Lipid production through the single-step microwave hydrolysis of macroalgae using the oleaginous yeast *Metschnikowia pulcherrima*

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Abstract

Macroalgae (seaweeds) represent an emerging resource for food and the production of commodity and specialty chemicals. In this study, a single-step microwave process was used to depolymerise a range of macroalgae native to the United Kingdom, producing a growth medium suitable for microbial fermentation. The medium contained a range of mono- and polysaccharides as well as macro- and micronutrients that could be metabolised by the oleaginous yeast *Metschnikowia pulcherrima*. Among twelve macroalgae species, the brown seaweeds exhibited the highest fermentation potential, especially the kelp *Saccharina latissima*. Applying a portfolio of ten native *M. pulcherrima* strains, yeast growth kinetics, as well as production of lipids and 2-phenylethanol were examined, with productivity and growth rate being strain dependent. On the 2 L scale, 6.9 g L^{-1} yeast biomass, a yield of 0.15 g L^{-1} with respect to supplied macroalgae, containing 37.2 % (w/w) lipid was achieved through utilisation of the proteins, mono- and polysaccharides from *S. latissima*, with no additional enzymes. In addition, the yeast degraded a range of fermentation inhibitors released upon microwave processing at high temperatures and long holding times. As macroalgae can be cultured to food grade, this system offers a novel, potentially low-cost route to edible microbial oils as well as a feedstock for oleochemicals.

Keywords: Microbial lipids, *Metschnikowia pulcherrima*, macroalgae, marine biorefinery, microwave treatment, *Saccharina latissima*

Introduction

Microbial lipids offer a credible feedstock for advanced biofuel production to reduce the impact of fossil fuels as well as a potentially more sustainable source of edible oil. The concept of a marine biorefinery includes the utilisation of marine plants for the provision of food, proteins, minerals, commodity and fine chemicals, biofuels and/or energy. Due to their fast growth, high protein content, high diversity of carbohydrates and low lignin content, macroalgae (seaweeds) are of particular interest for a marine biorefinery[1–3]. Macroalgae are generally classified as brown (*Phaeophyta*), green (*Chlorophyta*) or red (*Rhodophyta*) type relating to their photosynthetic pigments, usually perceptible in the phenotype.

In 2014, wild and cultivated macroalgae harvesting more than doubled to 28.4 million tonnes from 10.4 million in 2000.[4] Global production is overwhelmingly dominated by Asia (96.6 %), with America (1.7 %), Europe (1 %), Africa (0.6 %) and Oceania (0.1 %) accounting for the remaining continental production figures.[4,5] Production in America and Europe is dominated by wild harvesting, whereas the main method for production in Africa and Asia is through formal cultivation.[4] In the four years leading up to 2014, global red and brown (the predominant type produced in Europe) macroalgae production has increased by 84 % and 47 %, respectively, whilst green macroalgae production decreased by 30 %.[5]

Currently, the most common use of macroalgae is for food production. As a fuel or biorefinery feedstock macroalgae has the potential to compete with second generation lignocellulosic biomass such as crop residues or dedicated energy crops. Compared to terrestrial crops, marine plants do not require arable land, freshwater or fertilizer,[6] and furthermore convert sunlight more efficiently,[7] inducing their potential for carbon sequestration.[8] For cultivation in northern Europe towards bioethanol and biogas production, brown macroalgae *Laminaria digitata* yields associated greenhouse gas emissions of 45 kg

CO_2-equiv. per tonne of macroalgae produced.[9] This can be compared to cultivation of wheat straw (54 to 236 kg CO_2-equiv. per tonne[10]), miscanthus (51 kg CO_2-equiv. per tonne[11]) and SRC willow (138 kg CO_2-equiv. per tonne[11]). Environmental and techno-economic credentials for macroalgae cultivation can be further improved by integrating production into other established aquaculture activity. The potential for macroalgae as a major source for speciality and commodity products is significant; however, in the UK a bottleneck to expanding macroalgae biorefining activity is the lack of systematic wild feedstock appraisal, demonstration cultivation sites and pilot-scale downstream technology assessment.[5]

Current research has developed techniques to enhance macroalgae valorisation through collaterally extracting proteins[1] and/or utilising other available saccharides, for instance through purification[12] or microbial processing.[13–18] Whilst the high carbohydrate, sulphur and nitrogen content make macroalgae a promising feedstock for microbial fermentation within a biorefinery setting, pretreatment and fermentation within such as process should be cost efficient and sustainable, utilising a microbe with versatile characteristics and ideally yield high-value products to enhance the feasibility of such a process. Recent research for microbial macroalgae utilisation focussed on ethanol,[17–20] butanol[1,21] and biogas[15,16] production, with pretreatment often taking place via acid and/or enzymatic hydrolysis.

Depolymerisation via time- and energy-efficient[22] microwave processing has been employed successfully for a range of lignocellulosic feedstocks.[23,24] Considering the lack of lignin and the previous successful recovery of macroalgae constituents through microwave-assisted extraction,[18,25] this technology offers a potentially viable alternative to produce an inexpensive microbial growth medium from macroalgae.[18] However, the thermochemical treatment of biomass generally produces mainly oligosaccharides and a range of inhibitors. To this end, we recently reported on the oleaginous yeast *Metschnikowia pulcherrima* that can

metabolise a range of carbon sources including oligosaccharides and has a high inhibitor tolerance,[23,26] though the growth on macroalgae hydrolysate is yet to be assessed. This yeast demonstrates excellent suitability for industrial biotechnology since it produces a range of valuable metabolites, most prominently microbial lipids and 2-phenylethanol (2-PE), and it has the ability to outcompete other microbes through secretion of antimicrobial agents and iron sequestration.[26,27] Whilst there are a few reports of producing microbial lipids from macroalgae recently,[13,14,28,29] a system coupling low-energy microwave depolymerisation with *M. pulcherrima* offers additional benefits for a potentially more economic route to microbial lipid production.

Experimental

Chemicals were purchased from Sigma-Aldrich and Fisher Scientific, for biological culturing suitable for cell culture and for standards analytical grade. Centrifugations were performed at 1,680 × g and room temperature for 10 min (Rotina 380, Hettich) and lyophilisation at -40 °C and 60 mbar overnight (Modulyo, Thermo Savant). Fermentation vessels were sterilised with 70 % (v/v) ethanol, media freshly prepared and actions involving biological reagents handled aseptically.

Macroalgae preparation and hydrolysis

Twelve different macroalgae species were harvested from the South West UK coast in August and *Saccharina latissima* (SL, formerly *Laminaria saccharina*) additionally in May, washed, chopped to around 100 mm long pieces, flash frozen in liquid nitrogen, lyophilised and ground using a pestle and mortar (Table 1). The dried macroalgae was then suspended in deionised water at 5 % (w/v), 40 mL placed in 75 mL PTFE vials (CEM Corporation) equipped with a PTFE magnetic stirrer bar, and digested in a MARS 6 microwave digestion system (CEM Corporation) with 1,800 W. Microwave conditions ranged from 150 to 210 °C final temperature, 5 to 15 min ramping time and 0 to 10 min holding time (hereinafter as ramping+holding time). One macroalgae hydrolysate (SL, May, 190 °C, 5+0 min) was prepared as 50 mM L-(+)-tartaric acid solution (pKa 4.34, 25 °C) (pH 4 with NaOH). Another microwave hydrolysate (SL, May, 190 °C, 5+0 min) was subjected to enzymatic hydrolysis according to published procedure with slight modification.[30] Briefly, the enzyme preparation CellicCTec2 (Sigma-Aldrich) was added to the microwave hydrolysate without buffer (section S2) at 7 mg protein/g dried macroalgae and a solution of 20 mL incubated at 50 °C and 200 rpm in a shaking incubator (SI500, Stuart) for 20 h. Prior to fermentation, remaining

126 solids were removed from any hydrolysate by centrifugation to avoid interference with cell
127 growth assessment.

128

129 **Table 1.** Investigated macroalgae species, their type and notation. Macroalgae were harvested from
130 the South West UK coast in August, and *S. latissima* additionally in May.

Notation	Scientific name	Type
UL	*Ulva lactuca*	green
UI	*Ulva intestinalis*	green
JR	*Jania rubens*	red
PL	*Porphyra leucosticta*	red
DC	*Dilsea carnosa*	red
SC	*Soliera chordalis*	red
SS	*Stypocaulon scoparium*	brown
SM	*Sargassum muticum*	brown
AN	*Ascophyllum nodosum*	brown
HS	*Halidrys siliquosa*	brown
FS	*Fucus serratus*	brown
SL	*Saccharina latissima*	brown

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132

133 **Media, strains and culture conditions**

134 Ten *M. pulcherrima* strains were used: locally (Bath, UK) isolated from fruit and flowers
135 (section S1) ICS 1, 46 & 48; DH 3, 5, 10, 18 & 21; and commercially available NCYC 2580
136 & 3047 (National Collection of Yeast Cultures, Norfolk, UK). Strains were kept at -80 °C as
137 20 % (v/v) glycerol stocks, from which agar plates (YMD: yeast extract 10 g L^{-1}; malt extract
138 20 g L^{-1}; glucose 20 g L^{-1}; agar 15 g L^{-1}, pH 5; in deionised water) were inoculated,
139 incubated at 20 °C for 4 days, then kept at 4 °C and renewed every four weeks. Soy-malt
140 broth (SMB: soy peptone 30 g L^{-1}; malt extract 25 g L^{-1}; pH 5; in deionised water) was
141 inoculated with a single colony in unbaffled Erlenmeyer (shake) flasks, incubated for 24 h
142 and used as preculture for main cultures on macroalgae hydrolysate or nitrogen-limited broth
143 (NLB: KH_2PO_4 7 g L^{-1}; $(NH_4)_2SO_4$ 2 g L^{-1}; $NaHPO_4$ 1 g L^{-1}; $MgSO_4$ 7·H_2O 1.5 g L^{-1}; yeast
144 extract 1 g L^{-1}; carbon source 40 g L^{-1}; pH 5; in deionised water). For shake flask and stirred
145 tank reactor cultures preculture amounted to 2.5 % (v/v) of total culture volume, and for well
146 plate cultivations, preculture was diluted to an OD_{600} of 1 through addition of phosphate-
147 buffered saline (PBS, Oxoid) before inoculation. Working volume in shake flasks was 20 %

(v/v) of flask volume (100 mL) and their incubation took place on orbital shakers (Unimax 2010, Heidolph) at 180 rpm (unless specified otherwise) in temperature controlled cabinets (MLR-352-PE, Panasonic). All cultivations were carried out at 20 °C, balancing cell growth and lipid production with *M. pulcherrima*.[26]

Well-plate cultivations on macroalgae hydrolysate

In 96-well plates, 140 µL sterile filtered (0.22 µm, Millipore) macroalgae hydrolysate (August, 190 °C, 15+0 min) was inoculated with 10 µL of inoculum. Sealed with gas-permeable film to avoid evaporation, the inoculated well plate was incubated at 11 Hz and 3 mm amplitude (Multiskan FC, Thermo Scientific) for 72 h, with readings of OD_{600} performed semi-hourly. The OD_{600} of inoculum cultured on deionised water and non-inoculated macroalgae hydrolysates were subtracted from the final OD_{600}.

Shake flask cultivations on synthetic media and hydrolysate

In shake flasks, *M. pulcherrima* ICS 1 was cultured on NLB with fucose, rhamnose, arabinose, glucose, mannose, mannitol, xylose and galactose (each separately) until stationary stage, determined through daily OD_{600} readings. Fermentations with selected macroalgae (August, 190 °C, 15+0 min) and yeast strain combinations were carried out for 12 days with readings of OD_{600} on Day 2, 5, 8 and 12. Further fermentations were performed with *M. pulcherrima* ICS 1 on *S. latissima* (May) hydrolysate, hydrolysed at different microwave conditions, enzymatically pretreated, buffered, at shaking frequency of 220 rpm (each separately), until stationary stage, determined through daily OD_{600} readings.

Stirred tank reactor fermentations with mannitol and *S. latissima* hydrolysate

In 2 L FerMac 320 stirred tank reactors (Electrolab), *M. pulcherrima* ICS 1 was cultured on 1 L NLB with mannitol as well as *S. latissima* hydrolysate (May, 190 °C, 5+0 min) without sterility barrier. Prior to inoculation, 5 mL polypropylene glycol P 2,000 was added to control foaming, the pH lowered to 4 and kept constant with 5 M NaOH and 1 M HNO_3. Aeration with 0 to 3 L min^{-1} air through a sparger with 100 µm pores and agitation with 150 to 500 rpm kept the dissolved-oxygen (DO) concentration at 80 % air saturation (cascade PID control). Evaporation was minimised by a condenser (5 °C), but obtained concentrations rectified with respect to the amount of evaporated broth.

Analytical methods

Carbon, hydrogen and nitrogen content of dried macroalgae were determined with a CE440 Elemental Analyser (Exeter Analytical) (calibrated against acetanilide with S-benzyl-thioronium chloride internal standard), and further elemental analysis performed externally (Yara) via inductively coupled plasma (ICP) spectrometry. Briefly, dried macroalgae was digested in reverse aqua regia with a MARSXpress microwave digestion system (CEM Corporation), thereafter diluted, filtered and analysed on an axial Vista ICP (Varian). For determining hydrolysis solid residue, the hydrolysate solid and liquid phase were separated by filter paper (11 µm, Whatman) and the solid material oven-dried (Plus II Oven, Gallenkamp) at 105 °C until constant weight (B154, Mettler Toledo). Concentrations of monosaccharides, polyols, fermentation inhibitors, and 2-PE in hydrolysate and fermentation broth were assessed through high-performance liquid chromatography (HPLC) in a 1260 Infinity LC system (Agilent) (section S3). Total organic carbon (TOC) and total nitrogen (TN) analysis were carried out with an automated TOC-L analyser (Shimadzu) (section S3). Optical density of fermentation broth was assessed at 600 nm (OD_{600}) in a spectrophotometer (Spectronic

200, Thermo Fisher Scientific). For determination of yeast DCW, the culture was centrifuged, the supernatant set aside, the pellet re-suspended in deionised water, centrifugation repeated and supernatant discarded. Subsequently, the pellet was frozen (-80 °C), lyophilised and its dry weight gravimetrically assessed (B154, Mettler Toledo). Lipids were extracted with an adapted Bligh and Dyer method[31] and their fatty acid profile determined according to standard procedures (section S4).

Replication and statistical methods

Analysis of dried macroalgae and hydrolysates was performed in duplicates or triplicates and cultivations in singles to triplicates as stated in figure/table captions. The significance of differences in yeast growth characteristics was determined through one-way analysis of variance (ANOVA), normality and homogeneity tested through histograms, skewness-kurtosis, Shapiro-Wilk and Levene's test; and significantly different means identified through post-hoc analysis (Tukey), all carried out in SPSS Statistics (IBM).

Results and discussion

Suitability of macroalgae for microbial lipid fermentation

The macroalgae species investigated varied distinctly in their elemental composition, with carbon contents ranging from 15.0 % (w/w) in *Jania rubens*, through to 36.2 % (w/w) in *Porphyra leucosticta* (Figure 1). Seasonal compositional variation was observed with *S. latissima*, harvested in August and May (Figure 1). Macro- and micronutrients were abundant in all investigated species (Figure 1 & S1), demonstrating the suitability for microbial fermentation. However, the carbon-nitrogen (C/N) ratio of macroalgae varied between 9.4 and 34.0 g g^{-1} for *Soliera chordalis* and *S. latissima* (May), respectively (Figure 1), and most oleaginous yeasts typically require C/N ratios of above 30 g g^{-1} for reasonable lipid production, with other nutrients in excess. The C/N ratio for *S. latissima* has previously been reported lower in the winter months,[32,33] but specific harvesting location could have influenced this discrepancy.[32] Furthermore, phosphorus is in an excess with carbon-phosphorus (C/P) ratios of macroalgae ranging between 93.7 and 584.6 g g^{-1} (Figure 1).

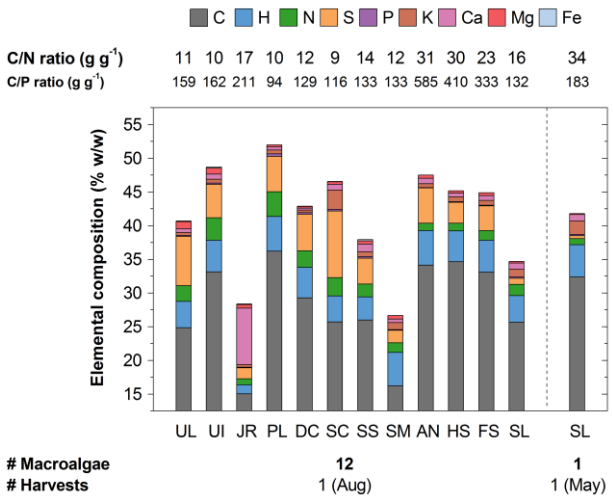

Figure 1. Macronutrients (semi-quantitively), carbon-nitrogen (C/N) and carbon-phosphorus (C/P) ratios (total carbon) of all species of dried macroalgae investigated (Table 1) (n=3,

mean). Twelve different macroalgae were harvested in August and *S. latissima* (SL) additionally in May.

Different species of macroalgae exhibit large differences in their susceptibility to undergo hydrothermal decomposition (Figure 2a). No correlation could be elucidated between the extent of decomposition and the elemental composition of the macroalgae. Milder microwave conditions resulted in lower hydrothermal decomposition, associated with lower carbon release into the hydrolysate (Figure 2). Microwave hydrothermal pretreatment was found to be highly suitable for *S. latissima*, where 69.6 to 85.2 % (w/w) of macroalgal carbon could be recovered into the hydrolysate (Figure 2b).

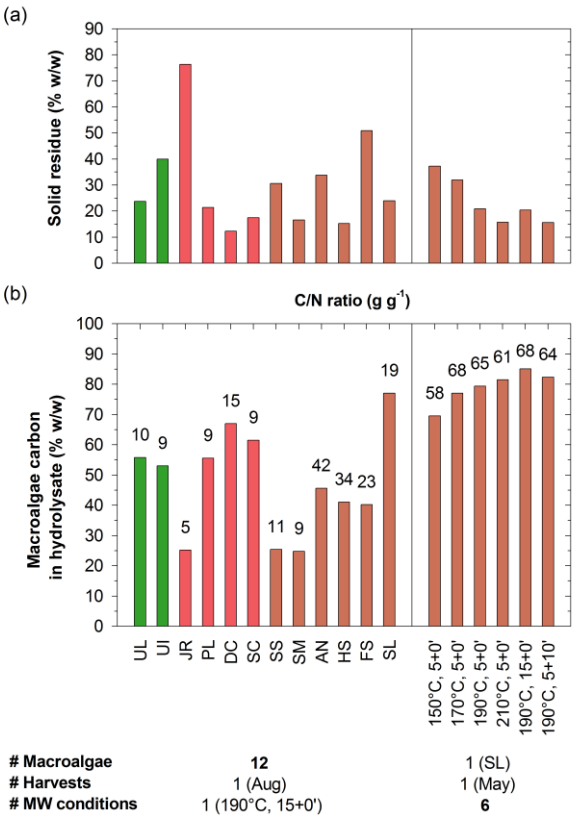

Figure 2. Solid residue (a) and efficiency of carbon release as well as carbon-nitrogen (C/N) ratio (total organic carbon) of the hydrolysate (b) for each species of dried macroalgae (Table 1) after microwave (MW) hydrothermal pretreatment (n=3, mean). Twelve different macroalgae were harvested in August and hydrolysed at 190 °C, 15+0 min, and *S. latissima* (SL), harvested in May, at six different MW conditions.

The different microwave release efficiencies of carbon and nitrogen (Figure 2b & S2) resulted in C/N ratios from 5.0 to 68.3 g g^{-1} for *J. rubens* and *S. latissima* (May), respectively, thus only in favour of oleaginous yeasts for certain macroalgae (Figure 2b). Specifically, *S. latissima* (May) hydrolysate indicated C/N ratios suitable for most oleaginous yeasts, given the entire TOC can be accessed.

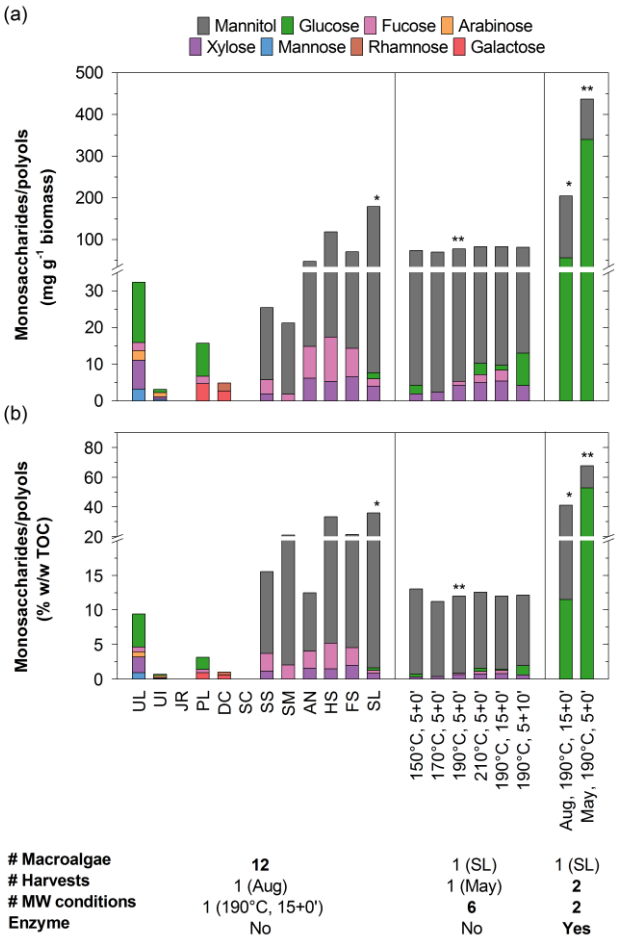

Figure 3. Monosaccharide and polyol content in all hydrolysates used in this study with respect to the dried macroalgae supplied (a) and their share of the total organic carbon (TOC) (b) (n=3, mean). The first data set depicts twelve macroalgae (August, Table 1), depolymerised through microwave pretreatment (190 °C, 15+0 min). The second set includes *S. latissima* (SL, May) depolymerised at six different microwave (MW) conditions. The third set involves SL (May & August), depolymerised through microwave (190 °C, 15+0 min and 5+0 min, respectively) and enzymatic pretreatment (50 °C, 20 h). Stars indicate the corresponding results prior to enzymatic pretreatment.

The percentage of monosaccharides and polyols comprising the hydrolysate TOC varied between macroalgae species, but also depended on harvesting time, as well as microwave conditions and additional enzymatic pretreatment (Figure 3). Dried *S. latissima* (August) constituted of over 17.1 % (w/w) mannitol, which complies with published data[33,34] and underlines its suitability for microbial cultivation. The considerable seasonal effect on macroalgae composition is demonstrated with hydrolysate of the same species harvested in May, containing 96.8 mg mannitol g^{-1} macroalgae (Figure 3a) – in line with observation in other studies, where mannitol concentration peaks typically between June and September,[33–35] constituting an ultimate carbon storage compound for growth in winter.[36–38] The increased presence of glucose in hydrolysate obtained with longer holding time (190 °C, 5+10 min) indicates that some polysaccharides were broken down into their constituents.

Through application of enzymes to degrade macroalgal structural (alginate, cellulose) and storage (laminarin) polysaccharides, as performed in many fermentation studies,[1,14,17,21] the monosaccharide yield for *S. latissima* (May) could be enhanced by 460 % (w/w) (Figure 3a). For certain macroalgae, however, single-step microwave pretreatment is sufficient to release monosaccharides: they were only increased by 14 % (w/w) through additional enzymatic pretreatment of *S. latissima* (August) hydrolysate (Figure 3a), removing the benefit of this additional step representing up to 20 % cost of the overall process.[39] Similarly, acid addition prior to microwave treatment to enhance monosaccharide yields may only be necessary for certain macroalgae such as *A. nodosum*[18], as comparable monosaccharide yields have been achieved with only water herein.

The results demonstrate that microwave processing can be applied to the feedstock effectively producing a fermentable medium containing polysaccharides, polyols and monosaccharides. To access the full range of carbon sources solubilised, coupling with a

suitable microorganism is necessary, to this end *M. pulcherrima* was selected due to the ability to catabolise certain oligosaccharides.[23]

M. pulcherrima's suitability for macroalgae fermentation

The suitability of *M. pulcherrima* for fermentation of macroalgae hydrolysates was assessed through its growth, lipid and 2-PE production on a range of macroalgae-specific carbon sources.[40] *M. pulcherrima* strain ICS 1 metabolised C6 monosaccharides glucose, mannose and galactose, polyol mannitol and C5 monosaccharide xylose (Figure 4).

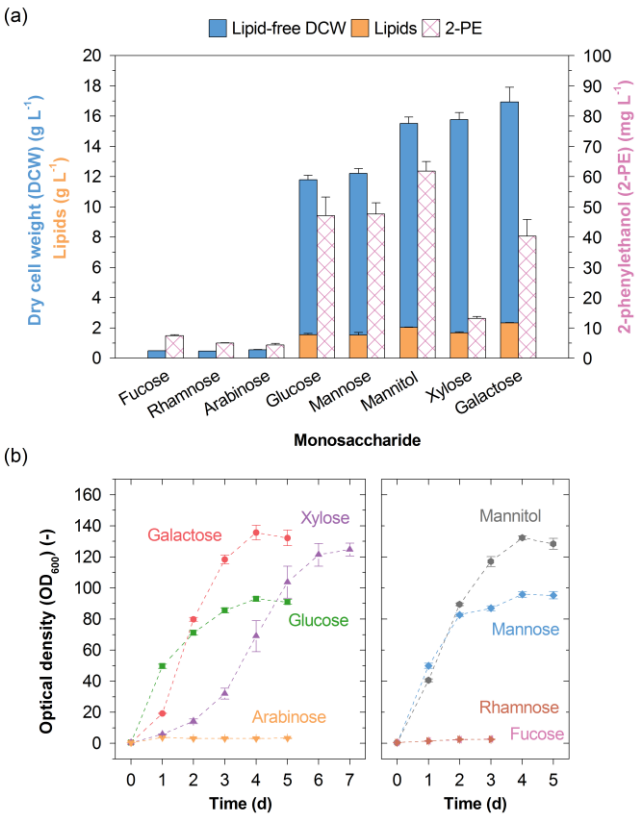

Figure 4. Final dry cell weight, lipids and 2-phenylethanol concentrations (a) and OD_{600} profiles (b) for shake flask fermentations (20 °C, 180 rpm) of *M. pulcherrima* ICS 1 on synthetic nitrogen-limited broth with 40 g L^{-1} of monosaccharides and polyols typically present in macroalgae (n = 3, mean ± SE). The yeast was cultivated until stationary stage.

The DCW increased when switching from glucose to any other assimilable carbon source, the highest biomass yield of 0.41 g g^{-1} being achieved with galactose. Importantly, the DCW increase was 32 % (w/w) using mannitol – the polyol prevalent in brown macroalgae and available in highest quantities in the produced microwave hydrolysate (Figure 3). Growth kinetics and lipid accumulation favour utilisation of C6 monosaccharides and polyols (t_{stat} = 4 d) compared to C5 monosaccharide xylose (t_{stat} = 7 d). Comparably slow assimilation of C5 monosaccharides is frequently observed with oleaginous yeasts and diverse effects on lipid production have been reported.[41,42] For *M. pulcherrima*, the lipid content was 10.7 % (w/w) below the average of 12.6 % (w/w). Similarly, 2-PE production was lowest for xylose (13.1 mg L^{-1}), compared to the highest of 61.8 mg L^{-1} for mannitol. A final pH of 1.9 (table S1), contributable to the nitrogen source being NH_4^+ upon which assimilation H^+ is released, together with the carbon source being fully utilised indicates that the yeast can grow under highly acidic conditions, a further mechanism to reduce bacterial contamination. A few carbon sources could not be assimilated under the given conditions, most prominently rhamnose, abundant in many green macroalgae such as *Ulva* spp.[1], but not highly present in the herein produced hydrolysates (Figure 3).

As a major constituent of the microwave hydrolysates (Figure 3), mannitol was chosen as the carbon source in a model system to investigate performance in controlled 2 L stirred tank reactors (figure S3). Compared to respective shake flask results, both biomass and lipid synthesis were increased, reaching yields of 0.55 g g^{-1} and 0.13 g g^{-1}, respectively (figure S3). Presumably the increased production on the larger scale was achieved through sustaining high dissolved oxygen throughout the fermentation, a major limitation in using shake flasks. Whilst the pH did not significantly influence final biomass and lipid production, emphasising the yeast's acidophility, 2-PE production decreased from 142 mg L^{-1} at pH 4 to 80 mg L^{-1} at

uncontrolled pH (table S2), demonstrating the importance of pH control on the 2-PE biochemical pathway.[43]

M. pulcherrima with different macroalgae species

With *M. pulcherrima* identified as suitable microorganism for bioconversion of macroalgae hydrolysates, the twelve macroalgae species (August) were screened in combination with alternate *M. pulcherrima* strains, and growth kinetics and attainable cell density assessed. Significantly, different yeast growth characteristics were observed on different macroalgae hydrolysates ($p < 0.001$) containing different (amounts and types of) saccharides, inhibitors and other growth compounds (Figure 1 & 3). Variation was also observed between the *M. pulcherrima* strains, although not significant ($p = 0.128$) (Figure 5).

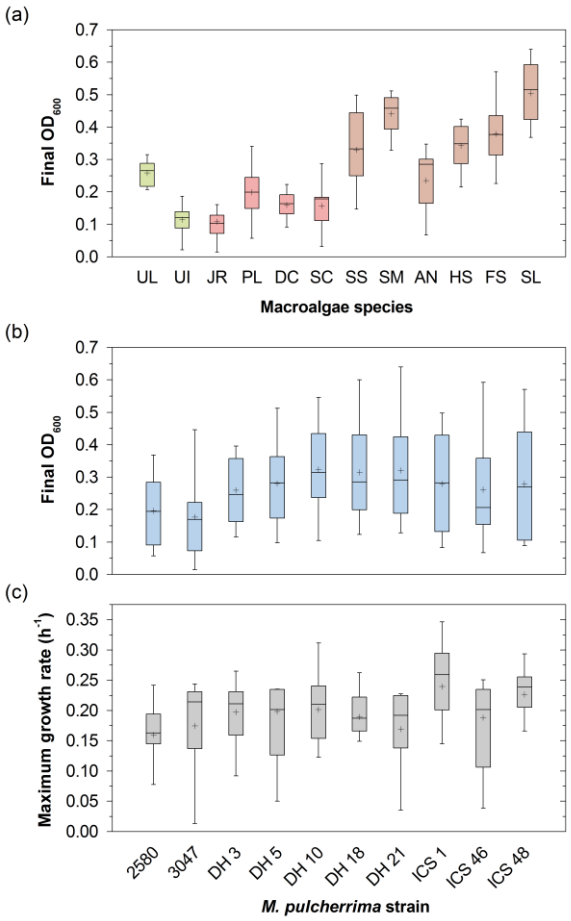

Figure 5. Final OD_{600} (a+b) and maximum growth rate (c) of ten *M. pulcherrima* strains grown on microwave hydrolysates (190 °C, 15+0 min) of twelve different macroalgae species (Table 1, August) in 96-well plates (20 °C) (n = 3). Contribution of inoculum and hydrolysate to the final OD_{600} were subtracted. Box plots indicate 25^{th} to 75^{th} percentile including median, + the mean, whiskers upper and lower adjacent values; and plot colours in (a) type of macroalgae species.

On average, highest OD_{600} of 0.50 was achieved on *S. latissima* and highest OD_{600} of 0.64 was observed in combination with DH 21 (Figure 5a+b). Final OD_{600} was dependent on macroalgae type, with best growth achieved on the brown macroalgae, averaging a final OD_{600} of 0.37, when compared to green (0.19) and red macroalgae (0.16). It has been argued that brown macroalgae represents a "principal feedstock" due to high carbohydrate contents, availability for mass-cultivation[6,44] and superior biosorbent characteristics[45] – despite their photosynthetic efficiency being generally lower than those of green and red macroalgae.[44] Amongst the best growing yeast strains are ICS 1 & 48, both of which achieved an averaged OD_{600} exceeding 0.3. Highest maximum averaged growth rate of 0.24 h^{-1} was achieved by ICS 1 (Figure 5c). Of note, flocculation of yeast cells was observed when growing DH 3 and 10 on *J. rubens* and *Ulva lactuca* hydrolysate, respectively (figure S4). This could be considered beneficial in a bioprocess where rapid settling of biomass is desired.

Scaling up to shake flasks, *M. pulcherrima* ICS 1 was selected to ferment the full range of macroalgae hydrolysates, based on favourable kinetics and balanced growth within each macroalgae type. As with 96-well plate cultures, highest growth was generally achieved on brown macroalgae hydrolysates, specifically *S. latissima*, yielding 5.65 g L^{-1} yeast biomass (Figure 6).

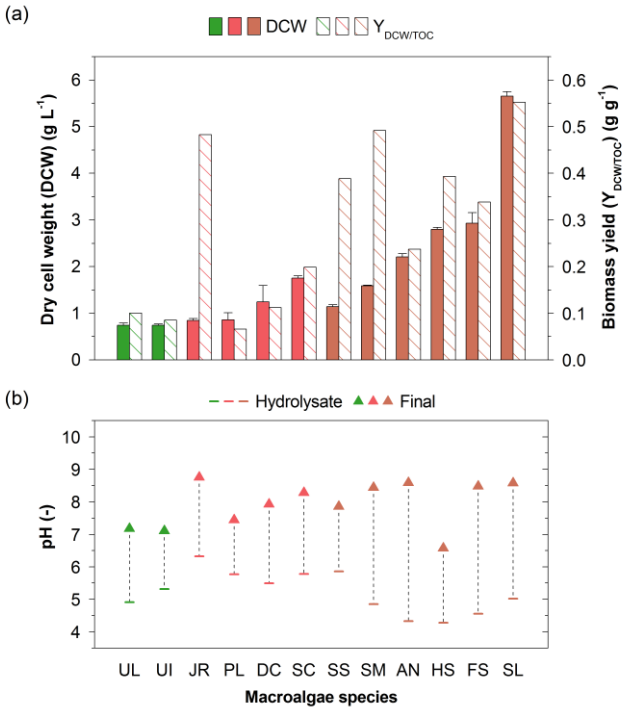

Figure 6. Dry cell weight and biomass yield with respect to total organic carbon (TOC) in the hydrolysate (a) and pH change (b) after 12-day shake flask fermentations (20 °C, 180 rpm) of *M. pulcherrima* ICS 1 on microwave hydrolysate (190 °C, 15+0 min) of different macroalgae species (Table 1, August) (n = 3, mean ± SE). Colours indicate type of macroalgae species.

OD_{600} measurements (figure S5) showed that 83 % of cell growth was achievable in the first two days, indicating that the gross of assimilable carbon sources is readily available under these conditions. In contrast to growth on NLB, a pH increase to neutral or slightly basic conditions was observed in all cases (Figure 6b), due to the yeast metabolising proteins and amino acids, whereby NH_4^+ is released into the medium.

To further narrow down the macroalgae/yeast strain combinations qualifying for potential larger scale fermentation, additional combinations were selected based on 96-well plate final cell densities, growth kinetics, and yeast flocculation (figure S6). Similar DCW values were achieved with other strains on *S. latissima* hydrolysate, including ICS 46 and DH 21 (5.29 to 5.68 g L^{-1}), indicating biochemical similarity between the strains in terms of their metabolic capability. While this might be unfavourable for directed evolution purposes, it is

beneficial from a stability point of view as – despite strain variation – the results are attainable with a range of *M. pulcherrima* wild type strains. Concentration of 2-PE ranged from 1.1 to 47.2 mg L^{-1}, with most yeast strains producing relatively minor amounts (figure S6). Importantly, distinct strain dependence was observed: for example, when grown on *S. latissima* hydrolysate ICS 1 & 46 produced just 7.8 and 5.1 mg L^{-1} 2-PE, respectively, but DH 21 produced 47.2 mg L^{-1} from the same hydrolysate (table S3, figure S6). This versatility of *M. pulcherrima* could become key in a biorefinery setting in which products may be prioritised depending on constantly shifting commercial attractiveness.

Under the given conditions, brown macroalgae constitute a superior substrate for fermentation with *M. pulcherrima*, with *S. latissima* standing out due to its high mannitol content. Its potential as a possible energy crop has been emphasised[6] and it has previously been utilised to produce both biogas[15,16] and bioethanol.[17] As natural resources of *S. latissima* (mainly north Atlantic and Pacific[37]) are limited and to avoid ecological damage, locations for commercial aquacultures are being explored.[46,47]

Factors influencing *M. pulcherrima* performance with *S. latissima*

Further shake flask fermentations were carried out with *S. latissima* (May) hydrolysate investigating the effect of harvesting time, microwave conditions, pH buffering and aeration. Generally lower cell growth in the subsequent sections is a consequence of the different harvesting time of the macroalgae.

Microwave conditions

The hydrothermal pretreatment conditions included different temperatures, ramping and holding time. The liberation of additional monosaccharides through longer ramping time (Figure 3) did not lead to enhanced growth nor lipid production, hence ramping time was

reduced to 5 min (Figure 7a). The breakdown of *S. latissima* polysaccharides through longer holding time (Figure 3) ultimately led to higher DCW, though degradation compounds caused an inhibitory effect which led to a lag time of up to 24 hours (Figure 7b). During fermentation, 5-HMF and furfural were nearly fully degraded by the yeast (figure S7), as similarly observed with other oleaginous yeast.[48] The proposed polysaccharide depolymerisation through microwave heating thus comes at the expense of inhibitor formation, a behaviour common to hydrolysates generated with most acid and thermal pretreatments.[20,44] Previously, *M. pulcherrima* has been demonstrated to have a high inhibitor tolerance,[49] indeed this is not necessarily a disadvantage as the hydrolysate would be less prone to contamination when utilised in an open system. A maximum lipid content of 24.7 % (w/w) was achieved at mild microwave conditions (150 °C, 5+0), with the lipid content negatively influenced at higher inhibitor concentrations (Figure 7 & S7).

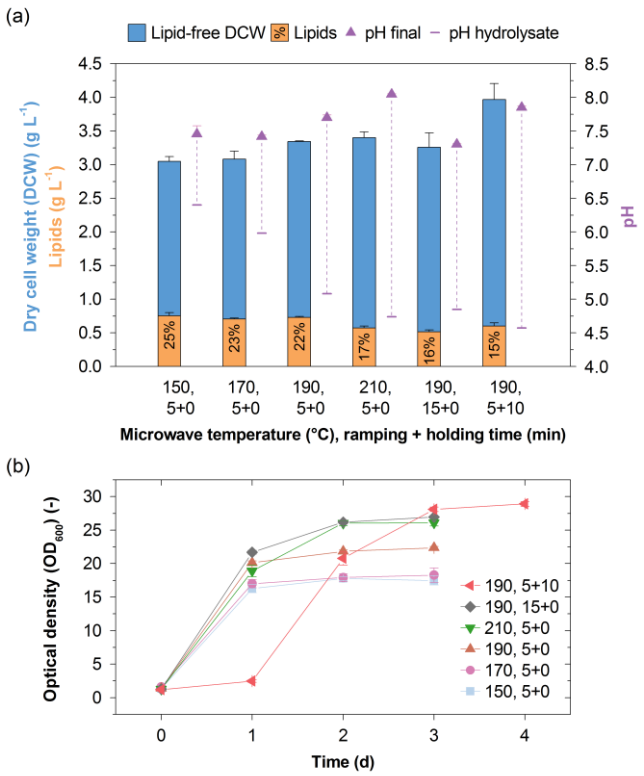

Figure 7. Growth of *M. pulcherrima* ICS 1 on macroalgae *S. latissima* (May) hydrolysed through microwave hydrothermal pretreatment at different target temperatures and ramping +

holding times, for 3 days in shake flasks (20 °C, 180 rpm) (n = 3, mean ± SE). (a) Dry cell weight and pH change. (b) OD_{600} profile (error bars supressed for clarity).

Culture conditions

Culture conditions were changed to approach controlled stirred tank fermentation, meaning the pH was buffered around pH 4 and aeration enhanced through higher shaking frequency. Whilst pH control enhanced growth, similar lipid concentrations could be obtained despite lower lipid content at pH mediated around 4 (table S4). Cell growth could furthermore be enhanced by 16 % (w/w) through increased oxygenation.

Enzymatic hydrolysis

Compared to results from simple microwave hydrolysate of *S. latissima* (May), biomass and lipid concentrations could be increased by 135 % (w/w) and 168 % (w/w), respectively, through additional enzyme pretreatment (table S5). The increase is not as high as additionally released glucose may suggest (460 % w/w), which is due to the yeast favouring mannitol (Figure 4), but also the catabolism of polymers, substantiated by the carbon assimilation with respect to monosaccharides being as high as 94.4 % (w/w) when cultured on microwave hydrolysed *S. latissima* (May) (figure S8). When comparing the macroalgal total carbon assimilation through yeast biomass between microwave hydrolysed *S. latissima* (August) and additionally enzyme hydrolysed *S. latissima* (May), similar values were obtained (0.23 and 0.20 g g^{-1}) (figure S8). Together with the monosaccharide analysis (Figure 3), this demonstrates that the seasonal composition of a single seaweed species is crucial in deciding whether an additional enzymatic pretreatment step is required.

Stirred tank reactor fermentation on *S. latissima* hydrolysate

Fermentation of macroalgae microwave hydrolysate was assessed on a 2 L stirred tank reactor scale to establish growth kinetics of macroalgae utilisation and investigate the viability of the proposed process under more controlled conditions (pH 4, DO 80 %). *S. latissima* microwave hydrolysate (May, 190 °C, 5+0 min) was selected from the shake flask results. During exponential stage, a maximum growth rate of 0.10 h^{-1} and corresponding doubling time of 6.7 h was recorded (figure S9), largely through assimilation of mannitol (Figure 8a). Moreover, the yeast catabolised proteins/amino acids, indicated by the attempted pH increase counteracted by HNO_3 addition from 12 to 41 h (figure S9), and polysaccharides (figure S10). With a final lipid content of 37.2 % (w/w), yeast biomass and lipid yields were 0.14 g g^{-1} and 0.05 g g^{-1} with respect to supplied dried macroalgae, and 0.21 g g^{-1} carbon deposition in the yeast biomass in relation to the macroalgal carbon. The more than 2-fold DCW increase compared to shake flask fermentations on the same hydrolysate can be largely contributed to sustained oxygen availability. The high lipid content together with the high nutrient availability in macroalgae also means that nutrient limitation may not be such a key factor in *M. pulcherrima* as with other oleaginous yeasts.[14] Saturation of produced lipids decreased with fermentation time, and the final product possessed similar composition to soybean oil (Figure 8b).

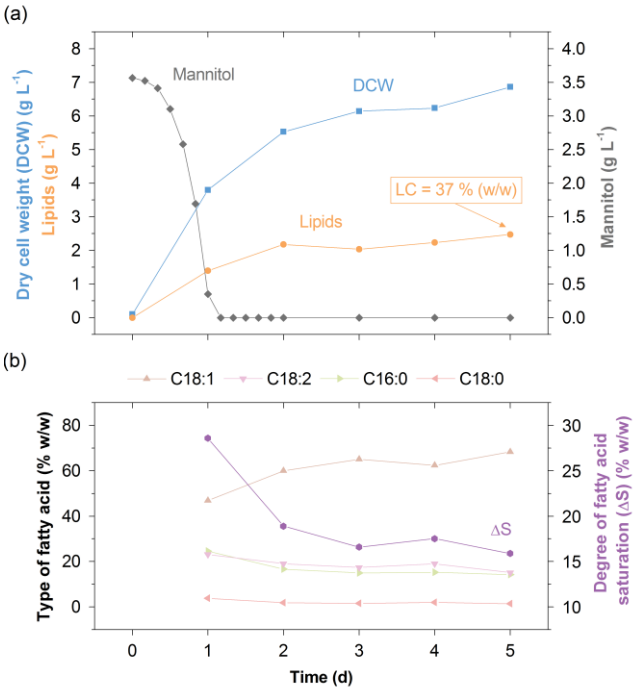

Figure 8. Dry cell weight, lipid and mannitol concentration (a) and fatty acid profile (b) in 2 L stirred tank reactor fermentation of *M. pulcherrima* ICS 1 on *S. latissima* microwave hydrolysate (May, 190 °C, 5+0 min) at pH 4, 20 °C and DO 80 % (n=1). LC: lipid content. Each data point is average value from two independent measurements (SD < 23 %).

The oleaginous yeast *M. pulcherrima* has shown versatile characteristics in breaking down macroalgae compounds, including growing on a wide pH range, degrading inhibitors and producing variable amounts of lipids and 2-PE. Although following microwave processing *M. pulcherrima* could degrade macroalgae polysaccharides, the majority remained in the fermentation broth, hindering higher biomass conversion ratios. To fully valorise the available polysaccharides, additional processing such as extraction or breakdown[1,29] may be considered or genetic modification to expand the metabolic repertoire may be necessary.[19] As non-sterility and the absence of supplementary enzyme addition potentially make the proposed process particularly low-cost, the benefit of those additional treatment must be economically assessed. Finally, the results emphasize the importance of using controlled reactors as part of an industrial biotechnology screening process.

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Synopsis

The proposed process provides a sustainable source of renewable edible oil from macroalgae through integration of low-energy microwave and microbial fermentation technology.

TOC/Abstract Graphic