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ORIGINAL ARTICLE

Optimizing Production Conditions of a Caspian Sea *Actinomycete* Exhibiting Promising Antibacterial Activity Against Clinically-important Pathogens Using the Two-Factor Interaction/ Minimum Run Resolution IV Method

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ABSTRACT

Natural products recovered from marine sediment have the potential for the treatment of various diseases. *Streptomyces* sp., strain MN38 which was previously isolated from the Caspian Sea of Iran was at first characterized based on its 16srRNA analysis and morphological properties. Two-factor Interaction/ Minimum Run Resolution IV method was employed to evaluate the influence of various potential factors on the strain's antibacterial activity using cost-effective substrates.

Twelve variables were considered effective for investigation, with an emphasis on assessing the antibacterial activity against *Staphylococcus aureus* and *Enterococcus faecalis* via the micro-dilution method. The findings indicate that a quadratic model and a second-order polynomial equation are statistically significant at the 95% confidence level due to a low P-value (< 0.0001) in this context.

As a result, A1BFe+C agar medium, incubated at 28°C for three days with a shaking speed of 200 rpm, using specific concentrations of starch (5.0 g/l), yeast extract (6.0 g/l), peptone (4.0 g/l), KBr (0.5 g/l), $CaCO_3$ (0.2 g/l), sea salt (15 g/l), and $Fe_2(SO_4)_3$ (0.003 g/l), along with an inoculum size of 3.0% v/v at a pH of 6, exhibits the enhanced antibacterial activity against *Staphylococcus aureus* and *Enterococcus faecalis* reached up to 69 and 166 (Bu/ml). It could be concluded that the MN38 of Caspian Sea sediments was a potent source of antimicrobial agent production and the production process was significantly optimized using mathematical methods.

Keywords: Caspian Sea, Marine *Streptomyces* optimization, Antibacterial activity, 2FI/ Min Run Res IV

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Introduction

The ongoing search for novel antimicrobial metabolites is driven by the urgent need to address the growing challenge of antimicrobial resistance (1). Natural compounds discovered from the different sources become good candidates to combat resistant pathogens (2). Many microorganisms with the capability of producing natural antibacterial agents live in marine environments (3) and one group of these microbes are marine *actinomycetes*. Marine *actinobacteria* are Gram-positive, aerobic bacteria, known for their spore formation and fungal growth patterns that grow on various substrates such as seaweed or dead algae. (4–6).

Previous research has shown that marine *Streptomyces* demonstrate a higher rate of discovery for bioactive compounds compared to their terrestrial counterparts, despite the limited scope of screening studies (7, 8). For instance, Mohseni et al. isolated some marine strains and evaluated their microbial inhibition by measuring the diameter of the inhibition zones (9,10). The MN38 strain exhibited notable antibacterial activity against *Staphylococcus aureus* (*S. aureus*) (ATCC 25923; 20.0±0.5mm), *Bacillus subtilis* (*B. subtilis*) (PTCC 1156; 27.0±0.2mm), and *Escherichia coli* (*E. coli*) (PTCC 1533; 11.0±1.4mm) which was selected for this study (4).

After the antibacterial screening test and finding producer strains, the next step is the production of bioactive agents in large amounts under optimized conditions. Thus, identifying the optimal growth condition for a particular strain becomes essential for future research. However, the numerous factors involved require a significant number of laboratory-scale experiments (11). Researchers have employed various statistical methods for media optimization. For instance, Chen et al. (12), and Wang et al. (13) applied Response Surface Methodology (RSM) for Medium Optimization for antibiotic production of *Bacillus sp. ZJUIBE-076* and *Xenorhabdus bovienii*, resulting in a 2.7 and 37.8-fold increase in antibacterial activity respectively.

Statistical experimental designs offer a valuable strategy for optimizing experimental efficiency. By reducing the number of necessary runs, these studies can significantly lower the costs associated with performance evaluation while maintaining a high level of confidence in the results. Designs such as minimum-run resolution IV allow for the identification of significant factors, which can subsequently be subjected to optimization models. In comparison with central composite and Box-Behnken, 2FI/Min Run Res IV design needs one experimental step to determine the influential factors and their optimum levels as well as suggesting a useful equation. Among the screening studies, such as fractional factorial design (FFD), Taguchi design (TD), and Plackett–Burman design (PBD), Min Run Res IV is of great importance in experimental design to facilitate, accelerate, and improve the antibacterial activity (14).

The present study was aimed to (i) characterize the antagonistic *Streptomyces*

sp. MN38 based on its 16S rRNA gene sequence and phenotypic characteristics and then (ii) enhance the antibiotic production produced by MN38 strain, using a successive optimization strategy, selection of media components that significantly influence the antibiotic production in the means of antibacterial activity using a minimum-run resolution IV (Min Run Res IV) design and optimization of these media components using the model and numerical optimization method with Min Run Res IV design.

Methods

Isolation and purification of *Streptomyces* sp., MN38 from the Caspian Sea

The *actinomycete* strain employed in this study, *Streptomyces* sp. MN38 was previously isolated from the Caspian Sea and preserved in the microbial laboratory of the School of Pharmacy, Mashhad University of Medical Sciences (10). The strain was maintained on sterile Starch Casein Agar (SCA) slants at 4°C. For long-term preservation, it was cultured on various International Streptomyces Project (ISP) media, including malt extract agar (ISP2), oatmeal agar (ISP3), and inorganic salts starch agar (ISP4), and incubated at 28 ± 2°C for 10-15 days.Glycerol was then added to achieve a final concentration of 15% (v/v) before the cultures were stored in a -20°C freezer for future use. Among the *actinomycetes* demonstrating antibacterial activity against various bacteria, the MN38 strain (GenBank: KF595306.1, available on https://www.ncbi.nlm.nih.gov/nuccore/KF595306.1) was chosen for further studies due to its previously reported effectiveness against Staphylococcus aureus (*S. aureus*) and *Enterococcus faecalis* (*E. faecalis*) (9,10).

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Morphological and physiological characteristics MN38 on different media

The morphological characteristics of MN38 were determined based on conventional protocols (15). Morphological and physiological Characteristics of MN38 have been determined on sterile plates via culturing on various media including Tryptic Soy Agar (TSA), Yeast Extract Malt Extract (YME), International Streptomyces Project-2 agar (ISP2), and A1BFe+C agar (Table S1). After 3-7 days of incubation at 28 ± 2 °C, their morphological properties including the absence or presence of aerial mycelia, colony reverse color, spore-bearing hyphae, and soluble pigments were studied. The plates were kept at 4 °C for further use.

DNA extraction and 16S rRNA sequencing

Genomic DNA was extracted from the potent isolates using a standard bead-beating method (16) Extraction of nucleic acids from marine bacteria is normally hampered by a thick. This paper presents procedures based on mechanical cell breakage to extract DNA from marine *actinomycetes*. Our DNA extraction protocol included using small bead (0.1mm) compositions for cell breakage. In this method, after adding phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]) lysis of cells in samples was done by bead beating for 1 min in a Mikrodismembrator U (Braun Ltd.).

Cell extracts were treated similarly except that a vortex mixer was employed for two 1-min periods to lyse cells. The quality and quantity of the extracted DNA were verified by spectrophotometry and agarose gel electrophoresis. The 16S rRNA gene was then amplified using universal bacterial 16S rRNA primers (PA 5′- GAGTTTGATCCTGGCTCAG-3′ and PH 5′-AGGAGGTGATCCAGCCGCA-3′). The PCR products were purified using the GeneJet PCR purification kit (Thermo Scientific, Lithuania) according to the manufacturer's instructions. Purified products were sequenced by GATC Biotech (Germany).

Phylogenetic analysis

Reference sequences were obtained from the National Centre for Biotechnology Information (NCBI) and the BLAST program (www.ncbi.nlm.nih.gov/blst) was used for DNA similarity analysis. Multiple sequence alignment was performed with ClustalX (17). Phylogenetic trees were created using MEGA5, a software for analyzing molecular evolution, to show the relationships between different groups. Neighbor-participation was the method used to construct these trees (18).

Determination of antibacterial activity

Indicator microorganisms

The indicator microorganisms, including Multi-Drug Resistant strains of *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella pneumoniae* ATCC 700603 besides control test strains of Staphylococcus aureus ATCC 25923, *Enterococcus faecalis* ATCC 29212 and *Escherichia coli* ATCC 25922 were maintained at minus 20 °C in glycerol stock. All the indicators were revived via inoculating on Mueller-Hinton Agar medium (MHA, Merck) followed by incubation at 37±2 °C for 24 hours (19).

Preliminary screening for antibacterial activity using the cross-streak method

MN38 strain, at first, was cultivated on three solid culture media, which were TSA, ISP2, and A1BFe+C agar. The inoculation process involved streaking the MN38 onto sterile MHA plates using a pure colony of each indicator microorganism as a seed. After being incubated at 28 ± 2°C for three to seven days, these cultures were used for further experiments. A pure colony of each indicator microorganism (*S. aureus*, *E. coli*, *P. aeruginosa*, *E. faecalis*, and *K. pneumoniae*) was transferred into sterile normal saline, reaching a visible turbidity and density of 1×10^6 CFU/ml. After adjusting to this required level, a cotton swab was used to distribute bacterial suspensions perpendicularly onto MHA plates in triplicate. These dishes were then incubated at 37 ± 2°C for twenty-four hours before assessing the inhibitory effect by measuring the diameter of zones surrounding each antagonist (20).

Selection of inoculum medium

Media Screening

Nine flasks, each containing 100 mL of either sterile liquid medium A1BFe+C, TSB (Tryptic Soy Broth), or YME (Yeast Extract Peptone Medium), were inoculated with a fresh spore suspension of MN38 colonies. The colonies displayed various morphological characteristics such as a tough or powdery texture, a dry or folded appearance, and branching filaments with or without aerial mycelia. These colonies were cultivated on different solid/liquid

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media combinations including ISP2/YME, ISP2/TSB, ISP2/A1BFe+C, TSA/YME, TSA/TSB, TSA/A1BFe+C, A1BFe+C agar/YME, A1BFe+C agar/TSB, and A1BFe+C agar/A1BFe+C (Figure 1). The inoculated flasks were incubated at 25 ± 2°C for either 7 or 10 days in a shaking incubator set to run at 180 rpm. Each experiment was conducted in triplicate, with results averaging from the three default trials.

Samples were collected into sterile centrifuge tubes after 7 or 10 days of incubation, and they were centrifuged for 5 minutes at 10,000×g (Sigma). A 0.2-µm membrane filter was then used to filter the clear cultures. To choose a suitable basal medium for further statistical optimization, the antibacterial activity of the crude extract against certain bacteria was further examined using the Kirby-Bauer disk diffusion method following the initial evaluation (21).

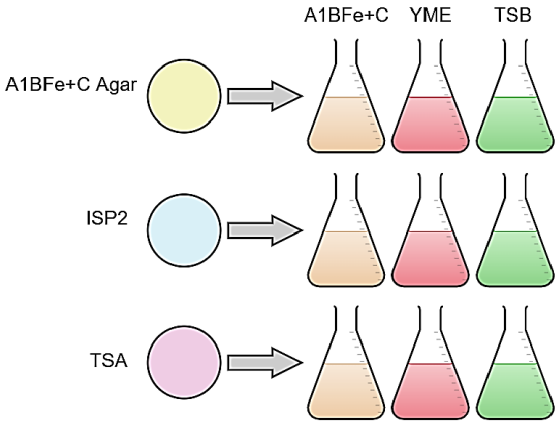

Figure 1. Transferring MN38 from various solid media to different liquid broth media (for 7-10 days cultivation) to evaluate the antibacterial activity of cell-free supernatant against five pathogens. Performed in triplicates. Diagram(s) created with Chemix (2024). Retrieved from https://chemix.org.

Secondary screening of antibacterial activity using Kirby–Bauer disk diffusion method

Following the extraction of the crude product through submerged fermentation, its antibacterial efficacy was evaluated against several clinically important bacteria, including *S. aureus*, *E. coli*, *P. aeruginosa*, *E. faecalis*, and K. pneumoniae. The Kirby-Bauer disk diffusion method was used for this evaluation. Once bacterial cultures reached a final cell density of approximately 1 to 1.5 × 10^6 CFU/ml within sterile normal saline, a sterile swab was used to uniformly disperse this bacterial suspension across the entire culture medium surface. Then, fifty to one hundred microliters of crude extract were subsequently placed on 6 mm sterile discs (Hi Media, India), allowed to dry, and then positioned onto MHA plates which had been seeded with indicator bacteria, previously. After being incubated overnight at 37 ± 2°C, these dishes were observed for antibacterial activity by measuring the zone of inhibition (ZOI) surrounding each disc. The experiments were carried out in triplicate and results were reported as an average from three trials (n=3). Sterile blank discs served as controls during this process.

Design of experiment for optimization of antibacterial compound production

Similar to Mujeli et al.'s (22) study, we selected the Min Run Res IV design which has been used as an approach to promote and accelerate antibacterial activity production. Design-Expert® software package v13 was employed for data analysis to screen out significant variables affecting the yield of antimicrobial compounds (Table S2). As the A1BFe+C medium showed the highest production of antibacterial activity, it was selected for further optimization. A total of twelve factors were examined, including temperature, incubation period, shaking speed, starch, yeast extract, peptone, KBr (potassium bromide), sea salt, $CaCO_3$ (calcium carbonate), $Fe_2(SO_4)_3$ (iron sulfate), inoculum size and pH. The 2FI/Min Run Res IV design was used to investigate the effect of these variables on antimicrobial compound production which led us to conduct a total of twenty-six experimental runs, with each experiment being carried out as triplicate sets (n=3) for every factor level tested (Table 1).

To prevent any bias from occurring during experiments, we made sure that each trial was performed separately. The high and low levels were represented by positive ones and negative ones respectively within our experimental design setup. The experimental data was analyzed using appropriate statistical methods and the overall significance of the model was assessed using ANOVA.

Photometric bioactivity assay

The antimicrobial activity of crude extracts obtained from 26 experimental trials of strain MN38 was evaluated using a standardized micro-dilution assay (23). After three or eight days depending on the

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specifics of each trial, samples from cultured medium were collected into sterilized centrifuge tubes and subjected to high-speed centrifuging at 10,000×g for a duration spanning five minutes to obtain clear supernatants.The cleared cultures were filtered through a 0.2µm membrane filter. Stock solutions of crude extract were prepared to a concentration of 180 µl dispersed in 180 µl MHB per well in a 96-well plate at two-fold serial dilution up to 2^{-8} (0.0078125) times the initial extract, and 0 as control. Meanwhile, a 24-hour culture of five indicator bacteria, of *S. aureus, E. Faecalis, K. pneumoniae, P. aeruginosa,* and *E. coli* were prepared using MHA for further analysis. Turbidity levels of these cultures ranged between 1-1.5×10^{6} CFU/ml which was adjusted using sterile normal saline solution as needed during experimentation processes. Broth microdilution assays were conducted via adding serial dilutions of crude extracts into MHB followed by inoculation of 20µl of bacterial suspension per well.

The positive control wells had only indicator bacteria in them, while the negative control wells contained a sterile medium. The bacterial growth of five pathogens was measured by the cloudiness of the broth, which was determined using a microplate reader (Model 680; Bio-Red, Saint Louis, USA). The optical density of each well was measured at 600 nm after 16 hours of incubation at 37±2°C. This measurement helped to identify whether the bacteria had been inhibited or killed. Values are means±SD from triplicate (n=3) determinations.The half-maximal inhibitory concentration (IC50) value was determined as the concentration of the product that reduced absorbance by 50% compared to the control.

A second-order polynomial regression analysis was performed to estimate the relationship between optical density (OD) and concentration. The IC_{50} concentration was calculated by multiplying the estimated IC50 value from the regression analysis by the appropriate dilution factor.

Table 1. Summary of twelve variables, their codes, and levels involved in 2FI/ Minimum-Run Resolution –IV used to optimize the antibacterial production of the active metabolite(s) produced by strain MN38.

NO	Parameter	Code	Unit	Low level (-1)	High level (+1)
1	Temperature	A	° C	20	28
2	Time	B	Days	3	8
3	Shaking speed	C	rpm	100	200
4	Starch	D	g/L	5	15
5	Yeast extract	E	g/L	1	6
6	Peptone	F	g/L	0.5	4
7	KBr	G	g/L	0.5	0.1
8	$CaCo_3$	H	g/L	0.2	2
9	Sea salt	J	g/L	15	45
10	$Fe_2 (SO_4)_3$	K	g/L	0.03	0.1
11	Inoculum Size	L	% V/V	3	10
12	pH	M	-	6	8

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Results

Culture Characteristics

For initial regeneration, MN38 needs a longer period in the incubator to grow. This process typically lasts at least 5 to 7 days, depending on the type of culture media used. In this context, the growth rate of MN38 bacteria was set up using all three mentioned solid media (TSA, ISP2 selective media, and A1BFe+C agar) in an incubator at a temperature of 28 ± 2°C. TSA and ISP2 selective media are convenient and effective for the isolation of rapidly growing MN38 bacteria compared to A1BFe+C agar. The growth, color of aerial mycelium, substrate mycelium color, and pigmentation of MN38 were summarized in Table 2.

Phylogenetic Characteristics

Figure 2 displays the phylogenetic tree of the MN38 strain. MN38 was posed with *Streptomyces* sp. NEAU-Y10 (JX110668) in a branch. MN3 (KF595305), *Streptomyces* ghanaensis strain KCTC 9882 (NR043366), and MN39 (KF595307) were found to be the neighbor strains. MN38 was posed with *Streptomyces* sp., GB1 (KF313073), and *Streptomyces* rochei strain HBUM 174697(FJ532458) as a single branch and shared 99% sequence similarity supported by a bootstrap value of 99.

Primary antibacterial screening

The MN38 strain grown on an un-optimized ISP2 medium, exhibited antibacterial activity against *S. aureus*, *E. faecalis*, and K. pneumoniae, whereas this strain cultured in an unoptimized A1BFe+C agar medium, showed antibacterial properties against *P. aeruginosa*, in addition to K. pneumoniae, *S. aureus*, and *E. faecalis*. Using the cross-streak method, the antimicrobial activity was observed only against K. pneumoniae when MN38 was grown on TSA medium (Table S3).

Secondary antibacterial screening

The type of medium used had a significant impact on the antibacterial activity of the MN38 strain (Table S3). Among the nine different growth media, the highest antibacterial activity against *S. aureus* (62.0 ± 0.2mm), *E. faecalis* (16.0 ± 0.2mm), and K. pneumoniae (10.0 ± 0.1 mm) was observed with the A1BFe+C agar/A1BFeC media, as determined by the Kirby–Bauer disk diffusion method. Consequently, A1BFe+C agar/ A1BFe+C media was selected for further optimization study using statistical approaches (Table S4a, S4b).

2FI/ Min Run Res IV statistical design

Bioassay against *S. aureus*

A 2FI/ Min Run Res- IV design was employed to evaluate and identify the most influential fermentation variables that influence antibacterial production by the MN38 strain. Twelve variables (incubation temperature, incubation time, shaking speed, starch, yeast extract, peptone, KBr, sea salt, $CaCO_3$, $Fe_2(SO_4)_3$, inoculum size, and pH) were evaluated through the 26-trial model with 3 (Table 3).

Results indicated that the maximum antibacterial production against *S. aureus* by MN38 which was 69.34 Bethesda Units per milliliter (BU/ml) was recorded in the 9th run with positive effects of shaking speed, yeast extract, peptone, $CaCO_3$, and $Fe_2(SO_4)_3$ and negative effect of incubation temperature, incubation time, starch, KBr, sea salt, inoculum size, and pH (Figure 3a).

The lowest antibacterial yield by MN38, 3.42 BU/mL was observed in run 3. Statistical analysis (Tables 4 and 5) reveals an F-value of 496.07, implying the significance of our predictive equation. The antibacterial activity data and the experimental design are also presented in Table 4. Regression analysis was conducted using Design Expert v13 to model the relationship between the experimental factors and antibacterial activity using a second-order polynomial equation. The resulting equation describes this relationship: Equation (1)

$$Y = 37.0319 + (-3.76896\times A) + (-4.60604\times B) + (6.19479\times C) + (3.26854\times D) + (3.51771\times E) + (9.57687\times F) + (0.533125\times G) + (1.66979\times H) + (7.25563\times J) + (1.12521\times K) + (1.34229\times L) + (4.04271\times M)$$

Where Y is the model equation activity (BU/ml) and A, B, C, D, E, F, G, H, J, K, L, and M were incubation temperature, incubation time, shaking speed, starch, yeast extract, peptone, KBr, $CaCO_3$, sea salt, $Fe_2(SO_4)_3$, inoculum size, and pH respectively. The ANOVA analysis (Table 4) showed that the fitted model was statistically significant. The predicted R2 value (0.9911) was in good agreement with the adjusted R2 value (0.9958), indicating that the model was a good fit for the data.

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Table 2. Growth characteristics of *Streptomyces* NM38 on different media.

Media	Growth	Colour of aerial mycelium	Colour of substrate mycelium	Presence of soluble pigments
ISP-2 agar	Excellent	grey	Light yellowish white	-
YEME	Excellent	-	-	Cinnamon
TSA	Excellent	white	Yellow-brown	
TSB	Excellent	-	-	Burnt brick
A1BFe+C.agar	Excellent	Pale grey	Pale white grey	-
A1BFe+C	Excellent	-	-	Creamy-white

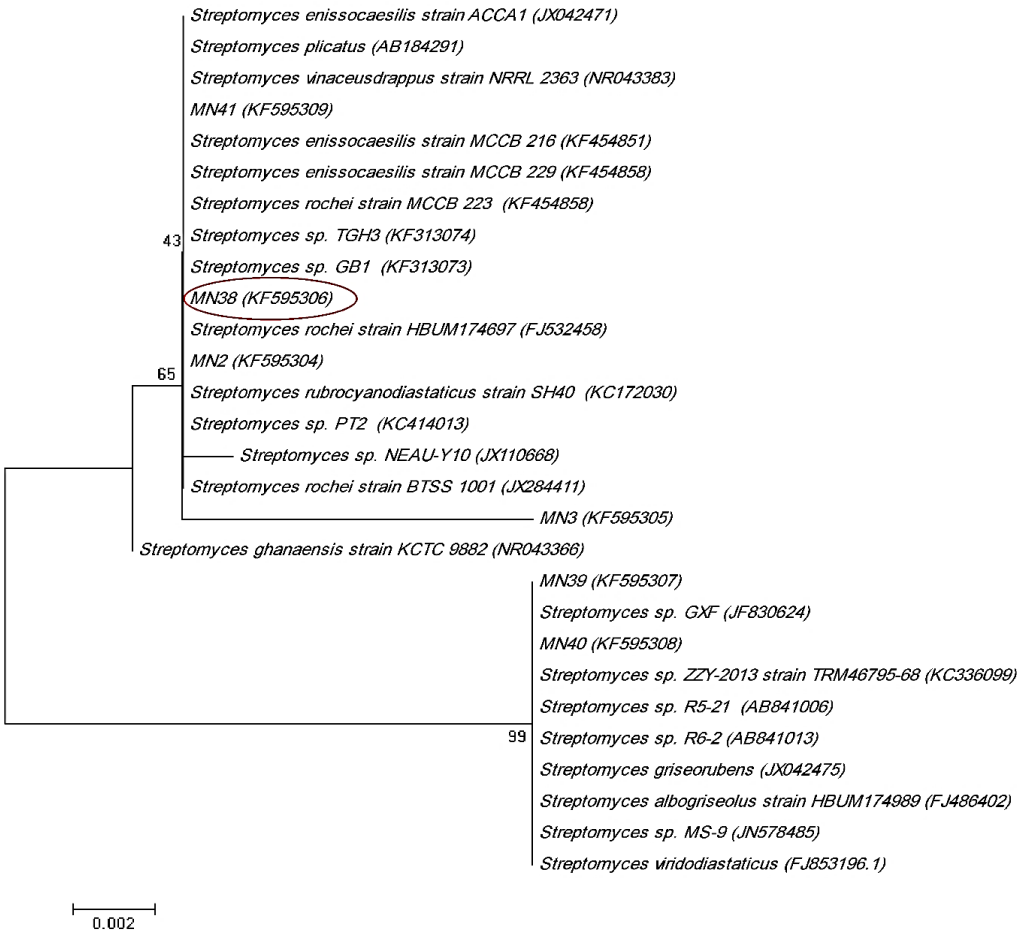

Figure 2. Phylogenetic analysis of *Streptomyces* strain MN38 and related species. The tree was inferred using the neighbor-joining method with 1000 bootstrap replicates. Bootstrap values greater than 50% are shown. *Streptomyces* sp. MN39 (KF595307) was used as an out group. The scale bar indicates the number of nucleotide substitutions per site.

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Table 3. Twenty-six trial 2FI/minimum-run resolution IV screening design for twelve variables affecting the antibacterial production. The table shows the value of twelve variables in each run and the results of antibacterial activity (BU/ml) obtained from the real experiments (Exp.) besides antibacterial activity (BU/ml) predicted and calculated from the formula (Pred.).

Run	Variables												Antibacterial activity against *S. aureus* (Bu/ml)		Antibacterial activity against *E. Faecalis* (BU/ml)	
	A	B	C	D	E	F	G	H	J	K	L	M	Exp	Pred	Exp	Pred
1	28	3	100	5	1	4	0.1	2	45	0.1	3	6	42.74	42.05	70	73.20
2	28	8	100	15	1	4	0.5	2	15	0.03	3	8	38.38	38.00	50	55.63
3	28	3	100	15	6	0.5	0.5	2	15	0.1	10	6	3.42	4.32	80	77.70
4	28	3	200	5	6	4	0.5	0.2	15	0.03	3	6	8.96	9.00	94	92.43
5	28	8	100	5	6	4	0.1	0.2	15	0.1	10	8	33.2	32.01	50	54.13
6	20	8	200	15	6	4	0.1	2	15	0.1	3	6	41.14	42.40	120	113.57
7	28	3	100	5	1	0.5	0.5	0.2	45	0.03	10	8	40.36	39.50	165	166.38
8	20	8	200	5	1	4	0.1	0.2	45	0.03	3	8	31.81	31.60	16	14.43
9	20	3	200	5	1	4	0.5	2	15	0.1	10	8	69.34	69.74	150	148.20
10	20	8	100	5	1	0.5	0.5	0.2	15	0.1	3	6	65.02	65.06	82	83.03
11	20	3	100	5	1	0.5	0.5	0.2	15	0.1	3	8	5.53	5.88	11	12.53
12	28	8	200	5	6	0.5	0.5	2	45	0.1	3	6	35.93	34.56	10	5.10
13	28	3	200	15	1	0.5	0.1	0.2	45	0.1	3	8	35.93	36.06	111	106.60
14	28	8	100	15	6	0.5	0.1	0.2	45	0.03	3	6	25.05	24.37	81	87.53
15	28	3	200	15	6	4	0.1	2	45	0.03	10	8	30.44	31.74	60	63.63
16	20	8	100	5	6	4	0.5	2	45	0.03	10	6	44.03	44.24	146	146.30
17	20	8	200	15	1	0.5	0.1	2	15	0.03	3	8	42.06	41.82	152	155.63
18	20	8	200	15	6	0.5	0.5	0.2	15	0.03	10	8	31.91	31.66	108	105.10
19	20	3	200	5	6	0.5	0.1	0.2	45	0.1	10	8	43.17	42.46	68	68.30
20	28	8	200	15	1	4	0.1	0.2	45	0.1	3	6	50.38	49.70	102	97.10

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Run	Variables												Antibacterial activity against *S. aureus* (Bu/ml)		Antibacterial activity against *E. Faecalis* (BU/ml)	
	A	B	C	D	E	F	G	H	J	K	L	M	Exp	Pred	Exp	Pred
21	20	3	100	15	1	4	0.1	0.2	15	0.03	10	6	31.98	32.24	100	95.10
22	20	8	100	15	1	0.5	0.1	2	45	0.1	10	8	67.33	68.18	47	47.30
23	20	3	200	15	1	0.5	0.5	2	45	0.03	3	6	41.03	42.33	115	113.43
24	28	8	200	5	1	0.5	0.1	2	15	0.03	10	6	29.1	29.82	38	47.16
25	28	3	100	5	6	4	0.5	0.2	45	0.1	10	6	33.39	33.38	1.5	-5.65
26	20	3	100	5	6	0.5	0.1	2	15	0.03	10	8	41.20	40.68	62	65.63

Exp: Experimental Response, Pred: Predicted Response

Table 4. Estimated effects and their coefficients for antibacterial compound production models by *Streptomyces* sp., MN38 against *S. aureus*. C.V. (%) = 2.80, AP = 89.3146, S.D.= 1.04, Mean = 37.03, R2 = 0.9978.

Source	Code	Sum of squares	df	Mean square	F-value	p-value	Result	Coefficient estimate
Model	-	6386.48	12	532.21	496.07	< 0.0001	significant	-
Time	B	531.31	1	531.31	495.23	< 0.0001 [b]	-	-4.61
Shaking Speed	C	961.05	1	961.05	895.79	< 0.0001 [a]	-	6.19
Starch	D	267.55	1	267.55	249.38	< 0.0001 [b]	-	-3.27
Yeast extract	E	309.89	1	309.89	288.85	< 0.0001 [a]	-	3.52
Peptone	F	2296.90	1	2296.90	2140.93	< 0.0001 [a]	-	9.58
KBr	G	355.74	1	355.74	331.59	< 0.0001 [b]	-	-0.5331
$CaCo_3$	H	531.31	1	531.31	495.23	< 0.0001 [a]	-	1.67
Sea Salt	J	961.05	1	961.05	895.79	< 0.0001 [b]	-	-7.26
$Fe_2(So_4)_3$	K	267.55	1	267.55	249.38	< 0.0001 [a]	-	1.13
Inoculum Size	L	309.89	1	309.89	288.85	< 0.0001 [b]	-	-1.34
pH	M	409.30	1	409.30	381.50	< 0.0001 [b]		-4.04
Residual	13.95	13	1.07	-	-	-	-	-
Cor Total	6400.43	25	-	-	-	-	-	-

a Significantly positive effect at P<0.05. b Significantly negative effect at P<0.05. CV: coefficient of variation, AP: Adequate precision, R2: determination of coefficient.

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Table 5. Estimated effects and their coefficients for antibacterial compound production models by *Streptomyces* sp., MN38 against *E. faecalis*. C.V (%) = 6.96, AP=45.2526, S.D= 5.60, Mean = 80.37, R2 = 0.9917

Source	Code	Sum of squares	do	Mean square	F-value	p-value	Result	Coefficient estimate
Model	-	52418.45	11	4765.31	152.20	< 0.0001	significant	-
Temp	A	5097.61	1	5097.61	162.82	< 0.0001 [a]	-	14.25
Time	B	15477.94	1	15477.94	494.36	< 0.0001 [b]	-	-24.84
Shaking Speed	C	344.37	1	344.37	11.00	0.0051 [a]	-	3.70
Starch	D	2992.16	1	2992.16	95.57	< 0.0001 [b]	-	-10.92
Yeast extract	E	136.18	1	136.18	4.35	0.0558 [C]	-	-2.33
Peptone	F	1031.92	1	1031.92	32.96	< 0.0001 [b]	-	-6.41
KBr	G	10893.94	1	10893.94	347.95	< 0.0001 [a]	-	20.84
$CaCo_3$	H	156.36	1	156.36	4.99	0.0422	-	2.50
Sea Salt	J	13317.04	1	13317.04	425.34	< 0.0001 [b]	-	-23.04
$Fe_2(So_4)_3$	K	1457.42	1	1457.42	46.55	< 0.0001 [b]	-	-7.62
Inoculum Size	L	1590.58	1	1590.58	50.80	< 0.0001 [a]	-	7.96
Residual	13.95	13	1.07	-	-	-	-	-
Cor Total	6400.43	25	-	-	-	-	-	-

a Significantly positive effect at P<0.05. b Significantly negative effect at P<0.05. CV: coefficient of variation, AP: Adequate precision, R2: determination of coefficient.

Bioassay against *E. faecalis*

Results indicated that the maximum antibacterial production against *E. faecalis* by *Streptomyces* sp., MN38 165.0 BU/ml was recorded in run 7 with positive effect of incubation temperature, shaking speed, KBr, and $CaCO_3$, inoculum size, low levels negative effect of incubation time, starch, peptone, sea salt, and $Fe_2(SO_4)_3$ (Figure 3b). The lowest antibiotic yield by MN38, 1.50 BU/mL, was observed in run 25 (Table 5). The significant variables against *E. faecalis* by MN38 including incubation temperature, incubation time, shaking speed, starch, peptone, KBr, $CaCO_3$, sea salt, $Fe_2(SO_4)_3$, and inoculum size were selected for further optimization by a Model and Numerical with 2FI/ Min Run Res- IV design.

The antibacterial activity and corresponding experimental data are presented in Table 5. Regression analysis was performed to fit the data to a second-order polynomial equation using Design Expert v13. The resulting equation describes the relationship between the experimental factors and the antibacterial activity: Equation (2)

$$Y= 80.3654 + (14.3229\times A) + (-24.9063\times B) + (3.63542\times C) + (-10.9896\times D) + (-6.34375\times F) + (20.9063\times G) + (2.42708\times H) + (-22.9688\times J) + (-7.55208\times K) + (8.03125\times L)$$

The statistical model was highly significant for antibiotic production by the MN38 strain, as evidenced by an F-value of 152.20 and P-values less than 0.0500. The predicted R2 (0.9733) and adjusted R2 (0.9852) values were in good agreement, indicating that the model was a good fit for the data. Yeast extract was found to be a non-significant variable.

Equation establishment

According to the guides from this procedure, the two-order polynomial equation (1) needs to be established to show the relationship between the response (S) and the main effects variables (X), as in the following equation (Equation 3):

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$$S = a_0 + \sum_{i-1}^{k} a_i x_i + \sum_{i-1}^{k} a_{ii} x_i^2 + \sum_{ij-1, j \neq i}^{k} a_{ij} x_i x_j$$

The model equation response (S) is a function of the intercept term (a0), linear coefficients (ai), quadratic coefficients (aij), and interaction coefficients (aii). These coefficients measure the effects of the variables xi, xixj, and x2i, respectively. The term xi, xj represents the first-order interaction between xi and xj (where i is less than j). The model's statistical adequacy was assessed using ANOVA.

3D response surface plots were created to visualize the relationship between the variables and antibacterial activity. The optimal levels of the variables for maximizing antibacterial activity were identified using a response optimizer tool.

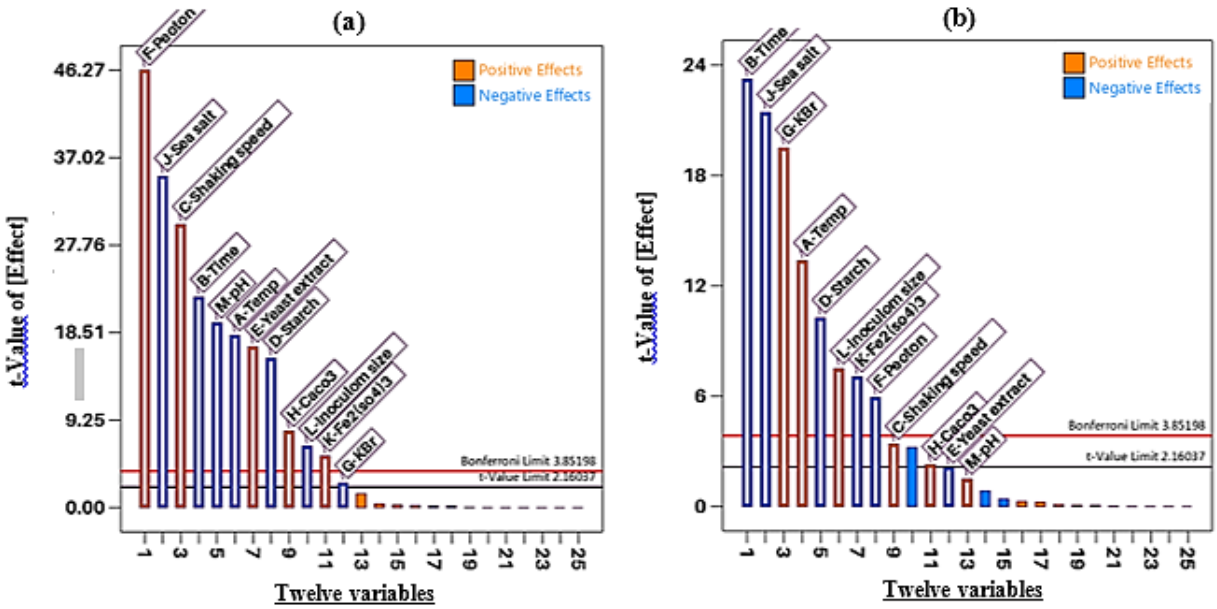

Figure 3. Effect of different factors on the production of the active compound by MN38 against *S. aureus* (a) and *E. faecalis* (b). Created using Design Expert 13. Here, the X axis shows the twelve variables tested, while the Y axis demonstrates their t-value of effect. The variables that possess a t-value more than the threshold, affect the production yield negatively (blue color) or positively (brown color).

Parameters analysis

The possible parameters consisted of incubation temperature (A); incubation period (B); shaking speed (C); starch (D); yeast extract (E); peptone (F); KBr (G); $CaCO_3$ (H); sea salt (J); $Fe_2(SO_4)_3$ (K); with inoculum size (L) at pH (M), for at both low (-1) and high (+1) levels for investigation of the data ranges, are shown in Table 1. Each experiment of antibacterial production was separately conducted based on the guides of minimum-run resolution IV. By establishing 2FI with the DX13 program, the ANOVA table allows the observed and predicted data to be analyzed. Thoroughly, according to Tables 4 and 5, estimated effects and their coefficients for two models were listed to find out the significance of parameters in two states, including a significant positive effect at $P<0.05$, and a significant negative effect at $P<0.05$. Apparently, among the twelve parameters that affected the production of bioactive compounds against *S. aureus*, incubation temperature (A), incubation time (B), starch (D), KBr (G), sea salt (J), inoculum size (L) and pH (M) had the significantly negative effect at $P<0.05$, while the shaking speed (C), pepton (F), yeast extract (E), $CaCO_3$ (H), and $Fe_2(SO_4)_3$ (K) affected the biomolecule production positively. As a result, decreasing the incubation temperature °C (A), incubation time (days) (B), shaking speed rpm (C), and pH (M), with decreasing concentration of sea salt g/L (J), starch g/L (D) and KBr g/L (G), besides increasing the concentration of the peptone g/L (F), yeast extract g/L (E), $CaCO_3$ g/L (H), and $Fe_2(SO_4)_3$ g/L (K) tended to improve the percentage of antibacterial compound production (BU/ml) (Table 4, Figure 3a). Instead of 12 variables, ten factors affected the production of

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antimicrobial agents produced by MN38 against *E. faecalis*. For example, the incubation time (B), starch (D), peptone (F), sea salt (J), and $Fe_2(SO_4)_3$ (K) had a significantly negative effect at $P<0.05$, while the incubation temperature (A), shaking speed (C), KBr (G), $CaCO_3$ (H), and inoculum size (L) positively enhanced the bioactive compound production. It is indicated that by increasing the shaking speed rpm (C), incubation temperature °C (A), inoculum size % (L), CaCO3 g/L (H), and concentration of the KBr g/L (G) with decreasing the incubation time, concentration of starch g/L (D), pepton g/L (G), sea salt g/L (J) and $Fe_2(SO_4)_3$ g/L (K), the antimicrobial biomolecule production will be improved (BU/ml) (Table 5, Figure 3b).

Analysis of the Optimization Process

A quadratic regression model was developed to assess the interactive effects of various factors and to optimize the conditions for enhanced antibacterial activity. Following the identification of optimal conditions, a confirmation experiment was conducted to validate the proposed model. These comprehensive optimization studies were undertaken to maximize the production of the antibacterial compounds. Table 3 exhibits both actual and predicted values for improved antibacterial activity against *S. aureus* and *E. faecalis*. The quadratic model's statistical significance was evaluated using ANOVA. P values, R2 coefficients, and AP ratios were calculated and the model was considered significant at a 95% confidence level based on these values. Studentized deleted residuals were used to assess the model's adequacy (Tables 4 and 5). The normal probability plot of residuals in Figures S1 and S2 shows an S-shaped pattern instead of a linear or normal pattern. However, it is suggested that transforming the residuals by dividing them by their estimated standard deviations could improve the analysis (24).The predicted value versus actual data shows how actual and predicted results (BU/ml) are correlated with the bioactive agent production produced by MN38 against *S. aureus* (Figure S3a) and *E. faecalis* (Figure S3b). It shows the results were predicted well using the suggested model equation with high correlation.

The predicted versus residuals is an important analysis to evaluate the model adequacy. Here, in plot Figure S4a and S4b show a random scatter, hence, most of the points were inside two limits ±3.85198 and ±3.78739, for the improved antibacterial activity against *S. aureus* and E. faecali, respectively, indicating the variance is a constant against the residuals' variables. In addition, Figure S4c and S4d confirm that the residuals for experimental runs do not follow a specific pattern. Three-dimensional and two-dimensional response surface graphs were generated to visualize the correlation between the experimental variables and the antibacterial activity. The response optimizer tool was employed to identify the optimal levels of the variables for maximizing antimicrobial activity. Figure 4 and S5 are the response surface 3D plots and 2D plots showing individual and interactivity effects of variables on the antibacterial activity of *Streptomyces* sp. MN38 against *E. faecalis* bacteria. Figure 4a and S5a shows the effects of incubation temperature and incubation time on the antibacterial activity of MN38 against *E. faecalis*. Although, at different temperatures, the response rate decreases with increasing time, increasing in temperature could decrease the downward trend in the results when the strain is incubated for more days. In the first three days of increasing temperature, there is a greater effect on the response and this effect will decrease with time, while the antibacterial activity increased with increasing of both the temperature and shaking speed (Figure 4b and S5b). When the effect of starch was analyzed (Figure S5c) the antibacterial activity decreased with the increase of starch concentration. Also at 5 g /L starch, the increase in temperature has a greater effect on the response and this effect decreases with the increase of starch. In addition, Figure. S5d depicted that the antibacterial activity of MN38 against *E. faecalis* increased with increasing concentration of $CaCO_3$ and incubation temperature.

Reducing peptone and increasing the shaking speed increases the response (Figure S5e), with the difference that the effect of the shaking speed is less than that of Peptone because increasing the shaking speed in the maximum and minimum possible concentration of Peptone will not make much difference on the response rate. Figure S5f shows that simultaneously increasing both shaking speed and inoculum size enhances the response; but compared to inoculum size, the effect is less pronounced as when the inoculum size reaches the maximum, in both shaking speed of 100 and 200 (rpm), maximum response value is achieved. Both seal salt and starch possess negative effects (Figure S5g) on antimicrobial

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production, when their concentration increased. Finally, the effect of increasing inoculum size in low peptone concentration has a greater effect on the response rate and this effect decreases with increasing concentration of peptone (Figure S5h). But in general, an increase in both parameters leads to a higher response.

Figure 5a and S6a shows the increasing antibacterial activity against *S. aureus* with increasing incubation temperature and time, while the effect of increasing the shaking speed will be more significant at 20°C, with the response rate reaching a maximum at a speed of 200 (rpm) and with increasing temperature, this effect will gradually decrease, and at 28°C and shaking speed of 100 (rpm) the minimum response was recorded (Figure 5b and S6b).

It should be noted that the effect of increasing the shaking speed is greater and more effective than the temperature. In addition, the effect of increasing peptone concentration at 20°C will be more significant, in such a way that at 4 g/l peptone, the response rate reaches the maximum, however, with increasing temperature, this effect gradually decreases thus and so at 28°C and 0.5 g/l peptone will have the lowest response. It should be noted that the effect of peptone increase is greater and more effective than temperature (Figure S6c). Antibacterial activity increases with increasing peptone and yeast extract. At 6 g/l yeast extract and 4 g/l peptone, the response rate will reach the maximum. In this interaction, the effect of Peptone will be greater than Yeast extract (Figure S6d). The active compound production improved with increasing sea salt and yeast extract. In this interaction, the effect of sea salt will be less than that of yeast extract (Figure S6e). On the contrary, the bioactivity with decreasing concentration of sea salt and inoculum size (Figure S6f). Finally, the antimicrobial activity was enhanced with increasing $Fe_2(SO_4)_3$ concentration and decreasing pH (Figure S6g).

Discussion

Nowadays, it has become increasingly important to pay closer attention to infectious diseases because of their significant threat to public health and the overall safety of communities worldwide (1). Since most antibiotic drugs are produced by microorganisms, it is worth studying marine *actinomycetes* as a natural source for antimicrobial production (R3). In this study, marine strain MN38, previously isolated from the Caspian Sea, shared 99% phylogenetic similarity with *Streptomyces* sp., GB1 (KF313073), and *Streptomyces rochei* strain HBUM 174697 (FJ532458). This strain revealed antibacterial activity against clinically important pathogens like *S. aureus*, *E. faecalis*, *P. aeruginosa*, and *K. pneumoniae* (table S3).

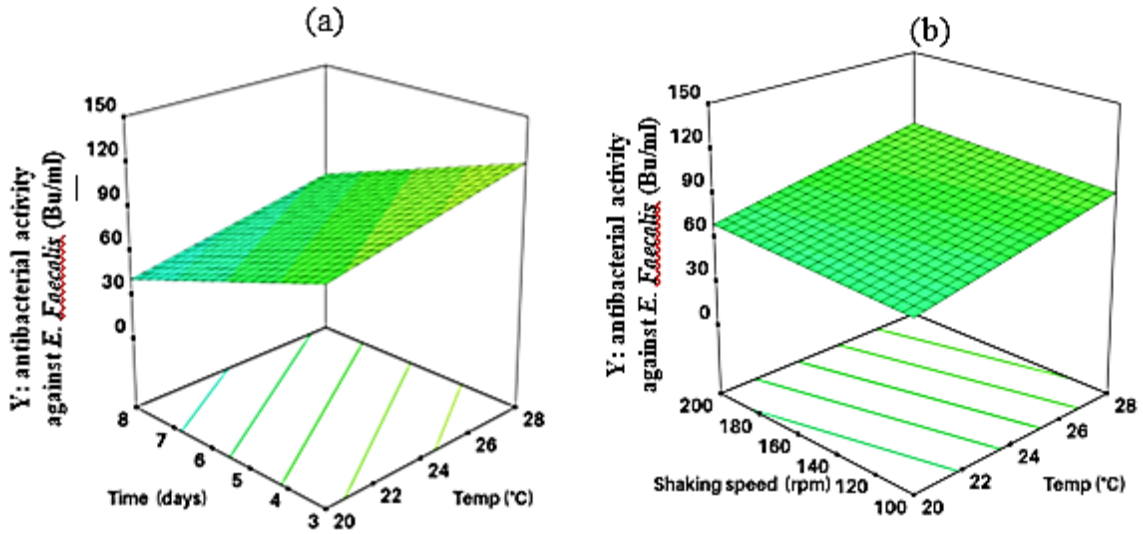

Figure 4. Plotting the response curve of various factors against *E. faecalis* and their relation. Fig 4a shows the effect of incubation temperature and incubation time on antibacterial activity. At different temperatures, the response rate decreases with increasing time. Fig 4b demonstrates the effect of shaking speed and incubation temperature on the antibacterial activity of MN38 against *E. faecalis*. The antibacterial activity increased with increasing both the temperature and shaking speed.

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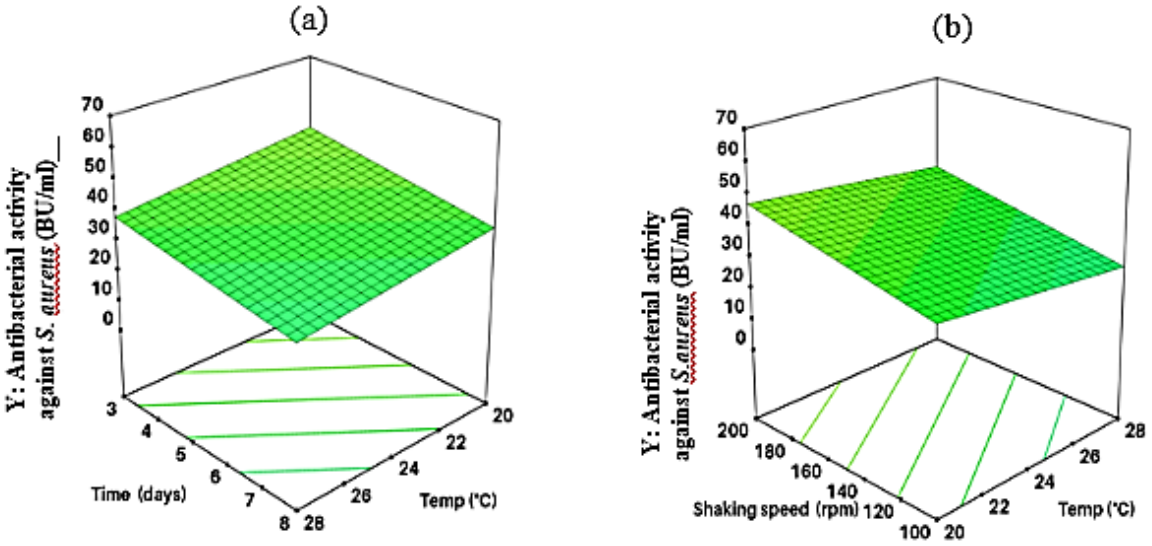

Fig. 5. Plotting the response curve of various factors against *S. aureus* and their relation. Fig 5a shows the effects of incubation temperature and incubation time on the antibacterial activity of MN38 against *S. aureus*. The antibacterial activity increased with increasing incubation temperature and incubation time. Fig 5b revealed the effects of shaking speed and incubation temperature on the antibacterial activity of MN38 against *S. aureus*. The effect of increasing the shaking speed will be more significant at 20°C, in such a way that the response rate reaches a maximum at a speed of 200 (rpm), and with increasing temperature, this effect will gradually decrease, and at 28°C and shaking speed of 100 (rpm) we will have the lowest answer. It should be noted that the effect of increasing the shaking speed is greater and more effective than the temperature.

Yang W et al. isolated S. griseorubens f8 from marine sediment of the Yellow Sea in China which produced an active compound with antibacterial activity against Staphylococcus aureus, *Klebsiella aerogenes* and *Proteus vulgaris* (25), while the crude extracts from marine strains SCSIO 64649T and SCSIO 03032 isolated from coral of South China sea exhibited only antifungal activity (26). This study identified an optimal condition for cultivating *Streptomyces* sp. MN38 to enhance the production of antibiotic compounds. An increase in time course (days) and sea salt concentration showed the most negative effect on the production of active metabolite(s) against both *S. aureus* and *E. faecalis* whereas the variables that positively affected the production were different (Figure. 3). For example, shaking speed (rpm) and pepton concentration exerted positive effect on the antibacterial activity of crude extract against *S. aureus*, significantly, but against *E. faecalis* the production of bioactive molecules was positively influenced by temperature (˚C) and KBr concentration. It could be concluded that the MN38 strain produced, at least, two active compounds. Norouzi H, et al. showed four variables starch concentration, pH, $CaCO_3$ concentration and Pepton concentration had the main effect on the production of antimicrobial agents active against *S. aureus* (27). Statistical analysis for Plackett-Burman experiment for the antimicrobial production from marine *Streptomyces* sp. MMM2 exhibited Temperature and starch concentration affected the production significantly (28). Our results revealed that the antibacterial agent production under optimal concentration reached up to 69.34 (BU/ml) and 166.38 (Bu/ml) against *S. aureus* and *E. faecalis*, respectively. According to the 2FI/ Min Run Res- IV model R^2 value was calculated at 0.9978 and 0.9917 when antimicrobial activity was studied against *S. aureus* and *E. faecalis*, respectively, indicating the total variation could be explained by model with 99.0% accuracy. The closer the R^2 value to 1.00, the more accuracy of the model to predict the response (29). Therefore, regarding the calculated R^2, the developed design optimized the culture conditions with high accuracy. A novel marine *Streptomyces* isolate, MN38, demonstrated potent antibacterial activity against both

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Gram-positive and Gram-negative pathogens. The highlight of this work was the optimization of culture conditions to enhance antimicrobial activity and demonstrating the possibility of using mathematical models to accelerate the optimization process. Our findings paved the way for further characterization of this strain and its bioactive compounds to use for clinical and pharmaceutical purposes.

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