fermentations at 30°C for a period of 150 hours using the yeasts *S. stipitis* and *K. marxianus*. For *S. stipitis*, peak ethanol production of about 0.3% (v/v) (2.4g/l) was observed at 144 hours of fermentation (Fig. 5a). Although this is a low yield of ethanol, the available rhamnose, mannose, galactose and xylose was completely consumed while there was a respective 94% and 92% utilization of

Table 3. Yeast sugar consumption during fermentation of brown seaweeds.

	L. digitata		*A. nodosum*	
Reducing sugar consumption	*S. stipitis*	*K. marxianus*	*S. stipitis*	*K. marxianus*
Glucose (%)	84	80	94	100
Mannose/Galactose/Xylose (%)	100	100	100	100
Rhamnose (%)	87	**44**	100	**29**
Fucose (%)	100	100	92	**58**

Specific sugar consumption below 60% listed in **bold** occurred in only *K. marxianus* cultures.

Table 4: Fermentation parameters of brown seaweeds using two non-conventional yeasts.

	L. digitata		*A. nodosum*	
Fermentation parameters	*S. stipitis*	*K. marxianus*	*S. stipitis*	*K. marxianus*
Yeast cells (10^6 cells/ml)	133	92	600	59
Total sugar (start)	29.3	29.3	15.45	15.45
Total sugar (end)	3.96	8.94	0.45	7.48
Total sugar consumed (g/l)	25.33	20.1	15.19	7.97
Sugar consumption (%)	86	69	97	52
Ethanol yield (g/L)	5.8	6.0	2.4	0.7
Theoretical Ethanol yield (g/L)	12.92	10.25	7.75	4.06
Efficiency (%)	45	59	31	17

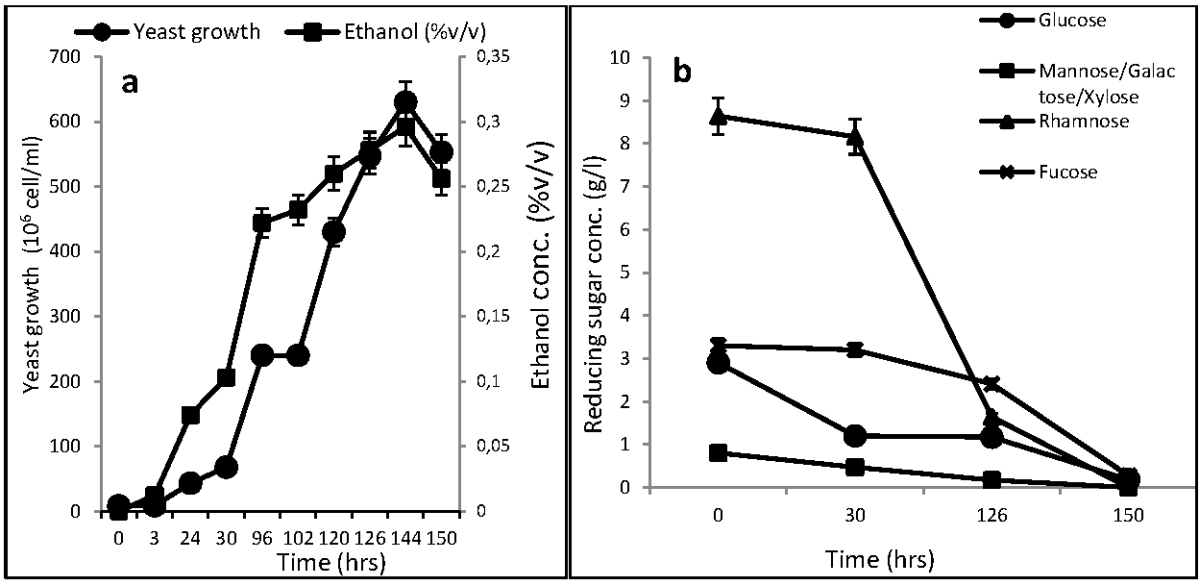

Fig. 5. Fermentation characteristics of *A. nodosum* hydrolysates with *S.stipitis* (a) Yeast' growth and ethanol production, (b) Specific sugar fermentation and utilisation in the cultures containing *A. nodosum* inoculated with *S. stipitis*. Viable yeast cell count was performed with haemocytometer. Ethanol concentration was measured by HPLC. Error bars represent standard error from the mean values of the duplicate samples.

glucose and fucose utilised by *S. stipitis* by the end of the 150 h fermentation process (Fig. 5b). For *K. marxianus*, peak ethanol production of 0.088% v/v (0.7 g/L) was produced at 96 h of fermentation (Fig. 6a) while the highest yeast growth (59 x 10^6 cells/ml) was recorded at 144 h of fermentation. The lower yields of ethanol (Fig. 6b) obtained are reflected in that fact that only about 52% of the total available sugar was used up during the fermentation process, with much of rhamnose (which forms the bulk of the reducing sugars), remaining unfermented by this yeast.

Fermentation of *A. nodosum* by both yeasts resulted in low yields of ethanol (Fig. 5a and 6a), due to low concentrations of sugars obtained following acid and enzyme hydrolysis of this seaweed. However, both yeasts showed high affinity for glucose and MGX [22] which they almost completely utilised. *S. stipitis* exhibited a wider range of sugar utilisation compared with *K. marxianus* as it utilised all the available rhamnose and most of the fucose present in the hydrolysate (Fig. 5b). Generally, sugar consumption was higher by *S. stipitis* (over 97%) irrespective of substrate than *K. marxianus* (about 52%). Rouhollah *et al.* [30] has previously reported the broader substrate specificity in *S. stipitis* compared with *K. marxianus* and *S. cerevisiae*. Our study (Table 4) has shown, using one-way ANOVA and Tukey's pairwise comparison, that *S. stipitis* grew much faster and produced over 3 times more ethanol than *K. marxianus* when *A. nodosum* was fermented.

The results of the fermentation of both seaweeds reveal higher ethanol yield from *L. digitata* than in *A. nodosum*, most likely due to higher sugar concentrations in the hydrolysate of the former. In fact, the amount of glucose released from *L. digitata* after enzyme hydrolysis was over 6 times higher than that of *A. nodosum* (Fig. 1 and 2). This accounted for most of the fermentable sugar available for fermentation as similar amounts of minor sugars (rhamnose, MGX and fucose) were produced from both seaweeds. Therefore, the relative abundance of glucose in *L. digitata* compared to *A. nodosum* may account for the higher ethanol yield obtained from *L. digitata* with both yeasts since glucose remains the best substrate for ethanol production (Fig. 7).

Various methods have been exploited to enhance ethanol production from biomass, including seaweeds. For example, Yanagisawa *et al.* [21] utilised a combination of approaches including washing of the seaweeds prior to drying and grinding. This was followed by saccharification involving acid and enzyme hydrolyses; a 2-stage saccharification procedure in which the hydrolysate from the primary saccharification was used as the hydrolysing liquid for a secondary saccharification after the removal of residue. Glucose concentrations of 43 g/L and 67.2 g/L were obtained from *Ulva pertusa* and *Alaria crassifolia*

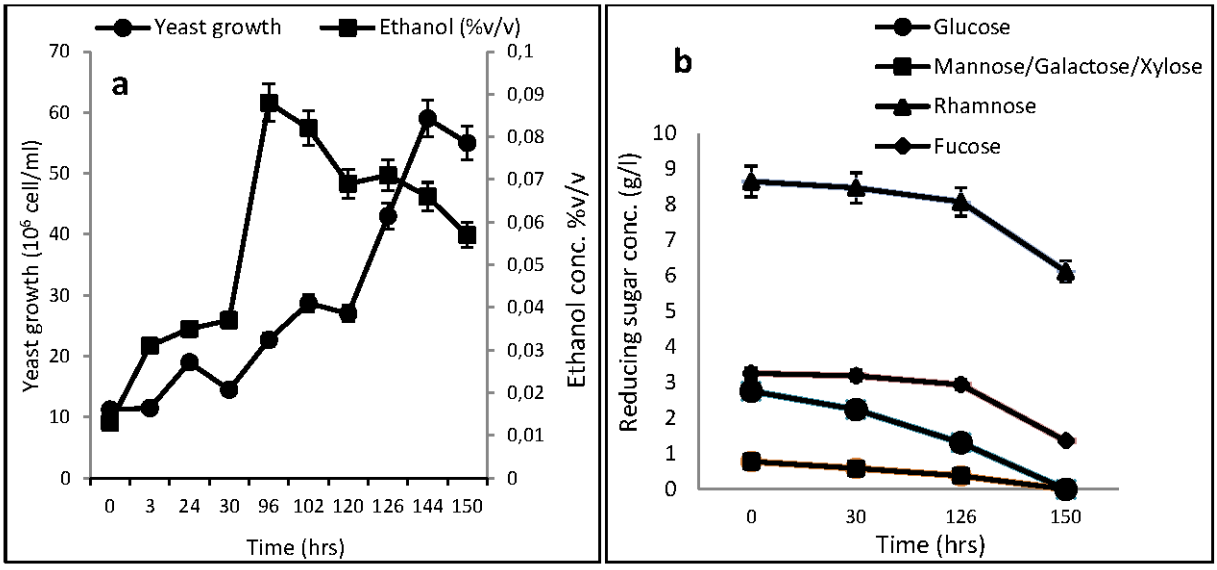

Fig. 6. Fermentation characteristics of *A. nodosum* hydrolysates with *K.*marxianus (a) Yeast growth and ethanol production, (b) Specific sugar fermentation and utilisation in the cultures containing *A. nodosum* inoculated with *K. marxianus*. Viable yeast cell count was performed with haemocytometer. Ethanol concentration was measured by HPLC. MGX = mannose, galactose and xylose measured together. Error bars represent standard error from the mean values of the duplicate samples.

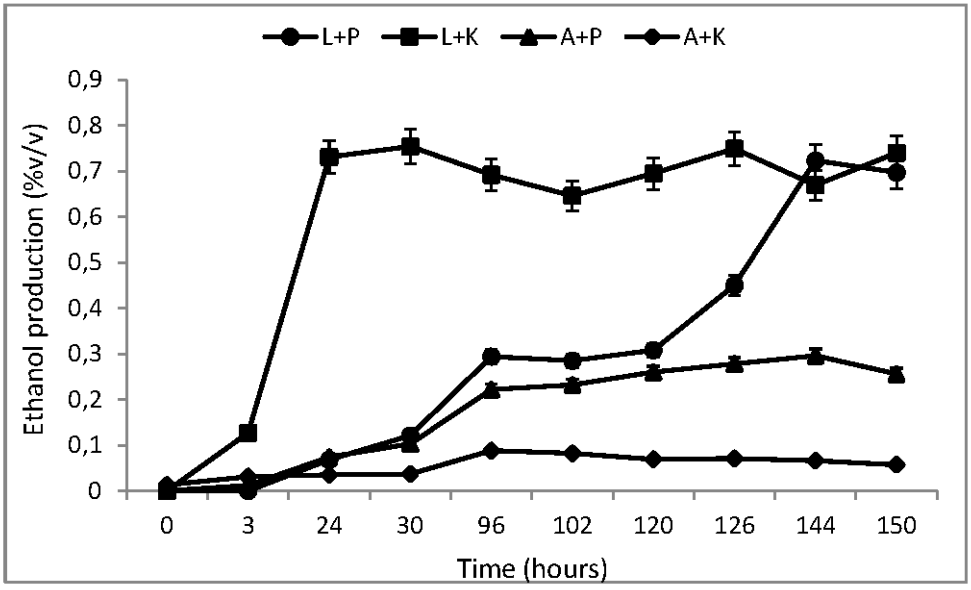

Fig. 7. Ethanol production during seaweed fermentation using two different yeasts. Ethanol concentration was measured by HPLC. (L+P = *L. digitata* using *S. stipitis*, L+K = *L. digitata* using *K. marxianus*, A+P = *A. nodosum* using *S.* stipitis, A+K = *A. nodosum* using *K. marxianus*). Error bars represent standard error from the mean values of the duplicate samples.

which upon complete fermentation produced 27.5 g/l and 25.6 g/L of ethanol, respectively [29]. Similarly, Kim *et al.* [40] investigated the effects of pre-treating algae (*L. japonica*) for ethanol fermentation using ethanologenic *E. coli* and reported ethanol production of 23-29 g/L, compared with only 7-9 g/L when using *S. cerevisiae*. In fact, *S. cerevisiae* appears ill suited for algal biomass fermentation. Trivedi *et al.* [41] attempted to enhance the efficiency of marine biomass bioethanol fermentation by exploiting the cellulase production potential of a marine fungus *Cladosporium sphaerospermum* through a solid

state fermentation of cellulose-rich *Ulva fasciata*. Ethanol production of 0.47 g/g reducing sugar and conversion efficiency of 93.81 %, was obtained, which is the highest reported in literature for marine biomass. An earlier report [42] involving the optimization of enzyme hydrolysis using commercial cellulase resulted in ethanol production of 0.45 g/g sugar with an efficiency of 88.2 %.

In the current study, although the fermentation of *L. digitata* hydrolysate with *K. marxianus* produced peak ethanol faster (Fig. 7), fermentation of *L. digitata* using *S. stipitis* would a be better option for commercial utilisation of seaweeds due to the wider range of sugars metabolised by this yeast. *L. digitata* is considered the better substrate of the two seaweeds owing to substantially higher release of sugars, especially glucose, which is the most important substrate for ethanol fermentation [4,9,35].

However, considering glucose and general sugar utilization by the two yeasts, the fermentation process appears inefficient. For example, the lack of complete utilization of glucose suggests some form of inhibition which may be due to small molecular weight compounds such as 5-hydroxy-methyl-furfural and levulinic acid generated during acid hydrolysis of the seaweeds. These compounds have been shown to have profound impact on the efficiency of ethanol production [20]. Moreover, other inhibitory materials present in the seaweeds feedstock (such as polyphenols, metals and salt) might also have contributed to the suppression of yeast growth and metabolism.

Furthermore, the generally low ethanol production efficiency (Table 4) may be due to specific fermentation conditions adopted. For example, when *S. stipitis* is used, efficient ethanol production from reducing sugar fermentation, depends on oxygen availability, with ethanol production occurring only when oxygen is limiting [24]. The fact that oxygen was not limiting in the present study could have resulted in lowered sugar consumption and reduced yeast fermentation performance.

4 Conclusion

This work has shown the potential of bioethanol production from selected brown seaweeds using appropriate yeast species for the fermentations. Although we found generally low ethanol yields, this may be due to the time of harvest of seaweeds utilised in this study as well as the fermentation conditions employed. It is therefore advisable for future bioethanol production from seaweeds in Northern Europe to consider harvesting around July and August for optimum bioethanol yield and also to optimise fermentation conditions in relation to the physiological requirements of the yeasts employed. The ethanol production potential of the two non-conventional yeasts employed in this study was highlighted with promising results from *S. stipitis* fermentation of *A. nodosum* hydrolysates. If enzymatic inhibition due to the high concentration of polyphenolic compounds present in *A. nodosum* could be alleviated, then potentially much higher yields of ethanol may be obtained. In addition, to be economically viable, marine biomass bioconversion technologies would require that higher value chemical commodities are co-produced during the process. The current interest in renewable biofuels resulting from environmental, political and economic pressures will continue to drive research into various ways of optimising bioethanol production from various sustainable feedstocks, including algal biomass.

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