Isolation of fungal strains for biodegradation and saccharification of microalgal biomass

DOI:

[10.1016/j.biombioe.2020.105547](https://doi.org/10.1016/j.biombioe.2020.105547)

Document Version

Accepted author manuscript

[Link to publication record in Manchester Research Explorer](#)

Citation for published version (APA):

Monjed, M., Robson, G., & Pittman, J. (2020). Isolation of fungal strains for biodegradation and saccharification of microalgal biomass. *Biomass & Bioenergy*, 137, [105547]. <https://doi.org/10.1016/j.biombioe.2020.105547>

Published in:

Biomass & Bioenergy

Citing this paper

Please note that where the full-text provided on Manchester Research Explorer is the Author Accepted Manuscript or Proof version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version.

General rights

Copyright and moral rights for the publications made accessible in the Research Explorer are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

Takedown policy

If you believe that this document breaches copyright please refer to the University of Manchester's Takedown Procedures [<http://man.ac.uk/04Y6Bo>] or contact uml.scholarlycommunications@manchester.ac.uk providing relevant details, so we can investigate your claim.

Isolation of fungal strains for biodegradation and saccharification of microalgal biomass

Mohammad Khalil Monjed ^{a,b}, Geoffrey D. Robson ^{b,1}, Jon K. Pittman ^{c,*}

^a Department of Biology, Faculty of Applied Science, Umm Al-Qura University, Makkah, Saudi Arabia

^b School of Biological Sciences, Faculty of Biology, Medicine and Health, The University of Manchester, Michael Smith Building, Oxford Road, Manchester M13 9PT, UK

^c Department of Earth and Environmental Sciences, School of Natural Sciences, Faculty of Science and Engineering, The University of Manchester, Michael Smith Building, Oxford Road, Manchester M13 9PT, UK

¹ Deceased

*Corresponding author.

Dr Jon Pittman, Department of Earth and Environmental Sciences, The University of Manchester, Michael Smith Building, Oxford Road, Manchester M13 9PT, UK; Tel: +44 161 275 5235; Email: jon.pittman@manchester.ac.uk

Orcid id: 0000-0001-7197-1494

Abstract

Products from microalgae have multiple commercial applications including for nutrition, health and fuel uses, but the breakage and release of products from biomass remains a challenge, particularly for microalgal strains with thick cell walls. Fungal strains were isolated due to their ability to degrade microalgal biomass of *Parachlorella hussii*, *Hindakia tetrachotoma* and *Jaagichlorella luteoviridis* that was buried in soil at 25°C for 8 weeks. Fungal isolates were identified by sequencing, with the three most ubiquitous strains identified as *Fusarium solani*, *Doratomyces nanus* and *Actinomucor elegans*. Crude enzyme extracts from these strains were used to quantify the saccharification of intact or lipid-extracted *Chlorella vulgaris* biomass. The *D. nanus* extract gave the highest saccharification efficiency of 57% from the intact biomass and 76% from the lipid-extracted biomass, without need for any pre-treatment. Outcomes of this study can improve the design of microbial enzymes to efficiently degrade microalgal biomass for various industrial applications.

Keywords: *Chlorella vulgaris*; *Doratomyces nanus*; fungal degradation; microalgal biomass; saccharification.

1. Introduction

The exploitation of microalgae for different industrial applications is being extensively evaluated, and include the uses of microalgal biomass and extracted chemicals and metabolites as pharmaceuticals, cosmetics, human or animal food supplements, and as a feedstock for various forms of biofuel [1-3]. Microalgae are particularly attractive due to their short generation time, high photosynthetic efficiency and ability to be cultivated in a sustainable manner [4]. Various types of biofuel can be generated from microalgal biomass including the conversion of lipids into biodiesel and the use of microalgal derived sugars for fermentation of bioethanol or biobutanol [5-7]. The evaluation of microalgal biomass for biofuel production has generated particular interest, especially because of the potential to shift the dependency away from fossil fuel usage for energy production, and because microalgae can be cultivated on non-arable land and therefore avoid competition with food crop cultivation [8, 9]. The sustainability and economic viability of microalgae cultivation can be further enhanced by providing recycled nutrients from wastewater sources, thus reducing the fertiliser and freshwater requirements [10]. For example, microalgae strains *Hindakia tetrachotoma*, *Parachlorella hussii*, and *Chlorella luteoviridis* were previously shown to be efficient at growing efficiently municipal wastewater and assimilate ammonium and phosphate from this source [11, 12].

However, there are still several obstacles that limit the affordable production of these bioproducts. In particular, biofuels derived from microalgae are low value and require efficiencies in many stages of production to be cost effective [13]. In addition, the thickness of the cell walls of many microalgae species, such as *Chlorella* spp., *Haematococcus pluvialis* and *Nannochloropsis* spp., is a major factor in the cost of extracting metabolites and chemicals [14, 15]. Therefore, there is a need to improve the efficiency of cell wall degradation in order to make the overall extraction and production of microalgal chemicals and metabolites commercially viable. Furthermore,

the consideration of microalgae as a biorefinery whereby multiple added-value components are utilised from the biomass, should increase the economic and sustainable viability of microalgal cultivation [2]. For example, following the extraction of lipids or pigments, residual carbohydrates in cell wall and starch materials may be used for biofuel generation after saccharification has been performed.

While amylose and amylopectin polymers from starch granules are a significant source of glucose sugars, microalgal cell walls, which may be composed of polymers such as cellulose, hemicellulose, chitin and pectin [16], are also a major source of various sugars, including rhamnose, xylose, mannose, galactose, glucose, glucuronic acid and glucosamine [17]. For example, a *Parachlorella kessleri* strain was shown to have a rigid fibrillar cell wall composed of glucosamine and an amorphous cell wall matrix with high abundance of xylose and glucose [18], while *Chlorella vulgaris* strains were found to also contain rigid glucosamine cell walls with an amorphous matrix rich in rhamnose and galactose but with lower proportions of glucose [17]. Various physical or chemical methods have been employed to degrade microalgae cell walls, including homogenisation, sonication, microwave treatment, heat treatment, and acid or alkaline lysis, all with variable efficiencies [19, 20]. Enzymatic degradation (either alone or in combination with physicochemical treatments) and saccharification has also been tested through use of various commercial enzymes, including cellulase, chitinase, β -glucuronidase, lysozyme, and pectinase [15, 20-22].

A criticism with the use of such commercial enzymes is that they have not been customised for the degradation of microalgal biomass. Therefore the aim of this study was to investigate the degradation of microalgal biomass by microorganisms that have been selected specifically for their ability to colonise and degrade this type of biomass. Consequently, this approach is more likely to produce degrading enzymes that are better adapted and more efficient for the break down and saccharification of microalgal biomass. Although bacteria can also play a role in degrading microalgal biomass [23],

this study only focussed on the isolation and identification of fungi since these organisms are regarded as an important source of biomass degrading enzymes [24]. Native filamentous fungal species were isolated using a soil-microalgal solid-state fermentation approach and then crude enzyme extracts from selected fungal strains were tested for efficient saccharification of intact or lipid-extracted *C. vulgaris* biomass. To our knowledge, this is the first study that has selected fungal strains from a microalgal carbon source and investigated their use in microalgal biomass saccharification.

2. Materials and methods

2.1. Microalgae strains and biomass production

Three microalgae strains were used to screen for fungal degraders. Strains of *P. hussii*, *H. tetrachotoma* and *Jaagichlorella luteoviridis* (recently reclassified from *C. luteoviridis* [25]) were previously isolated from municipal waste water treatment effluent [11]. The *P. hussii*, *H. tetrachotoma* and *J. luteoviridis* strains were maintained on Tris-acetate-phosphate (TAP) agar media, as described previously [11] at 25°C and pH 7.0 under 150 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ light intensity with a 16:8 h light/dark cycle and sub-cultured onto fresh media every two weeks. The strains were also maintained in liquid TAP medium at the same growth conditions with continuous agitation at 150 rpm on an orbital shaker. In order to produce sufficient amounts of microalgal biomass for degradation studies, strains were cultivated in 1.5 L of TAP media in 2 L Erlenmeyer flasks and incubated for up to 28 days before harvesting. Biomass was harvested by centrifugation at 11,000 g for 15 min. The pellet was suspended in distilled water and re-centrifuged to remove excess salts. Harvested biomass was collected in Petri dishes and dried at 45°C to constant weight. Dried biomass was ground in a mortar and pestle and stored dry and dark at room temperature until required. *C. vulgaris* was purchased

as dried powder (Chlorella Europe, Wych Cross, UK) for use in saccharification studies.

2.2. Burial of microalgal biomass in soil

Commercial topsoil (Online Turf, Ormskirk, UK) was sieved using a metal sieve with a pore size of 4 mm. Moisture content (% w/w) of the soil was determined by weighing 10 g of sieved soil in triplicate (w_{ti}) in empty pre-weighed Petri dishes. The dishes were then incubated at 50°C to constant weight and re-weighed (w_{tf}). The moisture content (% w/w) was calculated using the formula:

$$\text{Moisture content (\% w/w)} = \frac{(w_{ti}) - (w_{tf})}{(w_{ti})} \times 100 \quad (1)$$

Plastic 1 L containers (19.5 cm × 14 cm × 6 cm) were surface sterilised using 70% ethanol, air-dried and filled to two thirds of their maximum capacity with sieved soil. Nylon mesh bags (4 cm × 3 cm) with a pore size of ≤ 100 µm (Sigma-Aldrich, Dorset, UK) were filled with 500 mg of *P. hussii*, *H. tetrachotoma* or *J. luteoviridis* biomass and sealed. Five independent replicates for each strain were prepared then each mesh bag was placed at a depth of 1 cm from the surface of the soil. Each container was weighed and placed in a 25°C incubator. Control boxes of sieved soil, which contained no microalgal biomass were included as negative controls. Moisture content of the soil was adjusted every 2 weeks to 15% (w/w) using sterile distilled water by weighing the boxes and replacing any water lost by evaporation. Each week the mesh bags were carefully removed, excess surface soil was brushed off and the bags were weighed, using an analytical balance with accuracy to 1 mg (Mettler-Toledo, Leicester, UK), until the net weight of the microalgae samples reached approximately 50% of their initial weight.

2.3. Isolation of microalgal degrading fungal strains

Microalgae samples were removed from the mesh bags and the contents were serially diluted (at 10^{-1} - 10^{-6}) in sterile phosphate buffered saline (PBS) pH 7.0. Triplicate volumes of 0.1 mL from each dilution were then added onto potato dextrose agar (PDA) plates containing 50 $\mu\text{g mL}^{-1}$ chloramphenicol and 0.5 g L^{-1} deoxycholic acid sodium salt to suppress bacterial contamination and reduce the radial size of the colony forming units (CFUs). Plates were incubated at 25°C for 5-7 d. After the incubation period, the total viable count of the developed CFUs was performed using a colony counter. The CFUs were initially grouped according to their morphology, and then each was isolated and transferred aseptically onto the centre of a fresh PDA plate, supplemented with 50 $\mu\text{g mL}^{-1}$ chloramphenicol to avoid bacterial contamination. PDA plates were incubated at 25°C until the fungal isolate occupied most of the surface of the plate. Fungal spores from each isolate were cryopreserved at -80°C for later experiments, when they were recovered by growth on PDA plates at 25°C.

2.4. Genomic DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from fungal mycelia using the protocol described previously [26]. In order to genetically identify the fungal isolates, the ITS1-5.8S-ITS2 region of the fungal rRNA gene was amplified by PCR using the ITS5-F primer (5'-GGA AGT AAA AGT CGT AAC AAG G-3') and the ITS4-R primer (5'-TCC TCC GCT TAT TGA TAT GC-3') [27] and using the same PCR conditions as described previously [28]. In the cases where PCR failed, the ITS5-F primer was replaced by the ITS1-F primer (5'-CTT GGT CAT TTA GAG GAA GTA A-3') [29]. PCR products were visualised using agarose gel electrophoresis and bands of the expected size (~ 500-700 bp) were purified using a PCR Purification Kit (Qiagen, Manchester, UK) following the manufacturer's instructions. Samples were sequenced by Sanger sequencing at the

University of Manchester sequencing facility. All sequence reads were deposited in the NCBI GenBank database under the following accession numbers: MN535081 (isolate C. S1), MN535082 (isolate C. S4), MN535083 (isolate C. S5), MN535084 (isolate J.L S1), MN535085 (isolate J.L S2), MN535086 (isolate J.L S3), MN535087 (isolate J.L S5), MN535088 (isolate H.T S1), MN535089 (isolate H.T S2), MN535090 (isolate H.T S3), MN535091 (isolate H.T S6), MN535092 (isolate H.T S7), MN535093 (isolate P.H S4), MN535094 (isolate P.H S5), MN535095 (isolate P.H S6).

2.5. Bioinformatic analysis

ITS sequence reads were viewed using Finch TV v1.4.0 software (Geospiza, Seattle, WA, USA). Sequences were initially analysed by NCBI BLAST to determine the identical or most closely matched NCBI database sequences in order to identify identical or related strains and species. The identification by NCBI BLAST was further confirmed by the morphological characteristics of each isolate. Phylogenetic relationships of the isolated fungal sequences were compared with sequences of closely related fungi selected from NCBI GenBank database. All ITS sequences were aligned using ClustalW in the MEGA7 software. Aligned sequences were used to construct neighbour-joining trees and robustness of each branch was estimated by bootstrapping of 1,000 samples. Evolutionary analyses were conducted in MEGA7 [30].

2.6. Preparation of fungal enzyme extracts

The fungal strains *Fusarium solani* (J.L S1), *Actinomucor elegans* (J.L S2), and *Doratomyces nanus* (J.L S3) were propagated on a modified version of Mandel's enriched agar media [31], which was adapted by replacing cellulose with 0.5% (w/v) *C. vulgaris* biomass as the sole carbon source. Mycelia plugs (13 mm) made of the fungal isolates that had been grown on PDA plates were transferred to the centre of the Mandel's agar media plates (6 replicates per fungal strain) followed by incubation at

25°C until fungal growth covered the whole plate. The mycelia plugs were removed and the agar was roughly chopped using a sterile blade. Chopped fungal agar from two replicate plates were combined and extracted in a flask containing 25 mL of extraction buffer (100 mM sodium acetate, 20 mM sodium chloride and 0.02% (w/v) sodium azide, pH 5.5) followed by incubation on an orbital shaker at 180 rpm at room temperature for 2.25 h in aseptic conditions, as described previously [32]. Extracted enzymes were then filtered through double layered sterile tissue and filtrates were centrifuged at 12,470 g at 4°C for 10 min. The resultant supernatants were passed through a sterile syringe filter (0.22 µm) and stored at 4°C until required.

2.7. Saccharification of intact and lipid extracted microalgal biomass

To assess the efficiency of the isolated fungal enzyme extracts on a commercially used microalgal strain, and due to the high volume of biomass required for these saccharification experiments, commercially supplied dry *C. vulgaris* biomass was used. To obtain the lipid extracted *C. vulgaris* biomass, an extraction method was performed essentially as described [21], whereby 200 mL chloroform:methanol (1:2 ratio) was added to 30 g of intact *C. vulgaris* biomass in a glass beaker and mixed on a magnetic stirrer (Bibby Scientific, Stone, UK) overnight at 50°C in a fume hood. The sample was filtered using Whatman GF/C filter paper (diameter 47 mm, GE Healthcare, UK) then the remaining residual biomass was dried at 50°C until it reached a constant weight. Total lipid concentration from biomass was determined essentially as described previously [11].

The fungal crude enzyme extract degradation of *C. vulgaris* biomass was carried out in flat-bottom 96-well microtiter plates where 90 µL of *C. vulgaris* biomass (30 mg mL⁻¹) suspended and autoclaved in 50 mM sodium acetate buffer (pH 5.5) were added to wells along with 90 µL of enzyme extract and incubated at 37°C for 70 h. Control treatments to determine background baseline values contained only biomass without

enzyme extract or enzyme extract without biomass. Samples were taken periodically at different time points, centrifuged at 900 *g* for 10 min and the concentration of reducing sugars, which represents the enzymatic saccharification of the *C. vulgaris* biomass, were estimated in the supernatant as described [32] using 3,5-dinitrosalicylic acid (DNS) reagent prepared as described previously [33]. Glucose concentration was determined by comparison to a glucose standard (concentration range 0.25 – 5 mg mL⁻¹). For calculation of saccharification efficiency (%), the following formula was used:

$$\text{Saccharification efficiency (\%)} = \frac{\text{Reducing sugars in sample (mg/mL)}}{\text{Total carbohydrates in sample (mg/mL)}} \times 100 \quad (2)$$

In order to determine the efficiency of extracted fungal crude enzymes on liberating reducing sugars from *C. vulgaris* biomass, enzyme activity was determined in units (U) per mL of enzyme extract, where 1 U was defined as the amount of fungal crude extract to release 1 μ mol glucose per min from intact *C. vulgaris* biomass under the studied saccharification conditions. The total carbohydrate concentration of the biomass was determined by measuring the concentration of reducing sugars in biomass that was treated by acid hydrolysis in 0.5 M H₂SO₄ at 121°C for 20 min. Starch concentration in the *C. vulgaris* biomass was measured essentially as described previously [34] using a Megazyme Total Starch analysis kit (Megazyme International, Wicklow, Ireland). The concentration of cell wall derived sugars was determined using an adapted method [35] whereby 25 mg of dried biomass was digested in 1.5 mL of the acetic acid/nitric acid reagent (150 mL of 80% (v/v) CH₃COOH, 15 mL concentrated nitric acid (Fisher Scientific, Loughborough, UK)) at 100°C for 30 min then the residual biomass was washed in water, followed by incubation in 1.25 mL of 67% (v/v) H₂SO₄ at room temperature for 1 h by shaking to solubilise cell wall polymers. The samples were mixed with 1 mL of 0.2% (w/v) anthrone reagent (Merck, Hohenbrunn, Germany) in concentrated H₂SO₄ then incubated at 100°C for 16 min, cooled to room temperature

and the absorbance measured at 630 nm. Sugar concentration was determined by comparison to a cellulose standard (concentration range 20 – 200 µg mL⁻¹).

2.8. Statistical analysis

Statistical analysis of the data by ANOVA with the significance threshold set at p value < 0.05 was performed using GraphPad Prism version 7.01 for Windows (GraphPad Software, La Jolla, USA).

3. Results

3.1. Degradation of microalgal biomass in soil

Dried *J. luteoviridis*, *H. tetrachotoma* and *P. hussii* biomass was incubated in soil at 25°C for an 8 week time period with a moisture content maintained at 15% (w/v) to ensure the required humidity for microbial growth and microbial-derived degradation of the microalgal biomass. During the initial weeks of incubation, the water loss from the soil ranged between 20 and 35% but in the later weeks until the end of the incubation period there was less water loss (10 – 15%). Each microalgal strain sample displayed a significant decrease in biomass weight during the 8 weeks of incubation, indicative of the degradation process (Fig. 1). Occasional temporary increase in biomass was observed at some time points (at weeks 2 and 7 for *J. luteoviridis* and *H. tetrachotoma* and at week 4 for *P. hussii*), which was likely due to rehydration of the dried biomass when water was added to maintain the moisture content. By the end of incubation period at week 8, the percentage of biomass weight loss was 59.4% for *J. luteoviridis*, 45.4% for *H. tetrachotoma* and 63.7 % for *P. hussii* biomass (Fig. 1).

3.2. Quantification of fungal growth

For all three types of the degraded microalgal biomass, there were significantly higher ($p < 0.05$) total viable fungal counts present when compared to the negative control, which was soil without added biomass (Fig. 2). The highest viable fungal count was associated with the *P. hussii* biomass (2.5×10^7 CFU), which was the biomass that exhibited the largest degradation in terms of weight loss (Fig. 1). There was no significant difference in viable fungal counts between the residual biomass of *J. luteoviridis* and *H. tetrachotoma* ($p > 0.05$), while both of these strains exhibited significantly lower viable fungal loads than the *P. hussii* sample ($p < 0.05$).

3.3. Identification of isolated fungal strains

The quantification of fungal growth, and consequently the fungal strain isolation process, was conducted at the end of the 8-week incubation period. The recovered fungal strains from the different samples of residual microalgal biomass were identified based on morphological characteristics of recovered isolates on PDA plates (Fig. 3) followed by genetic identification by amplifying and sequencing the ITS1-5.8S-ITS2 region of fungal rRNA genes (Table 1). Phylogenetic analysis corroborated the taxonomic identification made by the BLAST analysis in nearly all cases (Fig. 4). All isolates were classified as filamentous fungi. More fungal isolates were recovered from *P. hussii* and *H. tetrachotoma* biomass although some strains were found to be identical species; for example, two strains of *F. solani* from the *P. hussii* biomass. One of the *F. solani* isolates (strain P.H S1) was also isolated from *H. tetrachotoma* biomass (Table 1). Likewise, a strain of *Circinella chinensis* was recovered from *P. hussii* and *H. tetrachotoma* biomass, while a strain of *Aphanoascus verrucosus* was isolated from biomass of all three microalgae. Most isolates belong to the Ascomycota phylum while only *A. elegans* and *C. chinensis* belong to the Zygomycota phylum. *F. solani* was the predominant species that was isolated from all sources of microalgal residual biomass,

which accounted for 60 – 70% of all fungal isolates. None of the fungal isolates extracted from the negative control (with no microalgal biomass) were identified in any of the microalgal biomass (Table 1).

Comparison of the sequences extracted from these isolates was conducted by pairwise sequence alignment and it was found that the *P. hussii* biomass *F. solani* isolates P.H S1 and P.H S5 have 99.6% sequence similarity, respectively, indicating that these pairs represented the same species although each individual isolate showed slight differences in their morphological characteristics (Fig. 3c). The phylogenetic analysis also indicated that all isolated *F. solani* strains were related (Fig. 4). The *Fusarium oxysporum* strain (P.H S6) isolated from *P. hussii* biomass did not group with other known *F. oxysporum* strains, possibly suggesting that the strain may have been mis-identified.

3.4. Saccharification of *Chlorella vulgaris* by fungal enzyme extracts

Fungal enzyme extracts were produced from *F. solani* J.L S1, *A. elegans* J.L S2, and *D. nanus* J.L S3, which were isolated from *J. luteoviridis* residual degraded biomass. These fungal strains were selected because it was hypothesised that strains that are able to degrade *J. luteoviridis* may also be efficient at saccharifying *C. vulgaris* biomass, since both species share taxonomic similarity [11]. The *C. vulgaris* biomass had a mean total carbohydrate content of 17.3% (w/w). When starch-derived sugars and cell wall-derived sugars were determined individually, the mean starch concentration was 5.8% (w/w) while the mean cell wall releasable sugar concentration was 10.4% (w/w). All fungal crude enzyme extracts tested were able to release reducing sugars from *C. vulgaris* biomass (Fig. 5). They gave enzyme activity values of 0.043 ± 0.002 U mL⁻¹ for the *A. elegans* extract, 0.053 ± 0.007 U mL⁻¹ for the *D. nanus* extract, and 0.044 ± 0.002 U mL⁻¹ for the *F. solani* extract. For intact *C. vulgaris* biomass, there were only very minor differences between the three extracts (Fig. 5a).

However, a significantly higher ($p < 0.05$) concentration of reducing sugar was released after 70 h of incubation with *D. nanus* extract, equivalent to 57% of total *C. vulgaris* carbohydrate, compared to the incubation with the extracts from *A. elegans* and *F. solani* (Table 2), which were not significantly different ($p > 0.05$).

The *C. vulgaris* biomass had a mean total lipid content of 14.7% (w/w). Further saccharification analysis was performed with the fungal crude enzyme extracts on the residual biomass following extraction of the lipids. The concentration of released reducing sugars from lipid-extracted *C. vulgaris* biomass was higher than from intact *C. vulgaris* biomass, and there was greater variation between the different fungal extracts (Fig. 5). There were consistently higher saccharification efficiency values for each extract when incubated with the lipid-extracted biomass (Table 2). Treating lipid-extracted *C. vulgaris* biomass with *D. nanus* crude extracts resulted in the release of 76% of carbohydrates, which was significantly higher ($p < 0.05$) compared to all other used fungal extracts.

4. Discussion

There are many species of fungi in all ecosystems that play an important role in the degradation and recycling of biomass. For example, indigenous fungi that are rich in soil have various roles in decomposing biomass while marine fungi can also play a role in plant and algal bioconversion [24, 36]. Although the microalgal biomass degradation that occurred in the soil may not have been completely due fungal activity, most fungal strains can grow within the temperature range (25 – 30°C) of the incubation conditions [37] and it was expected that the majority of the microalgal biomass degradation was due to fungal activity. To our knowledge, a soil incubation approach has not previously been used to identify novel fungal degraders of microalgal biomass and very little is known about fungal organisms that can mediate the degradation of microalgal material. Knowledge of fungal degradation of algal biomass

is mostly from marine environments, where marine fungi have been shown to be associated with and degrade green and brown seaweed [38-40].

The abundance of Ascomycota from this soil-microalgal fermentation screen suggests that they are efficient at colonising algae biomass. A previous analysis of a marine green macroalga *Flabellia petiolata* found that its natural fungal microbiome was also dominated by Ascomycota [40]. Very few studies have examined fungal species associated with Chlorophyta, especially microalgae, and while there is some evidence of a fungal (*Acremonium* sp.) symbiosis with *Chlorella sorokiniana* [41], there is no previous evidence of saprophytic fungi associating with microalgae. The predominance of *Fusarium* isolates in this study is interesting since most *Fusarium* species are soil saprophytes and are significant plant pathogens [42], and therefore their ability to penetrate host tissues via their enzymatic capacity can be exploited. For instance, the capacity of cell wall degrading enzymes of *F. oxysporum* to saccharify corn stalk and switchgrass [32] as well as lignin degradation by *F. solani* [43] has previously been demonstrated. The potential microalgal biomass degraders should be attached to the surface of the algal biomass for the degradation process to occur, as demonstrated previously in studies concerning fungal degradation of different polymers [28, 44, 45]. However, to ensure that the isolated fungal strains are mainly responsible for degradation of biomass and not simply present within the soil but with no role in degradation, future studies may need to monitor the isolation of fungal strains at earlier time points rather than only at the end of the process.

Previous studies that treated microalgal biomass with a cocktail of specified commercial cell wall degrading enzymes demonstrated the release of 10% of the carbohydrate sugars from *C. sorokiniana* biomass [20], and 76% of sugars from *C. vulgaris* biomass [46] for subsequent production of bioethanol. However, these extractions also required additional pre-treatment steps, such as use of bead beating, acid or alkaline pre-treatment or microwave radiation in order to extract most of the

sugars from the cells. Our experiments have demonstrated the efficient capability of fungal crude enzyme for releasing sugars from intact microalgal biomass when this is the only treatment method with no additional physicochemical pre-treatment steps needed. Other studies that used enzymatic pre-treatment for extracting microalgal sugars employed polymers such as cellulose, starch or pectin incorporated within the fungal growth media to trigger the excretion of enzymes by the degrading microorganism [47]. Secretion of degrading enzymes from the fungal strains in this study was achieved using a minimal media incorporated with intact microalgal biomass as a sole carbon source.

One previous study also used crude fungal enzyme extracts to degrade microalgal cells, although in this case the fungal strains, *Aspergillus lentulus* and *Rhizopus oryzae* that were used had been isolated from the environment and not isolated from a microalgal carbon source [48]. Consequently the sugar release using enzyme extracts from these strains was fairly modest (10 – 20% of total sugars), and while one sample of *A. lentulus* enzyme was able to release 44% of the total sugars, the saccharification efficiency was less than achieved here with the enzyme extract from *D. nanus*. Of the three sets of enzyme extract tested here, the *D. nanus* derived sample was the most efficient. This may suggest that the crude extract from the *D. nanus* isolate contained higher enzyme abundance or more efficient degrading enzymes that may be more compatible with the polysaccharides present in *C. vulgaris* compared to the enzyme extracts from *A. elegans* and *F. solani*.

Efficient utilisation of microalgal biomass will require exploitation of all available products for maximum efficiency [2], thus lipids can be extracted for uses such as for biodiesel production, while carbohydrates can then be released from the residual biomass such as for bioethanol production. Therefore we examined the saccharification of residual *C. vulgaris* biomass following lipid extraction and observed that the sugar release was higher from this biomass compared to the intact biomass. The lipid

extraction process included solvent extraction with a mixture of chloroform:methanol coupled with high temperature (40 – 50°C) and a drying process, therefore it is expected that the complexity of cell wall bonds will weaken. In support of this, it has been previously demonstrated that lipid extraction by chloroform:methanol solvent treatment elicits some cell wall damage as determined by the presence of disrupted and distorted cells, although this is not as damaging to cell wall structure as sonication, which causes substantial disruption of cells [49]. Future work will be needed to fully examine exactly how the cell wall structure changes in response to these treatments. We therefore suggest that weakening of the *C. vulgaris* cell wall caused by the solvent treatment may have facilitated the enzymatic degradation and explained the higher release of sugars compared to from the intact biomass, and therefore this could be considered as a pre-treatment.

In conclusion, we have demonstrated the ability to isolate fungal strains that can be sources of novel enzymes to efficiently break down and release sugars from microalgal biomass. Crude enzymes from *D. nanus* showed the highest saccharification efficiency followed by *A. elegans* and *F. solani*. The saccharification efficiency was significantly higher in lipid-extracted biomass than in intact biomass, indicating the benefit of utilising microalgal biomass for multiple products. The saccharification conditions were maintained at 37°C and pH 5.5 to ensure the compatibility of these conditions with subsequent fermentation steps, which is usually performed by *Saccharomyces cerevisiae*. As such, two separate steps for saccharification and fermentation could be avoided which would minimise any additional costs. The potential outcome from this research is to improve the saccharification efficiency of microalgal biomass without need for pre-treatment steps. Future analysis of the fungal secretomes will allow identification and purification of the enzymes responsible for the biomass degradation.

Acknowledgements

M. K. Monjed thanks Umm Al-Qura University, Ministry of Education, Saudi Arabia for providing a PhD scholarship. We are grateful to Dr Olumayowa Osundeko for providing microalgae strains.

References

- [1] D. Gangl, J.A.Z. Zedler, P.D. Rajakumar, E.M.R. Martinez, A. Riseley, A. Włodarczyk A, S. Purton, Y. Sakuragi, C.J. Howe, P.E. Jensen, C. Robinson, Biotechnological exploitation of microalgae, *J. Exp. Bot.* 66 (22) (2015) 6975-6990.
- [2] K.W. Chew, J.Y. Yap, P.L. Show, N.H. Suan, J.C. Juan, T.C. Ling, D.-J. Lee, J.-S. Chang, Microalgae biorefinery: High value products perspectives, *Bioresour. Technol.* 229 (2017) 53-62.
- [3] J. Ruiz, G. Olivieri, J. de Vree, R. Bosma, P. Willems, J.H. Reith, M.H.M. Eppink, D.M.M. Kleinegris, R.H. Wijffels, M.J. Barbosa, Towards industrial products from microalgae, *Energ. Environ. Sci.* 9 (10) (2016) 3036-3043.
- [4] M.I. Khan, J.H. Shin, J.D. Kim, The promising future of microalgae: current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products, *Microb. Cell Fact.* 17 (1) (2018) 36.
- [5] Y. Chisti, Biodiesel from microalgae, *Biotechnol. Adv.* 25 (3) (2007) 294-306.
- [6] R.P. John, G.S. Anisha, K.M. Nampoothiri, A. Pandey, Micro and macroalgal biomass: A renewable source for bioethanol, *Bioresour. Technol.* 102 (1) (2011) 186-193.
- [7] K. Gao, V. Orr, L. Rehmann, Butanol fermentation from microalgae-derived carbohydrates after ionic liquid extraction, *Bioresour. Technol.* 206 (2016) 77-85.
- [8] A. Demirbas, Biofuels securing the planet's future energy needs, *Energ. Convers. Manage.* 50 (9) (2009) 2239-2249.

468 [9] A. Singh, P.S. Nigam, J.D. Murphy, Renewable fuels from algae: An answer to
469 debatable land based fuels, *Bioresour. Technol.* 102 (1) (2011) 10-16.

470 [10] J.K. Pittman, A.P. Dean, O. Osundeko, The potential of sustainable algal biofuel
471 production using wastewater resources, *Bioresour. Technol.* 102 (1) (2011) 17-25.

472 [11] O. Osundeko, H. Davies, J.K. Pittman, Oxidative stress-tolerant microalgae
473 strains are highly efficient for biofuel feedstock production on wastewater, *Biomass
474 Bioenergy* 56 (2013) 284-294.

475 [12] O. Osundeko, J.K. Pittman, Implications of sludge liquor addition for
476 wastewater-based open pond cultivation of microalgae for biofuel generation and
477 pollutant remediation, *Bioresour. Technol.* 152 (2014) 355-363.

478 [13] Y. Chisti, Constraints to commercialization of algal fuels, *J. Biotechnol.* 167 (3)
479 (2013) 201-214.

480 [14] S.Y. Lee, J.M. Cho, Y.K. Chang, Y.-K. Oh, Cell disruption and lipid extraction
481 for microalgal biorefineries: A review, *Bioresour. Technol.* 244 (2017) 1317-1328.

482 [15] H.G. Gerken, B. Donohoe, E.P. Knoshaug, Enzymatic cell wall degradation of
483 *Chlorella vulgaris* and other microalgae for biofuels production, *Planta* 237 (1) (2013)
484 239-253.

485 [16] P.-H. Baudalet, G. Ricochon, M. Linder, L. Muniglia, A new insight into cell walls
486 of Chlorophyta, *Algal Res.* 25 (2017) 333-371.

487 [17] H. Takeda, Sugar composition of the cell wall and the taxonomy of *Chlorella*
488 (Chlorophyceae), *J. Phycol.* 27 (2) (1991) 224-232.

489 [18] Á.B. Juárez, C.G. Vélez, A.R. Iñiguez, D.E. Martínez, M.C. Rodríguez, M.S.
490 Vigna, M. del Carmen Ríos de Molina, A *Parachlorella kessleri* (Trebouxiophyceae,
491 Chlorophyta) strain from an extremely acidic geothermal pond in Argentina, *Phycologia*
492 50 (4) (2011) 413-421.

493 [19] R. Halim, R. Harun, M.K. Danquah, P.A. Webley, Microalgal cell disruption for
494 biofuel development, *Appl. Energ.* 91 (1) (2012) 116-121.

495 [20] D. Hernández, B. Riaño, M. Coca, M.C. García-González, Saccharification of
 496 carbohydrates in microalgal biomass by physical, chemical and enzymatic pre-
 497 treatments as a previous step for bioethanol production, Chem. Eng. J. 262 (2015)
 498 939-945.

499 [21] O.K. Lee, A.L. Kim, D.H. Seong, C.G. Lee, Y.T. Jung, J.W. Lee, E.Y. Lee,
 500 Chemo-enzymatic saccharification and bioethanol fermentation of lipid-extracted
 501 residual biomass of the microalga, *Dunaliella tertiolecta*, Bioresour. Technol. 132
 502 (2013) 197-201.

503 [22] A.-M. Aguirre, A. Bassi, Investigation of an integrated approach for bio-crude
 504 recovery and enzymatic hydrolysis of microalgae cellulose for glucose production, Can.
 505 J. Chem. Eng. 94 (2) (2016) 238-243.

506 [23] F. Lü, J. Ji, L. Shao, P. He, Bacterial bioaugmentation for improving methane
 507 and hydrogen production from microalgae, Biotechnol. Biofuels 6 (1) (2013) 92.

508 [24] J. van den Brink, R.P. de Vries, Fungal enzyme sets for plant polysaccharide
 509 degradation, Appl. Microbiol. Biotechnol. 91 (6) (2011) 1477.

510 [25] T. Darienko, T. Pröschold, The genus *Jaagichlorella* Reisi
 511 (Trebouxiophyceae, Chlorophyta) and its close relatives: an evolutionary puzzle,
 512 Phytotaxa 388 (1) (2019) 22.

513 [26] J. Feng, R. Hwang, K.F. Chang, S.F. Hwang, S.E. Strelkov, B.D. Gossen, Q.
 514 Zhou, An inexpensive method for extraction of genomic DNA from fungal mycelia, Can.
 515 J. Plant Pathol. 32 (3) (2010) 396-401.

516 [27] T.J. White, T. Bruns, S. Lee, J. Taylor, Amplification and direct sequencing of
 517 fungal ribosomal RNA genes for phylogenetics, in: M.A. Innis, D.H. Gelfand, J.J.
 518 Sninsky, T.J. White (Eds.), PCR Protocols, Academic Press, San Diego, 1990, pp. 315-
 519 322.

- [28] U. Zafar, A. Houlden, G.D. Robson, Fungal communities associated with the biodegradation of polyester polyurethane buried under compost at different temperatures, *Appl. Environ. Microbiol.* 79 (23) (2013) 7313.
- [29] M. Gardes, T.D. Bruns, ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts, *Mol. Ecol.* 2 (2) (1993) 113-118.
- [30] S. Kumar, G. Stecher, K. Tamura, MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets, *Mol. Biol. Evol.* 33 (7) (2016) 1870-1874.
- [31] P. Chand, A. Aruna, A.M. Maqsood, L.V. Rao, Novel mutation method for increased cellulase production, *J. Appl. Microbiol.* 98 (2) (2005) 318-323.
- [32] B.C. King, M.K. Donnelly, G.C. Bergstrom, L.P. Walker, D.M. Gibson, An optimized microplate assay system for quantitative evaluation of plant cell wall-degrading enzyme activity of fungal culture extracts, *Biotechnol. Bioeng.* 102 (4) (2009) 1033-1044.
- [33] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem.* 31 (3) (1959) 426-428.
- [34] A.K. Bajhaiya, A.P. Dean, T. Driver, D.K. Trivedi, N.J.W. Rattray, J.W. Allwood, R. Goodacre, J.K. Pittman, High-throughput metabolic screening of microalgae genetic variation in response to nutrient limitation, *Metabolomics* 12 (1) (2016) 9.
- [35] D.M. Updegraff, Semimicro determination of cellulose in biological materials, *Anal. Biochem.* 32 (3) (1969) 420-424.
- [36] L. Balabanova, L. Slepchenko, O. Son, L. Tekutyeva, Biotechnology potential of marine fungi degrading plant and algae polymeric substrates, *Front. Microbiol.* 9 (2018) 1527.
- [37] J. Pietikäinen, M. Pettersson, E. Bååth, Comparison of temperature effects on soil respiration and bacterial and fungal growth rates, *FEMS Microbiol. Ecol.* 52 (1) (2005) 49-58.

547 [38] N. Trivedi, C.R.K. Reddy, R. Radulovich, B. Jha, Solid state fermentation
548 (SSF)-derived cellulase for saccharification of the green seaweed *Ulva* for bioethanol
549 production, *Algal Res.* 9 (2015) 48-54.

550 [39] R.P. Singh, V. Gupta, P. Kumari, M. Kumar, C.R.K. Reddy, K. Prasad, B. Jha,
551 Purification and partial characterization of an extracellular alginate lyase from
552 *Aspergillus oryzae* isolated from brown seaweed, *J. Appl. Phycol.* 23 (4) (2011) 755-
553 762.

554 [40] G. Gnani, L. Garzoli, A. Poli, V. Prigione, G. Burgaud, G.C. Varese, The
555 culturable mycobiota of *Flabellia petiolata*: First survey of marine fungi associated to a
556 Mediterranean green alga, *PLOS ONE* 12 (4) (2017) e0175941.

557 [41] K. Watanabe, N. Takihana, H. Aoyagi, S. Hanada, Y. Watanabe, N. Ohmura, H.
558 Saiki, H. Tanaka, Symbiotic association in *Chlorella* culture, *FEMS Microbiol. Ecol.* 51
559 (2) (2005) 187-196.

560 [42] R. Dean, J.A.L. van Kan, Z.A. Pretorius, K.E. Hammond-Kosack, A. Di Pietro,
561 P.D. Spanu, J.J. Rudd, M. Dickman, R. Kahmann, J. Ellis, G.D. Foster, The Top 10
562 fungal pathogens in molecular plant pathology, *Mol. Plant Pathol.* 13 (4) (2012) 414-
563 430.

564 [43] V.V. Lozovaya, A.V. Lygin, O.V. Zernova, S. Li, J.M. Widholm, G.L. Hartman,
565 Lignin degradation by *Fusarium solani* f. sp. glycines, *Plant Dis.* 90 (1) (2006) 77-82.

566 [44] M. Karamanlioglu, A. Houlden, G.D. Robson, Isolation and characterisation of
567 fungal communities associated with degradation and growth on the surface of
568 poly(lactic) acid (PLA) in soil and compost, *Int. Biodeterior. Biodegrad.* 95 (2014) 301-
569 310.

570 [45] A.S. Al Hosni, J.K. Pittman, G.D. Robson, Microbial degradation of four
571 biodegradable polymers in soil and compost demonstrating polycaprolactone as an
572 ideal compostable plastic, *Waste Manage.* 97 (2019) 105-114.

573 [46] K.H. Kim, I.S. Choi, H.M. Kim, S.G. Wi, H.-J. Bae, Bioethanol production from
 574 the nutrient stress-induced microalga *Chlorella vulgaris* by enzymatic hydrolysis and
 575 immobilized yeast fermentation, *Bioresour. Technol.* 153 (2014) 47-54.

576 [47] S.-H. Ho, S.-W. Huang, C.-Y. Chen, T. Hasunuma, A. Kondo, J.-S. Chang,
 577 Bioethanol production using carbohydrate-rich microalgae biomass as feedstock,
 578 *Bioresour. Technol.* 135 (2013) 191-198.

579 [48] S.K. Prajapati, A. Bhattacharya, A. Malik, V.K. Vijay, Pretreatment of algal
 580 biomass using fungal crude enzymes, *Algal Res.* 8 (2015) 8-14.

581 [49] A. Ranjan, C. Patil, V.S. Moholkar, Mechanistic assessment of microalgal lipid
 582 extraction, *Ind. Eng. Chem. Res.* 49 (2010) 2979-2985.

583

Figures

Fig. 1 Microalgae biomass degradation in soil. (a) *J. luteoviridis* biomass. (b) *H. tetracontoma* biomass. (c) *P. hussii* biomass. Dried biomass was incubated in soil at 25°C for 8 weeks. Each data point represents the mean $\pm$ SEM of five independent replicates.

Fig. 2 Fungal growth on degraded microalgal biomass. Total viable fungal counts (CFU) on residual microalgal biomass that was incubated in soil for 8 weeks in comparison to a control (no biomass). Each sample was serially diluted in PBS and incubated on selective PDA plates at 25°C for up to 7 days. Each data point represents a mean $\pm$ SEM of three independent replicates. Asterisks above a bar indicate a significant difference ($p < 0.05$) compared to the control.

599

600 **Fig. 3** Fungal isolates recovered from degraded residues of microalgae biomass
 601 following incubation in soil at 25°C for 8 weeks. (a) *J. luteoviridis* biomass isolates. (b)
 602 *H. tetrachotoma* biomass isolates. (c) *P. hussii* biomass isolates.
 603

Fig. 4 Phylogenetic tree of fungal isolates recovered from degraded microalgal biomass. A triangle symbol indicates the isolates identified from microalgal biomass and a circle symbol indicates the isolates identified from control soil (no biomass). The microalgal biomass source is indicated (J.L: *J. luteoviridis*, H.T: *H. tetrachotoma*, P.H: *P. hussii*). The neighbour-joining tree is based on ITS1-5.8S-ITS2 DNA sequences. Comparison is made with selected sequences from known fungal strains from NCBI GenBank database with accession number provided. Bootstrap values are represented as percentages from 1000 replications.

614

615 **Fig. 5** Saccharification of *C. vulgaris* biomass by fungal crude enzyme extracts. (a)
 616 Treatment of intact biomass. (b) Treatment of lipid-extracted biomass. Biomass at a
 617 concentration of 1.5% (w/v) was incubated with enzyme extracts from *A. elegans*
 618 (strain J.L S2), *D. nanus* (strain J.L S3) and *F. solani* (strain J.L S1). Each data point
 619 represents a mean $\pm$ SEM of three independent replicates. Within each incubation time
 620 point that shows significantly different data, values that are significantly different do not
 621 share a lowercase letter ($p < 0.05$).

Table 1 Fungal taxa assignments identified using ITS sequencing of rRNA gene amplification from samples isolated from control conditions or from residual degraded microalgal biomass buried in soil at 25°C for 8 weeks. The closest sequence matches from the NCBI GenBank database determined by BLAST are shown.

Fungal isolate name	Isolate identification	Sequence identity % to closest match (Accession number of closest match)	Strength of growth on PDA plates ^a
Control (soil without added biomass)			
C. S1	<i>Arthrographis kalrae</i>	98% (HG004577.1)	+++
C. S4	<i>Aspergillus</i> sp.	99% (HQ631021.1)	++
C. S5	<i>Arthrographis kalrae</i>	99% (AB506810.1)	+
<i>Jaagichlorella luteoviridis</i> biomass			
J.L S1	<i>Fusarium solani</i>	99% (KP265368.1)	+++
J.L S2	<i>Actinomucor elegans</i>	99% (AY492091.1)	++
J.L S3	<i>Doratomyces nanus</i>	99% (FJ914668.1)	+
J.L S5	<i>Aphanoascus verrucosus</i>	99% (KP147971.1)	+
<i>Hindakia tetrachotoma</i> biomass			
H.T S1	<i>Fusarium solani</i>	99% (KM268688.1)	+++
H.T S2	<i>Circinella chinensis</i>	99% (JN205855.1)	++
H.T S3	<i>Actinomucor elegans</i>	99% (JQ683221.1)	++
H.T S4	<i>Aphanoascus verrucosus</i>	99% (KP147971.1)	+
H.T S6	<i>Fusarium oxysporum</i>	99% (GU566301.1)	+
H.T S7	<i>Doratomyces</i> sp.	99% (HQ832968.1)	+
<i>Parachlorella hussii</i> biomass			
P.H S1	<i>Fusarium solani</i>	99% (KM268688.1)	+++
P.H S2	<i>Aphanoascus verrucosus</i>	99% (KP147971.1)	++
P.H S4	<i>Penicillium citrinum</i>	99% (KP757562.1)	+
P.H S5	<i>Fusarium solani</i>	99% (HE974458.1)	+
P.H S6	<i>Fusarium oxysporum</i>	100% (KC201696.1)	+
P.H S7	<i>Circinella chinensis</i>	99% (JN205855.1)	+

^a indicates the relative growth dominance of identified isolates on PDA plates: +++ 60-70%; ++ 20-30%; + ≤ 5-10%.

Table 2 Concentration of released reducing sugars from intact or lipid extracted *C. vulgaris* microalgal biomass after 70 h of incubation with crude fungal enzyme extracts.

Enzyme extract fungal source	Sugar release from intact biomass (mg g ⁻¹)	Saccharification efficiency from intact biomass (%)	Sugar release from lipid-extracted biomass (mg g ⁻¹)	Saccharification efficiency from lipid-extracted biomass (%)
<i>A. elegans</i>	59.33 ± 4.67	34.2	92.03 ± 12.68	52.9
<i>D. nanus</i>	98.67 ± 21.33	56.8	131.35 ± 5.30	75.7
<i>F. solani</i>	58.67 ± 8.00	34.0	97.46 ± 7.33	56.0