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# Extracellular Polymeric Substances (EPS) produced by *Streptomyces* sp. biofilms: Chemical composition and anticancer properties

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ABSTRACT

The extracellular polymeric substances (EPS) have shown free radical scavenging and antitumor activity against both breast and colon cell lines. In this regard, actinobacteria have become an increasingly popular sources of EPS. Therefore, in this study four *Streptomyces* strains isolated from contaminated soil (M7, A5, A14 and MC1) were evaluated for determining its biofilm-forming capacity including under pesticide stress. In addition, chemical composition of EPS and its cytotoxic effects over 4T1 breast cancer cell and Caco-2 human tumor colon cells were evaluated. The results demonstrated that *Streptomyces* sp. A5 had the highest capability to develop biofilm more than other strains tested, even under pesticide stress. Moreover, this strain produced EPS with a total protein/total polysaccharide rate of 1.59 ± 0.05. On the other hand, cytotoxicity assays of EPS showed that *Streptomyces* sp. A5 display a higher toxic effect against 4T1 Breast cancer cells (96.2 ± 13.5 %), Caco-2 (73.9 ± 6.4 %) and low toxicity (29.9 % ± 9.1 %) against non-transformed intestinal cells (IEC-18). Data do not show cytotoxic effect relationship with biofilm-forming capabilities of strains, nor the chemical composition of EPS matrix. The gene that codes for polysaccharide deacetylase, parB-like and transRDD proteins, were identified. These results contribute to the knowledge about the variability of chemical composition and potential cytotoxic properties of EPS produced by *Streptomyces* biofilms. It proposes interesting future challenges for linking *Streptomyces*-based pesticide remediation technology with the development of new antitumor drugs.

## 1. Introduction

Bioremediation belongs to the environmental-friendly processes for the detoxification of harmful xenobiotics from soil, water, and air using microorganisms (bacteria, fungi, actinobacteria among others) or their enzymes (Alvarez et al., 2015; Tortella et al., 2015). During bioremediation process, hazardous chemicals are not applied and therefore, do not represent any direct environmental damage (Shah and Shah, 2020). In the bioremediation process are employed a diverse group of microorganisms for the degradation or treatment of pollutants such as, polycyclic aromatic hydrocarbons, volatile organic compounds, pesticides, heavy metals, radionuclides, crude oil, jet fuels, petroleum products and explosives (Cuozzo et al., 2009; Oliveira et al., 2015; Patel et al., 2017; Kapahi and Sachdeva, 2019).

In some instances, in natural soil or water environment, the bioremediation process is developed by complex microbial communities who live attached mainly over soil particles and plant surfaces, submerged in a self-produced extracellular polymeric substance (EPS), where an increased tolerance to drugs and other xenobiotics, including nutritive functions (*i.e.*, substrate, electron acceptors, trace nutrients improved intercellular communication (Betancur et al., 2017). The EPS are composed by compounds such as sugars, proteins, lipids, DNA, vesicles, or cellular fragments, and the changes of their composition will be function of different organisms and / or environmental conditions (Hobley et al., 2015; Crabbé et al., 2019). On the other hand, the highly hydrated EPS matrix supply protection to avoid desiccation, and at the same time offers better opportunities for different interactions, as well as facilitating horizontal gene transfer, and improving the metabolism of

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microorganisms (Desmond et al., 2018). The EPS are found to show a wide range of biological properties, including immunostimulant, anti-inflammatory, antioxidant and antitumor activity (Du et al., 2017; Wu et al., 2021; Wei et al., 2019). Microbial EPS present a wide range of uses in different fields including industrialized food production, agriculture and pharmaceuticals (Moscovici, 2015; Decho and Gutierrez, 2017).

The *Streptomyces* genus is characterized by biofilms formation in natural environment as well as, in medical or industrial environments. Although biofilms derived from some *Streptomyces* species have been associated with the deterioration of water quality, artworks and historical monuments as well as the human health (Vinogradova et al., 2015a), *Streptomyces* biofilms have also been used in biotechnological applications such as water purification systems, soil and water bioremediation (Vinogradoya et al., 2015b). All these properties are associated to several secondary metabolites produced by these microorganisms. It should be considered that *Streptomyces* regularly releases secondary metabolites with different structures and biological activities. (Selim et al., 2018). In this regard, inhibition of *Listeria monocytogenes* by biofilms from *Streptomyces* spp. (Kim et al., 2019) or inhibition of biofilm formation from *Xanthomonas oryzae* by anthranilamide produced by *Streptomyces* spp. (Ham and Kim, 2018) has been reported. Currently there are not known studies related to the production of EPS from *Streptomyces* isolated from soil with the capacity to inhibit the development of carcinogenic cell lines, hence the importance of our results (Davies-Bolorunduro et al., 2019; Abd-Elnaby et al., 2016).

Therefore, in this study, we evaluate free-living actinobacteria strains isolated from a contaminated soil, particularly *Streptomyces* sp., with a wide ability to degrade lindane in their capacity to form biofilm and including the antitumoral activity of its EPS biofilm-forming agents.

## 2. Materials and methods

### 2.1. General design

*Streptomyces* sp. A5, MC1, A14, and M7 isolated from Organochloride Pesticides (Ops)-contaminated soil (Fuentes et al., 2011) were used in this study. All strains were assayed for a) Biofilm growth ability, then EPS was extracted and tested for b) Chemical composition in terms of total polysaccharides, total protein, and total extracellular DNA and c) toxic effect over the following tumor model cells; Caco2 human colon cancer cells, 4TI breast cancer cells, and IEC-I8 non transformed intestinal cells.

### 2.2. Microorganisms and growth conditions

Minimal medium (MM) composed by (g/L): L-asparagine, 0.5; $K_2HPO_4$, 0.5; $MgSO_4{\cdot}7H_2O$, 0.2; $FeSO_4\ {\cdot}7H_2O$, 0.01; glucose, 1.0 was used for EPS production, and pH was adjusted to 7.0 prior to sterilization and supplemented with commercial standard of Lindane (98 % purity) at 100 μg $mL^{-1}$ (Cuozzo et al., 2009).

Tryptone Soy Broth (TSB) composed by (g/L): tryptone, 17.0; soy peptone, 3.0; NaCl, 5.0; $K_2HPO_4$, 2.5; glucose, 2.5, was used for the inoculum growth. The pH was adjusted to 7.3 before sterilization. Sterilization of culture media was carried out by autoclaving at 121 °C during 15 min. Cultures of actinobacteria were maintained at 30 °C on a rotary shaker at 240 rpm for 72 h, before starting biofilms assay.

### 2.3. Biofilm assay

The potential of each strain to form biofilm was evaluated by applying the microplate assay (Corte et al., 2019). Then, 200 μL of each inoculum (subculture in the experimental design, or resuspended cell pellet at different $OD_{540nm}$ values) was added to 5 mL of each medium (MM and TSB), and aliquots of 200 μL were placed in 96-well polystyrene microplates and incubated in dark for 72 h at 37 °C in incubator chamber. To quantify biofilm formation, the wells were washed with phosphate-buffered saline (g/L: 8 NaCl, 0.0002 KCl, 1.15 $Na_2HPO_4$, 0.2 $KH_2PO_4$; pH 7.4). The remaining attached bacteria were stained for 30 min with 200 μL 0.1 % (w/v) crystal violet. Excess stain was rinsed with 200 μL distilled water per well. The dye bound to the adherent biofilm was extracted with 200 μL ethanol 96 % (v/v). Aliquots (135 μL) from each well, which contains ethanol-solubilized, crystal violet-stained culture, were taken and placed in a different microplate for determination of $OD_{570}$ in a microplate spectrophotometer (VERSAmax, Molecular Devices, Sunnyvale, CA, USA; path length~0.4 cm for a volume of 135 μL). Each strain was assayed in at least three independent experiments, each with four replicates. Additionally, a sterile culture medium was always included as negative control.

### 2.4. Biofilm morphologic analysis

In separate experiments, actinobacterial strains were grown on TSB media at 30 °C on a rotary shaker at 240 rpm for 48 h in presence of circular glass slide, which were examined by scanning electron microscopy (SEM). Structures were fixed with 2 % glutaraldehyde in 0.1 mol $L^{-1}$ phosphate-buffered saline for 2 h at 4 °C and 1 % osmium tetroxide ($OsO_4$) in 0.1 mol $L^{-1}$ phosphate-buffered saline for 1 h at 4 °C and dehydrated through an ethanol series and 2-methyl-2-propanol, followed by platinum ion coating (E-1030; Hitachi, Tokyo, Japan). Specimens were examined with an SEM (S-4800; Hitachi) at an accelerating voltage of 1 kV.

### 2.5. EPS extraction

Biofilms were extracted from the membrane surface using a cell scraper and transferred into sterile glass beaker. According to D'Abzac et al. (2009) and Desmond et al. (2018), different EPS extraction tests were carried out to maximize its extraction and to avoid cell lysis (determined through of soluble ATP measure). Biofilm suspensions were sonicated for 120 s with 10 s rest intervals in an ice bath and a frequency of 46 KHz (three times repeated, 500 W Q55 Sonicator, Thomas Scientific). Thereafter, homogenized samples were centrifuged at 5000 rpm at 20 °C for 15 min (Laboratory Centrifuge, Digital 4400 R.P.M. Model RST-4M) to separate the EPS from cells. The soluble EPS supernatant was separated from the resuspended cell pellet and aliquots were taken from the extracellular fraction to determine total organic carbon content (Hach BioTector B3500c TOC Analyzer, USA). Samples were lyophilized and stored at 4 °C, prior to EPS analysis (Total polysaccharide, total Protein, and total extracellular DNA).

### 2.6. Total protein quantification

At 0.1 g of each EPS sample was taken and adjusted to 10 mL with sterile distilled water and stirred until complete dissolution. Then, 400 μL of sample was taken and 400 μL of Supry Ryby Stain Biofilm reagent (maintain 1:1 ratio) was added and maintained for 30 min, in dark. To validate the measurement, a white solution (sterile water plus reagent) and a solution of known concentration (1 mg $mL^{-1}$ protein) was prepared. In 96-well black microplates, 200 μL were added to each well (triplicate) of each sample incubated at the previous point. The absorbance was determined in a UV–vis 287 spectrophotometer (Infinite M200, TECAN, Männedorf, Switzerland) at a wavelength of 288 nm. Bovine serum antigen (BSA) was used as a control and the values were expressed in mg $C/m^2$ (conversion factor of 0.83 mg C/mg in BSA). The results were expressed as mg of total protein per g of EPS.

### 2.7. Extracellular DNA quantification

PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, USA) was used to extract the DNA, according to the protocol provided by the manufacturer (200 mg of EPS). Extracted DNA samples (about 60 μL) were

preserved under −20 °C. The fluorescence was measured at an excitation wavelength and an emission wavelength of 480 nm and 520 nm, respectively. The result was expressed as mg of total extracellular DNA (eDNA) per g of EPS.

### 2.8. Extraction of total polysaccharides

EPS samples (3 g) were diluted with ethanol (−80 °C, 96 % v/v) and centrifuged at 1381*g* for 3 min, and the precipitate was collected. The procedure was repeated until the precipitate was colorless, then suspended (500 mL of distilled water), heated to a boiling point for 40−45 min with constant stirring, allowed to stand for 1 h to allow the complete release of the polysaccharide in the water. After that, centrifuged (3107 *g*, 10 min, 4 °C) (this extraction process is repeated twice). Subsequently, the supernatant was removed, and to reduce volume it was concentrated in a rotary evaporator (40−45 °C). Precipitation of polysaccharides was performed according to Zhang et al. (1999) using cold absolute ethanol (3:1 ratio to the solution), followed by 15 min at -20 °C. After that, it was centrifuged at 1381 *g* for 5 min, and the polysaccharide (pellet) was stored at -20 °C until purification. The pellet was purified by mean of successive precipitation steps (ethanol 96 % v/v / 4 M NaCl centrifuged for 7 min at 2455 *g*). The purified polysaccharide was dialyzed using dialysis membranes (8000– 14,000 Da, Sigma), a 4 M NaCl solution for 4 h and under stirring, favoring ion exchange. After that, the membrane was emptied, the content was precipitated with 96 % ethanol and centrifuged for 7 min at 2455 *g*, the polysaccharide obtained was frozen and lyophilized for 24−48 h and introduced into vials for later use. A stock solution of 100 mg of glucose was used as a positive control for analysis determination (Smibert and Krieg, 1994). Absorbance was determined with a UV–vis spectrophotometer (Infinite M200, TECAN, Männedorf, Switzerland) with a wavelength of 625 nm. The result was expressed as mg of total protein per g of EPS.

### 2.9. Anticancer properties test

The colon cancer cell line Caco-2 from human, 4T1 Breast cancer cells, and IEC-18 non-transformed intestinal cells were proliferated in Medium with high glucose of Dulbecco's Modified Eagle's (DMEM, Gibco, Life Technologies, Grand Island, NY, USA), containing 10 % (v/v) fetal bovine serum (Natocor, Córdoba, Argentina), 100 μg $mL^{-1}$ of penicillin and 100 μg $mL^{-1}$ of streptomycin (Gibco), at 37 °C in an atmosphere with humidity and 5 % $CO_2$. The cells were grown until reaching 80–90 % of confluence within in vented tissue culture flasks of 25-$cm^2$, and then transferred to 96-well plates. The inhibition of Caco-2 colon cancer cells, 4T1 Breast cancer cells, and IEC-18 non-transformed intestinal cells viability by the samples (in the assays were added with individual biofilms or culture media), determined by two different methodologies: trypan blue exclusion and (4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay.

The trypan blue exclusion method is supported on that the dead cells do not possess intact cell membranes and therefore cannot exclude certain dyes, such as trypan blue. Cells ($1 \times 10^5$ cells/well) were seeded on a 96-well tissue culture plate with final volume of 1000 μL/well (*i.e.* 900 μL of DMEM together to 100 μL of the sample), for 24 h. Following treatment, non-attached and attached (trypsinized) cells were collected and 0.4 % trypan blue dye and cell suspension were combined in equal parts. In all cases the cells were counted in a Neubauer chamber. Always the assay was performed in triplicate, and the results expressed as the average percentage of dead cells ± standard deviation.

The MTT assay consists of the change of water-soluble MTT to an insoluble purple formazan, by mitochondrial dehydrogenases present in viable cells. Cells ($1 \times 10^4$ cells/well) were seeded on 96-well tissue culture plates and incubated during 24 h to allow attachment. The different samples analyzed were used at a dilution of 1/10 in the culture medium (10 μL of the sample to complete a final volume of 100 μL of medium) and incubated for 24 h. By well was added 10 μL of MTT reagent (2.5 mg/mL) and then incubated at 37 °C for 3 h. The medium was removed and replaced with 100 μL of dimethyl sulfoxide (DMSO) to dissolve the formazan crystals of purple color. Plates were incubated by shaken during 15 min and evaluated in a microplate reader at 570 nm. Data were expressed as a percentage absorbance of that of control cells incubated with DMEM.

### 2.10. Detection of Biofilm formation associated genes

DNA Extraction and quantification: It was performed according to previously described procedures (Walden et al., 2017). Samples of lyophilized were collected and then harvested by centrifugation (3000 rpm, by 5 min) at 4 °C for DNA extraction. PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, USA) was used to extract the DNA, according to the manufacturer's protocol. Extracted DNA samples (about 60 μL) were preserved under −20 °C for further analysis. The RAST database (www.rast.theseed.org/FIG/rast.cgi), was used to evaluate the percentages of identity and similarity of the genes associated with the production of biofilms.

### 2.11. Statistical analysis

EPS analysis: Extraction of total protein, total eDNA, and total polysaccharides was conducted in triplicate for each experimental treatment, including positive and negative control samples. The results were expressed in the average of mg per g of sample. For complete total mass balance, the fraction of the undetermined chemical composition of each sample was determined by subtracting the accumulated weight of total DNA, protein, and exopolysaccharides, from 1000 mg. Results of *In-vitro* cytotoxic assays were expressed as mean ± standard deviation of cells' viability percentage for each cell line.

Two paired samples *T*-test (Sokal and Rohlf, 1981) was used to test for significant differences ($P \leq 0.05$) between averages of strains biofilm growth (expressed as O.D. measured at 570 nm), total eADN, total protein, and total polysaccharides of produced EPS matrix. The same analysis was performed to search the effect of biofilm over the mean- ± standard deviation of cells' viability percentage for each cell line. Professional versions of R software (R Core Team, 2013) were used as an analysis platform.

## 3. Results and discussion

### 3.1. Screening of actinobacteria able to produce biofilms

The biofilm formation by MC1, A5, A14, and M7 strains in TBS culture media is shown in Fig. 1. As it can be seen, at 72 h of incubation, the production of biofilm by A5, MC1, M7, and A14 strains are

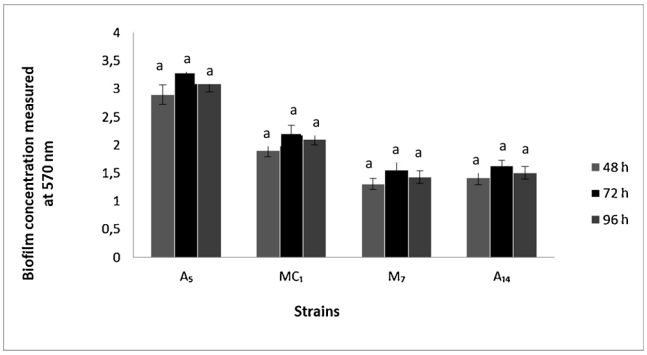


**Fig. 1.** Production of biofilm in TSB medium at 48, 72 and 96 h of the different *Streptomyces* sp. A5, MC1, A14, M7, Values without a common letter differ significantly ($P < 0.05$) for each cell line.

maximum with a value of the O.D. $_{570\ nm}$ of 3.28 ± 0.21, 2.2 ± 0.09, 1.55 ± 0.17, and 1.52 ± 0.09 nm respectively. Biofilm developed on TSB medium reached by A5 strain (Fig. 3C), was significantly high (*T*-test, p = 0.039).

At 96 h of incubation the O.D. $_{570\ nm}$, reached by strains A5, MC1, M7 was 3.18 ± 0.22, 2.1 ± 0.11, 1.45 ± 0.10, and 1.5 ± 0.08 nm respectively (Fig. 1). This drop-in reading indicated that the bacteria have begun the shedding stage inert surface where dynamic equilibrium is reached when the outermost layers of the biofilm begin to generate metabolically active and capable of dividing planktonic cells, which can colonize new surfaces as described by Busscher and Van der Mei (1997) and Flemming and Wingender (2010).

### 3.2. Relationship between capability for biofilm formation and growth conditions of selected Streptomyces strains in lindane

From the four strains evaluated, *Streptomyces* sp. A5 and MC1 reached the highest biofilm production (Fig. 1). Therefore, these two strains were evaluated in their capacity to produce biofilm in presence of lindane (100 μg/mL). As shown in Fig. 2A, *Streptomyces* sp. A5 maintains higher levels of biofilm production in all media, including under pesticide stress. The O.D. $_{570nm}$ for *Streptomyces* sp. A5 in TSB was 3.28 ± 0.15; in MM with glucose, it was 2.26 ± 0.18; in MM plus Lindane it was 2.1 ± 0.33 and MM alone it was 0.13 ± 0.03, reaching in all the cases (except controls) significant higher O.D. values (Test, p = 0.042) than MC1 strain, in equivalent experimental conditions (Fig. 2B). In both strain (A5 and MC1) biofilms grew in MM using lindane (100 μg/mL) as the only carbon source, although A5 strain shows better growth. Data agree as expected, both strains are already known for their ability to use lindane as the only carbon source. Accordingly, the pathway for lindane degradation has been already described on the same strain (Cuozzo et al., 2009). However, the A5, as well as MC1 strains, decrease biofilm development (measured as D.O $_{570nm}$ respect theirs corresponding control on MM plus glucose) in a 22.2 and 15.3 % respectively (significant in both cases *T*-test, p = 0.043).

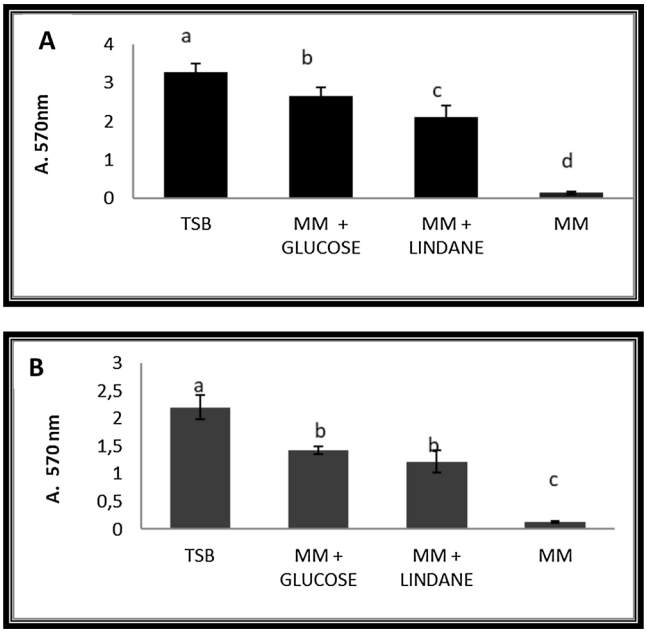


**Fig. 2.** Growth and production of biofilms of *Streptomyces* sp. A5 (A) and MC1 (B) in TSB medium, minimum medium with glucose, minimum medium with lindane and minimum medium without carbon source. Values without a common letter differ significantly (P < 0.05) for each cell line.

### 3.3. Ultrastructure of Streptomyces biofilm analyzed by SEM

According to literature in most biofilms, primary and secondary steps occur, and the production of EPS starts from 48 to 72 h period (Memariani et al., 2019). In Fig. 3, it is shown SEM images taken after 72-h incubation biofilm (attached on a circular glass slide) of *Streptomyces* sp. M7 growing in TSB medium (Fig. 3A), *Streptomyces* sp. A14 growing on MM plus lindane (Fig. 3B), *Streptomyces* sp. A5 developed in TSB (Fig. 3C) and *Streptomyces* sp. MC1 developed in TSB media. As can be seen, the EPS matrix has almost entirely disappeared in all pictures. Only 0.1–0.3 μm in diameter filaments can be seen. These structures are produced because of the dehydration steps required by SEM standard procedure, a product of which EPS matrix, becomes in a thin filament network surrounding attached bacterial cells (Gomes and Mergulhão, 2017). Despite this inconvenience, the filaments network may be used as an indirect qualitative parameter analysis of matrix robustness, to get an initial overview of biofilms. On the image showed in Fig. 3B, the EPS matrix in *Streptomyces* sp. A14 may be recognized in its dehydrated version, as a compact mass of greater brightness filaments. The Confocal microscopy procedure (not performed in this work) is a better way to examine EPS morphology, even the cells located deep inside the matrix (Hobley et al., 2015; Gomes and Mergulhão, 2017).

In relation to the results shown on the biofilm growth assay (Fig. 1), after 72 h the biofilm produced in TSB media by *Streptomyces* sp. A5 strain completely covers the surface of the discs with homogeneous layers of cells combined with stacked bacteria groups, showing channels inside of the formed structure (Fig. 3C). This type of structures provides porosity, providing flow of water, nutrients and ions, and speeding up the distribution, which it has been already described on soil biofilms (Tseng et al., 2013; Flemming et al., 2016; Kim et al., 2016; Rossi, 2020).

Cell morphology can be seen in Fig. 3D, where a microcolony (1–1.5 un length cells, forming short septate filaments) of *Streptomyces* sp. MC1 attached on coverslips, can be observed. In the same way, the morphological cell diversity showed in Fig. 3B and C, suggest some differential level of cellular activity, located on micro-anaerobic domains, which has been described in most of the biofilm (Flemming and Wingender, 2010; Banerjee et al., 2015; Desmond et al., 2018). It has been suggested that the growth rate of adherent cells is enhanced when a certain cell density is reached, whereas the growth rate drops at higher densities. This density-dependent growth may be explained by cell-cell signaling, resulting in physical or morphological changes of the biofilm bacteria (Rajput et al., 2015; Betancur et al., 2017; van Dissel and van Wezel, 2018).

### 3.4. Analysis of chemical composition of the biofilm

Table 1 shows the composition of total protein, eDNA, and total EPS of biofilms samples, by *Streptomyces* sp. A5, M7, MC1, and A14. The results shows that the total weight of EPS obtained from the M7 and A14 strains biofilms was not explained by the added weight of total eDNA, total protein, and total exopolysaccharides on the matrix. The 7.4 and 10.3 % (respectively) of its EPS chemical composition, were unknown (these values are not explained by an extended error on total protein, total eDNA, and total polysaccharide measurement). Other authors (Nocelli et al., 2016; Jamal et al., 2018; Nievas et al., 2021) have also seen this fact, to provide a complete biochemical profile of most EPS which has a highly variable composition and remains still a substantial analytical challenge (Zhang et al., 1999; Wang et al., 2003; Flemming and Wingender, 2010; Yamanaka et al., 2012; Pinto et al., 2019).

It is often difficult to purify EPS matrix constituents and differentiate them from other components such as cells or other macromolecules transiently associated with the EPS (Nielsen and Jahn, 1999; Sutherland, 2001; Vuong et al., 2004; Salwan and Sharma, 2020). It may be produced for at least two different reasons. On one side, the chemical composition of EPS is quite diverse (Chen and Wen, 2011; Felz et al., 2019; Shi and Liu, 2021) and some of them, may not be properly

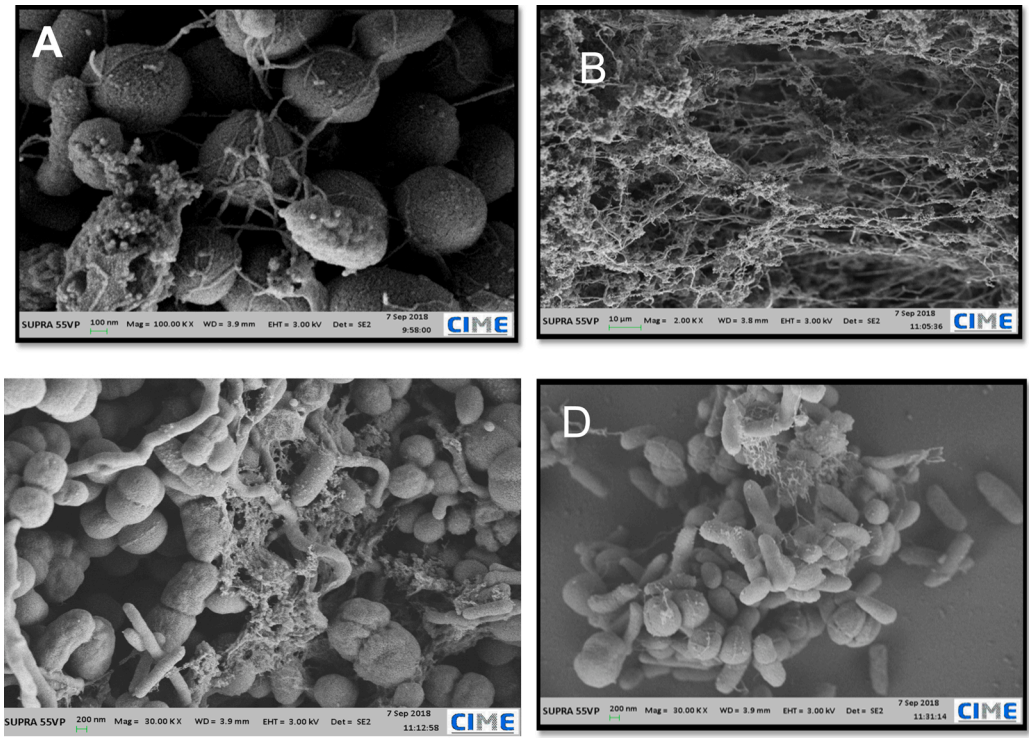


**Fig. 3.** SEM micrograph of the biofilm of *Streptomyces* sp. A) biofilm of *Streptomyces* sp. M7 grown in TSB medium developed on circular coverslips (Magnification 100.00 KX). B) biofilm of *Streptomyces* sp. A14 developed in minimal medium with lindane on circular coverslips (Magnification 100.00 KX). C) Biofilm of *Streptomyces* sp. A5 developed in TSB medium on circular coverslips (Magnification 30.00 KX). D) *Streptomyces* sp. MC1 developed in TSB medium on circular coverslips (Magnification 30.00 KX).

**Table 1**
Composition of EPS biofilms samples, by *Streptomyces* sp. A5, M7, MC1, and A14. Data expressed average ± Standard deviation (n = 3), Significant differences $P \leq 0.05$ (*).

| Treatment | mg / g sample (n = 3) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total Protein | | Total eDNA | | Total polysaccharides | | Total | | Undetermined Composition |
| | Average | ± Sd | Average | ± Sd | Average | ± Sd | Mean | ±Sd | Mean |
| *Streptomyces* sp. M7 | 331,6 | 9,9 | 2,0 | 0,2 | 592,4 | 11,8 | 926,0 | 15,4 | 74,0 |
| *Streptomyces* sp. A5 | 597,4 | 7,1 | 4,3 | 0,1 | 374,9 | 14,0 | 976,6 | 15,7 | 23,4 |
| *Streptomyces* sp. A14 | 376,7 | 4,4 | 2,8 | 0,0 | 516,9 | 21,6 | 896,4 | 22,1 | 103,6 |
| *Streptomyces* sp. MC1 | 649,2 | 7,5 | 6,7 | 0,5 | 319,0 | 20,0 | 974,9 | 21,4 | 25,1 |
| Positive Control | 104,72 | 0,35 | 1 | 0,01 | 100,18 | 0,33 | | | |
| Negative control | 0 | 0 | 0 | 0 | 0 | 0 | | | |

Results are expressed as mean ± SD of content. Values without a common letter differ significantly ($P < 0.05$) for each cell line.

determined by standards analytical methods, mainly due to some interference during protocols (Zhang et al., 1999; Nielsen and Jahn, 1999; Vuong et al., 2004; Selim et al., 2018). Carbohydrate chemical analyses remain difficult because of the diversity in sugar monomers, linkages, and unique structures present in the carbohydrate fraction of the EPS matrix material (Xu et al., 2011). Nonetheless, all EPS biopolymers are highly hydrated and form a matrix, which keeps the biofilm cells together and retains water. This matrix interacts with the surrounding environment, *e.g.*, by attaching biofilms to surfaces and through its sorption properties, which allow for sequestering of dissolved and particulate substances from the environment, incorporating them into EPS chemical composition (Felz et al., 2019). By other and if EPS matrix contains DNA, RNA and polysaccharide components from previously lysed cells, giving some chemical pollution, modifying protein, DNA and saccharides, proportions, with the inability to discern cellular from EPS components (Wu and Xi, 2009).

On the other side, the structure and components of EPS can be diverse depending on bacterial species, environmental conditions, nutrient availability, or can be self-regulated based on environmental stimuli such as shear, nutritional status, and functional interactions in complex biofilm communities or axenic cultures. (Flemming and Wingender, 2010; Flemming et al., 2016). Furthermore, RNA and polysaccharide expression quantities have been noted to change by organism or growth environments (Bober, 2005; Elliott et al., 2007), which can arise the importance of compounds not targeting in this study, as EPS lipids, and minerals composition (Flemming and Wingender, 2010; Desmond et al., 2018)

For example, the total-protein/total-polysaccharide/ ratio on EPS matrix (taken from Table 1) reach 0.55 ± 0.05; 1.59 ± 0.05; 0.72 ± 0.05; and 2.03 ± 0.07, produced by *Streptomyces* sp. A5, M7, MC1, and A14, respectively. Again, strains M7 and A14 have different behavior, reaching significantly higher ratios (*T*-test $p = 0.032$). Accordingly, both strains have also lesser biofilm development on standard assay (Fig. 1). This data agrees with Desmond et al. (2018), who found that the Biofilm EPS composition (total protein, polysaccharide, and eDNA) was manipulated by growing biofilms under contrasting nutrient conditions. Nutrient conditions consisted of (i) a nutrient-enriched condition with a nutrient ratio of 100:30:10 (C: N: P), (ii) a phosphorus limitation (C: N: P), ratio: 100:30:0), and (iii) a nitrogen limitation (C: N: P ratio: 100:0:10).

Data reveals those biofilms grown under P limiting conditions were characterized by EPS matrix with higher total protein/total polysaccharide ratios, dense and homogeneous physical structures with high concentrations of polysaccharides and DNA. Biofilm was grown under

nutrient-enriched or N limiting conditions were characterized by heterogeneous physical structures with lower concentrations of polysaccharides and DNA.

One widely accepted surrogate for total biofilm growth is total protein content. Assuming that protein content is approximately similar between cells, protein content has been found to correlate with the number of cells in biofilms of wetland microcosms (Corte et al., 2019). However, variability of protein production across species, age, and culture conditions may result in deviation from direct correlation with cell number making this a method to be used in conjunction with strict experiment controls and verified with more direct quantification methods (Salwan and Sharma, 2020).

Biofilms can be analyzed using DNA, RNA, and polysaccharide quantification (Desmond et al., 2018) it provides valuable data about the chemical diversity of natural biofilms, some clue about physiological state and age, and even antagonistic capabilities of the attached microbial community (Matz and Kjelleberg, 2005; Kim et al., 2016). However, this approach assumes that each cell will have similar DNA, RNA, and polysaccharide quantities per cell, it is advisable to provide these quantifications in tandem with more direct methods, such as CFU or cell counting (Bober, 2005; Elliott et al., 2007).

### 3.5. Determination of anticancer properties of EPS on transform cells

In Table 2 is showed that the exopolysaccharide extracted from *Streptomyces* sp. M7 strain caused highest cytotoxic effect on ISC-18 non-transformed intestinal cells (98.7 ± 5.7 %, Table 2), instead of *Streptomyces* sp. A5 arise the highest cytotoxic effect on 4T1 Breast cancer cells (96.2 ± 13.5 %), and *Streptomyces* sp. MC1 has the highest cytotoxic effect on Caco-2 human Tumor colon cancer cells (93.9 ± 1.9 %).

Analysis of overall data (Table 2), shown significant differences ($Chi^2$, $P \geq 0.05$) between the cytotoxic effect of EPS produced by M7 and M14 only when it was tested against 4T1 Breast Cancer cells (59.8 % and 84.5 % of cell viability, respectively). This may indicate that the unknown EPS compounds produced in the M7 EPS matrix (Table 1), maybe having some effect increasing the cytotoxic activity of this strain. The cytotoxicity of EPS on normal cell lines was lower than that observed with 5-FU (100 µg / mL) (Table 2).

The earliest polysaccharide recorded to have antitumor activity was isolated in 1943 from the bacterium *Serratia marcescens* and got to be distinctly known as Shear's polysaccharide. Wang et al. (2003) reported that exopolysaccharides produced from *Streptomyces* sp. 139 have immune-stimulatory, anti-tumoral, anti-rheumatic arthritis, and antioxidant activity. Manivasagan et al. (2013) extracted extracellular polysaccharides showing antioxidant activity from a marine strain of actinobacterium *Streptomyces violaceus* MM72. Furthermore, it was found that exopolysaccharides produced by *Bacillus* sp. and *Pseudomonas* sp. have cytotoxicity for both colon cancer and human breast cancer cell lines and are promising novel therapeutic agents, since are active at low concentration (Selim et al., 2018).

Data do not allow us to see some relationship between the chemical composition of EPS and the cytotoxic effect of each assayed EPS matrix. Pearson correlation Index was performed, and non-significant r values were detected.

### 3.6. Detection of Biofilm formation associated genes

Accordingly with biofilm-forming capabilities showed by all assayed strains (Table 2) the presence of potential genes associated with biofilms formation was in all assayed strains. The comparative analysis of the sequences determined in the genome of *Streptomyces* sp. M7, MC1 and A5 demonstrated that the gene encoding Polysaccharide deacetylase has a similarity between 38–57 % and an identity of between 24–34 % with the *Actinomyces oris* K20 gene (Mashimo et al., 2013), while the same gene in *Streptomyces* sp. A5 is 41–45 % and 25–32 % and in MC1 39–43 % and 25–29 % respectively. While for the gene parB-like partition protein for *Streptomyces* sp. M7 (similarity/identity) 75−64 %, respectively, in the case of *Streptomyces* sp. A5 (similarity/identity) 73−64 % and in the case of *Streptomyces* sp. MC1 (similarity / identity) 76−68 %; while for the AoriK_14165 gene for *Streptomyces* sp. M7 it was 76−63 %, for A5 77−64 % and for MC178 / 65 % respectively. Finally, the gene that codes for the transRDD proteins family in the case of *Streptomyces* sp. M7 was 46−31 %, for *Streptomyces* sp. A5 it was 46−31 % and for *Streptomyces* sp. MC1 46−31 %, respectively.

**Table 2**
*In vitro* cytotoxic assays of biofilms samples, by *Streptomyces* sp. A5, M7, MC1, and A14.

| Biofilms strains‡ | Caco-2 human Tumor colon cancer cells Mean ± SD | 4T1 Breast cancer cells Mean ± SD | IEC-18 non-transformed intestinal cells Mean ± SD |
|---|---|---|---|
| *Streptomyces* sp. M7 | 73.3 ± 3.2 [a] | 59.8 ± 1.5 [a] | 98.7 ± 5.7 [a] |
| *Streptomyces* sp. A5 | 73.9 ± 6.4 [a] | 96.2 ± 13.5 [b,c] | 29.9 ± 9.1 [b] |
| *Streptomyces* sp. A14 | 60.4 ± 1.9 [b] | 84.5 ± 3.2 [b,d] | 84.5 ± 5.9 [c] |
| *Streptomyces* sp. MC1 | 93.9 ± 1.9 [c] | 76.1 ± 3.8 [d] | 96.9 ± 3.1 [a] |
| 5-FU (100 µg/mL)# | 78.1 ± 2.2 [a] | 49.3 ± 0.1 [e] | 74.1 ± 1.8 [d] |
| Triton 1 %† | 2.5 ± 0.4 [d] | 4.2 ± 3.6 [f] | 4.6 ± 2.5 [e] |
| Non-treated cells* | 100 [c] | 100 [c] | 100 [a] |

Results are expressed as mean ± SD of cells' viability percentage for each cell line. Values without a common letter differ significantly ($P < 0.05$) for each cell line.

‡ Biofilms were assayed diluted ½ in the culture media.

* Non-treated cells were used as viability control (100 % viability).

† Triton was used as non-viability control.

# 5-Fluoruracil is a chemotherapy drug used as cytotoxic positive control (concentration 100 µg/mL).

## 4. Conclusion

From the results obtained it is possible to conclude that all evaluated *Streptomyces* strains displayed capability to develop biofilm. However, *Streptomyces* sp. A5 and MC1 develop the biofilm to a greater extent. On the other hand, although the capacity to form biofilm is reduced for both strains in presence the pesticide, *Streptomyces* sp. A5 was able to produce a two-fold more biofilm than MC1 strain. On the other hand, EPS from *Streptomyces* sp. A5 and MC1, showed a high cytotoxicity against Caco-2 and 4T1cancer cells. However, only EPS from *Streptomyces* sp. A5 cause a low cytotoxicity against IEC-18 non-transformed intestinal cells, demonstrating that actinobacteria have become increasingly popular sources of EPS. An inhibitory activity was observed towards cell lines similar to the positive control (5-fluorouracil). Although EPS did not show cytotoxicity on normal cell lines such as IEC-18, they will be considered in future studies to be able to propose another alternative for future treatments. In addition more studies are needed to know if EPS do not induce risk of septicemia. Therefore, the search for new EPS producing microorganisms is still promising.

## Authorship contribution statement

**Homero Urrutia**: Conceptualization, Methodology, data curation and analysis, Investigation. **Gonzalo Tortella**: Funding acquisition, Supervision, Conceptualization, Roles/Writing - original draft, Writing - review & editing. **Evangelina Sandoval**: Methodology and analysis. **Sergio Cuozzo**: Writing – review & editing, Funding acquisition.

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