Antibacterial and anticancer activity of marine *Streptomyces parvus*: optimization and application

Hanan Abd-Elnaby, Gehan Abo-Elala, Usama Abdel-Raouf, Abeer Abd-elwahab and Moaz Hamed

Introduction

Marine microorganisms, as a new and promising source of biologically active compounds, are currently of considerable interest.[1] They produce a variety of metabolites, some of which can be used for drug development. [2] These microorganisms are virtually unlimited sources of novel compounds with many therapeutic applications.[3] Actinomycetes hold a prominent position because they have a wide diversity and proven ability to produce new compounds. Further, the discovery of novel antibiotic and non-antibiotic lead molecules through microbial secondary metabolite screening is becoming increasingly important.[3]

The majority of actinomycetes can be found in both aquatic and terrestrial habitats.[4,5] The recent researchers screened intensively the marine plants, medicinal plants, sediments and soil environments of actinomycetes, in order to elucidate their bioactive molecules.[4,6–9] Although soils have been screened by the pharmaceutical industry for about 50 years, only a minuscule fraction of the surface of the globe has been sampled, and only a small fraction of actinomycetes taxa has been discovered.[10] Actinomycetes are the most economically and biotechnologically valuable prokaryotes. About half of the discovered bioactive secondary metabolites are produced by them.[11] Microorganisms have been reported to produce around 23,000 bioactive secondary metabolites and over 10,000 of these compounds are produced by actinomycetes, representing 45% of all bioactive microbial metabolites discovered.[12] Among actinomycetes, around 7600 compounds are produced by *Streptomyces* species.[8,13] Approximately 289 secondary metabolites from the marine-derived genus of *Streptomyces* are reported in the MarinLit database, covering a wide variety of chemical structures, including peptides, macrolides, lactones, indoles, terpenes and quinones.[14] These compounds show an extensive range of industrially useful activities, such as cytotoxic, antibacterial, antifungal, antimalarial,[15,16] anticancer,[16] immuno-suppressant,[16,17] anti-inflammatory, anthelmintic, herbicide, enzyme and others.[6,8]
The bioactive secondary metabolites from marine-derived *Streptomyces* have thus attracted increasing interest during the last decades.[18] Because of the excellent record of accomplishment of actinomycetes in this regard, a significant effort has been focused on the successful isolation of novel actinomycetes from terrestrial sources for drug screening programmes in the past 50 years. Recently, there has been a decrease in the rate of discovery of new compounds from terrestrial actinomycetes. On the other hand, there has been an increase in the rate of re-isolation of known compounds. Thus it is crucial for new groups of actinomycetes from unexplored or underexploited habitats to be pursued as sources of novel bioactive secondary metabolites.[19]

Although there is an extraordinary diversity of life in the terrestrial environment, the greatest biodiversity is localized in the oceans.[11,20] Marine actinomycetes are chemically rich sources of structurally diverse secondary metabolites.[14,21]

Looking at the wide importance of actinomycetes in various fields, the present study was undertaken to isolate actinomycetes from seawater and sediment samples along the sea shore of Suez Bay, Egypt. Also, an identification of the most potent isolate was conducted to assess its antibacterial and anticancer activities.

**Materials and methods**

**Actinomycetes isolation and growth conditions**

A total of 17 actinomycetes’ isolates representing different colony morphologies were isolated from seawater and sediment samples along the sea shore of Suez Bay, Egypt, from winter to autumn of 2012. Actinomycetes strains were cultivated and purified on starch–nitrate liquid medium,[22] which had the following constituents: 20 g L\(^{-1}\) starch, 1 g L\(^{-1}\) KNO\(_3\), 0.5 g L\(^{-1}\) K\(_2\)HPO\(_4\), 0.5 g L\(^{-1}\) MgSO\(_4\)
7H\(_2\)O, 0.05 g L\(^{-1}\) FeSO\(_4\) and 15 g L\(^{-1}\) agar (in case of solid medium), pH was adjusted to 7 and the incubation was conducted at 30–32 °C for 7 d.[23]

**Pathogenic indicators**

The bacterial pathogens used as target strains in the present investigation were *Aeromonas hydrophila*, *Vibrio damsela*, *Pseudomonas aeruginosa* ATCC 6739, *Escherichia coli* and *Staphylococcus aureus* ATCC 6538. These pathogens were kindly provided by the staff members of The National Institute of Oceanography and Fisheries, Egypt.

**Bacterial identification**

The promising actinomycetes’ isolate was cultured in starch–nitrate liquid medium for 7 d and genomic DNAs were extracted with the genomic DNA extraction protocol of GeneJET genomic DNA Purification Kit (Fermentas). The polymerase chain reaction (PCR), using Maxima Hot Start PCR Master Mix (Fermentas), was carried out in a thermal cycler (Multigene Optimax, Labnet International, Inc.). The PCR thermocycler was programmed as follows: 95 °C for 5 min for initial denaturation, 30 cycles at 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 10 min. The PCR mixture contained 25 pmol of each primer (Sigma Scientific Services Company, Egypt, 2013), 10 ng chromosomal DNA, 200 mmol L\(^{-1}\) dNTPs and 2.5 U of *Taq* polymerase in 50 μL of *Taq* polymerase buffer (10X standard *Taq* reaction buffer).

The PCR clean-up of the PCR product was performed by using GeneJET™ PCR Purification Kit (Fermentas) at Sigma Scientific Services Company, Egypt, 2013. The sequencing of the PCR product was made by the GATC Company by using ABI 3730xl DNA sequencer with universal primers (16S 27F and 16S 1492R), (5’-AGAGTTTGATCCTGGCTCAG-3’ and 5’-GGTTACCTTGGTACGACTT-3’). Genotypic characterization was performed using 16S sequence analysis. Multiple alignments with sequences of the most closely related members and calculations of levels of sequence similarity were carried out using BioEdit (software version 7).[24] Sequences of rRNA genes, for comparison, were obtained from the National Center for Biotechnology Information database.

**Electron microscopy studies**

For the electron microscopy, starch–nitrate agar medium was inoculated with spores of *S. parvus* strain and incubated for 14 d at 32 °C. A plug of the culture was removed and fixed in glutaraldehyde (2.5%, v/v), washed with water and post-fixed in osmium tetroxide (1%, w/v) for 1 h. The sample was washed twice with water, dehydrated in ascending ethanol concentrations (30%, 50%, 70%, 90% and 100%, v/v) and finally coated in gold and examined at 15–20 kV in JEOL JSM 5400 LV scanning electron microscope.

**Antibacterial activity**

The actinomycetes’ isolates that were studied were inoculated in flasks (250 mL) containing 50 mL of starch–nitrate medium. Seeded flasks were incubated at 30–32 °C on a rotary shaker at 200 rpm for 7 d. At the end of the incubation, the cultures were harvested, filtered and...
sterilized by ultrafiltration by using 0.22 μL sterilized filters and used as antimicrobial agents. The antagonistic activity of actinomycetes’ supernatants was detected by using well-cut diffusion technique, in which, 5-mm diameter wells were punched in marine nutrient agar (Oxoid Ltd, England) plates inoculated with bacterial pathogenic strains. Fifty microlitre of the tested supernatant was pipetted into each well. The plates were incubated at 30 °C for 24 h. Each set was prepared in duplicate. After incubation, the radius of the clear zone around each well was linearly measured in mm.[25,26]

Optimization of culture conditions

Plackett–Burman design

The Plackett–Burman experimental design [27] was used to evaluate the relative importance of the various factors involved in the production of antimicrobial agents by the selected actinomycete isolate S. parvus against the bacterial pathogen A. hydrophila. Seven independent variables were examined in this experiment and their settings are shown in Table 1. Each variable was tested in four runs at low level (-1) and in four trials at high level (+1). The eight different runs and the basal control (trial number 9 in Table 2) were performed in duplicate. The main effect of each variable on the inhibition zones’ diameter was determined by using Equation (1):

\[ E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-}) / N \] (1)

where \( E_{xi} \) is the variable main effect, \( M_{i+} \) and \( M_{i-} \) are the radiiuses of the clear zones around each well in the runs. The independent variable \( xi \) is present in the high and low concentrations, respectively, and \( N \) is the number of runs, divided by 2. By using Microsoft Excel, statistical \( t \)-values for equal unpaired samples were calculated [28] to determine the variable’s significance. From the main effect results, an optimized medium was predicted.

Verification experiment

A verification experiment was carried out in duplicate. The predicted optimum levels of the independent variables were examined and compared to the basal conditions setting (trial number 9 in Table 2) and the secondary metabolites production was detected by measuring the average of the inhibition zones’ diameter (mm).

Optimization of the incubation time and temperature

The effect of different incubation temperatures (25–35 °C) and different incubation periods (6–8 d) on the production of antimicrobial agent(s) by S. parvus strain against bacterial pathogen A. hydrophila was determined by using the optimized medium. The production of the antimicrobial agent(s) was detected by measuring the inhibition zones’ diameter (mm).

Imobilization technique

The immobilization was performed by using adsorption techniques, as described by Eikmeier and Rehm,[29] to enhance the production of the antimicrobial agents. Erlenmeyer flasks (250 mL capacity) containing 50 mL of the optimized medium and porous supporting materials (luffa pulp, pumice, sponge and tails), were inoculated with the selected actinomycete isolate. The flasks were then shaken slowly at 120 rpm for 7 d of incubation. The production of the antimicrobial agents was compared with control (free cells), by using well-cut diffusion technique. The production of the antimicrobial agent(s) was detected by measuring the inhibition zones’ diameter (mm).

Extraction of the active compound(s)

A 7-d culture of the selected strain was centrifuged (Centurion Scientific, United Kingdom) at 10,000 rpm for 15 min. The supernatant was extracted with equal volume of chloroform, n-butanol or ethyl acetate. After soaking and vigorously shaking for a complete extraction, the organic phase was collected and evaporated by using a rotary evaporator (Stuart, BIBBY Scientific Limited, United Kingdom), and then dissolved in the appropriate solvent. The antagonistic activity of the crude extract was tested by using the well-cut diffusion method.[30] The activity of the extracted antimicrobial
agent(s) was detected by measuring the inhibition zones’ diameter (mm).

**Estimation of the crude extract’s antibacterial activity**

The antimicrobial activity of the crude extract was estimated via comparison with some standard commercial antibiotics and was determined by disc diffusion method according to Bauer et al.[31] About 250 μg of the actinomycete crude extract was impregnated on sterile disc (Whatman No.1 filter paper was punched into discs) and dried under sterile conditions at room temperature. Three standard commercial antibiotic discs were used for the comparative study. The used antibiotics were 5 μg/disc methicillin, 2 μg/disc lincomycin and 30 μg/disc chloramphenicol (Oxoid Ltd, England). The discs were assayed onto the surfaces of the plates seeded with *A. hydrophila* (1 of the McFarland scale) as a target strain. The plates were incubated at 30 °C overnight. After incubation, the inhibition zone around each disc was linearly measured (mm). The assays were performed in duplicate.

**Antifouling activity**

Fouling bacteria (1 mL of seawater) were incubated with cover glass in 50 mL conical flask containing 20 mL of nutrient broth medium (Oxoid Ltd, England) at 28 °C for 24 h. About 0.1 g of the crude extracted substance was added into the flask as an antifouling agent. After dying with 0.4% crystal violet solution for 10 min, the cover glass was washed with water, air dried and observed under a microscope (OPTIKA microscopes, model: B-182, Italy).[32] A control flask (without crude extract) was used for comparison.

**Anticancer activity**

In order to evaluate the anticancer activity of *S. parvus* culture supernatant, several steps were undertaken: lyophilization (Telstar, Spain), cytotoxicity test and effect of the median inhibitory dose (IC50) on four different tumour cell lines: human liver cancer cell line (HepG2), mouse lymphoma cell line (EL-4), breast cancer cell line (MCF-7) and human colon cancer cell line (Caco-2).

The lyophilization process can be summarized in three general steps: freezing, primary drying and secondary drying. Filtrate of *S. parvus* culture (10 mL) was lyophilized and converted into powder by using a Telstar lyophilizer (Spain). Different concentrations (5, 25, 50, 75 and 100 μg mL⁻¹) of the powder were prepared and used in the second step (cytotoxicity test). To measure the cytotoxicity of the examined compounds, 5 × 10⁴ lymphocyte cells were seeded in 96-well plates and incubated in RPMI (Roswell Park Memorial Institute) 1640 medium supplemented with 200 μmol L⁻¹ L-glutamine and 10% foetal bovine serum (FBS) containing different concentrations (5, 25, 50, 75 and 100 μg mL⁻¹) of the tested compound and incubated for 72 h in 5% CO₂ incubator. Wells were washed with Hank’s balanced salt solution. Fraction of viable lymphocytes was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

In the MTT assay, yellow MTT is reduced to purple formazan in the mitochondria of viable cells. A quantity of 100 μL of the MTT working solution (0.5 mg mL⁻¹) was added to each well and incubated at 37 °C for 4 h. Next, media were removed, wells were washed with phosphate buffer saline and 100 μL of dimethyl sulphoxide was added to solubilize the formazan crystalline product. The absorbance was measured with a plate reader (Cell Star, USA) at 570 nm. The viable cells’ fraction was determined by dividing the absorbance of the cells treated with a test compound (test well) to that of the cells unexposed to any compounds (control well). Safe doses of different test compounds were determined from the GraphPad InStat software, as the compound concentration that caused 100% viability of normal cells (EC100) and was calculated from the equation of cell viability as follows: (absorbance of wells containing cells treated with the compound at different concentrations/absorbance of control well) × 100.

**Table 2. Experimental results of the applied Plackett–Burman design for seven cultural variables.**

| Trials | Starch | KN | K₂ | Mg | Fe | IS | pH | Inhibition zones (mm) against *A. hydrophila* |
|--------|--------|----|----|----|----|----|----|---------------------------------------------|
| 1      | 1      | 1  | 1  | 1  | 1  | 1  | 1  | 0                                            |
| 2      | 1      | -1 | -1 | 1  | 1  | 1  | 1  | 0                                            |
| 3      | -1     | 1  | -1 | -1 | -1 | 1  | 1  | 0                                            |
| 4      | 1      | 1  | -1 | 1  | -1 | -1 | 1  | 0                                            |
| 5      | -1     | -1 | 1  | 1  | -1 | 1  | 1  | 16                                           |
| 6      | 1      | -1 | 1  | -1 | -1 | 1  | -1 | 0                                            |
| 7      | -1     | 1  | 1  | 1  | -1 | 1  | -1 | 25                                           |
| 8      | 1      | 1  | 1  | 1  | 1  | 1  | 1  | 26                                           |
| 9      | 0      | 0  | 0  | 0  | 0  | 0  | 0  | 21                                           |

Burman design for seven cultural variables.
Cytotoxicity assay was performed on different human and mouse cancer cell lines by using the described method. The anticancer activity of the tested compound was assayed using four different cell lines (EI-4, HepG2, Caco-2 and MCF-7). Cells (15 × 10⁶, 4 × 10⁶, 3 × 10⁶ and 3 × 10⁶) were seeded in 96-well plates and incubated in 200 μL of RPMI 1640, RPMI 1640 high-glucose formulation Dulbecco’s modified eagle’s medium and RPMI 1640 media/10% FBS (Lonza, Switzerland), respectively, for 24 h in 5% CO₂ incubator for cell attachment. Next, the media were changed with the same media containing the safe dose of the tested compound and cells were incubated in that media for 72 h. Medium without any chemicals was used as a negative control. Fractions of viable cells were measured with MTT assay.[33] The inhibition of growth rate of tumour cells for each compound with different concentrations was calculated as follows: [100 − (absorbance of cells that were exposed to the compound at different concentrations/absorbance of control well)] × 100.

Antiviral activity

Qualitative in vitro anti-hepatitis C virus screening

HepG2 cells were washed twice with RPMI 1640 medium supplemented with 200 μmol L⁻¹ L-glutamine (Lonza, Switzerland) and 25 μmol L⁻¹ N-[2-hydroxyethyl] piperazine-N9-[2-ethanesulphonic acid] buffer (Lonza, Switzerland). The cells were suspended to 2 × 10⁵ cells mL⁻¹ in RPMI 1640 medium containing 10% FBS (Gibco-BRL). The cells were seeded in six-well plates and incubated for 24 h in an incubator (37 °C, 5% CO₂, 95% humidity) for cell attachment.

Cytotoxicity assay was performed on HepG2 cell line by using the previously described method. HepG2 cell culture was prepared as described by El-Hawash et al.[34] The cells were infected with 2% hepatitis C virus (HCV)-infected serum in RPMI culture medium containing 8% FBS. The tested compound was added at its non-lethal dose (IC50 dose of the compound in HEPG2 cells). Positive (infected untreated cells) and negative (uninfected untreated cells) control cultures were included. After 96 h of incubation at 37 °C, 5% CO₂ and 95% humidity, a second dose of the test compound was added. The cells were incubated for another 96 h, followed by a total RNA extraction. The positive strand and its replicating form (negative strand) were detected by reverse transcription polymerase chain reaction (RT-PCR) using HCV specific primers to the 5' untranslated region of the virus.

RNA extraction and RT-PCR of HCV RNA

Total RNA was extracted from HepG2 HCV-infected cells using the method described by El-Awady et al.[35] Briefly, culture cells were mixed with 500 μL of 4 mol L⁻¹ guanidinium isothiocyanate containing 25 mmol L⁻¹ sodium citrate, 0.5% (w/v) sarcosyl, 0.1 mol L⁻¹ β-mercaptoethanol and 100 μL sodium acetate. The lysed cells were mixed with an equal volume of phenol, chloroform and isomyl alcohol (25:24:1, pH 4). After vortexing (Thermolyne, USA) of the sample, the mixture was centrifuged at 14,000 rpm for 10 min at 4 °C. The aqueous layer was collected and mixed with an equal volume of isopropanol. After an overnight incubation at -20 °C, RNA was precipitated by centrifugation at 14,000 rpm for 30 min at 4 °C and the precipitated RNA was washed twice with 70% ethanol.

The complimentary DNA (cDNA) and the first PCR reaction of the nested PCR detection system for the HCV RNA was performed in a 50 μL volume single-step reaction using the Ready-To-Go RT-PCR beads (Amersham Pharmacia Biotech, USA), 10 μmol L⁻¹ from each of the RT-downstream primers (PCR forward primer and reverse primer P2) (5'-GGTGCCACGTCTACGAGACCTC-3' and 5'-AAC TAC TGT CTT CAC GCA GAA-3' and 5'-TGC TCA TGG TGC ACG GTC TA-3'). The thermal cycling protocol was as follows: 30 min at 42 °C for cDNA synthesis, followed by 5 min at 95 °C and 30 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C. The nested PCR amplification was performed in 50 μL reaction mixture containing 0.2 mmol L⁻¹ from each dNTP, 10 mmol L⁻¹ from each of the reverse and forward nested primers (5'-ACT CGG CTA GCA GTC TCG CG-3' and 5'-GTG CAG CCT CCA GGA CCC-3'), 2 U Tag DNA polymerase (Promega, USA) and 10 μL from the RT-PCR reaction product in a 1 x Tag buffer (10 mmol L⁻¹ Tris-HCl, 50 mmol L⁻¹ KCl and 1.5 mmol L⁻¹ MgCl₂, pH 8.3). The PCR product was separated by agarose gel electrophoresis (ClariScientific-INC, USA) by using 2.5% agarose gel that was impregnated with ethidium bromide at 90 V. A fragment of 174 bp was visualized by comparison with several molecular weight markers.

The gas–liquid chromatography mass spectra of the crude extract

Identification of the crude extract’s chemical constituents of the selected actinomycete isolate was done using high performance 5890 gas–liquid chromatography (GLC) (Hewlett Packard) coupled with 5989 B series mass spectrometer (MS) (Shimadzu, El, Japan). The percentage of each compound was calculated as the ratio of the peak area to the total chromatographic area.[37] The gas–liquid chromatography mass spectrometer (GC-MS) peaks were identified by comparison with several
reported data, their profiles and similarity percentages were detected from the Wiley 275 libraries.

Results and discussion

Screening for antibacterial activity

The screening for antagonistic activity revealed that 7 out of the 17 isolated marine actinomycetes isolates (41%) exhibited antagonistic effect against the tested bacterial pathogens (Table 3). Three isolates (18%) were active only against one bacterial pathogen and 23% of actinomycetes isolates were capable of inhibiting the growth of most of the tested bacterial pathogens. Isolate 8 exhibited good activities against S. aureus ATCC 6538, E. coli, P. aeruginosa ATCC 6739 and A. hydrophila and the inhibition zones were 14, 19, 20 and 20 mm, respectively. Isolate 8 was selected as a representative of this group and was subjected to further studies.

The results obtained in the present experiment confirmed the bioactive potential of marine actinomycetes’ strains. Patil et al. [38] reported that 77 isolates out of 104 were found to be inhibitory to at least one of the pathogens. Also, Patel et al. [39] reported that 79% of the actinomycetes isolates were active against at least one of the 18 tested pathogens. In a recent study, Rana and Salam [9] characterized 23 actinomycetes isolates, 7 of which showed significant inhibition of different Gram positive and Gram negative bacteria and also some fungi, with zones of inhibition up to 20 mm. Actinomycetes isolates have a broad spectrum of antimicrobial activities, and this is why it is of major importance to conduct further studies for the isolation of novel antimicrobial compounds. So far, quite a number of reports are available, in which such novel compounds have been obtained and studied.[4,9]

Molecular identification

The obtained sequence of the 16S rRNA gene indicated the promising actinomycete isolate from the present study (isolate 8 from sediment in Suez Bay, Egypt), which was similar to S. parvus with a maximum identity of 96%. The nucleotide sequence was deposited to GenBank sequence database with the following accession number: KP675949. Figure 1 shows the spore formation of S. parvus.

Patil et al. [38] found that the highest incidence of inhibitory isolates was isolated from sediment samples. All the isolates of antagonistic marine actinomycetes were identified to be Streptomyces. The genus Streptomyces seems to provide a wide variety of new antibiotics more than any other genus; hence, it is of great importance for both industrial applications and human health care.[40]

Optimization of culture conditions

In order to maximize the antimicrobial agent production by marine S. parvus, an experimental design and immobilization techniques were employed to optimize the culture conditions. Plackett–Burman design, an efficient technique for medium component optimization, was employed to identify the significant variables that enhance the antimicrobial agent production.[41] The results were achieved in an economical manner.[42] One of the advantages of the Plackett–Burman design is that it may rank the effects of different variables on the measured response, independently of their nature (either nutritional or physical factor) or sign (whether contributes positively or negatively).[43]

Table 3. The antibacterial activity of the actinomycetes isolates against some bacterial pathogens.

| Isolates’ codes | 1 | 5 | 6 | 7 | 8 | 9 | 14 |
|-----------------|---|---|---|---|---|---|----|
| Bacterial pathogens | 0 | 0 | 0 | 0 | 14 | 20 | 0  |
| S. aureus ATCC 6538 | 0 | 0 | 0 | 0 | 14 | 20 | 0  |
| E. coli | 0 | 0 | 0 | 0 | 19 | 13 | 0  |
| V. damsel | 0 | 15 | 0 | 10 | 0 | 20 | 0  |
| P. aeruginosa ATCC 6739 | 0 | 18 | 0 | 13 | 20 | 18 | 13 |
| A. hydrophila | 19 | 17 | 17 | 20 | 20 | 0 | 0  |

Figure 1. Scanning electron micrograph showing spore formation of S. parvus on starch–nitrate agar medium after 14 d of incubation.
Table 4. Statistical analyses of the Plackett–Burman experimental results.

| Variable          | Main effects | t-value $^*$ |
|-------------------|--------------|--------------|
| Starch            | -3.75        | 1.894        |
| KNO$_3$           | 8.75         | 2.015        |
| K$_2$HPO$_4$      | 16.75        | 3.1824       |
| MgSO$_4$$\cdot$7H$_2$O | 4.25     | 1.9431       |
| FeSO$_4$          | 8.75         | 2.015        |
| Inoculums size    | 4.25         | 1.9431       |
| pH                | 4.25         | 1.9431       |

Note: $^*$-value significant at the 1% level = 3.70; $^*$-value significant at the 5% level = 2.446; $^*$-value significant at the 10% level = 1.94; $^*$-value significant at the 20% level = 1.372; standard $^*$-values were obtained from statistical methods.[28]

(−) level of starch and the high (+) levels of K$_2$HPO$_4$, KNO$_3$, MgSO$_4$, FeSO$_4$, inoculum size and pH encouraged the production of antimicrobial agent(s) formed by S. parvus strain (Figure 2). Therefore, decreasing the starch concentration and simultaneously increasing the K$_2$HPO$_4$ and KNO$_3$ concentrations in the culture medium can increase the suppression of A. hydrophila with respect to production of the antimicrobial agent(s) by S. parvus. Moreover, the significant variables were identified by statistical analysis of the Plackett–Burman experiment by using the $^*$-test supported by Excel Microsoft Office to determine the statistical significance of the measured response and calculate the main effects for S. parvus (Table 4).

**Verification experiment**

To verify the obtained results from the statistical analysis of Plackett–Burman design, verification tests were performed in duplicate by using the predicted optimized medium against the basal conditions media. The growth of S. parvus on the optimized medium with the following composition: 10 g L$^{-1}$ starch, 1.5 g L$^{-1}$ KNO$_3$, 0.75 g L$^{-1}$ K$_2$HPO$_4$, 0.75 g L$^{-1}$ MgSO$_4$$\cdot$7H$_2$O, 0.015 g L$^{-1}$ FeSO$_4$, 2 mL (10$^3$ colony-forming units (CFU) mL$^{-1}$) inoculum size with pH 8 for 7 d of incubation, recorded larger inhibition zones (27 mm) than that in the case of the basal conditions, with an increase by 1.3-fold. This result confirmed the validity of the optimized medium.

Another data obtained by Wefky et al. [44] found that the production of the bioactive compounds by Enterococcus faecium was positively affected by increasing the incubation period, peptone, inoculum size, temperature, pH and seawater concentration; the optimized medium increased the inhibition zone by about 1.6-fold.

**Optimization of incubation time and temperature**

The effect of different incubation times (6–8 d) and incubation temperatures (25–35 °C) on the growth of S. parvus strain and the enhancement of the antimicrobial agent(s) production were evaluated. The optimum activity was detected after 7 d of incubation. On the other hand, the increase in the incubation temperatures enhanced the antimicrobial agent(s) production by S. parvus. The highest activity exhibited the largest inhibition zone of 29 mm and was observed in cultures previously incubated at 35 °C (Figure 3(A) and (B)).

El-Sersy and Abou-elela [45] reported that a marine strain, identified as Nocardia brasiliensis, produced a bioactive compound that exhibited the largest inhibition zone and the highest activity against the fish pathogen V. damsel, when the culture’s conditions were adjusted to pH 8 and the incubation was performed at 40 °C under static conditions. The increase in the incubation temperature led to an increase in the productivity and a higher production of the antagonistic compound.

**Cell immobilization**

In the most natural environments, the prevailing microbial lifestyle is in association with a surface, in a structure known as biofilm.[46] There are multiple advantages of using immobilized microbial cells to enhance productivity and recovery. Although whole cell immobilization is achieved by various methods of chemical coupling and cell entrapment, the only method that has been applied to a large scale is via biofilm formation on the surface of a solid carrier.[47]

It was observed that the activity of the immobilized living cells of S. parvus was higher than that of the free cells (except in the case of pumice). The results in Figure 4 revealed that biofilm of S. parvus on luffa pulp showed the highest activity in the production of the antimicrobial agent(s). It gave the highest diameter of the inhibition zone (32 mm), i.e. raised up the productivity by 1.45-fold. This result coincided with Abou-elela et al., [48] who reported that the production of bioactive compounds by immobilized Nocardiopsis aegyptia cells on

![Figure 2. Elucidation of the fermentation conditions affecting the production of antimicrobial agents by S. parvus.](image-url)
Luffa pulp was the highest and its yield was increased from 1.12- to 1.16-fold.

Cell immobilization biotechnology is a multidisciplinary area, shown to have an important impact on many scientific sub-disciplines, including biomedicine, pharmacology, cosmetology, food and agricultural sciences, beverage production, industrial waste treatment and analytical applications.[49]

### Antibacterial activity of S. parvus

The crude extracts of *S. parvus* resulting from each solvent were tested for their antimicrobial activities against *A. hydrophila*. The results revealed that n-butanol extract exhibited the highest inhibition zone (26 mm), followed by ethyl acetate extract (24 mm) and chloroform (23 mm) and thus n-butanol was chosen as the most efficient solvent for extracting the desired compounds.

The antibacterial activity of *S. parvus* crude extract against *A. hydrophila* was estimated via comparison with commercial standard antibiotics (Figure 5). The crude extract disc exhibited an inhibition zone of 15 mm, whereas the other antibiotic discs (30 µg chloramphenicol, 2 µg lincomycin and 5 µg methicillin) exhibited inhibition zones of 24, 22 and 0 mm, respectively.

### Anti-biofouling activity of S. parvus

Figure 6 shows the inhibitory activity of *S. parvus* crude extract on bacterial biofilm formation. The crude extract reduced the density of the bacterial cells and acted as an anti-biofouling agent.

The present study concluded that the marine *S. parvus* crude extract might be a potential source for the development of eco-friendly antifouling compounds, which might be a better alternative to the pollution causing synthetic antifoulants. This result agreed with the studies of Bavya et al. [52] on the antifouling effect of *S. filamentosus*, which reduced the biofilm formation on glass.

Actinomycetes produce most of the roughly 10,000 known antibiotics and 75%–80% of the antibiotics prescribed today are derived from the genus *Streptomyces*. [8] Marine derived actinomycetes have become recognized as a source of novel antibiotics.[50] Furthermore, marine natural products exhibit a wide range of biological activities.[51]
Anticancer activity of S. parvus

As clearly shown in Table 5, the effect of S. parvus’ culture supernatant on HepG2 tumour cell line exhibited a reasonable degree of anticancer activity, causing almost 53% inhibition after 72 h (Figure 7(A) and 7(B)). Upon using the EI-4 tumour cell line and MCF-7 cell line, the supernatant of the tested compound showed almost 56% (Figure 7(C) and 7(D)) and 57% (Figure 7(E) and 7(F)) inhibition, respectively, after 72 h by using the MTT assay. On the other hand, the supernatants of the tested compound showed the weakest anticancer activity (42%) towards Caco-2 cell line, as detected by the MTT assay (Figure 7(G) and 7(H)). Table 5 showed the lowest IC50 (the highest anticancer activity) against lymphoma cells and the highest IC50 (the lowest anticancer activity) against Caco-2 cell line.

In a similar study, Vijayabharathi et al. [53] reported that Steptomyces strain, isolated from humus soils in the Western Ghats, has an anticancer activity against HepG2 (hepatic carcinoma) and HeLa (cervical carcinoma) in vitro. Secondary metabolites from actinomycetes,

### Table 5. IC50 and percentage of growth inhibition of the tested compound’s safe dose against different cancer cell lines after 72 h using the MTT assay.

| Cell line | IC50  | Inhibition (%) | Time (h) |
|-----------|-------|----------------|----------|
| EI-4      | 16.48 | 56             | 72       |
| Caco-2    | 128.34| 42             | 72       |
| HepG2     | 75.25 | 53             | 72       |
| MCF-7     | 66.32 | 57             | 72       |

Figure 6. Photographs illustrating the antifouling effect of S. parvus’ crude extract on the fouling bacteria (A) and the effect without crude extract (B).

Figure 7. Growth inhibition effect of the tested S. parvus’ compound on different cancer cell lines. Comparison of treated (A) vs. untreated (B) HepG2 cancer cells; treated (C) vs. untreated (D) EI-4 cancer cells; treated (E) vs. untreated (F) MCF-7 cancer cells; treated (G) vs. untreated (H) Caco-2 cancer cells.
especially the genus *Streptomyces*, may be one of the most important sources for novel anticancer agents.\[54\] Also, Yip et al. \[54\] isolated three purified fractions from a novel strain, *Streptomyces* sp. H7372. In MCF-7 and MDA-MB-231 breast cancer cells, 31-2 (one of the fractions) induced a cytostatic (anti-proliferative) effect without causing cytotoxicity (cell death).

### Antiviral activity of *S. parvus*

As shown in Figure 8, the supernatant of the actinomycete strain *S. parvus* failed to inhibit the HCV replication. A fragment of 174 bp length was identified in the positive samples.

### GC mass spectra of the crude extract

The GC-MS of *S. parvus* extract determined the main constituents and the relative percentage of the identified compounds (Table 6). It was found that, the main constituents were ethane, 1,1-diethoxy; di-n-octyl phthalate; ethanol, 2,2-diethoxy; 9,12-ctadecadienoic acid, methyl ester (E,E) and benzoic acid (Figure 9).

### Conclusions

The marine environment is a good source for valued species that need to be explored. The aim of the present study was to explore our natural environments, searching for actinomycetes producing bioactive compounds.

**Table 6.** The chemical composition of *S. parvus*’ crude extract obtained by using GC-MS.

| Compound                                      | Retention time (min) | Molecular weight | Area | Similarity (%) |
|------------------------------------------------|----------------------|------------------|------|----------------|
| Ethane, 1,1-diethoxy                          | 3.8                  | 118              | 78%  | 97             |
| Ethanol, 2,2-diethoxy                         | 6.1                  | 134              | 4%   | 88             |
| Benzoic acid                                  | 8.9                  | 122              | 1%   | 83             |
| 9,12-octadecadienoic acid, methyl ester (E,E) | 16.6                 | 294              | 4%   | 93             |
| Di-n-octyl phthalate                          | 23.4                 | 390              | 13%  | 92             |

**Figure 8.** Nested PCR detection of anti-HCV activity of *S. parvus*’ extract.

Note: 200 bp ladder (Thermo Scientific, USA) (lane M), positive control ‘uninfected HCV cells’ (lane +), negative control ‘infected HCV cells’ (lane −) and 100 μg mL\(^{-1}\) of *S. parvus*’ extract (lane 4).

**Figure 9.** Gas—liquid chromatography mass spectra of *S. parvus*’ crude extract.

The major chemical component (ethane, 1,1-diethoxy) is reported to have a known biomedical value in the pharmacological fields, which is known to demonstrate valuable therapeutic uses, including anti-inflammatory, antipyretic, antithrombotic and analgesic effects.\[55\]

Also, chemical compounds, such as hexadecanoic acid, octadecatrienoic acid, pentadecanoic acid, heneicosanoic acid and oleic acid may be responsible for the antimicrobial properties of *Andrographis paniculata*’s extract.\[56\] In addition, Kumar et al. \[57\] concluded that compounds like hexadecanoic acid, methyl ester and 9,12-octadecadienoic acid (Z,Z)-, methyl ester have anticancer properties. Jaina et al. \[58\] reported that the 1,2-benzenedicarboxylic acid has insecticidal, pesticide and antitumour effects; the hexadecanoic acid has antioxidant, nematicide, hypcholesterolemic and pesticide effects and the 9,12-octadecadienoic acid has anticarcinogenic, antiatherogenic, antioxidant and anti-inflammatory effects. Furthermore, the synthetic methyl and propyl esters of p-hydroxybenzoic acid, the so-called parabens, are on the generally recognized as safe list for use in food at a maximum concentration of 0.02%.\[59\]
The findings of the present study suggested that the antagonistic marine *Streptomyces* isolates, especially *S. parvus* or the antibacterial compounds produced by it, could be used as antibiotics and antifoulants, which might have future applications in the aquaculture systems. In addition, the *S. parvus* might have future applications in the aquaculture systems. In addition, the *S. parvus* might have future applications in the aquaculture systems. In addition, the *S. parvus* might have future applications in the aquaculture systems. In addition, the *S. parvus* might have future applications in the aquaculture systems.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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