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An engineered polysaccharide lyase to combat harmful algal blooms

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ABSTRACT

A growing global population and industrialization have come at the cost of induced climate change and pollution of natural resources, resulting in formation of toxic algal blooms in fresh water sources. In the US alone, these blooms cost an estimated $1.5 billion dollars each year to remediate. Current methods to combat such blooms such as copper sulfate treatment are expensive, ineffective, and environmentally toxic, motivating development of biochemical algaecides as green alternatives. Our previous research led to identification of mutant polysaccharide lyase (PL) enzyme H208F derived from *Stenotrophomonas maltophilia* Smlt2602 (WT), which displays increased activity on the polysaccharide polyglucuronic acid. Polyglucuronic acid is a major component of the algal cell wall. This study focused on applying the two enzymes to a common bloom-forming, toxin-producing algae, *Microcystis aeruginosa*. We hypothesized the 2 enzymes would kill algae through disrupting the cell wall, with H208F displaying a higher killing efficacy over WT. A fluorescence assay in conjunction with live/dead staining was used to quantify killing of algae treated with each enzyme. SEM analysis was used to observe cell wall morphology in order to verify the killing mechanism. The mutant H208F was confirmed as having potential application as a biochemical, enzymatic algaecide with a 17% greater algicidal efficacy than the WT. SEM images revealed deformities in the algal cell wall, confirming the purported mechanism.

1. Introduction

In a world where the human population and industrialization is rapidly increasing and technology is becoming more widely accessible, the environment is coming under greater strain. One specific impact that has become increasingly prevalent in the past decade is the appearance of algal blooms in lakes, coastal waters, and other bodies of water [1]. Increased industrialized farming practices have led to an increase in runoff of fertilizers containing nitrates, phosphates and trace metals into water sources. Such compounds are the major nutrient requirements for algal and cyanobacterial growth [2]. As a result, excess amounts of these nutrients trigger harmful algal blooms (HABs), which are defined as uncontrolled growth of algae and cyanobacteria that release toxins and deplete dissolved oxygen levels [3]. Fig. 1 illustrates bloom formations observed at Seneca Lake, NY in July 2017 used in the current study. Both toxins and reduced oxygen levels have led to death of fish and other organisms in lakes and near coasts. The frequency and severity of HABs has increased in recent years, and it is suspected that a warming climate is another major factor in their formation [1]. Therefore, it can be expected that this trend will continue into the future.

Humans are affected both directly and indirectly by algal blooms. The harmful algae and cyanobacteria produce toxins such as microcystin, which is a potent hepatotoxin resistant to boiling [4]. *Microcystis aeruginosa* is the most common HAB forming, microcystin-producing organism in fresh water [5]. The concentration of microcystin in lakes due to recent blooms has been enough to kill fish, birds, and even dogs [1]. This toxin also affects humans directly. Consumption or even contact with this contaminated water from swimming or bathing can induce abdominal pain, vomiting and diarrhea, liver inflammation and hemorrhage, pneumonia, dermatitis and potential tumor growth promotion [6]. In 2014, HABs cut off drinking water to over 400,000 people in Toledo and Michigan with toxin levels reaching more than double the World Health Organization's threshold [7]. Therefore, this problem affects not only those who live near bodies of water, but also those who travel to such locations for vacation and recreation. However, the impact of this issue goes well beyond recreation. Fishing and shell fishing industries have been severely hindered by the toxins produced from algal blooms, which result in reduced catches

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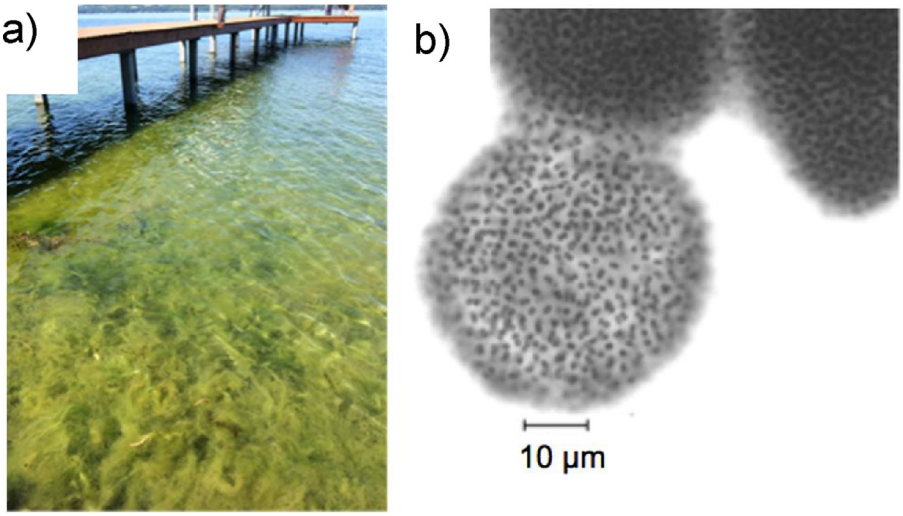


Fig. 1. (a) Algal blooms at Seneca Lake, NY have impacted recreation and fishing in the area. Samples of bloom-forming algae were collected from local agricultural pond water and isolated. (b) Observation of the colonies at 20× magnification revealed morphology similar to that of *M. aeruginosa* as described in the landcare research database. Colony size, cell shape, and cell diameter matched those characteristic of *M. aeruginosa.*

and accumulation of the toxins in their products [8]. Between 1987–1992, the economic impact of algal blooms was estimated at over $700 million, which is equivalent to over $1.5 billion today considering inflation [8]. However, HABs have become more prevalent and more frequent since 1992, so this cost today is likely even higher. This problem is not unique to the US as Australia, China and many other countries have struggled with HABs in recent years [1].

Current methods to remove HABs are often costly and impose additional adverse effects on the environment [9,10]. One such example is skimming, a physical means of removal whereby algae are skimmed from the surface using boats and then incinerated. However, this method only removes algae once blooms have already formed rather than preventing HAB formation. Furthermore, the remarkable thermal stability of toxins such as microcystin to incineration can cause release of toxins into the atmosphere, which is potentially just as harmful [9]. Other chemical treatments such as copper sulfate have toxic effects on other organisms in the ecosystem and disrupt the ecology potentially as much if not more than HABs do [9,10].

In terms of biological methods to control HABs, previously studied solutions include viruses, macrophytes, and bacteria [10–16]. One such study applied a chitinase-producing strain of *Chitinomonas prasina* as a bacterial algaecide to successfully inhibit growth of marine algae [10]. Another study explored algicidal properties of the bacterium isolate DHQ25, indicating its ability to lyse algal cells of the species *A. tamarense* [11]. However, translating the same impact of these results to more complex environments *versus* laboratory cultures remains an area of active research. For this reason, others have sought to identify specific compounds from predatory bacteria responsible for their anti-algal activity. One such study isolated palmitoleic acid from *Vibrio* sp. BS02, which when applied as an algaecide significantly inhibited growth of *A. tamarens* [12]. A separate study was able to isolate and characterize an algicidal protein from the DHQ25 bacterium, which had previously displayed an ability to inhibit growth of *A. tamarense* [15]. These and other studies suggest that specific chemical compounds and proteins also may be useful as anti-algal agents, although their mechanism of action remains an area of active research.

In this paper, we describe an alternate strategy to target HABs by utilizing engineered enzymes that degrade polysaccharides commonly found in algal and cyanobacterial cell walls. The algae cell wall is largely composed of various polysaccharides, with the most prominent of these being polyglucuronic acid [9]. An illustration of the cell wall structure and polysaccharide layers is presented in Fig. 3. Our previous work demonstrated that an engineered mutant (H208F) of the polysaccharide lyase (PL) Smlt2602 from *S. maltophilia* exhibited increased exolytic digestion of polyglucuronic acid (polyGlcA) as compared to wild-type (WT), thereby exhibiting dual activity against alginate and polyGlcA found in the cell wall of HAB forming organisms [17]. We tested the ability of H208F and WT to kill *M. aeruginosa*, and found H208F to be superior in terms of killing efficiency under standard growth conditions as determined using fluorescence microscopy, intrinsic fluorescence and direct observation in culture. Furthermore, SEM imaging indicates distortion of the cell wall with enzyme treatment, consistent with the proposed mechanism of action. Thus, we conclude that engineered PLs with activity against cell wall polysaccharides found in HAB forming organisms such as H208F are effective under standard growth conditions leading to HAB formation, and therefore are effective as potential algaecides to eliminate HABs.

2. Experimental

2.1. Enzyme expression

Expression plasmids and methods for enzyme overexpression have been described in detail previously [18]. In summary, Smlt2602 WT and mutant H208F were subcloned into pET28a vectors as BamHI-XhoI inserts, which includes a C-terminal hexahistidine tag for purification, and transformed into BL21 (DE3) pLysS cells by electroporation. Individual colonies were isolated from kanamycin-selective plates (50 μg/mL working concentration), inoculated in 20 mL LB cultures containing kanamycin, and grown to saturation at 37C with 200 rpm agitation in a shaking incubator (Innova R26). Cell mass from saturated cultures were collected by centrifugation at 3000 rpm for 10 min, spent media decanted, and resuspended in 200 mL of fresh LB media containing kanamycin in 1 L flasks. Cultures were switched to 20C for growth, and after 1 h, IPTG added to a final concentration of 1 mM to induce protein expression. Cells were harvested after 24 h growth at 20 °C with 200 rpm agitation by centrifugation at 3000 rpm for 10 min.

2.2. Enzyme purification

Cell pellets from 50 mL of induced culture were resuspended in 20 mL of lysis buffer (100 mm HEPES, 500 mm NaCl, 10% w/v glycerol, 10 mm imidazole). Sonication (Misonix 3000 Ultrasonic Cell Disruptor, 15 W, 20 min process time, 20 s on/20 s off pulses) was used to lyse the cells within the 20 mL of buffer on ice. The lysis mixture was centrifuged at 12000 rpm for 12 min, insoluble material was discarded and the soluble supernatant containing enzyme collected. The enzyme was purified using immobilized metal ion affinity chromatography (IMAC) with 15 mL of Profinity resin. The column was charged with a 0.2 M nickel chloride solution and equilibrated with two column volumes of lysis buffer prior to running the cell lysate. The column was initially washed with two column volumes of 10 mM imidazole and collected. Then, imidazole concentrations of 250 mM and 500 mM were used to elute the protein and 5 mL fractions collected. SDS-PAGE was used to confirm purification and purity of final enzyme product. Protein samples (40 μL) were mixed with 10 μL of 5 × Lammeli sample buffer and heated for 5 min at 90 °C before loading 25 μL onto a 4% stacking, 12% separating acrylamide gel with MES running buffer. Precision Plus Protein All Blue Standard (Bio-Rad) was used as a molecular weight standard. The gel was run at 100 V for 15 min and then at 175 V for 40 min. The gel was then stained with Coomassie Blue stain (1 g Coomassie Brilliant Blue (Bio-Rad), 1:4:5 acetic acid, methanol, double-distilled H_2O; ddH_2O) for 2 h and then destained with a solution of 1:2:7 acetic acid, methanol and ddH_2O.

Eluates containing purified protein were dialyzed for 24 h at 4C with a 7000 MWC ThermoFisher Snakeskin dialysis membrane in 4 L of 75 mM pH 8 phosphate buffer to yield a purified product. Enzyme concentration was then measured using absorbance at 280 nm with an extinction coefficient of 103710 M^{-1} cm^{-1}.

2.3. Enzyme activity assay

A thiobarbituric acid (TBA) assay was used to demonstrate the ability of both mutant H208F and WT Smlt2602 to cleave alginate [19]. 50 μL of 0.1 mg/mL enzyme solution was combined with 50 μL 1 M sodium phosphate buffer pH8, 200 μL of 2 mg/mL low molecular weight alginate and 200 μL distilled H_2O. After incubating at room temperature for 30 min, 63 μL of periodic acid (0.025 N HIO_4, 0.125 N H_2SO_4) was added and the solution was incubated at room temperature for 20 min. 125 μL of 2% sodium arsenite was added and the solution was incubated at room temperature for another 2 min. 0.5 mL of 0.3% TBA was added to the reaction mixture, which was then heated at 90 °C for 10 min and the degree of pink coloration was observed as a qualitative measure of enzyme activity.

2.4. Characterization and culturing of algae

Samples of algae were collected from local water sources near agricultural areas (Fig. 1) where blooms were observed. Colonies of algae were skimmed from the surface and stored in pond water. The colonies were first observed using bright field microscopy with a 40X dry objective and compared to standard images of ultracellullar structure in the Landcare Research online database [20]. Algae from that colony was then inoculated into Bristol Media (2.94 mM NaNO3, 0.17 mM CaCl2·2H2O, 0.3 mM MgSO4·7H2O, 0.43 mM K2HPO4, 1.29 mM KH2PO4, 0.43 mM NaCl). Algae were grown in a 12:12 h light dark cycle at 30 °C, 3% CO_2, and light intensity of 50 μmol photons m^{-2} s^{-1} with daily agitation [21]. Isolated algae were genotyped by extraction of algicidal genomic DNA as described by Singh et al. and sequencing the 16S rRNA gene (GenScript) [22].

2.5. Sample preparation

To test the killing efficacy of enzymes on algae, algae in growth media was adjusted to an optical density measured by absorbance at 600 nm of 0.3. 125 μL of adjusted algal suspension was added to 125 μL of 75 mM phosphate buffer as a negative control, or to 125 μL of 0.2 mg/mL enzyme solutions (WT, H208F) in 75 mM phosphate buffer, pH 8. Samples were incubated at room temperature for 24 h.

2.6. Autofluorescence plate assay

Serial dilutions of cultured algae were first created to verify a linear relationship between cell concentration and fluorescence. A Tecan M1000 microplate reader was used to measure fluorescence with excitation of 440 nm and emission of 680 nm as well as cell concentration by monitoring absorbance at 600 nm (OD600) [23–25]. Autofluorescence at 680 nm corresponds with an emission peak of Chlorophyll α, which corresponds directly with algae survival [10,25]. Autofluorescence was measured for 20 individual samples (n = 20) and used to calculate the mean and standard error of each group. Sample volume was fixed at 200 μL, and measurements made in black 96 well plate with clear bottom (Grenier). Standard error for the sample group was calculated for each experiment.

2.7. Live/dead staining and quantification

Sytox green dye was used to determine live/dead staining of enzyme-treated algae. Details of this method have been described previously for microalgae [25,26,24]. Sytox green is a cell-impermeable dye that can only bind to nucleic acids of cells with damaged cell walls [27]. 5 μL of 50 μM Sytox stock solution was added to 95 μL of algae-enzyme mixture (or algae and 75 mM phosphate buffer as a control), resulting in a final dye concentration of 5 μM. Samples were incubated at room temperature in the dark for 30 min. For quantification, 160 μL aliquots were transferred to quartz cuvettes and fluorescence emissions spectra collected from 510 to 600 nm with excitation at 504 nm using PTI Quantamaster fluorescence spectrophotometer. Intensity at 535 nm is indicative of Sytox-nucleic acid binding and thus extent of inactive algae present. Sytox fluorescence was measured for 10 individual sample preparations (n = 10). Fluorescence microscopy (Nikon TE 2000-S inverted microscope) was also used on stained samples to directly observe fluorescence of the cells at 20X magnification. Filters were adjusted to capture images of autofluorescence at 680 nm as well as fluorescence at 535 nm. Images were captured and merged using Spot software [28]. To quantify movement of cells observed in the control and treated samples, 60 s videos were captured and processed using Spot software. Net cell movement was measured per second and normalized by the number of cells present within the frame. CellProfiler particle tracking software was used to quantify cell movement [29]. Colonies (aggregates of greater than 3 cells) were excluded from calculations.

2.8. Scanning electron microscopy

In order to observe cell wall morphology after enzyme treatments, algae from each sample group was dried and sputter coated for 5 s with Iridium as described by Li et al. [10]. Secondary electron detection was used to view the samples a 4300SE SEM. A low gun voltage of 3 kV was used in order to prevent damage to the samples during observation, and a standard magnification between 15,000X and 20,000X was used to capture each Image [10,11]. Circularity

was then used to quantify degree of cell wall deformation and was calculated using the formula [30]:

$$Circularity = 4\pi A/P^2$$

where A and P are the area and perimeter of the cell respectively. A perfectly circular cell would have a circularity of 1, which decreases as cell perimeter increases relative to area. Cells that displayed a circularity of less than 0.88 were considered significantly damaged and the number of damaged cells for each sample group was counted. A similar analysis has previously been used in studies to quantify cell morphology [31,32].

3. Results

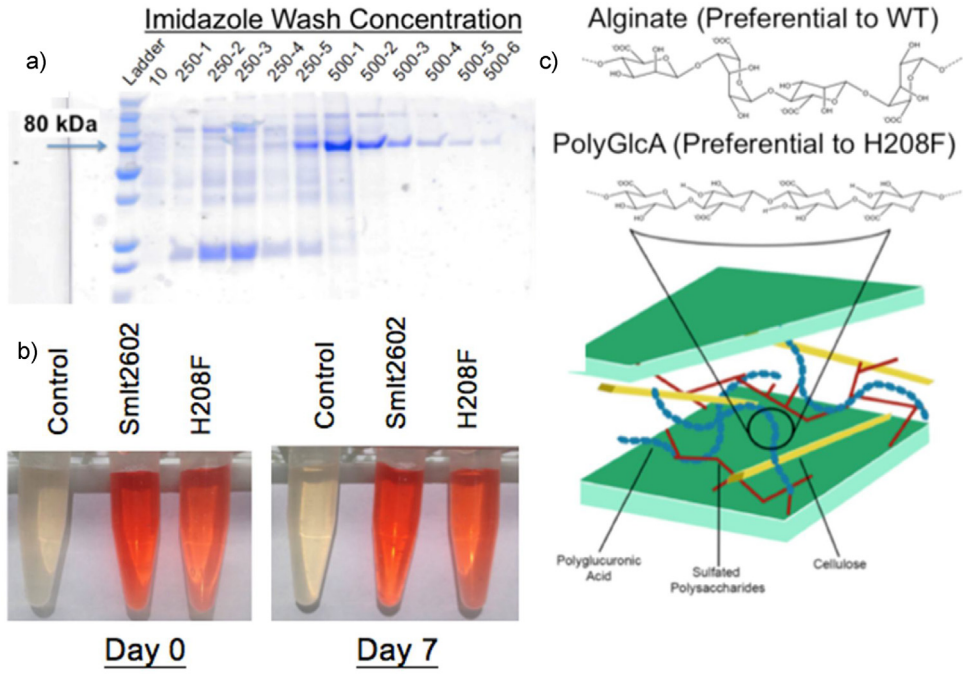
3.1. Isolation and identification of M. aeruginosa from field samples

To confirm that microorganisms isolated from actual blooms correspond to common toxin-forming cyanobacteria, and to also use samples representative of actual HABs, we isolated and processed material collected from area ponds (Fig. 1). After extraction, filtration and washing to isolate the HAB, we cultivated samples and used brightfield microscopy to characterize and sort initial isolates (Fig. 1). From this analysis, we were able to routinely identify blue-green, spherical algae with an average diameter of 8 μm that aggregated into spherical colonies, consistent with previous observation of overall morphology of *M. aeruginosa* [20]. 16s rRNA sequencing of genomic DNA isolated from cultivated samples also confirmed pond samples as *M. aeruginosa*. Thus, our results are consistent with literature indicating that *M. aeruginosa* is among the most common species contributing to HAB formation in ponds and other freshwater sources [6,7].

3.2. Determining killing efficiency of enzymes against M. aeruginosa

Both WT and mutant H208F were expressed and purified from *E. coli* BL21 cells using IMAC affinity chromatography; SDS-PAGE indicates purification to homogeneity of PLs H208F and the WT (Fig. 2). Bands at 80 kDa correspond with the molecular weight of these enzymes, which were collected in relatively high purity in the 500 mM Imidazole washes. After dialysis, protein activity was verified through a TBA assay on alginate. As indicated in previous work, *In vitro* activity of both purified enzymes against purified alginate and polyglucuronic acid (polyGlcA), both of which are major components of algae and cyanobacterial cell walls, indicates preference of H208F for polyGlcA *versus* alginate with nearly one order of magnitude improvement in specific activity over the WT [17]. A TBA assay was applied to both the WT and H208F to verify activity of the purified protein. Formation of a red adduct is indicative of polysaccharide lyase activity, leading to an unsaturated product reactive with TBA and D-GlcA (Fig. 2c), as compared to control [19]. Additionally, an enzyme sample left at room temperature for 30 days was assayed using alginate and demonstrated similar lyase activity as the initial sample, indicating long-term enzyme stability (Fig. 2b). In HAB forming organisms such as *M. aeruginosa*, polyGlcA is the predominant cell wall polysaccharide (Fig. 2c). The higher activity of H208F on this compound relative to the WT should lead to a higher degree of cleavage of polyGlcA and further breakdown of the algal cell wall (Fig. 2c).

Consistent with its expected role in preventing *M. aeruginosa* growth, treatment of samples grown in Bristol media with H208F and WT show significant reduction in cell growth *versus* treatment with buffer control by monitoring chlorophyll autofluorescence at 680 nm (Fig. 3). Cyanobacteria and other photoautotrophic organ-



ting to HAB formation in ponds and other freshwater sources[6,7].

Fig. 2. (a) SDS-PAGE was used to verify expression and purification of H208F and the WT. A band at 80 kDa for each wash concentration (10 mM, 250 mM, 500 mM) corresponds with the enzymes of interest. (b) TBA assay with alginate was used to measure enzyme activity at concentrations of 0.1 mg/mL, with the red adduct formed indicative of polysaccharide depolymerization. Enzyme activity was tested 7 days after incubation at 30C. Similar activity was observed, indicating the enzyme remained stable and active over this time period. (c) Proposed Algicidal Mechanism; The cell wall of algae is composed largely of polysaccharides. In blue-green algae specifically, one of the predominant structural polysaccharides is polyglucuronic acid. Previous work has identified mutant PL H208F from WT Smlt2602, which has a significantly higher specific activity on polyGlcA despite a lower specific activity on alginate. Therefore, it is proposed that H208F will display higher algicidal ability due to its specific activity in cleaving this key structural compound and converting it to D-GlcA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

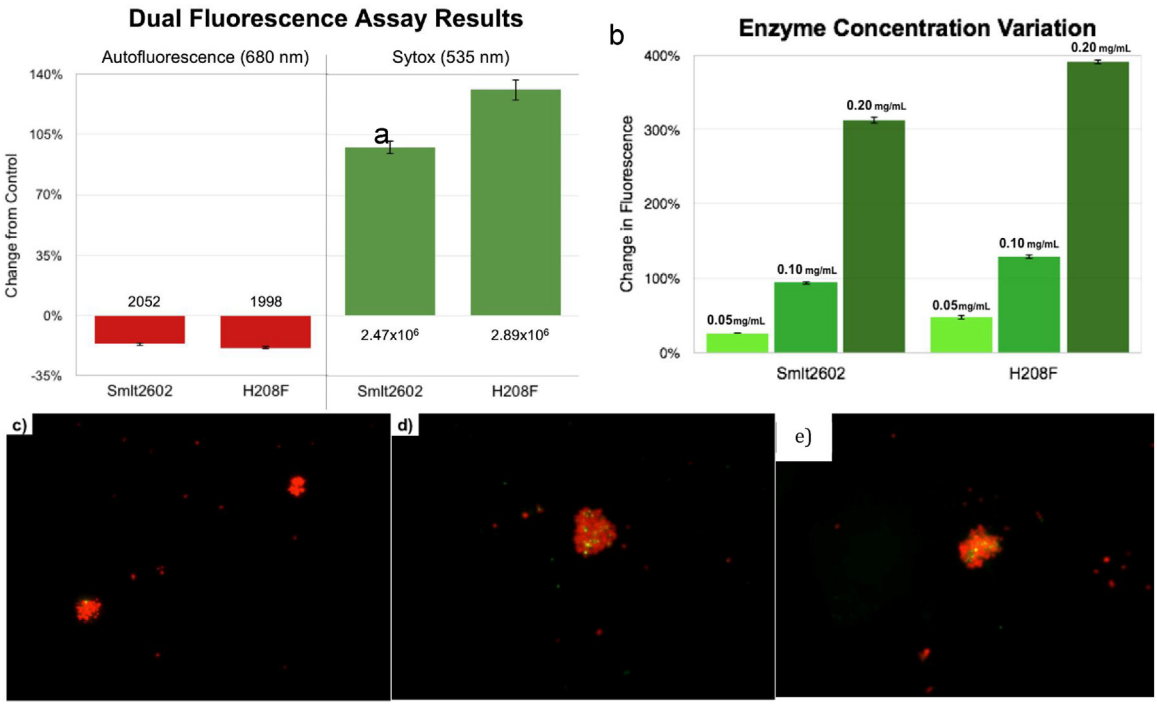


Fig. 3. Inhibitory effect of H208F and WT on algal growth Samples of *M. aeruginosa* were treated with H208F and WT enzyme, and two fluorescence-based assays were used to determine cell survival and death. Fluorescence microscopy images were collected using a 20× objective as described in Materials and Methods, with clusters of cells visible in images provided. Autofluorescesce at 680 nm was measured as an indicator of cell survival while Sytox Green nucleic acid stain and resulting fluorescence at 535 nm was used to measure cell permeabilization (a) Both the WT and H208F displayed a significant decrease in autofluorescence by 16.7% and 18.9% respectively compared to the control, indicative of cell inactivation. Both WT and H208F display a significant increase in Sytox fluorescence at 535 nm compared to the control, indicative of enhanced cell permeabilization, with H208F displaying 17% higher permeabilization than the WT. Concentration dependent results for H208F and the WT can be seen in Fig. 3b. Fluorescence images of Sytox-stained cells qualitatively support this data with fewer green cells appearing in the control images (c) compared to the WT (d) and H208F (e) treated samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

isms use light to generate energy for growth and produce a series of pigmented chlorophylls and other molecules required for light harvesting; thus fluorescence due to the presence of these molecules provides an indirect measure of cell density and viability [10]. Interestingly, both WT (16.7% reduction) and H208F (18.9% reduction) show similar, though significant reductions in autofluorescence as compared to control, indicating reduced cell viability. However, chlorophyll α is released by algae and persists in solution for longer time periods, which may contribute to a higher degree of fluorescence at 680 nm and less specific differences between H208F and WT. Furthermore, H208F and WT do not act directly on chlorophyll α but rather the cell wall, which may not entirely capture the cell-wall specific effects of these enzymes on polysaccharides such as alginate and polyglucuronic acid.

To independently assess cell viability in the presence of added enzyme, we used a cell staining/viability assay developed previously for microalgae using the cell-impermeable, fluorescent nucleic acid stain Sytox green. Unlike autofluorescence, which is an indirect measure of cell viability, Sytox green provide a direct measure of permeabilization of the cell wall, which is the expected effect (*i.e.*, increased permeabilization of the cell wall) for the purified enzymes [25,26,24]. We observe a significant increase in cell permeabilization for H208F *versus* WT compared to the control (Fig. 3). The control displayed an average maximum fluorescence at 535 nm of 1.251 million with standard error of 0.076 million. The WT and H208F displayed fluorescence intensities of 2.472 million and 2.891 million with standard errors of 0.046 million and 0.077 million respectively. This accounts for a 17% increase in permeabilization of H208F over WT. Representative fluorescence images (Fig. 3) for H208F also indicate qualitatively a greater number of individual cells staining green (indicative of Sytox binding) *versus* WT, again consistent with enhanced permeabilization of cells using H208F *versus* WT.

3.3. *Observing enzymatic mechanism of action against* M. aeruginosa

We used scanning electron microscopy (SEM) to visualize cells treated with H208F and compare them to untreated cells in order to observe differences in cell wall ultrastructure. 25 Independent SEM images were collected for algae with each enzyme treatment (0.1 mg/mL concentration, 24 h incubation), representing a population of over 100 individual cells observed. As noted in the initial identification and characterization of *M. aeruginosa* from pond water samples, individual cells have a spherical shape; thus, deviations from this spherical shape are one indication of distortion of the cell wall that can be quantified in order to classify cells observed by SEM. For WT, non-treated *M. aeruginosa* samples, the mean sphericity observed was 0.92 with a standard deviation of 0.04; a sphericity of 0.88 (one standard deviation below the mean) was selected as our cutoff for classifying cells as maintaining or losing cell wall integrity. As shown in Fig. 5, we observe a substantial increase in the number of distorted cells for H208F treated (76%) *versus* untreated (8%); representative images of untreated and treated cells are also given for comparison (Fig. 4). Of particular note, the H208F treated cells clearly show loss of cell wall integrity, sphericity and overall cell shape, which is again consistent with the expected mode of action for H208F in degrading the primary cell-wall polysaccharide polyGlcA (Fig. 2c) as well as the results of Sytox staining (Fig. 3).

We also observed loss of cell buoyancy in cell suspensions treated with WT and H208F, which would be expected in the case of loss of cell wall integrity and inability of the cell to maintain internal turgor pressure. As shown in Fig. 5, samples treated with H208F or WT show increased sedimentation over the course of the 24 h experimental treatment *versus* buffer control where no significant sedimentation occurs. When redispersed samples after sedimen-

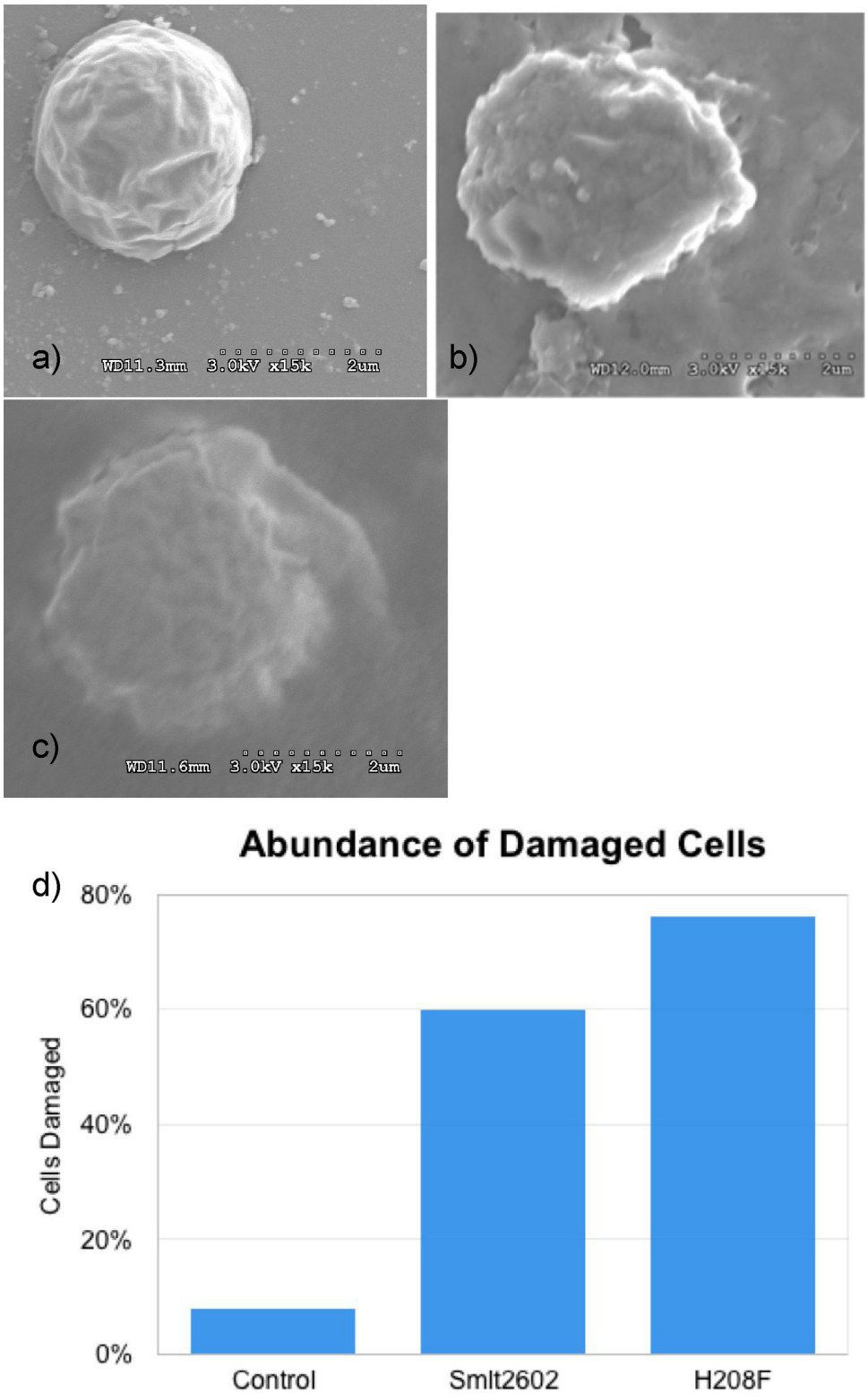


Fig. 4. SEM Images of Algae Morphology Control samples (a), *vs* samples treated with WT (b) and H208F (c). Clear deformation of the cell wall can be observed in samples treated with H208F. Loss of spherical form and collapse of cell wall in places indicate interference with cell wall integrity as a means by which H208F treatment of algae is consistent with loss of cell viability. (d) Out of 25 representative images, 76% of H208F treated samples displayed significant decrease in cell circularity below 0.88 while 60% of the WT and 8% of the control samples displayed such deformation.

tation were observed by brightfield microscopy, it was clear that in H208F treated samples a large fraction of the cells present were not motile *versus* untreated samples. We collected a series of cell sample images over the course of 1 min and assembled into videos, and used CellProfiler software to track non-colonial cell movement. Colonies (defined as 3 or more cells in an aggregate) were excluded from analysis. In the control (untreated) group, 11 of 19 cells were motile (58%) resulting in a net movement of 2.2 μm per cell per

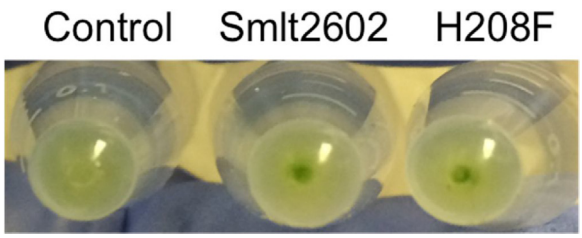


Fig. 5. Sedimentation of Algae Treated with PL Algae incubated with WT (middle) and H208F (right) for 24 h displayed significant increase in sedimentation compared to the control (left). Cell movement was measured in the control and H208F treated samples using videos captured during fluorescence microscopy, and are provided as supplemental videos (Appendix A. Supplementary data) in the web version of this article. While a significant degree of motion was contributed by 58% of cells observed cells in the control group, no cells moved in the treated sample.

second. Images were collected in an environment where vibrations in the sample stage occurred, which may influence the apparent motion of adherent cells. In the H208F treated sample, 0 of 21 cells displayed movement, resulting in no net movement. Thus, the observed sedimentation is consistent with loss of observed cell motility, and is also consistent with loss of cell viability due to enzymatic breakdown of the algal cell wall.

4. Discussion

Overall, our work indicates the ability of polysaccharide degrading enzymes to disrupt cell growth through degradation of the polysaccharide layer within the cell wall. Overall cell viability in terms of autofluorescence (Fig. 3) clearly indicates loss of cell density as compared to control, and comparison of this with Sytox green nucleic acid staining to determine cell permeability indicates this loss of viability correlates with increased cell permeability. Fluorescence microscopy further verified the significant difference in fluorescence between samples, while a complete loss of cell movement was observed in H208F treated samples compared to the control. Direct imaging of treated cells by SEM reveals a distorted morphology upon treatment with H208F, again consistent with loss of cell wall integrity. Moreover, this loss of cell wall integrity causes sedimentation and loss of buoyancy for cells grown in culture; in other words, disruption of internal turgor pressure that in turn prevents formation of vacuoles and other accessory organelles required for organisms to aggregate at the water-air interface to form HABs [33,34]. Moreover, there is specificity in terms of which polysaccharides are critical for maintaining cell wall integrity, although both alginate and polyglucuronic acid play important roles. In particular, H208F has enhanced activity against polyglucuronic acid, which is the main polysaccharide of the *M. aeruginoa* cell wall, and exhibits enhanced cell permeabilization *versus* WT (Fig. 3) [9,17]. Thus, strategies that target one or more major cell wall polysaccharides for algae, and use of lyases specific to said polysaccharides, are a potentially useful route to prevention and mitigation of overproliferation leading to HAB formation.

Our work also highlights a complementary and targeted approach as compared to other biochemical and biological tools developed to combat HABs. In particular, use of predatory bacteria such as chitinase-producing *Chitinomonas prasina* and an unidentified bacterium DHQ25 has demonstrated promising algicidal potential, including inhibition and removal of species *A. tamarense* [10,11]. However, one challenge in field applications is the ability to control constant production of proteins required. In most of these studies, a specific biomolecule or biomolecules responsible for the anti-algal effect has not been identified, which makes engineering an organism or enhancing expression of key biomolecules extrinsically difficult. In contrast, in the current study we have taken a direct approach in targeting cell wall polysaccharides known to be important for cell integrity (Fig. 2), and applying purified proteins directly to cell suspensions to determine a specific effect on algal inhibition (Figs. 3–5). Other studies have sought to use compounds isolated from organisms with anti-algal activity, including palmitoleic acid from *Vibrio* sp. BS02 and an algicidal protein from bacterium DHQ25 effective against *A. tamarens* [12,15]. While these studies successfully isolate a compound and demonstrate that it displays algicidal capability, specific mechanisms of action remain an area of active research. Moreover, biomolecules such as palmitoleic acid are consumed during cell lysis, whereas enzymes such as those described in this study, exhibit continuous anti-algal activity. Moreover, the long-term stability (Fig. 2) enables a way to not only eliminate HABs and harmful algae that overproliferate, but to have continuous inhibition of these algae. Additionally, the ability to engineer substrate specificity for different polysaccharides such as polyglucuronic acid (H208F) *versus* alginate (WT) [17], enables a targeted approach that can selectively inhibit harmful algae *versus* beneficial microbes required for a balanced ecosystem.

Overall, a biological algaecide with high activity, specificity and long-term stability addresses environmental concerns of chemical algaecides such as copper sulfate while providing a defined inhibitory effect on algal growth. In the case of HABs that routinely form in lakes and fresh water sources such as that used in this study (Fig. 1), an abundance of nitrates and phosphates causes increased growth of algae beginning in spring. In order to compete for light, which becomes a limiting growth factor, algae develop vacuoles in association with bloom formation in order to capture light at the surface of the water. This, in turn, limits light to deeper regions of the body of water and forces algae to develop vacuoles in order to effectively reach light [33,35]. This positive feedback cycle leads to the uncontrolled growth of algae that forms HABs, produces toxins and destroys the overall ecosystem. An approach using an enzymatic algaecide such as that described in this study could be applied to bodies of water in the spring using similar methods for dispersing chemical algaecides. Ideally, by inhibiting growth early on, the cycle of bloom formation could be stopped or at least slowed significantly. As a result, toxin levels can be maintained within a safe threshold in order to protect drinking water sources, preserve the health of fisheries, and ensure safety of recreational waters.

5. Conclusions

In summary, we have demonstrated that a polyglucuronic acid-selective lyase is capable of targeting the cell wall of and preventing growth of the harmful cyanobacteria *M. aeruginosa* responsible for HAB formation. Moreover, the use of a mutant lyase (H208F) specific to the major polysaccharide component of the cell wall is more effective than the parental (WT) enzyme, which is not selective for polyglucuronic acid *versus* alginate, although both H208F and WT are effective as algaecides. Whereas other biocontrol strategies utilize predatory bacteria, but without a clearly defined mechanism of action, we have demonstrated a rational strategy that targets specific chemical structures within the cell wall as a way to inhibit cyanobacterial growth. Thus, our work suggests polysaccharide composition of the algal cell wall presents a unique and specific target for engineering polysaccharide degrading enzymes as a way to inhibit algal cell growth and ultimately provide a green, non-toxic and targeted alternative to remove HABs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bej.2018.01.005.

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