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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 0.14 g g^{-1} with respect to the 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 renewable feedstock for oleochemicals.

1. 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 figs. [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

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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 [16], butanol [1] and biogas [15] production, with pretreatment often taking place via acid and/or enzymatic hydrolysis.

Depolymerisation via microwave processing has been employed successfully for a range of lignocellulosic feedstocks [19,20]. Compared to conventional heating techniques a microwave process is advantageous in terms of shorter reaction times, higher heating efficiencies and greater control [21,22]. Many examples highlighting the efficiency of microwave mediated reactions have been described, particularly in the areas of organic synthesis [23], polymers [24], and green chemistry [25]. Microwave heating is volumetric, which is very important for activation of solid materials such as macroalgae. Furthermore, microwave irradiation is a clean, cheap and convenient method in carbohydrate chemistry. In general, microwave heating for certain applications is more efficient than conventional heating and should be considered as an alternative and potentially faster, greener methodology. Microwave technology has been demonstrated at both pilot [26] and industrial scale [27,28]. Recently, microwave generators with power up to 100 kW became available making their industrial applications in such areas as food preparation, high quality ceramic formation and wood drying [22,29], commercially feasible.

Considering the lack of lignin and the previous successful recovery of macroalgae constituents through microwave-assisted extraction [16,30], this technology offers a potentially viable alternative to produce an inexpensive microbial growth medium from macroalgae [16]. 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 [19,20], 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). Microbial lipids can be used as a source of food, biofuels, surfactants or polymers, whereas 2-PE has a worldwide production of approximately 10,000 tonnes, though this is predominantly from non-renewable resources. The biological 2PE market is far smaller, though the product has several advantages in being food-grade, having a positive public image and not containing isomers that lead to a poor smell. Due to the minute amounts present in rose petals, and the inefficient extraction, the biological sourced 2PE retails for up to \$1000 kg^{-1} [31,32]. 2PE is also an antimicrobial and with this, as well as the production of other antimicbiral agents, *M. pulcherrima* has the ability to outcompete other microbes [33,34]. Aiming to achieve economic viability and promote sustainability, an imperative focus of oleaginous yeast research lies on the appraisal of low-cost [35], and renewable substrates [36], such as whey, industrial fats [37] or lignocellulosic biomass [20]. With macroalgae (potentially) embodying these characteristics [14], strong cases emphasising the aforementioned advantages over lignocellulosic biomass are made for utilisation in renewable energy production [2,3]. Whilst for these reasons there are a few reports of producing microbial lipids from macroalgae recently [13,14,17,18], coupling low-energy microwave depolymerisation with *M. pulcherrima* offers additional benefits for a potentially more economic route to microbial lipid production. Investigating the suitability of this novel system for development beyond laboratory scale, this study goes beyond previous studies through extensively considering the impact of species on the process.

2. Materials and methods

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 1680 × *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.

2.1. 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 1800 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 sub-

Table 1
Investigated macroalgae species, their type and notation. Macroalgae were harvested from 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

jected to enzymatic hydrolysis according to published procedure with slight modification [38]. 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 solids were removed from any hydrolysate by centrifugation to avoid interference with cell growth assessment.

2.2. Media, strains and culture conditions

Ten *M. pulcherrima* strains were used: locally (Bath, UK) isolated from fruit and flowers (Section S1) ICS 1, 46 & 48; DH 3, 5, 10, 18 & 21; and commercially available NCYC 2580 & 3047 (National Collection of Yeast Cultures, Norfolk, UK). Strains were kept at −80 °C as 20% (v/v) glycerol stocks, from which agar plates (YMD: yeast extract 10 g L^{-1}; malt extract 20 g L^{-1}; glucose 20 g L^{-1}; agar 15 g L^{-1}, pH 5; in deionised water) were inoculated, incubated at 20 °C for 4 days, then kept at 4 °C and renewed every four weeks. Soy-malt broth (SMB: soy peptone 30 g L^{-1}; malt extract 25 g L^{-1}; pH 5; in deionised water) was inoculated with a single colony in unbaffled Erlenmeyer (shake) flasks, incubated for 24 h and used as preculture for main cultures on macroalgae hydrolysate or nitrogen-limited broth (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 extract 1 g L^{-1}; carbon source 40 g L^{-1}; pH 5; in deionised water). For shake flask and stirred tank reactor cultures preculture amounted to 2.5% (v/v) of total culture volume, and for well plate cultivations, preculture was diluted to an OD_{600} of 1 through addition of phosphate-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* [34].

2.3. 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}. In the event of yeast flocculation, OD_{600} results were excluded and cell growth was assessed through DCW in shake flask cultivations.

2.4. 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, except where yeast flocculation occurred. 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.

2.5. 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 2000 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.

2.6. 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 (hydrogenated) monosaccharides, 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 [39] and their fatty acid profile determined according to standard procedures (Section S4).

2.7. 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).

3. Results and discussion

3.1. 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* (Fig. 1). Seasonal compositional variation was observed with *S. latissima*, har-

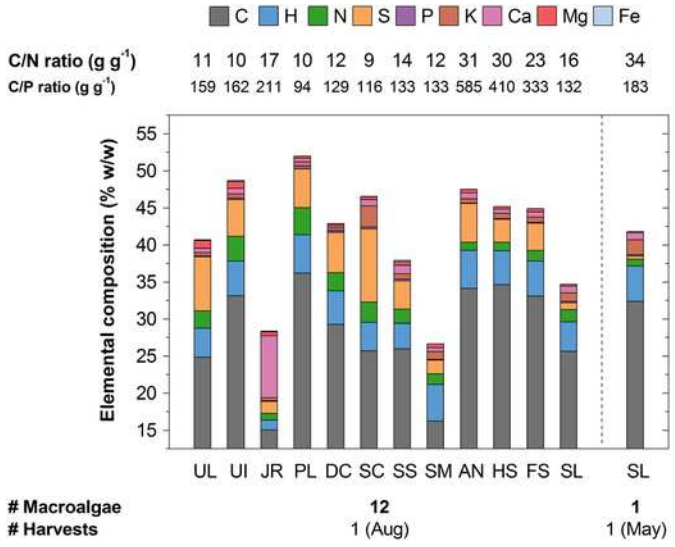

Fig. 1. Macroalgal elemental composition. Macronutrients, carbon-nitrogen (C/N) and carbon-phosphorus (C/P) ratios (total carbon) of all species of dried macroalgae investigated (semi-quantitatively) (n = 3, mean). Twelve different macroalgae (Table 1) were harvested in August and *S. latissima* (SL) additionally in May.

vested in August and May (Fig. 1). Macro- and micronutrients were abundant in all investigated species (Fig. 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 (Fig. 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 [40,41], but specific harvesting location could have influenced this discrepancy [40]. Furthermore, phosphorus is in an excess with carbon-phosphorus (C/P) ratios of macroalgae ranging between 93.7 and 584.6 g g^{-1} (Fig. 1).

Different species of macroalgae exhibit large differences in their susceptibility to undergo hydrothermal decomposition (Fig. 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 (Fig. 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 (Fig. 2b). This is considerably higher in comparison to lignocellulosic biomass such as wheat straw (~16% w/w [20]), presumably due to the absence of lignin.

The different microwave release efficiencies of carbon and nitrogen (Fig. 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 (Fig. 2b). Specifically, *S. latissima* (May) hydrolysate indicated C/N ratios suitable for most oleaginous yeasts, given the entire TOC can be accessed.

The percentage of (hydrogenated) monosaccharides comprising the hydrolysate TOC varied between macroalgae species, but also depended on harvesting time, as well as microwave conditions and additional enzymatic pretreatment (Fig. 3). The highest monosaccharide yield achieved with single-step microwave pretreatment was 179.5 mg g^{-1} macroalgae (95.7% w/w of which was mannitol) using *S. latissima* (August). Hence, dried *S. latissima* (August) constituted of over 17.1% (w/w) mannitol, which complies with published data [41,42] 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

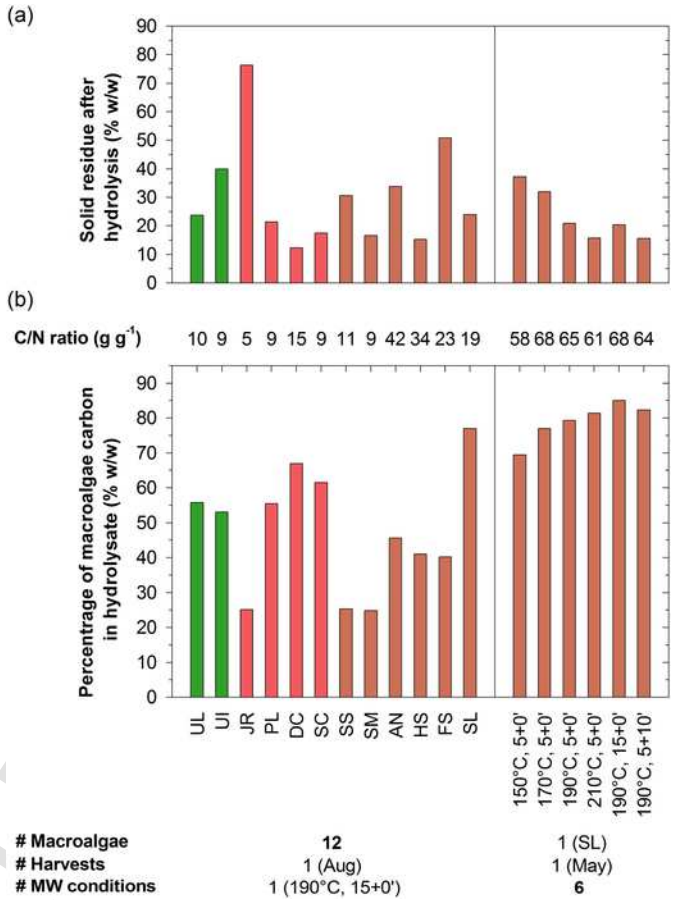

Fig. 2. Microwave hydrothermal pretreatment of macroalgae prior to microbial fermentation. (a) Solid residue and (b) efficiency of carbon release as well as carbon-nitrogen (C/N) ratio (total organic carbon) of the hydrolysate for each species of dried macroalgae after microwave (MW) pretreatment in aqueous phase (5% w/v) (n = 3, mean). Twelve different macroalgae (Table 1) were harvested in August and hydrolysed at 190 °C, 15 + 0 min, and *S. latissima* (SL), harvested in May, at six different MW conditions.

96.8 mg mannitol g^{-1} macroalgae (Fig. 3a) – in line with observation in other studies, where mannitol concentration peaks typically between June and September [41–43], constituting an ultimate carbon storage compound for growth in winter [44–46]. 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 [1,14,47,48], the monosaccharide yield for *S. latissima* (May) could be enhanced by 460% (w/w) to 436.8 mg g^{-1} macroalgae (Fig. 3a). For certain macroalgae, however, depending on their harvesting time, single-step microwave pretreatment is sufficient to release (hydrogenated) monosaccharides: they were only increased by 14% (w/w) through additional enzymatic pretreatment of *S. latissima* (August) hydrolysate (Fig. 3a), removing the benefit of this additional step representing up to 20% cost of the overall process [49]. Similarly, acid addition prior to microwave treatment to enhance monosaccharide yields may only be necessary for certain macroalgae such as *Ascophyllum nodosum* (October), with which under similar microwave conditions (150 °C, 5 min, 3.13% (w/v) solid loading) a monosaccharide yield of 136.0 mg g^{-1} macroalgae has been achieved using 0.4 M sulphuric acid to aid hydrolysis [16].

The results demonstrate that microwave processing can be applied to the feedstock effectively producing fermentable media containing

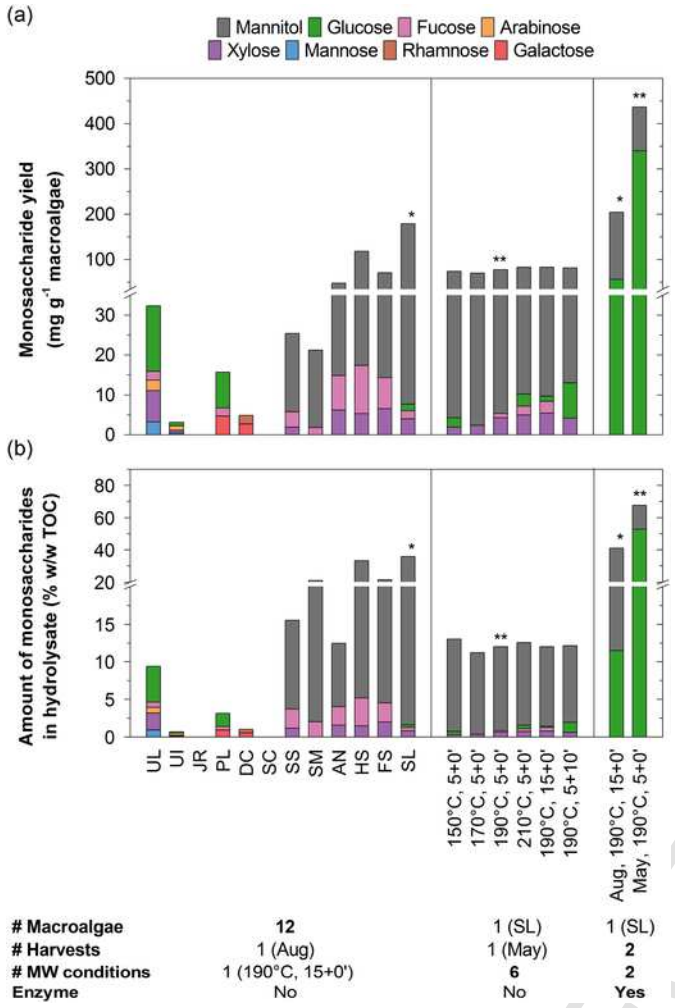

Fig. 3. Monosaccharide and alditol content in all hydrolysates used in this study. (a) With respect to the dried macroalgae supplied and (b) their share of the total organic carbon (TOC) (n = 3, mean). The first data set depicts twelve macroalgae (August, Table 1), depolymerised through microwave pretreatment (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. Stars indicate the corresponding results prior to enzymatic pretreatment.

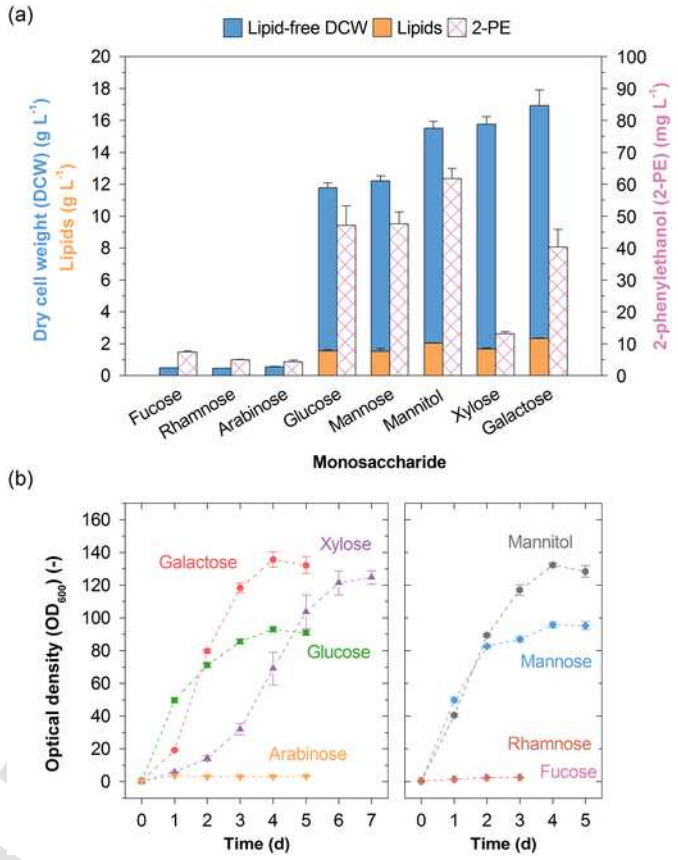

Fig. 4. Growth of *M. pulcherrima* on carbon sources typically present in macroalgae. (a) Final dry cell weight, lipids and 2-phenylethanol concentrations and (b) OD_{600} profiles for shake flask fermentations of *M. pulcherrima* strain ICS 1 on synthetic nitrogen-limited broth with 40 g L^{-1} of monosaccharides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

polysaccharides and (hydrogenated) 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 [19,20].

3.2. *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 [50]. *M. pulcherrima* strain ICS 1 metabolised C6 monosaccharides glucose, mannose and galactose, alditol mannitol and C5 monosaccharide xylose (Fig. 4).

The DCW increased when switching from glucose to any other 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 alditol prevalent in brown macroalgae and available in highest quantities in the produced microwave hydrolysate (Fig. 3). Growth kinetics and lipid accumulation favour utilisation of C6 (hydrogenated) monosaccharides (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 [51,52]. 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 (Fig. 3). Conclusively, *M. pulcherrima* is highly suitable for fermentation of hydrolysates specifically from brown macroalgae, superior to other oleaginous yeasts such as *Rhodosporidium toruloides*, which are limited in the uptake of certain macroalgae reducing sugars [14].

As a major constituent of the microwave hydrolysates (Fig. 3), mannitol was chosen as the carbon source in a model system to investigate performance in controlled 2 L stirred tank reactors (Fig. 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 (Fig. 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), demon-

strating the importance of pH control on the 2-PE biochemical pathway [32].

3.3. *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 (Figs. 1 & 3). Variation was also observed between the *M. pulcherrima* strains, although not significant ($p = 0.128$) (Fig. 5).

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 (Fig. 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 [6,53] and superior biosorbent characteristics [54] – despite their photosynthetic efficiency being generally lower than those of green and red macroalgae [53]. Among 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 (Fig. 5c). Of note, flocculation of yeast cells was observed when growing DH 3 and 10 on *J. rubens* and *Ulva lactuca* hydrolysate, respectively (Fig. 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 (Fig. 6).

OD_{600} measurements (Fig. 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 (Table S1), a pH increase to neutral or slightly basic conditions was observed in all cases (Fig. 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 (Fig. 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

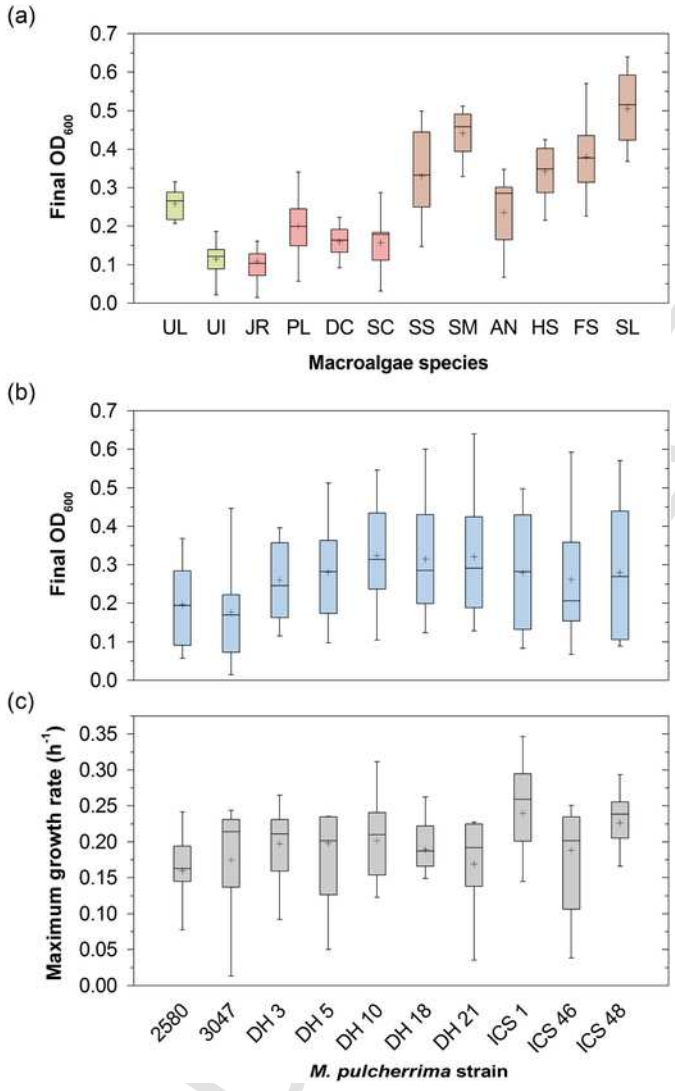

Fig. 5. Growth screening of ten *M. pulcherrima* strains in combination with microwave hydrolysates of twelve macroalgae species (10 × 12 array). Plotted are final OD_{600} and maximum growth rate of the yeast, with respect to (a) macroalgae species and (b + c) *M. pulcherrima* strains. The macroalgae (Table 1, August) microwave hydrolysates (190 °C, 15 + 0 min) were fermented in 96-well plates (n = 3). Box plots indicate 25th to 75th percentile including median, + the mean, whiskers upper and lower adjacent values; and plot colours in (a) type of macroalgae species.

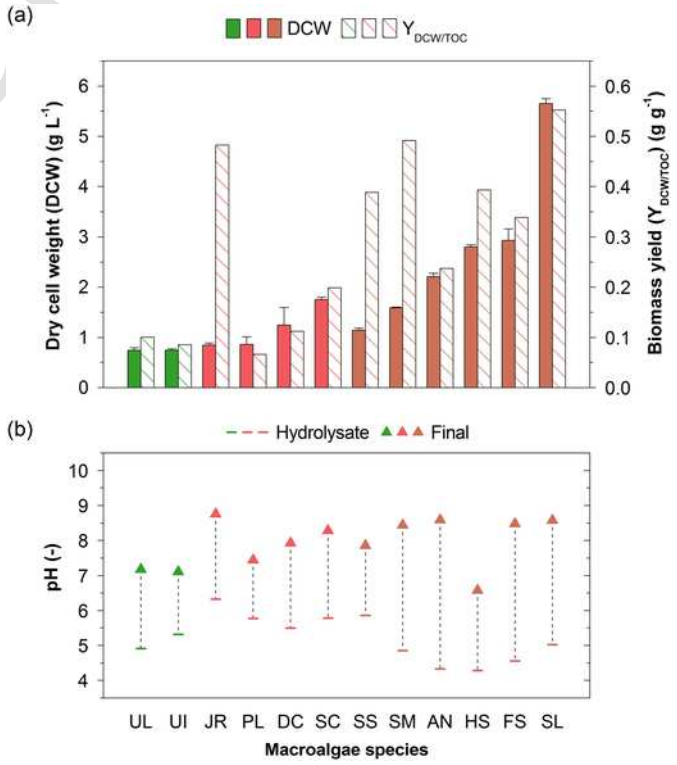

Fig. 6. Growth of *M. pulcherrima* ICS 1 on microwave hydrolysates of twelve macroalgae species at shake flask scale. (a) Dry cell weight and biomass yield with respect to total organic carbon (TOC) in the macroalgae (Table 1, August) hydrolysate (190 °C, 15 + 0 min) and (b) pH change after 12-day fermentation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

between the strains in terms of their metabolic capability. This 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 (Fig. 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, Fig. 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 [15,55] and bioethanol [47]. As natural resources of *S. latissima* (mainly north Atlantic and Pacific [45]) are limited and to avoid ecological damage, locations for commercial aquacultures are being explored [56,57].

3.4. 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.

The microwave conditions included different temperatures, ramping and holding time. The liberation of additional monosaccharides through longer ramping time (Fig. 3) did not lead to enhanced growth nor lipid production, hence ramping time was reduced to 5 min (Fig. 7a). The breakdown of *S. latissima* polysaccharides through longer holding time (Fig. 3) ultimately led to higher DCW, though degradation compounds caused an inhibitory effect which led to a lag time of up to 24 h (Fig. 7b). During fermentation, 5-HMF and furfural were nearly fully degraded by the yeast (Fig. S7), as similarly observed with other oleaginous yeast [58]. 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 [53,59]. Previously, *M. pulcherrima* has been demonstrated to have a high inhibitor tolerance [20,60], 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 (Fig. 7 & S7).

To approach controlled stirred tank fermentation, culture conditions were changed, 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.

Through additional enzyme pretreatment, biomass and lipid concentrations could be increased by 135% (w/w) and 168% (w/w), respectively (Table S5), compared to results from simple microwave hydrolysate of *S. latissima* (May) (Fig. 7). The increase is not as high as additionally released glucose may suggest (460% w/w), which is due to the yeast favouring mannitol (Fig. 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) (Fig. S8). When comparing the macroalgal total carbon assimilation through yeast biomass between microwave hydrolysed *S. latissima* (August) and additionally enzyme

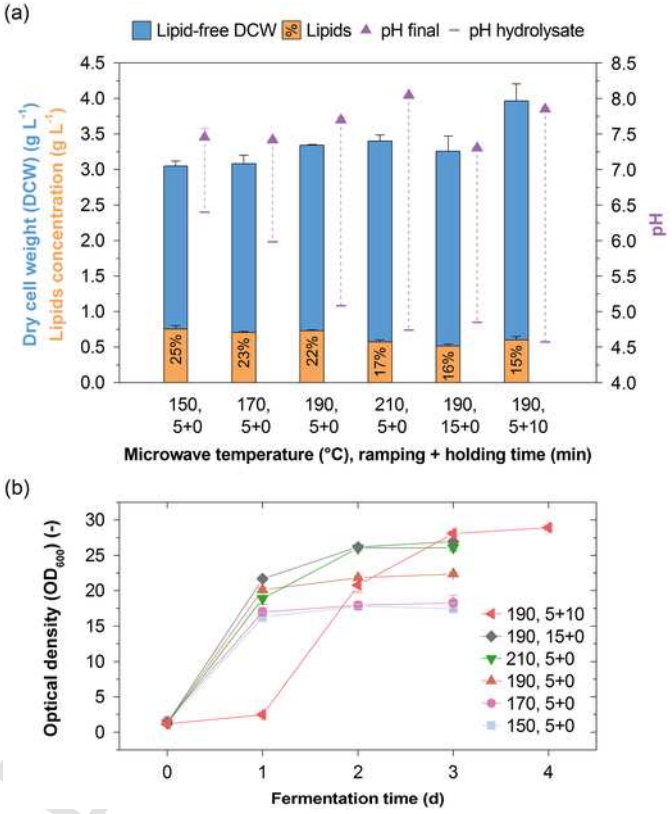

Fig. 7. Influence of microwave pretreatment conditions on *M. pulcherrima* growth on the macroalgae hydrolysate. Macroalgae *S. latissima* (May) was hydrolysed through microwave hydrothermal pretreatment at different target temperatures and ramping and holding times, and fermented by strain ICS 1 for 3 days in shake flasks (n = 3, mean ± SE). (a) Dry cell weight and pH change and (b) OD_{600} profile (error bars suppressed for clarity).

hydrolysed *S. latissima* (May), similar values were obtained (0.23 and 0.20 g g^{-1}) (Fig. S8). Together with the monosaccharide analysis (Fig. 3), this demonstrates that the seasonal composition of a single seaweed species is crucial in deciding whether an additional enzymatic pretreatment step is required.

3.5. 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 (Fig. S9), largely through assimilation of mannitol (Fig. 8a). Moreover, the yeast catabolised proteins/amino acids, indicated by the attempted pH increase counteracted by HNO_3 addition from 12 to 41 h (Fig. S9), and certain polysaccharides (Fig. S10). However, maximum rate of polysaccharides assimilation is estimated at only around 8% (w/w) compared to mannitol (0.34 g L^{-1} h^{-1}). With a final lipid content of 37.2% (w/w), yeast biomass yield was 0.14 g g^{-1} macroalgae, lipid yields 0.05 g g^{-1} macroalgae or 0.61 g g^{-1} (hydrogenated) monosaccharides, and 0.21 g g^{-1} macroalgal carbon was deposited in the yeast biomass. The >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 oleagi-

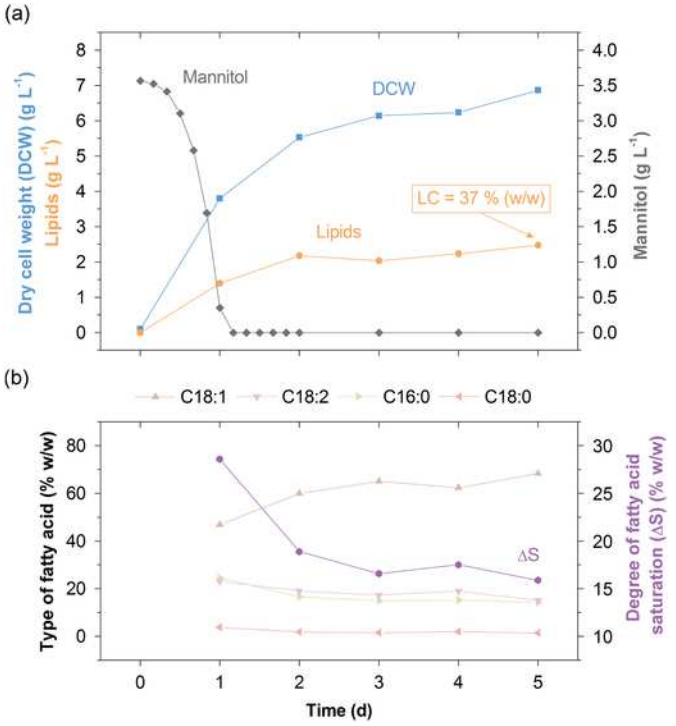

Fig. 8. Increasing scale of *M. pulcherrima* fermentation on *S. latissima* microwave hydrolysate to 2 L stirred tank reactor. (a) Dry cell weight, lipid and mannitol concentration and (b) fatty acid profile of lipid. Strain ICS 1 was fermented on the hydrolysate (190 °C, 5 + 0 min) of *S. latissima* (May) under high oxygen availability (DO 80%) (n = 1). LC: lipid content. Each data point is average value from two independent measurements (SD < 23%).

nous yeasts [14]. Saturation of produced lipids decreased with fermentation time, and the final product possessed similar composition to soybean oil (Fig. 8b).

The obtained lipid concentration of 2.6 g L^{-1} for *M. pulcherrima* grown on macroalgae hydrolysate is superior to those on hydrolysates of wheat straw (1.2 g L^{-1}) and distiller's dried grains with solubles (1.7 g L^{-1}), of which all were hydrolysed through microwave hydrothermal pretreatment [20]. In comparison with other (oleaginous) yeasts, the results place *M. pulcherrima* as highly suitable for valorisation of macroalgae hydrolysates: ethanol yields through fermentation of *Saccharomyces cerevisiae* on *A. nodosum* microwave hydrolysate (0.02 g g^{-1} macroalgae), and also lipid yields with respect to monosaccharides with *R. toruloides* on *Laminaria* residue acid + enzyme hydrolysate (0.16 g g^{-1} total reducing sugars) [14] and *Cutaneotrichosporon oleaginosus* on L. *digitata* (March/June) enzyme hydrolysate (0.32 g g^{-1} monosaccharides) [17] were lower. However, higher overall valorisation of macroalgae to lipids has been reported (0.21 g g^{-1} macroalgae) [17], mostly contingent on the different harvesting time and nearly full hydrolysis of poly- into monosaccharides (< 95% w/w) through the application of 72 h enzymatic pretreatment (monosaccharide yield 650 mg g^{-1} macroalgae). Whilst the time of this hydrolysis method is considerable higher, and the treatment more cost-intensive these results emphasize that further work is necessary to optimise microwave hydrothermal pretreatment of macroalgae, but also enhance metabolism of polysaccharides in *M. pulcherrima*, prior to moving this promising process beyond laboratory scale. Likewise, the integration of this process into a marine biorefinery should be investigated, where particularly the solids from hydrothermal pretreatment can be utilised as biochar [16] and polysaccharides such as alginate remaining after fermentation can be extracted [3,14,30].

4. Conclusions

In rapid hydrothermal microwave pretreatment of macroalgae carbon efficiencies of up to 85.2% (w/w) have been achieved, however a large fraction of this carbon remained locked in polysaccharides. The oleaginous yeast *M. pulcherrima* has shown versatile characteristics in breaking down macroalgae compounds under industrial conditions, including growing on a wide pH range and degrading inhibitors, whilst producing commercially relevant amounts of lipids and 2-PE. Although following microwave processing *M. pulcherrima* could degrade macroalgae polysaccharides, a substantial amount remained in the fermentation broth, hindering higher biomass conversion ratios. To fully valorise the available polysaccharides, additional processing such as extraction or breakdown [1,17] may be considered. As non-sterility and the absence of supplementary enzymes potentially make the proposed process particularly low-cost, the benefit of those additional treatment must be economically assessed. Indeed, genetic modification [61] of *M. pulcherrima* to expand its metabolic repertoire or mixed community culture [13,18] may provide a low-cost option to improve process economics. Finally, the results emphasize the importance of using controlled reactors as part of an industrial biotechnology screening process and provide further credibility to the burgeoning marine biorefinery concept.

Conflict of interest

None of the authors listed have a conflict of interest with this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2019.101411.

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