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ORIGINAL ARTICLE

Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production

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Abstract Cell walls of microalgae consist of a polysaccharide and glycoprotein matrix providing the cells with a formidable defense against its environment. We characterized enzymes that can digest the cell wall and weaken this defense for the purpose of protoplasting or lipid extraction. A growth inhibition screen demonstrated that chitinase, lysozyme, pectinase, sulfatase, β-glucuronidase, and laminarinase had the broadest effect across the various *Chlorella* strains tested and also inhibited *Nannochloropsis* and *Nannochloris* strains. *Chlorella* is typically most sensitive to chitinases and lysozymes, both enzymes that degrade polymers containing *N*-acetylglucosamine. Using a fluorescent DNA stain, we developed rapid methodology to quantify changes in permeability in response to enzyme digestion and found that treatment with lysozyme in conjunction with other enzymes has a drastic effect on cell permeability. Transmission electron microscopy of enzymatically treated *Chlorella vulgaris* indicates that lysozyme degrades the outer surface of the cell wall and removes hair-like fibers protruding from the surface, which differs from the activity of chitinase. This action on the outer surface of the cell causes visible protuberances on the cell surface and presumably leads to the increased settling rate when cells are treated with lysozyme. We demonstrate radical ultrastructural changes to the cell wall in response to treatment with various enzyme combinations which, in some cases, causes a greater than twofold increase in the thickness of the cell wall. The enzymes characterized in this study should prove useful in the engineering and extraction of oils from microalgae.

Keywords Growth inhibition · Permeability · Lysozyme · Nitrogen depletion

Abbreviations

NREL	National renewable energy laboratory
TFA	Triflouroacetic acid
TLS	Trilaminar structure
TEM	Transmission electron microscopy
CCAP	Culture collection of algae and protozoa
NCMA	Formerly CCMP, Provasoli-Guillard National Center for marine algae and microbiota
ATCC	American type culture collection
UTEX	Culture collection of algae at the University of Texas at Austin
ASP	NREL Aquatic species program
mBBM	Modified bold's basal medium
ASW	Artificial seawater
PBS	Phosphate buffered saline
SEM	Scanning electron microscopy

Introduction

As algae are increasingly being considered as a potential feedstock to meet a portion of our liquid transportation fuels

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demand, many hurdles to the development of an algal biomass to liquid transportation fuel process have been indentified (Scott et al. 2010; Wijffels and Barbosa 2010; Knoshaug and Darzins 2011). A major hurdle that has generated much interest from the industrial and research communities is the extraction of the internal lipid bodies from algal cells. Typically, lipid extraction methods use solvents such as toluene, hexane, butanol, ethanol, methanol or ionic liquids to extract lipids from intact, chemically treated or mechanically ruptured cells (Pienkos and Darzins 2009; Shen et al. 2009; Brennan and Owende 2010; Lee et al. 2010; Siddiquee and Rohani 2011; Kim et al. 2012). Due to the chemically complex and structurally robust nature of algal cell walls, many current extraction processes require large chemical or energy loads. Technoeconomic analysis suggests that for an algae-to-biofuel process to be profitable, the post-extraction algal biomass must also be utilized as either materials recycled back into the process or as a feedstock for further conversion to saleable co-product (Davis et al. 2011). Enzymatic degradation of the algal cell walls prior to lipid extraction has the potential to facilitate both lipid extraction and post-extraction use of the algal biomass. Weakened cell walls could reduce solvent and energy inputs needed for lipid extraction by either liberating cell wall mono or polysaccharides or by improving accessibility of cell wall polymers to microorganisms, enzymes or reagents involved in downstream algal biomass processing such as anaerobic digestion, fermentation or direct conversion. At the National Renewable Energy Laboratory (NREL), *Chlorella vulgaris* UTEX395 has been established as our model green alga, because it grows rapidly, can attain a high lipid content (>45 % of cell dry weight), has large scale cultivation history, and has reports of successful genetic transformation (Guarnieri et al. 2012). The transcriptome and proteome of this organism have recently been revealed under nitrogen-replete and -deplete conditions (Guarnieri et al. 2011), and a genome sequencing project is also underway.

Microalgal cell walls are complex and poorly understood (Popper and Tuohy 2010). The *Chlorella* intraspecies variation in cell walls as well as variations observed in a single strain grown under different conditions can be dramatic, and thus it is difficult to predict which of the compounds noted below will be present in any one strain. While some *Chlorella* species have only a single microfibrillar layer, others have two layers with the microfibrillar layer proximal to the cytoplasmic membrane and a mono or trilaminar outer layer (Yamada and Sakaguchi 1982). The cell walls of *C. vulgaris* and other green microalgae are known to have rigid wall components embedded within a more plastic polymeric matrix. This matrix is defined as the fraction that is hydrolyzable in 1 N NaOH or 2 M triflouroacetic acid (TFA) and contains uronic acids, rhamnose, arabinose, fucose, xylose, mannose, galactose, and glucose. The rigid, TFA-resistant cell wall is either glucosamine or a glucose-mannose polymer (Takeda 1991). The predominant amino sugar found in the rigid cell wall of *Chlorella* Pbi is *N*-acetylglucosamine present as a chitin-like glycan. Chitin (poly-β-1,4-D-*N*-acetylglucosamine), a polymer commonly found in shellfish, insects, fungi, worms, and mushrooms which can be deacetylated, either partially or fully, to produce chitosan (poly-β-1,4-D-glucosamine). Strong acid (6 N HCl) was needed to hydrolyze the rigid cell wall of a *Chlorella* sp., which removed the acetyl group from *N*-acetylglucosamine leaving glucosamine while hydrolysis with strong sulfuric acid failed to release detectable neutral sugars suggesting that this strain of *Chlorella* does not have cellulose in the rigid cell wall. Digestion of the rigid wall with chitinase released *N*-acetylglucosamine yet complete digestion required a chitosanase, which indicates that glucosamine polymers are also present in the rigid wall of *Chlorella* Pbi (Kapaun and Reisser 1995). Based on these previous studies, the genus *Chlorella* was in-part defined as containing glucosamine as the common dominant cell wall polymer (Huss et al. 1999). The neutral cell wall sugars of symbiotic *Chlorella* strains contain decreasing amounts glucose, rhamnose, arabinose, mannose, xylose, fucose, and galactose, respectively (Kapaun et al. 1992). Other than the neutral sugars ($\sim$25 %), the cell wall contained 15–20 % uronic acids, 10–15 % glucosamine, and 6–10 % protein. This cell wall composition was reported to be similar to the glucosamine containing cell wall composition of the non-symbiotic algae *C. vulgaris*, *Chlorella sorokiniana*, and *Chlorella kessleri*; however, the authors were unable to determine the composition of 30–44 % of the cell wall (Kapaun et al. 1992).

Unusual and previously undescribed polymers have also been isolated from *C. vulgaris* cell walls. Two rhamnose containing polymers and a β-D-galactan polymer were isolated (Ogawa et al. 1998, 1999, 2001), and a number of unidentified sugars that in some cases can be present in substantial amounts (Burczyk et al. 1995). Other sugars include 2-*O*-methyl-L-rhamnose and 3-*O*-methyl-L-rhamnose, identified as residues in an acidic polysaccharide of *C. vulgaris* (Ogawa et al. 1997). The cell walls of *C. vulgaris* are readily stained by Ruthenium, Red which typically adheres to pectin-like substances or acidic sugars (Takeda 1991). The presence of pectin has also been demonstrated in *C. vulgaris* and has been shown to increase on extended exposure to alkaline pH of 9.5 (Malis-Arad et al. 1980).

Algal cell walls are highly persistent in sediments rich in organic matter preserved in terrestrial and marine environments. Algaenan is postulated as being the reason for the persistance of algal cell walls (Derenne et al. 1992; Gelin et al. 1999). Initially described as plant lignin-like

and also likened to the sporopollenin of spores and pollen grains, algaenan has been suggested to be responsible for the resistance of algae to bacterial degradation (Gunnison and Alexander 1975). Typically, algaenan, an aliphatic, non-hydrolyzable polymer is located in a thin (10–20 nm) outer wall layer with a trilaminar structure (TLS). Two electron-dense layers surround a less dense layer, which survives drastic chemical treatment typically involving multiple extractions with KOH, strong acids or trifluoroacetic acid (Gelin et al. 1999). The main building blocks of algaenan are described as $C_{30–34}$ mono- or di- unsaturated ω-hydroxy fatty acids which, through a combination of ester and ether linkages, create a very strong and recalcitrant structure similar to cutin (Blokker et al. 1998) and lacking in carotenoid moieties (Derenne et al. 1992). *C. vulgaris* lacks a typical TLS cell wall structure (Corre et al. 1996) and was found to be more sensitive to detergents than a species, *Chlorella emersonii*, having a TLS outer cell wall structure. However, as the cells aged, they become more resistant to detergents, particularly *C. vulgaris* (Corre et al. 1996). Algaenan from *Botryococcus braunii* race A has been suggested to change in composition, mainly in the reduction of terminal methyl groups, depending on growth stage and conditions (Simpson et al. 2003).

Enzymatic digestion of algal cell walls has been previously studied as a methodology to make proto or spheroplasts of algal cells usually as a step in cell preparation for genetic transformation. Naked protoplasts, termed spheroplasts, of *Chlorella saccharophila* and *Chlorella ellipsoidea* were made using an enzyme mix containing cellulase, hemicellulase, and pectinase activities (Braun and Aach 1975). However, these green algae, which are presumed not to have an outer layer or sporopollenin (now known as algaenan), required up to 90 h to display any effect from the enzymes. The cells became very sensitive to physical pressures, bursting simply from the pressure of a cover slip placed on a microscope slide (Braun and Aach 1975). Similarly, protoplasts were made from *C. vulgaris* K-73122 using acromopeptidase, cellulase, chitosanase, gluczyme, and uskizyme; however, only $\sim$88 % of the cells were considered osmotically labile (Honjoh et al. 2003). Protoplasts were also made in *C. ellipsoidea*, *C. vulgaris*, and *C. saccharophila* using three different enzyme mixes containing cellulase, macerozyme, pectinase, cellulysin, and driselase, although 7 of the 12 strains tested showed no protoplast formation with any of the formulations (Yamada and Sakaguchi 1982).

Bacterial degradation was examined by incubating *C. emersonii* (TLS outer wall) or *C. vulgaris* (non-TLS outer wall) with two types of aerobic heterotrophic bacteria. After 4 months, there was no bacterial attachment to or penetration into the algal cells, indicating the effects of degradation must be due to extracellular enzymes produced by the bacteria (Afi et al. 1996). In addition, similar changes to the internal contents of the algal cells indicate that the TLS layer did not protect against the diffusion of enzymes into algal cells possessing this cell wall structure even though the cell wall architecture remained un-degraded. Similarly, as *Chlamydomonas reinhardtii* or *Selenastrum capricornutum* cells aged and become nitrogen or phosphate limited, they became resistant to digestion by grazers suggesting the layers in the cell wall became thicker and more protective to enzymatic digestion in the gut (Van Donk et al. 1997). Snail gut enzymes (glusulase) have been observed to digest the inner, less electron-dense cell wall layer, but did not affect the TLS layer in *Chlorella fusca* cell wall fragments. When glusulase and a wide variety of other enzymes, including lysozyme, were tried on intact cells, there was no effect on the cell walls as judged by transmission electron microscopy (TEM) imaging (Atkinson et al. 1972). *C. fusca* contains a TLS outer layer enclosing all the other cell wall layers and is impervious to enzymatic attack or penetration by the enzymes to attack other internal layers. Interestingly, during cell reproduction, the inner non-TLS component was degraded by internal enzymes (Atkinson et al. 1972) and enzymes with the activities of β-D-fucosidase, β-D-mannosidase, and β-D-glucosidase were identified that play roles in the disintegration of the mother cell wall (sporangium) to release autospores (Walter and Aach 1987). These enzymes released oligomers from the inner wall to loosen the cell wall rather than completely degrade it while not impacting the outer TLS wall. Similarly, subtilase-like serine proteases perform this function in *C. reinhardtii* and *Volvox carteri* (Fukada et al. 2006; Kubo et al. 2009).

In the work presented here, we sought to define the extent of degradation that a variety of enzymatic activities have on the cell walls of *C. vulgaris*. As the cell wall provides the main protective barrier between the algae and its surroundings, a detailed characterization of enzymes that act on the wall could enable the rational design of an enzyme cocktail that would allow efficient degradation of the wall. Removal of the wall could allow more efficient transformation technologies to be developed, conversion of these cell wall sugars into a more usable substrate for downstream processes, and potentially ease lipid extraction from these organisms.

Materials and methods

Strains, enzymes, and growth conditions

We acquired the following strains from established algae collections [Culture Collection of Algae and Protozoa

(CCAP), Provasoli-Guillard National Center for Marine Algae and Microbiota (NCMA), formerly CCMP], American Type Culture Collection (ATCC) and the Culture Collection of Algae at The University of Texas at Austin (UTEX), *C. emersonii* CCAP 211/11N, *Chlorella variabilis* NC64A (ATCC 50258), *C. vulgaris* UTEX 26, 30, 259, 265, 395, 396, 1803, 1809, 1811, 2714, and CCAP 211/11B, *Phaeodactylum tricornutum* CCMP 632, and *S. capricornutum* UTEX 1648. We also used the following strains from the NREL Aquatic Species Program (ASP) collection *Ankistrodesmus falcatus* ANKIS1, *Chlorella* sp. CHLOR1, *Franceia* sp. FRANC1, *Nannochloris* sp. NANNO2, *Nannochloropsis* sp. NANNP2, and *Oocystis pusilla* OOCYS1 (Barclay et al. 1987; Sheehan et al. 1998).

Achromopeptidase, alginate lyase, α-amylase (960 FAU/mL), chitinase, chitosanase (25.9 U/mL), chondroitinase ABC (5 U/mL), drieselase, β-galactosidase (1,000 U/mL), β-glucosidase, hemicellulase, heparinase II (10 U/mL), hesperidinase, hyaluronidase, kitalase, laminarinase, lyticase, lysing enzymes from *Aspergillus* sp., lysozyme, mutanolysin (10 U/mL), pectinase (3,000 U/mL), pectolyase, phospholipase A_1 (10 kU/mL), phospholipase A_2, proteinase K, sulphatase (3.37 mg/mL), and trypsin were purchased from Sigma. β-glucuronidase (140 U/mL) was purchased from Roche. Neuraminidase (50 kU/mL), β-*N*-acetylhexosaminidase (5 kU/mL), β-*N*-acetylglucosaminidase (4 kU/mL), α-1-6 mannosidase (32 kU/mL), α-2-3 neuraminidase (50 kU/mL), 1-2,3 mannosidase (40 kU/mL), and endo-α-*N*-acetylgalactosaminidase (40,000 kU/mL) were purchased from New England Biolabs. Glusulase was purchased from NEN (10,000 U sulfatase and 90,000 U β-glucuronidase/mL). Zymolyase (10 mg/mL) was purchased from ZymoResearch. Macerozyme was purchased from RPI corp. Cellulase was purchased from Onozuka. Stock concentrations of enzymes were 20 mg/mL unless otherwise noted.

Chlorella and *Selenastrum* were grown in a modified Bold's Basal medium (mBBM) (3 mM $NaNO_3$, 170 μM $CaCl_2{\cdot}2\ H_2O$, 304 μM $MgSO_4{\cdot}7H_2O$, 431 μM K_2HPO_4, 1.3 mM KH_2PO_4, 428 μM NaCl, 12 μM Na_2EDTA, 2.2 μM $FeCl_3{\cdot}6H_2O$, 1.2 μM $MnCl_2{\cdot}4H_2O$, 220 nM $ZnSO_4{\cdot}7H_2O$, 50 nM $CoCl_2{\cdot}6H_2O$, 99 nM $Na_2MoO_4{\cdot}2H_2O$, 6.4 μM $CuSO_4{\cdot}5H_2O$, 184 μM H_3BO_3) (UTEX media recipe for Modified Bold 3N medium). *C. variabilis* NC64A was grown on mBBM containing Proteose Peptone [0.1 % (w/v)] and sucrose [0.5 % (w/v)] (Van Etten et al. 1991). *Nannochloris*, *Nannochloropsis*, and *Oocystis* were grown in SERI Type I media (3 mM $NaNO_3$, 20.2 mM $MgCl_2{\cdot}6H_2O$, 2.6 mM KCl, 2.2 mM $NaHCO_3$, 36.2 mM NaCl, 12.4 mM $CaSO_4{\cdot}1/2H_2O$, 3 nM thiamine-HCl, 8.2 nM biotin, 0.8 nM cobalamin B_{12}, 0.6 mM KH_2PO_4, 81 uM Na_2EDTA, 5.4 uM $FeCl_3{\cdot}6H_2O$, 553 uM H_3BO_3, 22 uM $MnCl_2{\cdot}4H_2O$, 2.2 uM $ZnCl_2$, 0.55 uM $CoCl_2{\cdot}6H_2O$) (Barclay et al. 1987). *Ankistrodesmus* was grown in SERI Type II media (3 mM $NaNO_3$, 0.252 mM $CaCl_2$, 9.6 mM $MgCl_2{\cdot}6H_2O$, 18.8 mM Na_2SO_4, 6.3 mM KCl, 14.4 mM $NaHCO_3$, 2.2 mM Na_2CO_3, 25.9 mM NaCl, 3 nM thiamine-HCl, 8.2 nM biotin, 0.8 nM cobalamin B_{12}, 0.6 mM KH_2PO_4, 81 uM Na_2EDTA, 5.4 uM $FeCl_3{\cdot}6H_2O$, 553 uM H_3BO_3, 22 uM $MnCl_2{\cdot}4H_2O$, 2.2 uM $ZnCl_2$, 0.55 uM $CoCl_2{\cdot}6H_2O$) (Barclay et al. 1987). *Franceia* was grown in artificial seawater (ASW) medium at 10 % of full strength (47.8 mM NaCl, 3 mM $MgSO_4{\cdot}7H_2O$, 2.6 mM $MgCl_2{\cdot}6H_2O$, 1 mM $CaCl_2$, 1 mM KCl, 3 mM $NaNO_3$, 2.4 mM $NaHCO_3$, 1 mM $Na_2SiO_3{\cdot}9\ H_2O$, 97 uM H_3BO_3, 37 uM NaH_2PO_4, 78 uM Na_2EDTA, 12 uM $FeC_6H_5O_7$, 40 nM $CuSO_4{\cdot}5H_2O$, 77 nM $ZnSO_4{\cdot}7H_2O$, 42 nM $CoCl_2{\cdot}6H_2O$, 25 nM $Na_2MoO_4{\cdot}2H_2O$, 1.1 uM $MnSO_4{\cdot}H_2O$, 5 mM HEPES, 300 nM thiamine-HCl, 2 nM biotin, 0.4 nM cobalamin B_{12}) modified from Brown (1982). *Phaeodactylum* was grown in a defined version of f/2 medium (445 mM NaCl, 32.5 mM $MgCl_2$, 18.3 mM $MgSO_4$, 8.8 mM $CaSO_4{\cdot}1/2H_2O$, 9.4 mM KCl, 1.4 mM NaBr, 883 uM $NaNO_3$, 36.2 uM $NaH_2PO_4{\cdot}H_2O$, 300 nM thiamine-HCl, 2 nM biotin, 0.4 nM cobalamin B_{12}, 11.7 uM Na_2EDTA, 11.7 uM $FeCl_3{\cdot}6H_2O$, 910 nM $MnCl_2{\cdot}4H_2O$, 40 nM $CuSO_4{\cdot}5H_2O$, 77 nM $ZnSO_4{\cdot}7H_2O$, 42 nM $CoCl_2{\cdot}6H_2O$, 25 nM $Na_2MoO_4{\cdot}2H_2O$) based on (Glover 1977). Plate media contained 15 g/L agar. Cultures were grown in shake flasks at 25 °C with 16 h light and 8 h dark cycles with cool white fluorescent illumination (200 μmol photons /m^2 s) in an incubator supplemented with a 5 % CO_2/air mixture.

Enzyme inhibition assay

To assay growth inhibition due to enzymatic activity, 200 μL of algal cells normalized to an OD_{750} of 1.0 was mixed with 4 mL of media containing 7.5 g/L agar, which was at 42 °C and poured on a petri plate containing 15 g/L agar. Once hardened, 10 μL of enzyme stock was then spotted on the top agar and plates were incubated in the light at 23 °C for 5 days. As a negative control for enzymatic activity, enzymes were heat denatured at 100 °C for 10 min and spotted on plates.

18S DNA sequencing

Genomic DNA for the 18S ribosomal subunit was amplified by colony PCR using the 360FE: 5′-CGGAGARGG MGCMTGAGA-3′ forward and 1391RE: 5′-GGGCGGT GTGTACAARGRG-3′ reverse primers (Dawson and Pace 2002). Briefly, cells were boiled for 10 min in 2X Phusion GC buffer. After boiling, primers (0.5 uM final concentration), dNTPs (0.2 mM final concentration), Phusion DNA polymerase (Finnzymes, 0.01 U final concentration), and water were added to the reaction mixture such that the

final concentration of Phusion GC buffer was 1X. Thermocycler conditions were as follows: initial denaturation 98 °C for 30 s, followed by 29 cycles of 98 °C for 10 s, 56 °C for 30 s, 72 °C for 2 min, with a final extension of 72 °C for 10 min. Amplified products were electrophoresed on a 0.8 % agarose gel, purified using a QIAquick Gel Extraction Kit (Qiagen), and sequenced using the above primers.

Flow cytometry

Cell permeability assays were performed on the Amnis ImagestreamX using SYTOX Green DNA staining dye (Invitrogen S7020). To 1 mL of 1 OD_{750} standardized actively growing algal culture, 25 µL of enzyme stock was added. The cells were tumbled overnight at room temperature. After 20 h, 1 µL of SYTOX Green (200 uM) was added, incubated for 2 min, and loaded on the ImagestreamX where 20,000 cells were imaged. Samples were excited using a 488 nm laser and 660–740 nm (chlorophyll) and 480–560 nm (Sytox) emission data as well as bright field image data were collected. Populations were gated for in-focus cells and analyzed for permeability.

Transmission electron microscopy

Algal cells were digested by tumbling overnight at room temperature having 25 µL of enzyme stock per mL of algal cells. After which, 150 mM mannitol was added to cell suspension and cells were centrifuged at 3,000×*g* for 2 min. Cells were frozen with a Leica EM PACT2 high pressure freezing system and fixed in 2 % OsO_4 with 0.1 % uranyl acetate. Samples were rinsed with bone dry acetone and subjected to a freeze substitution using a Leica AFS Freeze Substitution system (4 days at −80 °C, 1 day at −20 °C, 1 day at 4 °C, 2 h at room temperature, and rinsed three times in dry acetone) followed by embedding in EPON812 at 60 °C for 48 h. Thin sections of ~60 nm thickness were obtained with a Diatome diamond knife on a Leica EM UTC ultramicrotome (Leica, Wetzlar, Germany), placed on 0.5 % Formvar coated copper slot grids, and stained with 2 % uranyl acetate and Reynolds lead citrate for 6 min each. Stained sections were then visualized using an FEI Tecnai G2 20 Twin 200 kV LaB6 TEM (FEI, Hillsboro, OR) controlled by Serial EM program (http://bio3d.colorado.edu/SerialEM/index.html).

Scanning electron microscopy

Algal cells were digested by tumbling overnight at room temperature having 25 µL of enzyme stock per mL of algal cells. Cells were centrifuged at 2,000×*g* for 1 min. Primary fixation involved resuspension of cells in 2.5 % glutaraldehyde in 0.2 M phosphate buffered saline (PBS) at 30 °C. Cells were then rinsed in 0.2 M PBS at 30 °C followed by secondary fixation in 1 % osmium tetroxide in 0.2 M PBS at 30 °C. Cells were again rinsed in 0.2 M PBS at 30 °C followed by a final water rinse. Samples were then freeze-dried, sputter coated with 5 nM iridium, and imaged using a FEI Quanta FEG 400 scanning electron microscope (SEM) at an accelerating voltage of 30 kV. Images were obtained at 25,000× magnification.

Statistical analysis

All experiments were performed in biological triplicate to ensure reproducibility. Experimental results were obtained as mean value ± SD and reported error bars on the figures. Electron microscopy figures were assembled from representative micrographs that display the characteristic features observed in each condition. In total >1,100 micrographs, each often capturing multiple cells, were individually analyzed between 25 and 79 micrographs for each enzyme treatment condition.

Results

Enzymes inhibit algal growth

To begin this exploration of enzyme activity against algal cell walls, we developed a reasonable throughput growth inhibition assay on agar plates, where the ability to inhibit growth was compared to heat denatured enzyme. Growth inhibition suggests the enzyme is either degrading the cell wall during construction or the enzyme interferes with precursor generation prior to assembly into the cell wall. While no individual enzymes affected growth on all strains tested, chitinase, lysozyme, and pectinase had the broadest effect on the *Chlorella* strains (Table 1). In addition, chitosanase, β-glucuronidase, laminarinase, sulfatase, and trypsin inhibited growth of the majority of *Chlorella* strains. *C. vulgaris* UTEX395 was also inhibited by mutanolysin and proteinase K. Alginate lyase showed peculiar results in that a halo of increased growth typically would form around the spotted area. We interpreted this to be an effect of the release of mono-saccharides from the agar substrate near the area, where the enzyme was spotted. As such, we could not conclude that alginate lyase inhibited growth using a plate-based assay for most strains, though *Chlorella* sp. CHLORI and *C. emersonii* CCAP211/11N had obvious zones of inhibition (data not shown). Of the *Chlorella* strains we tested, only *C. emersonii* CCAP211/11N showed sensitivity to cellulase and had only very minor inhibition by drieselase and macerozyme, which both contain a cellulase. Of the 14 strains of

Table 1 Growth inhibition of different *Chlorella* species and strains by a variety of enzymes

Enzyme	*Chlorella* strains (ASP, UTEX or CCAP designation)													
	CHLOR1	26	30	259	265	395	396	1803	1809	1811	2714	CCAP211/11B	CCAP211/11N	NC64A
Cellulase	–	–	–	–	–	–	–	–	–	–	–	–	++	–
Chitinase	–	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Chitosanase	++	–	+	+/–	–	+	+/–	–	–	+/–	+	++	++	+
Driselase	–	–	–	–	–	–	–	–	–	–	–	–	+/–	–
β-Glucosidase	–	–	–	–	–	–	–	–	–	–	–	–	+	–
β-Glucuronidase	+	+/–	++	+/–	–	++	–	++	–	–	+++	+++	+++	–
Hyaluronidase	–	–	+/–	–	–	–	–	–	–	–	–	–	+	–
Laminarinase	–	++	++	+++	–	+++	–	–	+/–	+/–	++	+++	+	+++
Lysozyme	–	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++
Lyticase	–	–	–	–	–	–	–	–	–	–	–	–	–	+++
Macerozyme	–	–	–	–	–	–	–	–	–	–	–	–	+/–	
Pectinase	–	++	++	+++	+++	++	+++	+++	++	–	+++	+++	+++	+++
Pectolyase	+++	+++	++	++	–	–	–	+	+	–	–	++	–	–
Sulphatase	++	+/–	++	+/–	–	++	––	++	–	–	++	+++	+/–	+
Trypsin	–	+++	+	–	–	+	++	–	–	+/–	+++	+++	–	–
Zymolyase	–	–	–	–	–	–	–	–	–	–	–	–	–	–

–, no growth inhibition; +++, complete growth inhibition

Chlorella tested, no two strains had the same pattern of inhibition. In all cases, heat denatured enzymes did not impact growth in our assay, indicating the growth inhibition was due to the enzymatic activity alone and not from other potentially toxic components of the enzyme preparations (data not shown). Having observed clear differences in growth inhibition we wanted to determine, if all of these strains were in fact *C. vulgaris*. The most widely employed method to study diversity is to sequence the small ribosomal subunit RNA gene (18S for eukaryotes and 16S for prokaryotes) (Fawley et al. 2004). Subjecting the ten UTEX *C. vulgaris* strains and *C. vulgaris* CCAP211/11B to 18S, DNA analysis showed that only UTEX2714 had four base pair differences over the 1,259 base pairs checked of the 18S DNA sequence, while the rest of the strains showed a 100 % identity (data not shown).

Of the non-*Chlorella* strains tested, we observed a large range of sensitivity to various enzymatic activities (Table 2). Some strains such as *Ankistrodesmus* and *Franceia* were resistant to almost all enzymes tested, whereas *Nannochloris* and *Nannochloropsis* displayed significant sensitivities to a number of enzymes. Interestingly, *Nannochloris* and *Nannochloropsis* displayed patterns of inhibition that are nearly identical to one another with the only difference between the two strains being the sensitivity of *Nannochloropsis* to chitosanase.

In addition, β-*N*-acetylglucosaminidase, β-*N*-acetylhexosaminidase, acromopeptidase, chondroitinase ABC, β-galactosidase, hesperinidase, Endo-α-*N*-acetylgalactosaminidase, heparinase II, hemicellulase, lysing enzymes from *Rhizoctonia solani* (kitalase) and *Aspergillus* sp., α-1-6 mannosidase, 1-2-3 mannosidase, neuraminidase, α-2-3 neuraminidase, phospholipase A_1, and phospholipase A_2 were found not to have any effect on growth of any of the algal species tested.

Enzymes allow penetration of DNA stain

After finding enzymes that inhibited growth, we examined their effects on cell walls of actively growing cultures. To process a large number of enzymes individually and in combination, we developed a rapid assay that would give statistically relevant results. We postulated that degradation of the cell wall may have dramatic effects of the cell membrane as well, which could lead to an altered permeability barrier. Cell permeability due to enzymatic disruption of the wall was visualized using an imaging flow cytometer, the ImagestreamX. We used the fluorescent signal of a DNA staining dye, SYTOX Green, that normally cannot pass the cell wall/membrane barrier to indicate permeability (Veldhuis et al. 1997; Sato et al. 2004). During data acquisition, algal cells were positively defined by their chlorophyll autofluorescence. A minimum

Table 2 Growth inhibition of non-*Chlorella* algal species by a variety of enzymes

Enzyme	Strains (ASP, UTEX, or CCMP designation)						
	ANKIS1	FRANC1	NANNO2	NANNP2	OOCYS1	CCMP632	UTEX1648
Cellulase	−	−	−	−	++	−	−
Chitinase	−	−	+++	+++	+++	++	−
Chitosanase	−	−	−	++	−	−	−
Driselase	−	−	−	−	−	−	−
β-Glucosidase	−	−	++	++	−	−	−
β-Glucuronidase	−	−	++	++	−	+/−	+++
Hyaluronidase	−	−	++	++	−	++	−
Laminarinase	+/−	−	−	−	−	−	−
Lysozyme	−	−	+++	+++	−	−	+/−
Lyticase	−	−	+++	+++	−	−	−
Macerozyme	−	−	−	−	−	−	−
Pectinase	−	−	++	++	−	++	++
Pectolyase	−	−	−	−	−	−	+++
Sulphatase	−	−	+++	+++	−	−	+++
Trypsin	−	−	+++	+++	−	−	−
Zymolyase	−	−	++	++	−	−	++

−, no growth inhibition; +++, complete growth inhibition

chlorophyll autofluorescence was set to eliminate potential false positives from bacteria and debris present in the culture. The resultant data were analyzed by setting a minimum SYTOX Green fluorescent intensity threshold, such that the majority of untreated cells (Fig. 1a) had fluorescence intensity lower than the threshold. Cells with a fluorescent intensity above this threshold were considered permeable to the DNA dye as indicated by cells treated with lysozyme (Fig. 1b) or lysozyme and sulfatase (Fig. 1c). Images (Fig. 1d–f) from representative regions (R1, R2, or R3) of a particular condition are also presented to visualize cells typically found in particular regions of the dot plots. Region R1 was typically representative of single, permeable cells. Cells in region R2 showed higher chlorophyll intensity and typically represented permeable cells that were clustered. Cells in region 3 were typically single, non-permeable cells. When enzymes were used individually, only lysozyme gave a significant increase in permeability to SYTOX Green (Fig. 1g). When individual enzymes were coupled with lysozyme, a slight increase in cell permeability was observed for chitinase, chitosanase, β-glucuronidase, pectolyase and trypsin, while a more dramatic increase was observed with laminarinase, lyticase, phospholipase A_1, sulfatase, and the glusulase enzymes (β-glucuronidase and sulfatase). The combination of lysozyme and sulfatase had the greatest effect and permeabilized >96 % of the population (Fig. 1g). When additional enzymatic activities were added to the combination of lysozyme, β-glucuronidase, and sulfatase, there were not any additional increases in permeability (data not shown). After overnight digestion with lysozyme or glusulase, we streaked the cells to agar plates and growth was noted after a few days. Growth was not as dense as without enzyme treatment, but this result does indicate that a portion of the population survived digestion with lysozyme or glusulase as only ~8 or ~2 %, respectively, became permeable to the DNA dye. However, when cells were digested with lysozyme and glusulase, no growth was noted after extended incubation. The lack of growth is in agreement with our permeability data, which shows 96 % permeability for lysozyme and sulfatase (an enzyme present in glusulase).

Having developed and demonstrated a technique to rapidly identify enzymes capable of affecting the permeability of *C. vulgaris* UTEX395, we applied this technique to other microalgal strains. As with *C. vulgaris* UTEX395, *Nannochloropsis* sp. NANNP2 was sensitive to lysozyme and the majority of the cells were permeabilized by the lysozyme and sulfatase combination. Similarly, when lysozyme was coupled with trypsin or lyticase, dramatic increases in permeabilization were also observed. Permeabilization of *S. capricornutum* UTEX1648, however, was dramatically different. No changes in permeability in response to individual enzymes were noted, yet roughly $^1/_3$ and $^2/_3$ of the cells were permeabilized using lysozyme and sulfatase or lysozyme and lyticase, respectively. Minor increases in permeabilization (~10 %) were noted for lysozyme and cellulase, lysozyme and pectinase, or lysozyme and trypsin (data not show).

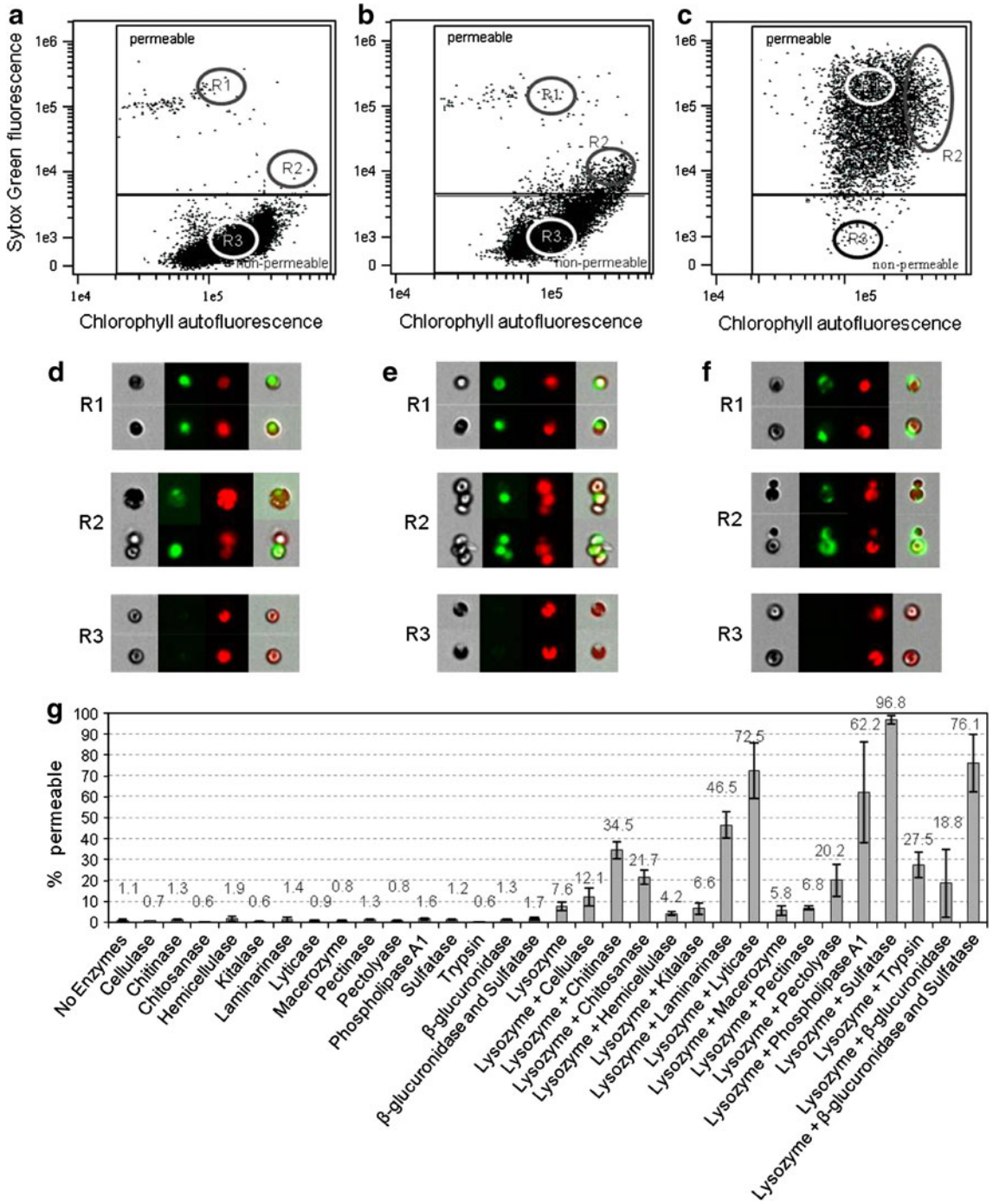

Fig. 1 ImagestreamX dot plot analysis example and images of *C. vulgaris* UTEX395 cells, (**a**, **d**) without enzyme treatment (**b**, **e**) lysozyme digested, and (**c**, **f**) lysozyme and sulfatase digested. Cells were illuminated with a white LED for bright field and a 488 nm laser for fluorescence. Images, in order from *left* to *right*, were collected in the bright field, 480–560 nm emission (Sytox green fluorescence), and 660–740 nm emission (chlorophyll autofluorescence) channels, followed by a merged image of all three channels. Images were taken from their representative regions as denoted by R1, R2, and R3 on the *dot plots*. **g** *Bar graph* showing the percent of the population that becomes permeable with a given enzyme treatment with *error bars* representing standard error for $n = 3$ experiments

Transmission electron microscopy reveals details of enzymatically digested *Chlorella vulgaris* cell walls

Growth inhibition and permeability data illustrated enzymes that can potentially degrade the cell wall of *C. vulgaris*. Using TEM, we further characterized the effects individual enzymes have on the cell wall ultrastructure. Two distinct layers are present in the *C. vulgaris* cell wall with the inner layer having much lower electron density than the outer layer. In addition, long hair-like

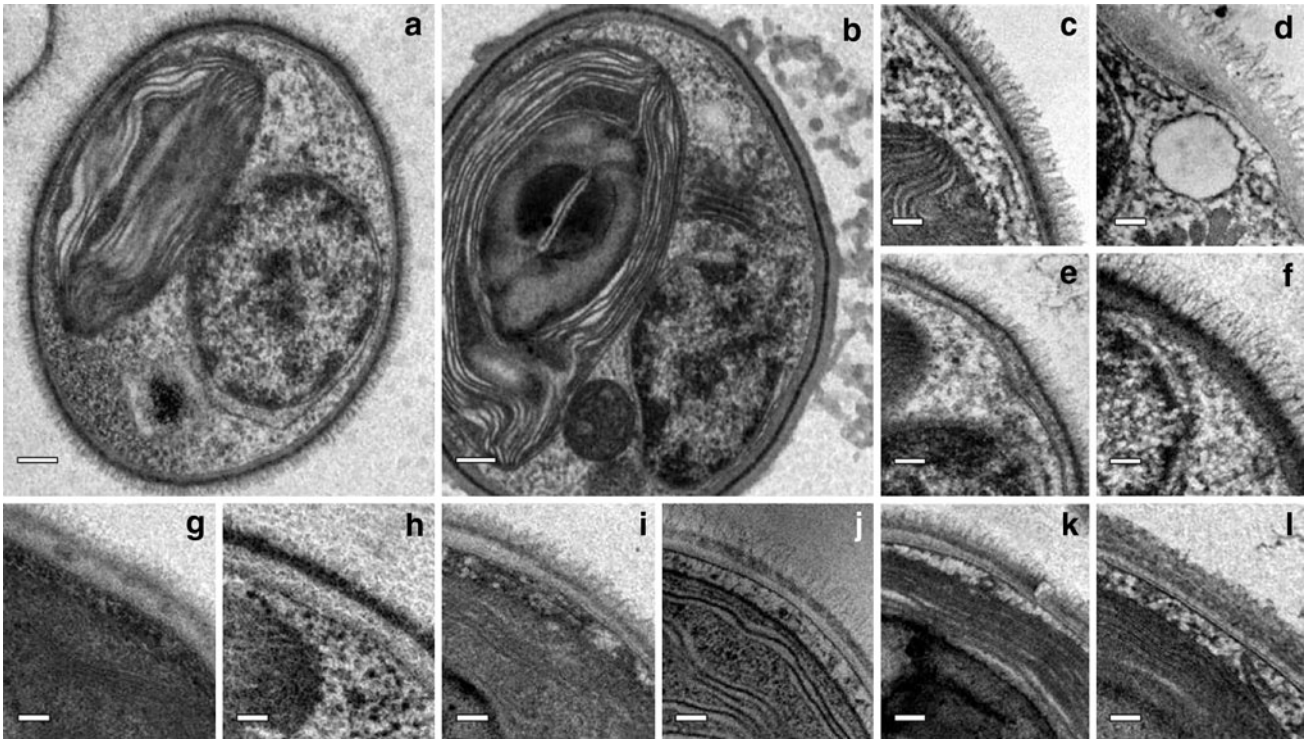

Fig. 2 Transmission electron micrographs of *C. vulgaris* UTEX395 digested with **a** no enzyme, **b** lysozyme, **c** no enzyme, **d** chitinase, **e** chitosanase, **f** laminarinase, **g** trypsin, **h** sulfatase, **i** β-glucuronidase, **j** pectinase, **k** cellulase, **l** phospholipase A_1. *Scale bar* 200 nm (**a, b**), 100 nm (**c–l**)

fibers protrude from the outer layer (Fig. 2a, c). In agreement with the growth inhibition and permeability data displayed in Table 1 and Fig. 1, we found that lysozyme had a dramatic effect on the cell wall of *C. vulgaris*. Lysozyme, an enzyme that typically acts on bacterial peptidoglycan, a polymer of β-1,4 linked *N*-acetylglucosamine and *N*-acetylmuramic acid, seems to swell the outer surface of the electron-dense layer and lower the density of the outermost portion such that there are now three distinct layers. Lysozyme also removes the hair-like fibers that protrude from the surface of the cell (Figs. 2b and 3a). Graves et al. (1999) demonstrated that these hair-like fibers on the surface of *C. variabilis* NC64A are hyaluron, a linear polysaccharide chain composed of alternating β-1,4-glucuronic acid and β-1,3-*N*-acetylglucosamine groups, and may play a role in preventing superinfection of the PBCV-1 virus. Curiously, chitinase, which degrades poly-β-1,4-D-*N*-acetylglucosamine, seemed to give a different pattern of digestion to the *C. vulgaris* wall, not affecting the hair-like fibers, but causing a general decrease in electron density of the outer wall similar to lysozyme (Fig. 2d). Chitosanase (Fig. 2e) caused a minor thinning of the electron-dense outer region of the cell wall, while laminarinase (Fig. 2f) caused a slight increase in density of the inner wall. Trypsin digestion caused a general decrease in density of the outer wall in a fashion similar to that of chitinase and an increase in cell wall thickness (Fig. 2g). While sulfatase, β-glucuronidase, pectinase, and cellulase caused no systematic changes to the ultrastructure of the *C. vulgaris* cell wall (Fig. 2h–k), we were surprised to see quite dramatic changes in the wall in response to phospholipase A_1. Phospholipase A_1 digestion removed the hair-like fibers and caused a general widening of the outer wall resulting in visible stratification of layers in the outer wall (Fig. 2l).

Increased permeability when certain enzymes are coupled with lysozyme, and the ability of lysozyme to degrade the outer surface of the *C. vulgaris* cell led us to hypothesize that lysozyme is opening up the cell wall for further degradation by enzymes that cannot normally cross the outer wall of *C. vulgaris* to find substrates buried within the wall. When lysozyme was coupled with chitinase, we saw a dramatic change in the cell wall ultrastructure. The cell wall more than doubled in width and the electron-dense outer wall was thinned to a greater degree than lysozyme alone (Fig. 3b). When lysozyme was added in conjunction with some enzymes such as cellulase, pectinase or phospholipase A_1, we noticed that the outer wall no longer maintained a smooth interaction with the inner wall. The outer wall seems to "push in" and displace the membrane toward the center of the cell (Fig. 3c, d, g). When coupled with lysozyme, chitosanase, trypsin, and β-glucuronidase showed no drastic effect over that of lysozyme alone (Fig. 3e, h, i), whereas sulfatase and β-glucuronidase with

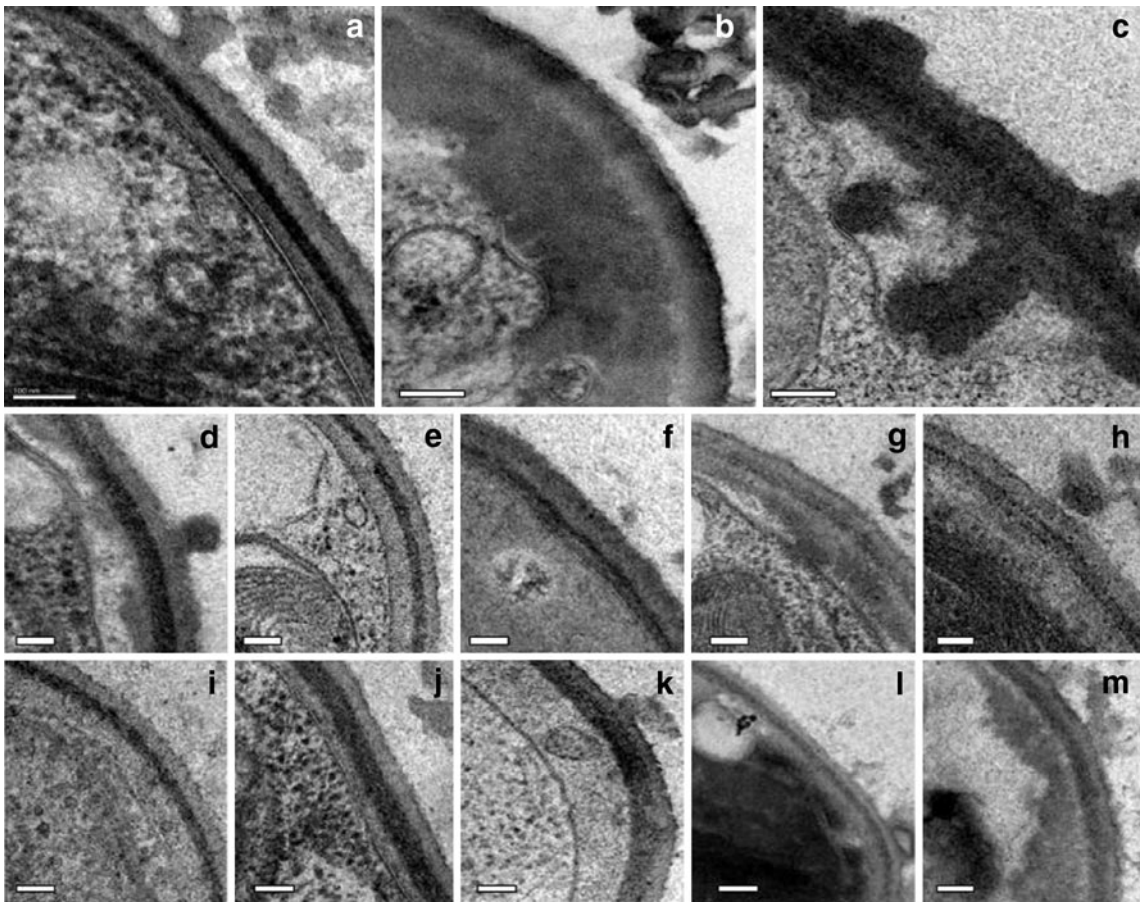

Fig. 3 Transmission electron micrographs of *C. vulgaris* UTEX395 digested with lysozyme and the additional enzyme, **a** no additional enzyme, **b** chitinase, **c** cellulase, **d** pectinase, **e** chitosanase, **f** laminarinase, **g** phospholipase A_1, **h** trypsin, **i** β-glucuronidase, **j** sulfatase, **k** β-glucuronidase and sulfatase, **l** β-glucuronidase, sulfatase, and cellulase, **m** β-glucuronidase, sulfatase, cellulase, and phospholipase A_1. *Scale bar* 200 nm (**a**–**c**), 100 nm (**d**–**m**)

sulfatase widened the electron-dense outer layer of the wall (Fig. 3j, k). When lysozyme was added with laminarinase, the electron-dense outer region appeared closer to the membrane (Fig. 3f). This enzyme combination gave large amounts of broken cells and cell debris in some preparations as did lysozyme with chitinase and lysozyme with sulfatase (data not shown). Addition of multiple enzymes with lysozyme had dramatic effects on the ultrastructure of the wall (Fig. 3l, m) and location of the wall relative to the cell membrane, while also increasing the electron density of the inner wall. No enzyme combination was able to completely remove the electron-dense outer wall.

Nitrogen depletion changes cell wall morphology

Because *C. vulgaris* is considered a possible strain for production of biodiesel, we also sought to analyze the effects of these enzymes in the high oil phase. It is very likely that the composition of the cell wall could change dramatically under nitrogen deprivation conditions. Using TEM, we analyzed the effects of lysozyme in conjunction with a number of enzymes on the cell wall of *C. vulgaris*. Under extreme nitrogen deprivation, we noticed significant changes to the ultrastructure of the cell wall. We observed that the cell walls no longer display the hair-like fibers on the surface of the cell and the electron-dense region is now proximal to the cell membrane (Fig. 4a, b). Addition of lysozyme with up to six additional enzymes decreased this electron density now in the inner wall, yet there seems to be very little ultrastructural changes to the nitrogen-deplete *C. vulgaris* cells when digested with the same enzymes that have dramatic effects on the nitrogen-replete cells (Fig. 4c–e). Quantification of the cell wall thickness for Figs. 2, 3, and 4 is displayed in Fig. 5.

Scanning electron microscopy reveals extracellular material causing aggregation and settling of *C. vulgaris*

When enzymatically digesting algal cells, it was noticed that cells treated with lysozyme or combinations of enzymes that included lysozyme, settled much more rapidly than untreated cells. We used SEM to visualize the cell

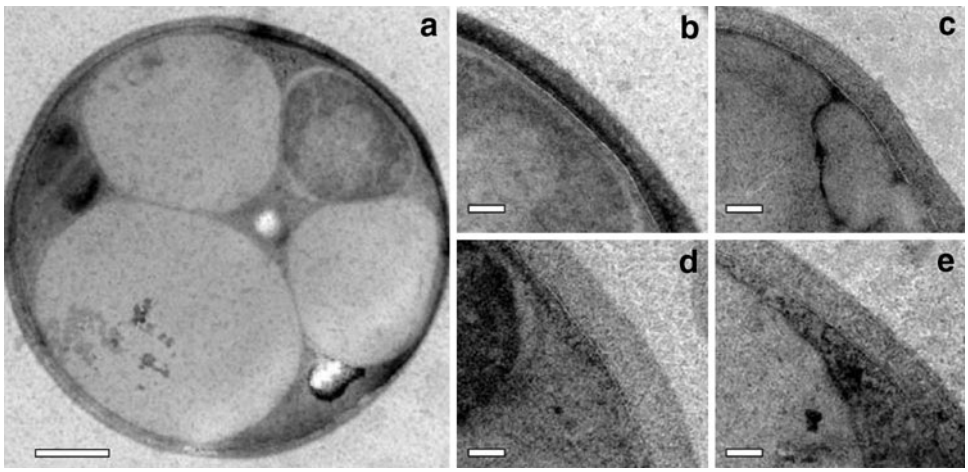

Fig. 4 Transmission electron micrographs of *C. vulgaris* UTEX395 after nitrogen deprivation and digested with **a** no enzyme, **b** no enzyme, **c** lysozyme and laminarinase, **d** lysozyme, laminarinase, *β*-glucuronidase, sulfatase, cellulase, and chitinase, **e** lysozyme, laminarinase, *β*-glucuronidase, sulfatase, cellulase, chitinase, and phospholipase A_1. *Scale bar* 500 nm (**a**), 100 nm (**b**–**e**)

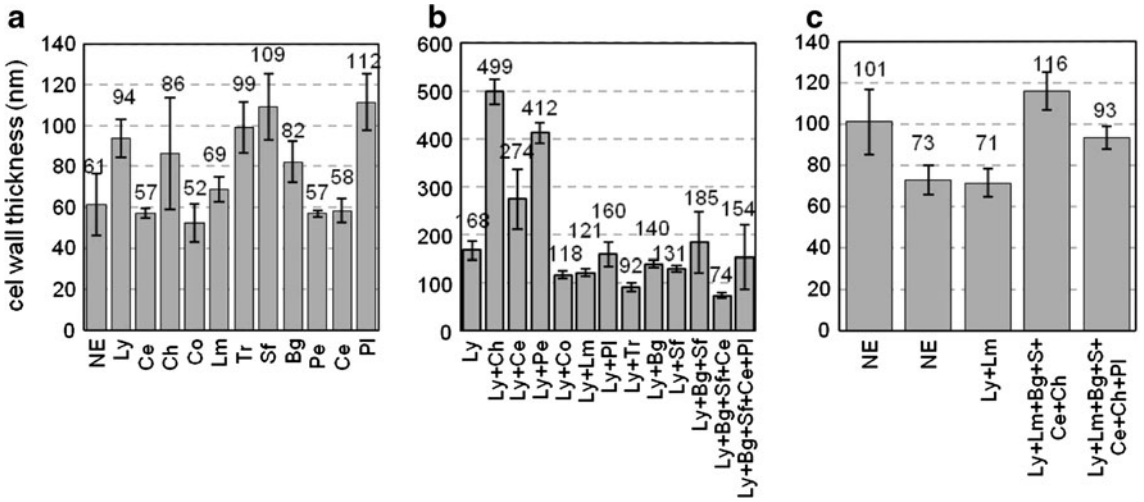

Fig. 5 Quantification of cell wall thickness from images in **a** Fig. 1, **b** Fig. 2 and **c** Fig. 3. *NE* no enzymes, *Ly* lysozyme, *Ch* chitinase, *Ce* cellulase, *Pe* pectinase, *Co* chitosanase, *Lm* laminarinase, *Pl* phospholipase A_1, *Tr* trypsin, *Bg* *β*-glucuronidase, *Sf* sulfatase. Wall thickness was measured from the plasma membrane to the outer surface of the cell in each image and is an average of at least 75 measurements ($n \geq 75$)

surface of enzymatically treated cells. Sputter coating of the cells prevented visualization of the hair-like fibers on the surface of the cells as seen in the TEM micrographs (Fig. 2). Treatment with lysozyme led to the aggregation of fibrous materials on the surface of the cells without any obvious visible changes to the cell walls themselves (Fig. 6a, b). Treatment with laminarinase (Fig. 6c) gave very minor aggregations on the cell surface, while sulfatase (Fig. 6d) had no visible effects on the cells. When lysozyme was coupled with laminarinase or sulfatase, there was a drastic increase in irregular surface aggregations over that of lysozyme or laminarinase alone. This material is clearly seen bridging between nearby cells (Fig. 6e, f). Even with the drastic increase in extracellular materials, the cells still appear intact with little or no visible changes in the underlying cell wall layers. This dislodged and aggregated extracellular material is likely the reason that lysozyme treated cells settle faster and clearly the inter-cellular bridging would lead to rapid settling, as it is very similar to the effective mechanism when using polymeric polyelectrolyte flocculants such as chitosan (Divakaran and Pillai 2002). We postulate that the aggregations are materials having peptidoglycan-like bonds sensitive to lysozyme that are being sloughed off the surface in thin layers, too small to visualize with SEM, yet visible in TEM (Fig. 7).

Discussion

The cell wall of *C. vulgaris* and other algae presents a formidable barrier necessary for survival in aquatic environments. Unfortunately, this barrier affects certain processes of interest in algal biotechnology such as genetic transformation, fermentation, anaerobic digestion, and oil

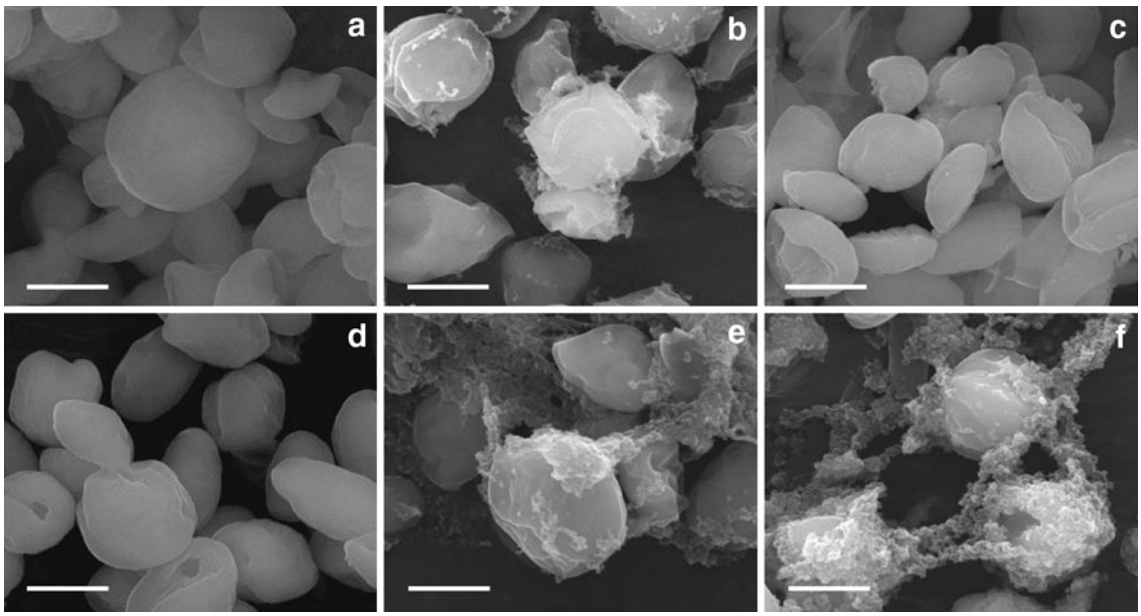

Fig. 6 Scanning electron micrographs of *C. vulgaris* UTEX395 cells treated with **a** no enzyme, **b** lysozyme, **c** laminarinase, **d** sulfatase, **e** lysozyme and sulfatase, **f** lysozyme and laminarinase. *Scale bars* 2 μm

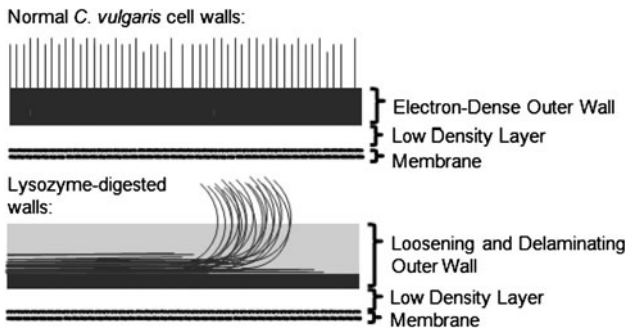

Fig. 7 Schematic depiction of the action of lysozyme on the cell wall of *C. vulgaris*. Normal walls consist of an electron-dense outer wall and a less dense inner wall. On digestion with lysozyme, there is a thinning and delaminating of the outer wall and loss of hair-like fibers from the surface of the cell

extraction. For biofuels production from terrestrial plant biomass, reducing recalcitrant substrates, such as cellulose, to more facile substrates, enzymatic saccharification is a vital step. Enzymatically treating algal cell walls will likely be required to realize the same level of utility. Thus, assessing the impact of enzymes on algal cell wall degradation is a critical first step to utilizing algae and algal biomass more efficiently. We have demonstrated two methodologies to rapidly assess the efficacy of enzymes toward algal cell walls, growth inhibition and fluorescent cytometry. The ability of an enzyme to inhibit growth indicates that the substrate for the enzyme is present either in the cell wall as it is being built or as a pre-cursor that is prevented from attaching properly. The cell wall and cytoplasmic membrane prevent enzymes from crossing into the cell. We thus expect growth inhibition by enzymes to be due to the cells inability to properly construct a cell wall during growth or cell division. A second rapid methodology for assessing activity of enzymes toward algal cell walls is the use of fluorescent imaging flow cytometry. This methodology allows rapid quantification of changes in cell permeability caused by cell wall disruption. In addition to these screening methods, we employed SEM and TEM to further characterize the action of enzymes on algal cell walls.

Our survey of commercially available enzymes having activity on *C. vulgaris* cell walls revealed several candidate enzymes of which lysozyme gives the most dramatic effect. Although enzymes other than lysozyme are capable of complete growth inhibition, there is no effect on the permeability of mature cells when used singly. Lysozyme appears to be required to expose sensitive substrates in the mature cell, whereas these same substrates are accessible during growth. An example of this enzyme class is chitinase. Chitinase completely blocks growth similar to lysozyme yet has no effect on permeability of mature cells. However, treating mature cells with lysozyme and chitinase together results in a dramatic increase in permeability far above that of either lysozyme or chitinase alone. The differing effects of lysozyme and chitinase indicate that these enzymes have different activities toward the *C. vulgaris* cell wall, though both enzymes degrade polymers of *N*-acetylglucosamine. Lysozymes are frequently characterized as chitinases, while chitinases typically also have the ability to break down peptidoglycan, but there is no obvious sequence similarity between these enzymes (Monzingo et al. 1996). Still there is evidence for a

β-hairpin type structure that is common to lysozymes, chitinases, and chitosanases (Wohlkonig et al. 2010). The permeability data show that by treating the cell walls initially with lysozyme, chitinase is then better able to interact with its target substrate to synergistically increase permeability (34.5 %) greatly above either chitinase (1.3 %) or lysozyme (7.6 %). This protective quality of a polymer sensitive only to lysozyme remains true with other enzymes used individually and having a synergistic effect when used in combination with lysozyme, particularly with sulfatase or β-glucuronidase. The activity of lysozyme on the outer surface of the cell as observed by TEM and SEM supports the theory that digesting first with lysozyme removes a protective outer layer allowing other enzymes to reach their target substrates. In addition to *C. vulgaris*, we also see a sensitivity of *Nannochloropsis* and *Nannochloris* strains to lysozyme and chitinase, while *O. pusilla* OOCYS1 and *P. tricornutum* CCMP 632 were sensitive to only chitinase. We tested the permeability of *Nannochloropsis* strains to the Sytox DNA stain after treatment with chitinase and saw a dramatic increase in permeability (data not shown). The ability of chitinase and lysozyme to act on the cell walls of *Nannochloropsis* is indicative of an *N*-acetylglucosamine containing polymer in the cell wall of these important biodiesel producing organisms.

Only two species, *C. emersonii* CCAP211/11N and *O. pusilla* OOCYS1, showed any sensitivity to cellulase or enzyme mixes containing cellulase such as driselase and macerozyme. In support of previous literature indicating a lack of cellulose in the cell walls of *C. vulgaris* (Kapaun and Reisser 1995), we found that cellulase did not have any effect on growth and minimal increase in permeability in *C. vulgaris.* We also noticed that cells digested with lysozyme and cellulase were slightly more irregular than the cells digested with lysozyme alone, indicating there may be a small amount of cellulose or other similar polymers that cellulase can hydrolyze. As these effects of cellulase are minor, cellulose does not appear to play a major role in cell wall integrity or permeability in most of the algal species and strains tested. This agrees with the work of Takeda (1991), who demonstrated that most *C. vulgaris* strains contain little to no glucose in the cell wall. Still, there may be a polymer of glucose in the wall at certain stages of the life cycle. Only *C. emersonii* CCAP211/11N and *O. pusilla* OOCYS1 showed sensitivity to cellulase at the growth inhibition level. Some strains of *Chlorella* were sensitive during growth to laminarinase which catalyzes the endohydrolysis of 1,3- or 1,4-linkages in β-D-glucans when the glucose residue whose reducing group is involved in the linkage to be hydrolyzed is itself substituted at C-3. Interestingly, *Nannochloropsis* sp. NANNP2 and *Nannochloris* sp. NANNO2 were sensitive to lyticase and zymolyase. Both enzyme mixes contain β-1-3 glucanase activity, yet lyticase and zymolyase do not affect *Chlorella* cell walls during growth. Conversely, *S. capricornutum* UTEX1648 was only sensitive to zymolyase. These results suggest that these enzymes are quite specific for their substrates, as the well-characterized laminarinase appears to be or that the target polymer is buried or obscured, such that it requires the secondary component activity to access it which could be the protease activity in the case of zymolyase and UTEX1648.

One approach we took toward finding enzymes that degrade algal walls was to look at the enzymes that are in the glusulase mixture. Glusulase is an extract from the stomach of the snail *Helix pomatia* and contains β-glucuronidase and sulfatase enzymes. β-glucuronidase activity toward *Chlorella* is not surprising, as uronic acids have been found in *Chlorella* cell walls (Takeda 1991). It is well established that many algal-derived polymers such as carageenan and agar (McCandless and Craigie 1979) or ulvan (Jaulneau et al. 2011) contain sulfate groups (Ray and Lahaye 1995). With *C. vulgaris* it is possible that this sulfate group is added on the *N*-acetylglucosamine residues similar to heparin sulfate or keratan sulfate. Removal of sulfate groups may free up the polysaccharide for degradation by other enzymes or may make the polymer more cationic such that it no longer forms stabilizing salt bridges. However, sulfatase alone still also requires removal of the protective lysozyme-susceptible layer to be effective.

The curious activity of phospholipase A_1 digestion toward *Chlorella* cell walls indicates that a substrate for this enzyme is buried within the cell wall. Phospholipase A_1 removes the 1-acyl chain from phospholipids. The action of this enzyme on the *Chlorella* wall may be due to the removal of lipid anchors in the wall to ultimately loosen the compaction of the wall. Alternatively, the activity of this enzyme may be functioning toward algaenan or an algaenan-like polymer that could be part of the cell wall. The long aliphatic chains of algaenan could be structurally similar to a lipid and phospholipase A_1 could be hydrolyzing this molecule.

Our data highlight an interesting phenomenon with respect to interspecies variation of enzyme sensitivities among *Chlorella* strains. This variation in growth inhibition between strains by enzymes suggests different cell wall matrices in the ten *C. vulgaris* strains tested. Though 18S rDNA sequencing indicates that these strains are all *C. vulgaris*, there are substantial differences at the phenotypic level. With the complete genomes of these organisms currently unavailable, one cannot be certain, if the changes between strains are due to genomic-level changes in machinery used to construct the cell wall or due to regulatory changes in gene expression in response to the environment. The fact that these *C. vulgaris* strains were all isolated from different microenvironments, which they

have presumably adapted to, could lead to differential gene expression patterns when all strains are put into similar conditions. In addition, within a single strain of algae, there appears to be extreme responses to minuscule changes in the environment. We noted some high variability in the cell wall permeability study data which may be indicative of subtle differences in cell wall composition and architecture due to minute differences in growth conditions during replicate experiments. Previous studies suggested that algal cell walls change dramatically as the cell ages, such that an older culture subjected to the same enzymatic treatment is more resistant (Van Donk et al. 1997). The range of enzymatic activities showing high degrees of variability in their impact on cell wall permeability between our replicate experiments suggests that no one single, easily identifiable component is changing, but rather wholesale changes in cell wall composition may be occurring.

The changes in cell walls in response to the environment are highlighted with the TEM images of cell walls after nitrogen depletion (Fig. 4). Changes in ultrastructure for nitrogen-deplete cells may be due to the cells scavenging the amino sugars from the wall to use as a source of nitrogen. This would help explain the extended period of growth for *C. vulgaris* when cells are transferred to nitrogen-free medium (Guarnieri et al. 2011). The cell walls of nitrogen-deplete cells look similar to lysozyme digested cells (Figs. 3a, 4a) and lack exterior hairs. The cells could be resorbing this layer, which contains *N*-acetylglucosamine as a source of nitrogen under nitrogen deprivation conditions. Loss of hair-like fibers supports this hypothesis since hyaluron contains *N*-acetylglucosamine and has been postulated to be the major component making up these hairs (Graves et al. 1999).

In conclusion, we have found that mature *Chlorella* cell walls are protected by a durable polymer of unknown composition that is sensitive to lysozyme treatment. Lysozyme alone does not have a huge impact in permeabilizing mature cells, yet by removing this protective layer, other substrates become available to a wide range of differing enzymatic activities. This layer must be established late in the growth or maturing stage of individual cells as growing cells are readily susceptible to a variety of enzymes causing slow or a complete block in growth. Our results show that in methodologies or processes using intact algal cells or residual algal biomass, enzymatic treatment can have large impacts of the permeability of the algal cell walls and may be useful in optimization. Certainly lysozyme and certain other enzymes can play a role in the facilitated utilization of algae or algal biomass.

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