Characterisation of algicidal bacterial exometabolites against the lipid-accumulating diatom *Skeletonema sp.*

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Abstract

Microalgae are of increasing interest due to their occurrence in the environment as harmful algal blooms and as a source of biomass for the production of fine and bulk chemicals. A method for the low cost disruption of algal biomass for environmental remediation or bioprocessing is desirable. Naturally-occurring algal lytic agents from bacteria could provide a cost-effective and environmentally desirable solution. A screen for algal lytic agents against a range of marine microalgae has identified two strains of algicidal bacteria isolated from the coastal region of the Western English Channel. Both strains (designated EC-1 and EC-2) showed significant algicidal activity against *Skeletonema sp.* and were identified as members of *Alteromonas sp*. and *Maribacter sp*. respectively. Characterisation of the two bioactivities revealed that they are small extracellular metabolites displaying thermal and acid stability. Purification of the EC-1 activity to homogeneity and initial structural analysis has identified it as a putative peptide with a mass of 1266 amu.

Key words algicidal bacteria; *Skeletonema;* metabolites; autolysis

1.1 Introduction

Bloom-forming microalgae are a major source of environmental distress which can be the cause of environmental damage by eutrophication whilst also being a source of risk to human health through the formation of harmful algal blooms [1] which are capable of generating exo-metabolites toxic to humans [2]. The existence of naturally-occurring lytic agents which could be used to disrupt microalgal blooms is of increasing interest in the developing field of environmental biotechnology. The induction of algal cell lysis by viruses [3] and bacteria [4] is a well-known phenomenon and could provide a low-cost natural method for disrupting cell structures and so controlling microalgal growth. The recent interest in large-scale growth of microalga for the production of bulk and speciality chemicals, including biofuels, provides a further interest in developing low cost lysis methods which could be adapted for use in bioprocessing [5].

A large number of bacterial species have been identified which release algae-lysing agents but to date only a small number of such compounds have been characterised in detail and these often show a limited spectrum of activity against microalgae. Most efforts have focussed on identifying bacterial strains capable of killing harmful algal blooms which produce toxic chemicals. Examples include bacillamide, a novel anti-algal compound active against the harmful dinoflagellate *C. polykrikoides* [6] as well as polyunsaturated fatty acids from the seaweed *Ulva fasciata,* which are active against *Heterosigma akashiwo* [7]. Recently it has been reported that derivatives of the thiazoldinedione class of anti-diabetic drug have potent activity against *C. polykrikoides* and *H. akashiwo* [8]. Similar anti-algal results have been reported for peptides with anti-microbial activity derived from *Helicobacter pylori* [9].

As part of an on-going programme of research to investigate marine resources for biotechnological applications, water samples from the L4 monitoring site located in the Western English Channel (http://www.westernchannelobservatory.org.uk/) were screened to identify algae-lysing bacteria and viruses for use in bioprocessing and bioremediation. Two bacterial strains are described that produce alga-specific lytic agents primarily active against the bloom-forming and lipid-accumulating diatom *Skeletonema sp.* which can form dense, largely monospecific blooms in temperate waters such as the North Adriatic Sea [10]. The isolation and preliminary characterisation of one lytic agent is presented and its relevance to population dynamics and the persistence of advantageous genotypes in the marine environment discussed.

2. Materials and Methods

2.1 Chemicals

All chemicals were of analytical grade and purchased from Sigma Chemical Company (Poole, Dorset, UK) or Fisher Scientific Ltd (UK).

2.2 *Strains*

Algal strains (*Skeletonema sp.* CCAP1077/1B, *Tetraselmis chui* CCAP8/6, *Pavlova lutheri* CCAP931/3, *Dunaliella salina* CCAP19/3, *Nannochloropsis salina* CCAP849/2, *Isochrysis galbana* CCAP949/1, *Tetraselmis suecica* CCAP66/38, *Thalassiosira pseudonana* CCAP1085/12) were obtained from the Culture Collection of Algae and Protozoa (Scottish Association for Marine Science, Dunstaffnage, U.K.) and cultivated in f/2 medium [11] under white fluorescent light (50 µmol photons m^{-2} s^{-1}) using a 12: 12h light-dark cycle at 20 ℃. The composition of f/2 medium was: each litre of filtered seawater contained 75 mg $NaNO_3$, 5 mg NaH_2PO_4·H2O, 4.36 mg

Na_2EDTA, 3.15 mg $FeCl_3 \cdot 6H_2O$, 0.01 mg $CuSO_4 \cdot 5H_2O$, 0.022 mg $ZnSO_4 \cdot 7H2O$, 0.01 mg $CoCl_2 \cdot 6H2O$, 0.18 mg $MnCl_2 \cdot 4H_2O$, 0.006mg $Na_2MoO_4 \cdot 2H_2O$, 0.1 mg thiamine· HCl, 0.5 µg biotin, and 0.5 µg vitamin B_{12}. For growth of diatoms f/2 medium was supplemented with 30 mg $Na_2SiO_3.9H_2O$ (f/2-Si medium).

Bacterial strains were isolated from water samples collected at the L4 site (50°15.0'N; 4°13.0'W) of the Western Channel Observatory near Plymouth, UK and cultured in marine broth: 5.0 g Bacto peptone and 1.0 g yeast extract made up to 1 litre in filtered seawater. Strains were purified to homogeneity using standard microbiological sub-culturing techniques. 16S rDNA genes were amplified and sequenced using standard conditions and primers [12].

2.3 *Effects of strains EC-1 and EC-2 against Skeletonema sp.*

Bacterial strains were cultured at 20℃ in marine broth with orbital shaking at 130 rpm for 3 days (10^7 cells mL^{-1}) and 3 mL^{-1} of bacterial culture mixed with 50 mL^{-1} logarithmic-phase cultures of *Skeletonema sp.* The numbers of viable algal cells was determined by microscopic examination based on cell morphology and by cell count using a haemocytometer at a magnification of ×40 every two days. All experiments were performed in triplicate.

2.4 *Activity range of strains EC-1 and EC-2 against seven other algal species*

The relative sensitivity of different algae to algicidal bacteria was investigated using the following algal species with each species tested in triplicate: *Tetraselmis chui, Pavlova lutheri, Dunaliella salina, Nannochloropsis salina, Isochrysis galbana, Tetraselmis suecica, Thalassiosira pseudonana* and cultured in f/2-Si agar medium. Bacterial cultures were filtered to remove cells and filtrates (6% v/v) added to the algal cultures. Algicidal activities were assessed by viable cell count, as described in Section 2.3, with a minimum of 3 independent counts per flask and data compared

directly with the untreated control. Average cell counts were used to calculate the percentage cell death following treatment with lytic agent.

2.5 *The characteristics of the algicidal activity*

Pellets and cell-free extracts were prepared by centrifugation of bacterial cultures for 8 min at 8000 *g*. The pellets were suspended in fresh f/2 medium whereas the supernatants were then passed through a 0.2µm syringe filter (to remove any remaining cells) before addition to algal cultures. Viable algal cell numbers were counted every 2 days as described above.

2.6 *Role of bacterial growth on metabolite production*

Single colonies of bacterial strains were each cultured in 500 mL marine broth for 7 days, and 50mL samples were taken out for metabolites extraction every day. Cell-free medium was prepared and XAD-16 resin added for 16 h with shaking to capture metabolites. The resin was recovered, washed with de-ionised water and bound substances eluted with 20 mL ethyl acetate. Samples were evaporated to dryness and re-dissolved in de-ionised water. The paper disk method [13] was initially used to measure the activities of extracts versus *Skeletonema sp.* Test plates were incubated for 24 h at 20℃ and the diameters for zones of clearance measured. Extracts were then re-tested as described in Section 2.3 to determine the percentage algicidal activity.

2.7*Microscopic observations of Skeletonema sp. in cultures with EC-1 metabolite*

An EC-1 extract was added into *Skeletonema sp.* cultures (10^6 cells mL^{-1}) and samples were taken for detailed microscopic observation after 0, 2, 4, 8, and 16 h incubation.

2.8 *Stability of the algicidal substances*

Cell-free medium was autoclaved at 121℃ for 15min., 6 % v/v added to actively growing *Skeletonema sp.*, and algal cell viability determined after 5 days. Additionally, metabolite extracts were heated at 90℃ for 15 min and residual

activities determined using the paper disk diffusion method. Metabolite extracts were also treated with 0.1M HCL and 0.1M NaOH at a concentration of 10% (v/v) for 20 min., neutralised, and residual algicidal activity measured by the disc diffusion method.

2.9 *Purification of the algicidal activity EC-1*

XAD-16 resin-captured metabolite was eluted with ethyl acetate and the resultant eluent dried and the metabolite mixture resuspended in water. The extract was treated with Dowex X-1 resin to further remove impurities and the resulting (unbound) fraction applied directly to a Supelco Ascentis C18 (15cm x 4.6mm, 3µm) reverse phase column and eluted with a 10-90% water/methanol gradient. Three peaks were detected at 215 nm, collected, dried, and tested for bioactivity. Algicidal activity was detected in the third peak. Samples were analysed by MALD-TOFMS and LC-ESI-MS at the University of Manchester, U.K.

3. Results

3.1 *Strain identification*

Sequencing of 16s rDNA revealed that Strains EC-1 (GenBank KT461670) and EC-2 (GenBank KT461671) are highly similar to *Alteromonas sp*. (e-value of 0.0) and *Maribacter sp*.(e-value of 0.0), respectively, based on BLAST analysis of the rDNA sequence [14].

3.2 *Algicidal activities of the two bacterial strains versus Skeletonema sp.*

The algicidal activities of the two bacterial strains EC-1 and EC-2 against *Skeletonema sp.* were evaluated over a period of 10 days by cell count with a minimum of 3 independent cultures per experimental condition (Fig. 1). Initial experiments were performed several times to confirm the observation. In contrast to

control cells which showed a steady increase in cell number over this period, no intact cells were observed in the test samples at 10 days, although the survival of a few cells cannot be excluded.

3.3 *Algicidal activity of strains EC-1 and EC-2 against other algal species*

The activity of the metabolite extracts from strains EC1 and EC2 were tested against seven other microalgal species under similar test conditions to *Skeletonema sp*. (*Tetraselmis chui, Pavlova lutheri, Dunaliella salina, Nannochloropsis salina, Isochrysis galbana, Tetraselmis suecica, Thalassiosira pseudonana*) to establish the lytic profile of the metabolites. Strain EC-1 showed algicidal activity against *Dunaliella salina* and *Isochrysis galbana* (8.3 ± 2.1 % and 41.6 ± 1.4 % cell death) whereas Strain EC-2 demonstrated activity versus both *Isochrysis galbana* (12.5 ± 2.1 %) and *Pavlova lutheri* (12.5 ± 2.3 %). Results are expressed as percentage cell death compared to untreated controls.

3.4 *Initial characterisation of the algicidal substances*

The algicidal effects of different components of strain EC-1 and EC-2 against *Skeletonema sp.* are shown in Fig. 2. A 0.2 µm filtrate of strain EC-1 (ensuring removal of whole cells) decreased the number of intact algal cells effectively (Fig. 2a), however, the bacterial cell pellets when resuspended in a culture of *Skeletonema sp.* had no algicidal effects, showing no difference in growth when compared with the control. Similarly for EC-2 pellets (Fig. 2b), algicidal activity against *Skeletonema sp.* was detected in the supernatant and filtrate, but not in the pellet. This confirmed that the bioactivity was extracellular in nature.

3.5 *Growth phase-specific production of the algicidal bioactives by EC-1 and EC-2*

To determine when the bacteria produced the algicidal bioactive compound, bacteria were grown and samples removed for analysis at regular intervals. Bioactivity versus

growth phase was plotted for both strains (Fig. 3) with maximal activity detected in mid-logarythmic phase (EC-1) or early stationary phase (EC-2), respectively.

3.6 *Stability of the algicidal compounds to heat and pH*

The thermal and pH stability of the algicidal compounds was tested. Thermal stability was tested in two ways: ethyl acetate extracts of spent medium (enriched for bioactivity) were heated at 90℃ for 15min and residual activity detected; or cell-free spent medium was directly autoclaved for 20 min and tested with no further purification. The algicidal activities of metabolite and broth culture against *Skeletonema sp.* were assessed by cell count after either 24 h incubation (ethyl acetate-enriched extracts) or 5 days incubation (cell-free spent medium). Both strains produce a bioactive molecule which is heat-stable with comparable activities between treated and untreated samples in all instances (Fig. 4).

The effect of pH on the stability of each compound was also assessed following exposure to either 0.1 M HCl or 0.1 M NaOH solution as shown in Fig. 5. For strain EC-1 the metabolite treated with 0.1 M HCl and 0.1 M NaOH caused 60 ± 1.7 % and 9 ± 1.3 % cell death of the culture, respectively, whilst untreated sample caused 72 ± 1.2 % of cells to die under the test conditions (Fig. 5a). The activity of strain EC-2 metabolite under the same assay conditions when treated with 0.1 M HCl was 11.2 ± 2.3 % cell death whilst the control (untreated metabolite) was 89.2 ± 1.7 % cell death (Fig. 5b). Treatment with 0.1 M NaOH resulted in total inactivation of the molecule (no cell death detected).

The results indicate that the algicidal bioactivity is heat stable with both compounds producing similar responses to this stress but differences in response following exposure to acidic and alkaline conditions indicate that the molecules are dissimilar.

3.7 *Microscopic observations of Skeletonema. sp. in cultures with EC-1 metabolite*

Microscopic studies of the effect of EC-1 extract on the cell structure of *Skeletonema sp.* cells after 4 h is shown in Figure 6. Intact cells (Fig. 6a) show signs of disintegration after 4 hours with some cells losing shape and cell wall integrity (Fig. 6b) and evidence of membrane-blebbing, perhaps indicative that the metabolite activates a programmed cell death pathway. There is a concomitant decrease in the chlorophyll fluorescence after 8 hours and complete lysis by 16 hours (data not shown).

3.8 *Characterization of the algicidal activities*

Metabolite extracts were initially size fractionated using a 5,000 molecular weight cut-off filter unit. Both compounds were detected in the flow-through indicating that the compounds have a molecular weight of less than 5,000 and thus are unlikely to be a protein. The lytic bioactivity of strain EC-1 was investigated in more detail and bioactivity-guided fractionation performed. MS analysis of the purified molecule generated major peaks of mass $[M+H^+]$ 1266.88 a.m.u. and 634.15, respectively (Fig. 7). LC-MS/MS fragmentation analysis generated mass peaks consistent with the presence of amino acids but attempts to sequence the peptide using conventional Edman degradation failed to produce any significant peaks, indicating that the peptide may be cyclic in nature. Further work to elucidate the structure of the algicidal molecule and its mode of action is required.

4. Discussion

Algae-lysing bacteria have been isolated from many bacterial genera against a number of different microalgae but only one strain with activity against the diatom *Skeletonema sp.* has been previously reported [15] and the lytic agent was identified as a protease. This study has identified two bacterial strains (EC1, an *Alteromonas sp*.,

and EC2,a *Maribacter sp*.) from the Western English Channel both of which produce algicidal compounds with strong activity against *Skeletonema.* Previous studies have described strains of *Alteromonas* which are capable of causing algal lysis [16, 17] indicating that this species may play an important role in the natural dissolution of algal blooms in the environment. Both strains EC-1 and EC-2 showed significant and rapid algicidal activity against *Skeletonema.* Previous studies indicate that two general mechanisms are used by bacteria to lyse algae, involving either a direct mechanism where whole bacteria must be present for successful lysis, or an indirect mechanism where an extracellular product is responsible for cell lysis [18, 19]. To understand which mechanism is used by EC-1 and EC-2, bacteria were grown and cell-free spent medium and cell pellets tested separately. In both cases bioactivity was detected in the spent medium (Fig. 2) and simple size-fractionation showed both products to be less than 5,000 molecular weight confirming that the lytic agents were small, soluble molecules and unlikely to be enzymes. This is further supported by the heat stability displayed by both samples (Fig. 4). To assess the breadth of algicidal activity displayed by EC-1 and EC-2 a further 7 species of microalgae were tested for sensitivity. Not all species tested were sensitive but a range of algicidal activity was detected for both samples with EC-1 favouring *Skeletonema sp. > I. galbana >D. salina* whereas EC-2 favoured *Skeletonema sp. > I. galbana = P. lutheri*. This indicates that the two products are distinct and this is further supported by their differing responses to short-term exposure to acid and alkali conditions (Fig.5). No anti-microbial activity was detected when tested against a range of human pathogens (data not shown). Interestingly there is no obvious phylogenetic relationship between species which display sensitivity to the two bioactives although both target *Isochrysis galbana* (haptophyte) to differing degrees as well as *Skeletonema sp.* (diatom).

The product of EC-1 was selected for further analysis due to its greater stability under acidic and alkaline conditions. Microscopic examination of cells exposed to extract revealed features of membrane blebbing and condensation of internal structures after 4 h exposure (Fig. 6). This indicates that EC-1 may induce a mechanism of programmed cell death which is consistent with previous studies demonstrating that *Skeletonema* encodes a cell death mechanism involving a gene product (Death Specific Protein, *DSP1*) that induces autolysis when the cells are stressed [20]. Further analysis of *DSP1* indicated its expression is modulated by the messenger molecule nitrous oxide and that light intensity is one trigger of stress-induced autolysis [21].

The product was purified from spent medium and initial analysis using LC-ESI-MS generated a product of 1266.88 amu [$M+H^+$] whilst a second product with half the mass (634.15 amu) was also detected. Attempts to further characterise EC-1 by N-terminal sequencing and MS/MS analysis did not generate any definitive data suggesting that EC-1 is either modified at its N-terminus or a cyclic peptide whose composition may include neutral amino acids (data not shown).

It is interesting to note that *Skeletonema sp.* CCAP1077/1B was originally isolated from the L4 site of the Western English Channel and that the two strains described here (EC-1 and EC-2) may have evolved a toxin which is predominantly targeted at this bloom-forming alga as a method of controlling excessive algal growth which would lead to nutrient depletion. Algicidal metabolite production is triggered when the bacterial cultures approach stationary phase probably due to the deprivation of key nutrients (Fig. 3), which would arise if an algal bloom was forming. Bloom dissolution through autolysis induction of the *Skeletonema* by algicidal metabolites would both release nutrients from the dying cells and halt further depletion of

293 nutrients from the surrounding environment allowing the proliferation of the bacteria
294 to continue. The L4 time-series extends over many decades [22] and long-term
295 monitoring has seen a decline in the general population of *Skeletonema* in Plymouth
296 Sound with no recent blooms of note (C. Widdecombe, personal communication).
297 The persistence of these bacterial strains and their retention of algicidal activity
298 predominantly targeted to *Skeletonema sp*. in the English Channel further supports the
299 notion that, once acquired, these secondary metabolic pathways (i.e. antibiotic activity)
300 are stably maintained in the genome. This further highlights the dynamic and
301 competitive environment which exists in the sea and the potential for discovering new
302 and useful natural products with applications in biotechnology and management of the
303 environment.
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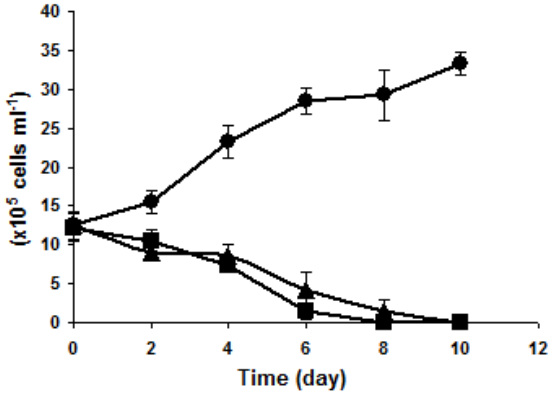
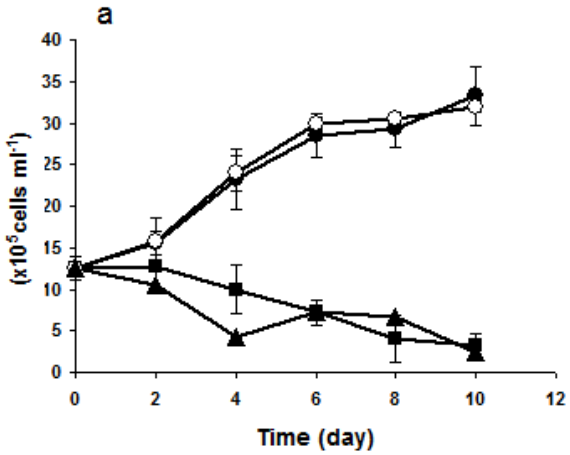


Fig. 1. Algicidal activities of two bacterial strains against *Skeletonema sp.* (●, control; ■, EC-1; ▲, EC-2).



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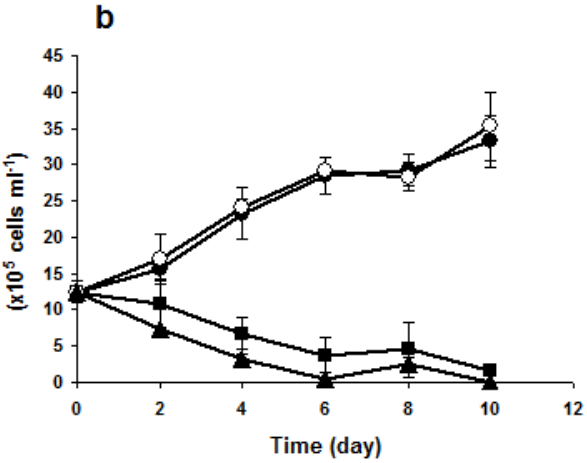

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389 **Fig. 2 Algicidal effects of the bacterial strains EC-1 (a) and EC-2 (b) following**
390 **different treatment methods (●, control; ◯, pellet; ■, supernatant; ▲, filtrate).**

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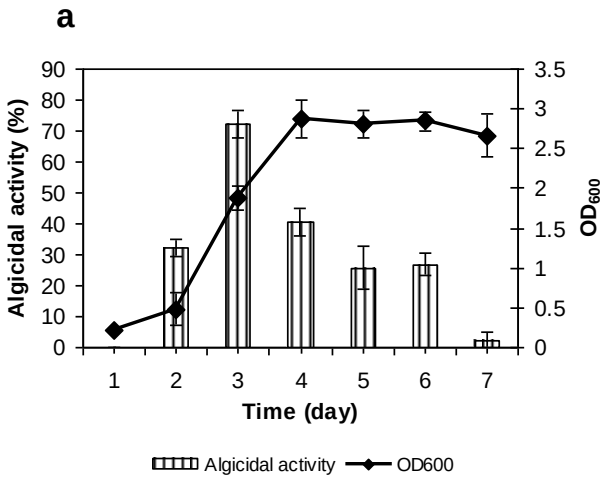

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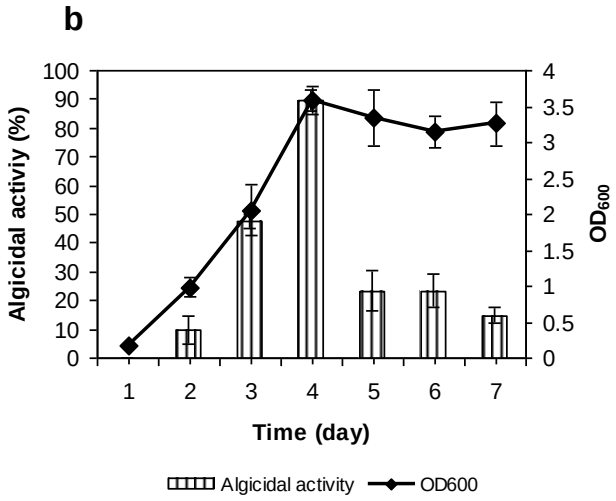

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394 **Fig. 3 Algicidal activity of the metabolites of two bacterial strains against**
395 ***Skeletonema sp.*** (a) EC-1 growth versus production of lytic activity. (b) EC-2
396 growth versus production of lytic activity. Activities are expressed as a percentage of
397 control (uninoculated) medium.
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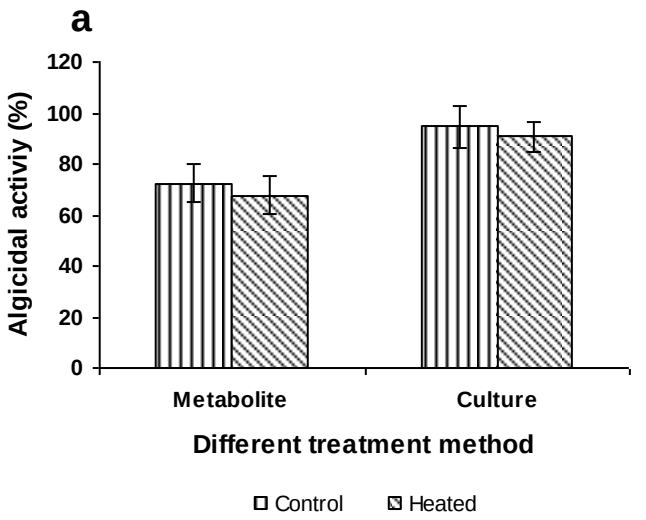

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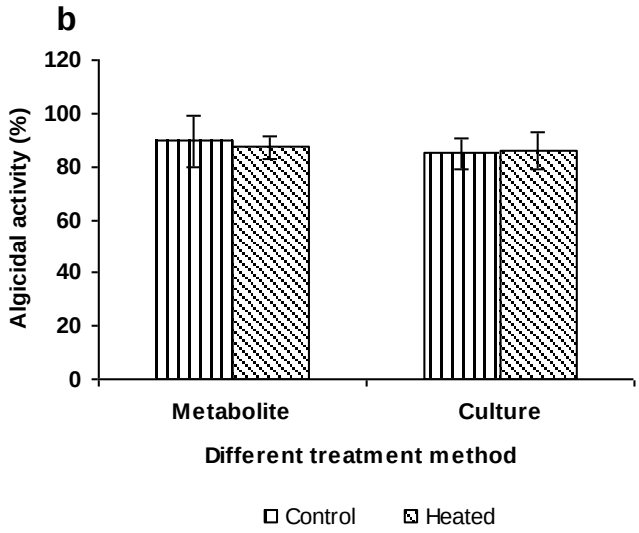

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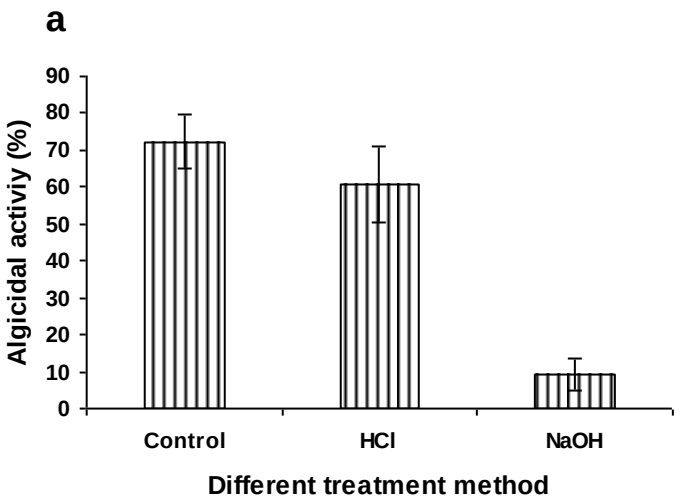
404 **Fig. 4 Algicidal activities of metabolites before and after heat treatment.** The

405 percentage cell death caused by cell-free extracts either as spent medium or enriched

406 resin-captured metabolite is shown for strain EC-1 (a) and strain EC-2 (b).

407



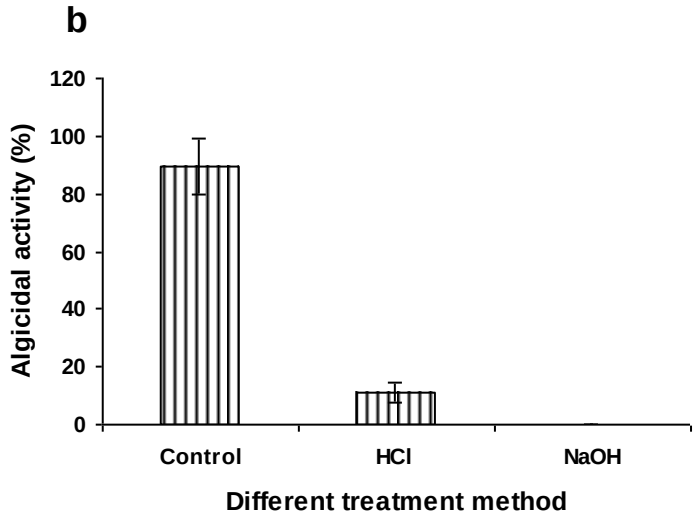


Fig. 5 Remaining algicidal activities of the metabolites from strain EC-1 (a) and strain EC-2 (b) following acidic and alkaline treatments compared to untreated control.

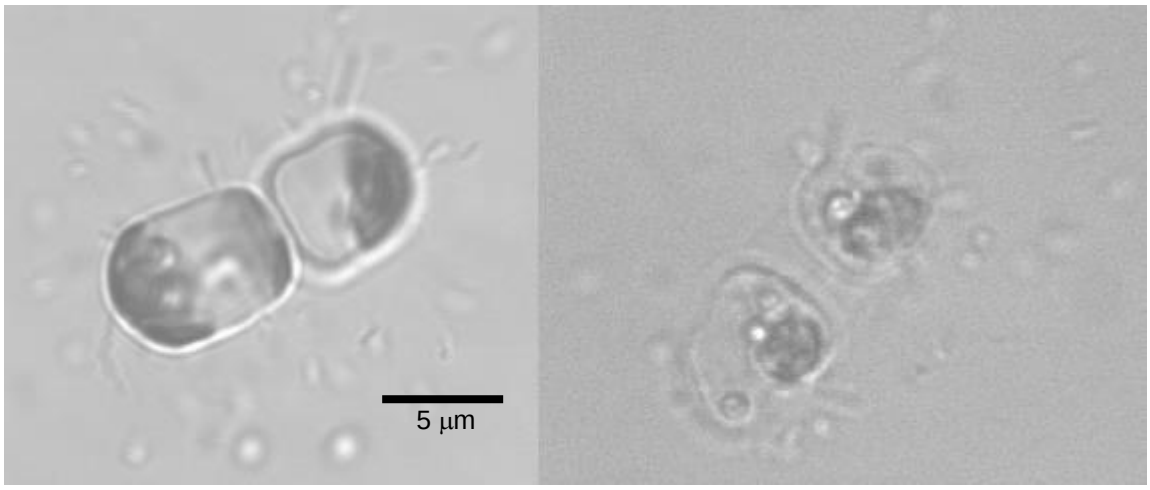


Fig. 6 Microscopic observations of *Skeletonema sp.* in cultures in the absence (left) and presence (right) of EC-1 extract. Exposure to EC1 extract for 4h has resulted in condensation of the cell and evidence of membrane-blebbing.

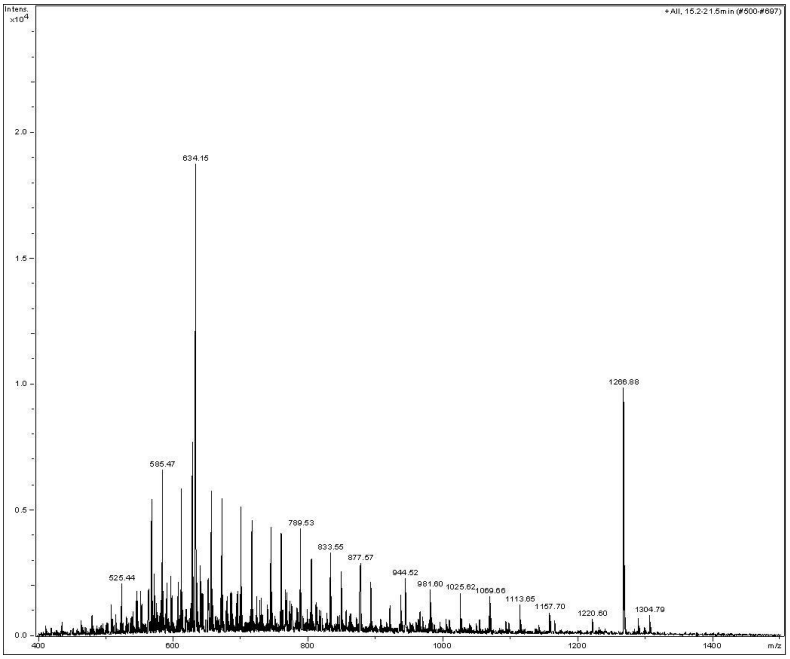


Fig. 7 LC-ESI-MS analysis of purified EC-1 bioactivity showing major peaks at 1267 and 634 amu respectively.