ARTÍCULO CORTO

Actividad biológica de biomasa, ficocianina y exopolisacáridos de una cepa nativa de *Spirulina subsalsa*, cultivada en un medio salino de bajo costo

Biological activity of biomass, phycocyanin and exopolysaccharides from a native strain of *Spirulina subsalsa*, grown in low cost saline medium

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RESUMEN

Con el objetivo de evaluar la actividad biológica de la biomasa, extractos de ficocianina y exopolisacáridos de *S. subsalsa*, se realizaron cultivos axénicos a 9 ‰ de salinidad mediante la adición de agua de mar y en un medio salino de bajo costo: [N]=14 mM. El cultivo se realizó en ambiente externo (agitación manual). Los cultivos fueron centrifugados cuando alcanzaron las fases de crecimiento exponencial y estacionario. El filtrado se utilizó para obtener los exopolisacáridos por precipitación con acetona, y la biomasa se utilizó para obtener los extractos en diferentes solventes. La ficocianina se extrajo en agua. Se evaluó la actividad frente a *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus cereus*, *Micrococcus luteus* y *Staphylococcus aureus*, mediante la técnica de difusión en agar. Los resultados mostraron que sólo los extractos etanólicos de biomasa, cosechados en fase estacionaria, tuvieron actividad antibacteriana leve a moderada contra *Bacillus cereus* y *Staphylococcus aureus*. También se evaluó el efecto antifúngico de los extractos. Los extractos acuosos (exopolisacáridos y ficobiliproteínas) y los extractos etanólico, clorofórmico y hexánico de *S. subsalsa*, no inhibieron el crecimiento de *Rhizopus orizae* y *Aspergillus niger*. La toxicidad de los extractos se probó utilizando los crustáceos *Artemia franciscana* y *Daphnia magna*. Los extractos de exopolisacáridos y biomasa resultaron ser inocuos, pero los extractos acuosos de ficocianina mostraron actividad citotóxica significativa contra *A. franciscana* ($CL_{50\ \text{fase exponencial}}$ = 1,69 µg mL^{-1}; $CL_{50\ \text{fase estacionaria}}$ = 2,59 µg mL^{-1}). Los resultados sugieren que la cepa nativa *S. subsalsa*, cultivada en medio enriquecido con agua de mar y en un medio salino de bajo costo tiene potencial para producir compuestos bioactivos con propiedades antimicrobianas.

Palabras clave: *Spirulina subsalsa*, actividad antimicrobial, citotoxicidad.

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ABSTRACT

To evaluate the biological activity of biomass, phycocyanin extracts, and exopolysaccharides from *S. subsalsa*, axenic cultures were conducted at 9 ‰ salinity by adding seawater and using a low-cost saline medium: [N]=14 mM. The cultivation was performed in an external environment and was manually stirred. The cyanobacteria were centrifuged when they reached the exponential and stationary growth phases. The filtrate was used to obtain the exopolysaccharides by acetone precipitation, and the biomass was used to obtain the extracts in the different solvents. Phycocyanin was extracted in water. The activity against *Escherichia coli, Pseudomonas aeruginosa*, *Bacillus cereus, Micrococcus luteus* and *Staphylococcus aureus*, using the agar diffusion technique was evaluated. The results showed that only the ethanolic biomass extracts, harvested in stationary phase, had mild to moderate antibacterial activity against *Bacillus cereus* and *Staphylococcus aureus*. The antifungal effect of the extracts was also evaluated. The aqueous extracts (exopolysaccharides and phycobiliproteins) and ethanolic, chloroformic and hexane extracts of *S. subsalsa,* did not inhibit the growth of *Rhizopus orizae* and *Aspergillus niger*. The toxicity of the extracts was tested using the crustaceans *Artemia franciscana* and *Daphnia magna*. *The* extracts of exopolysaccharides and biomass were found to be innocuous, but the aqueous extracts of phycocyanin showed significant cytotoxic activity against *A. franciscana* ($LC_{50\ exponential\ phase}$= 1.69 µg mL^{-1}; $LC_{50\ stationary\ phase}$= 2.59 µg mL^{-1}). The results suggest that the native strain of *S. subsalsa*, cultivated in a seawater enriched medium and in a low-cost saline medium has potential to produce bioactive compounds with antimicrobial properties.

Key Words: *Spirulina subsalsa*, antimicrobial activity, citotoxicity.

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INTRODUCTION

Cyanobacteria are a diverse group of photosynthetic prokaryotic organisms that are found in a variety of habitats, including ponds, soils, rocks, bark, sea, and freshwater (Issa *et al.,* 2013). To compete for space, nutrients, and light, and to avoid being consumed by herbivores, cyanobacteria have developed defense mechanisms, including the synthesis of bioactive compounds such as antibiotics, algaecides, and cytotoxic agents.

Some secondary metabolites extracted from cyanobacteria have been evaluated to determine their importance in the pharmaceutical and medical industries (Schaeffer and Krylov, 2000; Prashantkumar et al., 2006; Ramamurthy and Raveendran, 2009; Seyidoglu *et al.*, 2017; Villalobos and Hernández, 2019; García-Ishimine *et al.*, 2020). Several studies report that cyanobacteria species have been studied for their antimicrobial properties, including *Anabaena variabilis* (Ma and Led, 2000), *Trichodesmium erythraeum* (Thillairajasekar *et al.*, 2004), *Nostoc commune* (El-Sheekh *et al.*, 2006), *Microcystis aeruginosa*, *Anabaena flos-aquae* (Khairy and El-Kassas, 2010), *Nostoc* spp. (Nowruzi *et al.*, 2018), and *Spirulina platensis* (Abdel-Moneim *et al.*, 2022).

The biotechnological potential of *Spirulina subsalsa* has been little studied. However, this cyanobacterium has been used as a bioremediation agent for pollutants and industrial waste (Chakraborty *et al.*, 2014; Wang *et al.*, 2014; Jiang *et al.*, 2015). Additionally, it has been used as a biosensor for assessing the toxicity of estuarine waters (Campanella *et al.*, 2001). This species has also been considered a source of polyhydroxyalkanoates (PHA), environmentally friendly biopolymers which may be applied in the production of implants and artificial tissues (Shrivastav *et al.*, 2010). Mazur-Marzec *et al.* (2015) and Szubert *et al.* (2018) have reported that *S. subsalsa* produces biologically active metabolites such as commercially important enzymes and unknown enzymes, as well as agents that promote the growth of several bacterial strains.

It is relevant to develop research on the biotechnological use of a native strain of *Spirulina subsalsa*, isolated from the Turimiquire dam in Sucre state, Venezuela. The objective of this study is to explore the bioactivity of the metabolites produced by this cyanobacterium.

MATERIALS AND METHODS

Conditions of cultivation and production of the biomass and exopolysaccharides of the native strain of *Spirulina subsalsa*.

A native strain of *Spirulina subsalsa* was isolated from Clavellino Reservoir in Sucre State, Venezuela (coordinates: 10° 19' to 10° 23' N, 63° 35' to 63° 40' W). This strain has been deposited in the Algae Germplasm Bank of the Instituto Oceanográfico de Venezuela, Universidad de Oriente, under the code BGAUDO 161. It was previously cultivated in seawater (9 ‰) following the method outlined by Faucher *et al.* (1979).

Cultures were grown axenically for 30 days in an outdoor environment (manually shaken) in sextuplicate, using 2 L flasks containing 1500 mL of culture medium.

The medium contained a concentration of 14 mM nitrate, 36 µM phosphate, 95.23 mM sodium bicarbonate, 13 µM Fe, and 0.9 µM Mn.

Environmental physicochemical parameters were recorded daily. Temperature was measured using a Sealog electronic thermograph (Vemco Ltd., Halifax, Canada) submerged in one of the cultures, while pH was measured with a Denver pH-meter. Cultures were harvested in the exponential and stationary phases (3 replicates on each phase) by filtering through a permaline sleeve with 30-40 µm pores. The filtrate was used to extract exopolysaccharides following the methodology of Vicente *et al*. (2004). Harvested biomass was stored at -20 °C for subsequent compound extraction and biological activity tests, including antibacterial, antifungal, and cytotoxic assays.

Extracts

Crude extracts of *Spirulina subsalsa* biomass were prepared by a four-solvent extraction method. The microalgal biomass was separately macerated with hexane, chloroform, ethanol, and water. The extraction was carried out in the dark, at 4 °C, for 48 h. Each extract was centrifuged (2000 g, 15 min). The organic extracts were concentrated in a rotary evaporator (40 °C), and the aqueous extract was concentrated by lyophilization. The extracts were stored at freezing temperatures (- 20 ºC) for later use in biological activity tests.

The phycocyanin extracts were obtained according to the methodology described by Sharma *et al*. (2014) and Murugan and Rajesh (2014), with modification by Romero *et al*. (2017). These extracts were suspended in water and the exopolysaccharides were dissolved in acetone. The biological activity was subsequently evaluated.

Biological activities

Antibacterial activity

The antibacterial effect of the crude extracts of biomass, phycocyanin and exopolysaccharide was detected by agar diffusion technique (Bauer *et al*., 1966), for which gram-negative bacterial strains: *Escherichia coli* (ATCC 10536), *Pseudomonas aeruginosa* (ATCC 9027), and gram-positive: *Bacillus cereus* (ATCC 9634), *Micrococcus luteus* (Bioanalysis 33-8-10) and *Staphylococcus aureus* (ATCC 6538) were used.

Petri dishes were filled with 15-20 mL of Müller-Hinton agar and inoculated with a standardized bacterial suspension. The concentration of the bacterial suspension was adjusted to match a 0.5 McFarland standard. Sterile Whatman No. 3 filter paper discs with 10 mm in diameter were impregnated with 25 µL of the extracts to be evaluated. The extracts were prepared at 40 mg mL^{-1}, which provides a final concentration of 1000 µg of extract per disk. The plates were pre-incubated at 4°C, for 12 h, to allow for greater diffusion of the extract. They were then incubated at 37°C, for 48 h, to allow bacterial growth. The appearance of inhibition halos around the discs indicated antibacterial activity. The diameter of the zones of bacterial lysis was measured in millimeters. Paper discs impregnated with 10 µL of the different solvents used to prepare the extracts were used as negative controls. Positive controls with paper discs impregnated with commercial antibiotics were also included.

Antifungal activity

The antifungal properties of crude biomass, phycocyanin and exopolysaccharides extracts were evaluated by the technique described by Madubunyi (1995). The phytopathogenic fungal strains (*Rhizopus orizae* and *Aspergillus niger*) were incubated in separated tubes containing potato dextrose agar (PDA) for one week, at room temperature. After this period, 10 mL of sterile distilled water was added to each of tubes and shaken vigorously to dislodge the spores. The resulting mixture was filtered through sterile gauze to prepare suspensions in sterile saline. The suspensions were re-diluted and one drop of each dilution was used for spore counting in Neubauer chamber to standardize the inoculum to 1×10^{8} mL^{-1} spores. The conidial suspensions (asexual spores) were inoculated onto Petri dishes containing PDA agar using sterile swabs. Finally, 10 mm diameter Whatman No. 3 filter paper discs, impregnated with 25 µL of the extracts evaluated (40 mg mL^{-1}) were placed on the agar obtaining a final concentration of 1000 µg of extract per disk. Discs impregnated with 25 µL of solvent served as negative control. Plates were incubated for 48 hours at room temperature. Antifungal activity was evaluated by measuring the diameter of the inhibition halo (mm), with a graduated ruler.

Toxic activity

The toxicity of the crude extracts of biomass, phycocyanin and exopolysaccharides was determined using the crustaceans *Artemia franciscana* and *Daphnia magna*.

The toxicity of the extracts was evaluated using *Artemia franciscana* (Meyer *et al*., 1982). This method involves exposing brine shrimp nauplii to the extracts and measuring the mortality percentage after 24 hours (obtained according to the methodology of Sorgeloos *et al*. (1986). By testing different concentrations of the extracts, the median lethal concentration (LC_{50}) can be determined. The LC_{50} is the concentration of the extract that kills 50% of the nauplii and is used to assess the toxicity of

the extract. Extracts with an LC_{50} of less than or equal to 30 µg mL^{-1} are considered to be bioactive.

For this purpose, a standard solution was prepared by dissolving 50 mg of the sample in 0.5 mL of solvent and 4.5 mL of filtered seawater. Serial dilutions were prepared from this standard solution (100, 10, 1, 0.1, and 0.01 µg mL^{-1}) in 9 mL vials. Ten nauplii of *Artemia franciscana* were introduced into each of the experimental vials. For each concentration, four replicates and four control vials were established.

After 48 hours, mortality of the organisms was assessed using a stereoscopic microscope. The data collected underwent LC_{50} calculation via a software designed by Stephan (1977), utilizing four methods: Binomial, Moving Average, Probit and Logit with 95% reliability limits, each method was described in standard protocols for toxicity bioassays with aquatic species (Rodríguez and Esclapés, 1995), with 95% reliability limits.

Daphnia magna was chosen for this study due to its routine use as a model organism in toxicity bioassays (Goulden *et al.*, 1982). Its selection is attributed to its ease of handling and cultivation in a laboratory setting, as well as its sensitivity in quantifying the effects of xenobiotics. *D. magna* was nourished with *Scenedesmus quadricauda*, which was grown in medium f/2 (Guilard, 1975), with a nitrogen concentration of 8 mmol L^{-1}, a temperature of 25 ± 1 °C, 2000 lux lighting, a 24:0 photoperiod, and constant aeration at 200 mL min^{-1}. The organism was cultured in hard water (distilled water with a high concentration of carbonated salts), which had been sterilized in an autoclave at 15 psi/120 °C for 15 minutes.

The experimental procedure involved exposing *D. magna* neonates to varying concentrations of the extracts under investigation and determining the toxicity based on organism mortality (LC_{50} of the extracts), following the recommendations of Lennuk *et al.* (2015). Specifically, ten neonates of *D. magna* (from the same cohort) were exposed to different concentrations of the various extracts (1000; 100; 10; 1; 0.1; 0.01 and 0 µg mL^{-1}), in triplicate in 50 mL flasks. The experiment was conducted with four replicates and four controls. After of 48 hours, mortality was assessed using a stereoscopic microscope. The data obtained were used to calculate the LC_{50}, similar to the procedure used for *A. franciscana*.

RESULTS AND DISCUSSION

Environmental parameters

The temperature of the cultures varied between 25.1-31.3 °C and the pH varied from 8.3 to 10.1 at the end of the test.

Exopolysaccharides and pigments

The highest exopolysaccharides (58.5 %) and phycocyanin (25.8 µg mL^{-1}) contents were accumulated in the stationary phase, in *S. subsalsa*, cultivated in low-cost saline medium and harvested in the exponential and stationary phases of growth, as reported by other authors like Romero *et al.* (2019).

Antibacterial activity

Antibacterial activity was witnessed only on the ethanolic extract of the biomass harvested in the stationary phase, in comparison to extracts of *S. subsalsa* evaluated (including aqueous exopolysaccharides and phycocyanin, as well as ethanolic, chloroformic, and hexane biomass). This activity was particularly observed against *Bacillus cereus* and *Staphylococcus aureus*, with inhibition halos measuring 15.1 ± 1.2 mm and 11.2 ± 1.1 mm respectively.

Based on the quantitative scale proposed by Monks *et al.* (2002), this extract can be classified as having mild to moderate antibacterial activity. This scale is based on antibiogram studies and categorizes activity as follows: 0 mm indicates no activity, 11-14 mm indicates mild activity, 15-18 mm indicates moderate activity, and anything greater than 18 mm indicates strong activity.

The "mild-moderate" antibacterial activity against *Bacillus cereus* and *Sthaphylococcus aureus*, exhibited by the ethanol extract of *Spirulina subsalsa* biomass harvested in the stationary phase, aligns with findings reported by Kaushik and Chauhan (2008). They found that various extracts of differing polarities from *Spirulina platensis* were active against both Gram-positive and Gram-negative bacteria. Notably, the alcoholic extracts of this cyanobacterium's biomass inhibit the growth of *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Salmonella typhi.*

Additionally, Parisi *et al.* (2009) and Challouf *et al.* (2011) observed significant antimicrobial activity of the methanolic extracts of *Spirulina platensis* against various pathogenic bacteria. Other authors like Abdel-Moneim

et al. (2022) report that methanolic, acetone and hexane extracts of *Spirulina platensis* biomass succeed in inhibiting the growth of *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes* and *Escherichia coli*, *Salmonella typhi* and *Klebsiella pneumonia*. These findings underscore the potential of *Spirulina subsalsa* as a source of antibacterial agents.

The bioactivity exhibited by the ethanolic extracts of the *S. subsalsa* biomass could possibly be attributed to the presence of polyphenols, such as phenolic acids, phenylpropanoids, flavonoids and quinones. This suggestion aligns with the findings of Ozdemir *et al*. (2004), who proposed that these types of compounds are soluble in polar solvents. This is further reported by Roopchand *et al*. (2013), who reported a higher yield of total polyphenols in vegetables when using aqueous ethanol. Contrary to the ethanolic extracts of the biomass, the phycobiliprotein and exopolysaccharide extracts of *S. subsalsa* did not exhibit any activity against the evaluated bacteria. These findings contrast with those published by Sarada *et al*. (2011) and Najdenski *et al*. (2013), who reported antibacterial activity of phycobiliprotein extracts isolated from *Arthrospira fusiformis* against several bacterial strains including *Streptococcus pyogenes*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus*. Similarly, Kaushik and Chauhan (2008) pointed out that the extract of exopolysaccharides from *Spirulina platensis* cultures was able to inhibit *E. coli*.

Hamad *et al*. (2023) reported that phycobiliprotein extracts from *Arthrospira fusiformis* (commercially known as *Spirulina platensis*) did not have an antibacterial effect against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Shigella sonnei*. However, they inhibited the growth of *Salmonella typhi*, *Proteus vulgaris*, *Escherichia coli* and *Salmonella typhimurium* bacteria.

The observed discrepancy in results could potentially be attributed to the extraction procedures employed. It is possible that certain compounds were either lost or retained in the biomass due to their solubility in solvents other than those used in this study. This could result in a reduction of bioactivity due to the low concentration of compounds in the disks used. This perspective aligns with the findings of Szubert *et al.* (2018), who noted that low concentrations of *Spirulina subsalsa* CCNP1310 extracts may not exhibit biological activity, even against certain enzymes such as trypsin and thrombin.

Antifungal activity

The aqueous (exopolysaccharides and phycobiliproteins), ethanol, chloroform, and hexane extracts of *S. subsalsa* did not exhibit any antifungal effects against the phytopathogenic fungi *Rhizopus orizae* and *Aspergillus niger*. These findings align with the results presented by Rania *et al*. (2008), who evaluated the impact of methanolic and hexane extracts of *Spirulina platensis* on the growth of four fungal strains: *Candida kefyr*, *Aspergillus fumigatus*, and *Aspergillus niger*.

Although most authors report that *Spirulina platensis* and *Spirulina maxima* extracts have antifungal activity, this activity is closely related to the presence of phenolic compounds, which are a constituent of secondary metabolites present in *Spirulina* biomass (Parisi *et al*., 2009; Moraes *et al*., 2011; Ansari *et al*., 2013). Studies have reported the antifungal activity of *Spirulina* extracts grown on commercial media such as Zarrouk and BG-11 (Peeler *et al*., 1989; Ansari *et al*., 2013; Battah *et al*., 2014; Usharani *et al.,* 2015), at pH 8.0, and using the Soxhlet extraction method, which is one of the most efficient extraction methods. These conditions are different from those used in the present study, which confirms the findings of other authors who have suggested that there is a synergistic relationship between culture conditions and the production of bioactive compounds (Patterson *et al*., 1991; Morton *et al*., 1994).

Another factor to consider, and perhaps the most important of all, is that the cyanobacterium isolated was identified as *Spirulina subsalsa*, for which there are no reports of antifungal activity. This is probably due to taxonomic differences between *S. subsalsa* and other *Spirulina* species. Hamad *et al.* (2023) reported an antimicrobial effect of phycobiliprotein extracts obtained from *Arthrospira fusiformis* (*Spirulina platensis*) against the filamentous fungi *Aspergillus niger*, *Aspergillus flavus* and *Candida albicans*. This suggests that the antifungal activity of *Spirulina* may be due to the presence of other bioactive compounds in addition to phenolic compounds.

Toxic activity

Aqueous phycocyanin extracts showed cytotoxic activity only against *A. franciscana*. Those from the exponential phase presented an LC_{50} of 1.69 µg mL^{-1} and those from the stationary phase an LC_{50} of 2.59 µg mL^{-1}. These results suggest that said cytotoxicity is significant, since the LC_{50} values are less than 30 µg mL^{-1}, as proposed by Meyer *et al.* (1982).

Aqueous phycocyanin extracts showed cytotoxic activity only against the brine shrimp *Artemia franciscana*. The extracts from the exponential phase had an LC_{50} of 1.69 µg ml^{-1}, while those from the stationary phase had an LC_{50} of 2.59 µg mL^{-1}. These LC_{50} values are significantly lower than the 30 µg mL^{-1} LC_{50} report-

ed by Meyer *et al*. (1982), suggesting that the aqueous phycocyanin extracts have significant cytotoxic activity against *A. franciscana*.

Cytotoxic activity

Aqueous extracts of exopolysaccharides, as well as ethanolic, chloroformic, and hexane extracts of *S. subsalsa*, were found to be non-toxic against *A. franciscana* and *D. magna*. However, aqueous extracts of phycocyanin demonstrated significant cytotoxic activity against *A. franciscana*. These findings align with those of other researchers who have evaluated the cytotoxic activity of *Spirulina* using human cells. Szubert *et al*. (2018) obtained extracts from *S. subsalsa* and reported that these extracts did not exhibit strong inhibitory activity against T47D breast cancer cells. Until this study, no cytotoxic activity had been reported for the metabolites isolated from this species. Nonetheless, they recommended continuing phytochemical studies, as *S. subsalsa* could potentially serve as a valuable source of cytotoxic agents.
The lack of antimicrobial and cytotoxic activity in the aqueous extracts of exopolysaccharides, as well as ethanolic, chloroform and hexane of *S. subsalsa* might initially suggest discouraging results. This is due to inability to inhibit the growth of Gram-negative strains: *Escherichia coli*, *Pseudomonas aeruginosa*, and Gram positive: *Bacillus cereus*, *Micrococcus luteus* and *Staphylococcus aureus*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, and fungi: *Rhizopus orizae* and *Aspergillus niger*. However, these findings indicate that further research with this strain of *Spirulina subsalsa* is warranted. Different culture conditions and extraction systems could reveal the presence of bioactive compounds.

Another significant finding is that the evaluated extracts promoted the growth and development of *Artemia* sp. and *Daphnia magna*. This could be attributed to cyanobacteria of the genus *Spirulina* being a rich source of antioxidant compounds, which are beneficial for growth and reproduction of zooplankton cultures as suggested by Johnston *et al*. (2018), Kandathil *et al*. (2019) y Mezgebu *et al*. (2024).

CONCLUSIONS

The ethanol extract of *S. subsalsa* biomass, cultivated in saline conditions and harvested during the stationary phase, demonstrated mild to moderate antibacterial activity against *B. cereus* and *S. aureus*. However, no inhibitory effects were observed against the fungi *R. orizae* and *A. niger* using aqueous and variable polarity extracts of *S. subsalsa*. These extracts were non-toxic to *A. franciscana* nauplii and *D. magna* neonates. Notably, only the phycocyanin extracts exhibited cytotoxic activity against *A. franciscana*. The results suggest that the native strain *S. subsalsa*, cultivated in a seawater enriched medium and in a low-cost saline medium has potential to produce bioactive compounds with antimicrobial properties. This finding represents an opportunity for further study related to its possible application in aquaculture, phycology, biotechnology among other fields of study.

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