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The Bioactivity of Actinomycetes Isolate *Streptomyces variabilis* H2 Against Some Bacterial Pathogens: Optimization and Applications

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ABSTRACT

The most valuable prokaryotes, both biotechnologically and monetarily, are actinomycetes. *S. variabilis* H2 demonstrated a broad spectrum of antagonistic effects against all tested Gram-negative and Gram-positive bacteria, exhibiting inhibition zones of 18mm against *Escherichia coli*, 16mm against *Bacillus subtilis*, 16mm against *Staphylococcus aureus*, 14mm against *Pseudomonas aeruginosa*, 14mm against *Enterococcus faecalis*, and 12mm against *Klebsiella pneumoniae*. Using Plackett-Burman design and one variable at a time approach and testing against the six different bacterial pathogens, one sought a maximal synthesis of the bioactive chemical. The highest antagonistic activity of *S. variabilis* H2 metabolites was observed against *E. coli*, where productivity increased by up to 1.3-fold when the strain was grown in an optimized medium composed of: starch (30g/ L), KNO_3 (1.5g/ L), K_2HPO_4 (0.75g/ L), $MgSO_4 \cdot 7H_2O$ (0.25g/ L), $FeSO_4$ (0.015g/ L), NaCl (5.0g/ L), and an inoculum size of 2mL (10^3 colony-forming units/mL) for 7 days at 37°C and pH 8. Moreover, the anticancer activity of *S. variabilis* H2 crude extract was tested against three different cell lines: Lung carcinoma cells, Hepatocellular carcinoma cells and breast carcinoma cells. The inhibition activities were 61.57 and 44.51% for lung and Hepatocellular cells, respectively. In addition, the *S. variabilis* H2 crude extract acted as antifungal and anti-biofouling agent, but it failed to act as antiviral agent. The primary components of the crude extract of *S. variabilis* H2 were identified using gas-liquid chromatography-mass spectrometry (GC-MS). The identified compounds included phthalic acid, di(2-propylpentyl) ester, octadecanoic acid methyl ester, and hexadecenoic acid methyl ester.

INTRODUCTION

When it comes to fighting drug-resistant bacteria, one of the best sources of antibiotics is microbial secondary metabolites. Many different kinds of microbes, including actinobacteria, create these crucial secondary metabolites **(Siddharth *et al.*, 2020; Siro *et al.*, 2022)**. Among the many different kinds of microorganisms found in

nature, actinomycetes are aerobic, free-living creatures most commonly found in soil and water **(Isik *et al.*, 2014; Siddharth *et al.*, 2020; Zhang *et al.*, 2020; Ngamcharungchit *et al.*, 2023)**.

Among prokaryotes, actinomycetes are the most useful from a biotechnological and commercial perspectives. They are responsible for producing around 50% of the bioactive secondary metabolites that have been found **(Lam, 2006)**. More than 10,000 of the almost 23,000 bioactive secondary metabolites generated by bacteria come from actinomycetes; these molecules account for 45 percent of all bioactive microbial metabolites known to exist at this point **(Shin, 2013; Muazi *et al.*, 2023)**. Around 7600 different chemicals are produced by *Streptomyces* species, which are classified as actinomycetes **(Antunes *et al.*, 2014)**.

Streptomyces sp. and other actinobacteria are renowned as "noble factories" due to their wide variety of biologically active compounds they produce. These compounds have various medical applications, including anti-tumor, immunomodulatory, anti-fungal, antiviral, antithrombotic, and enzyme inhibitors **(Ahmad *et al.*, 2017; Shubha *et al.*, 2017; Ngamcharungchit *et al.*, 2023)**. Finding novel antibiotic and non-antibiotic compounds by means of microbial secondary metabolite screening is becoming ever more important. Nearly ten percent of the bioactive compounds are antibiotics, and antibiotics with other bioactivities from microbial origin. Antibiotic substances are characterized by their high physiological effectiveness in low concentrations. The action of antibiotic substances is selective, meaning that each antibiotic is biologically effective only against certain pathogens or groups of pathogens, without significantly affecting other important living organisms **(Dezfully & Ramanayaka, 2015)**.

The secondary metabolites from the genus *Streptomyces* encompass a broad spectrum of chemical structures, including peptides, macrolides, lactones, indoles, terpenes, quinones, aminoglycosides, and glycopeptides **(Abd-Elnaby *et al.*, 2016; Salem & Ali, 2023)**.

The present study was undertaken to test the activity of *S. variabilis* H2 against some bacterial pathogens in addition to scaling up the production of the bioactive compound (s) by optimization of the growth conditions and some applications of *S. variabilis* H2 crude extract.

MATERIALS AND METHODS

Actinomycete and growth conditions

The actinomycete strain (*Streptomyces variabilis*) used in the present study was cultured on starch nitrate medium **(Waksman, 1959)** which had the following constituents: gl^{-1}: Starch, 20; KNO_3, 2; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; NaCl, 0.5;CaCO3, 3 and agar, 20 (in case of solid medium) for 7 days.

Antibacterial activity of *S. variabilis* H2

In order to determine if the *S. variabilis* H2 strains might limit the growth of indicator pathogenic bacteria, the well diffusion technique was employed. 50µl of the cell free supernatant from the tested strains was added to each well. After being incubated at 30°C for the night, the diameter of the clear zone surrounding each well was measured in millimeters **(El-Masry *et al.*, 2002)**. We performed each experiment twice and averaged the results. The indicator pathogenic bacteria used were Gram-positive (*Staphylococcus aureus* ATCC25923, *Enterococcus fecalis*ATCC29212 and *Bacillus subtilis* ATCC6633) and Gram-negative (*Escherichia coli* ATCC8739, *Pseudomonas aeruginosa* ATCC9027 and *Klebsiella pneumonia* ATCC13883). The staff members at the National Institute of Oceanography and Fisheries (NIOF), which is located in Alexandria, Egypt, were gracious enough to supply these pathogens without any hesitation.

Enhancement of the bioactive compounds produced

Statistical design for optimization of the culture conditions

Several parameters related in the generation of the antimicrobial agents by *S. variabilis* H2 were evaluated using the Plackett-Burman experimental design **(Plackett & Burman, 1946)**. Table (1) displays the experimental setup and the seven independent variables that were considered. Each column in Table (2) represents a different variable, while the rows reflect the eight separate tests (with row 9 being the basal control trial). An extreme (+) or extreme (-) concentration was examined for every nutritional variable. Using the following equation, we were able to ascertain the primary impact of each variable:

$$E\ xi = (<Mi^{+} - \sum Mi^{-}) / N$$

Where, The mean diameter of the clear zone around each well in the trials is denoted as $M\ i^{+}$ and $M\ i^{-}$, and Exi is the variable main effect. N is the number of trials divided by 2, and the independent variable (xi) was present in both the high and low concentrations. **Snedecor and Cochran (1980)** used Microsoft Excel to calculate statistical t-values for equal unpaired samples, which were used to determine the variable's significance. The findings of the major effect predicted an optimum medium.

Verification experiment

Conducting duplicates of a verification experiment, the average production of the secondary metabolites was calculated, while the expected ideal levels of the independent variables were studied and matched with the basal conditions setting.

Table 1. Independent variables affecting bioactive compound (s) production

Factor (gl^{-1})	Symbol	Level		
		-1	0	+1
Starch	Starch	10.0	20.0	30.0
KNO_3	KN	0.5	1.0	1.5
K_2HPO_4	K_2	0.25	0.5	0.75
$MgSO_4.7H_2O$	Mg	0.25	0.5	0.75
$FeSO_4$	Fe	0.005	0.01	0.015
Inoculum size	IS	0.5	1.0	2.0
NaCl	NaCl	2.5	5.0	7.5

* Inoculum size was added in ml from 7 days culture (10^3 CFU/ml)

Table 2. The Plackett-Burman experimental design for 7 factors

Trials	Starch	KN	K2	Mg	Fe	IS	NaCl
1	-	+	+	+	-	-	-
2	+	+	-	-	-	-	+
3	+	-	+	-	-	+	-
4	-	-	-	+	-	+	+
5	+	-	-	+	+	-	-
6	-	-	+	-	+	-	+
7	-	+	-	-	+	+	-
8	+	+	+	+	+	+	+
9	0	0	0	0	0	0	0

Optimization of pH and temperature

The effects of different incubation temperatures (25-60 °C) and different pH (5-9) on bioactive compound(s) production were determined, using optimized medium.

Separation of the active component(s)

The clear supernatant was extracted using several solvent systems, including ethyl acetate, hexane, and chloroform. The solvents were added to the filtrate in a 1:1 (v/v) ratio and were vigorously shaken for thorough extraction. After collecting the organic phase, a rotary evaporator was used to evaporate it under low pressure. This process continued until a thick, dried material was obtained **(Abd-Elnaby *et al.*, 2016)**.

The extracts obtained from various solvents with antibacterial properties were evaluated against *E. coli*. Further studies identified the bioactive compounds extracted using the most effective solvent.

Other applications

Anti-fungal activity

A 10mg/ ml solution of the dried material was diluted in dimethyl sulfoxide (DMSO) and tested for antimicrobial activity using the agar diffusion method. The results were calculated by subtraction the diameter of solvent inhibition zone from sample inhibition zone. Additionally, gentamycin was used as control **(Tenover, 2017)** and potato dextrose medium was used for the fungal cultivation.

Antifouling activity

The fouling bacteria, which were found in 1 milliliter of seawater, were kept at 28 degrees Celsius for 24 hours in a 50-milliliter conical flask with 20 milliliters of nutritional broth medium and covered with glass. As an antifouling agent, approximately 0.1g of the crudely extracted material was applied to the flask. For comparison, a flask was utilized that did not contain crude extract. The incubation period was followed by 10 minutes of dying the cover glass with a 0.4% crystal violet solution. Afterward, the cover glass was wetted, allowed to dry naturally, and subsequently examined microscopically

(Bavya *et al.*, 2011). The antifouling activity was determined according to the degree of loss in bacterial aggregates of cells.

Antiviral activity

For the purpose of determining whether or not *S. variabilis* H2 crude extract inhibits Adenovirus replication, the method used the maximum non-toxic concentration (MNTC) on antiviral assay against Adeno virus. Determination of crude extract cytotoxicity on Vero cell and the procedure of MTT assay was processed according to **Abd-Elnaby *et al.* (2016)**.

Anticancer activity

In order to evaluate the anticancer activity of *S. variabilis* H2 crude extract on three different tumor cell lines: Lung carcinoma cells (A-549), hepatocellular carcinoma cells (HepG-2) and breast carcinoma cells (MCF-7), the cytotoxicity test (MTT assay) and effect of the median inhibitory dose (IC50) were undertaken according to the method of **Siddiqui *et al.* (2015)**.

GC- mass spectra of the bioactive compounds

The chemical composition of the crude extract from the most potent isolate was analyzed at the central lab of the National Institute of Oceanography and Fisheries (NIOF) in Alexandria, Egypt. The identification of chemical constituents in the fractionated extracts was performed using a gas-liquid chromatography-mass spectrometry (GC-MS) model 17A Shimadzu **(Kumar *et al.*, 2010)**. The proportion of each compound was calculated as the ratio of the peak area to the total chromatographic area. Identification of the GC-MS peaks was achieved by comparing the data with profiles reported in the Wiley 275 libraries.

RESULTS AND DISCUSSION

Bioactivity of *S. variabilis* H2

Actinomycetes, especially *Streptomyces* are inexhaustible source and prolific producers of novel antimicrobial agents **(Takahashi & Nakashima, 2018)**. We first tested the bioactivity of *S. variabilis* H2 against six reference bacterial infections to see how well it worked against them (Fig. 1). Against all of the studied bacterial pathogens, *S. variabilis* H2 displayed inhibition zones ranging from 12 to 18mm and exhibited a broad spectrum of antagonistic effects against both Gram-negative and Gram-positive bacteria. Several investigations have provided support for the present findings **(Patel *et al.*, 2014; Abd-Elnaby *et al.*, 2016; Salem *et al.*, 2023)**.

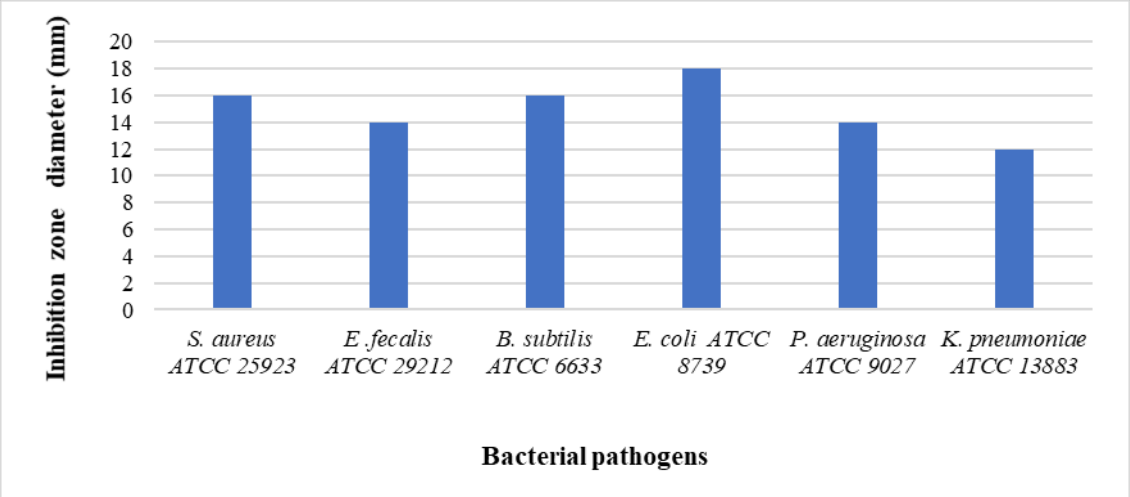

Fig. 1. Mean diameters of inhibition zones (mm) induced by *S. variabilis* H2 against some reference's bacterial pathogens

Enhancement of the production of bioactive compound(s) by *S. variabilis* H2

Improvement of the bioactive compounds production in the present study depends on two tools, the statistical experimental designs and one variable at a time.

Optimizing the culture conditions through statistical design

One effective method for optimizing medium-sized components is the Blackett-Burman design. It was used to find the important factors that improved bioactive chemical production **(El-Sharouny *et al.*, 2015)**. An affordable method was used to accomplish the outcomes **(Xiong & Dongsheng, 2007)**. The Plackett-Burman design has the advantage of being able to rank the impacts of various variables on the measured response, regardless of their nature (physical or dietary factors) or sign (whether they contribute positively or negatively) **(Youssef & Berekaa, 2009; Abd-Elnaby *et al.*, 2016)**. Moreover, it was used to assess the significant effect of starch nitrate agar medium components, as well as other cultural factors such as inoculum size and NaCl, on the production of bioactive compounds produced by *S. variabilis* H2 and tested against various pathogens (*E. coli, P. aeruginosa, B. subtilis, E. faecalis, K. pneumonia,* and *S. aureus*). Examined concentrations of medium components at basal control (0), high level (+) and low level (-) are shown in Table (1). The applied Plackett- Burman experimental design for seven cultural variables and the inhibition zones for the eight tested trails and the basal (trail 9) are illustrated in Table (3)

.

Table 3. The experimental results of the applied Placket-Burman design for seven cultural variables in eight tested trials and basal medium (The matrix of the trials is shown in Table (2)).

Trial no.	Mean diameters of inhibition zones (mm)					
	E. coli	*P. aeruginosa*	*B. subtilis*	*E. faecalis*	*K. pneumoniae*	*S. aureus*
1	19	0	15	10	0	12
2	27	14	19	13	0	12
3	26	0	10	13	0	12
4	10	0	15	10	0	23
5	30	0	10	14	12	0
6	26	0	15	12	0	12
7	0	19	0	13	16	18
8	0	15	22	14	12	0
9	18	14	16	14	12	16

The major influence of each component on the generation of bioactive chemicals, as evidenced by inhibition zones against each pathogen, and the calculated t-values for each variable are displayed in Table (4). The primary effects of the examined factors on the diameter of the inhibition zone were determined through the analysis of these designs. The data demonstrated that the primary effects of the factors under investigation varied depending on the target pathogen. Main effects calculated from results showed that, the negative (–) level of $MgSO_4$ encouraged the production of the antimicrobial agent(s) formed by *S. variabilis* H2, while the positive levels of starch, KNO_3, K_2HPO_4, $FeSO_4$ and inoculum size supported the production of bioactive compounds tested against *E. coli* (Fig. 2). Moreover, the *t*-values represented in Table (4) support the results. Pareto chart of Plackett-Burman showed the effect of different factors on the production of bioactive compounds by *S. variabilis* H2, rationalizing the effect of each variable on the production (Fig. 3). Media with various formulas were expected to be near optimal for producing bioactive chemicals evaluated against various pathogenic bacteria, as indicated in Table (5). Based on these results, a medium of the following formulation (gl^{-1}) was predicted to be near optimum for production of antimicrobial agents against *E. coli*: starch, 30; KNO_3, 1.5; K_2HPO_4, 0.75; $MgSO_4$, 0.25; $FeSO_4$, 0.015; NaCl, 5 and inoculum size of 2ml.

Table 4. Statistical analyses of the Plackett-Burman experimental results

E. coli		
Variable	Main effect	t-value*
Starch	1.44	3.65
KNO_3	0.26	0.58
K_2HPO_4	2.65	4.24
$MgSO_4.7H2O$	-1.63	-4.33
$FeSO_4$	1.125	3.00
Inoculum size	1.88	3.00
NaCl	0.00	65535

P. aeruginosa		
Variable	Main effect	t-value*
Starch	0.58	0.24
KNO_3	1.16	0.43
K2HPO4	-0.16	-0.04
$MgSO_4.7H_2O$	-2.25	-1
$FeSO_4$	6.00	2.66
Inoculum size	1.25	0.55
NaCl	0.00	65535

B. subtilis		
Variable	Main effect	t-value*
Starch	4.416	1.86
KNO_3	-1.666	-0.61
K_2HPO_4	-1.833	-0.48
$MgSO_4.7H_2O$	2.25	1.00
$FeSO_4$	0.75	0.33
Inoculum size	2.00	0.88
NaCl	0.00	65535

E. fecalis		
Variable	Main effect	t-value*
Starch	-0.19	-1.47
KNO_3	-0.01	-0.09
K_2HPO_4	0.59	2.86
$MgSO_4.7H_2O$	-0.37	-3.00
$FeSO_4$	0.12	1.00
Inoculum size	1.12	9.00
NaCl	0.00	65535

K. pneumoniae		
Variable	Main effect	t-value*
Starch	-2.55	-1.21
KNO_3	0.88	0.36
K_2HPO_4	2.77	0.83
$MgSO_4.7H_2O$	1.0	0.50
$FeSO_4$	2.0	1.00
Inoculum size	1.00	0.50
NaCl	0.00	65535

S. arues		
Variable	Main effect	t-value*
Starch	0.30	0.13
KNO_3	1.48	0.58
K_2HPO_4	-4.90	-1.38
$MgSO4.7H_2O$	-2.37	-1.11
$FeSO_4$	-0.62	-0.29
Inoculum size	-5.12	-2.41
NaCl	0.00	65535

t-value significant at the 1% level=3.70
*t-value significant at the 5% level=2.45

*t-value significant at the 10% level=1.94
*t-value significant at the 20% level=1.37

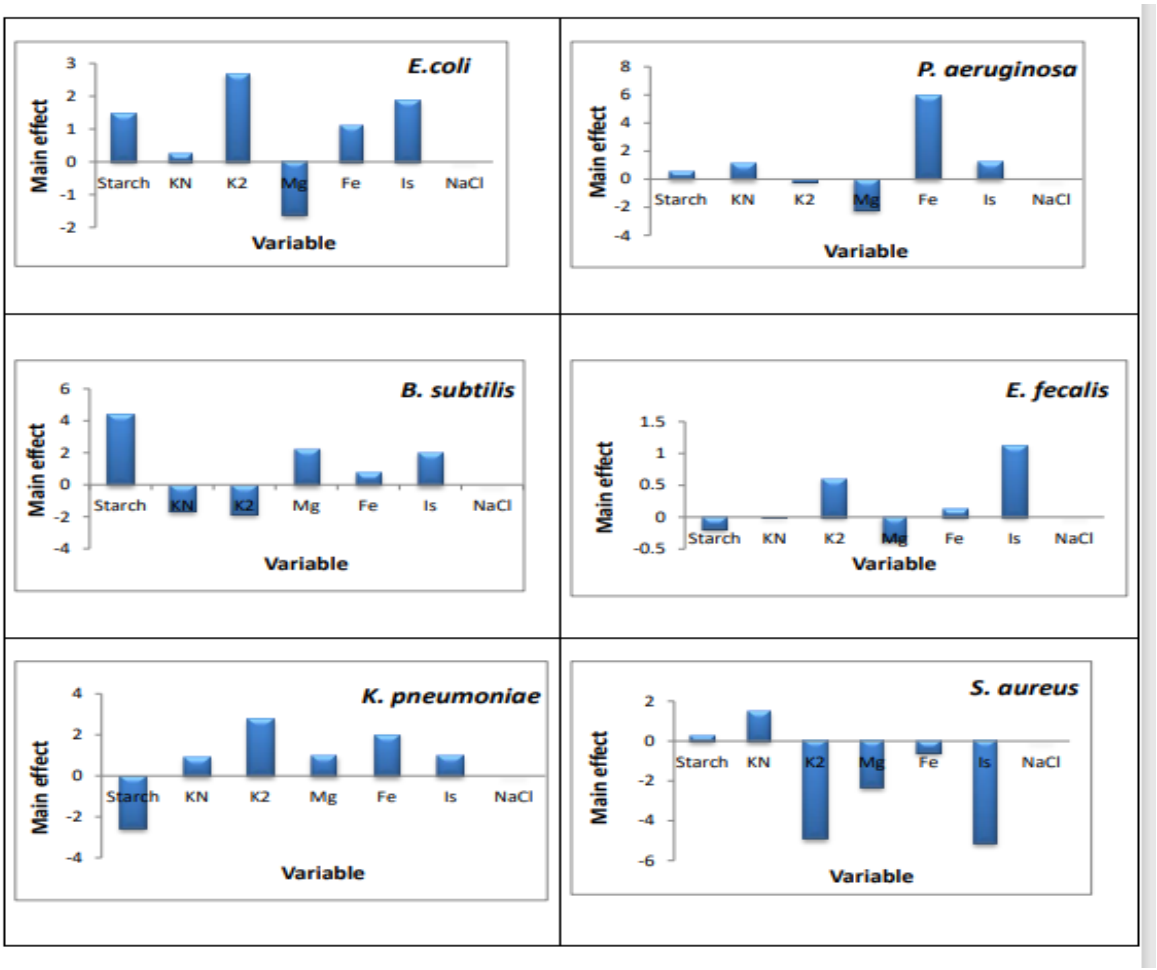

Fig. 2. Main effects for bioactive compounds produced by *S. variabilis* H2 and tested against different pathogenic bacteria

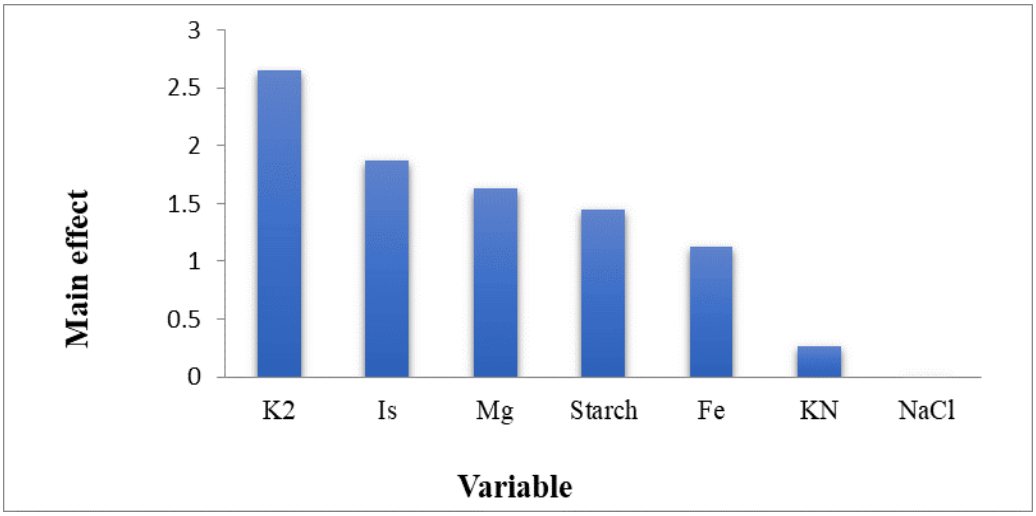

Fig. 3. Pareto chart of Plackett-Burman showing the effect of different factors on bioactive compounds production by *S. variabilis* H2 and tested against *E. coli*

Table 5. Basal medium and predicted formula for verified medium to be near optimum for production of bioactive compounds by *S. variabilis* H2 and tested against different pathogenic bacteria

Variable	**Basal medium**	**Verified medium**					
(g l^{-1})		*E. coli*	*P. aeruginosa*	*B. subtilis*	*E. faecalis*	*K. pneumoniae*	*S. aureus*
Starch	20.0	30.0	30.0	30.0	10.0	10.0	30
KNO_3	1.0	1.5	1.5	0.5	0.5	1.5	1.5
K_2HPO_4	0.5	0.75	0.25	0.25	0.75	0.75	0.25
$MgSO_4.7H_2O$	0.5	0.25	0.25	0.75	0.25	0.75	0.25
$FeSO_4$	0.01	0.25	0.015	0.015	0.015	0.015	0.005
Inoculum size (ml)	1.0	2.0	2.0	2.0	2.0	2.0	0.5
NaCl	5.0	5.0	5.0	5.0	5.0	5.0	5

Verification experiment

The produced optimized medium was validated by conducting a second set of experiments to confirm the optimization outcome. During a 7-day growth experiment, *S. variabilis* H2 was grown on the optimized medium. The results showed that the metabolites had the strongest antagonistic effect against *E. coli*. Compared to the case when the actinomycete was cultivated in the basal medium, a larger inhibition zone of 18mm was observed with an increase of 1.29 times. The improved medium was proven true by this outcome (Fig. 4).

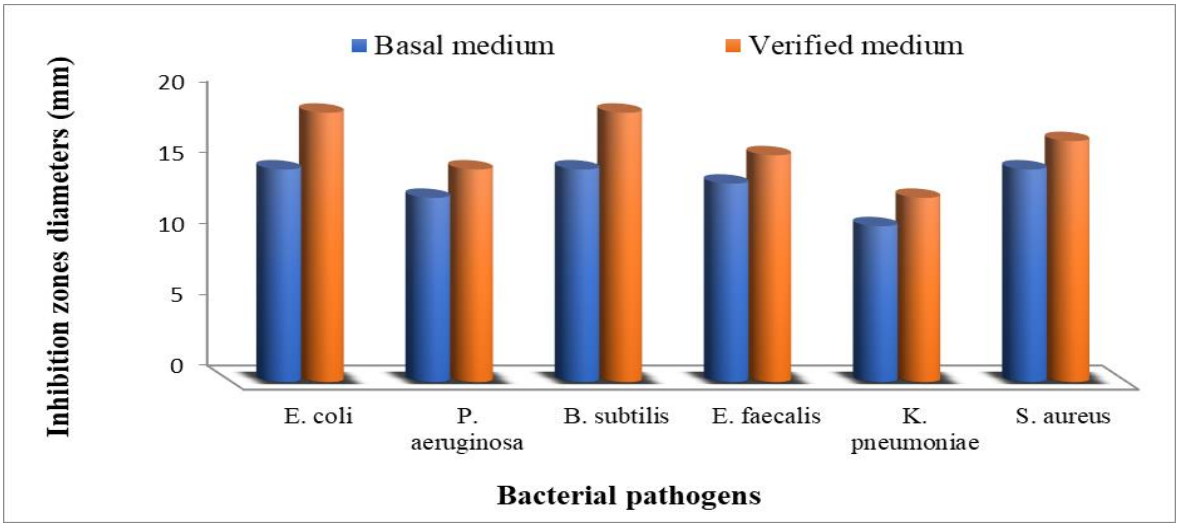

Fig. 4. A verification experiment for the activity of bioactive compounds produced by *S. variabilis* H2, grown on basal versus verified media and tested against different bacterial pathogens

Another data obtained by **Abd-Elnaby *et al*. (2016)** demonstrated that the low (-1) level of starch and the high (+1) levels of K_2HPO_4, KNO_3, $MgSO_4$, $FeSO_4$, inoculum size and pH enhanced the production of antimicrobial agent(s) formed by *Streptomyces parvus* and tested against *Aeromonas hydrophila*, the optimized medium formulated increased the diameter of inhibition zone by about 1.6-fold. Additionally, **Hamed *et al.* (2019)** showed that the growth of *Streptomyces* sp. MK388207 under the optimized culture conditions led to a 1.4-fold increase in its antimicrobial activity.

One variable at a time

One factor at a time method was used to choose the dynamic variables, this method depended on studying one factor, while the other variables were constant.

Effect of pH

One of the most important ecological factors is pH. The growth of *S. variabilis* H2 on the optimized medium at different pH levels (5–9) was tested to enhance the production of bioactive compounds against *E. coli*. The data indicated that the optimum pH was 8, where the inhibition zone reached 20mm (Fig. 5).

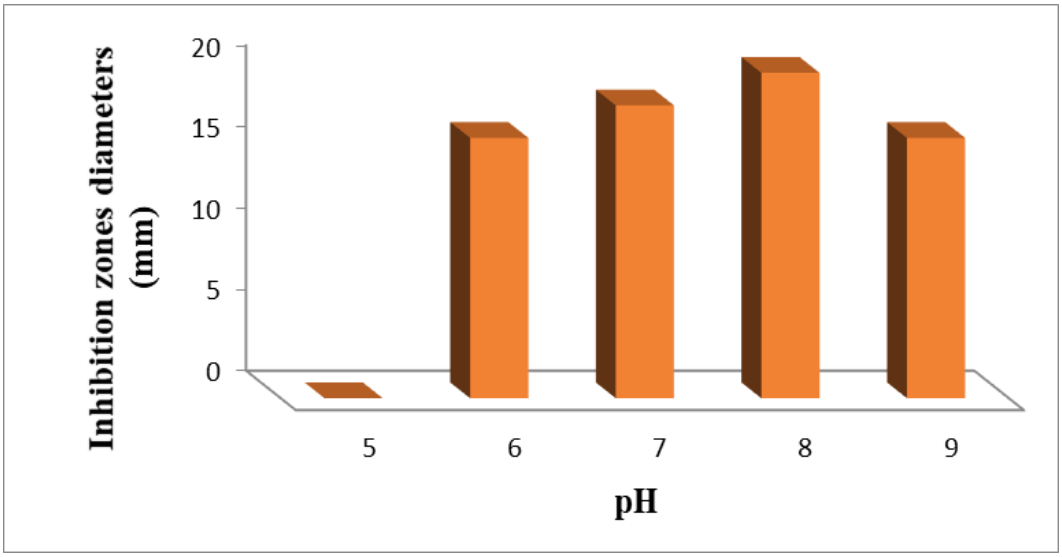

Fig. 5. Effect of different pH on the production of bioactive compounds by *S. variabilis* H2

Previous study done by **Abd-Elnaby *et al*. (2016)** tested the effect of different pH levels on the bioactive compounds production *by Streptomyces parvus* against the bacterial pathogen *Aeromonas hydrophila*. The results explained that the produced bioactive secondary metabolites exhibited the highest activity when the culture's conditions were adjusted to pH 8. Similar results were obtained by **El-sersy and Abou-Elela (2006)**.

Effect of temperature

Temperature plays an important role in the life of organisms and affects their metabolism. Effect of different incubation temperatures (25, 30, 37 and 45°C) on growth

of *S. variabilis* H2 (on verified medium) and production of antimicrobial agent(s) against *E.coli*, was evaluated. The highest inhibition (21mm zone diameter) was observed in cultures cultivated at 37°C (Fig. 6).

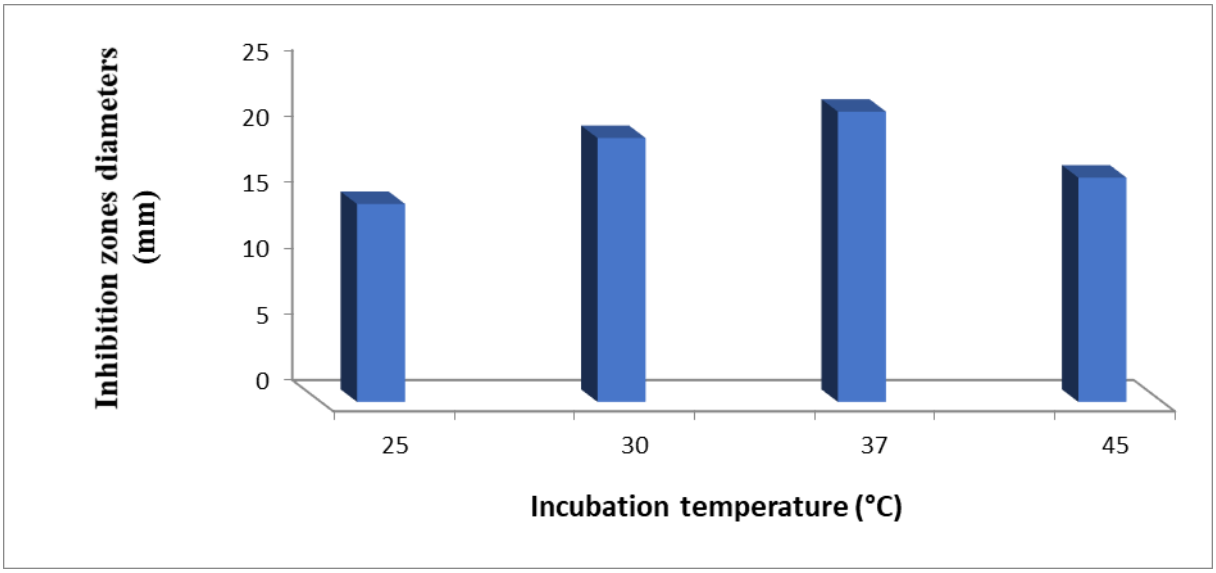

Fig. 6. Effect of incubation temperature on bioactive compounds production by *S. variabilis* H2

Increasing the incubation temperature caused *Streptomyces parvus* to create more bioactive chemicals, according to a prior study by **Abd-Elnaby *et al.* (2016)**. The cultures showed the greatest inhibition zone (29mm) and highest activity when incubated at 35°C. Additionally, **El-Sersy and Abou-elela (2006)** noted that, when the incubation temperature was changed to 40°C, marine *Nocardia brasiliensis* had the strongest inhibitory action against the fish disease *Vibrio damsela*. They concluded that raising the incubation temperature improved the antibacterial agent production.

Extraction of the bioactive compounds from *S. variabilis* H2

The crude bioactive chemicals generated from *S. variabilis* H2 were extracted using ethyl acetate, n-hexane, and chloroform, and their efficacy was evaluated. Each solvent's crude extract was independently evaluated for its antibacterial effectiveness against *Escherichia coli*; the pathogen that was most severely impacted. Fig. (7) summarizes the screening test results, which showed that the most potent solvent for extracting the necessary bioactive components was the ethyl acetate crude extract of *S. variabilis* H2. It also showed the best antibacterial activity, with an inhibitory zone diameter of 21mm. However, when chloroform and hexane were used, there was no antibacterial action and it was extremely weak (Fig. 7). Accordingly, ethyl acetate was chosen as the best solvent for this study because of how well it extracted the target chemical.

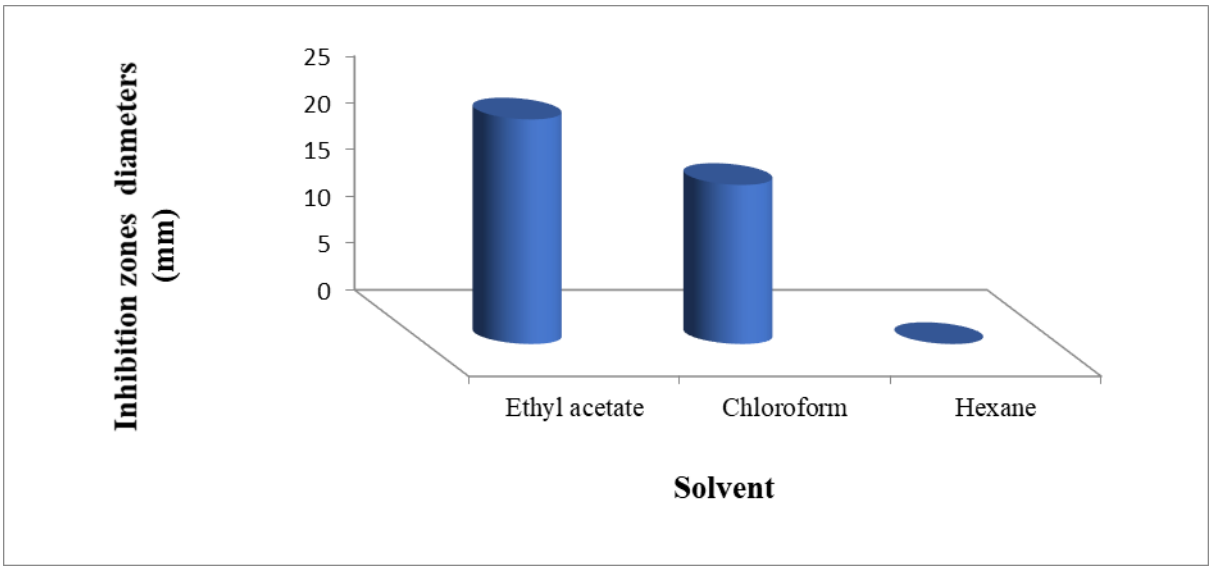

Fig. 7. Antibacterial activity of different solvent extracts produced by *S. variabilis* H2 and tested against *E. coli*

The antimicrobial activity of *Streptomyces parvus* crude extracts resulting from three solvent (chloroform, n-butanol and ethyl acetate) were tested against *Aeromonas hydrophila* in terms of inhibitory zone, and the results demonstrated that n-butanol extract had the largest at 26mm, followed by ethyl acetate extract at 24mm, and chloroform at 23mm **(Abd-Elnaby *et al.*, 2016)**.

Applications of *S. variabilis* H2 crude extract

A wide taxonomic range of actinomycetes especially *Streptomyces* have the ability to produce bioactive compounds with biological activities such as antifungal, antibacterial, antiviral, immunosuppressant and anticancer **(Devi, 2011)**.

Antifungal activity of *S. variabilis* H2

The *S. variabilis* H2 crude extract had potential activity against tested pathogenic fungi and the inhibition zones ranged from 11mm (against *Aspergillus flavus*) to 25mm (against *Candida glabrata*). The inhibition zones due to the bioactivity of *S. variabilis* H2 crude extract was near to the inhibition zones recorded for gentamycin which tested as control (Fig. 8). **Bharti *et al.* (2010)** reported that a total of 316 actinomycetes were isolated from 69 soil samples. Of the 316 isolates tested, 98 (31.01%) showed antifungal activity against at least one pathogen. Six of the 98 active isolates showed antimicrobial activity against *Candida albicans*, *Candida gypseum*, *Candida flavus*, and *Candida fumigatus*; seven isolates exhibited activity against all of the fungal infections that were examined. When compared to other genera, *Streptomyces's* antagonistic potential stood out.

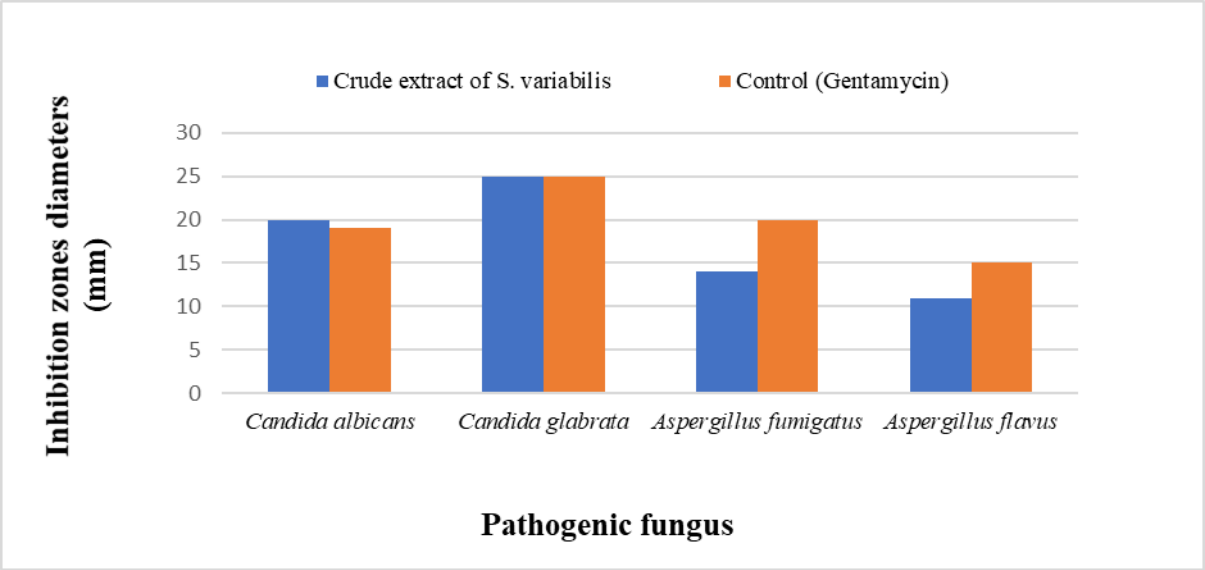

Fig. 8. Inhibition zones diameters (mm) due to *S. variabilis* H2 crude extract tested against some fungal pathogens

Antifouling activity of *S. variabilis* H2

The result of the inhibitory activity of bioactive compounds produced by *S. variabilis* H2 on bacterial biofilm formation is illustrated in Fig. (9). The crude extract from *S. variabilis* reduced the bacterial cells' density and acted as an anti-biofouling agent. In contrast to manufactured antifoulants, which contribute to environmental degradation, the prepared extract showed promise as a source of environmentally acceptable antifouling chemicals, according to the present study. This finding is consistent with the results of **Bavya *et al.* (2011)**, who studied the antifouling effect of *S. filamentosus*, which reduced the formation of biofilm on glass. Moreover, **Abd-Elnaby *et al.* (2016)** discussed that the N-butanol extract from *S. parvus* inhibited a biofilm formation on cover slip. The treated cover slip showed a smaller number of organisms than that of control cover slip; this means that strain showed good antifouling activity.

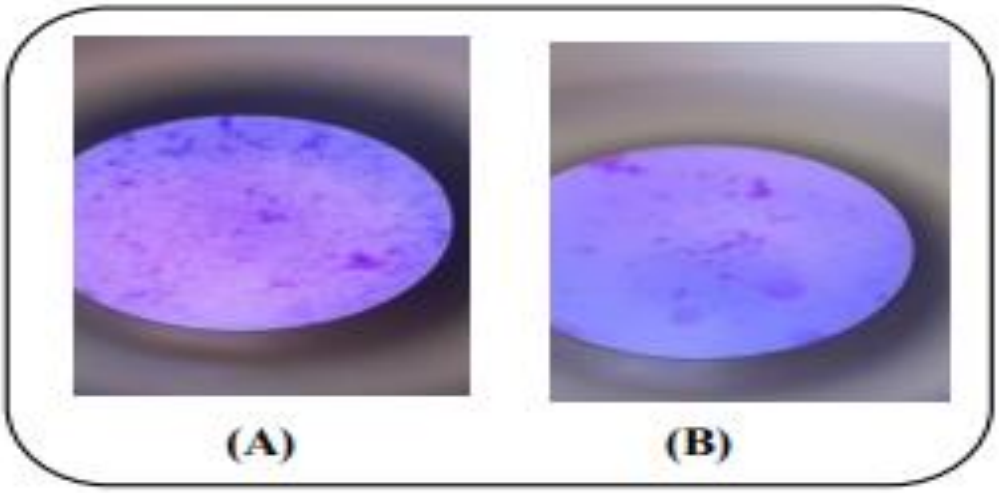

Fig. 9. Photographs illustrating the antifouling effect of *S. variabilis* H2 crude extract **(A)** in the absence of crude extract and **(B)** in the presence of crude extract

Antiviral activity of *S. variabilis* H2

The MTT assay was used to screen the in *vitro* anti-Adeno virus. The VERO cell (ATCC ccl-81) cytotoxicity study showed that the crude extract had a maximum non-toxic concentration (MNTC) of 2500µg/ ml. The adenovirus antiviral test utilized this MNTC. With a viral activity of 98.7 percent at MNTC, the crude extract of the actinomycete *S. Variabilis* H2 was unable to prevent Adenovirus replication (Table 6). This finding is in line with a previous research that found no anti-viral effects of *S. parvuscrude* extract when applied to HCV **(Abd-Elnaby *et al.*, 2016)**.

Table 6. The percentage of growth inhibition of *S. variabilis* H2 crude extract at different doses against Adeno virus using the MTT assay

Test	Conc. ug/ml	Mean OD	Viability	Toxicity	Viral activity %	Anti-viral effect %
Control Vero cells		0.329	100	0		
Adeno virus		0.144	43.769	56.231	100	0
	2500	0.146333	44.47822	55.52178	98.73874	1.261655924
Tested sample	1250	0.145333	44.17427	55.82573	99.27928	0.721115354
	625	0.145	44.07295	55.92705	99.45946	0.540935164

Anticancer activity of *S. variabilis* H2

To begin determining the anticancer activity, the toxicity of the crude extract was tested. Then, the extract's activity was assessed against three distinct cell lines: A-549, which represents lung carcinoma; HepG-2, which represents hepatocellular carcinoma; and MCF-7, which represents breast carcinoma. Some cell lines' viability percentages decreased in a dose-dependent manner, according to dimethylthiazole diphenyl treazlioum bromide (MTT) assay results (Table 7). The anticancer activity against A549 cells was maximum at an IC50 of 2166.97µg/ ml, whereas the lowest anticancer activity against the HepG2 cell line was 6738.95µg/ ml. According to the results, two different tumor cell lines were satisfactorily affected by the crude extract of *S. variabilis* H2. The anticancer activity of the crude extract of *S. variabilis* H2 on the A-549 tumor cell line was rather high, as demonstrated in Table (7). At a concentration of 2500µg/ ml, which is close to the IC50, the extract caused a nearly 61.57% inhibition. Additionally, at a concentration of 5000µg/ mL, which is below the IC50 value identified by the MTT assay, the crude extract of S. variabilis H2 inhibited 44.51% of HepG-2 cancer cells. However, the MTT assay revealed that the tested crude extract had no effect on the Mcf-7 cancer cell line, indicating that *S. variabilis* H2 lacked anticancer activity (Fig. 10). Furthermore, the evaluated cancer cell lines' morphology was changed by the *S. variabilis* H2 crude extract. A related study found that crude extract of *Streptomyces parvus* had anticancer activity when tested against four distinct cell lines: one for each of human liver, breast, murine lymphoma, and colon cancer-related cells. Inhibitory actions ranged from 42 - 57 to 56 - 53% **(Abd-Elnaby *et al.*, 2016)**. Isolated *Sterptomyces* strains from Western Ghats humus soils were also reported by **Vijayabharathi *et al.* (2011)**. This particular strain showed anticancer properties when tested *in vitro* against HepG-2 and HeLa cervical carcinomas. Additionally, three fractions of *Streptomyces* sp. H7372, a new strain, were identified by **Yip *et al.* (2010)**. They found that 31-2, one of the fractions, had an anti-proliferative and cytostatic effect on MCF-7 and MDA-MB-231 breast cancer cells, without inducing cytotoxicity **(Yip *et al.*, 2010)**. One of the most significant sources for new anticancer drugs are the secondary metabolites of the *Streptomyces* genus. According to **Nguyen *et al.* (2020)**, *Streptomyces* sp. VN1 was found to create a number of secondary metabolites, one of which was an anticancer chemical that was not naturally occurring.

Table 7. IC50 and percentage of growth inhibition of *S. variabilis* H2crude extract at different doses against A549, HepG-2 and Mcf-7 cancer cell lines using the MTT assay

A549 cancer cell lines						
ID	Conc. (µg/ml)	Mean (O D)	ST.E	Viability %	Toxicity %	IC50
A-549	1:2	0.334	0.006429	100	0	µg/ml
Sample	10000	0.024	0.001528	7.185628743	92.81437126	2166.97
	5000	0.053	0.007	15.86826347	84.13173653	
	2500	0.128333	0.005364	38.42315369	61.57684631	
	1250	0.275333	0.006333	82.43512974	17.56487026	
	625	0.332333	0.005207	99.500998	0.499001996	
	312.5	0.332333	0.002404	99.500998	0.499001996	
HepG-2 cancer cell lines						
ID	Conc. (µg/ml)	Mean (OD)	ST.E	Viability %	Toxicity %	IC_{50}
HepG-2	1:2	0.357	0.003606	100	0	µg/ml
Sample	10000	0.073667	0.006009	22.05588822	77.94411178	6738.95
	5000	0.185333	0.005548	55.48902196	44.51097804	
	2500	0.328333	0.003528	98.30339321	1.696606786	
	1250	0.338333	0.007535	101.2974052	0	
	625	0.33	0.003464	98.80239521	1.19760479	
	312.5	0.335667	0.002333	100.499002	0	
Mcf-7cancer cell line						
ID	Conc. (µg/ml)	Mean (OD)	ST.E	Viability %	Toxicity %	IC50
Mcf-7	1:2	0.373	0.004726	100	0	µg/ml
Sample	10000	0.363667	0.006741	97.49776586	2.502234138	-------
	5000	0.367	0.004359	98.39142091	1.608579088	
	2500	0.374333	0.005239	100.357462	0	
	1250	0.366667	0.004333	98.30205541	1.697944593	
	625	0.373667	0.002848	100.178731	0	
	312.5	0.374333	0.002028	100.357462	0	

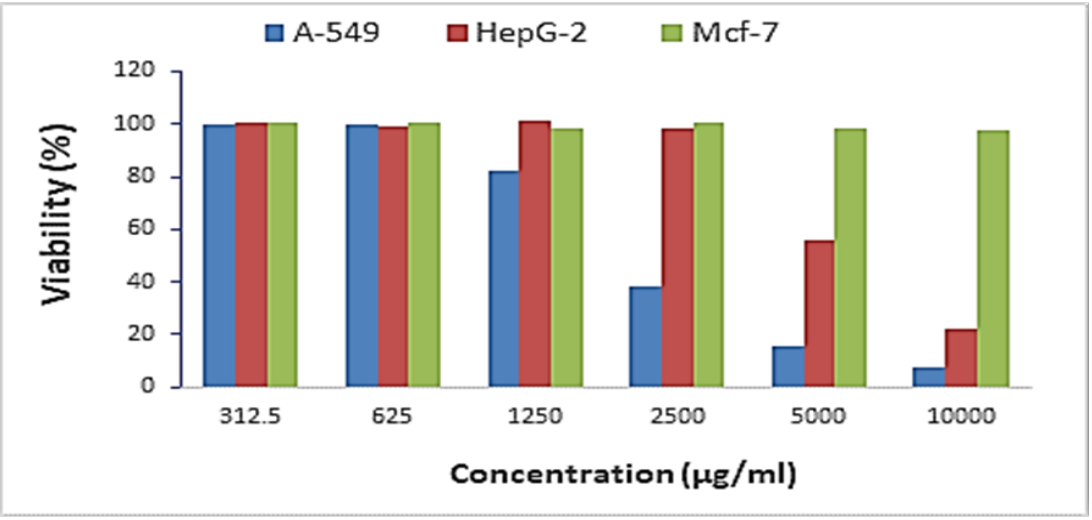

Fig. 10. Cytotoxicity of *S. variabilis* H2 crude extract against lung cancer cell line (A-549), hepatocellular cancer cell line (HepG-2) and breast cancer cell line (MCF-7)

Gas-liquid chromatography mass spectra (GC-MS) of the crude extract

The GC-MS of *S. variabilis* H2 crude extract determined the main constitutes and the relative abundance of the identified compounds (Fig. 11). It was found that, the main constituents were hexadecanoic acid, methyl ester, 5-Hydroxymethylfurfural, Phthalic acid, di(2-propylpentyl) ester and octadecanoic acid, methyl ester (Table 8 & Fig. 12). Reports indicate that the primary chemical component has biomedical relevance in the pharmacological domains, suggesting promising therapeutic applications. The antibacterial capabilities could be attributed to chemical components such hexadecanoic acid, octadecatrienoic acid, pentadecanoic acid, heneicosanoic acid, and oleic acid **(Mishra *et al.*, 2009)**. In addition, **Kumar *et al.* (2010)** came to the conclusion that certain substances had anticancer characteristics, such as hexadecanoic acid, methyl ester and 9,12- octadecadienoic acid (Z,Z)-menthol. Both hexadecanoic acid and 9,12-octadecadienoic acid have beneficial impacts on health, with the former having antioxidant, nematicide, hypocholesterolemic, and pesticide properties and the latter having anticarcinogenic, antiatherogenic, antioxidant, and anti-inflammatory properties **(Jain *et al.*, 2012)**. Furthermore, **Zhao *et al.* (2013)** reported the antioxidant and antiproliferative activities of 5-Hydroxymethylfurfural. Recently, **Erwin *et al.* (2020)** reported that *Streptomyces lasalocidi* sp., an actinomycete isolated from soil, produces the polyether antibiotic lasalocid.

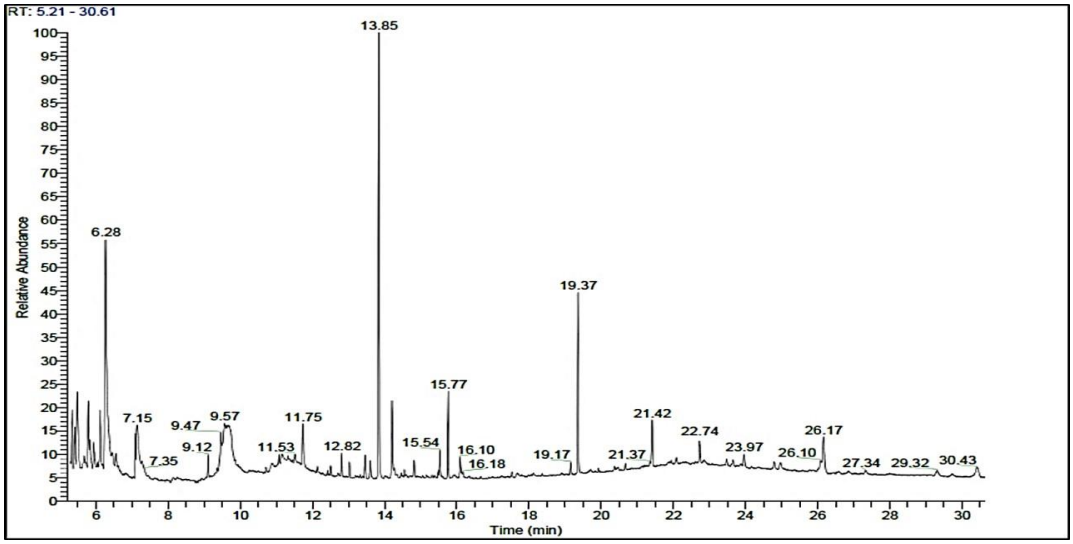

Fig. 11. Gas-liquid chromatography mass spectra of *S. variabilis* H2 crude extract

Table 8. The main constituents of the chemical composition for *S. variabilis* H2 crude extract using GC-MS

Compound	Retention time (min.)	Formula	Molecular weight	Relative abundance
Hexadecanoic acid, methyl ester	13.85	$C_{17}H_{34}O_2$	270	99
5-Hydroxymethylfurfural	6.28	$C_6H_6O_3$	126	56
Phthalic acid, di(2-propylpentyl) ester	19.37	$C_{24}H_{38}O_4$	390	45
Octadecanoic acid, methyl ester	15.77	$C_{19}H_{38}O_2$	298	23

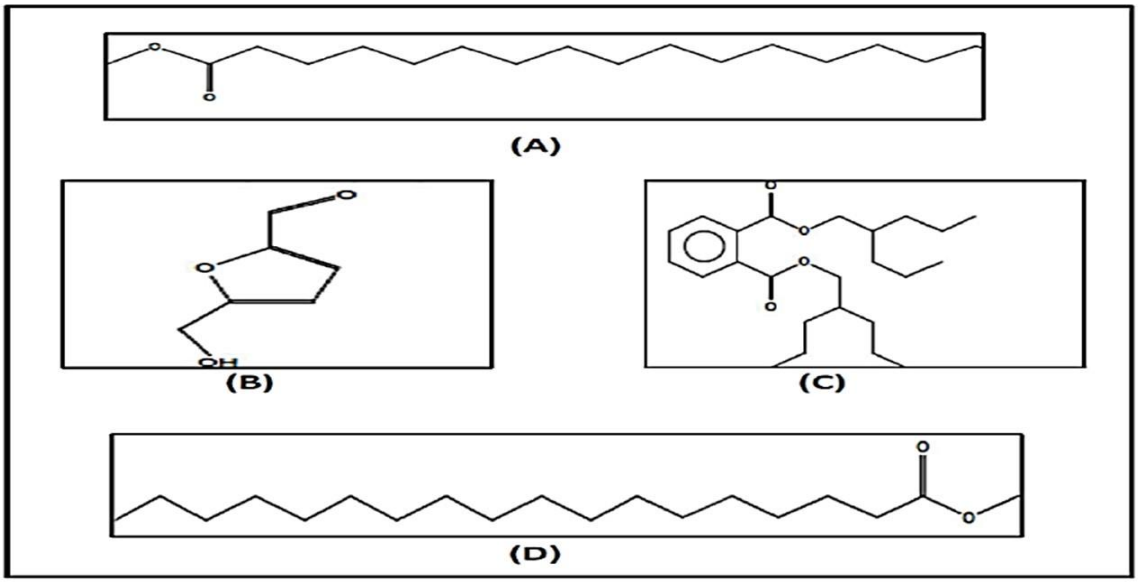

Fig. 12. The main constituents of *S. variabilis* H2 crude extract. **(A):** Hexadecanoic acid, methyl ester, **(B):** 5-Hydroxymethylfurfural, **(C):** Phthalic acid, di(2-propylpentyl) ester and **(D):** Octadecanoic acid, methyl ester

CONCLUSION

The biotechnological potential of *Streptomyces variabilis* H2 was highlighted by its great antibacterial activity against several Gram-negative and Gram-positive bacteria. Notably, it showed the strongest inhibition against *E. coli*. The production of bioactive compounds was increased by 1.3 times when growth conditions were optimized using the Plackett-Burman design. In addition to its antifungal and anti-biofouling capabilities, the crude extract of *S. variabilis* H2 demonstrated significant anticancer activity, namely against cell lines representing lung and hepatocellular carcinoma. Nevertheless, it was ineffective against viruses. The chemical diversity and potential for additional applications in biotechnology and medicine are highlighted by the identification of key bioactive chemicals in the extract, which include phthalic acid, di(2-propylpentyl) ester, octadecanoic acid, methyl ester, and hexadecenoic acid, methyl ester.

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