Application of biosurfactant from *Bacillus subtilis* C9 for controlling cladoceran grazers in algal cultivation systems

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Open algal cultivation platforms often suffer crop losses to herbivorous grazers that have potential to devastate biomass production within a few days. While a number of studies suggest synthetic chemicals as control agents for voracious algal grazers, environmental and safety concerns associated with the use of these chemicals encourage the exploration of alternative biological control agents. We hereby propose the application of a biosurfactant produced by *Bacillus subtilis* C9 (referred to as C9-biosurfactant) for controlling cladoceran grazers commonly found in algal cultivation systems. The results indicated that C9-biosurfactant completely eradicated *Daphnia pulex* and *Moina macrocopa* within 24 hours when concentrations were equal to or exceeded 6 mg/L. Moreover, supplying C9-biosurfactant into the cultures of selected algal species with and without cladoceran grazers indicated no adverse effect of C9-biosurfactant on the growth and lipid productivity of algal crops, while cladocerans were selectively controlled by C9-biosurfactant even under the presence of their prey. These results thus indicate that C9-biosurfactant could be an effective biocontrol agent for cladoceran grazers at industrial algal cultivation.
cost-effective strategies for controlling unpredictable blooms of algal grazers because loss of even a single algal reactor in an array can dramatically impact overall facility productivity and yield3,19,20.

Synthetic chemicals have thus been explored to mitigate algal crop losses from zooplankton grazers in algal production systems17–21. For example, the disinfectant hypochlorite was used to control the rotifer Brachionus calyciflorus in laboratory cultures of the microalga Chlorella kessleri19,21,22. In addition, Wang et al. outlined the lethal concentrations of three synthetic pesticides on B. calyciflorus and Daphnia pulex23. Synthetic compounds, however, may have negative impacts on non-target organisms and/or adjacent ecosystems24,25. Therefore, sustainable cost-effective strategies that control growth and proliferation of grazing zooplanktons in algal cultivation platforms without the use of synthetic chemicals are needed17,26–30.

In terrestrial agriculture, microbial secondary metabolites have been regarded as promissory alternatives to synthetic pesticides because of their diverse metabolites with high biological activities31,32. In particular, Bacillus spp. lead active research and development efforts because the strains of Bacillus are known to excrete peptides and lipopeptides, such as fungicin, iturin, bacillomycin, and others that have antifungal, antibacterial, and high surfactant activity33. Although a variety of biological control products based on Bacillus species are available for agronomic use to control agricultural pests, few studies explored the direct application of the secondary metabolites of Bacillus in the systems targeting to grow algal biomass33.

Previously, Kim et al. isolated Bacillus subtilis C9 that produces surfactin, a lipopeptide biosurfactant with antibacterial, antiviral, and anti-biofilm forming activities34,35. While B. subtilis and its biosurfactant were known to exhibit biocontrol activities against mosquito pupae and phytopathogenic fungi36–39, the purpose of this study was to explore the biocontrol activity of recovered biosurfactant from the culture of B. subtilis C9 (referred hereupon as C9-biosurfactant) on common cladoceran grazers in algal cultivation platforms34,39. Grazer-introduced cultures were first subjected to different concentrations of C9-biosurfactant to test whether cladoceran grazers were effectively controlled by C9-biosurfactant. Because the industrial applicability of any biocontrol compound must assure its non-toxicity to non-target species, we further explored the influence of supplemental C9-biosurfactant on the growth and/or lipid productivity of two microalgal strains with and without the presence of cladoceran grazers.

Results and Discussion

Recovery and identification of C9-biosurfactant. The average surface tension of cell-free supernatants from triplicate cultures of B. subtilis C9 after a 72-hour-long cultivation period was 29.8 ± 1.1 mN/m, and the average concentration of acid precipitate in the respective culture supernatant was 0.9 ± 0.2 g/L. 0.3 ± 0.1 g/L of C9-biosurfactant was then recovered from acid precipitates by dissolving C9-biosurfactant in deionized water and freeze-drying the biosurfactant solution. Our subsequent mass spectrometry analysis of C9-biosurfactant exhibited the highest peak at m/z 1058.67, where the highest peak of HPLC-grade surfactin from B. subtilis was also observed (Fig. S1). While the molecular ion species of surfactin can be detected in either protonated or sodium adduct forms, Kim et al. also reported a high peak of C9-biosurfactant at m/z 1058, where the sodium adduct of surfactin is putatively present34,39,40. In addition, the CMD values of aqueous solutions of C9-biosurfactant and HPLC-grade surfactin at an identical concentration of 0.3 g/L were 50 and 60, indicating that the relative proportion of surfactin in the biosurfactant was roughly 83% (Figs S2 and S3). These results thus indicated the production of biosurfactant in the culture of B. subtilis C9 and further confirmed surfactin as the main surfactant compound of C9-biosurfactant34,35,40. Although C9-biosurfactant tested in this study was obtained from triplicated batch cultivation of B. subtilis C9, it should be also noted that the concentration of surfactin in the culture of B. subtilis C9 could substantially vary under different cultivation conditions, and direct quantification of surfactin in C9-biosurfactant will be required especially when determining the application doses of C9-biosurfactant in industrial algal cultivation41.

C9-biosurfactant acts as an effective control agent for cladoceran Daphnia and Moina. While a diverse array of physical, biological, and chemical measures were recently explored to successfully control blooms of algal grazers39, our results indicated that C9-biosurfactant can also be implemented to eradicate cladoceran grazers in industrial algal cultivation platforms. The results showed 6 mg/L of C9-biosurfactant was enough to cause complete mortality of both Daphnia pulex and Moina macrocopa within 12 hours (Fig. 1). Our subsequent test with HPLC-grade surfactin from Bacillus subtilis also indicated the complete mortality of cladocerans within 12 hours at concentrations equal to or above 6 mg/L, confirming that the main surfactant compound of C9-biosurfactant resulted in the mortality of algal grazers (Figs S2 and S3).

The results further suggested that the cladoceran-control activity of C9-biosurfactant is dependent on the concentration of C9-biosurfactant. In particular, the data obtained 1 hour after the application of C9-biosurfactant exhibited substantial increases in the percent mortalities of both cladocerans with increasing concentrations of C9-biosurfactant (Fig. 1). While Vollenbroich et al. similarly reported that the antiviral activity of surfactin substantially increased with increasing surfactin concentration42, dose-dependent mortality response of cladocerans may provide important information that can be exploited to achieve successful cladoceran-control in a shorter period of time. Nonetheless, complete eradication of cladoceran grazers within 1 day following the application of C9-biosurfactant at all tested concentrations indicates C9-biosurfactant can act as an effective biocontrol agent for cladoceran algal grazers in industrial algal cultivation systems, especially because invading cladoceran grazers often require at least 1–2 days to substantially consume dominant microalgal species32,39,43.

Although this study was focused on demonstrating the biocontrol activity of C9-biosurfactant on cladoceran grazers, microzooplanktons, including rotifers and ciliated protists, are also known to detrimentally contaminate industrial algal cultivation platforms. Indeed, order-of-magnitude reductions in microalgal biomass have been well acknowledged for ciliate-dominated zooplankton in commercial Spirulina (Arthrospira) cultivation sites and for rotifer-dominated zooplankton in High Rate Algal Ponds (HRAPs)32,29,39,43,44. Such devastatingly high level of
algal crop losses will impede making algae-based products a commercial reality, thus exploring the effectiveness of C9-biosurfactant in controlling other frequent algal grazers will be vitally important to further support the implementation of C9-biosurfactant at industrial algal cultivation.

Indeed, previous studies have reported that surfactants generally exhibit biocontrol activities against different organisms. Geetha et al., for example, noted the mosquito pupicidal activity exhibited by the biosurfactant of Bacillus subtilis, and Manonmani et al. further confirmed that the culture supernatant of Bacillus subtilis was found to kill both larval and pupal stage of mosquito species of Anopheles stephensi, Culex quinquefasciatus, and Aedes aegyti, while a significantly lesser amount of biosurfactant was required to kill the mosquito pupae. In addition, Lechuga et al. explored the toxicity of different surfactants on the luminescent bacterium Vibrio fischeri, and reported that V. fischeri was more sensitive to the toxic effects of the surfactants than was cladoceran Daphnia. Nonetheless, it is evident that the cladoceran-control activity of C9-biosurfactant bears substantial industrial potential for managing blooms of cladoceran grazers in algal cultivation platforms, especially given that large-bodied cladocerans are typically thought to control microalgal production much more effectively than other small-bodied herbivorous grazers because their algal ingestion is less influenced by the size of resident algal strains.

Although the exact mode of action of allelopathic C9-biosurfactant on cladoceran grazers requires further exploration, it was speculated that a reduction in the surface tension of the water caused by C9-biosurfactant was likely to require more energetic costs during swimming that may have substantially contributed to the observed cladoceran mortality. Vollenbroich et al. reported the disintegration of mycoplasma membranes after treating mycoplasma-contaminated mammalian cells with surfactin, and concluded that the disintegration of bacterial membrane was due to a physicochemical interaction of the membrane-active surfactant with the outer part of the lipid membrane bilayer, whereas the low cytotoxicity of surfactin for mammalian cells was observed. Although the cladoceran-control activity of C9-biosurfactant is likely to depend on the life cycle and nutritional status of cladocerans, a physicochemical interaction of surfactin with the carapace of cladocerans may also be an important contributor to the observed cladoceran mortality.

C9-biosurfactant does not negatively influence the biomass and lipid productivity of algal crops. Any potential biocontrol agents in agriculture should be tested for their toxicity to crop species prior to warranting their application in agricultural fields. Likewise, the application of C9-biosurfactant in algal cultivation systems cannot be justified without evaluating the influence of C9-biosurfactant on the growth and productivity of selected algal crops. The results of our flasks-scale experiments, however, clearly indicated no growth inhibiting effect of C9-biosurfactant on Chlorella sp. HS2 and Scenedesmus deserticola JD052 (Fig. 2 and Table 1).
The specific growth rates of each algal species were not statistically different under varying concentrations of C9-biosurfactant (p-values > 0.05), except the culture of \textit{Chlorella} sp. HS2 amended with 60 mg/L of C9-biosurfactant, which had a lower specific growth rate than that of the control without C9-biosurfactant (p-value < 0.05) (Table 1). The well-plate experiments similarly indicated that the supplemental surfactin did not negatively influence the growth of \textit{Chlorella} sp. HS2 and \textit{Scenedesmus deserticola} JD052, except in the case of adding 60 mg/L of surfactin into the culture of \textit{Chlorella} sp. HS2 where the final cell density was only about half that of the control with no supplemental surfactin (Fig. S7). These results indicated no growth inhibiting effect of C9-biosurfactant and surfactin on the selected \textit{Chlorella} and \textit{Scenedesmus} strains at the concentrations below 60 mg/L, which were enough to cause complete cladoceran mortality (Fig. 1).

The DCW and lipid content of \textit{Chlorella} sp. HS2 and \textit{Scenedesmus deserticola} JD052 substantially increased under 6 and 20 mg/L treatments of C9-biosurfactant (Fig. 3). While no substantial increase in final DCW was measured under 60 mg/L of C9-biosurfactant for either algal strain, increasing the concentration of C9-biosurfactant up to 60 mg/L continuously enhanced the lipid content of \textit{Scenedesmus deserticola} JD052 (Fig. 3). In contrast, the lipid content of \textit{Chlorella} sp. HS2 was highest in the cultures amended with 6 mg/L of C9-biosurfactant, and the \textit{Chlorella} cultures amended with 60 mg/L of C9-biosurfactant exhibited no considerable difference in the lipid content compared to that of the control with no supplemental C9-biosurfactant (Fig. 3). The subsequent FAME analysis further indicated minor changes (i.e., 1–5%) in the proportion of each type of fatty acid under varying concentrations of C9-biosurfactant: oleic (C18:1n9c) and \(\alpha\)-linolenic (C18:3n3) acids exhibited substantial shifts in the cultures of \textit{Scenedesmus deserticola} JD052 and the additive C9-biosurfactant substantially influenced the proportions of palmitic (C16:0) and linoleic (C18:2n6c) acids in the cultures of \textit{Chlorella} sp. HS2 (Tables 2 and 3).

The lipid productivities of \textit{Chlorella} and \textit{Scenedesmus} were significantly enhanced with the supplemental C9-biosurfactant (p-values < 0.05) in most cases (but see \textit{Chlorella} cultures amended with 60 mg/L of C9-biosurfactant – Fig. 4). The highest lipid productivities of \textit{Chlorella} sp. HS2 and \textit{Scenedesmus deserticola} JD052 were 0.178 and 0.128 g/L/day at C9-biosurfactant concentrations of 6 and 20 mg/L, respectively. Such increments

|               | 0 mg/L of C9-biosurfactant | 6 mg/L of C9-biosurfactant | 20 mg/L of C9-biosurfactant | 60 mg/L of C9-biosurfactant |
|---------------|----------------------------|----------------------------|-----------------------------|-----------------------------|
| \textit{Chlorella} sp. HS2 | 1.55 ± 0.21               | 1.52 ± 0.28               | 1.56 ± 0.42                 | 1.20 ± 0.11                 |
| \textit{Scenedesmus deserticola} JD052 | 1.11 ± 0.21               | 1.18 ± 0.34               | 1.08 ± 0.27                 | 0.929 ± 0.18                |

Table 1. Specific growth rates of \textit{Chlorella} sp. HS2 and \textit{Scenedesmus deserticola} JD052 under varying concentrations of C9-biosurfactant in 250-mL flask cultures. Data are presented as means ±95% confidence intervals.

![Figure 2](https://www.nature.com/scientificreports/)
corresponded to 35 and 51% of increases in the lipid productivities compared to the control cultures of each algal strain. While the amount of triacylglycerols (TAG) in harvested algal biomass would be a direct indicator for understanding the influence of supplemental biosurfactant on the bioenergy potential of selected algal strains, Griffiths and Harrison emphasized that lipid productivity itself could be a key characteristic for choosing algal species for biodiesel production52. These results thus suggest that the additive C9-biosurfactant does not compromise the growth or lipid productivity of the selected algal crops, and further indicate the possibility of utilizing C9-biosurfactant to promote the bioenergy potential of harvested biomass at industrial algal cultivation.

The increased lipid productivities of *Chlorella* sp. HS2 and *Scenedesmus deserticola* JD052 may be resulted from increased nutrient uptake into cell bodies with the additive C9-biosurfactant. Taoka et al., for example, reported ca. 4% increase in the total lipid content of *Thraustochytrium aureum* with the addition of 1% Tween 80, a non-ionic surfactant, and speculated that the addition of surfactant likely interfered with the permeability of cell membranes and enhanced the nutritional uptakes into the cell body53. The authors reported significant increases in the proportions of both saturated and monounsaturated fatty acids, while the percentage of unsaturated fatty acids decreased with the addition of Tween 8053. Our results, however, indicated the opposite trend:

Figure 3. Dry cell weight (g/L) and lipid content (% DW) of harvested biomass of *Chlorella* sp. HS2 (a) and *Scenedesmus deserticola* JD052 (b) following the harvest of 250-mL flask cultures grown under varying concentrations of C9-biosurfactant in batch cultivation mode. Error bars represent the standard deviation from the mean for triplicate independent measurements.

| Fatty acid                  | Proportion (%) of fatty acid |
|-----------------------------|------------------------------|
|                             | 0 mg/L of C9-biosurfactant  | 6 mg/L of C9-biosurfactant | 20 mg/L of C9-biosurfactant | 60 mg/L of C9-biosurfactant |
| Palmitate (C16:0)           | 29.66 ± 1.34                 | 26.79 ± 1.71                | 26.44 ± 0.80                 | 25.18 ± 0.37                 |
| Palmitoleate (C16:1)        | 0.30 ± 0.03                  | 0.00 ± 0.00                 | 0.28 ± 0.40                  | 0.65 ± 0.91                  |
| Hexadecatrienoate (C16:3)   | 5.46 ± 0.54                  | 6.55 ± 0.72                 | 7.66 ± 1.72                  | 7.29 ± 0.36                  |
| Stearate (C18:0)            | 1.80 ± 0.21                  | 2.15 ± 0.19                 | 1.56 ± 0.40                  | 1.61 ± 0.15                  |
| Oleate (C18:1n9c)           | 24.72 ± 0.91                 | 23.51 ± 1.62                | 21.55 ± 2.63                 | 21.46 ± 0.85                 |
| Linoleate (C18:2n6c)        | 27.00 ± 1.16                 | 30.96 ± 1.70                | 30.72 ± 1.36                 | 32.29 ± 0.31                 |
| Linolenate (C18:3n3c)       | 3.34 ± 0.23                  | 3.67 ± 0.49                 | 4.18 ± 0.78                  | 4.11 ± 0.26                  |
| Saturated (%)               | 31.46 ± 1.55                 | 28.94 ± 1.90                | 28.00 ± 1.20                 | 26.79 ± 0.30                 |
| Monounsaturated (%)         | 25.02 ± 0.48                 | 23.51 ± 1.62                | 21.83 ± 3.03                 | 22.10 ± 1.76                 |
| Polyunsaturated (%)         | 35.80 ± 1.93                 | 41.18 ± 2.90                | 42.57 ± 3.86                 | 43.68 ± 0.93                 |
| Others (%)                  | 7.71 ± 0.10                  | 6.37 ± 0.62                 | 7.60 ± 0.37                  | 7.42 ± 0.62                  |
| Total (%)                   | 100                          | 100                         | 100                          | 100                          |

Table 2. Fatty acid composition of *Chlorella* sp. HS2 under varying concentrations of C9-biosurfactant. Data are presented as means with the standard deviation.
the proportions of saturated and mono-unsaturated fatty acids were generally declined with increasing concentration of C9-biosurfactant, whereas the proportion of unsaturated fatty acids tended to increase with increasing C9-biosurfactant concentration mainly due to substantial increase in the proportion of polyunsaturated fatty acids (Tables 2 and 3). The increase in the relative proportion of unsaturated fatty acids has been acknowledged as a compensating response to the decreased thylakoid membrane fluidity under low temperature conditions for a green alga, *Tetraselmis* sp., and an increase in the level of polyunsaturated fatty acids particularly was suggested as a shift in the structure of membrane-forming lipids to spatially increase membrane fluidity that potentially enables more efficient exchange of metabolites54–56. Although few studies addressed the influence of biosurfactant

| Fatty acid          | Proportion (%) of fatty acid |
|---------------------|-----------------------------|
|                     | 0 mg/L of C9-biosurfactant | 6 mg/L of C9-biosurfactant | 20 mg/L of C9-biosurfactant | 60 mg/L of C9-biosurfactant |
| Palmitate (C16:0)   | 21.92 ± 0.38                | 20.70 ± 0.14                | 20.13 ± 0.14                | 19.30 ± 0.26                |
| Palmitoleate (C16:1)| 4.00 ± 0.11                 | 4.41 ± 0.05                 | 3.49 ± 0.40                 | 3.07 ± 0.44                 |
| Hexadecatrienoate (C16:3) | 5.31 ± 0.12     | 5.51 ± 0.18                 | 5.64 ± 0.17                 | 5.44 ± 0.26                 |
| Stearate (C18:0)    | 4.67 ± 0.18                 | 3.30 ± 0.11                 | 4.15 ± 0.55                 | 3.90 ± 0.18                 |
| Oleate (C18:1n9c)   | 21.79 ± 0.34                | 20.58 ± 0.23                | 20.34 ± 1.42                | 18.51 ± 0.57                |
| Linoleate (C18:2n6c)| 14.46 ± 0.28                | 15.09 ± 0.08                | 16.25 ± 0.67                | 16.72 ± 0.77                |
| Linolenate (C18:3n3c)| 13.55 ± 0.39                | 15.29 ± 0.60                | 15.07 ± 0.66                | 17.24 ± 0.13                |
| Saturated (%)       | 26.59 ± 0.56                | 24.00 ± 0.25                | 24.28 ± 0.41                | 23.20 ± 0.08                |
| Monounsaturated (%) | 25.79 ± 0.45                | 24.99 ± 0.28                | 23.83 ± 1.83                | 21.58 ± 1.01                |
| Polyunsaturated (%) | 33.32 ± 0.79                | 35.89 ± 0.35                | 36.96 ± 1.16                | 39.41 ± 0.39                |
| Others (%)          | 14.29 ± 1.79                | 15.12 ± 0.32                | 14.92 ± 0.26                | 15.80 ± 0.54                |
| Total (%)           | 100                         | 100                         | 100                         | 100                         |

Table 3. Fatty acid composition of *Scenedesmus deserticola* JD052 under varying concentrations of C9-biosurfactant. Data are presented as means with the standard deviation.

Figure 4. Lipid productivity of the cultures of *Chlorella* sp. HS2 (a) and *Scenedesmus deserticola* JD052 (b). Lipid productivity was calculated by multiplying lipid content (%) with corresponding biomass productivity after harvesting flask cultures treated with varying concentrations of C9-biosurfactant at the end of either 6 or 7 day-long batch cultivation period. Error bars indicate 95% confidence intervals for the mean.
on algal membrane fluidity, more careful evaluation on the mechanisms involved in possible shifts in membrane fluidity will be necessary to clearly understand algal response to the additive biosurfactant.

It should be, however, noted that algal response to biosurfactant is closely dependent on the type of biosurfactant and the algal strains that are being cultivated. For example, Rieß and Grimme reported that diethyleneglycol monohexadecylether (C16E2), a nonionic surfactant, was shown to act as an instantaneous inhibitor of cell growth and reproduction of *Chlorella fusca*, whereas a cationic surfactant cetyltrimethylammonium chloride (CTAC) was shown to induce only minor acute effects on photosynthesis and respiration. A recent study further tested the toxicity of alkylglucosides on the freshwater alga, *Selenastrum capricornutum* and reported that these non-ionic surfactants are relatively innocuous to *Selenastrum* after a 72-hour algal growth inhibition test, while substantial toxicity of these surfactants were confirmed with cladoceran *Daphnia magna*. In addition, Ahn et al. confirmed that the additive surfactin slightly inhibited the growth of *Chlorella vulgaris* and a *Scenedesmus* species, suggesting the possibility that two algal strains tested in this study had intrinsically greater tolerance against high surface activity of surfactin than other algal species. These authors, however, also noted that the growth of *Navicula* sp. seemed to be stimulated with the additive surfactin. Therefore, the influence of C9-biosurfactant on a wide variety of algal crop species should be systematically evaluated throughout a long-term operation to successfully achieve the industrial application of C9-biosurfactant as an effective biocontrol agent.

**Applying C9-biosurfactant in industrial algal cultivation platforms.** While the results presented in Fig. 1 were based on the cases in which algal cells were not provided throughout the experimental period, it is true that prey availability is not limiting for the growth of algal grazers in industrial algal cultivation platforms. It is thus vitally important to evaluate the effectiveness of C9-biosurfactant in controlling cladoceran grazers in the presence of algal prey because the nutritional status of cladocerans can substantially influence the cladoceran-control activity of C9-biosurfactant.

Although Fig. 1 indicated the complete eradication of cladocerans within 12 hours at the C9-biosurfactant concentration of 6 mg/L, the results clearly showed that more than 12 hours were required to completely eradicate cladoceran grazers with 6 mg/L of C9-biosurfactant under the presence of algal prey (Figs 5b and 6b). In particular, the respective percent mortalities of *Daphnia pulex* were 45 and 60% in the cultures of *Chlorella* sp. HS2 and *Scenedesmus deserticola* JD052, and 40 and 70% of the respective percent mortalities of *Moina macrocopa* were observed in the cultures of *Chlorella* sp. HS2 and *Scenedesmus deserticola* JD052 after treating with 6 mg/L of C9-biosurfactant for 12 hours (Figs 5b and 6b). The delayed mortality response of cladoceran grazers at the C9-biosurfactant concentration of 6 mg/L was reflected in lower algal cell densities than those of the tube cultures amended with higher C9-biosurfactant concentrations (Figs 5a and 6a), and no algal growth limiting effect of C9-biosurfactant was observed at the C9-biosurfactant concentrations above 6 mg/L (Figs 5a and 6a). These results indicate that longer exposure of C9-biosurfactant to cladoceran grazers is likely to be necessary to
achieve complete eradication in the presence of algal prey, and reaffirm that the additive C9-biosurfactant does not substantially limit the growth of two selected algal strains.

Although the complete eradication of cladoceran grazers was observed within 12 hours upon the application of C9-biosurfactant at the concentrations of 20 and 60 mg/L, the percent mortalities of cladoceran grazers were greater in the cultures of *Scenedesmus* after 12 hours under 6 mg/L of C9-biosurfactant than in the cultures of *Chlorella*. Such differences in cladoceran mortality may be the results of different ingestion rate of algal grazers by the size of algal prey. Cho et al., for example, tested the ingestion rate of *Daphnia* sp. with different algal strains and reported that the large-sized microalga, *Pediastrum* sp. was most resistant to the grazing pressure of *Daphnia*, while relatively smaller unicellular algal species such as *Parachlorella* sp. were effectively grazed by the cladoceran grazer. Cell size and long axis length of microalgae were particularly hypothesized as master traits that inherently control other ecological traits related to the trends in competition and facilitation, especially because size confers a defense against grazing. Indeed, the cell density of *Chlorella* sp. HS2 was more drastically decreased than that of *Scenedesmus deserticola* JD052 under the presence of cladoceran grazers without supplemental C9-biosurfactant (Figs 5a and 6a). It is therefore likely that small-sized unicellular *Chlorella* sp. HS2 was more effectively ingested by cladoceran grazers than colonial *Scenedesmus deserticola* JD052, and potential differences in algal ingestion efficiency seemed to contribute to the lower percent mortalities of cladoceran grazers in the culture of *Chlorella* sp. HS2 under 6 mg/L of C9-biosurfactant. These results thus reemphasize potential differences in resistance against grazing between different algal species, and further suggest the importance of carefully selecting algal crop species to effectively achieve crop protection against grazing consumers because the extent of crop loss to algal grazers may significantly vary with the choice of algal crop species.

Although the biosurfactants of *Bacillus subtilis* are known to withstand a wide range of pH values, exposure to long hours of sunlight, UV radiation, as well as moist heat up to 121 °C, continuous or semi-continuous operation of algal reactors may necessitate greater inputs of C9-biosurfactant to successfully control algal grazers due to typically greater biomass-specific grazing rates than biomass-specific growth rates in continuous cultures. In addition, the exposure regime of biocontrol agent (i.e., continuous vs. pulse exposure) was known to play a critical role in determining the effectiveness of selected control agents. While the complete eradication of cladoceran grazers may not be desirable because of the potential ecological imbalances and the establishment of possibly unmanageable alien grazers, future research will need to test the cladoceran-control activity of C9-biosurfactant under varying operational conditions with high-frequency monitoring of ecological communities to assure the industrial applicability of C9-biosurfactant. Furthermore, the environmental impact of the possible release of biosurfactant to waterbodies should be evaluated across different trophic levels to ensure safe and reliable application of C9-biosurfactant, although biosurfactants are generally considered to be less toxic and more biodegradable than synthetic surfactants.
It has to be also noted that the yield of biosurfactant from the culture of *B. subtilis* C9 can be substantially enhanced by optimizing growth medium or selecting cost-effective growth substrate. Kim *et al.*, for example, carefully optimized both macro- and micro-nutrients of the growth medium of *B. subtilis* C9 and achieved the production of C9-biosurfactant at the concentration of 7.0 g/L. Furthermore, Gudina *et al.* reported 1.3 g/L of average biosurfactant production from *B. subtilis* #573 grown in corn steep liquor (CSL) with the low surface tension values between 29.7 and 31.0 mN/m of cell-free supernatants without dilution. Moreover, Fox and Bala tested waste potato substrates as a low-cost carbon source for surfactant production by *B. subtilis ATCC 2133*, and observed a significant drop of surface tension with the recovered biosurfactant. High yield of biosurfactant from *B. subtilis* with agro-waste particularly indicates the possibility of achieving the production of C9-biosurfactant in a cost-effective manner; further investigation into producing C9-biosurfactant with waste nutrient sources could substantially contribute to the industrial application of C9-biosurfactant as an effective biocontrol agent.

Although there is unlikely to be a one-size-fits-all solution for managing unpredictable blooms of algal grazers at industrial algal biomass production, the results of this study clearly supported the potential of C9-biosurfactant as an effective biocontrol agent for cladoceran algal grazers. The additive C9-biosurfactant, however, did not inhibit the growth and lipid productivity of *Chlorella* sp. HS2 and *Scenedesmus deserticola* JD052, supporting the non-toxicity of C9-biosurfactant to the selected algal crops. While managing algal pests in industrial algal cultivation platforms is likely to require the harmonious utilization of carefully screened control strategies, combining the C9-biosurfactant with other effective biocontrol measures will be needed to successfully reduce or completely eliminate algal grazers. Additionally, we urge more careful investigations into optimizing the application strategy of C9-biosurfactant under varying outdoor cultivation conditions at the scales more relevant to industrial algal cultivation.

Methods

**Strain selection.** *Bacterial strain.* Kim *et al.* previously isolated *Bacillus subtilis* C9 that produces an effective biosurfactant by the method of oil-film collapsing assay. The biosurfactant of *B. subtilis* C9 was determined to be a lipopeptide consisting of a C14–15 fatty acid tail linked to a peptide moiety consisting of 7 amino acid residues that was identical to the peptide moiety of surfactin. While the lipopeptides produced by *Bacillus* strains are considered as the main compounds involved in the biocontrol activities of *Bacillus* on phytopathogens, surfactin is particularly known for its remarkable surfactant properties and interactions with artificial and biomembrane systems (e.g., bacterial protoplasts or enveloped viruses) that were postulated to give rise to its antibiotic activities.

*Cladoceran algal grazers.* While the zooplankton communities of algal cultivation systems are likely to substantially vary across different sites and seasons, *Daphnia pulex* and *Moina macrocopa* were selected as model cladoceran grazers based on their blooms during our year-long operation of wastewater-fed open algal ponds. Laboratory clones of *Daphnia pulex* and *Moina macrocopa* were obtained from a local supplier (Biozoo Biological Supply Co. Ltd, Korea) and Gyeongsang National University (Tongyeong, Korea), respectively. Cladoceran cultures were then maintained at 25 °C in 500–mL beakers at the light intensity of 150 µmol·m−2·s−1 and 14: 10 L/D photoperiod in COMBO medium. Each zooplankton culture was fed with either *Chlorella* sp. HS2 or *Scenedesmus deserticola* JD052 at the algal cell density of 1 × 10^6 cells/mL. Cladoceran density at each culture was maintained above 300 individuals/L and each beaker was gently shaken twice a day to ensure that algal cells stayed in suspension.

*Microalgal strains.* While our year-long operation of wastewater-fed open ponds indicated the year-round presence of *Chlorella* and *Scenedesmus* at the algal cell density of 1 × 10^6 cells/mL. *Chlorella* sp. HS2 and *Scenedesmus deserticola* JD052 were previously isolated from local wastewater sources. The bacteria-free seed culture of each of these algal strains was subsequently obtained by treating xenic algal cultures in BG-11 medium with ultrasonication and micropicking. Because the species of *Chlorella* and *Scenedesmus* are particularly recognized for their industrial potential for the production of biofuels and bioproducts, we selected these isolated strains to evaluate the influence of C9-biosurfactant on the growth and productivity of algal crops. Prior to the inoculation, samples withdrawn from bacteria-free seed cultures of two algal strains were streaked onto five different types of agar plates that contained organic carbon sources (i.e., YM, R2A, LB, TSA, and BG-11 + 100 ppm of glucose) to assure the absence of culturable bacteria.

**Production and recovery of biosurfactant from Bacillus subtilis C9.** The stock culture of *B. subtilis* C9 was stored at −80 °C in Luria-Bertani (LB) medium supplemented with 20% (v/v) of glycerol. Upon streaking the stock culture on LB agar plates, an inoculum was prepared by inoculating a single colony of *B. subtilis* C9 into 10-mL of LB broth in 125-mL Erlenmeyer flask that was subsequently cultivated at 37 °C for 12 hours. The 12-hour-old inoculum was used to seed 200-mL of LB broth medium supplemented with 5 g/L of glucose in each of triplicated 1-L culture flasks; the initial optical density of each culture following inoculation was 0.05 at 600 nm and three culture flasks were incubated at 37 °C for 72 hours on an orbital shaker at 170 rpm. The composition of LB medium was (g/L): NaCl 10.0; tryptone 10.0; yeast extract 5.0.

The recovery of C9-biosurfactant was performed as described in previous studies. Briefly, the cultures were first centrifuged at 7500 rpm for 20 minutes to remove bacterial cells after a 72-hour-long cultivation period. Upon confirming the production of biosurfactant by measuring the surface tension of culture broth with a ring tension meter at 25 °C (KR10ST; Kruss, Hambrug, Germany), the cell-free supernatants were adjusted to pH 2 with 6 M HCl and were subsequently incubated overnight at 4 °C to promote the precipitation of biosurfactant. The precipitates were then collected by centrifugation at 10000 rpm for 20 minutes at 4 °C. Afterwards, the biosurfactant of *B. subtilis* C9 was dissolved in a minimal amount of demineralized water and biosurfactant solution
was subsequently lyophilized after adjusting pH to 7 using 1 M NaOH. The freeze-dried products were weighed to calculate the yield of biosurfactant prior to storing at −20 °C. A known volume of culture medium was used to dissolve the recovered biosurfactant of \( B. \ subtilis \) C9 to explore its biocontrol activity against cladoceran algal grazers and its influence on the growth and productivity of selected algal strains under different concentration gradients. Although direct quantification of surfactin in C9-biosurfactant was not performed in this study, the relative proportion of surfactin in C9-biosurfactant was estimated by comparing critical micelle dilution (CMD) of aqueous solutions of C9-biosurfactant and HPLC-grade surfactin at an identical concentration of 0.3 g/L. CMD was the dilution necessary to reach the point where the surface tension starts to dramatically increase, and was known to be proportional to the amount of surfactin present in the original sample.

**Experimental design.** Assessment of the biocontrol activity of C9-biosurfactant against cladoceran algal grazers. Chlorella-fed adult cladocerans were first gently harvested by filtering the liquid with a 500 μm mesh filter and carefully rinsed with clean COMBO medium to remove any debris. Subsequently, each of 10-mL glass tubes received 10 individuals of either *Daphnia pulex* or *Moina macrocopa*. The average adult body sizes of *Daphnia pulex* and *Moina macrocopa* were 1.8 ± 0.3 mm and 1.2 ± 0.2 mm, respectively. Each glass tube was then tested at C9-biosurfactant concentrations of 0, 6, 20, and 60 mg/L in plain COMBO medium. Alive and dead individuals were counted thrice after 1, 12, and 24 hr by considering the cladocerans settled onto the bottom of glass tube without visual movement after gentle shaking as dead. In addition to the recovered C9-biosurfactant, HPLC-grade surfactin from *B. subtilis* (Sigma-Aldrich, St. Louis, MO, USA) was tested under identical experimental conditions at four concentrations (0, 6, 20, and 60 mg/L) in plain COMBO medium to confirm whether the main surfactant compound of C9-biosurfactant negatively influenced the growth of selected algal grazers.

**Influence of C9-biosurfactant on the growth of selected algal crops.** The influence of C9-biosurfactant on the growth of *Chlorella* sp. HS2 and *Scenedesmus deserticola* JD052 was tested to determine whether C9-biosurfactant negatively influenced the growth and productivity of selected algal strains. Prior to inoculation, triplicated 250-mL Erlenmeyer flasks were subjected to four different concentrations of C9-biosurfactant (0, 6, 20, and 60 mg/L) in plain BG-11 medium. Either *Chlorella* sp. HS2 or *Scenedesmus deserticola* JD052 was then inoculated into each flask culture at the cell density of 1 × 10⁶ cells/mL with the final working volume of 100-mL. Each flask culture was supplemented with 5% CO₂ through an autoclavable aeration tube with an inner diameter of 1 mm at 0.1 volume gas per volume culture per minute (vvm), and all cultures were shaken at 100 rpm on an orbital shaker under continuous light at the intensity of 150 μmol·m⁻²·s⁻¹. All experiments were performed in a temperature-controlled room at 25 ± 1 °C under the light intensity of 150 μmol·m⁻²·s⁻¹, and the pH level of each tube culture ranged between 7.0 and 8.0 throughout the experimental period, ensuring no significant influence of ammonia toxicity on the mortality of algal grazers.

**Exploring the biocontrol activity of C9-biosurfactant in grazer-introduced algal cultures.** Because blooms of cladoceran grazers in algal cultivation systems will occur under the presence of algal crops, the grazer-control activity of C9-biosurfactant was further evaluated by monitoring the percent mortality of cladoceran grazer in algal cultures at different concentrations of C9-biosurfactant. Each of 10-mL glass tubes was first inoculated with either *Chlorella* sp. HS2 or *Scenedesmus deserticola* JD052 at the cell density of 1 × 10⁶ cells/mL, and subsequently received 10 individuals of either *Daphnia pulex* or *Moina macrocapa* that were carefully rinsed with clean COMBO medium after being fed for at least 48 hours with the identical algal strain of the culture they were added. Each of triplicated tube cultures was then subjected to four different concentrations of C9-biosurfactant (0, 6, 20, and 60 mg/L) in plain COMBO medium at 25 ± 1 °C under the light intensity of 150 μmol·m⁻²·s⁻¹. Both alive and dead cladocerans were counted every 12 hours, and algal cell density in each culture was monitored daily throughout a 72-hour-long experimental period. In addition, the growth of each algal strain without the presence of cladoceran grazers was monitored for 72 hours in triplicated tube cultures in plain COMBO medium under identical light and temperature conditions. Because dissolved CO₂ and agitation may negatively influence the survival of cladocerans by limiting the exchange of CO₂ and exerting shear stress, all tube cultures were only gently shaken twice a day without supplemental CO₂ to ensure that algal cells stayed in suspension.

**Analytical methods.** Identification of C9-biosurfactant by mass spectrometry. While previous studies indicated a lipopeptide surfactin as the main surfactant compound of C9-biosurfactant, the recovered C9-biosurfactant and HPLC-grade surfactin from *Bacillus subtilis* (Sigma-Aldrich, St. Louis, MO, USA) were submitted to mass spectrometry analysis after dissolving each compound into methanol at the concentration of 1 mg/L. High-resolution mass spectra were collected using a Bruker microTOF-Q II mass spectrometer (Bruker Daltonics, Bremen, Germany) in ESI positive ion mode over the mass range of m/z 50−2000 under the following optimized settings: end plate offset, −500 V; capillary, 4800 V; nebulizer gas, 0.4 bar; dry gas, 4.0 liters/min; dry gas temperature, 180 °C. The obtained spectra of C9-biosurfactant and HPLC-grade surfactin were subsequently compared to assure the presence of surfactin in the recovered C9-biosurfactant.
Algal growth measurement. Culture growth was measured by either counting cell numbers (i.e., microalgal cell density) under a Nikon FX-A microscope (Optical Analysis Corp., Nishua, Japan) with a hemocytometer (C-chip, NanoEnTek, USA) or measuring optical density at 680 nm using a microplate reader (BioTek, Bad Friedrichshall, Germany). The dry cell weight (DCW) was also determined by filtration onto pre-weighed GF/C filters (Whatman, UK). After rinsing with distilled water, the filters were dried at 105 °C overnight. The filters were then reweighed after drying and the DCW was calculated from the difference between the filter weight with and without algal biomass. In addition, the specific growth rate of each flask culture was calculated during exponential growth phase as:

$$\mu = \ln \left( \frac{C_2}{C_1} \times \frac{1}{t_2 - t_1} \right)$$  \hspace{1cm} (1)

where $\mu$ was the specific growth rate, and $C_1$ and $C_2$ were culture densities at time 1 ($t_1$) and time 2 ($t_2$), respectively.

Lipid content and fatty acid methyl ester (FAME) composition of harvested algal biomass. Each of 250-mL flask cultures was harvested at the end of batch cultivation period by removing supernatant after centrifugation at 4000 rpm for 10 min, and the harvested biomass was subsequently lyophilized and stored in the freezer at −70 °C until further analysis. The lipid content of harvested biomass was analyzed by extracting total lipids from freeze-dried biomass with chloroform-methanol (2:1 (v/v)) following a slightly modified version of Bligh and Dyer’s method. FAME composition of lyophilized biomass was analyzed using a gas chromatograph (Shimadzu GC-2010, Japan). Each of 50 mg samples was first placed into capped test tubes, saponified with 1 mL of a saturated KOH-CH$_3$OH solution at 75 °C for 10 min, and submitted to methanalysis with 5% HCl in methanol at 75 °C for another 10 min. Fatty acids containing phase was subsequently separated by adding 2 mL of distilled water and recovered. The components were identified by comparing their retention times and peak areas with those of FAME Mix, C8-C24 (18918-1AMP, Supelco, Sigma-Aldrich, St. Louis, MO, USA).% mortality = \left( \frac{n_0 - n_t}{n_0} \right) \times 100

Algal grazer mortality. The % mortality of cladoceran grazer in each tube culture was calculated as:

where $n_0$ was the total number of cladoceran grazer at time 0, and $n_t$ was the number of alive individuals at each time point. The individuals settled onto the bottom of tube culture without visual movement after gentle shaking were considered as dead, and no newborn cladocerans were observed across all experimental treatments.

Statistical analysis. The results were presented as either mean values or mean values ± standard deviation from at least triplicated independent measurements unless denoted otherwise. The statistical significance of differences in specific growth rate and lipid productivity between treatments was evaluated by using 95% confidence intervals for the mean.

Data availability. The datasets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

References

1. Day, J. G., Slocombe, S. P. & Stanley, M. S. Overcoming biological constraints to enable the exploitation of microalgae for biofuels. Bioresour. Technol. 109, 245–251 (2012).
2. Chisti, Y. Biodiesel from microalgae. Biotechnol. Adv. 25, 294–306 (2007).
3. Hu, Q. et al. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J. 54, 621–639 (2008).
4. Chen, C.-Y., Yeh, K.-L., Aisyah, R., Lee, D.-J. & Chang, J.-S. Cultivation, photobioreactor design and harvesting of microalgae for technological challenges. Ind. Biotechnol. 102, 71–81 (2011).
5. Lammers, P. J. et al. Review of the cultivation program within the National Alliance for Advanced Biofuels and Bioproducts. Algal Res. 22, 166–186 (2017).
6. Paté, R., Klise, G. & Wu, B. Resource demand implications for US algae biofuels production scale-up. Appl. Energy 88, 3377–3388 (2011).
7. Yun, J.-H. et al. Hybrid operation of photobioreactor and wastewater-fed open raceway ponds enhances the dominance of target algal species and algal biomass production. Algal Res. 29, 319–329 (2018).
8. Kakarla, R. et al. Application of high-salinity stress for enhancing the lipid productivity of Chlorella sorokiniana HS1 in a two-phase process. J. Microbiol. 56, 56–64 (2018).
9. Greenwell, H., Laurens, L., Shields, R., Lovitt, R. & Flynn, K. Placing microalgae on the biofuels priority list: a review of the technological challenges. J. R. Soc. Interface 7, 703–726 (2010).
10. McBride, R. C. et al. Contamination management in low cost open algae ponds for biofuels production. Ind. Biotechnol. 10, 221–227 (2014).
11. Peng, L., Lan, C.-Q., Zhang, Z., Sarch, C. & Laporte, M. Control of protozoa contamination and lipid accumulation in Neochloris oleoabundans culture: effects of pH and dissolved inorganic carbon. Bioresour. Technol. 197, 143–151 (2015).
12. Day, J. G., Gong, Y. & Hu, Q. Microzooplanktonic grazers—a potentially devastating threat to the commercial success of microalgal mass culture. Algal Res. 27, 356–365 (2017).
13. Cauchie, H., Hoffmann, L., Jaspar-Versali, M., Salvia, M. & Thomé, J. Daphnia magna Straus living in an aerated sewage lagoon as a source of chitin: ecological aspects. Belg. J. Zool (1995).
14. Cho, D.-H. et al. Microalgal diversity fosters stable biomass productivity in open ponds treating wastewater. Sci. Rep. 7, 1979 (2017).
15. White, R. L. & Ryan, R. A. Long-term cultivation of algae in open-raceway ponds: lessons from the field. Ind. Biotechnol. 11, 213–220 (2015).
16. Richardson, J. W. et al. A financial assessment of two alternative cultivation systems and their contributions to algae biofuel economic viability. Algal Res. 4, 96–104 (2014).
17. Beyter, D. et al. Diversity, productivity, and stability of an industrial microbial ecosystem. Appl. Environ. Microbiol. 82, 2494–2505 (2016).
18. Lincoln, E., Hall, T. & Koopman, B. Zooplankton control in mass algal cultures. Aquaculture 32, 331–337 (1983).
19. Van Ginkel, S. W. et al. The prevention of saltwater algal pond contamination using the electron transport chain disruptor, rotenone. Algal Res. 18, 209–212 (2016).
20. Zmora, O. R. A. Microalgae for aquaculture: microalgae production for aquaculture in Handbook of Microalgal Culture: Biotechnology and Applied Phycology (eds Richmond, A. & Ha, Q.) 365–379 (Blackwell Publishing, 2013).
21. Park, S. et al. The selective use of hypochlorite to prevent pond crashes for algae-biofuel production. Water Environ. Res. 88, 70–78 (2016).
22. Montemezzani, V., Duggan, I. C., Hogg, I. D. & Cracks, R. J. Screening of potential zooplankton control technologies for wastewater treatment High Rate Algal Ponds. Algal Res. 22, 1–13 (2017).
23. Wang, H., Zhang, W., Chen, I., Wang, J. & Liu, T. The contamination and control of biological pollutants in mass cultivation of microalgae. Bioreour. Technol. 128, 745–750 (2013).
24. Pimentel, D. & Edwards, C. A. Pesticides and ecosystems. BioScience 32, 595–600 (1982).
25. Fox, J. E., Gulledge, J., Engelsena, T., Burrow, M. E. & McLachlana, J. A. Pesticides reduce symbiotic efficiency of nitrogen-fixing rhizobia and host plants. Proc. Natl. Acad. Sci. USA 104, 10282–10287 (2007).
26. Kazamia, E., Albritt, D. C. & Smith, A. G. Synthetic ecology—a way forward for sustainable algal biofuel production? J. Biotechnol. 162, 163–169 (2012).
27. Montemezzani, V., Duggan, I. C., Hogg, I. D. & Cracks, R. J. A review of potential methods for zooplankton control in wastewater treatment High Rate Algal Ponds and algal production raceways. Algal Res. 11, 211–226 (2015).
28. Shurin, J. B. et al. Industrial-strength ecology: trade-offs and opportunities in algal biofuel production. Ecol. Lett. 16, 1393–1404 (2013).
29. Smith, V. H. & Crews, T. Applying ecological principles of crop cultivation in large-scale algal biomass production. Algal Res. 4, 23–34 (2014).
30. Yun, J.-H., Smith, V. H., La, H.-J. & Yang, S. K. Towards managing food-web structure and algal crop diversity in industrial-scale algal biomass production. Curr. Biotechnol. 5, 118–129 (2016).
31. Deravel, J. et al. Mycosubtilin and surfactin are efficient, low ecotoxity molecules for the biocontrol of lettuce downy mildew. Appl. Microbiol. Biotechnol. 98, 6255–6264 (2014).
32. Thakore, Y. The biopesticide market for global agricultural use. Ind. Biotechnol. 2, 194–208 (2006).
33. Correa, O. S. & Soria, M. A. Potential of Bacilli for biocontrol and its exploration in sustainable agriculture in Plant Growth and Health Promoting Bacteria (ed. Maheshwari, D.) 197–209 (Springer, 2010).
34. Kim, H.-S. et al. A lipopeptide biosurfactant produced by Bacillus subtilis C9 selected through the oil-film-collapsing assay. J. Microbiol. Biotechnol. 7, 180–188 (1997).
35. Kim, H.-S. et al. Production and properties of a lipopeptide biosurfactant from Bacillus subtilis C9. J. Ferment. Bioeng. 84, 41–46 (1997).
36. Geetha, I. & Manonmani, A. Surfactin: a novel mosquitocidal biosurfactant produced by Bacillus subtilis sp. subtilis (VCRC-B471) and influence of abiotic factors on its pupicidal efficacy. Lett. Appl. Microbiol. 51, 406–412 (2010).
37. Geetha, I., Manonmani, A. & Paily, K. Identification and characterization of a mosquito pupicidal metabolite of a Bacillus subtilis subsp. subtilis strain. Appl. Microbiol. Biotechnol. 86, 1737–1744 (2010).
38. Geetha, I., Prabakaran, G., Paily, K., Manonmani, A. & Balarakam, K. Characterisation of three mosquitocidal Bacillus strains isolated from mangrove forest. Biol. Control 42, 34–40 (2007).
39. Montemezzani, V., Duggan, I. C., Hogg, I. D. & Cracks, R. J. Zooplankton community influence on seasonal performance and microagal dominance in wastewater treatment High Rate Algal Ponds. Algal Res. 17, 168–184 (2016).
40. Song, Y., Talaty, N., Datsenko, K., Wanner, B. L. & Cooks, R. G. In vivo recognition of Bacillus subtilis by desorption electrospray ionization mass spectrometry (DESI-MS). Analyst 134, 838–841 (2009).
41. Gudinia, E. J., Fernandes, E. C., Rodrigues, A. I., Teixeira, J. A. & Rodrigues, L. R. Biosurfactant production by Bacillus subtilis using corn steep liquor as culture medium. Front. Microbiol. 6 (2015).
42. Vollenbroich, D., Ozel, M., Vater, J., Kamp, R. M. & Pauli, G. Mechanism of inactivation of enveloped viruses by the biosurfactant surfactin from Bacillus subtilis. Biogical 25, 289–297 (1997).
43. Montemezzani, V., Duggan, I. C., Hogg, I. D. & Cracks, R. J. Control of zooplankton populations in a wastewater treatment High Rate Algal Pond using overnight CO2 asphyxiation. Algal Res. 26, 250–264 (2017).
44. Yuan, D. et al. Biodiversity and distribution of microzooplankton in Spirulina (Arthrospira) platensis mass cultures throughout China. Algal Res. 30, 38–49 (2018).
45. Manonmani, A. M., Geetha, I. & Bhuvaneswari, S. Enhanced production of mosquitocidal cyclic lipopeptide from Bacillus subtilis subsp. subtilis. Indian J. Med. Res. 134, 476 (2011).
46. Lechuga, M., Fernández-Serrano, M., Jurado, E., Núñez-Olea, J. & Ríos, F. Acute toxicity of anionic and non-ionic surfactants to aquatic organisms. Ecotoxicol. Environm. Saf. 125, 1–8 (2016).
47. Steiner, C. F. Context-dependent effects of Daphnia pulex on pond ecosystem function: observational and experimental evidence. Oecologia 131, 549–558 (2002).
48. Vollenbroich, D., Pauli, G., Ozel, M. & Vater, J. Antimycoplasm properties and application in cell culture of surfactin, a lipopeptide antibiotic from Bacillus subtilis. Appl. Environ. Microbiol. 63, 44–49 (1997).
49. Vollenbroich, D., Pauli, G., Ozel, M., Vater, J. & Shapiro, J. An examination of lipid reserves and the nutritional status of Daphnia pulex fed Aphaniizomenon flos-aquae. Limnol. Oceanogr. 29, 1137–1140 (1984).
50. Sterner, R. W., Hagemeier, D. D., Smith, W. L. & Smith, R. F. Phytoplankton nutrient limitation and food quality for Daphnia. Limnol. Oceanogr. 38, 857–871 (1993).
51. Buchoux, S. et al. Surfactin-triggered small vesicle formation of negatively charged membranes: a novel membrane-lysis mechanism. Biophys. J. 95, 3840–3849 (2008).
52. Griffiths, M. J. & Harrison, M. T. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. J. Appl. Phycol. 21, 493–507 (2009).
53. Taoka, Y. et al. Effect of Tween 80 on the growth, lipid accumulation and fatty acid composition of Thraustochytrium aureum ATCC 34504. J. Biosci. Bioeng. 111, 420–424 (2011).
54. Boelen, P., van Dijk, R., Damste, J. S. J., Rijpstra, W. I. C. & Buma, A. G. On the potential application of polar and temperate marine microalgae for EPA and DHA production. AMB Expr 3, 26 (2013).
55. Shin, H. et al. Genome-wide transcriptome analysis revealed organelle specific responses to temperature variations in algae. Sci. Rep. 6, 37770 (2016).
56. Suzuki, I., Kos, A. D., Kanesaki, Y., Mikami, K. & Murata, N. The pathway for perception and transduction of low-temperature signals in Synecystis. EMBO J. 19, 1327–1334 (2000).
57. Rie, M. & Grimme, L. Studies on surfactant toxicity to the freshwater alga Chlorella fusca: a common mode of action? Sci. Total Environ. 134, 551–558 (1993).
58. Ahn, C.-Y. et al. Selective control of cyanobacteria by surfactin-containing culture broth of Bacillus subtilis C1. Biotechnol. Lett. 25, 1137–1142 (2003).
59. Flynn, K. J., Kenny, P. & Mitra, A. Minimising losses to predation during microalgal cultivation. *J. Appl. Physiol.* 1–12 (2017).
60. Edwards, K. F., Thomas, M. K., Klausmeier, C. A. & Litchman, E. Allometric scaling and taxonomic variation in nutrient utilization traits and maximum growth rate of phytoplankton. *Limnol. Oceanogr.* 57, 554–566 (2012).
61. Litchman, E. & Klausmeier, C. A. Trait-based community ecology of phytoplankton. *Annu. Rev. Ecol. Evol. Syst.* 39, 615–639 (2008).
62. Narwani, A. et al. Common ancestry is a poor predictor of competitive traits in freshwater green algae. *PLoS One* 10, e0137085 (2015).
63. Hansen, P. I., Bjørnsen, P. K. & Hansen, B. W. Zooplankton grazing and growth: scaling within the 2–2,000-μm body size range. *Limnol. Oceanogr.* 45, 1891–1891 (2000).
64. Duquesne, S., Reynaldi, S. & Liess, M. Effects of the organophosphate paraaxon-methyl on survival and reproduction of *Daphnia magna*: importance of exposure duration and recovery. *Environ. Toxicol. Chem.* 25, 1196–1196 (2006).
65. Fox, S. L. & Balá, G. A. Production of surfactant from *Bacillus subtilis* ATCC 21332 using potato substrates. *Bioresour. Technol.* 75, 235–240 (2000).
66. Jain, D., Collins-Thompson, D., Lee, H. & Trevors, J. A drop-collapsing test for screening surfactant-producing microorganisms. *J. Microbiol. Methods* 13, 271–279 (1991).
67. Ulbrich, C., Kluge, B., Palacz, Z. & Vater, J. Cell-free biosynthesis of surfactin, a cyclic lipopeptide produced by *Bacillus subtilis*. *Biochemistry* 30, 6503–6508 (1991).
68. Ongena, M. & Jacques, P. *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol.* 16, 115–125 (2008).
69. Kilham, S. S., Kreeger, D. A., Lynn, S. G., Goulden, C. E. & Herrera, L. COMBO: a defined freshwater culture medium for algae and zooplankton. *Hydrobiologia* 377, 147–159 (1998).
70. Cho, D.-H. et al. Organic carbon, influent microbial diversity and temperature strongly influence algal diversity and biomass in raceway ponds treating raw municipal wastewater. *Bioresour. Technol.* 191, 481–487 (2015).
71. Cho, D. H. et al. Novel approach for the development of axenic microalgal cultures from environmental samples. *J. Phycol.* 49, 802–810 (2013).
72. Pathak, K. V. & Keharia, H. Identification of surfactins and iturins produced by potent fungal antagonist, *Bacillus subtilis* K1 isolated from aerial roots of banyan (*Ficus benghalensis*) tree using mass spectrometry. *J. Biotech.* 4, 283–295 (2014).
73. Cooper, D., Macdonald, C., Duff, S. & Kosaric, N. Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. *Appl. Environ. Microbiol.* 42, 408–412 (1981).
74. Thomas, P. K., Dunn, G. P., Passero, M. & Feris, K. P. Free ammonia offers algal crop protection from predators in dairy wastewater and ammonium-rich media. *Bioresour. Technol.* 243, 724–730 (2017).
75. Bligh, E. G. & Dyer, W. J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37, 911–917 (1959).
76. Lee, J.-Y., Yoo, C., Jun, S.-Y., Ahn, C.-Y. & Oh, H.-M. Comparison of several methods for effective lipid extraction from microalgae. *Bioresour. Technol.* 101, 575–577 (2010).
77. Kang, Z. et al. A cost analysis of microalgal biomass and biodiesel production in open raceways treating municipal wastewater and under optimum light wavelength. *J. Biotechnol.* 25, 109–118 (2015).

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