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Lipopeptide produced by the bacteria *Bacillus mojavensis* with activity against the phytopathogenic fungus *Colletotrichum gloeosporioides* Penz & Sacc var. Minor Simmonds

Lipopeptido producido por la Bacteria *Bacillus mojavensis* con actividad contra el hongo fitopatógeno *Colletotrichum gloeosporioides* Penz & Sacc var. Minor Simmonds

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Abstract

The objective of this work was to determine the antifungal activity against *Colletotrichum gloeosporioides* Penz & Sacc var. minor Simmonds of the lipopeptide(s) isolated and purified from the culture of the marine bacterium *Bacillus mojavensis* (MC3B-22). The results will show that the extraction method with ammonium sulfate was the one that extracted the largest amount of biosurfactants with a yield of 3.1243 g/L compared to the acid precipitation method (0.3173 g/L). The crude extract in the presence of 103 conidia/mL of the fungus *Colletotrichum gloeosporioides* did not present inhibition, but the semi-purified extract obtained with ammonium sulfate reached a minimum inhibitory concentration of 12.5 µg/mL. In conclusion, the optimal method for extracting the biosurfactant was 40% ammonium sulfate, with methanol being a suitable solvent to semi-purify and obtain a minimum inhibitory concentration of 25 µg/mL against C. *gloeosporioides*.

Biosurfactant, Lipopeptide, *Bacillus mojavensis*

Resumen

El objetivo de este trabajo fue determinar la actividad antifúngica contra *Colletotrichum gloeosporioides* Penz & Sacc var. minor Simmonds del o los lipopéptido(s) aislado(s) y purificado(s) del cultivo de la bacteria marina *Bacillus mojavensis* (MC3B-22). En los resultados se determinó que el método de extracción con sulfato de amonio fue el que extrajo la mayor cantidad de biosurfactantes con un rendimiento de 3.1243 g/L en comparación con el método de precipitación ácida (0.3173 g/L). El extracto crudo en presencia de 103 conidios/mL del hongo *Colletotrichum gloeosporioides* no presentó inhibición, pero el extracto semipurificado obtenido con sulfato de amonio alcanzó una concentración mínima inhibitoria de 12.5 µg/mL. En conclusión, el método óptimo para la extracción del biosurfactante fue el sulfato de amonio al 40% siendo el metanol un solvente adecuado para semipurificar y obtener una concentración mínima inhibitoria de 25 µg/mL contra *C. gloeosporioides*.

Biosurfactante, Lipopetido, *Bacillus mojavensis*

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Introduction

Marine microorganisms have evolved particular metabolic and physiological capabilities to adapt to extreme habitats and in response produce metabolites that are generally not expressed in land-based microorganisms. For this reason, the marine habitat is a potential source for the search for new compounds such as antibiotics, antioxidants, bioemulsifiers, biosurfactants, enzymes, drugs, anionic surfactants, vitamins and other commercially important compounds (Sarubbo *et al*., 2022 and Satpute *et al*., 2010).

Synthetic surfactants have generated, in 2008, a market growth of 13 million tonnes worldwide equivalent to $23.9 billion, while in 2009 it increased by 2 % and is expected to increase by a further 3-5 % by 2025 (Mordor 2022 and Reznick *et al*., 2010). However, these surfactants are derived from petroleum, so there is great interest in the search for and application of bioactive marine compounds, such as biosurfactants, which are of great importance, due to their structural and functional diversity as well as their multiple properties, such as their antimicrobial and antiviral activity, plant disease biocontrol agents, high biodegradability and selectivity, low toxicity, environmental compatibility, detergency, dispersion, emulsification, specific under high temperature, pH and salinity conditions, foaming, wetting and solubilisation of hydrophobic compounds. (Nawazish 2022, Perfumo *et al*., 2010, Tapati and Dabashis, 2022,). Another advantage of biosurfactants is that they are obtained by microbial fermentation processes, where inexpensive substrates can be used and culture conditions can be controlled (Desai and Banat, 1997).

Biosurfactants, produced by a wide variety of microorganisms, are classified by their chemical composition into: a) high molecular weight compounds consisting of polysaccharides, lipopolysaccharides, lipoproteins, among others and b) low molecular weight compounds such as glycolipids, polypeptides, lipopeptides, among others (Ekambaram et al 2022; Smyth et al., 2010). Lipopeptides produced by the genus Bacillus have been studied for their broad spectrum of antimicrobial activity, and especially for their antifungal activity (Banat *et al*., 2010).

This is the case of lipopeptides belonging to the iturin family, which show remarkable haemolytic and antifungal activity in vitro. On the other hand, phengicins, although they do not have high haemolytic activity like surfactins and iturins, do have good antifungal activity (Ongena and Jacques, 2007).

Therefore, the aim of this work was to determine the antifungal activity against Colletotrichum gloeosporioides Penz & Sacc var. minor Simmonds of the lipopeptide(s) isolated and purified from the culture of the marine bacterium Bacillus mojavensis (MC3B-22).

Description of the method

Micro-organism

The antagonistic strain B. mojavensis (MC3B-22) used in this work is preserved in deep freeze at -80°C in the Collection of Microbial Environmental Cultures (CCMA) of the Department of Environmental Microbiology and Biotechnology (DEMAB).

Culture conditions

A 24-hour pre-culture of strain B. mojavensis (MC3B-22) in Luria Bertani Miller broth (Fluka) supplemented with sea salts (Sigma) (LBMSM) was performed at 25°C, 140 rpm and constant light. After the time had elapsed, the pre-culture was adjusted to an optical density (OD) of 3 at 520 nm. Ten millilitres of the pre-culture, previously adjusted, was inoculated into blafeated Erlenmeyer flasks containing Luria Bertani Miller broth. The conditions established for the culture were the same as those used for the pre-inoculation. The fermentation time for obtaining the maximum crude extract was 84 hours. Subsequently, the cell-free supernatant (SLC) was obtained by centrifugation (Eppendorf Ultracentrifuge 5810-R) of the culture medium at 4000 rpm at 4°C and filtered with 0.45 μm Millipore membranes (Durán, 2010).

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Biosurfactant extraction

Acid extraction: SLC was adjusted to an acidic pH with 6 M HCl and allowed to stand overnight at 4°C for complete precipitation of the biosurfactant. Subsequently, it was centrifuged at 4000 rpm for 45 minutes at 4°C and the precipitate was collected. The precipitate was resuspended in basified water and then frozen and lyophilised (Labconco). The yield was obtained with respect to the volume of the medium and expressed in gr/L.

Extraction with ammonium sulphate: SLC cooled to (4-5°C) and under slow stirring was added to ammonium sulphate, until a final concentration of 40% was obtained. The extraction was kept under refrigeration and stirring overnight. Hours later it was centrifuged at 4000 rpm for 3 minutes and the packet was suspended in 5 mL of water, frozen and freeze-dried. The yield was estimated with respect to the volume of the medium and expressed in g/L.

Semi-purification of crude extracts

Pure methanol was added to part of the crude extracts obtained from the different extraction methods and the methanolic fraction obtained was transferred to another vial, previously weighed, to determine the yield of the semi-purified extracts.

Biosurfactant activity: collapsed droplet (CG) technique

Three polystyrene microplate coverslips (12.7 x 8.5 cm) of 96 microwells were washed three times with hot water, 96% ethanol and distilled water, and allowed to dry. Subsequently, 2 µL of mineral oil was added to each plate and allowed to stand for 24 hours to allow the oil to spread homogeneously over the surface of the wells. After the stabilisation time, 5 µL of the crude and semi-purified extracts were added at a concentration of 1mg/100 µL, positive control sodium dodecyl sulphate (SDS) at 1.84 mg/mL and negative control DMSO-SS (50/50 v/v). The assay was performed three times in quintuplicate. The shape and size of the droplet was observed on the microplates after one minute of applying the extracts and measured with a vernier under a stereo microscope. An increase of the droplet larger than 1 mm with respect to the negative control was considered to have biosurfactant activity (Youssef *et al*., 2004).

Purification of the semi-purified extract by preparative thin-layer chromatography

The semi-purified extract was dissolved in methanol to a concentration of 5% and 250 µL was applied to the whole plate. The plate was eluted with the nBuOH: MeOH: H_2O system (3:2:1), allowed to dry and then scraped off the silica gel in the area of the Rf where haemolytic activity was observed. The entire scraping was placed in a beaker containing 50 mL of chloroform: methanol ($CHCl_3$: MeOH), 2:1, and left to stir in this solvent system for extraction of the metabolites from the silica. The mixture was filtered to separate the silica and the solvent was concentrated under reduced pressure with a rotary evaporator. The excess solvent was allowed to dry and its yield was determined and expressed as mg/mL (*Satpute et al*., 2010).

Determination of the minimum inhibitory concentration by the microdilution method

For this assay, a suspension of conidia was obtained at 3.5 X 105 conidia/mL of C. gloeosporioides (ATCC 42374) in RPMI 1640 medium with L-glutamine, without bicarbonate and supplemented with 165 mM 3-(n-morpholino)-propanesulfonic acid (MOPS) and adjusted to pH 7.0. The assay was performed in a sterile 96-well microplate.

The semi-purified fractions were dissolved in DMSO-SS to a final concentration of 4 mg/mL. 10 µL (40 µg) were transferred to the first well previously containing 190 µL of RPMI 1640 medium, carefully mixed until a homogeneous suspension was obtained and 100 µL were transferred to the next well containing 100 µL of RPMI 1640 medium, and so on up to well 12. All wells were then inoculated with 100 µL of the conidial suspension. The assay was performed in triplicate and the microplate was incubated at 25°C for 48 hours.

The 24 hours was determined as the cut-off time of the assay, since at that time the inverted microscope observation showed total germination of the conidia (germination is considered when the size of the hyphae coming out of the spore is one and a half times larger than the conidium) and when developing with TTC the reaction is strongly observed. The addition of TTC to each well of the microplate allowed visualisation of the MIC of the extracts, which coincided with the observation with the inverted microscope.

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Results

In this research work, three biosurfactant extraction methodologies were used: acid extraction, ethyl acetate extraction and ammonium sulphate extraction. These techniques allowed to establish that the ammonium sulphate extraction was the one that presented the highest amount of biosurfactants with a yield of 3.1243 g/L compared to the acid precipitation (0.3173 g/L) and ethyl acetate (0.110 g/L) methods.

Confirmation of the surfactant nature of each extract was performed by the collapsed droplet assay with an increase of the droplet in the semi-purified extracts as visualised in Figure 1 (Dehghan-Noudeh et al., 2005; Youssef et al., 2004).

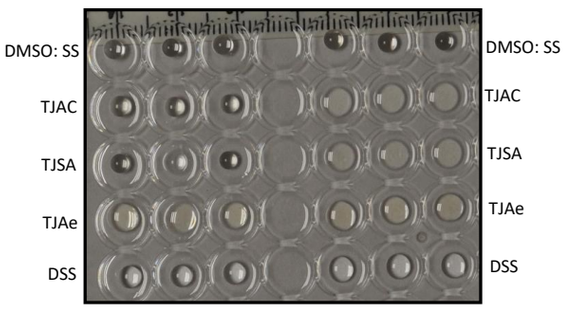

Figure 1 Biosurfactant activity of crude and semi-purified extracts. Negative control: DMSO: SS (Sodium Dimethyl Sulphate with Salt Solution (1:1)), Positive control: DDS (Sodium Dodecyl Sulphate), TJAC: extract obtained by acid extraction, TJSA: extract obtained by extraction with ammonium sulphate, TJAe: extract obtained with ethyl acetate

The determination of the active metabolites was determined by thin layer chromatography and haemolytic bioautography. This methodology allowed the Rf corresponding to the areas with haemolytic activity of the extracts to be located after 12 hours of incubation. Simultaneously to this assay, another plate was run to reveal the presence of peptides with ninhydrin, as well as cupric sulphate in phosphoric acid to reveal the presence of lipids (Figure 2).

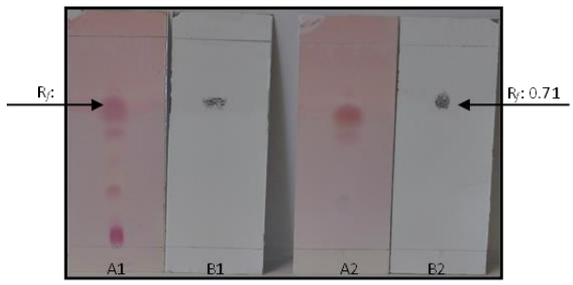

Figure 2. Thin layer chromatography of the semi-purified (A1 and B1) and purified (A2 and B2) extract developed with ninhydrin (A1 and A2) and with 10% cupric sulphate in 8% phosphoric acid (B1 and B2)

The determination of the minimum inhibitory concentration (MIC) and observation under inverted microscope and interpretation using the NCCLS numerical scale showed the following results: None of the tested concentrations of the TJSA extract inhibited the growth of the fungus, therefore, the MIC corresponds to a value higher than 100 µg/mL. These high values may be associated with the presence of impurities present in the crude extract. This was evident when the crude extract was evaluated, as no germination of the conidia of C. gloesoporioides ATCC 42327 was observed at a concentration of 12.5 µg/mL. These results demonstrate the sensitivity of this pathogen against this antifungal agent.

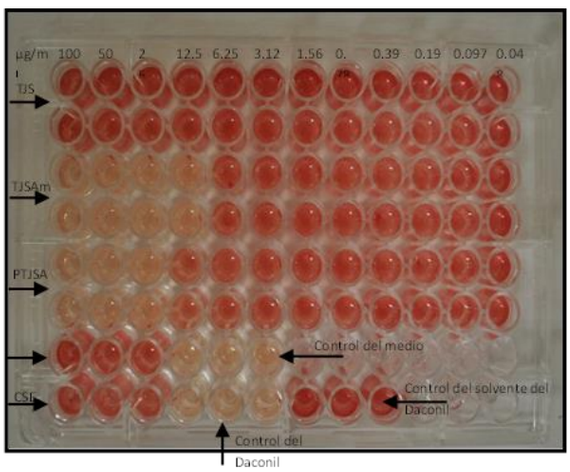

Figure 3 MIC (Minimum inhibitory concentration) of TJSA, TJSAm and PTJSAm extract. CCH: fungal growth control, CSE: extract solvent control. Daconil: positive control

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Conclusions

As conclusions we can mention that the optimal method for the extraction of the biosurfactant produced by *B. mojavensis* was determined as precipitation with 40% ammonium sulphate, the biosurfactant is of lipopeptidic nature and methanol was a suitable solvent for the semi-purification since the lipopeptide remained in this fraction increasing the haemolytic and antifungal activity. After successive purifications the total yield obtained by preparative thin layer chromatography was 92.5 mg/L and the minimum inhibitory concentration of the purified extract was 25 µg/mL against C. gloeosporioides.

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