International Journal of Oceans and Oceanography
ISSN 0973-2667 Vol.1 No.1 (2006), pp. 141-150

http://www.ripublication.com/ijoo.htm

Antagonistic effect of marine *Nocardia brasiliensis* against the fish pathogen *Vibrio damsela*: Application of Plackett-Burman experimental design to evaluate factors affecting the production of the antibacterial agent.

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Abstract

Sixteen actinomycete strains were tested for their potentiality to produce bioactive compounds that inhibit the growth of the fish pathogen *Vibrio damsela*. A marine strain identified as *Nocardia brasiliensis* produced a compound that showed the largest inhibition zone and highest activity against the tested organism. High level of $MgSO_4 7H_2O$ and low levels of all other components in the culture medium supported the production of the antagonistic compound as evaluated by the application of Plackett- Burman experimental design. Cultures adjusted to pH 8, incubated at 40°C under static condition lead to higher production of the antagonistic compound. Antagonistic activity of the compound produced from the optimized culture conditions against *Vibrio damsela,* showed 2.9 fold increase than that obtained from the basal medium. The inhibitory substance was found to be a low molecular weight compound, heat stable and resistant to proteolytic enzymes.

Key words: Antagonism- *Nocardia brasiliensis* - *Vibrio damsela* - Plackett- Burman design.

Introduction

Marine microorganisms are excellent source for antimicrobial compounds (1). Serious attempts to reach the vast potential of marine organisms as sources of bioactive metabolites that may be directly utilized as drugs or serve as lead structures for drug development started in the late 1960 (2).

Actinomycetes, are prolific producers of antibiotics and other industrially useful secondary metabolites (3, 4, 5, 6). The majority of studies was done with soil actinomycetes, whereas those from marine source did not attract much attention although marine environment could be a source of rare and uncommon bacterial groups and promising sources of bioactive compounds.

Experimental designs are excellent techniques for the optimization of cultures conditions to achieve optimal production (7, 8, 9). One of the most promising sources of bioactive compounds is *Nocardia brasiliensis,* which was reported as an antibiotic producer (10, 11).

El-Max fish farm is one of the most important natural fish farm in Alexandria, Egypt. This brackish water fish farm is characterized by the presence of three types of fish sp. *Tilapia sp.*, *Anguilla sp.* and *Mulet sp.* Lately, this farm has been suffered from die-off of large amounts of fish due to the dominant fish pathogen *Vibrio damsela* which caused the intestinal infections in most fishes (12).

Therefore, it is aimed in the present study to investigate the antagonistic effect of marine *Nocardia brasiliensis* against the fish pathogen *Vibrio damsela*. It is also aimed to apply the Plackett-Burman design (13) to evaluate the relative importance of medium constituents on the production of the compound and to determine some other factors affecting its efficiency.

Materials and Methods

Organisms and maintenance

A number of actinomycetes strains (29 strains) were selectively isolated from marine sediments on starch nitrate medium (14) of the following composition (g/l): Starch 20; KNO_3 1; K_2HPO_4 0.5; $MgSO_47H_2O$ 0.5; NaCl 0.5; Fe SO_4 0.01; agar 15. All strains were screened for their potentiality to produce a bioactive compound against the fish pathogen target strain *Vibrio damsela*. The actinomycete *Nocardia brasiliensis* was selectively chosen owing to its potentiality against *Vibrio damsela*. The fish pathogen *Vibrio damsela* was isolated from El-Max fish farm and was maintained on nutrient agar medium (Sigma chemical company, St louis,USA). Both strains were identified following Bergy's Manual of Systematic Bacteriology (15) by Al-Azhar University Fermentation Biotechnology & Applied Microbiology (Ferm.BAM) Center, Egypt.

Electron microscopy

For Scanning Electron Microscopy (SEM), the cover slip technique was performed (16). The cover slip was cut with a glass file with a suitable fragment of growth, mounted on a specimen stub, and coated with gold palladium under vacuum. The cover slip was examined with SEM, Jeol ISM-5300, operating at 10 KV at the Central Laboratory, Faculty of Science, Alexandria University.

Preparation of antagonistic agent

Nocardia brasiliensis was allowed to grow in a portion (50 ml) of starch nitrate broth (14) dispensed in 250 ml Erlenmeyer flask. The flask was inoculated with seven day old slant of *Nocardia brasiliensis* and incubated at 30 ºC for seven days under static condition. Cells were then removed by centrifugation at 10000 x g for ten minutes, the supernatant was used to study the antagonistic effect against *Vibrio damsela*.

Well-cut diffusion technique

Supernatant previously obtained was used to determine its suppressive effect on the fish pathogen *Vibrio damsela*. Wells were punched out using a sterile 0.5 cm cork borer in nutrient agar plates inoculated with 10^5 cells of *Vibrio damsela*, and each of the well bottoms was sealed with two drops of sterile water agar. Fifty microlitre of the actinomycete supernatant previously prepared were transferred into each well. All plates were incubated at 37 ºC for 24 hours. After the incubation period, the radius of the clear zone around each well (y) and the radius of the well (x) were measured in mm, where dividing y^2 over x^2 determines an absolute unit of activity (AU / ml) for the inhibition zone obeying the following equation: $AU/ml= y^2/x^2$ (17).

Evaluation of nutritional effects

The Plackett-Burman experimental design (13) was used to evaluate the relative importance of

various nutrients for the production of antimicrobial agents in liquid culture. The independent variables examined in this experiment and their setting are shown in Table 1. 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 effect of each variable was determined with the following equation:

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

Where E_{xi} 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. A main effect figure with a positive sign indicates that the high concentration of this variable is near to optimum and a negative sign indicates that the low concentration of this variable is nearer to optimum. Using Microsoft Excel, statistical *t*-values for equal unpaired samples were calculated for determination of variable significance.

Table1. Screening for growth factors affecting *Nocardia brasiliensis* antagonistic activity and their levels in the Plackett-Burman experiment.

Factor (g/l)	Symbol	Level		
		-1	0	+1
Starch	**Star**	15	20	25
KNO_3	**KN**	0.5	1	1.5
K_2HPO_4	**K_2**	0.25	0.5	1.5
$MgSO_4.7\ H_2O$	**Mg**	0.25	0.5	1.5
NaCl	**Na**	0.0	0.5	0.75
$FeSO_4$	**Fe**	0.0	0.01	0.015
***Inoculum size**	**IS**	0.5	1	2

* Inoculum size was added in ml of 7 days culture filtrate

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

Trials	Factors							Inhibition zone (mm)
	Star	KN	K_2	Mg	Na	Fe	IS	
1	-1	-1	-1	1	1	1	-1	12
2	1	-1	-1	-1	-1	1	1	0
3	-1	1	-1	-1	1	-1	1	0
4	1	1	-1	1	-1	-1	-1	12
5	-1	-1	1	1	-1	-1	1	10
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	0
9	0	0	0	0	0	0	0	10

*See Table 1 for explanation of factor symbols

Effect of pH, temperature and aeration

To study the effect of some factors, such as pH, temperature and aeration on the production of antagonistic agent, portions (50ml) of the culture formula obtained from the experimental design were inoculated with *Nocardia brasiliensis* and incubated at different pH's (6,7 and 8) or temperatures (20,30, 40, and 50 °C) for 4 days. Set of flasks were incubated under shaked condition (200 rpm) to evaluate the importance of aeration. Supernatant obtained from each experiment were tested using well cut diffusion technique on agar plates against *Vibrio damsela*.

Preliminary characterization of the antibacterial component in cell free supernatant of *Nocardia brasiliensis*

Ultra filtration of the cell free supernatant of *Nocardia brasiliensis* was carried out using a low protein binding regenerated cellulose membrane (molecular weight cut off 10,000 D, UFP2, NIKON, Millipore, Japan) and the residue was washed by passing 1ml of phosphate buffered saline. The residue was resuspended in one ml of saline and tested for antibacterial activity by well diffusion method (18).

The sensitivity of the antibacterial principle to proteolytic enzymes was tested using pepsin and trypsin at level of 50 and 100 mg/ml, respectively. Mixture of culture supernatant of the culture medium and enzyme was incubated for 1h at 30°C and tested for antibacterial activity by the well diffusion method.

The thermotolerance of the antibacterial component was tested by the well diffusion after heating the cell free supernatant in a boiling water bath for 5 min, 15 min, 30 min and 1 hour.

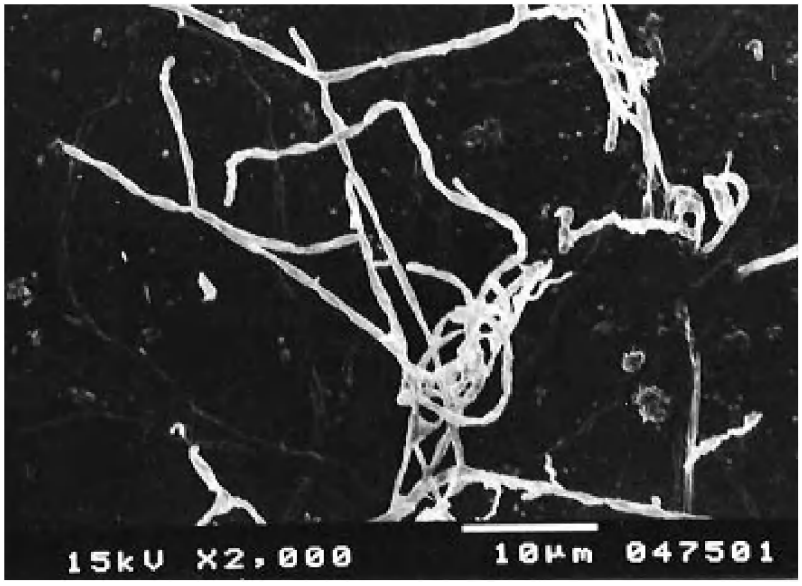

Figure 1: Scanning Electron Micrograph showing *Nocardia brasiliensis* filamentous structure, after 14 days of incubation on starch nitrate agar medium (bar, 10 µm).

Results

Isolation and identification

Sixteen bacterial strains of marine actinomycete were screened for their capacity to produce antagonistic agents that control the growth of the fish pathogen *Vibrio damsela.* Al- Azhar University Fermentation Biotechnology and Applied Microbiology (Ferm.BAM) Center, Egypt, identified the selected actinomycetes strain as *Nocardia brasiliensis* which is characterized by

yellowish brown color substrate mycelium with pink gray aerial mycelium carrying chain of conidia. It is non motile with no diffusible pigments. The cell wall hydrolysate contained meso-Diaminopimelic acid with arabinose and galactose as diagnostic sugars.

Scanning electron microscopy (Fig.1) showed the mycelia structure of *Nocardia brasiliensis*. This strain was selected as the most potent. This was indicated by the inhibition zone (10 mm) formed when the supernatant of this strain was introduced into a well in an agar plate inoculated with *Vibrio damsela*.

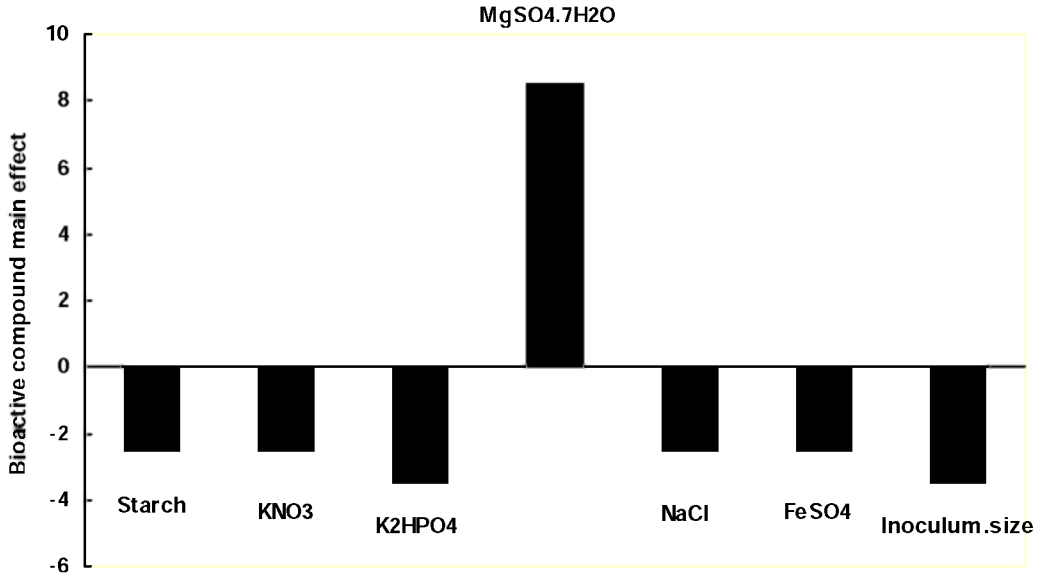

Figure 2: Effect of culture conditions on antagonistic agent production.

Factors affecting antimicrobial agent production

The design of the experiment, together with the observed inhibition zone diameters are given in Table 2. The inhibition zone diameters (mm) obtained by experimental trials (in triplicates) were found to be in the range of 10-12 mm. The mean values of the main effect of the examined factors on the inhibition zone diameter were calculated and presented graphically in Fig.2. Based on these results, it is conceivable that the – levels of starch, KNO_3, K_2HPO_4 NaCl, $FeSO_4$ and inoculum's size, encouraged the production of the antimicrobial agent formed by *Nocardia brasiliensis*. While the + levels of $MgSO_47H_2O$ supports its production. Moreover, the *t*-value represented in Table 3, supports this observation. Interaction of $MgSO_47H_2O$ concentration with low level of NaCl Fig. 3 (a) and with low levels of K_2HPO_4 Fig. 3 (b), both increase the inhibition zone diameter.

According to data obtained, a near optimum producer culture was formulated as follows: (g/l) Starch 15; KNO_3 0.5; K_2HPO_4 0.25; $MgSO_47H_2O$ 1.5 with inoculum size of 0.5 ml. This approach verified the validity of the applied design. A verification experiment was applied to evaluate the basal versus the optimized medium. Data in Table 4 show about 1.3 fold increase in the inhibition zone diameter and about 1.7 fold increase in the activity unit with optimized when compared with the control basal culture. The production of the antimicrobial agent after 4 days of incubation did not significantly differ from that achieved after seven days of incubation (data not shown).

Effect of temperature, pH and aeration

The effect of pH and temperature on growth of *Nocardia brasiliensis* and production of antagonistic agent against *Vibrio damsela* were evaluated. The actinomycete grew well at pHs 6,7

and 8 and temperature range 20-40 ºC. The highest antagonistic activities were observed in cultures previously adjusted to pH8 (11.65 AU) and incubated at 40ºC. Compared to inoculated under static condition, shake cultures resulted in negligible activities (data not shown).

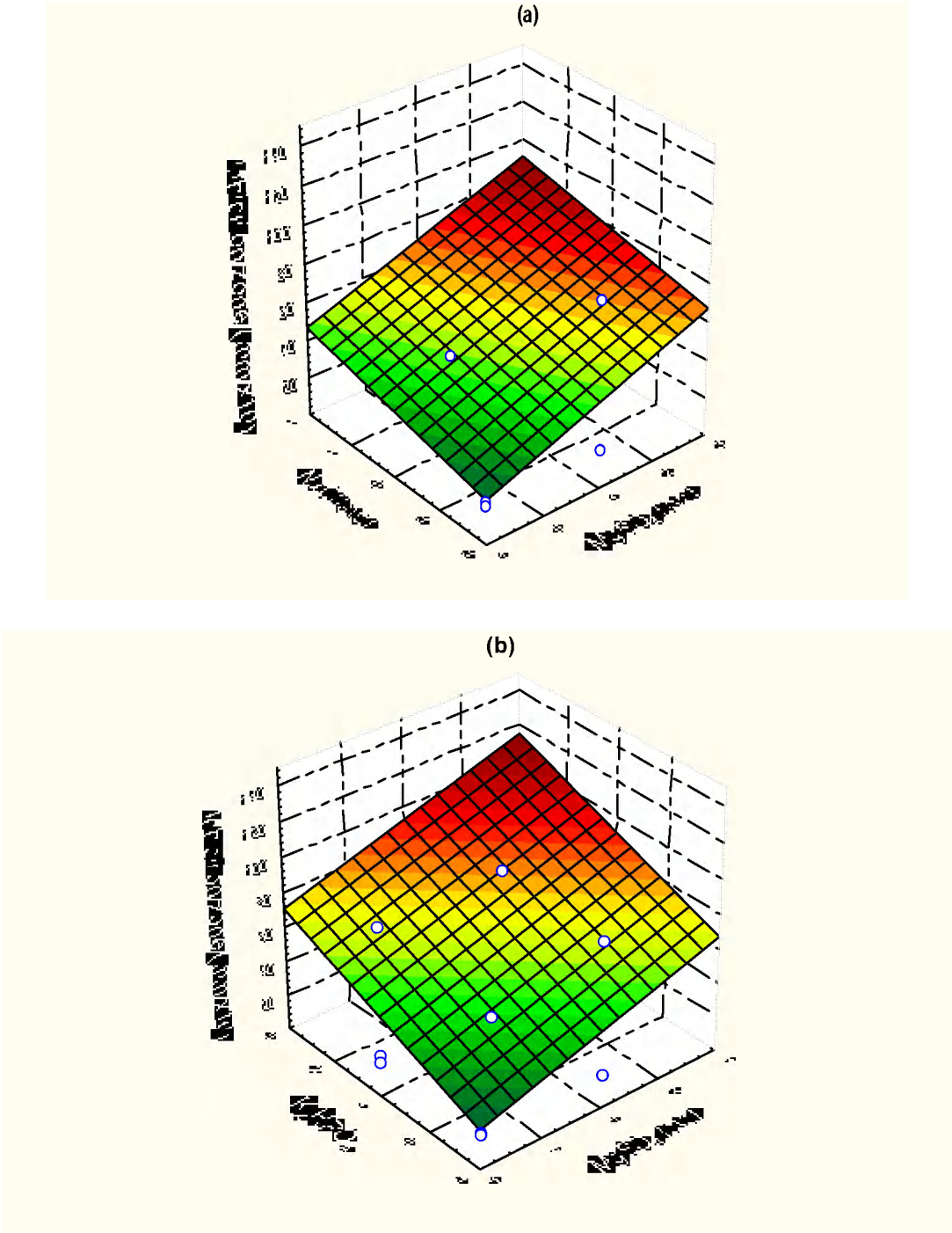

Figure 3. The interaction of $MgSO_4$ concentration with NaCl level (a) and with K_2HPO_4 level (b) with respect to inhibition zone diameter of antimicrobial agent based on the Plackett Burman results.

Table 3. Statistical analysis of the Plackett-Burman experiment.

Variable	Inhibition zone (mm)	
	Main effect	*t*-value
Starch	-2.5	-0.569
KNO_3	-2.5	0.569
K_2HPO_4	-3.5	-0.819
$MgSO_4.7\ H_2O$	8.5	**2.9**
NaCl	-2.5	**1**
$FeSO_4$	-2.5	**1**
Inoculum size	-3.5	-0.819

*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 (Cochran & Snedecor, 1989).

Table 4. A verification experiment: Activity of antagonistic agent production by *Nocardia brasiliensis* grown on basal versus optimized medium.

Response	Basal medium	Optimized medium[1]
Inhibition zone (mm)	10 ± 0.002	13 ±2.2
Activity unit (AU)	4 ±0.32	6.8 ±0.012

[1]An optimum medium formula was predicted according to the results obtained from the Plackett-Burman experiment. Cultures adjusted to pH 7.0 and incubated at 30 °C for four days.

Table 5. Effect of incubation temperature of *Nocardia brasiliensis* on the antagonistic activity using optimized medium.

Temperature	20 °C	30°C	40°C
Inhibition zone (mm)	10	13	15
Activity unit (AU)	4	6.76	9

Table 6. Effect of pH on antimicrobial agent activity using optimized medium.

pH	6	7	8
Inhibition zone (mm)	11	15	17
Activity unit (AU)	4.84	9	11.56

Preliminary characterization of the antibacterial component in cell free supernatant of *Nocardia brasiliensis*

Cell free supernatant was ultra filtered using cut off at 10 KD, the filtrate did not have any effect thus indicating that the inhibitory component has a molecular weight of less than 10 KD.

The proteolytic enzymes trypsin and pepsin used at 100 and 50 mg/ml, respectively, did not affect the activity of the antibacterial component in the cell free supernatant and the zone of inhibition observed was equal to the untreated cell free supernatant that served as control. Study of the heat stability of the antibacterial principle showed that even when heated in a water bath at 100° C for an hour, there was no loss of activity.

Discussion

In spite of the large number of bioactive products produced by terrestrial microorganisms (17, 19, 20), serious attempts are now going to reach the vast potential of marine microorganisms as sources of bioactive compounds (1, 21, 22, 23). The isolation of rare and uncommon actinomycetes has become an increasingly important part of natural product discovery (6, 24).

In this study, a marine actinomycete identified as *Nocardia brasiliensis* was found to produce one or more compound(s) showing antagonistic effect to the fish pathogen *Vibrio damsela.* This actinomycete was previously reported as antibiotic producer (10, 11). To climb the region of the optimum and minimizing the time of production, the Plackett Burman design was employed. This experimental design technique was successfully employed in enzyme production and other optimization experiments (7, 8, 9), but up to our knowledge this is the first report on its application for optimization of culture conditions for antibiotic production.

The data obtained show that $MgSO_47H_2O$ which was significant at the 0.5% level, were the factor that affected the production of the bioactive agent produced by *Nocardia brasiliensis*. Increasing the levels of this compound yielded the highest antagonistic effect against *Vibrio damsela*. On the other hand, the increase in starch, KNO_3, K_2HPO_4 NaCl, Fe SO_4 levels and inoculum size had a negative effect on the production of the metabolite formed. Therefore, we have reached the conclusion that to achieve the highest antagonistic effect against *Vibrio damsela*, the actinomycete *Nocardia brasiliensis* needs to be grown on a medium of the following composition (g/l): Starch 15; KNO_3 0.5; K_2HPO_4 0.25; $MgSO_47H_2O$ 1.5 and of pH 8 using an inoculum of 0.5 ml and incubating cultures at 40°C under static condition. Under such conditions, the natural product produced by *Nocardia brasiliensis* showed activity of 11.65 AU/ml (i.e. 2.9 fold increase from the results obtained with basal medium) after four days of incubation. This recommend that this design co-op with our aim of this work.

The results of the preliminary characterization of the antibacterial component suggests that it is a low molecular weight, non-proteinaceous heat stable compound. We expect that this antimicrobial agent is an antibiotic, since most actinomycetes are excellent sources in producing several antibiotics. Imamura *et al* (25)studied a novel antimycin antibiotics and uranchimycins A and B produced by marine actinomycetes. Also Akiranemoto *et al* (11) investigated a new terpenoid antibiotic from pathogenic *Nocardia brasiliensis*. Moreover Mikami *et al* (10) studied the production of Erythromycin E by pathogenic *Nocardia brasiliensis*.

Future studies are needed to investigate the structure of such natural product. In conclusion, it can be stated that the marine actinomycetes strain *Nocardia brasiliensis* has the properties of a biocontrol agent for use in controlling the fish pathogens *V. damsela* in fish farms with higher production by using Placket- Burman design.

Acknowledgments

Authors express their great gratitude to Prof. Dr. Soraya Sabry, Faculty of Science Alexandria University, for her help in revising the manuscript.

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