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Research Note

Evaluation of the antibacterial activity of different species of phytoplankton

Evaluación de la actividad antibacteriana de diferentes especies de fitoplancton

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Abstract.- The antibiotic activity of organic extracts from cultures of six species of phytoplankton, *Synechococcus elongatus*, *Synechocystis* sp., *Amphiprora paludosa*, *Porphyridium cruentum*, *Chaetoceros muelleri* and *Dunaliella tertiolecta* was examined. The bacteria used were Gram(-) strains, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, and the Gram(+) strains were *Bacillus subtilis*, *Staphylococcus aureus*, and *Microccocus luteus*. All extracts inhibited the growth of *B. subtilis* and effected the generation of inhibition halos, ranging in diameter from 7.06 to 15.23 mm, for several bacteria species. Wide-spectrum antibacterial activity was observed in extracts from *S. elongatus*, *Synechocystis* sp., *A. paludosa*, *P. cruentum*, and *C. muelleri*. Thus, this five phytoplankton species can be used to control bacteria.

Key words: Antibiotics, microalgae cultures, cyanobacteriae

Introduction

Marine planktonic algae are potentially new sources of specific antibacterial compounds (Patil *et al*. 2009). Diatoms are the major groups of phytoplanktonic class whithin algae, in which most organic compounds found in them with antibacterial activity have been pigments, lipids, and carbohydrates (Shimizu 1993), cyanobacteria (blue-green algae) have unique, biologically active secondary metabolites due to their ecological and morphological diversity (Kreitlow *et al*. 1999). Cyanobacteria proved to be an excellent source of natural metabolites, possessing antibacterial, antifungal, and cytotoxic-cytostatic activity (Patil *et al*. 2009). The chemical structures of these cyanobacterial metabolites have been determined, including polyketides, amides, alkaloids, lactones, peptides, and lipopeptides (Singh *et al.* 1999).

The use of antimicrobial agents in aquaculture can increase the prevalence of bacteria that are resistant to the organisms that are maintained in the culture; such bacteria could be transmitted and cause infections in humans on consumption of cultured organisms (WHO 2006). In aquaculture, the indiscriminate use of antibiotics influences the rates of diseases and resistance. Several international organizations have restricted the use of certain antibiotics in organisms that are produced for human consumption (FDA 2009).

The antagonism between microbes is a naturally occurring phenomenon through which pathogens are killed or decline in number in the aquaculture environment (Maeda *et al.* 1997). The identification and development of antibiotics are needed continuously due to the increase in resistant pathogens, the evolution of emergent diseases, the existence of naturally resistant bacteria, and the observed toxicity of current medicinal compounds (Demain 1999). In aquaculture, certain bacterial populations govern this equilibrium through their interactions with various pathogenic agents. An understanding of the specific interactions between bacteria and microalgae will allow us to optimize productive systems in aquaculture (Riquelme & Avendaño 2003). Thus, our aim was to examine the antibacterial properties of several strains of marine phytoplankton for their potential use in aquaculture.

Material and methods

Marine phytoplankton strains *Amphiprora paludosa* var. *hyalina*, *Synechococcus elongatus*, and *Synechocystis* sp. were obtained from 'Laboratorio de Microalgas, Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE), Ensenada, México'. *Dunaliella tertiolecta* and *Chaetoceros muelleri* strains were obtained from the Oyster Center of Sonora (COES), and

Porphyridium cruentum was acquired from the Institute of Applied Microbiology at the University of Tokyo, Japan.

Cultures were maintained in nonaxenic, monospecies batch cultures in triplicate. The bacterial counts of all phytoplankton strains were lower than 1 x 10^6 cells mL^{-1}. Cultures were grown in batches in 2.8-L Fernbach flasks that contained 2 L of medium (Guillard 1975), where in silicate and vitamin concentrations were doubled for diatoms and cyanobacteria, respectively. Cultures were maintained at 20 ± 2°C with continuous light at 75 μE $m^{-2}s^{-1}$ and shaken manually every 12 h, no oxygen was supplied by aeration.

To generate the growth curves, optical density (OD) was measured daily at 550 nm on a Hach DR/4000 UV-VIS spectrophotometer. Cell concentrations were determined using a hemacytometer, and size was measured by light microscopy. The cell concentrations were used to obtain the exponential growth rate (μ) and mean doubling time (G) (Fogg and Thake 1987). The number of colony-forming units (CFU) was determined in triplicate sets of each cultured microalgal strain using the method of Colwell *et al.* (1975).

Bacterial strains were obtained from the bacterial culture collection of the Faculty of Chemistry Science and Engineering at 'Universidad Autónoma de Baja California' (UABC), Tijuana, México. The Gram(-) strains were *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*, and the Gram(+) strains were *Bacillus subtilis*, *Staphylococcus aureus*, and *Micrococcus luteus*. Bacteria were precultured at 37°C for 12 h in Petri dishes in 10 mL Luria-Bertani agar (LB agar) medium (Maniatis *et al*. 1982).

The microalgae and cyanobacteria cultures were harvested on day 12 during the stationary phase, and the cells were concentrated by centrifugation at 3500 *g* for 15 min at 20ºC. The total cell biomass of each cultured strain was lyophilized, and the dry weight value was obtained gravimetrically (Table 1).

The cells were disrupted with an ultrasonic processor at 80 MHz for 2 min with 3 3-second intervals and extracted twice with 10 mL hexane; during the extractions, flasks were shaken at 150 rpm for 2 h in the dark at 20ºC (CLSI 2006). Pellets from the hexane extraction were reextracted with dichloromethane and methanol, as described above. The solvents were evaporated under a nitrogen stream. The dry microalgal extracts were dissolved in 1 mL dimethyl sulfoxide (DMSO).

The antimicrobial tests were performed in vitro on LB agar plates by diffusion method (Collins *et al*. 1989). The plates were inoculated with a standardized suspension of each bacterial strain (1.0 OD, measured at 600 nm as the McFarland equivalent concentration of 10^8 CFU mL^{-1}) and maintained at 37ºC (CLSI 2006).

The inhibition of bacterial growth was assessed in triplicate using sterilized 6-mm-diameter paper disks (S-&-S Filter-Paper-Disks) that contained 30 μL of algal extract in DMSO. Carbenicillin (500 μg mL^{-1}), a broad-spectrum antibiotic, was used as a positive control, and seawater and DMSO were used as negative controls (Al-Saimary *et al.* 2007). The inhibition zone was defined as the diameters of the area in which no bacterial growth was detected (CLSI 2006). Measurements were recorded after 24 h of incubation at 37ºC. Pre-assays were run to

Table 1. Mean cell size, cell density of the inocula and at harvest, exponential growth rate (μ), mean doubling time (G), dry weight, and colony-forming units (CFU) of marine microalgae and cyanobacterial strains maintained in batch cultures / Tamaño promedio de la célula, densidad de células en el inóculo y en la cosecha, tasa de crecimiento exponencial (μ), tiempo de duplicación (G), peso seco y unidades formadoras de colonias (CFU) de microalgas y cianobacterias marinas mantenidas en cultivos estáticos

Strain	Cell size μm	Cell density (inoculated) cells mL^{-1} 10^6	Cell density (harvested) cells mL^{-1} 10^6	μ divisions day^{-1}	G	Dry weight mg mL^{-1}	CFU CFU x 10^3 mL^{-1}
Synechococcus elongatus	1 x 2	0.06	24.3	0.7	1.4	1.1	n.d.
Synechocystis sp.	1.3 x 2	0.06	28.6	0.7	1.4	0.7	n.d.
Amphiprora paludosa	5.9 x 2.7	0.02	4.6	0.6	1.6	0.4	0.6
Dunaliella tertiolecta	8-11 x 14-16	0.02	1.8	0.5	2.0	0.5	3.0
Chaetoceros muelleri	8-12 x 7-10	0.02	2.6	0.5	2.0	0.9	2.6
Porphyridium cruentum	5 x 8	0.02	9.7	0.7	1.4	1.3	0.5

n.d. not detected

determine the optimal time and growth of the bacterial strains. Microalgal extracts were preabsorbed for 4 h at 20ºC to increase assay sensitivity.

To assess the differences in measurements of the inhibition zone, we performed one-way analysis of variance (ANOVA). Significant differences were analyzed using Tukey´s a posteriori test. We considered all of the assumptions of the statistical test (Zar 1996). All statistical analyses were performed using STATISTICA, version 6.0 with $\alpha = 0.05$.

Results and Discussion

The cultured microalgae and cyanobacteria strains showed taxon-dependent differences in cell size, density, dry weight, and growth rate (Fig. 1, Table 1). As expected, cyanobacteria cultures were the smallest and had the highest density, and *Dunaliella tertiolecta* cultures had the largest cell size and the lowest density. The *Amphiprora paludosa* strain had the lowest cellular mass, expressed as dry weight (Table 1).

In the antibacterial activity assays, certain parameters of growth, such as growth phase, cell density, exponential growth rate, mean doubling time, and dry weight, were relevant, because these variables can influence the antibacterial activity mentioned above. All microalgae cultures had CFU values that were lower than 3.0×10^3 mL^{-1} (Table 1).

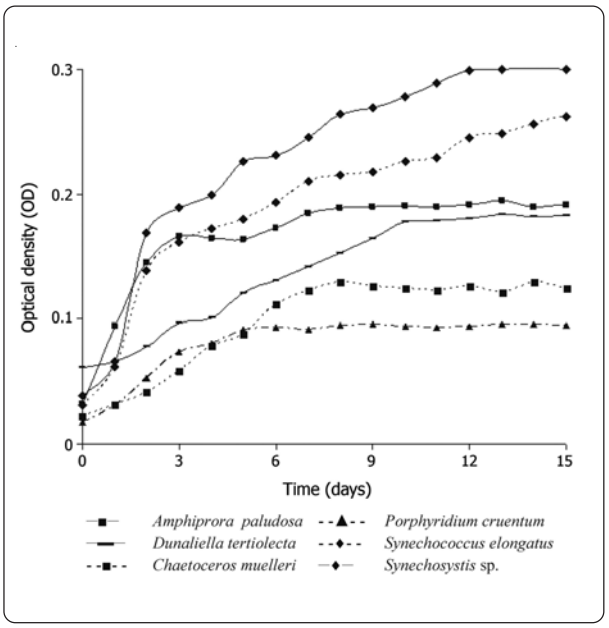

Figure 1. Growth of batch cultures of marine microalgae and cyanobacteria according to the mean optical density (OD) from triplicates measurements / Crecimiento de cultivos estáticos de microalgas y cianobacterias marinas según el valor de densidad óptica promedio (OD) de triplicados

Based on the screen, the *Chaetoceros muelleri* methanolic extract had the most potent antibacterial activity against *B. subtilis* and *S. aureus*, generating inhibition halos of 8.53-15.86 mm (Table 2). Of the 18 algal extracts that we tested, *S. elongatus*, *Synechocystis* sp., *A. paludosa*, and *P. cruentum* had antibacterial activity against Gram(+) and Gram(-) bacteria. Antibacterial activity against *M. luteus* or *E. coli* was not detected in any extract (Table 2).

The cyanobacterial extracts from *S. elongatus* and *Synechocystis* sp. had antibacterial activity against *B. subtilis* and *S. aureus* (inhibition halos: 7.96-9.86 mm) and the Gram(-) *K. pneumoniae* (inhibition halos: 9.76-9.93 mm) (Table 2).

Extracts from the benthic diatom *A. paludosa* generated inhibition halos of 7.60-11.80 mm against *B. subtilis* and *S. aureus*. Against *P. aeruginosa*, only the methanol extract had antibacterial activity (mean value 10.53 mm) (Table 2).

Extracts of the Rhodophyceae *P. cruentum* in all 3 solvents inhibited the growth of *B. subtilis* (inhibition halos of 7.06-9.80 mm). Only the dichloromethane extract was active against *P. aeruginosa* and *K. pneumoniae*, effecting mean inhibition halos of 9.83 and 11.00 mm, respectively (Table 2).

The extracts that we used might contain compounds that have antibacterial activity. To identify and evaluate the activity of individual compounds, they must undergo further purification and separation. Microalgae comprise a diverse group of photosynthetic organisms that have successfully adapted their metabolism to survive in different habitats. The ability to withstand environmental stress is related to their ability to produce a vast array of secondary metabolites, which have considerable value in the biotechnology, aquaculture, health, and food industries (Andersen 1996). Antibacterial activity has been observed predominantly in the Bacillariophycea Class, the most notable species of which are *Chaetoceros*, *Nitzschia*, and *Thalassiosira*. Strain selection is important, because biological activities can differ between strains of the same species (Pesando 1990).

Our results confirm the reported antibacterial properties of *Chaetoceros* spp. and *P. cruentum* (Pesando 1990, Ördög *et al*. 2004). Our data suggest that the antibacterial

Table 2. Mean inhibition halos in organic extracts from batch cultures of marine cyanobacteria and microalgae using hexane (H), dichloromethane (D), and methanol (M). Standard deviation are shown in brackets. a,b,c values indicate statistical significance (ANOVA and Tukey a posteriori test, $P < 0.05$: a<b<c) / Valores promedio de los halos de inhibición en los extractos orgánicos de cultivos estáticos de cianobacterias y macroalgas marinas utilizando hexano (H), diclorometano (D), y metanol (M). La desviación estándard se indica en paréntesis. Valores a,b,c indican diferencias estadísticas (ANDEVA y prueba a posteriori de Tukey, $P < 0,05$: a<b<c)

Strains	Extracts	*Staphylococcus aureus*	*Bacillus subtilis*	*Micrococcus luteus*	*Escherichia coli*	*Klebsiella pneumoniae*	*Pseudomonas auruginosa*
Synechococcus elongatus	H	7.96 (0.41) a	8.83 (0.32) a	-	-	9.93 (0.11) a	-
	D	9.86 (0.15) c	9.23 (0.09) b	-	-	9.76 (0.25) a	-
	M	8.66 (0.37) b	8.63 (0.35) a	-	-	-	-
Synechosystis sp.	H	9.56 (0.37) a	8.20 (0.20) a	-	-	9.83 (0.28) a	-
	D	9.33 (0.57) a	9.76 (0.25) b	-	-	9.86 (0.23) a	-
	M	9.40 (0.60) a	9.36 (0.55) b	-	-	-	-
Amphiprora paludosa	H	8.66 (0.40) a	11.76 (0.40) c	-	-	-	-
	D	10.76 (0.25) b	10.13 (0.75) b	-	-	-	-
	M	11.80 (0.52) c	7.60 (0.40) a	-	-	-	10.53 (0.41)
Dunaliella tertiolecta	H	10.00 (1.03) a	11.60 (0.60) c	-	-	-	-
	D	9.23 (0.25) a	8.93 (0.11) a	-	-	-	-
	M	9.70 (0.26) a	10.06 (0.11) b	-	-	-	-
Chaetoceros muelleri	H	8.96 (0.32) b	8.53 (0.41) a	-	-	-	-
	D	8.70 (0.36) a	9.70 (0.26) b	-	-	-	-
	M	15.86 (0.30) c	15.23 (0.32) c	-	-	-	-
Porphyridium cruentum	H	-	9.80 (0.62) b	-	-	-	-
	D	-	7.06 (0.40) a	-	-	11.00 (0.20)	9.83 (0.32)
	M	-	7.20 (0.26) a	-	-	-	-
Negative Control		-	-	-	-	-	-
Positive Control		13.13 (0.72)	16.36 (0.11)	10.56 (0.66)	12.00 (0.20)	10.26 (0.11)	13.03 (0.25)

activities of these strains are primarily Gram(+)-specific, perhaps due to the additional membrane that exists in Gram(-) bacteria. The active compound in the extracts might encounter difficulties in penetrating the additional membrane, which contains porins that impede the influx of drugs and a multiple-drug pump efflux system that expels most drugs from the bacteria, a well-documented phenomenon (Schmidt & Hansen 2001).

One of the cyanobacteria (*Synechococcus elongatus*) that we used were isolated from commercial aquaculture farms from a shrimp farm in Nayarit, México (Aguilar-May & Sánchez-Saavedra 2007) and *Synechosystis* sp. from an abalone farm in Eréndira, Baja California, México. The antibacterial activity of local *S. elongatus* and *Synechosystis* sp. strains had not been reported.

Cyanobacteria have the ability to synthesize antibiotic compounds could benefit their high growth rate and survival under extreme conditions in their competitive natural environment (Patil *et al.* 2009). This capacity might be useful for the isolation and production of new antibacterial compounds. For example, they could be used as a probiotic in shrimp farms to eliminate pathogenic bacteria, such as members of the genus *Vibrio*. *Synechococcus elongatus* has been used to control eutrophication and remove nitrogen and phosphorous by immobilization onto chitosan cells (Aguilar-May & Sánchez-Saavedra 2009) and as food for postlarval shrimp (Sánchez-Saavedra & Moreno-Pérez 2009[1]).

Our data implicate the novel antibacterial activity of the benthic diatom *Amphiprora paludosa*, which is a common strain that is used as feed for postlarval abalone cultures in México (Correa *et al.* 2009). Concentrated cultures of *A. paludosa* can be stored at 4°C in the dark for two months. Under these conditions, the cells maintain their viability due to their tolerance to low temperatures and antibacterial activity, as evidenced by their low CFU

[1]Sánchez-Saavedra MP & MPA Moreno-Pérez. 2009. Effect of monospecies and mixed diets on the growth and survival of shrimp larvae *Litopenaeus vannamei* (Bonne, 1931). World Aquaculture Symposium, Veracruz, México, 25 al 29 de septiembre. p. 93.

values (1 x 10^4 mL^{-1}) in storage cultures and low bacterial colony counts by epifluorescence microscopy (Sánchez-Saavedra 2006).

Dunaliella tertiolecta, *Choetoceros muelleri*, and *Porphyridium cruentum* strains showed antibacterial activity against *B. subtilis*, and only the dichloromethane extract of *P. cruentum* inhibited the growth of *K. pneumoniae* and *P. auruginosa*. Antibacterial activity of *P. cruentum* has been reported against *Vibrio* spp. (Cooper *et al.* 1983), and *C. muelleri* has been tested against *Vibrio* and *Pseudomonas* (Pesando 1990). Ethanolic *D. salina* extracts have shown antimicrobial activity against *S. aureus* and *E. coli*. In these extracts, 15 volatile compounds and several fatty acids (primarily palmitic, α-linolenic, and oleic acids) were identified (Herrero *et al*. 2006).

Antibacterial activity, observed in diatoms *Chaetoceros* and *Skeletonema*, has been associated with several fatty acids, primarily those with 10 carbon atoms, which induced lysis in bacterial protoplasts (Naviner *et al.* 1999). The strains *S. elongatus*, *Synechosystis* sp., *A. paludosa*, *C. muelleri*, and *P. cruentum* can potentially be used to control Gram(+) and Gram(-) bacteria. Our results showed that several microalgal strains could constitute a biological method of bacterial control under controlled culture conditions due to their antibacterial activity. Yet, the specificity of the chemical compounds that are produced from the microalgae cultures for a specific bacterial group must be considered. All antibacterial activities in the microalgae cultures were attributed to the cells -not to the bacteria- based on the low or undetectable CFU counts from the microalgal culture samples. In the sea, the bacterial concentration approaches 10^6 cells mL^{-1} and exceeds 10^6 cells mL^{-1} in aquaculture systems (Maeda *et al*. 1997).

In conclusion, this work demonstrates the vital role of certain marine microalgae and cyanobacteria in the production of antibacterial substances and lays the foundation for future applications by the aquaculture and pharmaceutical industries. Future studies should identify the compounds that mediate the antibacterial activity that we have observed.

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