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Distribution of Actionmycetes in Suez Gulf (Egypt) and Optimization of the Nanoparticles Production of Some Isolates

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Abstract: Sediment samples were collected from the shallow areas of the Suez Gulf during winter 2014. Eight locations were selected for this study included El-Tour, RasSidr, Suez North, Suez Middle, AdabiaHarbour, Ain Sokhna, RasGharib and RasShokheir. The physico- chemical characters of the samples were determined. The highest count of the acteniomycetes recorded in Adabia harbor (22cfu/gm) and the lowest count recorded in Ain sokhna station (6cfu/gm). Out of 41 actinomycetes isolates, only 11 isolates showed antimicrobial activities against the bacterial pathogens *Bacillus subtillus*6633, *Staphylococcus aureus* 25923, *Pseudomonas aeruginosa* 902 , *Bacillus cereus*, *Salmonella typhimurium* 14028, *Escherichia coli* 19404, and *Vibrio damsela*. Two isolates identified by molecular techniques as *Streptomyces rochei* HMM13 and *Streptomyces* sp., these two isolates were able to produce gold (0.035, 0.013), silver (0.376, 0.355) and zinc (4.080) nanoparticles. Plackett-Burman Design were applied for optimizing the production of nanoparticles by *Streptomyces rochei* HMM13 against *S. typhimurium* 14028, *E-coli* 19404 and *Vibrio damsela* .The productivity increased by 1.16 , 1.13 and 1.32 fold, respectively.

Keywords: Acteniomycetes, Nanoparticles, Antimicrobial activity, Plackett- burman design, Optimization, characterization, green synthesis**.**

1 Introduction

Marine environments present an invaluable source of new natural products that may hold important leads for future drug discovery and development [1,2]**.** These environments are still in their infancy for isolation of new microbes that can produce pharmaceutically valuable metabolites [3,4].

Actinomycetes are Gram-positive filamentous spore formers with high G+C (>55%) content of DNA. They are free living saprophytic bacteria forming a major group of soil population. *Actinobacteria* are widely distributed in terrestrial and aquatic ecosystems, especially in soil [5]. Around 23,000 bioactive secondary metabolites produced by microorganisms have been reported and actinomycetes alone produce 10,000 of these compounds. Many of these secondary metabolites are potent antibiotics, which has made *Streptomycetes* the primary antibiotic-producing organisms exploited by the pharmaceutical industry [6].

The reduction of metal ions using biological systems leads to the formation of size controlled, stable, and dispersed nanoparticles, which possesses attractive physicochemical properties [7, 8]. Biosynthesis of nanoparticles as the name indicates help in the synthesis of very complex reaction within a fraction of minutes have now taken up the attention towards synthesis grievance the need of environmentally benign technologies in material science[9]. The biosynthesis of nanoparticles by microorganism is a green and eco-friendly technology [10].

Actinomycetes are efficient candidates for metal nanoparticles production extracellularly and intracellularly. The synthesis of nanoparticles by actinomycetesre presents good stability and polydispersity. Also, actinomycetes possess important biocidal activity against different pathogens [11, 12].

Optimization of the growth conditions, such as media components, pH, temperature, substrate concentration and inoculums size will not only support the growth but also enhance the productivity and monitor the rate of enzyme activity which affects the synthesis of nanoparticles [11].

Therefore, the present preliminary study was aimed to evaluate the distribution of marine actinomycetes in sediment of Suez-Gulf, Egypt, and optimize the culture conditions using Plackett–Burman Design to enhance the production of nanoparticles by some isolates.

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2 Materials and Methods

2.1 Sampling Sites

Sediment samples were collected from the shallow areas of the Suez Gulf during winter, 2014. The eight locations selected for this study included El-Tour, RasSidr, Suez North, Suez Middle, AdabiaHarbour, Ain Sokhna, RasGharib and RasShokheir. They are distributed along the Suez Gulf as shown in (Figure1).Samples were immediately transported to the laboratory in an ice box for bacteriological analysis which was always completed within 24 h [13].

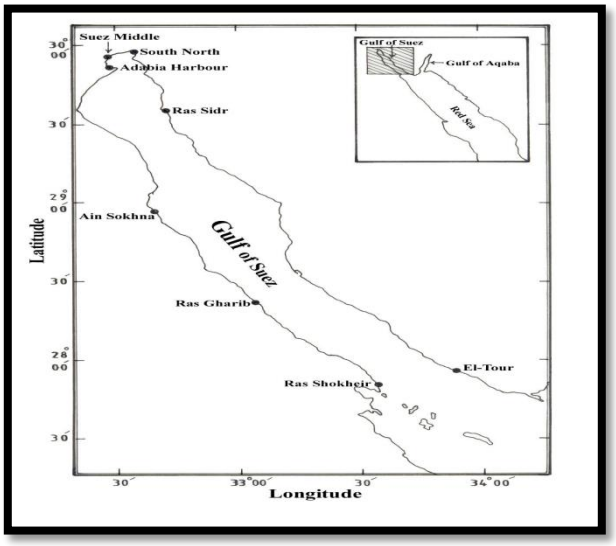


Fig. 1: Sampling sites along the Suez Gulf

2.2 ActinomycetesIsolation and Enumeration

The sediment samples were collected from the shallow areas of the Suez Gulf at depth (0-10 cm). The collected sediment samples were stored in ice box and then transported to the laboratory for bacteriological analysis which completed within 24 h[13].A suspension of sediment samples was prepared by taken 10g from each sample and then added to 30 ml sterilized sea water, shacked for 20 min. Starch nitrate agar medium was provided with 75 and 25µg ml^{-1} of filter sterilized cycloheximide and nystatin respectively to minimize fungal contamination. All plates inoculated with 1 ml of the suspension. A triplicate set of dishes was used incubated for 7 days at 32 °C [14].

2.3Physicochemical Characterization of Sediment Samples

Physical parameters as temperature, pH and salinity were measured in sediment samples. Also chemical characters as dissolved oxygen [15], dissolved phosphate [16], dissolved nitrate [17], dissolved nitrite [16], dissolved ammonia [18] and organic matter [19]were measured using standard methods.

2.4 Preparation of Cell Free Microbial Extract

The culture medium was prepared, sterilizedand inoculated with fresh culture of the actinomycetes isolates. The cultured flasks were incubated at 32 ºC for 7 days. After incubation time the cultureswere centrifuged at 12000 rpm and theirsupernatant was used for further experiments [20].

2.4.1Biological Synthesis of Nanoparticles by ActinomycetesIsolates

2.4.2 Biosynthesis of Silver Nanoparticles (AgNPs)

Eachsupernatant from actinomycetesisolateswere challenged with 0.5-2.0 mM silver nitrate ($AgNO_3$) with (pH 6.5 – 9.5)as precursor and the flasks were incubated at 37°C in a shaker under dark condition and observed for color change.The reduction of Ag^+ ions was monitored by sampling an aliquot (2 ml) of thesolution at intervals of 24 h and measuring the UV-Vis spectra in range of 300 to 650 nm by using UV-Vis spectrophotometer (Double Beam Spectrophotometer 6800 JENWAY) of the solution[21].

2.4.3 Biosynthesis of Gold Nanoparticles (AuNPs)

Typical reaction mixtures contained equal volumesof actinomycetessupernatant and 0.5, 0.1 and 2.0 mM from chloroauric acid solution ($HAuCl_4$). The reactionmixtures were incubated at room temperature for 4 h in dark condition. The effect of pH on AuNPs synthesis was studiedby adjusting pH of reaction mixture to 2, 3, 4 and 5.Control experiments were conducted with uninoculated media, to check for the role of bacteria in the synthesis of nanoparticles. The absorption spectrum of AuNPs was determinedin range of200-700 nm by using UV-Vis spectrophotometer (Double Beam Spectrophotometer 6800 JENWAY)[22].

2.4.4Biosynthesis of Zinc Nanoparticles (ZnNPs)

Actinomycetes supernatant was added separately to the reaction vessel containing (50, 100 and 150) mM zinc sulfatesolution (v/v). The reaction was carried out in light conditions for 24 h,at 37 °C, pH: (4-7) in rotary shaker with 120 rpm. Supernatant is further used for UV-Visible spectrum by UV-Vis spectrophotometer (Double Beam Spectrophotometer 6800 JENWAY) between 300 to 600 nm at room temperature [23].

2.5 Antimicrobial Assay for Biosynthesized Nanoparticles

Antimicrobial activity of biosynthesized nanoparticles was analyzed for comparative study using agar well diffusion method. The used bacterial indicators were *Bacillussubtillus*6633, *Staphylococcus aureus* 25923, *Pseudomonas aeruginosa* 9027, *Bacillus cereus*, *Salmonella typhimurium* 14028, *Escherichia coli* 19404and *Vibrio damsela*. 100 µl of biosynthesized nanoparticles were used as a test sample against the pathogens[21].

2.6 Characterization of Silver Nanoparticles

2.6.1UV-Visible Spectroscopy Analysis

A small aliquot was drawn from the reaction mixture and a spectrum was taken on a wavelength from 300-600nm on UV-Vis spectrophotometer (Double Beam Spectrophotometer 6800 JENWAY). The reduction of metallic Ag^+ ions was monitored by measuring the UV- Vis spectrum after reaction. [24].

2.6.2X-ray Microanalysis

50 µl of the nanoparticles samples were fixed on the specimen stubs and then the samples were examined under x-ray microanalyzer (Module Oxford 6587 INCA x-sigh) attached to JEOL JSM-5500LV scanning electron microscopy at 20 KeV after gold coating using SPI-Module sputter coater[25].

2.6.3 Fourier Transform-Infrared Spectroscopy (FTIR)

A known weight of sample (1 mg) was taken in a mortar and pestle and ground with 2.5 mg of dry potassium bromide (KBr).The samples were scanned using infrared in the range of 4000 to 400/cm using Fourier Transform Infrared Spectrometer. The spectral data obtained were compared with the reference chart to identify the functional groups present in the sample [26].

2.6.4 Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was used to observe the size, shape and morphology of the resultant nanoparticles. A specimen for SEM sample was made by casting a drop of suspension on a carbon-coated copper grid and the excess solution was removed by tissue paper and allowed to air dry at room temperature [26].

2.7 Identification of the Most Potent Strain

The promising actinomycetes' isolate was cultured in starch-nitrate liquid medium for seven days and genomic DNAs were extracted with the genomic DNA extraction protocol of Gene Jet genomic DNA purification Kit (Fermentas).Polymerase chain reaction (PCR) using Maxima Hot Start PCR Master Mix (Fermentas).The amplifications were carried out in a thermal cycler (Multigene Optimax, Labnet international, Inc). The PCR thermocycler was programmed as follow: 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 10min. The PCR mixture contained 25 pmol of each primer, 10 ngchromosomal DNA, 200 mmol/LdNTPs 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 Gene JET™ 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 sequence with universal primers (16S 27F and 16S1492R),(5′AGAGTTTGATCCTGGCTCAG-3′ and 5′-GGTTACCTTGTTACGACTT-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) [27].Sequences of rRNA genes, for comparison, were obtained from the National Center for Biotechnology Information (NCBI) database.

2.8Enhancement of the Silver Nanoparticles Production by the Most Potent Isolate

2.8.1Plackett- Burman Design

The Plackett-Burman experimental design [28]was used to evaluate the relative importance of various factors involved in the production of silver nanoparticles by the selected actinomycetes isolate. The independent variables examined in this experiment and their settings are shown in (Table1). Seven variables shown in (Table 2) were used. The rows in Table 2 represent the 8 different experiments (row no. 9 represents the basal control trial) and each column represents a different variable. For each nutrient variable, a high (+) or low (-) concentration was tested. The main effect of each variable was determined with the following equation:

$$E\,xi = (\sum M_{i+} - \sum M_{i-}) / N$$

Where Exi is the variable main effect, M_{i+} and $M_{i-}$are the radius of the clear zone around each well in the trials. The independent variable (xi) was present in the high and low concentrations, respectively, and N is the number of trials divided by 2. Using Microsoft Excel, statistical t-values for equal unpaired samples were calculated according to Cochran and Snedecor [29] using Microsoft excels to determine the variable significance. From main effect results an optimized medium was predicted.

Verification Experiment: A verification experiment was carried out in duplicates, the predicted optimum levels of the independent variables were examined and compared to the basal conditions setting and the average production of the secondary metabolites was calculated.

Table 1: Independent variables affecting antimicrobial agent(s) production

Factor	Symbol	Level		
		-1	0	+1
Starch (g/l)	Starch	10	20	30
KNO_3(g/l)	KN	0.5	1.0	1.5
K_2HPO_4(g/l)	K_2	0.25	0.5	0.75
$MgSO_4.7H_2O$(g/l)	Mg	0.25	0.5	0.75
$FeSO_4$(g/l)	Fe	0.005	0.01	0.015
Temperature	Temp	33	35	37
pH	pH	6	7	8

3

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

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

Trials	St	KN	K_2	Mg	Fe	pH	Temp
1	-	-	-	+	+	+	-
2	+	-	-	-	-	+	+
3	-	+	-	-	+	-	+
4	+	+	-	+	-	-	-
5	-	-	+	+	-	-	+
6	+	-	+	-	+	-	-
7	-	+	+	-	-	+	-
8	+	+	+	+	+	+	+
9	0	0	0	0	0	0	0

3 Results and Discussion

3.1Physical and Chemical Characters of Sediment Samples

Sediment samples collected in winter, 2014 from different along Sues Gulf were analyzed for physical and chemical properties. The data in Table 3 indicates that, temperature ranged from 17.6 ºC in Suez North to 19.6ºC in El-Tour ,this may be due to several factors such as, air temperature and winds in this time of year. Hamed [30]stated that, temperature of surface water in the Gulf of Suez ranged between 16.5ºC to 19.5 ºC during winter. The pH values showed tendency toward alkalinity (>8).The pH of seawater is relatively constant due to the presence of carbonate, bicarbonate ions and varied from7.8 to 8.3 in polluted water it falls outside this range [31]**.**Sediment samples exhibited pH range from 8.14 to 8.25. Salinity values of samples ranged from 40.2‰ in El-Tour to 41.9‰ in Suez Middle. The chemical parameters including dissolved phosphate, nitrate, nitrite, ammonia and organic matter varied according to the sampling sites as shown in (Table 3).The highest dissolved phosphate in samples was shown at Ras Gharib site (3.88 µg at.PO_4-P/l), whiles the lowest concentration of phosphate at Ain Sokhna site (0.14 µg at.PO_4-P/l) an observation previously reported by Fahmy*et al.*[32] attributed this to the flourishing of phytoplankton which consumes this element.Organic matter in samples showed the highest value at RasGharib (7.12 mg/l), while the lowest value showed at Ain Sokhna (0.71 mg/l) with low population density at that time of the year.Nitrate level showed more or less comparable values which ranged from 0.12 µg at.NO_3-N/l at RasShokair site to 45.61 µg at.NO_3-N/lat Suez Middle site.These results may be due to discharge of sewage wastes of nitrate from bottom sediments into overlying water. According to WHO [33]the nitrite existence in water sources due to fertilizer use decayed vegetable and animal matter, domestic effluents, sewage sludge disposal to land, industrial discharges, and leaches from refuse dumps and atmospheric washout. Dissolved nitrite measured in samples revealed a high value (2.22 µg at. NO_2-N/l) in samples of RasGharib site compared to other sampling sites and that results agree with Hamed [30]whostudied the surface concentration of nitrite in the Gulf of Suez and northern part of the Red Sea and he found that, the maximum concentration was 0.47 µmol/L during winter at RasAdabiya while the low concentration was recorded at Gafton station during summer (0.02 µmol/L). Ammonia concentration at RasGharib site was the much higher (47.2 µg at. NH_3-N/l) and El-Tour site recorded the much lower (0.3µg at. NH_3-N/l). Such concentration of ammonia is an indicator of the presence of pollutants of high activity and this is may be attributed to human activities of diverse origin and presence of water treatment planned of wastewater of RasGharib near these station, these results disagreed with that of Hamed[30]who reported that, the maximum surface ammonia concentration was 6.86 µmol/L during winter at RasAdabiya.

3.2 ActinomycetesViable Counts in Sediment Samples

The actinomycetes count was represented as colony forming unit (CFU/gm) for sediment samples. As shown in (Table 4), the highest count of actinomycetes recoded in AdabiaHarbour
Station (22 CFU /gm), while the lowest count detected in Ain Sokhna station (6 CFU /gm).The best marine sources of actinomycetes identified are sediments, from which their isolation is well
Documented [13].The number and variety of actinomycetes present in any sample would be significantly influenced by geographical location, temperature; pH, organic matter content, agricultural activities, aeration, nutrient availability and moisture content [34].

Table 3: Physico-chemical parameters of sediment samples during winter, 2014

Sampling site	Physico-chemical parameters								
	Temp °C	pH	Salinity ‰	Dissolved oxygen (mg O_2/l)	Dissolved phosphate (µg at.PO_4-P /l)	Dissolved nitrate (µgat.NO_3 N /l)	Dissolved nitrite (µg at. NO_2-N /l)	Dissolved ammonia (µg at. NH_3-N /l)	Organic matter (mg/l)
El-Tour	19.6	8.22	40.2	8.6	0.17	0.26	0.18	0.3	2.25
RasSidr	18.7	8.14	40.8	8.2	0.36	0.29	0.14	0.7	5.12
AdabiaHarbour	17.9	8.16	41.2	8.9	0.28	2.25	0.80	7.8	3.11
Suez North	17.6	8.14	41.7	8.8	0.17	18.15	0.77	15.0	2.63
Suez Middle	18.1	8.22	41.9	9.8	0.19	45.61	0.59	13.2	4.8
Ain Sokhna	18.1	8.24	41.8	8.6	0.14	0.27	0.09	4.8	0.71
RasGharib	18.9	8.20	40.4	9.9	3.88	2.20	2.22	47.2	7.12
RasShokair	19.2	8.25	41.1	8.9	0.28	0.12	0.15	0.9	4.51

Table 4: Actinomycets count (CFU /g) of sediment samples collected from Suez Gulf during winter, 2014.

Site	Count (CFU/ gm)	Selected isolates
El-Tour	8	Isolates numbers 1- 4
RasSidr	15	isolates numbers 5 & 6
AdabiaHarbour	22	Isolates numbers 7 – 22
Suez North	20	Isolates numbers 23 – 28
Suez Middle	15	Isolates numbers 29-34
Ain Sokhna	6	Isolates numbers 35 & 36
RasGharib	18	Isolates number 37
RasShokair	15	Isolates number 38 – 41

3.3 Statistical Analysis

The stepwise multiple regression was applied to reflect the relationship between the environmental factors and the abundance of the recorded actinomycetes, the analysis illustrated that: the actinomycetes were affected by temperature and PO_4. The model equation was: Actinomycetes= 94.299 - 4.169 temp. - 2.239 PO_4.Abou-elela *et al.,* [35] reported that, there was a highly significant relationship between total heterotrophs in sea Water and water temperature, dissolved nitrate and nitrite. Variations in the organic contents, nutrients and the pollution levels led to variations in the density and frequency of bacteria between water and sediments**.**

3.4Bioactivity of the Isolates

A total of 41 actinomycetes isolates were subjected to primary screening of their antagonistic effect against some bacterial pathogens.Only 11 showed antimicrobial activities against the bacterial pathogens*Bacillussubtillus*6633, *Staphylococcus aureus* 25923, *Pseudomonas aeruginosa*

9027, *Bacillus cereus*, *Salmonella typhimurium* 14028, *Escherichia coli* 19404and*Vibrio damsela*,the resulted inhibition zones were measured in terms of mean diameter of inhibition zones (mm) as recorded in (Table 5).Almost 80 % of the world's antibiotics are known to come from actinomycetes, mostly from the genera *Streptomyces* and *Micromonospora* [36].Marine actinomycetes are particularly attractive because they have the high potency required for bioactive compounds to be effective in the marine environment, due to the diluting effect of sea water. Members of the actinomycetes, which live in marine environment, are poorly understood and only few reports are available. Actinomycetesrepresent attractive source for isolation of novel microorganisms and production of potent bioactive secondary metabolites[37].

3.5 Screening of ActinomycetesIsolates to Biosynthesis Metal Nanoparticles

3.5.1 Biosynthesis of Silver Nanoparticles

Isolates 13 and 38showed ability to synthesis AgNPs at 1mM of silver nitrate and pH 8.5 after 5 days of incubation by color change from yellow to dark brown, (Table 6, Figure 2). While at concentrations of 0.5 and 2 mM $AgNO_3$, there was no color development .The absorption spectrum of the AgNPs showed a surface plasmon absorption band with a maximum of 410 nm, indicating the presence of AgNPs. The sharp narrow absorption peak located at 410nm for AgNPs was observed in the present study, (Figure 3) for strains 13 and 38, respectively. This was primarily observed by taking reading in UV-spectrophotometer at 410 nm in 1mM concentration as shown in (Table 7).The selected actinomycetes were screened for antibacterial efficacy by agar well diffusion method, then we have investigated extracellular biosynthesis of silver nanoparticles of $AgNO_3$as recommended byDeepa *et al.*[26].The exact reaction mechanism leading to the biosynthesis of silver nanoparticles is believe that NADH-dependent reductase involving in reduction of silver ions[38].Zeinat*etal.*,[39]recorded nearly result for thisstudy , the absorption spectra of AgNPs synthesized by two *streptomyces* species showed a surface Plasmon absorption band with maximum of 417 nm indicating the resonance of AgNPs.ThisIsolates 13 and 38showed ability to synthesis AgNPs at 1mM of silver nitrate and pH 8.5 after 5 days of incubation by color change from yellow to dark brown, (Table 6, Figure 2). While at concentrations of 0.5 and 2 mM $AgNO_3$, there was no color development .The absorption spectrum of the AgNPs showed a surface plasmon absorption band with a maximum of 410 nm, indicating the presence of AgNPs. The sharp narrow absorption peak located at 410nm for AgNPs was observed in the present study, (Figure 3) for strains 13 and 38, respectively. This was primarily observed by taking reading in UV-spectrophotometer at 410 nm in 1mM concentration as shown in (Table 7).The selected actinomycetes were screenedfor antibacterial efficacy by agar welldiffusion method, then we have investigated extracellular biosynthesis of silver nanoparticles of $AgNO_3$as recommended byDeepa *et al.*[26].The exact reaction mechanism leading to the biosynthesis of silver nanoparticles is believe that NADH-dependent reductase involving in reduction of silver ions[38].Zeinat*et al.* [39] recorded nearly result for thisstudy , the absorption spectra of AgNPs synthesized by two *streptomyces* species showed a surface Plasmon absorption band with maximum of 417 nmindicating the resonance of AgNPs.This observation is in good agreement withother studies [40,41].

Table 5: Inhibition zone (mm) of the isolates against some bacterial pathogens.

Pathogens	Isolate code										
	3	**8**	**9**	**10**	**13**	**15**	**17**	**22**	**23**	**38**	**41**
B. subtillus 6633	15.0	0.0	0.0	0.0	**16.0**	15.0	0.0	0.0	0.0	**0.0**	0.0
S. aureus 25923	0.0	0.0	0.0	12.0	**18.0**	0.0	0.0	0.0	0.0	**15.0**	0.0
P. aeruginosa 9027	0.0	0.0	0.0	0.0	**0.0**	0.0	0.0	0.0	0.0	**0.0**	22.0
B. cereus	0.0	0.0	0.0	0.0	**14.0**	12.0	0.0	0.0	0.0	**0.0**	0.0
S. typhimurium 14028	20.0	15.0	16.0	14.0	**16.0**	18.0	25.0	15.0	0.0	**15.0**	15.0
E. coli 19404	0.0	0.0	0.0	0.0	**16.0**	0.0	0.0	0.0	20.0	**16.0**	0.0
V. damsela	18.0	20.0	10.0	**13.0**	0.0	0.0	18.0	0.0	**0.0**	0.0	

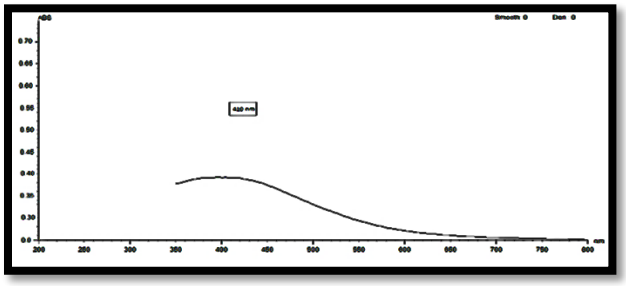
Table 6: Optimum conditions for silver nanoparticles detection ($AgNO_3$ concentrations and pH range) after 5 days of incubation-Ve (No change in color), +Ve (Changed color).

Strains	$AgNO_3$ conc.	0.5 mM				1 mM				2 mM			
	pH	6.5	7.5	8.5	9.5	6.5	7.5	8.5	9.5	6.5	7.5	8.5	9.5
13		-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	+Ve	-Ve	-Ve	-Ve	-Ve	-Ve
38		-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	+Ve	-Ve	-Ve	-Ve	-Ve	-Ve

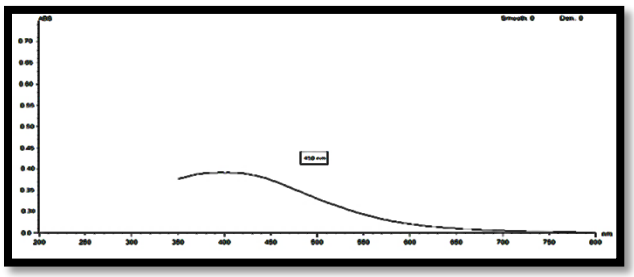
Table 7: UV spectrophotometer readings ofAgNPs for supernatant of strains 13 and 38 after treatment with 1 mM of $AgNO_3$.

Isolate code	Absorbance at 410 nm
13	0.376
38	0.355

Fig.2: Cell filtrate of actinomycetes isolates (13 and 38) treated with (1Mm) at the beginning of incubation (right flask).



Isolate 13



Isolate 38

Fig. 3: UV-Vis absorption spectrum of Ag NPs synthesized by strain 13 and 38after treated with 1mM $AgNO_3$solution.

3.5.2 Biosynthesis of Gold Nanoparticles

Isolates 13 and 38 have ability to biosynthesis gold nanoparticles at 2 mM of $HAuCl_4$ and pH 4 after 4 h (Table 8). As shown in Figure 4, the visualized color of the solution changed fromyellow to pink i.e. showed ability to synthesis gold nanoparticles. The formation of gold nanoparticles was monitoredby UV-Visible spectroscopy by recording the spectrabetween 200-800 nm and simultaneously monitoring the appearance of the characteristic peak of gold nanoparticles at 530-550 nm using a double beam spectrophotometer (Figure 5). This was primarily observed by taking reading in UV-spectrophotometer as shown in (Table 9). Prakash *et al.*,[22] reported that, the reaction mixtures developed a range of colors within 2 h of incubation under different conditions indicating the synthesis of a variety of gold nanoparticles from *Streptomyces* sp. NK52. Also,Soltani*et al.*,[42] mentioned that, the use of *Streptomycesfulvissimus* isolate U in the extracellular synthesis of gold nano-particles. Green synthesis of metal nano-particles using soil actinomysetes bacteria is an ecofriendly green process.

3.5.3 Biosynthesis of Zinc Nanoparticles

Only one isolate has the ability to synthesis zinc nanoparticles (Table 10). Primary confirmation for biosynthesis of zinc oxide nanoparticles from isolate 13 was recorded by visual observation, the color of the medium changed from yellowish to bright yellow. The characteristic surface showed plasmon absorption band with a maximum of 309 nm. Bright yellow color arises due to excitation of surface plasmon vibrations in the zinc nanoparticles. The reduction of zinc was subjected to analysis by using the UV-Vis spectrophotometer. Absorption spectra of Zn NPs formed in the reaction media

has absorbance peak at 309 nm by isolate 13 (Table 11 and Figure 6).In similar study; thebiosynthetic route using *S. nematodiphila* (CAA) has been developed for the zinc sulfide nanoparticle production. During the visual observation, the color of the culture supernatant incubated with zinc sulfate changed from yellow to whitish yellow. The appearance of a whitish Yellow color in the zinc sulfate-treated flask suggested the formation of zinc sulfide nanoparticles. The UV–Vis spectra were recorded broad peak which located between 380 to 400 nm and the strong absorbance centered at 390 nm [43].

Table 8: Optimum conditions for nanoparticles detection ($HAuCl_4$ concentrations and pH range) after 4h of incubation.

Strain	$HAuCl_4$ Conc	0.5 mM				1 mM				2 mM			
	pH	4	5	6	7	4	5	6	7	4	5	6	7
13		-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	+Ve	-Ve	-Ve	-Ve
38		-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	+Ve	-Ve	-Ve	-Ve

Table 9: UV spectrophotometer readings of nanoparticles produced from different strains at 2 mM of $HAuCl_4$.

Isolate code	Absorbance
13	0.035
38	0.013

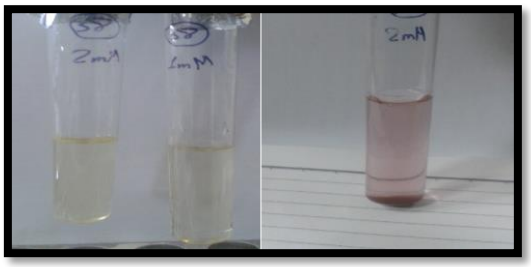
Table10: Optimum conditions for nanoparticles detection ($ZnSO_4$ concentrations and pH range)after 24h of incubation.

Isolate code	$ZnSO_4$ conc.	50 mM				100 mM				150 mM			
	pH	**4**	**5**	**6**	**7**	**4**	**5**	**6**	**7**	**4**	**5**	**6**	**7**
13		-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	**+Ve**	-Ve	-Ve	-Ve	-Ve
38		-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve

Table 11: UV spectrophotometer readings of supernatant of isolates 13 at 100 mMZnNPssolution.

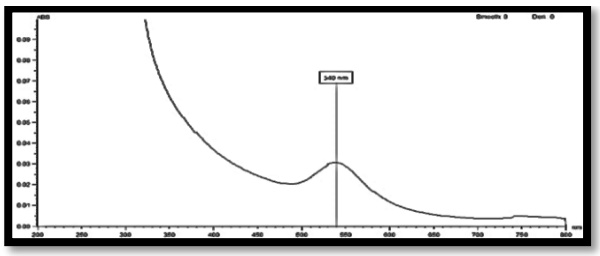
Isolate code	Absorbance
13	4.850

Isolate13

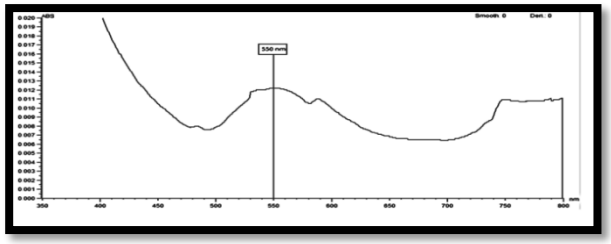


Isolate 38

Fig. 4: Cell filtrate of most isolates which treated with (2mM) of $HAuCl_4$ at the beginning of incubation and after 4h.

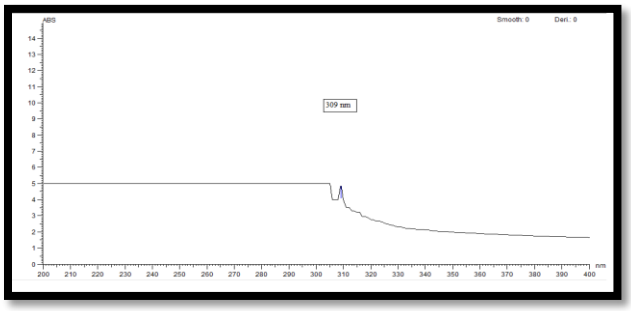


Isolate13



Isolate 38

Fig. 5: UV-Vis absorption spectrum of AuNPs synthesized by strains (13and 38) treated with 2mM $HAuCl_4$ solution.



Isolate 13

Fig. 6: UV-visible spectrum (Zn nanoparticles) by isolate 13 extract treated with100 mM zinc sulfate.

3.6 Antibacterial Activity of Nano Particles Production

The antibacterial activity of silver nanoparticles which produced form isolates 13 and 38 were investigated against the previously used bacterial pathogens. silver nanoparticles at 1mM concentration which produced from isolate 13 showed moderate activity against *Bacillus subtillus*6633 , *Staphylococcus aureus* 25923 , *Pseudomonasaeruginosa*9027and*Salmonella typhimurium* 14028(18mm), lower activity against *Bacillus cereus, Escherichia coli 19404* and *Vibrio damsela* (16 mm). Silver nanoparticles from isolate 38 showed lower activities than that produced from isolate 13. Theactivitiesranged from 13mm against *Vibriodamsel*to18mm *against Staphylococcus aureus* 25923. No effect on two pathogens, *Pseudomonas aeruginosa* 9027 *and Escherichia coli* 19404.The biosynthesized AgNPs by both *Streptomyces* species proved effective against the tested bacteria but the inhibitory effect varied from one another. *Klebsiella, E.coli, Bacilluscereus*, and *S.aureus* were more affected by AgNPs compared to *Salmonella*, *Pseudomonas*, *Moraxella*, *Acientobacter*, *Enterococcus*,and*S.pneumonia*[39]. Also, Sadhasivam*et al.*[44]reported antimicrobial activity of biosynthesized AgNPs from *Streptomyceshygroscopicus* against *B.subtilis, E. coli, E. fecalis*and*C.albicans.* In another hand the antibacterial activity of gold nanoparticles which produced form strain 13 showed activity against *Staphylococcus aureus* 25923, *Salmonella typhimurium*14028 and *Vibrio damsel* (16, 25 and 20 mm) respectively, while gold nanoparticles which produced form strain 38 affected on *Pseudomonas aeruginosa* 9027 and*Salmonella typhimurium*14028 (23 and 20) respectively. Shahzadi*et al.*,[45] reported that, gold nanoparticles 6–40 nm in size exhibited high antibacterial activity, the mechanism of this activity was found to be size- and dose-dependent. It was more influential against Gram-negative bacteria. The antibacterial mechanism of AuNPs against four pathogenic bacteria demonstrated that AuNPs can be the next therapy against this enteric bacterium. In antibacterial activity testing by well-diffusionmethod, the actinomycete biomass exposed to $HAuCl_4$ showed inhibition zone size of 14 and 20 mm against *S. aureus* and *E. coli*, respectively, but theuntreatedactinomycete biomass showed no inhibition[46].

Zinc nanoparticles produced from isolate 13 showed reasonable activity against only*Escherichia coli* 19404(36 mm) and*Salmonella typhimurium* 14028 (34 mm)and no effect on the other pathogens**.** Noor &Halah[47]reported that, zinc oxide nanoparticles had strong antibacterial activity and could inhibit one of the most important pathogenic bacteria *P. aeruginosa.*

3.7 Molecular Phylogeny of the Selected Isolates

Based on the obtained results, actionmycetes isolates 13 and 38 wereselected for identification and molecular phylogenetic analysis. Phylogenetic analysis based on 16S rDNA sequence comparison for establishing phylogenetic and evolutionary relationships among organisms [34].Genomic DNA was prepared, and the gen coding for 16S Rr NA was partially amplified using the universal primers

Primers	Sequence (5` to 3`)
16S 27F	**AGAGTTTGATCCTGGCTCAG**
16S 1492R	**GGTTACCTTGTTACGACTT**

The produced amplicons of the selected isolate were detected using agarose gel electrophoresis as shown in Figure 7.

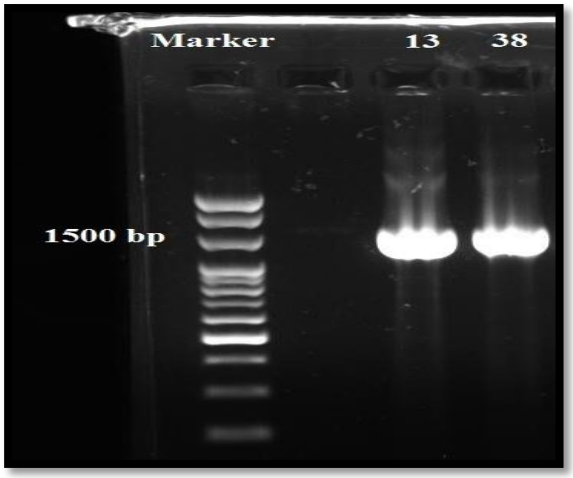


Fig. 7: Agarose gel electrophoreses of the amplified 16S rRNA gene of isolates 13 and 38.

The sequencing data utilizing this strategy (ABI 3730xl) was 1500 base pair. as shown in (figure 7). This sequence was aligned with other sequences of related actinomycetes on the database to determine its phylogenetic relationship to other actinomycetes. According to 16S rRNA gene sequence analysis of isolates 13 and 38 compared with those which gave the highest homology using Blast search computer based program. The resulting data indicated that, the isolates 13was similarto*Streptomyces rochei* with 99 %

similarity percentage and the isolate 38 similar to *Streptomyces*spwith91% similarity percentage. The sequence of strain 13 was deposited in Gene-Bank as ***Streptomyces rochei* HMM13**with accession number **KR108310**, and strain 38 deposited in Gene-Bank as***Streptomyces* sp. MHM38**with accession number**KU764745**and the phylogenetic tree was constructed as shown in (Figure 8 and 9).Several studies reported the potential of *Streptomyces*sp.Inbiosynthesized of silver nanoparticles [48,49,50].

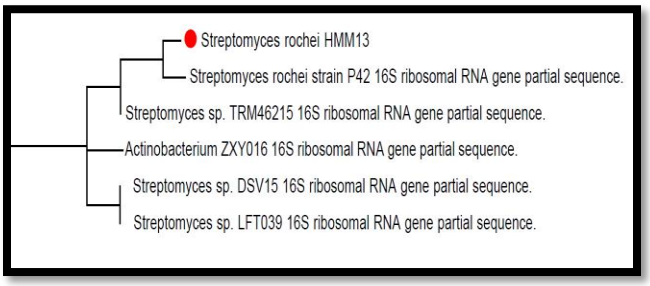


Fig. 8: Phylogenetic tree of *Streptomyces rochei* HMM13 based on partial sequencing of 16S rRNA.

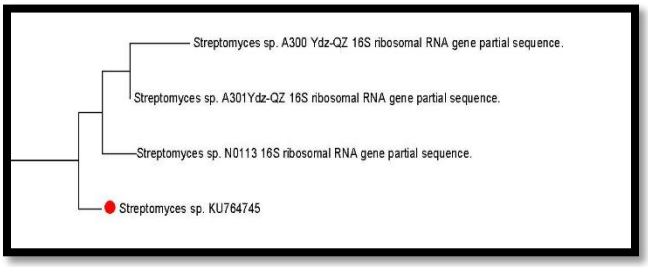


Fig.9: Phylogenetic tree of *Streptomyces* sp MHM38 based on partial sequencing of 16S Rrna.

3.8 Characterization of Silver Nanoparticles Synthesizedby Streptomyces Rochei HMM13

The biosynthesized AgNPs were achieved by addition of silver nitrate (1mM) to the culture supernatant and monitored by color change after incubation then confirmed by UV–Visible spectrophotometer, X-ray, Fourier transform-infrared spectroscopy and scanning electron microscopy. The results of characterization were reported in previous study byAbd-Elnaby*et al.*,[51], where, the obtained AgNPs are spherical in shape with a particle size of 22–85 nm.

3.9Optimization of the Culture Conditions for Streptomyces Rochei HMM13 by Plackett–Burman Design

Plackett-Burman design has been employed to evaluate the significant effect of starch nitrate agar medium components for production of silver nanoparticles by*Streptomyces rochei* HMM13and application of AgNPs as antibacterial agent against, *Salmonella typhimurium* 14028, *Escherichia coli* 19404, and *Vibrio damsela*.Statistical experimental designs are powerful tools for searching the key factors rapidly form a multivariable system and minimizing the error in determining the effect of parameters and the results are achieved in an economical manner[52].The experimental results of the applied Placket-Burman design for seven cultural variables against *S. typhimurium* 14028 was illustrated in (Tables12).

Statistical analysis of the data (t- test) showed that, among the examined environmental factors,Starch,$MgSO_4.7H_2O$, KNO_3 and temperature were the most significant independent variable that affects the nanoparticles production and consequently, the inhibition zone diameter against *S. typhimurium* 14028 (Table 13).

The main effects of the examined factors on the inhibition zone diameter were calculated and represented in Figure 10. Based on these results, the positive (+) level of starch, KNO_3, K_2HPO_4 and pH, in addition to the negative level (-) of $MgSO_4.7H_2O$, $FeSO_4$ and temperature supported the production. Moreover, the *t*-value represented in Table 14 supports this observation. Interaction of starch and $MgSO_4.7H_2O$, increase the inhibition zone diameter. This approach verified the validity of the applied design. A verification experiment was applied to evaluate the basal versus the optimized medium.

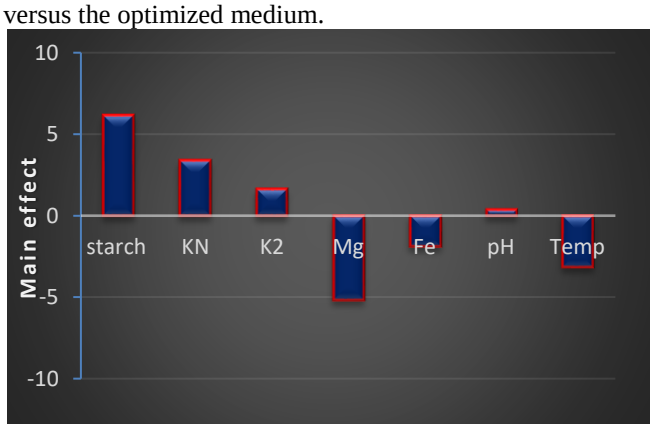


Fig. 10: Elucidation of fermentation conditions affecting the production of AgNPs by *Streptomyces rochei* HMM13 against *Salmonella typhimurium* 14028.

Verification experiment: A verification experiment was applied to evaluate the basal versus the optimized medium. Data in (Table 14) and Figure 11 showed that about 22 (mm) inhibition zone diameterafter incubation with 1.2 fold increase when compared to the control basal medium 18 (mm), on another hand , the uv spectrophotometer reading from verified medium showing rise up to 0.435 nm compared with the control basal medium 0.376nm (1.16 fold increase). The composition of the verified medium as follows (g/l): Starch, 30g;KNO_3, 1.5g; K_2HPO_4, 0.75g; $MgSO_4.7H_2O$, 0.25g; $FeSO_4$, 0.005; pH, 6 and Temperature33°C. The experimental results of the applied Placket-Burman design for seven cultural variables against *E-coli* 19404 were illustrated in Tables15.

Table12: The experimental results of the applied Placket-Burman design for seven cultural variables (application on *S. typhimurium* 14028).

Trials	Starch	KN	K_2	Mg	Fe	pH	Temp	Abs.	AgNPs inhibition zone (mm) Against*S. typhimurium*
1	-	-	-	+	+	+	-	0.177	0
2	+	-	-	-	-	+	+	0.340	20
3	-	+	-	-	+	-	+	0.230	10
4	+	+	-	+	-	-	-	0.430	22
5	-	-	+	+	-	-	+	0.210	0
6	+	-	+	-	+	-	-	0.380	25
7	-	+	+	-	-	+	-	0.455	24
8	+	+	+	+	+	+	+	0.380	16
9	0	0	0	0	0	0	0	0.298	16

Table 13: Statistical analyses of the Plackett-Burman experimental results.

Variable	Main effect	t-value*
Starch	6.12	13.36
KNO_3	3.37	7.36
K_2HPO_4	1.62	3.54
$MgSO_4.7H_2O$	-5.12	-11.18
$FeSO_4$	-1.87	-4.09
pH	0.37	0.81
Temperature	-3.12	-6.81

Table14: A verification experiment: Inhibition zone of silver NPs produced from *S. rochei*HMM13against *S. typhimurium* 14028grown on basal versus verified medium.

Medium	Inhibition zone (mm)	Abs. at 410nm
Basal medium with $AgNO_3$	18	0.376
Verified medium with $AgNO_3$	22	0.435

Table 15: The experimental results of the applied Placket-Burman design for seven cultural variables (application on *E-coli* 19404)

Trials	Starch	KN	K_2	Mg	Fe	pH	Temp	Abs.	AgNPs Inhibition (mm) *E- coli* 19404
1	-	-	-	+	+	+	-	0.210	0
2	+	-	-	-	-	+	+	0.380	20
3	-	+	-	-	+	-	+	0.240	10
4	+	+	-	+	-	-	-	0.470	20
5	-	-	+	+	-	-	+	0.270	0
6	+	-	+	-	+	-	-	0.480	20
7	-	+	+	-	-	+	-	0.410	20
8	+	+	+	+	+	+	+	0.430	0
9	0	0	0	0	0	0	0	0.320	16

Fig. 11: Inhibition zone diameter of Ag NPs of *S.rochei* HMM13grown on verified medium against *S. typhimurium* 14028.

Statistical analysis of the data (t- test) showed that, among the examined environmental factors,$MgSO_4.7H_2O$, temperature, starch and $FeSO_4$was the most significant independent variable that affect the suppression percentage of *Escherichia coli* 19404 (Table16).

Table 16: Statistical analyses of the Plackett-Burman experimental results.

Variable	Main effect	t-value*
Starch	3.75	1.0
KNO_3	1.25	0.33
K_2HPO_4	-1.25	-0.33
$MgSO_4.7H_2O$	-6.25	-1.66
$FeSO_4$	-3.75	-1.0
pH	-1.25	-0.33
Temperature	-3.75	-1.0

The main effects of the examined factors on the inhibition zone diameter were calculated and represented in Figure 12. Based on these results, the positive level (+) of starch and KNO_3 and the negative (-) level of the other variables supports the production. Moreover the *t*-value represented in Table 16 supports this observation.

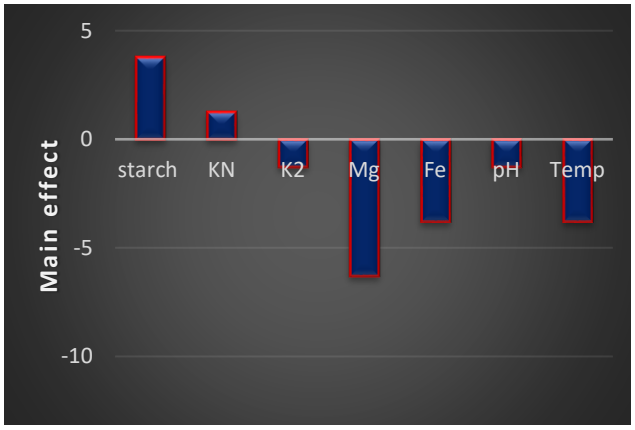


Fig. 12: Elucidation of fermentation conditions affecting the production of AgNPs by *Streptomyces rochei* HMM13 against*Escherichia coli* 19404.

Verification experiment: A verification experiment was applied to evaluate the basal versus the optimized medium. Data in Table 17 showed that about 20 (mm) inhibition zone diameterwith 1.25 fold increase when compared to the control basal medium 16 (mm), Figure 13was achieved the UV spectrophotometer readings from verified medium showing rise up to 0.487 nm i.e. 1.13 fold increasecompared with the control basal medium (0.376 nm). The composition of the verified medium as follows (g/l): Starch, 30g;KNO_3, 1.5g; K_2HPO_4, 0.75g; $MgSO_4.7H_2O$, 0.25g; $FeSO_4$, 0.005; pH, 6 and temperature33°C.

Table17: A verification experiment: Activity of silver NPs production from *S. rochei*HMM13against *Escherichia coli* 19404grown on basal versus verified medium.

Medium	Inhibition zone (mm)	Abs. at 410 nm
Basal medium with $AgNO_3$	16	0.376
Verified medium with $AgNO_3$	20	0.487

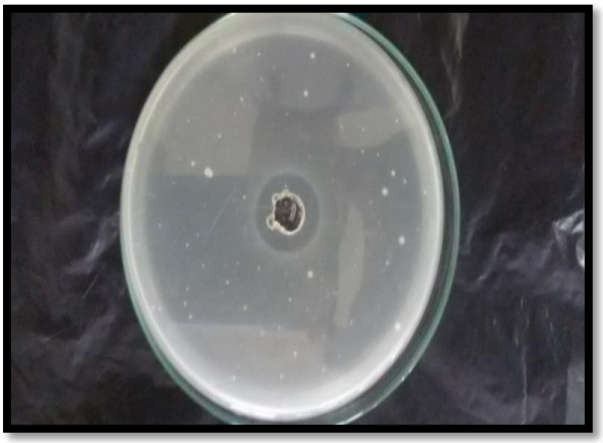


Fig. 13: Inhibition zone diameter of Ag NPs of *S.rochei* HMM13grown on verified medium against *Escherichia coli* 19404.

The experimental results of the applied Placket-Burman design for seven cultural variables against *Vibrio damsela* was illustrated in (Table18)

Statistical analysis of the data (t- test) showed that, among the examined environmental factors, starch was the most significant independent variable that affect the suppression percentage of *Vibrio damsela*(Table 19).

The main effects of the examined factors on the inhibition zone diameter were calculated and represented in Figure 14. Based on these results, the positive (+) level of all variables supports the production except $MgSO_4.7H_2O$, $FeSO_4$ and temperature which must be taken with (-) levels. Moreover, the *t*-value represented in Table 19 supports this observation.

Table 18: The experimental results of the applied Placket-Burman design for seven cultural variables (Application on *Vibrio damsela*)

Trials	ST	KN	K_2	Mg	Fe	pH	Tem	Abs.	AgNPs inhibition zone (mm) against *Vibrio damsela*
1	-	-	-	+	+	+	-	0.270	0
2	+	-	-	-	-	+	+	0.450	24
3	-	+	-	-	+	-	+	0.310	0
4	+	+	-	+	-	-	-	0.470	24
5	-	-	+	+	-	-	+	0.288	0
6	+	-	+	-	+	-	-	0.490	25
7	-	+	+	-	-	+	-	0.476	22
8	+	+	+	+	+	+	+	0.360	15
9	0	0	0	0	0	0	0	0.326	13

Table 19: Statistical analyses of the Plackett-Burman experimental results.

Variable	Main effect	t-value*
Starch	8.25	33.0
KNO_3	1.5	6.0
K_2HPO_4	1.75	7.0
$MgSO_4.7H_2O$	-4.0	-16.0
$FeSO_4$	-3.75	-15.0
pH	1.5	6.0
Temperature	-4.0	-16.0

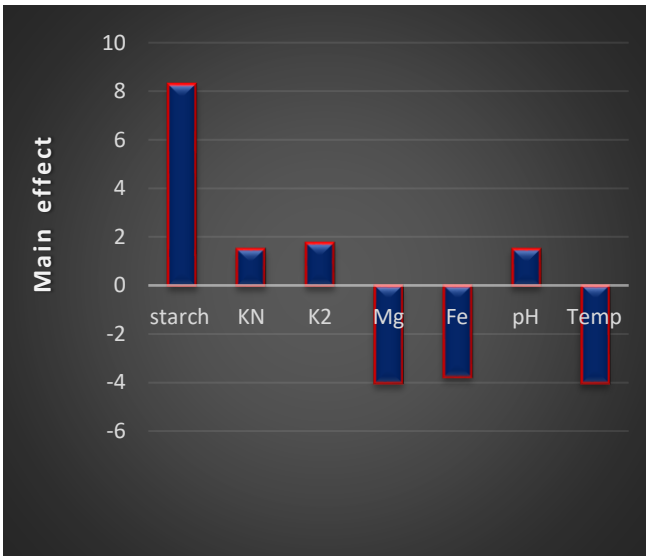


Fig. 14: Elucidation of fermentation conditions affecting the production of AgNPs by *Streptomyces rochei*HMM13 against *Vibriodamsel.*

Verification experiment: A verification experiment was applied to evaluate the basal versus the optimized medium. Data in (Table 20) shows that about 19 (mm) inhibition zone diameter achieved with 1.19 fold increase when compared to the control basal medium 16 (mm) Figure 15.The UV spectrophotometer readings from verified medium showed that 0.497 nm about(1.32 fold increase) compared with the control basal medium 0.376 nm was detected. The composition of the verified medium as follows (g/l): Starch, 30g; KNO_3, 1.5g; K_2HPO_4, 0.75g; $MgSO_4.7H_2O$, 0.25g; $FeSO_4$, 0.005; pH, 8 and temperature33°C.

Table 20: A verification experiment: Inhibition zone of silver NPs produced from *S. rochei*HMM13against *Vibrio damsela*grown on basal versus verified medium.

Medium	Inhibition zone (mm)	Abs at 410 nm
Basal medium with $AgNO_3$	16	0.376
Verified medium with $AgNO_3$	19	0.497

El-Naggar*et al.*, [53] reported, statistical optimization of fermentation conditions using Plackett-Burman design and Box-Behnken design appears to be a valuable tool for the productionofAgNPs by *Streptomycesnarbonensis* SSHH-1E, about 4.5 fold increase in AgNPs production was achieved with the following optimized factors: inoculums

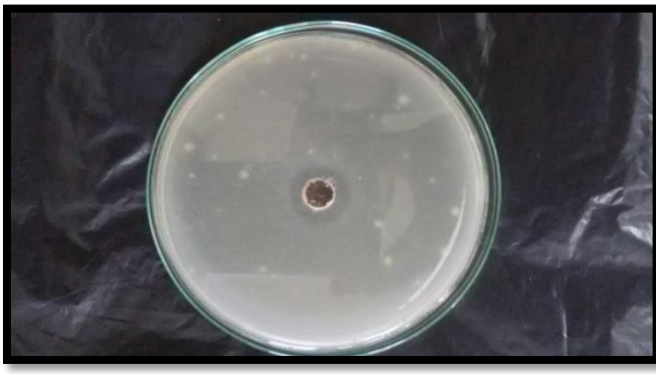


Fig. 15: Inhibition zone diameter of Ag NPs of *S. rochei* HMM13 grown on verified medium against *Vibrio damsela*.

age (48 h), peptone (0.5 g/ L), and pH value (8).Statistical optimizationof fermentation conditions using Plackett-Burman design and Box-Behnkendesignappears to be a valuable tool for the production of AgNPs by *Streptomyces viridodiastaticus*SSHH-1. Initial screening of production parameters was performed using a Plackett- Burman design and the variables with statistically significance effects on AgNPs production were identified. Among the14 variables tested, inoculums age, medium volume, and peptone concentration were identified as the most significant factors for AgNPs production (confidence level above 99%). These variables were selected for further optimization studies using a Box-Behnken design. The statistical optimization by RSM resulted in a 4.43- fold increase in the production of AgNPs by *Streptomyces viridodiastaticus*[12]**.**

4 Conclusions

Streptomyces rochei HMM13 is an excellent microbial resource for the synthesis of AgNPs. The surface plasmon resonance band at 400nm in UV-visible spectrum indicates the primary evidence for synthesis of AgNPs. Statistical optimization of fermentation conditions using Plackett-Burman design appears to be a valuable tool for the production of AgNPs by *Streptomyces rochei* HMM13.

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