



Screening of several microalgae revealed biopesticide properties of *Chlorella sorokiniana* against the strawberry pathogen *Phytophthora cactorum*

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Abstract

Present agricultural production typically depends on the intensive use of synthetic pesticides with potentially harmful consequences for humans and the environment. To ensure food security for the rapidly rising global population it is necessary to develop more sustainable alternatives to synthetic pesticides. Microalgae possess a large diversity in antimicrobial compounds and are considered one of the most promising sustainable sources of novel biopesticides. Antimicrobial activities of 15 microalgae strains were investigated against a selection of seven common plant pathogens relevant to agricultural production. Several microalgae were identified possessing antimicrobial activity, with an extract of *Chlorella sorokiniana* showing the strongest growth inhibition of the plant pathogen *Phytophthora cactorum*. Different pre-treatments like freeze-drying, solvents with different polarities, and extraction methods were analyzed in regards to the level of antimicrobial activity of *C. sorokiniana*. The best *C. sorokiniana* extract demonstrated potential for biopesticide application on strawberry leaves infected with *P. cactorum*. This study reveals the potential of microalgae as natural biopesticide for organic or more sustainable regular agriculture.

Keywords Biopesticide · Microalgae · *Phytophthora cactorum* · Strawberry · Sustainable agriculture

Introduction

The rapid rise in global population and accompanying increasing food demand resulted in the intensive use of synthetic pesticides to control loss of harvest caused by plant pathogens. Synthetic chemical pesticides are cost-effective, easy to apply and provide quick results. Their introduction has significantly reduced pre-harvest losses from 50 – 80% to an average of 30% (Oerke 2006; Alexandratos and Bruinsma 2012). Although synthetic pesticides offer a solution for almost every plant pathogen, thus providing farmers with simple and accessible solutions, over time many pesticides have gradually lost their effectiveness due to the development of resistance (Isman 2019). Resistance in pathogens can develop due to singular or polygenic evolutionary adaptations in the pesticide binding site or detoxification process (Matzrafi

2019). Furthermore, environmental conditions like e.g., drought or temperature changes can also alter the absorption and detoxification processes of the pesticides. Moreover, the long half-life of pesticides in soil and water can lead to their dispersion throughout the ecosystem, impacting various organisms (Carvalho 2017). The deleterious effects of synthetic pesticides on human health and the environment requires the development of new pesticide formulations (Marrone 2019). In line with this, the ‘Farm to Fork’ strategy of the European Commission sets a target of 50% reduction in the use of chemical pesticides by 2030 (European Commission 2020). The combined requirement to reduce the use of pesticides while still minimizing crop losses has led to the development of Integrated Pest Management (IPM). In IPM, lower amounts of a diversified array of chemical and biological pesticides, along with other crop management methods, are applied, resulting in improved crop quality and yield (Isman 2019; Marrone 2019).

In this regard, the development and implementation of environmentally friendly biopesticides will foster more sustainable agriculture (Costa et al. 2019; Fenibo et al. 2021). Biopesticides offer better resistance management

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due to their multiple modes of action i.e., inhibiting the growth, nutrition, development, or reproduction of plant pathogens (Mnif and Ghribi 2015; Sparks and Nauen 2015; Dar et al. 2021). These multiple modes of action reduce the probability of the targeted plant pathogens acquiring resistance. Biopesticides generally are applied in low concentrations and are rapidly biodegraded (Oguh et al. 2019). Thus, they pose lower risks to consumers, workers and the environment and can be used in organic farming, throughout the growth cycle (Marrone 2019). Several different sources of biopesticides have already been explored and are available on the market, e.g., beneficial fungi, bacteria, and plant extracts (Isman 2019; Marrone 2019).

In recent years, the use of microalgae as biopesticides has been suggested to reduce or even replace the use of synthetic chemicals for pest control (Righini and Roberti 2019; La Bella et al. 2022). Many microalgae are known to produce allelopathic chemicals with antimicrobial or antifungal activity (Ördög et al. 2004; Leflaive and Ten-Hage 2007; Shishido et al. 2015; Falaise et al. 2016; Swain et al. 2017). In this regard, carotenoid and phenolic extracts of *Nannochloropsis* sp. have been found to inhibit the mycelial growth of *Fusarium graminearum* (Scaglioni et al. 2019). The cyanobacterium *Anabaena* sp. shows antimicrobial activity against *Botrytis cinerea*, the cause of postharvest gray mold on various crops (Righini et al. 2019). Several strains of *Chlorella* have been shown to function as natural fungicide against a number of pathogenic apple rot fungi (Vehapi et al. 2020). Furthermore, extracts obtained from *Tetrademus obliquus*, *Chlorella protothecoides*, and *Chlorella vulgaris* cultivated in wastewaters have demonstrated antimicrobial effects, offering a potential integrated approach for developing biopesticides and bioremediation of wastewaters (Ferreira et al. 2021; Ranglová et al. 2021). Several other studies have validated the antimicrobial activity of different solvent extracts e.g., methanol or acetone, of *C. vulgaris* on a diverse range of plant pathogenic fungi (Al-Nazwani et al. 2021; Dinev et al. 2021; Perveen et al. 2022). It is important to note that most studies are performed on cyanobacteria, and therefore the antimicrobial activity of eukaryotic microalgae is still underrepresented in research (Shishido et al. 2015; Swain et al. 2017).

To identify microalgae with biopesticide potential it is important to screen against different plant pathogens that are relevant to agriculture. The plant pathogenic fungi *Fusarium oxysporum*, *Fusarium graminearum*, and *Botrytis cinerea* have devastating effects on various crops as diverse as tomato, cotton, and banana but especially on cereals (Dean et al. 2012). *Verticillium albo-atrum* is another fungus that causes severe wilting in crops including cotton, tomatoes, potatoes, peppers, and ornamentals (Pegg and Brady 2002). Other common pathogens are *Alternaria solani* and *Pythium ultimum*. *A. solani* causes early blight and fruit rot of tomatoes, potatoes, and peppers (Strandberg 1992), while *P.*

ultimum is an oomycete responsible for damping off and root rot of cabbage, carrot, cucumber, turfgrass, and wheat (Martin and Loper 1999). Finally, *Phytophthora cactorum* is a worldwide spread pathogen that causes root and collar rot, fruit rots, cankers, leaf blights and wilts in over 200 plant species. This oomycete is a threat to strawberry and birch production, particularly in Finland, leading to significant yield losses (Lilja et al. 2011). Not only *P. cactorum* but also the other aforementioned selection of plant pathogens are relevant to both, global and Finnish agriculture.

In this work, we screened 15 microalgal strains for their biopesticide effect against a selection of agriculturally relevant plant pathogens. Through this initial rapid screening, we revealed that an extract of *Chlorella sorokiniana* showed the most promising biopesticide potential against *P. cactorum*. We further evaluated different extraction methods using *C. sorokiniana* and successfully verified the biopesticide functionality of the best aqueous extract on strawberry leaves infected with *P. cactorum*.

Material and methods

Microalgae strains and maintenance

A total of 15 strains of microalgae were employed in the present study (Table 1). *Chlamydomonas reinhardtii* was obtained from the Chlamydomonas Resource Center (CC; Department of Plant and Microbial Biology at the University of Minnesota, USA), while the rest of the strains were obtained from the Norwegian Culture Collection of Algae (NORCCA). The strains were maintained in 100 mL Erlenmeyer flasks with 50 mL of recommended growth medium (Table 1) according to the culture collection guidelines (*<https://norcca.scrol.net/norcca-algal-culture-medium>; **Instant Ocean, Aquarium Systems, France), under continuous low light ($\sim 10 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ photosynthetic photon flux density (PPFD)), at room temperature ($\sim 22 \text{ }^\circ\text{C}$).

Plant pathogens and maintenance

Five plant pathogenic fungi (*Fusarium oxysporum*, *Fusarium graminearum*, *Alternaria solani*, *Verticillium albo-atrum*, and *Botrytis cinerea*) and two plant pathogenic oomycetes (*Phytophthora cactorum*, and *Pythium ultimum*) were used in the present study. The *Fusarium* species were provided by Dr. Tapani Yli-Mattila (University of Turku, Finland), while the remaining fungi and oomycetes species were obtained from the Belgian Coordinated Collections of Microorganisms (BCCM). The fungi were maintained in Potato Dextrose Agar plates (PDA; Scharlau, Spain) and the oomycetes were kept on PDA plates supplemented with 200 mL L⁻¹

Table 1 Microalgae strains screened for antimicrobial activity, their culture collection ID and growth medium

| Taxonomy | ID | Growth medium ¹ |
|-----------------------------------|--------------|--|
| <i>Selenastrum</i> sp. | K-1877 | Z8 (Kotai 1972) |
| <i>Klebsormidium flaccidum</i> | NIVA-CHL 80 | Z8 |
| <i>Tribonema</i> sp. | NIVA-1/84 | Z8 |
| <i>Scenedesmus</i> sp. | NIVA-CHL 99 | Z8 |
| <i>Tetradesmus obliquus</i> | NIVA-CHL 107 | Z8 |
| <i>Monoraphidium contortum</i> | NIVA-CHL 100 | Z8 |
| <i>Haematococcus lacustris</i> | K-0084 | Z8 |
| <i>Porphyridium purpureum</i> | NIVA-1/92 | 50% Z8 in Seawater ² |
| <i>Chlorella vulgaris</i> | NIVA-CHL 19 | Z8 |
| <i>Chlorella</i> sp. | NIVA-CHL 69 | Z8 |
| <i>Chlorella</i> sp. | NIVA-CHL 125 | Z8 |
| <i>Chlorella sorokiniana</i> | NIVA-CHL 176 | Z8 |
| <i>Tetraselmis subcordiformis</i> | NIVA-2/94 | 50% Z8 in Seawater ² + Vitamins |
| <i>Coelastrum</i> sp. | K-0559 | Z8 + Vitamins |
| <i>Chlamydomonas reinhardtii</i> | CC-124 | Z8 |

¹<https://norcca.scrol.net/norcca-algal-culture-medium>; ²Instant Ocean, Aquarium Systems, France

tomato juice. The plates were incubated under constant darkness at 25 °C.

Microalgae screening for antimicrobial activity

The entire workflow, including screening, antimicrobial tests, and later analysis of different extraction procedures is visualized in Fig. 1. For screening trials, the strains (Table 1) were cultivated under low energy conditions (~ 22 °C, continuous 10 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ PPFD, no air bubbling, shaken by hand once daily) for 4 weeks in 30 mL of their respective growth medium. After 4 weeks of growth, the biomass was harvested by centrifugation (12,400 $\times g$ rpm, 8 min). The supernatants were discarded and the pellets of each species were resuspended in 15 mL sterile Milli-Q water (MQ) with a concentration of 100 g L⁻¹ (wet weight). Cell disruption was achieved by 3 cycles of 11 min freezing at -20 °C and 20 min thawing at 4 °C. Short cycles were chosen to avoid degradation of biomass, while still breaking the cells. The crude aqueous suspension was stored at -20 °C and vortexed for a minute to homogenize before use. To avoid bacterial growth on the plates, chloramphenicol (10 mg mL⁻¹) was added to extracts and negative control (MQ).

The antimicrobial activity of different microalgae species was assessed by plating 100 μL of each aqueous crude extract on the surface of the fresh PDA or PDA + tomato plates. Sterile Milli-Q water was used for the control plates. A small mycelial disc was placed at the center of the plates and incubated at 25 °C for 7 days. To minimize diffusion effects on the efficacy of the microalgae extracts, the entire plate was covered with crude extract to ensure direct contact between the mycelia and crude extract. The experiments

were performed in triplicates ($n = 3$). The antimicrobial activity was determined by growth inhibition based on the diameter of the plant pathogens against controls and categorized as slight (+; < 50% growth inhibition), strong (++; 50–70% growth inhibition), and very strong (+++; > 70% growth inhibition). For examples of each category see supplemental material (Fig. S1). The plates were monitored daily. The maximum growth inhibition was observed on day 4 and used for evaluation.

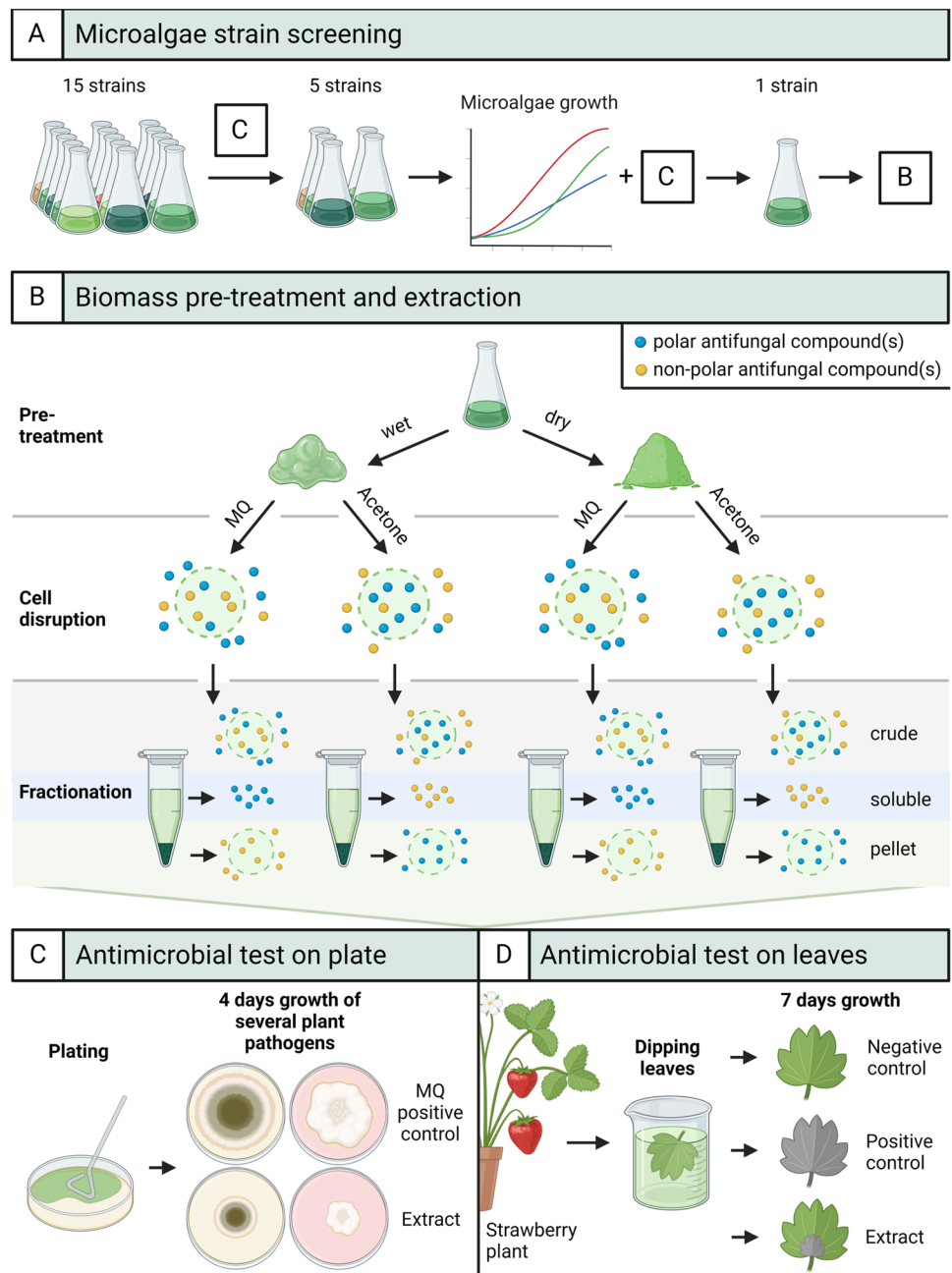
Effect of intensive growth conditions on antimicrobial activity

Five promising microalgae species were grown under intensive (higher biomass producing) conditions with 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ PPFD continuous light (20 °C, mixing at 120 rpm) in the presence of 1% CO₂ in standard BG11 growth medium (pH 7.5) (Rippka et al. 1979). The growth of the cultures was monitored daily by measuring optical density at 750 nm. After 2 weeks, when the cultures reached the late exponential / early stationary phase, the biomass was harvested by centrifugation and the crude aqueous extracts were prepared as described above. The growth and testing for antimicrobial activity of the selected microalgae strains were performed in triplicates ($n = 3$) as described in screening trials. The growth inhibition (%) was calculated using Eq. 1:

$$\text{Inhibition (\%)} = 100 - (\text{PD}/\text{CD} \times 100) \quad (1)$$

where *PD* and *CD* correspond to the diameter of the plant pathogen growth in the microalgae extract treated plate and in the control (MQ) plate, respectively.

Fig. 1 Schematic workflow of the screening and optimization processes to identify microalgae with the possibility for biopesticide application. **(A)** Strain selection cascade. **(B)** Biomass pre-treatment and extract fractionation. **(C)** The in vitro antimicrobial test on plate was used for rapid screening and identification of the most active extract fraction. **(D)** The antimicrobial test on detached strawberry leaves was performed with the most active microalgae extract fraction



C. sorokiniana extraction and optimization processes

C. sorokiniana, showing the best growth and antimicrobial activity was cultivated in 5-L glass bottles with 3.5 L of growth medium (BG11, pH=7.5), aerated with 1% CO₂ at 20 °C under 200 μmol photons m⁻² s⁻¹ continuous light. Experiments were performed with three biological replicates. After 2 weeks of growth, the cultures were harvested and the supernatant was discarded. One half of the biomass was used fresh (wet route), while the other half was freeze-dried (Martin Christ, Germany) (dry route).

Two solvents, acetone (medium polarity) and MQ water (high polarity), were tested to determine the solvent effect. The suspensions for extraction were prepared with adequate biomass amounts in cold acetone or sterile MQ water to reach a final concentration of 200, 100, and 50 g L⁻¹ for the wet route trials, which corresponds to 16, 8, and 4 g L⁻¹ for the dry route.

The cellular disruption was performed using a bead beater (Bullet Blender Storm 24, Next Advance, USA) with glass beads (diameter 425–600 μm, Sigma, USA) for 5 min at maximum speed. This step was repeated three times to ensure maximum extraction efficiency. The disrupted cell

suspensions with no centrifugation, which includes all the cellular debris (pellet) and the supernatant, were used for “crude extract” trials. For trials of “supernatant (soluble) fraction”, the disrupted cell suspensions were centrifuged ($15,000 \times g$, 5 min) and the supernatant was collected. The residual pellet after removing the supernatant was resuspended and stored for “pellet fraction” trials. To prevent any toxicity to the plant pathogens caused by acetone, the wet and dry route samples extracted with acetone were evaporated under a gentle stream of N_2 . Once dried, the fractions were resuspended in the corresponding volume of sterile MQ + 10% DMSO. The extracts were stored at $-20^\circ C$ for further use. Each fraction was plated individually to observe the antimicrobial activity following the procedure described in Section [Microalgae screening for antimicrobial activity](#). The plates were monitored daily and the growth inhibition was determined as described in Section [Effect of intensive growth conditions on antimicrobial activity](#).

Evaluation of antimicrobial activity of *C. sorokiniana* extract on detached strawberry leaves

To estimate the potential biopesticide effect of the extract, we utilized the method of detached leaves (Eikemo et al. 2000). To this end, young leaves were cut from fully developed strawberry plants (*Fragaria x ananassa*) and the petioles were trimmed at 4 cm length. The whole leaves ($n=5$) were then disinfected with 2% sodium hypochlorite for 5 min, washed three times with MQ water, air dried in sterile conditions, and submerged in extract for 1 min. Sterile MQ water was used for control leaves. After drying under sterile airflow conditions, mycelial discs (ca. 2 mm in diameter) of 1-week-old *P. cactorum* culture were inoculated into slits of 5 mm at the petiole base and secured by Parafilm. The leaves were then placed into sterile Petri dishes on moist filter paper with MQ water and stored in a dark chamber at $25^\circ C$. The pathogen progression was recorded by a 64 Mpx camera every 24 h. Visible symptoms (dark coloration) were analyzed by Image J software on images on the seventh day after inoculation, when necrosis reached ca 80% of the leaf area in the infected control group. Recently, a similar image-based method of scoring fungal symptom severity was successfully applied on excised *Arabidopsis* leaves (Pavicic et al. 2021). Leaf damage index (LDI) was calculated as $LDI = (\text{leaf area} - \text{green area}) / \text{leaf area}$. The experiment was repeated for extracts from three biological replicates of *C. sorokiniana*.

Statistics

The statistical analyses were performed using SPSS software (version 28) and the means were tested for normality and homoscedasticity before running a one-way analysis of variance (ANOVA). Significant differences between means were

further analyzed by either Tukey (for plate assay) or Fisher’s least significant differences (for leaf assay) post-hoc tests and are presented with a probability level $*P \leq 0.05$, $**P < 0.01$, and $***P < 0.005$.

Results

Rapid screening for antimicrobial activity

The aqueous crude extracts (see Section [Microalgae screening for antimicrobial activity](#)) acquired from 15 algae strains (see Section [Microalgae strains and maintenance](#)) were tested for their antimicrobial activity against five plant pathogenic fungi and two plant pathogenic oomycetes (see Section [Plant pathogens and maintenance](#)). The pathogens were selected based on their relevance to crop infections in both global and specifically in Finnish agriculture, e.g., cereals, tomatoes, cucumbers, potatoes, strawberries, and ornamentals. The rapid screening revealed that the crude extract of seven strains shows considerable antimicrobial activity to specific pathogens but not to every pathogen tested (Table 2). However, no activity was observed with the extracts of eight strains against all the tested pathogens (Table 2).

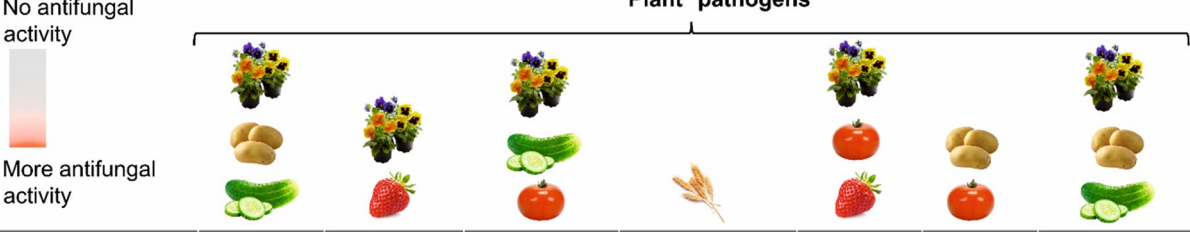
Seven algal extracts had different levels of antimicrobial activity to the specific chosen pathogens. Among all extracts studied, *C. sorokiniana* crude extract demonstrated the broadest antimicrobial activity spectrum showing a strong growth inhibition of *Alternaria* and *P. ultimum* and a very strong inhibition of *P. cactorum* growth. Furthermore, *C. sorokiniana* crude extracts slightly inhibited the growth of *Verticillium*, *F. oxysporum*, and *F. graminearum*. Nevertheless, no antimicrobial activity was observed against *B. cinerea* even with *C. sorokiniana* extract. *H. lacustris* extracts also show strong inhibition of *P. cactorum* and slight inhibition of *F. oxysporum* and *Alternaria*. Crude extracts of *Coelastrum*, *T. subcordiformis*, and *C. vulgaris* all show slight antimicrobial activity only against *P. ultimum* and *P. cactorum*. Only slight inhibition of the growth of *P. cactorum* was observed with the crude extracts of *C. reinhardtii* and *Tribonema* sp.

Effect of CO_2 and nitrate-rich media on growth and antimicrobial activity of promising microalgal strains

Following the screening, five promising microalgae strains (*C. vulgaris*, *C. sorokiniana*, *T. subcordiformis*, *Coelastrum* sp., and *H. lacustris*) showing inhibition or suppression of the growth of the pathogens were selected for monitoring the effect of intensive cultivation (higher biomass yielding) conditions on antimicrobial activity. The growth performances of five strains and corresponding growth inhibition of the aqueous algal extracts against the pathogens are presented in Fig. 2.

Table 2 Antimicrobial activity of aqueous crude extracts of screened microalgae strains. Screening was performed on PDA (+tomato) plates coated with crude microalgae extracts or MQ and mycelial discs of the plant pathogenic fungi and oomycetes were placed in the middle. Growth was compared on day 4 of cultivation. Antimicro-

bial activity was estimated on the basis of growth inhibition against the MQ treated plate (control) and categorized as no (-), slight (+), strong (++) and very strong (+++) for each of the tested plant pathogens ($n=3$). No growth inhibition is indicated as (-). Exemplary target crops for each tested plant pathogen are shown in the table



| Strains | Plant pathogens | | | | | | |
|----------------------------|-------------------|--------------------|---------------------|-----------------------|-------------------|------------------|----------------------|
| | <i>P. ultimum</i> | <i>P. cactorum</i> | <i>F. oxysporum</i> | <i>F. graminearum</i> | <i>B. cinerea</i> | <i>A. solani</i> | <i>V. albo-atrum</i> |
| <i>Chlorella</i> sp. (69) | - | - | - | - | - | - | - |
| <i>Chlorella</i> sp. (125) | - | - | - | - | - | - | - |
| <i>K. flaccidum</i> | - | - | - | - | - | - | - |
| <i>M. contortum</i> | - | - | - | - | - | - | - |
| <i>P. purpureum</i> | - | - | - | - | - | - | - |
| <i>Scenedesmus</i> sp. | - | - | - | - | - | - | - |
| <i>Selenastrum</i> sp. | - | - | - | - | - | - | - |
| <i>T. obliquus</i> | - | - | - | - | - | - | - |
| <i>Tribonema</i> sp. | - | + | - | - | - | - | - |
| <i>C. reinhardtii</i> | - | + | - | - | - | - | - |
| <i>C. vulgaris</i> | + | + | - | - | - | - | - |
| <i>Coelastrum</i> sp. | + | + | - | - | - | - | - |
| <i>T. subcordiformis</i> | + | + | - | - | - | - | - |
| <i>H. lacustris</i> | - | ++ | + | - | - | + | - |
| <i>C. sorokiniana</i> | ++ | +++ | + | + | - | ++ | + |

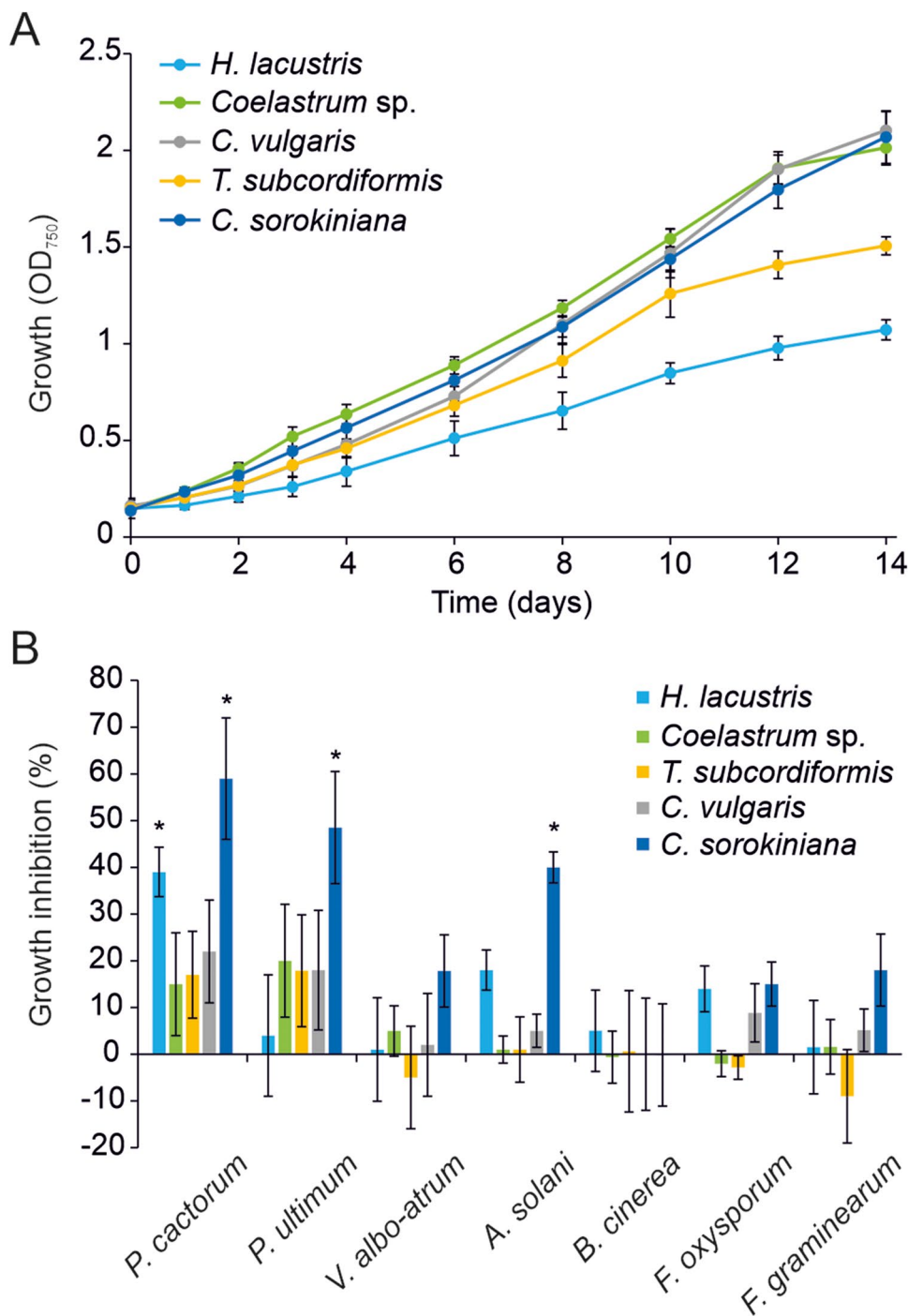
All cultures displayed a linear growth between day 1 and day 5 (Fig. 2A). Only *H. lacustris* demonstrated a short lag phase between day 0 and day 1. After 2 weeks of growth, all strains reached the late exponential or early stationary growth phase and were harvested to obtain crude extracts for testing antimicrobial activity. The growth of *Coelastrum* sp., *C. sorokiniana*, and *C. vulgaris* was the highest with OD_{750} of 2.07 ± 0.13 at the end of cultivation (Fig. 2A). On the other hand, *T. subcordiformis* achieved only an OD_{750} of 1.51 ± 0.04 and *H. lacustris* exhibited the slowest growth, only reaching OD_{750} of 1.07 ± 0.05 by day 14 under studied conditions.

The antimicrobial activity assay demonstrated that aqueous extracts of *H. lacustris* and *C. sorokiniana* suppress the growth of *P. cactorum* significantly by $39 \pm 5\%$ and $59 \pm 13\%$, respectively ($*P < 0.05$, Fig. 2B). In comparison, *T. subcordiformis*, *Coelastrum* sp. and *C. vulgaris* extracts showed low to moderate suppression of *P. cactorum* growth with no statistical significance. Furthermore, *C. sorokiniana* extracts showed the highest inhibition for *P. ultimum* ($49 \pm 19\%$) when compared to the rest of the algal extracts. *C. sorokiniana* crude

extracts were also able to significantly suppress the growth of *A. solani* by $40 \pm 3\%$ whereas *H. lacustris* showed a moderate suppression ($18 \pm 4\%$) with no statistical significance.

The results obtained with the extracts of microalgae grown in intensive culture conditions follow the trends observed in the initial screening results. *C. sorokiniana* extracts showed very strong (+++) and strong (++) antimicrobial activity against *P. cactorum*, *P. ultimum*, and *A. solani*, respectively (Table 2). The reproducible strong (++) antimicrobial activity of *H. lacustris* extract against *P. cactorum* was also observed. However, the slight (+) inhibitory activity shown by several microalgae strains during the rapid screening did not lead to significant growth suppression of plant pathogens when the microalgae were grown in intensive cultivation conditions (compare Table 2 and Fig. 2B). Considering both, the rapid growth and strong antimicrobial activity against three plant pathogens, with *P. cactorum* being inhibited the strongest, *C. sorokiniana* was chosen as the best strain for further investigation.

Fig. 2 Growth of the five selected microalgae strains with the most promising antimicrobial activity. *H. lacustris*, *T. subcordiformis*, *Coelastrum* sp, *C. sorokiniana*, and *C. vulgaris* cultures were cultivated for 2 weeks in intensive cultivation conditions. (A) Growth was followed by measuring OD₇₅₀. (B) Antimicrobial activity of the selected microalgae strains. PDA (+tomato) plates were coated with crude microalgae extracts or MQ and mycelial discs of the plant pathogenic fungi and oomycetes were placed in the middle. Growth was compared on day 4 of cultivation in 25 °C. The MQ control (no growth inhibition) was set to 0%. Data points at each day are shown as mean ± SE ($n=3$). Asterisks indicate significant differences according to one-way ANOVA with Tukey's correction ($P<0.05$)



Comparison of extraction procedures applied to *C. sorokiniana*

In order to identify the influence of downstream processing parameters on the antimicrobial activity, the extracts were performed with the most promising strain *C. sorokiniana* by applying different biomass concentrations, pre-treatment, extraction solvent, and fractionation (see Fig. 1, Section C. [sorokiniana extraction and optimization processes](#)).

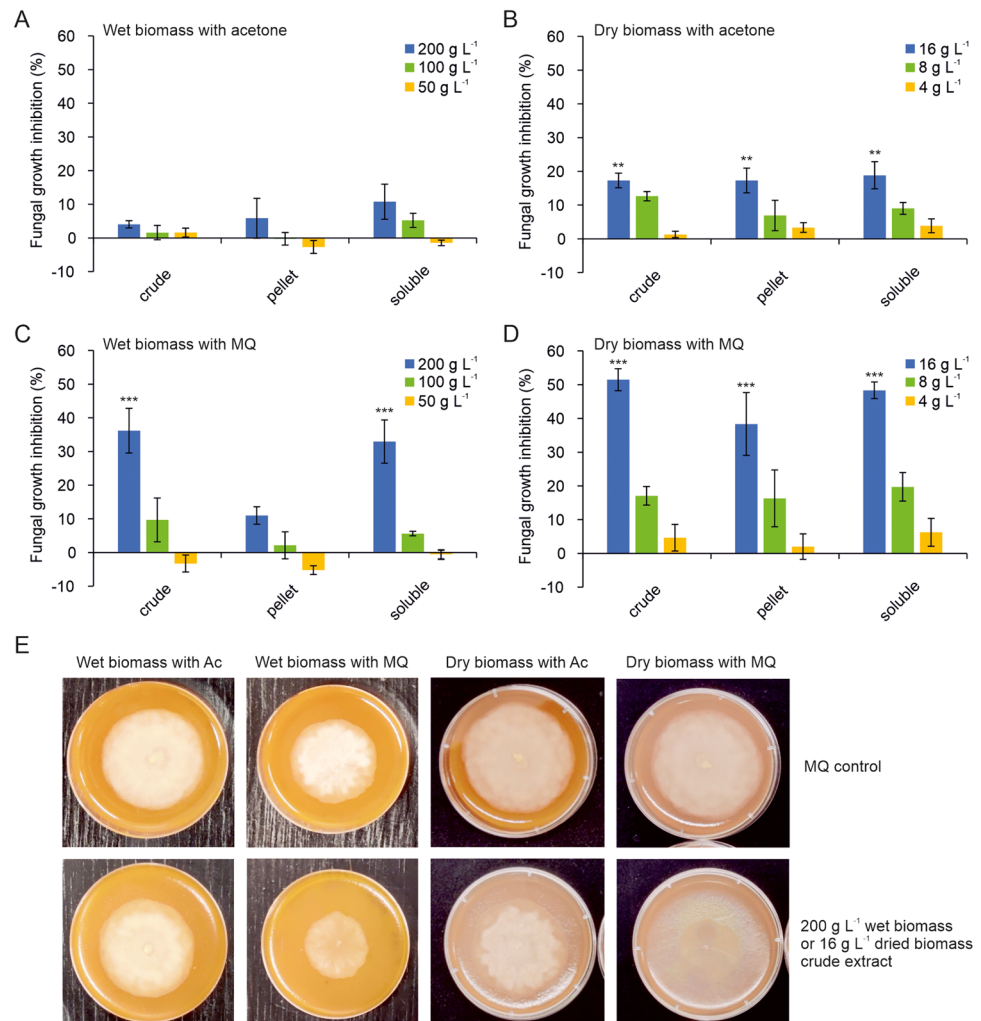
Through correlating the weight of the wet and freeze-dried biomass to each other, it was possible to estimate that the concentration of the crude extracts used in the rapid screening, 100 g L⁻¹ wet biomass, equals a concentration of 8.2 ± 0.1 g L⁻¹ dried biomass. To be able to directly compare the effect of the biomass pre-treatment on the antimicrobial activity of the extracts, the corresponding amount of wet (100 g L⁻¹) and dried (8 g L⁻¹) biomass was used for further extraction.

The antimicrobial activity was measured using 36 different extracts of *C. sorokiniana* against *P. cactorum* (Fig. 3). The results show that there is an apparent difference in the antimicrobial activity of the acetone extracts obtained from wet and dry biomass (Fig. 3A and B). No significant growth inhibition was achieved in any of the acetone extract fractions obtained from 200, 100, or 50 g L⁻¹ wet biomass (Figs. 3A and S2). Moderate antimicrobial activity was detected in the crude, pellet and soluble fractions of the acetone extracts from 16 g L⁻¹ dry biomass with the crude fraction inhibiting *P. cactorum* growth by 17 ± 2%, pellet fraction inhibiting the growth by 17 ± 3.5%, and the soluble fraction inhibiting the growth by 19 ± 4% (***P* < 0.01; Figs. 3B and S3). The acetone extracts with the lower concentrations of 8 or 4 g L⁻¹ dried biomass also showed up to 12 ± 1.5% growth suppression of *P. cactorum* however without statistical significance (Fig. 3B).

Whether the biomass is dry or wet, the results indicate that, in general, aqueous extracts show a better growth suppression of *P. cactorum* (Fig. 3C and D). The crude and soluble fractions of the aqueous extract obtained from 200 g L⁻¹ wet

biomass inhibited the growth of *P. cactorum* by 36 ± 6.5% and 32 ± 6.5%, respectively (***P* < 0.005; Figs. 3C and S4). In lower concentrations of aqueous extracts acquired from wet biomass, none of the fractions showed a significant inhibition against *P. cactorum*. The crude fraction of the 100 g L⁻¹ wet biomass extract merely suppressed the growth by 10 ± 6.5% (Fig. 3C). This is in contrast to the rapid screening results, where a crude aqueous extract of 100 g L⁻¹ wet biomass yielded 59 ± 13% growth inhibition of *P. cactorum* (Fig. 2B). The difference between the two extracts can be found in the method of cell disruption: freeze-thawing during rapid screening and bead-milling during the extraction test. The results indicate that the extract from bead-milling is ~sixfold less effective than the extract obtained by freeze-thawing. The aqueous extracts of 16 g L⁻¹ dried biomass exhibited the highest growth inhibition with 51 ± 3% for crude, 38 ± 9% for pellet, and 48 ± 2.5% for the soluble fraction (***P* < 0.005; Figs. 3D and S5). However, all fractions with lower dried biomass concentrations of 8 or 4 g L⁻¹ did not significantly suppress the growth of *P. cactorum* (Fig. 3D).

Fig. 3 Growth inhibition of *P. cactorum* exposed to different *C. sorokiniana* extracts. Wet biomass with acetone (A) and MQ solvent (C), respectively; dry biomass with acetone (B) and MQ solvent (D), respectively. The wet (200, 100, and 50 g L⁻¹) and dried (16, 8, and 4 g L⁻¹) biomass was used for extraction and every extract has been fractionated into crude, pellet and soluble fractions. (E) PDA + tomato plates were coated with 100 µL extracts or MQ as negative control and growth was observed for 7 days with maximum inhibition on day 4. Data are shown as mean ± SE (*n* = 3). ***P* < 0.01, ****P* < 0.005; one way ANOVA with Tukey correction



The results demonstrated that the growth inhibition of *P. cactorum* is concentration dependent with the highest biomass concentration (200 g L^{-1} wet and 16 g L^{-1} dry, respectively) exhibiting the strongest inhibition, the middle biomass concentration (100 g L^{-1} wet and 8 g L^{-1} dry, respectively) showing only slight inhibition, and the lowest biomass concentration (50 g L^{-1} wet and 4 g L^{-1} dry, respectively) exhibiting no inhibition (Fig. 3B, C, and D). The biomass concentration dependency of the growth inhibition verifies that, indeed, the *C. sorokiniana* extracts possess antimicrobial activity. The outcomes of the different pre-treatment and solvent comparison revealed that antimicrobial activity was favorable in the freeze-dried samples extracted with water.

Evaluation of antimicrobial activity of *C. sorokiniana* extracts on detached strawberry leaves

To further verify the possible biopesticide effect of the most promising *C. sorokiniana* extract, the soluble fraction of the aqueous extract from dried biomass was tested in detached strawberry (*Fragaria × ananassa*) leaves. This extract was

chosen because it possesses, together with the corresponding crude extract, the best performing antimicrobial activity under studied conditions (Fig. 3D). Detached strawberry leaves were dipped either into the microalgae extract or MQ as control, and infected with *P. cactorum* at the base of the petiole. The damage of leaves was evaluated based on RGB images on day 7 after infection (Fig. S6). Most of the non-treated infected leaves (MQ+P) were necrotized, while the non-infected control leaves (MQ) were mostly green except for small areas (Fig. 4B and D, respectively). The leaves treated with the *C. sorokiniana* extract slowed down the progression of the infection (Fig. 4C). Only small areas close to the stem and inner leaf were necrotized. The non-treated infected leaves had an overall average Leaf Damage Index (LDI) of 0.71 ± 0.11 , which was reduced to 0.32 ± 0.17 and 0.28 ± 0.19 with the best-performing extracts E1 and E2, respectively ($*P < 0.05$; Fig. 4A). This corresponds to a reduction of ~60% of the necrotized area when aqueous *C. sorokiniana* extracts were applied. The non-infected control leaves displayed a LDI of 0.17 ± 0.04 , which can be ascribed to non-specific airborne infection.

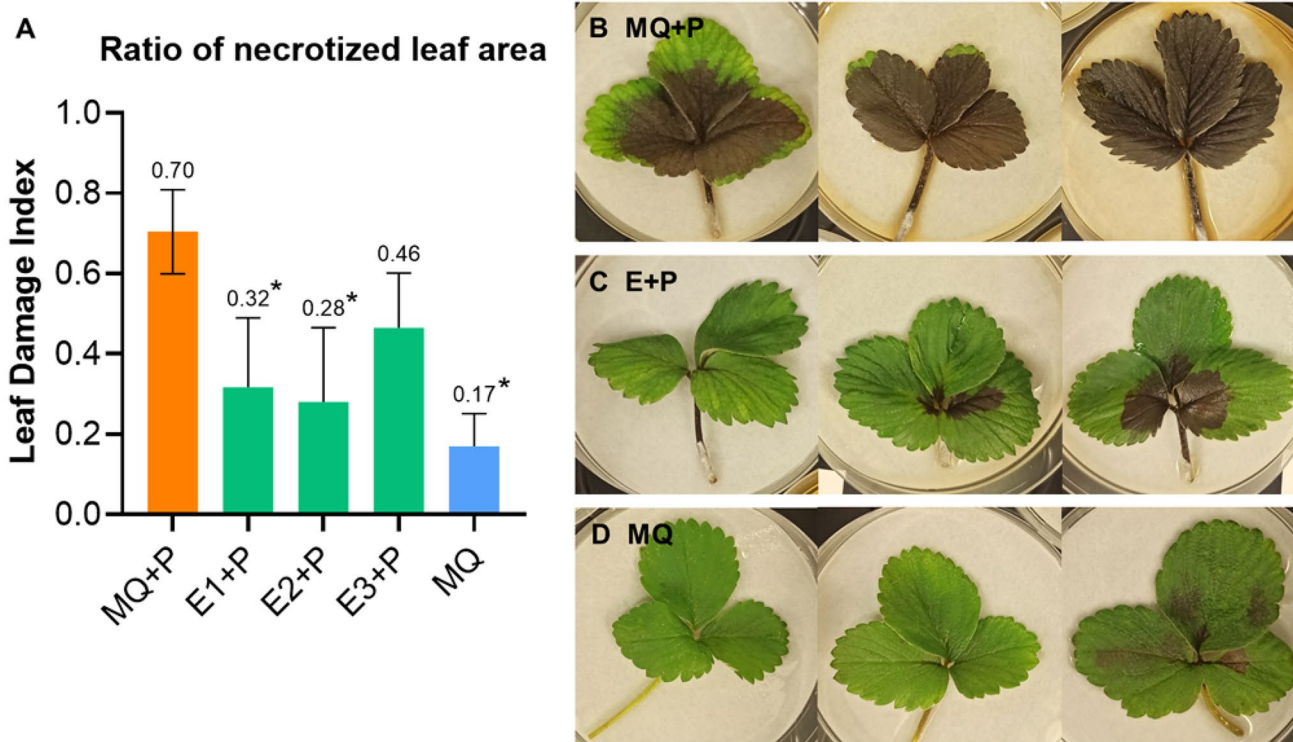


Fig. 4 Effect of an aqueous extract of *C. sorokiniana* on strawberry leaves infected with *P. cactorum*. Detached leaves were dipped into extracts from 3 biological microalgal replicates (E1, E2, E3) or MQ as control. Mycelial discs were inserted into incised petioles of the leaves, which then were incubated at $25 \text{ }^{\circ}\text{C}$ on moist filter paper. Infection progression was monitored by imaging every day. (A) Leaf Damage Index, LDI of non-infected control (MQ), non-treated infected leaves (MQ+P), and the infected leaves previously treated

with extracts of 3 microalgal extract replicates (Ex+P). (B) Non-treated leaves infected with *P. cactorum*, (C) leaves treated with extracts and infected with *P. cactorum*, (D) control leaves (dipped in MQ-water). Data points represent \pm SE of 5 technical replicates ($n=5$). Asterisks indicate significant differences to the non-treated infected leaves (MQ+P), according to one-way ANOVA followed by Fisher's LSD test ($P < 0.05$)

Discussion

Screening of microalgae strains demonstrates antimicrobial activity of several strains with *C. sorokiniana* effectively inhibiting *P. cactorum*

Microalgae produce a variety of bioactive compounds that offer advantages with antibacterial, antiviral, and antifungal properties. These photoautotrophic microorganisms are highly diverse and are present in many different habitats. Due to this extreme biodiversity, most of the available strains have not yet been explored for their antimicrobial activity. However, the general abundance of antimicrobial activity in microalgae appears to be significant, making microalgae an attractive resource for biopesticides (Righini and Roberti 2019).

The microalgae strains identified with promising antimicrobial activity of crude aqueous extracts in this study are *C. vulgaris*, *T. subcordiformis*, *Coelastrum* sp., *H. lacustris*, and *C. sorokiniana* (Table 2 and Fig. 2). The genus *Chlorella* is one of the most often identified microalgae with antimicrobial activity (Vehapi et al. 2020; Al-Nazwani et al. 2021; Dinev et al. 2021; Perveen et al. 2022). However, direct comparison of antimicrobial properties found in certain microalgae strains from the literature to strains from the same genus can be misleading. Our rapid screening results of several strains belonging to the *Chlorella* genus showed significant differences in the presence of antimicrobial activity against the screened plant pathogenic species. Two strains of *Chlorella* sp. did not show any antimicrobial activity, while *C. vulgaris* slightly inhibited *P. ultimum* and *P. cactorum*, and *C. sorokiniana* demonstrated a ubiquitous antimicrobial activity against most of the tested plant pathogens (Table 2). This indicates that there is often a strong specificity between the microalgae and the pathogen strain. The strong target specificity of natural biopesticides is one of the advantages over synthetic pesticides because of the comparatively fewer impacts on beneficial organisms and less adaptive resistance of the plant pathogen (Marrone 2019; Fenibo et al. 2021). Microalgal biopesticides will not provide a one-fits-all solution; instead, the huge diversity of microalgae will provide a variety of biopesticides to choose from, depending on the occurrence of different plant pathogens.

In this study, the *C. sorokiniana* extract showed the most promising biopesticide potential against *P. cactorum* (Table 2 and Fig. 2), a common plant pathogenic oomycete infecting various globally important crops, including apple, strawberry, and even ornamental plants. In Finland, *P. cactorum* greatly impacts strawberry production by causing crown rot, wilt, and leather rot on fruits in cultivated strawberry *Fragaria × ananassa* (Lilja et al. 2011). *P. cactorum* is soil-borne and the oospores are difficult to

eliminate once it infested the soil. The motile oospores can survive for several years and spread, particularly during wet periods and through fruit transportation. With the ban on the use of the traditional synthetic pesticide methyl bromide, alternative solutions for pest control in strawberry production are needed (Hautsalo et al. 2016). The application of *C. sorokiniana* extracts efficiently inhibits the growth of *P. cactorum* on strawberry leaves (Fig. 4) offering a potential sustainable approach for pest control against *P. cactorum* in strawberries.

Pre-treatment and extraction methods determine the level of antimicrobial activity

Recent studies indicate that the antimicrobial activity of various microalgae stems from a wide range of bioactive compounds with distinct chemical characteristics. Some suggested bioactive compounds include phenolic compounds (e.g. flavonoids, chlorogenic acid), carotenoid pigments (e.g. astaxanthin, β -carotene, canthaxanthin, neoxanthin, violaxanthin or zeaxanthin), as well as fatty acids (Ördög 2004; Leflaive and TenHage 2007; Shishido et al. 2015; Falaise et al. 2016; Swain et al. 2017). This suggests that the antimicrobial activity not only varies depending on the specific microalgal strains and pathogens involved but also on factors such as pre-treatment methods, solvent, and the extraction method used.

In this study, we compared freeze-drying with wet biomass as pre-treatment method. The drying process likely caused more efficient cell disruption leading to higher antimicrobial activity (compare Fig. 3A and C with B and D, respectively). The freeze-drying process alone often results in cell wall disruption, leakage of cell content, and thus enhancing extraction efficiency (Show et al. 2015). Additionally, a study showed that the drying process aids the extraction process when non-polar solvents are used. This is because the residual water present in wet biomass acts as a barrier between the cells and the solvent preventing efficient extraction (Ghasemi Naghdi et al. 2014). Indeed, all the acetone extract fractions from dried biomass exhibited greater antimicrobial activity than those obtained from wet biomass (Fig. 3B).

Efficient cell wall disruption is an important step in the processing of microalgal biomass and requires special attention. In our initial rapid screening, freeze-thawing was used for lysing the cells. This method is generally considered a gentle but efficient way to break cells and is used in numerous laboratory applications (Shehadul Islam et al. 2017). However, freeze-thawing can be a time-consuming process. In contrast, bead-milling is a fast and scalable method for cell disruption in microalgae (Corrêa et al. 2020). Surprisingly, when we compared the antimicrobial activity of crude aqueous extracts obtained

from 100 g L⁻¹ wet biomass using bead-milling instead of freeze-thawing, we observed a sixfold reduction in activity (compare Figs. 2B and C). This suggests that bead-milling may result in less efficient cell disruption in our specific experimental setup. A study testing various cell disruption techniques on the cyanobacterium *Microcystis aeruginosa* also demonstrated that freeze-thawing was a more efficient cell disruption method than bead-milling (Geada et al. 2019). Another possible explanation for the reduced antimicrobial activity is that the bioactive compound(s) may be sensitive to shearing forces or oxidation processes during bead-milling. Previous research has suggested that the activity of certain bioactive compounds can be reduced in bead-milled microalgae extracts (Stirk et al. 2020). However, we discovered that by doubling the biomass concentration and employing freeze-drying as a pre-treatment, the antimicrobial activity of the bead-milled aqueous extracts reached similar levels to the freeze-thawed extracts (compare Figs. 2B and 3D). In other words, further adjustments in pre-treatment and extraction methods can enhance the biopesticide potential of promising microalgal strains.

Several studies have described that the pathogen inhibition is dependent on the concentration of the microalgal extracts (Ferreira et al. 2021; Ranglová et al. 2021). Similarly, we have observed that *C. sorokiniana* extracts at 200 g L⁻¹ wet biomass and 16 g L⁻¹ dried biomass inhibit the growth of *P. cactorum* more effectively than the corresponding lower concentrations (Fig. 3), implying clear dose dependence. However, increased concentrations of microalgae extracts can also inhibit plant growth (Bumandalai and Tserennadmid 2019), and thus future studies should test these higher extract concentrations on plant growth as well.

The nature of the antimicrobial compound(s) in *C. sorokiniana* responsible for the inhibition of *P. cactorum* has not yet been described. Thus, more attention was given to different fractionations of the crude extract. In addition to water, acetone was selected as another solvent to dissolve nonpolar compounds reliably. The method is inexpensive, readily available, non-toxic and has already been successfully used for *C. sorokiniana* (Valcareggi Morcelli et al. 2021). However, the test of different pre-treatment and extraction methods revealed that aqueous *C. sorokiniana* extracts are more effective antimicrobial agents than acetone extracts (compare Fig. 3A and B with C and D, respectively). This suggests that the bioactive compound(s) are rather water-soluble. Hence, some known antimicrobial compounds found in microalgae can be excluded like e.g., terpenes (Vehapi et al. 2020) and antioxidant pigments (Scaglioni et al. 2019) that are mainly extracted by nonpolar solvents. This opens the possibility of creating a full value-chain multiproduct biorefinery concept (Eppink et al. 2021). The aqueous extraction of *C. sorokiniana* withholds a lot of

residual microalgal biomass, which is not water-soluble and it is very likely that valuable compounds like lipids, fatty acids, pigments, vitamins or antioxidants are retained in the debris (Koutra et al. 2022).

Curiously, the freeze-drying process led to the retention of some antimicrobial activity in the pellet fractions of both, aqueous and acetone extracts (Fig. 3B and D). This could be due to problems with the resolubilization of the bioactive compound(s) after the drying process and thus, the compound(s) remain in the pellet. Exploring possible applications for the residual biomass together with the optimization of biopesticide extraction requires further examination. A better solution for breaking the cells in wet biomass will not only enhance the extraction yield but can also eliminate the energy expensive step to dry the biomass and thus, reduce costs dramatically (Dasan et al. 2019).

Furthermore, the aqueous extracts of *C. sorokiniana* likely contain a wide mix of bioactive compounds with synergetic function. Major water-soluble compounds are polysaccharides, which are considered to enhance plant performance and resilience (Chanda et al. 2019). A study showed that the application of living biomass of *Chlorella fusca* on strawberry plants increased plant health and reduced *F. oxysporum* wilt, demonstrating a successful application of microalgae as biostimulant and biocontrol agent (Kim et al. 2020). With being used as not only biopesticide but also biostimulant enhancing the natural defense response in plants, microalgae are exceptionally suitable for Integrated Pest Management (IPM) and organic farming.

Conclusion

This study presents rapid screenings for antimicrobial activity and strong evidence of a functional biopesticide effect in strawberry leaves. The biopesticide screening of a relatively small selection of microalgae has revealed that five out of 15 microalgal strains (> 30%) showed antimicrobial activity against seven plant pathogens. The application of the most promising aqueous *C. sorokiniana* extract on strawberry leaves successfully impeded an infection caused by the common plant pathogen *P. cactorum*. Albeit the in planta efficacy of a *C. sorokiniana* as biopesticide, its action mechanism, and possible additional biostimulant effects need further investigation, our results demonstrate a budding potential of microalgae for developing natural biopesticides. Additionally, the vast biodiversity of microalgae opens up numerous opportunities for exploring their bioactivity not only as biopesticides but also as biostimulants, biofertilizers and in other applications. These applications encompass a wide range of areas, including agronomic interests, human nutrition, the energy industry, and other fields.

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Authors' contributions Y.A. conceived the research; All authors contributed to design of research; M.J. performed majority of the experiments. J.S. performed the extractions and E.C. performed the analysis in detached leaves. All authors analyzed and interpreted the data. M.J. prepared the first draft with significant contribution from all authors. All authors read and approved the final manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are included in this published article and its supplementary information files. Further information is available from the corresponding author on reasonable request.

Declarations

Competing interests Not applicable.

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