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Statistical optimization of fermentation conditions for bioactive compounds production by marine *Acinetobacter* sp. EM11 against the fish pathogen *Vibrio alginolyticus* MK170250

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ABSTRACT

Thirty-two marine bacterial strains were isolated from marine samples (seawater and sediments) collected from the Mediterranean Sea, Egypt. They were evaluated for their antimicrobial activity against the fish pathogen *Vibrio alginolyticus* MK170250, using the agar well-cut diffusion technique. The marine bacterial isolates EM11 showed significant activity against *V. alginolyticus* MK170250 with an inhibition zone of (10 mm). The isolate EM11 was phenotypically characterized and molecularly identified through 16S rRNA gene sequence analysis as *Acinetobacter indicus*. Placket-Burman and Box-Behnken experimental designs were applied to optimize the fermentation conditions and increase the bioactive compounds' productivity. The optimized medium was formulated as follows: (gl^{-1}) Peptone 7 g; beef extract: 2 g; inoculum's size (ml) 0.5; sea water concentration: 75%; pH: 6.02; temperature (°C) 40 for 24h incubation period. A verification experiment was applied to evaluate the basal versus the optimized medium. The optimized growth condition successfully increased the inhibition zone about 3.65-fold with an inhibition zone of 36.5 mm in diameter. To the best of our knowledge, this is the first report of *Acinetobacter indicus* species from marine sediments and its anti-fish pathogen activity.

1. INTRODUCTION

The *Vibrio* species represent a large percentage of the natural microbiota in marine ecosystems **(Urakawa & Rivera, 2006)**. This is mainly due to its wide host range as it may exist in a relationship with zooplankton, shellfish, shrimp and fishes. The *Vibrio* species coexist with the host as a mutualistic symbiont or an opportunistic pathogen. In the latter case, the drawbacks are intense on the aquaculture industry **(Austin & Austin, 2007)**. Moreover, Vibriosis, a human illness, may develop post consumption of contaminated seafood meal. In Egypt, where aquaculture production is considered the largest in Africa and 11th of the global market **(El-Sayed *et al.*, 2015; Abd El Tawab *et al.*, 2018)** there is a constant need to identify natural marine pathogens of *Vibrio* sp., which forms the top threat of aquaculture industry. Diverse *Vibrio* species are reported as

fish disease-causing opportunistic pathogens, including *V. alginolyticus*, *V. harveyi* and *V. parahaemolyticus* **(Jin *et al*., 2004; Liu *et al*., 2016)**.

The disease inducing ability of these *Vibrio* species are attributed to diverse factors. For instance, *V. alginolyticus* is known to produce extracellular natural products with a fish lethal effect. Previous studies reported the ability of *V. alginolyticus* to produce several enzymes, such as haemolysin, chitinase, lecithinase, protease, lipase and **(Jin *et al*., 2004)**. The arsenal of enzymes and other unknown factors produced by this pathogen make its treatment a difficult process. On the other hand, the marine habitats are rich with diverse beneficial bioactive substances produced by marine invertebrates and algae. These natural products are produced by these organisms for several reasons, such as exiling predators and competitors, or serving as mating attractants or as dye for the expulsion or attraction of the surrounding organisms. Recently, the modern marine biotechnology has been expanded to explore the marine fungi and bacteria and its secondary metabolites produced under adjusted *in vitro* conditions. Notably, many bacterial species are known as potent producers of bioactive substances with various and unique chemical structure, and hence promising potentials in marine market as antibiotics, antivirals, anti-inflammatory and anticancer drugs **(Waters *et al*., 2010; Imhoff *et al*., 2011; Petersen *et al*., 2020)**.

Among the newly explored natural bioactive substances-producing marine bacterial genera *Acinetobacter* is recognized. This genus is a member of family Moraxellaceae, characterized by having Gammaproteo bacteria, Gram-negative, strict aerobic, catalase-positive, oxidase-negative and non-motile bacteria **(Malhotra *et al*., 2012; Bunnoy *et al*., 2019)**. Although described as non-motile, they possess pilli on their cell membranes **(Harding *et al*., 2013)**. Ironically, most of the genus members have been isolated from human specimens and are described as opportunistic pathogens. However recently, many beneficial *Acinetobacter* species were isolated from diverse environmental habitats, such as soil, water, food, air, horse faeces and honey bees **(Carr *et al*., 2003; Krizova *et al*., 2014; Poppel *et al*., 2015; Bunnoy *et al*., 2019)**. Interestingly, it was only recently reported from a marine coastal-mud sample **(Anjum *et al*., 2019)**. The resilience of this genus members to withstand such wide and vibrant habitats attracted the attention towards possible future biotechnological applications of *Acinetobacter* sp. Indeed, the big number and structurally unique natural substances produced by *Acinetobacter* members are outstanding. For example, *Acinetobacter* sp. ZZ1725 produce alkaloids indolepyrazines A and B **(Anjum *et al*., 2019)**. In addition, *A. haemolyticus* ATCC 17906 and *A. baumannii* are both siderophores-producing bacteria **(Okujo *et al*., 1994; Yamamoto *et al*., 1994)**. While, *A. baumannii* produces polysaccharides **(Haseley *et al*., 1997)** and anti-fungal volatile compounds **(Kadhim, 2016)**.

Therefore, future research is expected to identify other unknown metabolites. It is worth to mention that, the production process of these antimicrobial metabolites derived from marine bacteria is not as easy as it may sound. A careful adjustment of each fermentation medium component is required. Otherwise, usually the achieved antimicrobial activity won't be as promising as it could be. Diverse statistical designs can be applied to achieve this target. Remarkably, Plackett-Burman and Box-Behnken are efficient statistical designs in optimizing the antimicrobial metabolites production by diverse bacterial species **(Yun *et al.*, 2018)**, by which the concentration of each medium component, pH, temperature, inoculum's size, etc are determined. Moreover, careful storage of adjusted fermentation medium is needed to avoid any change in the obtained antimicrobial activity **(Yun *et al.*, 2018)**.

In the current study, the marine *A. indicus* EM11 isolation, its characterization and statistical optimization of its growth and/or bioactive compounds production were addressed. This work would present the prerequisite study required for future exploration for the aquaculture sustainability and its preservation from fish pathogen *V. alginolyticus.* To the best of our knowledge, this is the first report of *A. indicus* species from marine sediments and its anti-fish pathogen activity.

2. MATERIALS AND METHODS

2.1. Isolation of marine bacteria

Different marine samples (sea water and sediments) were collected from the Mediterranean Sea, Alexandria, Egypt in sterile containers and delivered to the laboratory in icebox. The water sample of l mL or 1 g in case of sediment samples was added to 9 mL of sterile distilled water and serial dilutions were prepared; an aount of 1 mL of each dilution was plated on nutrient agar medium prepared using filtered aged sea water (28 g l^{-1}, sterilized by autoclaving at 121°C for 15 min) and incubated at 37°C for 24h **(Zaghloul *et al.*, 2021)**. Different colonies were picked up for purification by streaking on a nutrient agar medium. A number of 32 purified isolates were obtained and stored as glycerol stokes for further investigation.

2.2. Screening for antibacterial activity

The antibacterial activities of the obtained isolates were examined against the indicator pathogen *V. alginolyticus* MK170250 using the agar well diffusion assay. *V. alginolyticus* MK170250 was isolated from captive-bred adult dark-spotted stingrays (*Himantura uarnak*) housed at the Hurghada Grand Aquarium (Egypt) through members of the Fish Diseases Laboratory at the National Institute of Oceanography and Fisheries, Red Sea Branch. Briefly, each isolate was grown in nutrient broth medium prepared by filtered aged sea water (13 gl^{-1}, sterilized by autoclaving at 121°C for 15min) for 24h at 37°C. Then, the cells were removed by centrifugation at 4000 g for 10min, and the supernatants were sterilized by syringe filter (0.45 μm). The nutrient agar prepared by

distilled water (28 gl^{-1}, sterilized by autoclaving at 121°C for 15 min) was inoculated with 100 µl of 10^6 CFU mL^{-1} culture of *V. alginolyticus* MK170250. Wells of 8 mm were punched in the inoculated agar plate. Then, 100 µl of the cell free culture supernatant of each isolate was added to the wells, and the plates were incubated at 37°C for 24h. The diameter of inhibition zone around each well was measured. The experiment was conducted in triplicates, and the results were represented as mean experiment ± SD **(Zaghloul & Ibrahim, 2019)**.

2.3. Molecular taxonomy and phylogenetic analysis

Isolate EM11 gave the highest antibacterial activity and it was molecularly identified through sequencing of 16S rRNA encoding gene. Briefly, the genomic DNA of the marine isolate EM11 was isolated using DNA extraction kit (QIAGEN, German) according to the manufacturer instructions. The extracted DNA was then analyzed by electrophoresis in 1% agarose gel, dissolved in TAE buffer containing 1 µg mL^{-1} ethidium bromide (Sigma, USA). The DNA was mixed with the loading day and loaded in the prepared gel soaked in TAE buffer, then the voltage (90 v cm^{-1}) was applied. At the end, the DNA was visualized by the UV transilluminator (Bio-Rad, USA) **(Zaghloul *et al*., 2021)**. The 16S rRNA gene was amplified by polymerase chain reaction (PCR), and the PCR product was sequenced through the sequence facility offered by Applied Biotechnology Company, Egypt. The obtained sequence was BLASTed (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the latest release of the GenBank database. Moreover, it was submitted to the National Centre for Biotechnology Information (NCBI), and the phylogenetic analysis was constructed through www.phylogeny.fr website **(Amer *et al*., 2020).**

2.4. Optimization of the bioactive compounds production

2.4.1. Statistical design for optimization of bioactive compounds by isolate EM11

The Plackett-Burman experimental design **(Plackett & Burman, 1946)** was conducted to show how far the medium components were necessary to produce the bioactive compounds by the selected isolate (EM11) using nutrient broth medium. The Plackett-Burman design matrix had seven independent variables (Table 1) in eight combinations (Table 2). The baseline control was in row No. 9. High (+1) and low (−1) values were evaluated for each component. Each experiment was repeated twice, and the mean of these values was used as the response. Both trials were conducted in triplicate. The main effect of each variable was estimated using the following equation:

$$E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-}) / N$$

Where; Exi is the variable main effect; the bioactive production radiuses for the tests were Mi+ and Mi-, where the independent variables were present in high and low concentrations, respectively, and N was used to measure the statistical t-values of the

equal unpaired samples to determine the variable mean through dividing by two, using Microsoft Excel 2019.

Table 1. Independent variables affecting production of the bioactive compounds and their levels in the Plackett- Burman design

Factor	Symbol	Level		
		-1	**0**	**1**
Peptone (gl^{-1})	P	2.5	5	7.5
Beef extract (gl^{-1})	B	1.5	3	4.5
Inoculum's size (mL)	IS	0.5	1	1.5
Sea water concentration (%)	S. W	10	50	100
pH	pH	6	7	8
Temperature (^oC)	T	30	37	40
Incubation period (h)	IP	12	24	48

* Inoculum's size was added in mL of 24 h culture filtrate (10^6 CFU mL^{-1})

Table 2. The experimental results of the applied Placket-Burman design for seven cultural variables

Trail	P	B	IS	S. W	pH	Temp	IP
1	-1	-1	-1	1	1	1	-1
2	1	-1	-1	-1	-1	1	1
3	-1	1	-1	-1	1	-1	1
4	1	1	-1	1	-1	-1	-1
5	-1	-1	1	1	-1	-1	1
6	1	-1	1	-1	1	-1	-1
7	-1	1	1	-1	-1	1	-1
8	1	1	1	1	1	1	1
9	0	0	0	0	0	0	0

2.4.2. Experimental verification

Verification tests were conducted with double standards using the predicted optimized medium to validate the Plackett-Burman design statistical analysis results. The production of bioactive compounds was measured by dividing the activity against fish pathogen *V. alginolyticus* MK170250.

2.4.3. Optimization of culture conditions using Box-Behnken design

After assessing the relative importance of separate variables, the four most important variables were chosen to determine the optimum levels for the bioactive compounds production (Table 3). The Box-Behnken design (BBD) was used **(Box & Behnken, 1960; Agrawal *et al*., 2020)**. This optimization method includes three key steps; coefficient estimation of a mathematical model, response prediction and model adequacy verification. The four significant variables elucidated using the Plackett-

Burman experimental design for the selected best bioactive compounds produced by the isolate *A. indicus* EM11 were (beef extract X_1, sea water concentration X_2, pH X_3 and incubation period X_4). The center and tall levels of each variable were assigned as −1, 0, and +1, individually. A framework was built for the 27 trials, alongside the normal values for the four variables. The experiments were performed in triplicate, and the mean values were determined for bioactive compounds. The connection between the free factors and response functions was correlated using a second-order polynomial to actuate the optimal point. The condition of the four components was calculated using the formula below:

$$Y=B_0+B_1X_1+B_2X_2+B_3X_3+B_4X_4+B_{12}X_1X_2+B_{13}X_1X_3+B_{14}X_1X_4+B_{23}X_2X_3+B_{24}X_2X_4+B_{34}X_3X_4 +B_{11}X_{11}+B_{22}X_{22}+B_{33}X_{33}+B_{44}+X_{44}$$

Where; Y is the anticipated response; β_0 is the show constant; X_1, X_2, X_3, and X_4 are the free factors; β_1, β_2, β_3, and β_4 are the direct coefficients; β_{12}, β_{13}, β_{23}, and β_{24} are the cross-product coefficients, and β_{11}, β_{22}, β_{33}, and β_{44} are the quadratic coefficients. Microsoft Excel 2019 was used to examine the experimental data collected using regression analysis**.**

2.4.4. Statistical analysis

Numerous straight relapses were made utilizing Microsoft Excel predictions to determine the significance of the bioactive compounds in terms of t-value, *P*-value and confidence level. The level of significance (P-value) was resolved using the student's t-test. Every single impact t-test assesses the probability that finding the observed effect was pure chance. If this is highly unlikely, then the effect is thought to be caused by the variable when it is below the accepted level, such as 5%. The confidence level reflects a percentage of the *P*-value. The activities were assessed using the Microsoft Excel solver add-in program. Each response was simultaneously visualized in three-dimensional graphics created, using STATISTICA 10.0 software for the four largest independent factors.

2.4.5. Model verification

Experimentally, optimal conditions were verified from the optimization experiments. The predictions were examined and compared to the basic conditions, near-optimal conditions, and conditions different to the optimum levels of the independent variables.

Table 3. The Box-Behnken experimental design for four factors

x1	*x2*	*x3*	*x4*	x1*x2	x1*x3	x1*x4	x2*x3	x2*x4	x3*x4	x1*x1	x2*x2	x3*x3	x4*x4
0	1	0	1	0	0	0	0	1	0	0	1	0	1
1	−1	0	0	−1	0	0	0	0	0	1	1	0	0
0	0	1	−1	0	0	0	0	0	−1	0	0	1	1
1	0	0	1	0	0	1	0	0	0	1	0	0	1
0	1	−1	0	0	0	0	−1	0	0	0	1	1	0
0	0	−1	1	0	0	0	0	0	−1	0	0	1	1
0	0	1	1	0	0	0	0	0	1	0	0	1	1
1	0	1	0	0	1	0	0	0	0	1	0	1	0
−1	0	1	0	0	−1	0	0	0	0	1	0	1	0
0	0	−1	−1	0	0	0	0	0	1	0	0	1	1
0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	1	0	−1	0	0	0	0	−1	0	0	1	0	1
−1	0	0	1	0	0	−1	0	0	0	1	0	0	1
0	1	1	0	0	0	0	1	0	0	0	1	1	0
1	1	0	0	1	0	0	0	0	0	1	1	0	0
0	−1	0	−1	0	0	0	0	1	0	0	1	0	1
1	0	0	−1	0	0	−1	0	0	0	1	0	0	1
−1	0	−1	0	0	1	0	0	0	0	1	0	1	0
−1	−1	0	0	1	0	0	0	0	0	1	1	0	0
−1	0	0	−1	0	0	1	0	0	0	1	0	0	1
0	−1	−1	0	0	0	0	1	0	0	0	1	1	0
0	−1	1	0	0	0	0	−1	0	0	0	1	1	0
1	0	−1	0	0	−1	0	0	0	0	1	0	1	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	−1	0	1	0	0	0	0	−1	0	0	1	0	1
−1	1	0	0	−1	0	0	0	0	0	1	1	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0

2.5. Ethics Statement

The authors confirmed that the ethical policies of the journal, as noted on the journal's author guidelines page, were adhered to. No ethical approval was required as no animal experiments were conducted in this study.

3. RESULTS

3.1. Isolation of marine bacteria

Thirty two bacterial isolates were purified from the marine samples collected. The isolates were screened for antibacterial activity against *Vibrio. alginolyticus*, and they showed different levels of activity as presented in Table (4). Isolate EM11 (Fig. 1) shows the highest activity against the *V. alginolyticus* so it was further identified using 16s

rRNA encoding gene sequencing homology analysis by BLAST program offered by the NCBI. The sequenced DNA fragment had 97% sequence similarity to *Acinetobacter indicus***,** and the sequence was submitted to the GenBank with the accession number of MW413325. In addition, the phylogenetic tree of isolate EM11 is represented in Fig. (2).

Table 4. Antimicrobial activity represented as diameter of inhibition zones in mm of the marine isolates against *Vibrio alginolyticus* MK170250

Isolate No	**Inhibition zone (mm)**	**Isolate No**	**Inhibition zone (mm)**	**Isolate No**	**Inhibition zone (mm)**	**Isolate No**	**Inhibition zone (mm)**
EM1	-ve	EM9	6 ± 0.22	EM17	-ve	EM25	-ve
EM2	-ve	EM10	7 ± 0.12	EM18	-ve	EM26	-ve
EM3	9 ± 0.47	EM11	10 ± 0.15	EM19	-ve	EM27	-ve
EM4	-ve	EM12	-ve	EM20	-ve	EM28	-ve
EM5	-ve	EM13	8 ± 0.06	EM21	-ve	EM29	7 ± 0.05
EM6	8 ± 0.14	EM14	8 ± 0.10	EM22	-ve	EM30	-ve
EM7	-ve	EM15	-ve	EM23	7 ± 0.21	EM31	9 ± 0.31
EM8	-ve	EM16	-ve	EM24	6 ± 0.05	EM32	-ve

-ve: negative result. Results are expressed as mean ± SD (n = 3), where mean is significant at $p <$ 0:05.

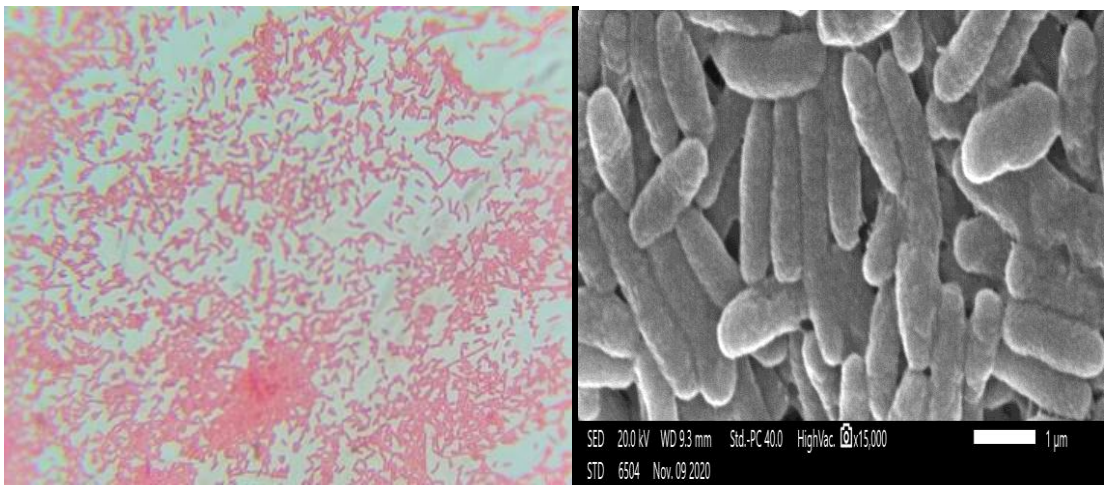


Fig. 1. Gram staining (Left) and scanning electon microscopy (Right) of the marine isolate *Acinetobacter indicus* EM11

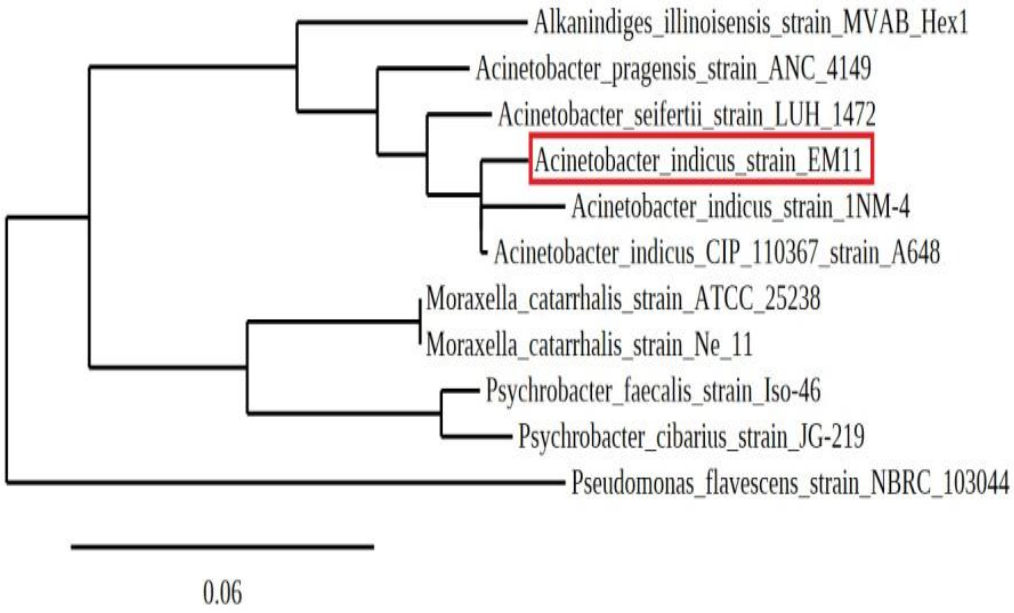


Fig. 2. Phylogenetic analysis of *Acinetobacter indicus* EM11

3.2. Statistical design for optimization of bioactive compounds by *Acinetobacter indicus* EM11 against fish pathogen *Vibrio alginolyticus* MK170250

Plackett-Burman design was employed to evaluate the significant effect of nutrient agar medium components for production of bioactive compounds from *A. indicus* EM11 against fish pathogen *V. alginolyticus* MK170250. The components of nutrient agar medium selected for the production of experimental bioactive compounds, in addition to some other cultural factors including sea water concentration, pH, temperature and incubation period, each of which were examined at two levels (-, +) as shown in Table (1). The experimental results of the applied Placket-Burman design for seven cultural variables against *V. alginolyticus* MK170250 are illustrated in Table (5). Statistical analysis of the data (t-test) showed that, among the examined environmental factors, sea water concentration, beef extract, incubation period and pH were the most significant independent variables affecting the bioactive compounds production and subsequently, the inhibition zone diameter against *V. alginolyticus* MK170250 (Table 6). The main effects of the examined factors on the inhibition zone diameter were calculated and are represented in Fig. (3). Based on those results, the positive (+) level of sea water concentration, and the incubation period, in addition to the negative level (-) of beef extract and the pH value supported the production. Moreover, the *t*-value represented in Table (6) supports this observation. This approach verified the validity of the applied design. Hence, a verification experiment was applied to evaluate the basal versus the optimized medium.

Table 5. The experimental results of the applied Placket-Burman design by *Acinetobacter indicus* for seven cultural variables against *Vibrio alginolyticus* MK170250

Trial	P	B	IS	S. W	pH	Temp	IP	Inhibition zone (mm)
1	-1	-1	-1	1	1	1	-1	12
2	1	-1	-1	-1	-1	1	1	18
3	-1	1	-1	-1	1	-1	1	0
4	1	1	-1	1	-1	-1	-1	10
5	-1	-1	1	1	-1	-1	1	16
6	1	-1	1	-1	1	-1	-1	0
7	-1	1	1	-1	-1	1	-1	0
8	1	1	1	1	1	1	1	12
9	0	0	0	0	0	0	0	10

Table 6. Statistical analyses of the Plackett-Burman experimental results

Variable	Main effect	*t*-value*
Peptone (gl^{-1})	1.5	3
Beef extract (gl^{-1})	-3	-6
Inoculum's size (mL)	-1.5	-3
Sea water concentration (%)	4	8
pH	-2.5	-5
Temperature (^oC)	2	4
Incubation period (h)	3	6

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

t-value significant at the 5% level = 2.446

t-value significant at the 10% level = 1.94

t-value significant at the 20% level =1.372

Standard *t*-values are obtained from Statistical Methods (Snedecor and William, 1989).

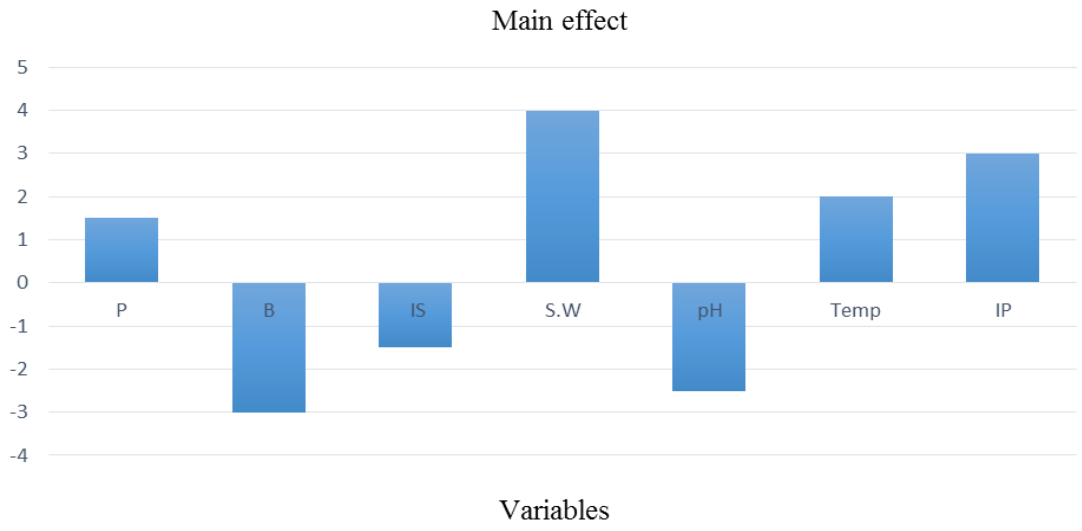
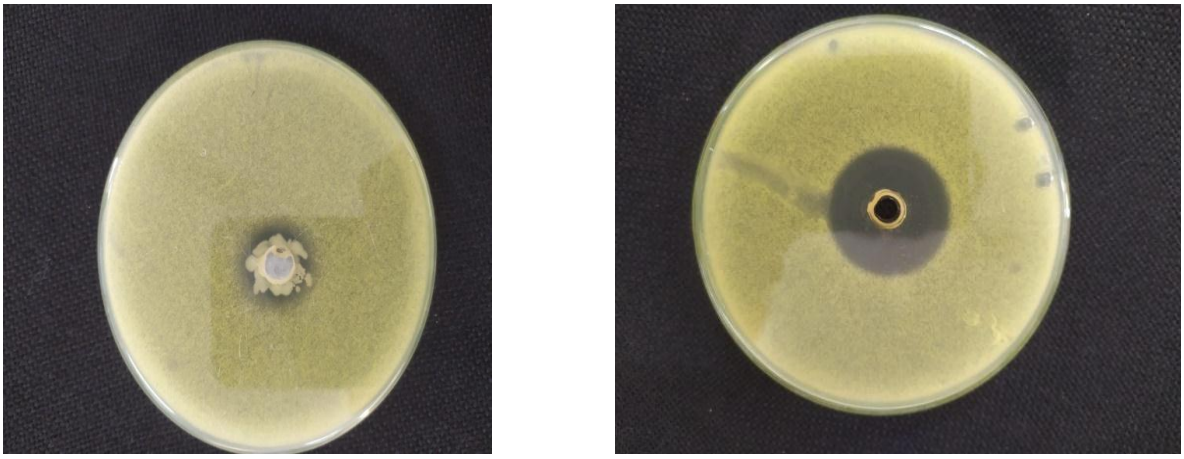


Fig. 3. Elucidation of fermentation conditions affecting the production of bioactive compounds by *Acinetobacter indicus* EM11 against *Vibrio alginolyticus* MK170250

3.3. Verification experiment

Results showed that, a near optimum production medium was formulated as follows: (gl^{-1}) Peptone 7; beef extract 1.5; inoculum's size (mL) 0.5; sea water concentration (%) 100; pH 6 and Temperature (°C) 40 for 48h incubation period. A verification experiment was applied to compare the predicted optimum levels of the independent variables and the basal conditions. The cultivation of isolate *A. indicus* EM11 in the verified medium, adjusted to pH 6 for 48 h, resulted in a 2.8-fold increase in the inhibition zone diameter compared to the basal conditions (Fig. 4).



(A) (B)

Fig. 4 Inhibition zone diameter of *Acinetobacter indicus* EM11 supernatant grown on basal medium **(A)** compared with the verified medium **(B)** against *Vibrio alginolyticus* MK170250

3.4. Optimization of the culture conditions using Box-Behnken design

To identify the optimum response region for bioactive compounds production, the significant independent variables (beef extract X_1, sea water concentration X_2, pH X_3 and incubation period X_4), suggested by the Plackett-Burman design were further investigated at three levels (-, 0, +) as shown in the Table (7) in Box-Behnken design. Table (8) presents the design matrix of the variables and the response of each trial. To predict the optimal point, a second order polynomial function was fitted to the experimental response results (non-linear optimization algorithm)

$$\mathbf{Y= 28.66+ 9.5X_1 -4.08X_2 + 6X_3 + 8.25X_4 – 14.5X_1X_2 + 3X_1X_3 - 7X_1X_4 - 6X_2X_3 + 10.25X_2X_4 + 9X_3X_4 - 1.95X_{11} – 8.58X_{22} – 14.2X_{33} - 1.83X_{44}}$$

On the model level, the correlation measures for estimating the regression equation are the multiple correlation coefficient *R* and the determination coefficient R^2. In this experiment, the value of R^2 was 0.889 for the bioactive compound's efficacy on test pathogen *V. alginolyticus* MK170250, indicating a high degree of correlation between the experimental and the predicted values. The optimal levels of the four factors (Table 3) obtained from the maximum point of the polynomial model were estimated using the *solver* function of the Microsoft Excel 2010 tools, and were recorded as follows: (beef extract: 2 g, sea water concentration: 75%, pH: 6.02 and incubation period 24h), with a predicted inhibition zone of 36 mm. In addition, Figs. (5A-F) show the simultaneous effects of the four most significant independent factors on each response, using three-dimensional graphs generated by STATISTICA 10.0 software. As shown in the surface plots of the Box-Behnken design variations in beef extract (X_1), sea water concentration (X_2), pH (X_3) and incubation period (X_4) within the examined concentration ranges and under the present experimental conditions, were clearly effective. Figs. (A-F) suggest that the increase in beef extract concentration to a value of (2 gl^{-1}), with high level of incubation period will promote the bioactive compound's efficacy. On the other hand, the higher level of the bioactive compound's production and higher diameter of inhibition zone were attained with the decrease of sea water concentration.

Table 7. The three levels of significant independent variables used in Box-Behnken factorial experimental design for bioactive compounds production by *Acinetobacter indicus* EM11 isolate EM11

Level	Beef extract X_1	Sea water concentration X_2	pH X_3	Incubation period X_4
1	2 g	150%	7	72 h
0	1.5 g	100%	6	48 h
-1	1 g	75%	5	24 h

Table 8. Box-Behnken factorial experimental design for bioactive compounds production by *Acinetobacter indicus* isolate EM11 against *Vibrio alginolyticus* MK170250

Ex	Beef extract X_1	Sea water concentration X_2	pH X_3	Incubation period X_4	Inhibition zone (mm)
1	0	1	0	1	36
2	1	-1	0	0	34
3	0	0	1	-1	0
4	1	0	0	1	36
5	0	1	-1	0	0
6	0	0	-1	1	0
7	0	0	1	1	36
8	1	0	1	0	38
9	-1	0	1	0	0
10	0	0	-1	-1	0
11	0	0	0	0	34
12	0	1	0	-1	0
13	-1	0	0	1	30
14	0	1	1	0	0
15	1	1	0	0	0
16	0	-1	0	-1	28
17	1	0	0	-1	34
18	-1	0	-1	0	0
19	-1	-1	0	0	0
20	-1	0	0	-1	0
21	0	-1	-1	0	0
22	0	-1	1	0	24
23	1	0	-1	0	26
24	0	0	0	0	24
25	0	-1	0	1	23
26	-1	1	0	0	24
27	0	0	0	0	28

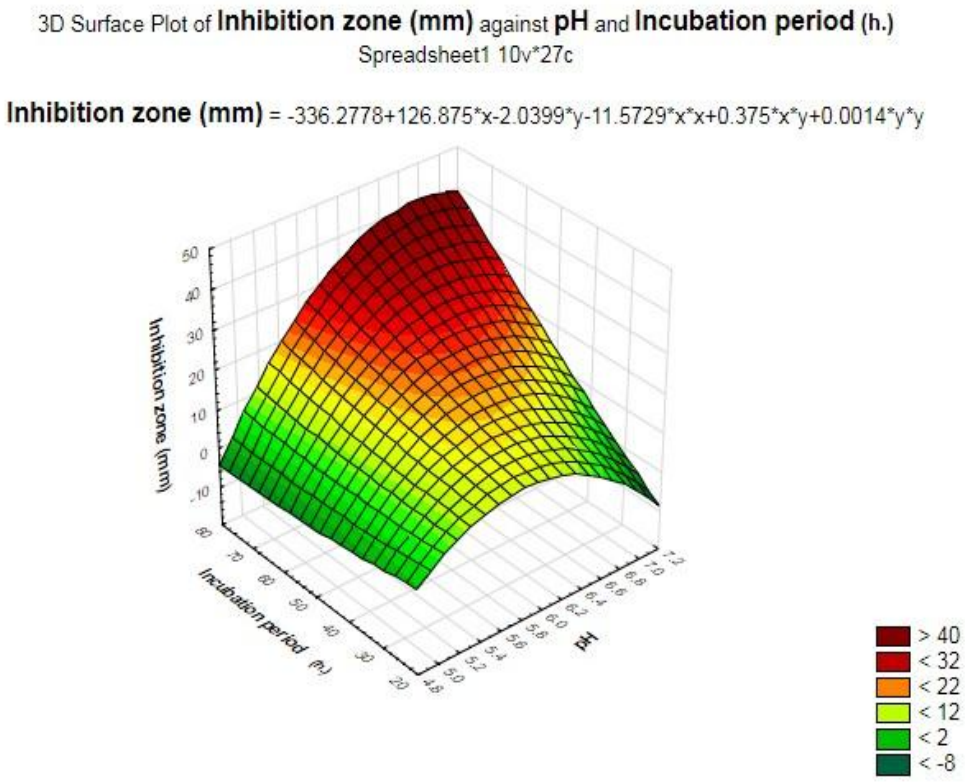


Fig. 5A. The interaction of pH with incubation period as independent variables affecting inhibition zone diameter

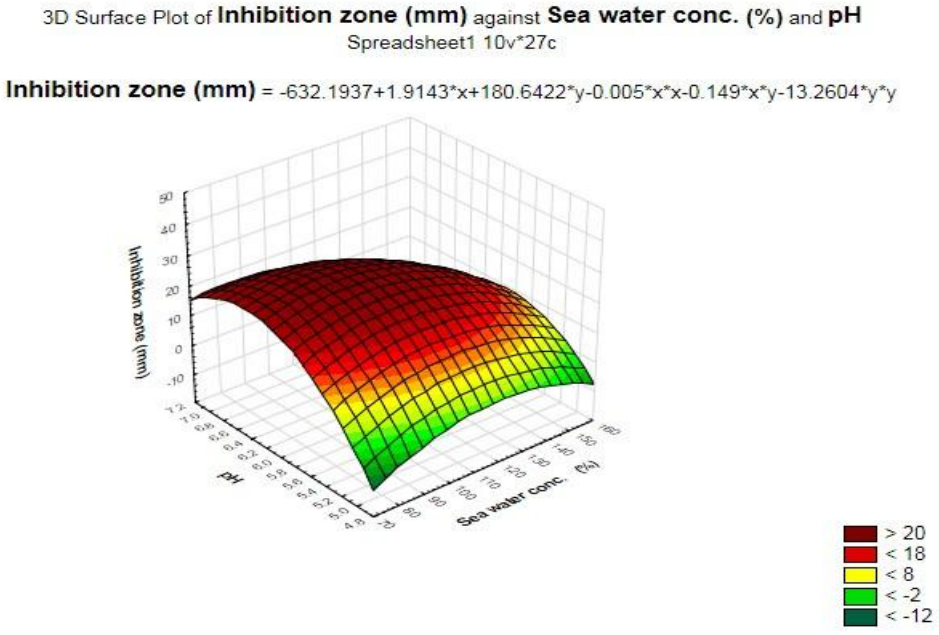


Fig. 5B. The interaction of sea water concentration with pH as independent variables affecting inhibition zone diameter

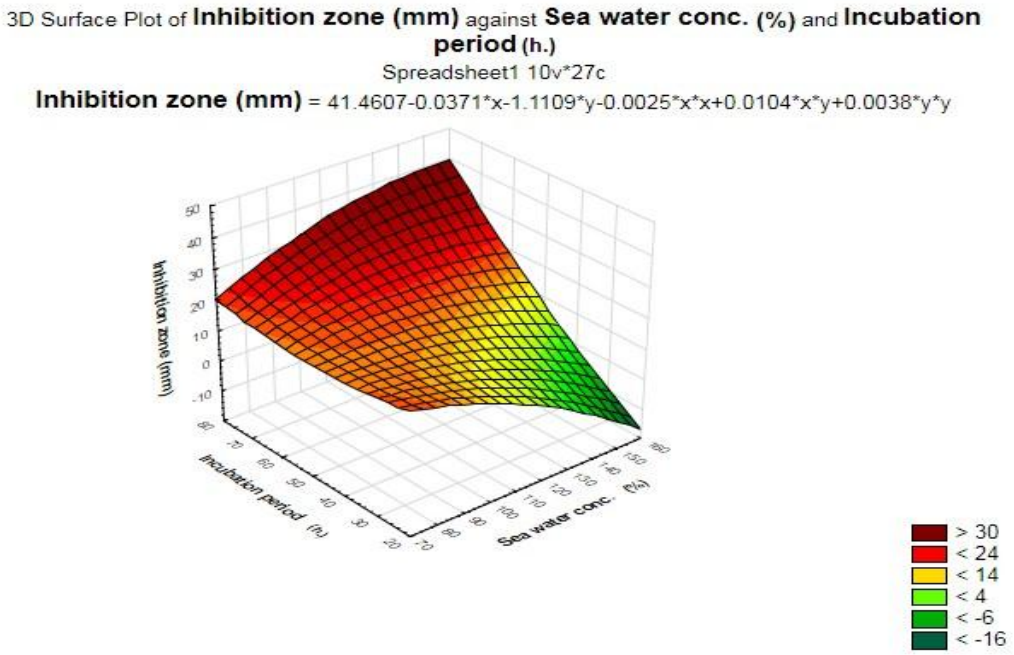


Fig. 5C. The interaction of sea water concentration with incubation period as independent variables affecting inhibition zone diameter

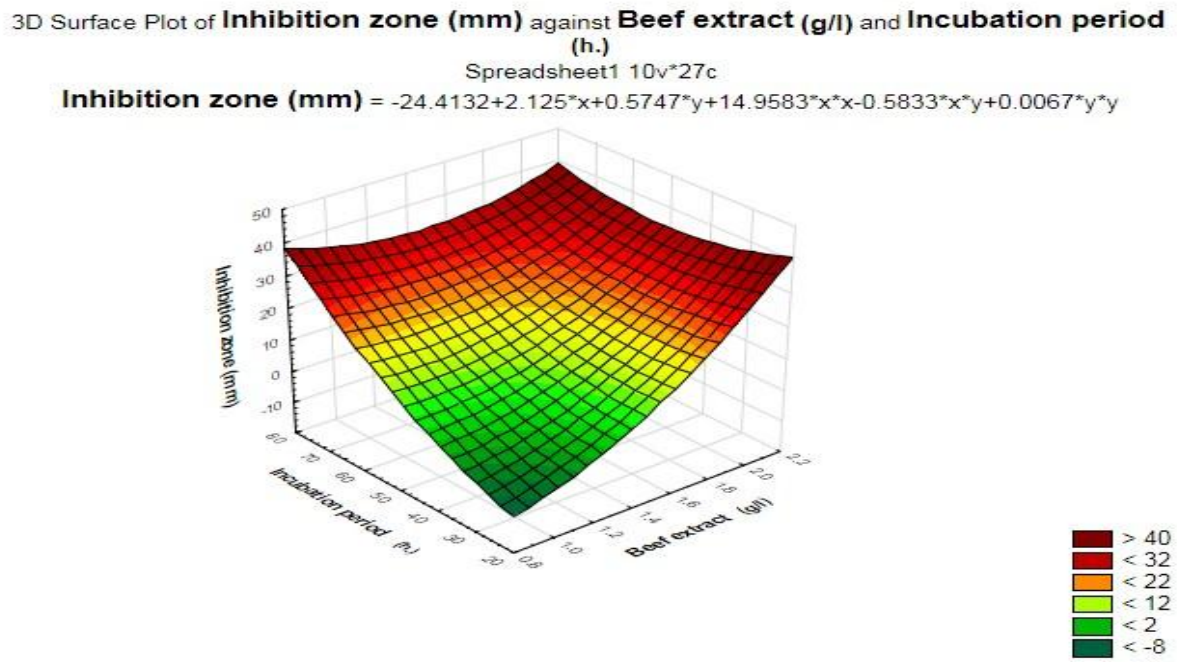


Fig.5D. The interaction of beef extract with incubation period as independent variables affecting inhibition zone diameter

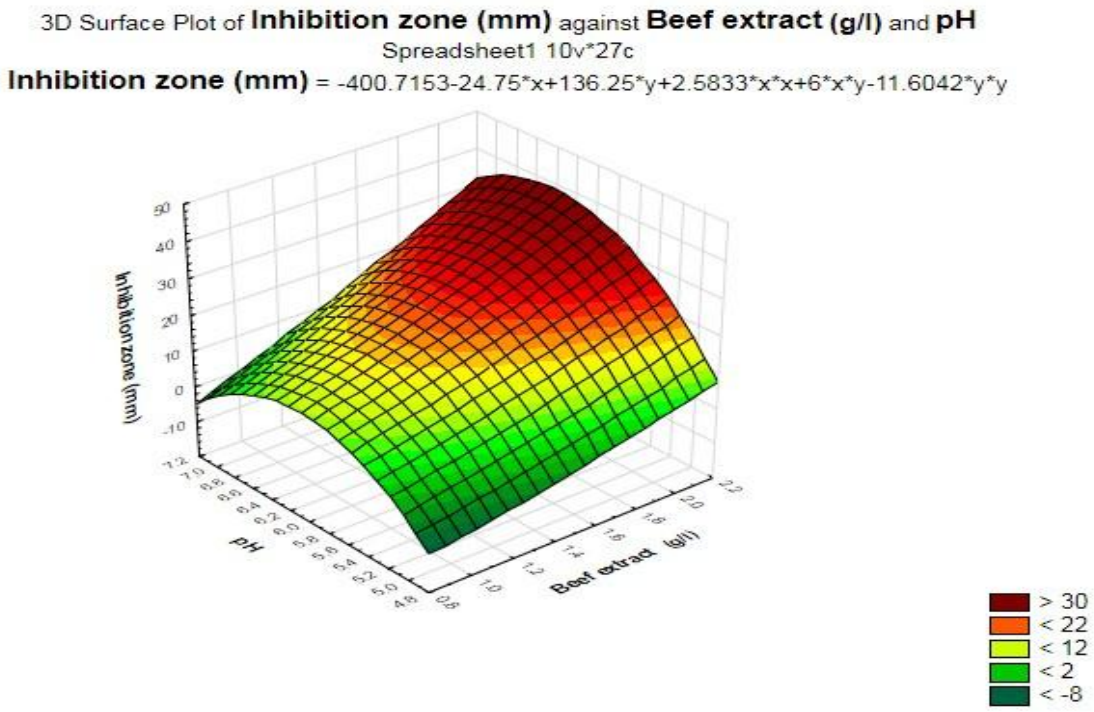


Fig.5E. The interaction of beef extract with pH as independent variables affecting inhibition zone diameter.

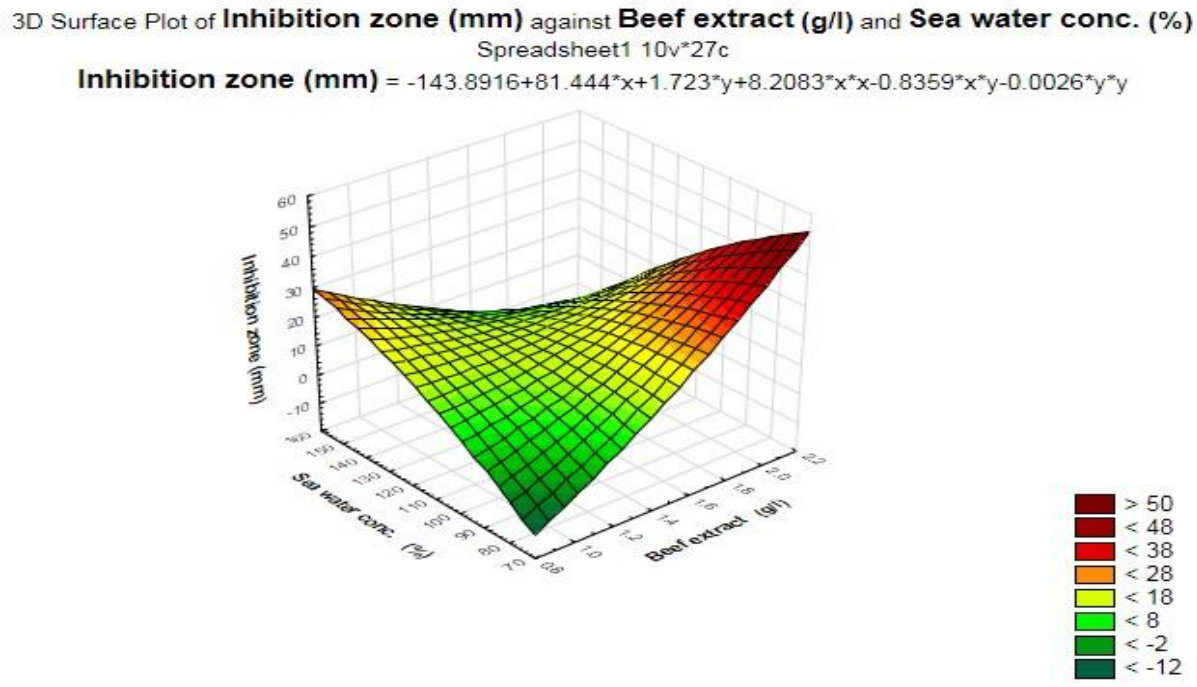


Fig.5F. The interaction of beef extract with sea water as independent variables affecting inhibition zone diameter.

Verification experiment

The optimal condition realized from the optimization experiment were verified experimentally and compared to the data calculated from the model. The estimated inhibition zone of 36.5 mm (Fig. 6); whereas, the predicted value from the polynomial model was 54.008 mm. This shows 67.58% validity of the predicted model. The good correlation between the predicted and experimental values under optimal condition proves the accuracy and validity of the model. Thus, it was predicted that in order to have the highest production of bioactive compounds by *A. indicus* EM11, the medium formula should be formulated as follows (gl^{-1}): peptone 7 g; beef extract: 2 g; inoculum's size (mL) 0.5; sea water concentration: 75%; pH: 6.02; temperature (°C) 40 for 24h. A verification experiment was applied to evaluate the basal versus the optimized medium. The results showed that, about 36.5 (mm) inhibition zone diameter with 3.65-fold increase were determined when compared to the control basal medium of 10 (mm).

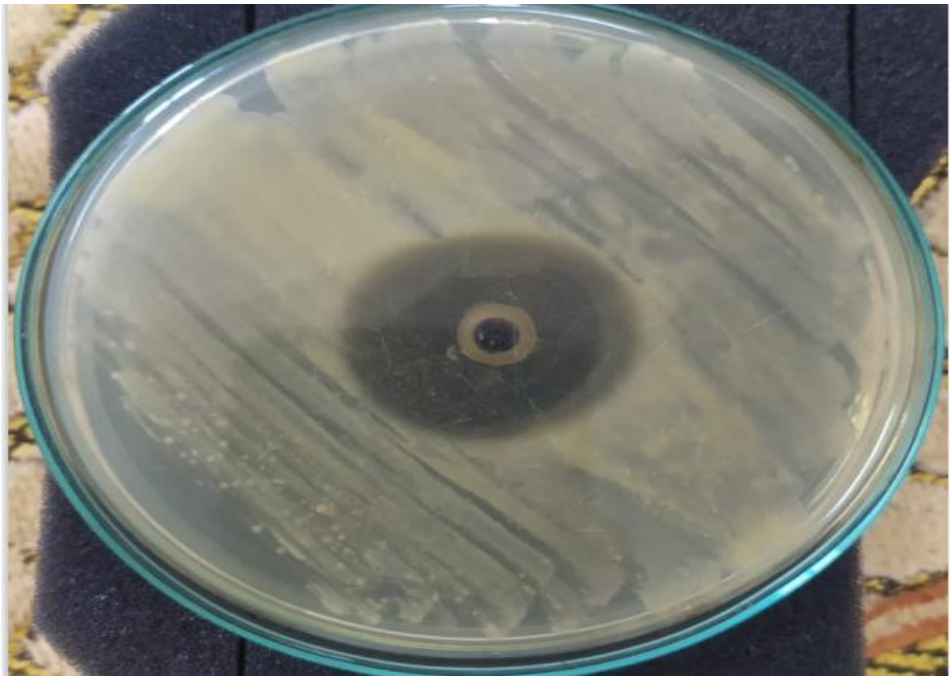


Fig. 6. Inhibition zone diameter of *Acinetobacter indicus* isolate EM11 supernatant grown on verified final medium against *Vibrio alginolyticus* MK170250

4. DISCUSSION

The treatment of aquaculture population by antibiotics is not limited to the diseased members, and therefore, the development of resistant microbial strains is a concurrent problem that ultimately leads to altered microbial composition and disease outbreaks. Hence, searching for novel bioactive compounds with anti-fish pathogens activity produced by marine adapted strains is urgently needed. Generally, the marine

habitats represent a treasure for natural products discovery **(Gulder & Moore, 2009)**. In the current study, the high antagonistic activity of marine isolate EM11 was observed against the fish pathogen *V. alginolyticus*, the causative agent of vibrosis.

The EM11 isolate was identified using 16S rRNA as *A. indicus*. To optimize the EM11 antagonistic activity, the Plackett-Burman was used, followed by Box-Behnken statistical designs to adjust the growth medium culturing variables. These factors included pH, temperature, incubation period, inoculum's size, sea water, peptone and beef extract concentrations. Based on the Plackett-Burman statistical design, a near optimum production medium was formulated as follows (gl^{-1}): peptone 7; beef extract 1.5; inoculum's size (mL) 0.5; sea water concentration (%) 100; pH 6 and temperature (°C) 40 for 48 h incubation period. This verified growth medium improved the inhibition zone diameter by 2.8-fold. Consequently, it was concluded that increasing the sea water concentration and incubation period were positively affecting *A. indicus* EM11 bioactive compounds production.

Conversely, the positive levels of beef extract (gl^{-1}) and pH (Table 1) induced a negative effect. The positive impact of sea water concentration was expected as *A. indicus* EM11 is a marine isolate adapted to sea water and high salt concentrations. The positive effect of prolonging the incubation period to 48h could be associated with the origin of this marine isolate, where the norm is a dormant or non-growing state **(Joint *et al.*, 2010)**. Thus, a lengthy incubation period is reported by other marine biologists to improve the cultivation of marine origin isolates **(Alain & Querellou, 2009; Joint *et al.*, 2010)**. On the contrary, the negative effect of beef extract concentration on *A. indicus* EM11 may be explained by the fact that the sea water habitats are known to be scarce in nutrients for instance, the nutrients level may drop to nanomolar range or even lower **(Ma *et al.*, 2014)**.

Therefore, the enriched supply of nutrients and vitamins induced by extra beef extract addition may contradict with these natural conditions. In terms of pH, the slight acidic level (pH 6) was found to support the antagonistic activity of *A. indicus* EM11. On the other hand, the Box-Behnken statistical design demonstrated that the medium formula should be formulated as follows (gl^{-1}): peptone 7 g; beef extract: 2 g; inoculum's size (mL) 0.5; sea water concentration: 75%; pH: 6.02; temperature (°C) 40 for 24h. Hence, the accurate adjustment of the three factors; namely, beef extract amount, sea water concentration and incubation period improved the antagonistic activity of *A. indicus* EM11 and increased the zone of inhibition by 3.6-fold. Specifically, the slight increase in the beef extract (2 instead of 1.5 gl^{-1}); the decrease of sea water concentration (75 instead of 100%), and the limitation of the incubation period to one instead of two days were the golden parameters to boost the antimicrobial metabolites production by *A. indicus* EM11.

The antimicrobial activity of *Acinetobacter* species can be attributed to the production of different metabolites as reported in previous studies. **Anjum *et al*. (2019)** reported the production of alkaloids indolepyrazines A and B from coastal mud marine-sourced *Acinetobacter* sp. ZZ1725. The produced alkaloids showed inhibitory activity against MRSA, *E coli* and *Candida albicans*. Moreover, **Okujo *et al*. (1994)** and **Yamamoto *et al*. (1994)** reported the production of siderophores from *A. haemolyticus* ATCC 17906 and *A. baumannii*, respectively. The produced siderophores were identified as acinetoferrin, a citrate-based dihydroxamate siderophore and acinetobactin, respectively. Whereas, the production of other chemically different antimicrobial structures was reported. In this context, the production of polysaccharides **(Haseley *et al*., 1997)** and the anti-*Aspergillus flavus* volatile compounds by *A. baumannii* **(Kadhim, 2016)** were recognized. In addition, the production of antimicrobial cyclo (Pro-Tyr) and cyclo (Pro-Leu) was observed for *A. calcoaceticus*, a commensal of entomopathogenic *Steinernema* sp. Finally, the wide range of *Acinetobacter* sp. isolation sources including soil, water, coastal mud, food, air, horse faeces, and honey bees can be explained on the molecular level. For example, the *A. indicus* genome is predicted to possess 3,054 genes of which 2,808 are protein-coding genes. The functional genome annotation demonstrated that 316 genes are assigned to amino acid and derivative metabolism, while 235 genes are identified to belong to co-factor, vitamin, prosthetic group and pigment subsystems **(Vettath *et al*., 2018)**. Therefore, it is not surprising that *Acinetobacter* species members can grow both on fresh and sea water sources. It is worth-noting that, a previous report confirmed that *Acinetobacter* sp., isolated from the skin of bighead catfish was capable to grow in saline conditions and was antagonistic to fresh and marine fish pathogens **(Bunnoy *et al*., 2019)**.

In addition to being a public health threat, the *Vibrio* population has piqued the interest of microbiologists and zoonotic disease experts all over the world. *V. alginolyticus* is one of the Vibrio's responsible for lethal diseases in both fish and humans according to epidemiology.

CONCLUSION

This study highlighted that marine *A. indicus* EM11 has promising antimicrobial activity against *V. alginolyticus*, and this activity was successfully optimized using Placket-Burman and Box-Behnken experimental designs, increasing it by 3.65-fold. Moreover, the study indicates that the marine habitats are enriched with novel bacterial isolates and novel antimicrobial metabolites that haven't been explored yet. In part, this is due to the difficulty of marine bacterial isolation, maintenance and optimization *in vitro* culturing conditions. However, continuous efforts have been worldly exerted revolutionizing this field. In addition, the future of marine-derived antimicrobial drugs is

remarkably flourishing giving hope to overcome serious diseases and antibiotics resistance.

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