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SCREENING STUDY FOR ANTIBACTERIAL ACTIVITY FROM MARINE AND FRESHWATER MICROALGAE

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ABSTRACT

Nowadays, several pathogenic bacteria present resistance against common antibiotics, being necessary the search new drugs with activity against resistant pathogens. The capacity of microalgae to produce diverse secondary metabolites has been widely reported. Here we searched the antibacterial activity of several organic extracts from different microalgae. We used five marine diatoms and a consortium of freshwater Chlorophyceae obtained from urban wastewater treatment. Target organisms were both Gram-negative (*Pseudomonas fluorescens*, *Serratia marcescens* and *Escherichia coli*) and Gram-positive (*Micrococcus luteus, Bacillus subtilis* and *Staphylococcus epidermidis*) human pathogenic bacterial strains. Antibacterial activity was evaluated by agar diffusion assays. *P. fluorescens, S. marcescens* and *S. epidermidis* were inhibited by many of the extracts tested, while *E. coli, M. luteus* and *B. subtilis* were found completely resistant. These results corroborate the potential use of microalgae extracts as a source of new antibiotic compound and open the possibility of use of wastewater cultured algae.

KEYWORDS: **Antibacterial activity; antibiogram; agar diffusion assay; microalgae;**

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INTRODUCTION

Microalgae embrace an extremely diverse life group not only morphologically or taxonomically but also from a metabolic point of view. This variety suggests the occurrence of many secondary metabolites of interest[1] and this has been pointed out by several reviews through the recent literature[2,3]. Although these organisms have been used with different purposes since 1950's, recent efforts are focused in the finding of novel compounds that might lead to therapeutically useful agents[4,5]. Despite the vertiginous progress in human medicine, infectious diseases caused by bacteria, fungi and viruses are still a major threat to public health. Their impact is particularly important in developing countries but the actual globalization, extreme weather events and human migration crisis in different areas makes this as a potential global problem. Additionally, the continuous evolution of microbial pathogens towards antibiotic resistance demands the development of new antimicrobial compounds. In this context, microalgae constitute a potentially source of novel antibacterial compounds[6] and a good choice for combating antibiotic resistant bacteria and fungal infections areas[7]. Several species of microalgae have shown antibacterial activities, attributed to different chemical classes of metabolites such as indoles, terpenes, phenols, volatile halogenated hydrocarbons or long chains unsaturated fatty acids [8,9,10,11]. These substances of algal origin offer the possibility to synthesize new drugs that provide an alternative against resistant pathogens to antibiotics known to date[12]. Our aim in this work was to provide information about the antibacterial activity of different organic extracts of marine microalgae freshly isolated in a coastal area site in the Southern Iberian Peninsula (Bay of Cádiz) and also strains isolated from wastewater treatments. The use of marine native strains is a prerequisite to ensure adaptability of these algae to future outdoor massive production in this geographical area. Additionally, the use of strains that grown in wastewater gives value to a biomass used for water depuration, which is a cheap and environmentally friendly way to generate algal biomass.

MATERIAL AND METHODS

Microalgal strains

Five different strains of marine diatoms and a consortium of freshwater Chlorophyceae were used. Diatoms strains were *Skeletonema marinoi*, *Thalassiosira sp.* and *Chaetoceros sp*. These strains were freshly isolated from the Bay of Cádiz (Southern Spain). Additionally, two strains of CCAP 1052/1A *Phaeodactylum tricornutum* derived from two different collections, one strain from Alfred Wegener Institute (Bremerhaven, Germany) (hereafter Pt 1) and strain from the Molecular Biology of Photosynthetic Organisms Institute (Paris, France) (hereafter strain Pt2) were also used. A consortium of freshwater Chlorophyceae composed by *Coelastrum sp., Scenedesmus quadricauda and Selenastrum sp.* collected from wastewater treatment was also used.

Bacterial strains

Bacterial strains were derived from the Spanish Type Culture Collection. Three Gram-negative strains (*Pseudomonas fluorescens* 378, *Serratia marcescens* 846 and *Escherichia coli* 434) and three Gram-positive bacteria (*Micrococcus luteus* 245*, Bacillus subtilis* 35 and *Staphylococcus epidermidis* 231) were used in this study. These bacterial cultures were maintained in petri dishes with solid LB medium at 26ºC or 37ºC depending on the optimum growth documented in previous literature.

Microalgae cultures

Microalgae cultures were performed in sterilized 1 L flasks. After isolation of diatoms strain by micropipetting and dilution. Unialgal diatom cultures obtained were grown using natural, filtered through 0.2 µm and autoclaved natural seawater, enriched additionally with silicate and f/2 medium[13]. Cultures were maintained at 19 ºC and light:dark cycle of 14:10 hours under 75 µmol quanta m^{-2} s^{-1}. Chlorophyceae were cultured using filtered 0.45 µm wastewater.

Biomass concentration

Exponential growing microalgae were collected and concentrated by centrifugation at 1200 g for15 minutes. Obtained pellets were frozen in liquid N_2 and stored at -80ºC until extraction. Previously, a cell count was performed by a Neubauer chamber to determine the volumes to be employed for the pellets (Table 1).

Preparation of Algal extracts

Different kind of algal extracts were performed to test antibacterial activity for all strains.

Methanol extracts

These extracts followed the procedure described by Desbois[14]. The frozen pellets were thawed, washed once in 1 mL sterile 3.2% NaCl and after centrifugation at 3000 rpm for 5 minutes, pellet was re-suspended in 6 mL of a mix methanol:water (5:1). Then, cells were lysed by sonication on ice for 2 minutes. Lysates were kept on ice and agitated 1 hour on an orbital mixer at 160 r.p.m before removal of cell debris by centrifugation at 4800 r.p.m for 15 minutes. The supernatant was aspirated and introduced in a glass vial. Extracts were then evaporated and conserve at -80 ºC until their use.

Hexane extracts

The frozen pellets were unthawed and 2 ml of hexane were added[15].Cells were them lysed by sonication on ice for 5 minutes and were centrifuged at 4800 r.p.m for 15 minutes. The supernatants were aspirated and introduced in a glass vial. This procedure was repeated and the new supernatant was added at the glass vial. Later, the extracts were evaporated and conserve at -80 ºC until their use.

Ethanol extracts

For ethanol extraction, the frozen pellets were unthawed and 2 mL of ethanol were added[16]. Cells were lysed by sonication on ice for 5 minutes and were centrifuged at 4800 rpm for 15 minutes. The supernatants were aspirated and introduced in a glass vial. Then, the extracts were evaporated and conserve at -80 ºC until their use.

Water:methanol:hexane extracts

These mixed solvent is used for the extraction of polyunsaturated aldehydes (PUAs)[17]. The frozen pellets were unthawed and immersed in added 1.5 mL of Pentafluorobenzylhydroxilamina (PFBHA). These compound transform volatile aldehydes into this compound becomes volatile aldehydes in stable compounds. First a hexane sample was sonicated for 5 minutes for wash the sonicator and this procedure was repeated between samples. Samples were sonicated for 5 minutes and were left for 1 hour at room temperature for stabilization. The extraction was performed with a mix of water:methanol:hexane (2:1:2). The pellets were added to a funnel. Water, methanol and hexane were added at the funnel and acidified with several drops of sulfuric acid. It was covered, stirred and degassed for 1 minute. The funnel was then placed on the sedimentation column and was expected for 30 minutes to separate phases. Later, the extracts were evaporated and conserve at -80 ºC until their use.

Bacterial culture

The day before performing the antibacterial assays, bacterial strains were inoculated from petri dishes into liquid LB medium and maintained 24 hours at 26 ºC with continuous agitation.

Antibacterial assay

Antibacterial activity of algal extracts was tested by agar diffusion assay[18]. Seventy two Petri dishes containing Müeller Hinton agar seeded with inoculum of bacterial strains were used. The different extracts were dissolved in ethanol, in two increasing concentrations (C1 and C2). Concentration C1 was dissolved in 1 mL of ethanol and concentration C2 was dissolved in 200 µL of ethanol.For evaluating the activity of the extracts, sterile glass fiber filter discs (5.5 mm diameter) were performed. These filters were embedded in the different extracts and ethanol was evaporated at room temperature. The discs were then placed on test plates inoculated with bacterial strains. A disc loaded with sole ethanol was prepared as a control. The plates were incubated at dark and at 26 ºC or 37 ºC during 48 hours. Extracts containing antibacterial components produced distinct, clear and circular zones of inhibition around the filter discs and this positive activity, if existed, was quantified by measuring the inhibition halos obtained after 24 and 48 hours.

RESULTS

The zone of inhibition of bacteria around the disc was measured and the assay was scored in Tables 2, 3 and 4. Five extracts from the twenty four extracts showed antibacterial activity in the bioassay of concentration C1. Four extracts were ethanolic extracts and one was PUAs extract. Microalgal strains showed positive effects were: *P. tricornutum* (Pt 1), *S. marinoi*, *Thalassiosira sp.*and the consortium of freshwater microalgae Chlorophyceae against the bacterial strains: *Staphylococcus epidermidis* (Gram-positive), *Serratia marcescens y Pseudomonas fluorescens* (Gram-negative). Ethanol extract of *P. tricornutum* (Pt 1) showed a stronger activity against *S.epidermidis* at this concentration, compared to the rest of extracts, which showed less activity. For the higher concentration, C2, seventeen extracts from the twenty four extracts showed antibacterial activity and all of the microalgal strains showed positive effects. Five extracts were methanolic extracts, two hexane extracts, five ethanolic extracts and five PUAs extracts. Ethanol extract of both strains of *P. tricornutum*m and *Thalassiosira sp.* showed a stronger activity against *P. fluorescens* and *S. marcescens*; PUAs extract of *P. tricornutum* strain 2 showed a stronger activity against *S. marcescens* and methanol extract of *S. marinoi* showed a stronger activity against *S. epidermidis* at concentration C2. No solvent or medium control produced zones of inhibition. Sometimes, bacteria develop detoxification systems that degrade the active molecule, which can be observed by an inhibition halo decrease in time. The growth of *E. coli, M. luteus* and *B. subtilis* was not inhibited by any extract.

Table 1
Cell concentration and culture volume employed for extractions.

Specie	Centrifuged volume (mL)	Cell density in culture (cell·mL^{-1})		Cell density in pellet (cell·mL^{-1})	
Phaeodactylum tricornutum strain AWI	80	$8,9 \cdot 10^6$		$712 \cdot 10^6$	
Phaeodactylum tricornutum strain Pt	80	$7,2 \cdot 10^6$		$576 \cdot 10^6$	
Chaetoceros sp.	320	$9,5 \cdot 10^4$		$30,4 \cdot 10^6$	
	240	$16,2 \cdot 10^4$		$38,88 \cdot 10^6$	
	190	$21,75 \cdot 10^4$		$41,325 \cdot 10^6$	
Skeletonema marinoi	80	$2,4 \cdot 10^6$		$192 \cdot 10^6$	
Thalassiosira sp.	240	$4,25 \cdot 10^4$		$10,2 \cdot 10^6$	
	240	$2 \cdot 10^4$		$4,8 \cdot 10^6$	
	240	$2,25 \cdot 10^4$		$5,4 \cdot 10^6$	
Chlorphyceae consortium	80	Sp. 1	$1,3 \cdot 10^6$	Sp. 1	$104 \cdot 10^6$
		Sp. 2	$4,6 \cdot 10^4$	Sp. 2	$3,68 \cdot 10^6$
		Sp. 3	$2,22 \cdot 10^6$	Sp. 3	$177,6 \cdot 10^6$

Sp. 1 →Coelastrum sp., Sp.2 →Scenedesmus quadricauda y Sp. 3 →Selenastrum sp.

Table 2

Positive antibacterial activity of several extracts of different microalgae after 24 h and 48 h of exposition Concentration C1. Activity was quantified in the basis of diameter of the inhibition zone (D) in mm.

Organism	Extract	Target pathogen bacteria		
		S. epidermidis	*S. marcescens*	*P. fluorescens*
		24 h		
Phaeodactylum tricornutum strain AWI	Ethanol	+++	-	+
Skeletonema marinoi	Ethanol	+	-	+
Thalassiosira sp.	Ethanol	+	+	-
Chlorophyceae consorptium	Ethanol	-	++	-
	PUAs	-	+	-
		48 h		
Phaeodactylum tricornutum strain Pt	Ethanol	+++	-	+
Skeletonema marinoi	Ethanol	+	-	+
Thalassiosira sp.	Ethanol	+	++	-
Chlorophyceae consortium	Ethanol	-	++	-
	PUAs	-	++	-

(-): No activity, (+): D<7mm, (++): 6<D<8.5 mm, (+++): D>8.5 mm. D: diameter of the inhibition zone in millimeters.

Table 3

Positive antibacterial activity of several extracts of different microalgae after 24 h of exposition Concentration C2. Activity was quantified in the basis of diameter of the inhibition zone (D) in mm.

Organism	Extract	Target pathogen bacteria		
		S.epidermidis	*S. marcescens*	*P. fluorescens*
		24 h		
Phaeodactylum tricornutum strain 1	Methanol	-	++	-
	Ethanol	++	+	+++
	PUAs	-	+	-
Phaeodactylum tricornutum strain 2	Ethanol	-	+++	+
	PUAs	-	+++	+
Chaetoceros sp.	Methanol	++	-	+
	Ethanol	++	-	-
	PUAs	++	-	-
Skeletonema marinoi	Methanol	+++	+	+
	Hexane	-	+	-
	Ethanol	++	-	+
	PUAs	+	-	-
Thalassiosira sp.	Methanol	-	++	-
	Hexane	-	+	-
	Ethanol	-	+++	-
	PUAs	-	++	-
Chlorophyceae consortium	Methanol	-	+	-

(-): No activity, (+): D<7mm, (++): 6<D<8.5 mm, (+++): D>8.5 mm. D: diameter of the inhibition zone in millimeters

Table 4

Positive antibacterial activity of several extracts of different microalgae after 48 h of exposition Concentration C2. Activity was quantified in the basis of diameter of the inhibition zone (D) in mm.

Organism	Extract	Target pathogen bacteria		
		S. epidermidis	*S. marcescens*	*P. fluorescens*
		48 h		
Phaeodactylum tricornutum strain 1	Methanol	-	++	-
	Ethanol	+	+	++
	PUAs	-	+	-
Phaeodactylum tricornutum strain 2	Ethanol	-	+++	-
	PUAs	-	+++	-
Table 3 cont.				
Chaetoceros sp.	Methanol	+	-	+
	Ethanol	+	-	-
	PUAs	+	-	-
Skeletonema marinoi	Methanol	++	++	+
	Hexane	-	+	-
	Ethanol	++	-	+
	PUAs	+	-	-
Thalassiosira sp.	Methanol	-	++	-
	Hexane	-	+	-
	Ethanol	-	+++	-
	PUAs	-	++	-
Chlorophyceae consortium	Methanol	-	+	-

(-): No activity, (+): D<7mm, (++): 6<D<8.5 mm, (+++): D>8.5 mm. D: diameter of the inhibition zone in millimeters.

DISCUSSION

In the screening of biologically active substances in microalgae by means of the antimicrobial activity test, we detected antibacterial activity in all species of microalgae against three bacterial strains (*Staphylococcus epidermidis, Serratia marcescens and Pseudomonas fluorescens*), while other three remained totally resistant to the extracts (*Escherichia coli, Micrococcus luteus* and *Bacillus subtilis*). Ranges of inhibition halos obtained varied from 6 to 11.5 mm using a very low microalgal biomass (< 1 mg) but comparable to that obtained from other microalgal species and near the values obtained for extracts obtained for higher macroalgal biomass. Higher biomass extract would ensure higher inhibition. In the case of *P. tricornutum*, it is known that methanolic extract of this specie contains EPA (eicosapentanoic acid) that is responsible for growth inhibition of several potential human pathogens as *Bacillus* strains or *Staphylococcus aureus*[19, 20]. This was probably responsible for microbial inhibition in our experiments. We also found that ethanolic extracts could extract these fatty acids and inhibit growth of *S. marcescens* strain. Differences observed among both *P. tricornutum* strains could be consequence of variety of morphotypes abundances in these strains since trirradiate morphotype, less abundant in strain Pt ENRS, produce more quantity of EPA[14]. Freshly isolated marine diatoms as *Chaetoceros sp.* or *S. marinoi*, showed antimicrobial activity for both groups of bacteria (Gram-positive and Gram-negative) and specially against Gram-positive strains, usually more resistant to classical antibiotics. This is interesting since reveal that autoctonous algal strains could be an interesting source of new antibiotics. Positive results against *S. epidermidis*, *S. marcescens* and *P. fluorescens* are important because its pathogenic activity causing human diseases [21,22,23,24,25]. Both diatom genus, *Chaetoceros* and *Skeletonema,* are cosmopolitan and widely distributed in the oceanic coastal areas. *Skeletonema* species are common phytoplankters, especially in coastal estuarine and marine environments where they often form dense blooms[26]offering a wide extended areas of source for local strains. Regarding the results obtained from the consortium of Chlorophyceae we found positive inhibition against *S. marcescens* (Table 2 and 3) at three different extracts (Ethanol, PUAs and Methanol). This has been observed by other authors [27, 20, 28], however the novelty of our results is the provenance of microalgae, since they grew in wastewater. We demonstrate that algal biomass used for wastewater treatments could be recollected for extracting potentially useful extracts, which is an interesting and valuable result for using algal biomass.

CONCLUSION

Autoctonous marine microalgae could serve as a source of antibiotic for different geographical area around the world. The autoctonous origin gives facilities for outdoor production in open system, since cells are adapted to that climatological area. Additionally, freshwater microalgae grown in urban wastewater can offer new and cheap grown possibilities for the screening of antibacterial substances.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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