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Production of Antimicrobial Agent from Marine Bacteria Isolated from Mediterranean

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Abstract: One hundred and forty marine isolates were obtained from various marine samples at different depths including seawater, sediment, algae, coral and rhizosphere of mangrove from both Mediterranean and Red sea, Egypt. After screening of the marine microbial isolates for the antimicrobial activity against pathogenic test strains; one of the best isolates that showed activity against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* was selected for both phenotypic and phylogenetic analysis. The results showed that the bacterium is Gram positive bacilli and it showed 99% homology with *Bacillus licheniformis.* The optimum antibiotic biosynthesis was obtained after 72 h of incubation period, agitation speeds at 180 rpm, initial fermentation pH at 7.0, incubation temperature at 30°C, inoculums size of 0.5 % (v/v), using glucose as a carbon source at concentration of 0.2% (w/v) and soybean as a nitrogen source at concentration of 1 % (w/v).

Key words: Isolation, screening of antimicrobial activity, API 50 CHB, 16S rDNA gene sequencing, optimization studies.

INTRODUCTION

Marine microorganisms have become an important point of study in searching for novel antibiotics. This is consequent to the decrease in discoveries from terrestrial microbial sources, as well as, the emergence of antibiotic resistant clinical pathogens such as *Mycobacterium tuberculosis*, *Enterococcus*, *Pseudomonas sp.*, *Streptococcus pneumoniae*, and *Staphylococcus aureus* led to constant need to find new sources of effective antibiotics.

Marine bacteria of the genus *Bacillus* are known to produce antimicrobial metabolites which have been reported including macrolactin F, 7-*O*- succinylmacrolactin F and 7-*O*-succinylmacrolactin A, *from Bacillus sp.* (Sc026) (Jaruchoktaweechai *et al.*, 2000), new thiopeptide compounds from *Bacillus cereus* QN03323 (Nagai *et al.*, 2003), and three bacteriocin-like peptides named Lichenin, Bacillocin 490 and P40 produced by *B. licheniformis* strain P40, (Cladera-Olivera *et al.*, 2004).

The present study was undertaken to isolate different species of marine bacteria, screening of their antimicrobial activity, identification of one of the best marine bacterial strain using various techniques and optimization of the antibiotic productivity by the selected strain.

MATERIALS AND METHODS

Bacterial Strain:

Samples were collected during winter and summer 2007 from different regions of both Mediterranean and Red sea, Egypt. Seawater samples were collected in polypropylene bottles at about 8 meters depth. Algae samples were collected at 1 meter depth. Sediment samples are collected from seashore and from depth about 2 meters. Hard corals samples were collected from depth about 16 meters, while the roots of the mangrove were excised. The solid samples were washed three times with sterile seawater to remove loosely attached bacteria. The sample was then placed in another 100 ml of sterile seawater and homogenized by shaking at 200 rpm for 15 minutes and a serial dilution was performed. Finally, 50 µl of the supernatant of each dilution was inoculated on: Marine agar 2216 E (Difco laboratories) (Oppenheimer and ZoBell, 1952) and ISP-2 agar medium (Pridham *et al.,* 1956). The pure culture was preserved by freezing in 20% glycerol (v/v) at -70°C for long- term storage.

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Screening of Isolates for Antimicrobial Activity:

In primary screening, determination of the antimicrobial activity of pure isolates was done by plug agar method on Nutrient agar (NA) using eight microbial test strains. They were *Escherichia coli* (*ATCC-25922), *Pseudomonas aeruginosa* (ATCC-27953), *Proteus vulgaris* (ATCC-13315), *klebsiella pneumoniae* (ATCC-13883), *Enterobacter cloacae* (ATCC-13047), *Staphylococcus aureus* (ATCC-29213), *Enterococcus faecalis (*ATCC-29212) and *Candida albicans* (Clinical isolate). Moreover, the active marine microbial isolates obtained from the primary screening were subjected to secondary screening using supernatant of the growth cultures under shaking conditions and tested by agar diffusion method against the previously mentioned pathogenic strains.

Morphological and Biochemical Characteristics:

The isolated strain was identified morphologically (shape, gram staining, spore staining and motility), biochemically (the API 50 CHB system (Bio Merieux, France)

DNA Extraction, PCR Amplification, Sequencing of 16s rDNA and Phylogenetic Analysis:

The strain was cultivated in the liquid alkaline medium overnight at 37 °C at 180 rpm. Three ml of growth culture was centrifuged and the pellets were washed twice with 0.01 M phosphate buffer, pH 7.5. The bacterial chromosomal DNA was extracted using UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA) following the manufacturer's instructions. 16S-rDNA was amplified by PCR (Mullis and Falloona, 1987) using universal forward primer 16F27 (5′- AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer 16R1525 (5′- AAG GAG GTG ATC CAG CCG CA-3′) derived from *E. coli* 16S-rDNA sequence (Lane, 1991) synthesized by MWG Biotech AG. The reaction mixture of 50 µl contained at least 100 ng of genomic DNA (in 10 mM Tris-HCl, pH 8), 0.2 µM of each primer and PCR Supermix High fidelity (Taq & Go, Promega, CA). PCR reaction was performed in a peltier thermal cycler PTC-200 using the following conditions: initial denaturation (5 min at 95°C), followed by 30 cycles of denaturation (30 s at 95°C), primer annealing (30 s at 52°C), primer extension (1.5 min at 70°C), and final extension (5 min at 70°C). The PCR products were mixed with 3 µl of loading dye solution (MBI Fermentas, St. Leon-Rot, Germany, 5: 1) and were separated in a 0.8 % (w/v) agarose gel electrophoresis in 1x TAE buffer, pH 8.0 (40 mM Tris-acetate, 1 mM EDTA). The gel was placed in ethidium bromide solution (1 µg/ml) for 30 min and was placed on an ultra violet transilluminator to visualize the DNA. The amplified 16S-rDNA products were sliced off from the agarose with sterile razor blade. The DNA was purified from the agarose by QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) following manufacturer's instructions and sequenced on an ABI 377 automated sequencer using the PRISM Ready Reaction Kit (Applied BioSystems, Foster City, CA). Sequence data were analyzed by comparison with 16S rRNA genes in the GenBank database. The nearest relative of the organism was obtained by BLAST searches (Altschul *et al*., 1997) and aligned with close relatives using the Clustal W software. A phylogenetic tree was constructed with MEGA version 5.0 using the neighbor-joining method (Tamura *et al.*, 2007).

Optimum Conditions for Antibiotic Production:

The effects of different agitation speeds (0-200 rpm), incubation temperatures (15-50°C), initial pH (3-9), inoculums size (0.25- 22% (v/v)), carbon and nitrogen sources and incubation period (8-192 h) were examined on the productivity of the antimicrobial substance by the active marine isolate. The fermentation parameters were determined,

a. Production Medium:

About 10 % (v/v) of an overnight preculture of the active marine isolate was added in separate flasks of ISP-2 broth medium (Pridham *et al.,* 1956).

b. Fermentation Parameters:

Growth of the bacterial cells of the marine isolate was evaluated by measuring of the optical density of the culture. The samples were diluted with 10-fold and measured by spectrometer (UV. Spectrophotometer, Lambda 16, Perkin-Elmer, Langen) at 600 nm ($O.D_{600}$). Measurement of inhibition zone (I.Z.) and determination of final pH using pH radiometer (pH M 62 Copenhagen, Denmark) were also done.

c. Agar Diffusion Method:

A cell suspension of the test microorganism *Staphylococcus aureus* prepared by a physiological saline (NaCl 9.0 g/1) was adjusted to 0.125 A° at 625 nm (equivalent to a McFarland 0.5). Each 20 ml nutrient agar medium was seeded with 100 µl of the previously mentioned cellular suspension. The wells were cut using a sterile cork borer of 9 mm diameter and 100 µl of supernatant (centrifuged at 6000 g for 10 min) was loaded into each well.

Results:

Screening of Isolates for Antimicrobial Activity:

As a Primary screening, about 67 microbial strains showed antimicrobial activity against at least one of the mentioned test organisms. While the secondary screening revealed seven active isolates which were able to produce the antimicrobial substance under shaking conditions. Isolate No. 18 was able to produce the highest activity of the antibacterial substance against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* after 72 h of incubation. Therefore, isolate No 18 was chosen for identification and further physiological studies.

Identification of Marine Isolate No. 18:

The marine isolate No. 18 showed is gram positive, motile spore former bacterial rod the biochemical characteristic results are shown in table 1.

Moreover, the 16S rDNA gene sequencing revealed that high similarity of 99 % with *Bacillus licheniformis.* In consequence, it was nominated as *B. licheniformis* NRC-18. The evolutionary history was inferred using the Neighbor-Joining method (Saitou N. and Nei M., 1987). The optimal tree with the sum of branch length = 0.06733462 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The analysis involved 8 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 919 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 Fig. 1 (Tamura *et al.,* 2007).

***Optimization of Physiological and Cultivation Conditions for Maximum Antimicrobial Production of B. Licheniformis NRC-18*:**

Effect of Different Agitation Speeds:

Maximum I.Z. of 24 mm was detected at 180 r.p.m. No further increase in the antimicrobial production was detected by raising the agitation speed up to 200 rpm. However, the results showed thus, as the shaking speed increased from 65 to 200, the final pH of the fermentation medium shifted towards the alkalinity and reached 9 at the 180 r.p.m. Moreover, the growth trend of the microorganism was monitored and was found to increase gradually as the cultivation of the latter was changed from static to shaking condition.

Effect of Initial Fermentation Medium pH Values:

Maximum activity was detected at pH7.

Effect of Different Incubation Temperatures:

Temperatures ranging between 30 and 35 °C resulted in optimum antimicrobial production since the inhibition zones of 24 mm. Increasing the temperature up to 50 °C resulted in a gradual decrease in the inhibition zone recorded reaching 15 mm at incubation temperature (50 °C).

Effect of Different Inoculum Sizes:

The inoculum size of 0.5 % (v/v) gave the maximum antimicrobial production since the I. Z. reached 31 mm. Further increase in inoculum size increased the cell growth of *B. licheniformis* NRC-18 but with decrease of the antimicrobial production (Fig. 2).

Effect of Different Carbon Sources:

The addition of glucose at concentration of 0.2% w/v as a sole carbon source to the fermentation medium resulted in a maximum antimicrobial activity since the inhibition zone detected reached 33 mm (Fig. 3).

Table 1: Biochemical characterization of the selected marine strain.

Test	Result	Test	Result
Glycerol	+	Melibiose	-
Ribose	+	Sucrose	+
D-xylose	?	Inulin	-
Galactose	+	Raffinose	-
Glucose	+	Starch	-
Fructose	+	Xylitol	-
Mannose	+	D-Tagatose	+
Sorbose	-	Gluconate	-
Dulcitol	-	ONPG	+
Inositol	-	ADH	-
Mannitol	+	Citrate	+
Arbutin	+	Urease	-
Esculin	+	Indole	-
Cellobiose	+	V-P	+
Maltose	?	Gelatin	+
Lactose	?	Nitrate	?

Abbreviations: ONPG, o-nitrophenyl-b-D-galactopyranoside; ADH, arginine dehydrolase; VP, Voges-Proskauer.
Results are given as follows: +, positive reactions; ?, intermediate reactions; -, negative reactions (48-h incubation at 37°C).

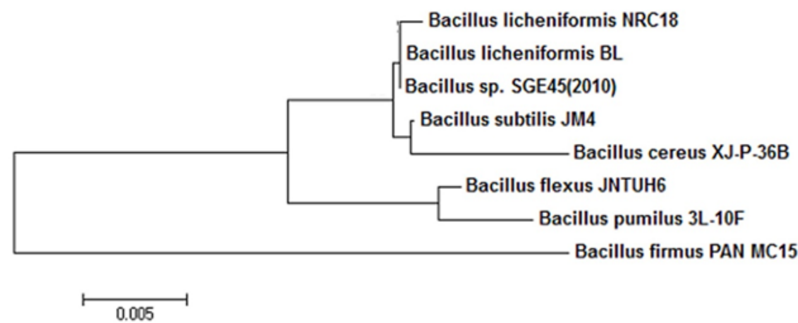

Fig. 1: Phylogenetic tree of the isolated strain and its closest Bacillus strains based on 16S rRNA gene sequences. Scale bar indicates 0.5% fixed point mutations per nucleotide base.

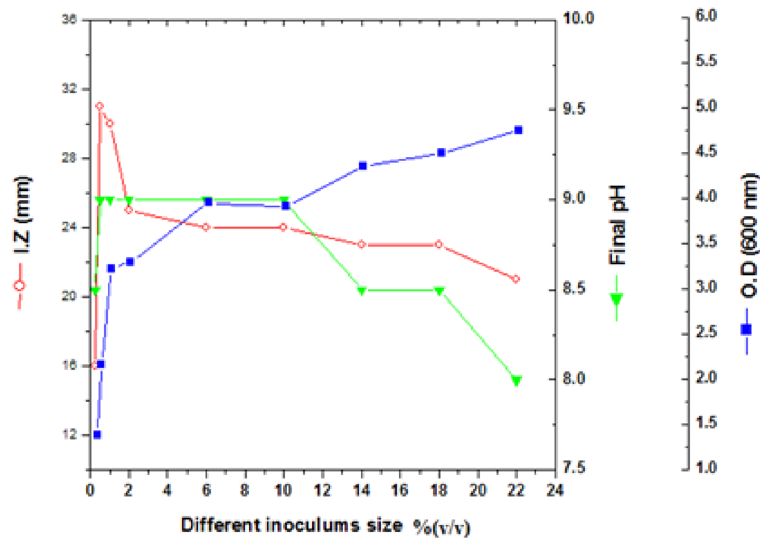

Fig. 2: Effect of different inoculum sizes on the cell growth and antimicrobial production of B. licheniformis NRC-18 against S. aureus

Effect of Different Nitrogen Sources:

Soybean at concentration of 1% (w/v) showed its superiority for the antimicrobial biosynthesis over the other investigated nitrogen sources since it resulted in I.Z. of 38 mm (Fig. 4).

Effect of Different Incubation Period:

The antimicrobial metabolite production by the strain using optimized fermentation medium under optimum culture conditions was started after 16 h of incubation and a rapid enhancement of antimicrobial production as the incubation period increased up to 72 h reaching a maximum inhibition zone of 38 mm after which the production remained stable forming a plateau till 120 h then rapid decline in activity occurred at 144 h (Fig. 5).

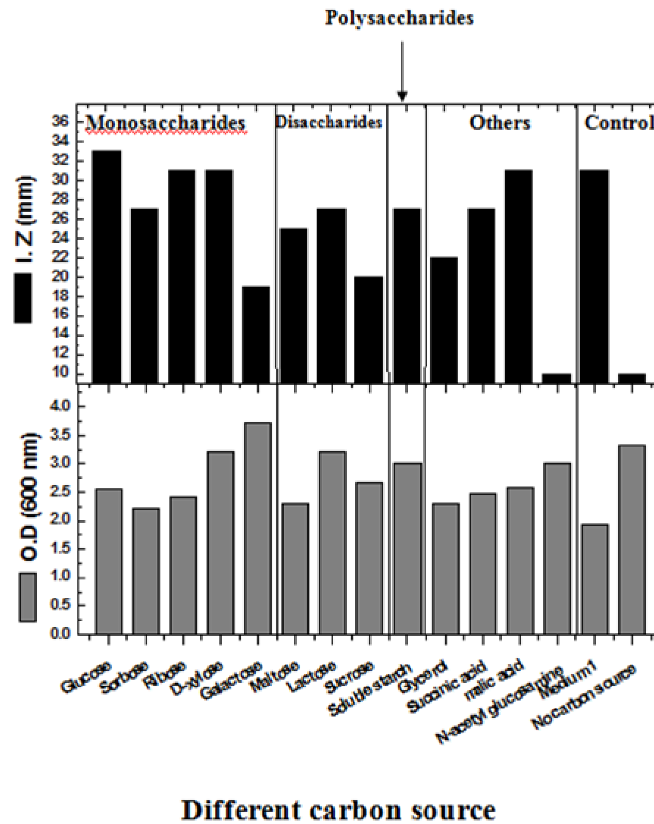

Fig. 3: Effect of different carbon sources on the cell growth and antimicrobial production of B. licheniformis NRC-18 against S. aureus

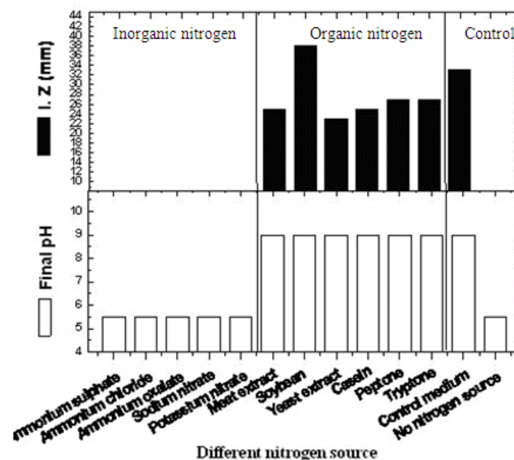

Fig. 4: Effect of different nitrogen sources on the antimicrobial production of B. licheniformis NRC-18 against S. aureus

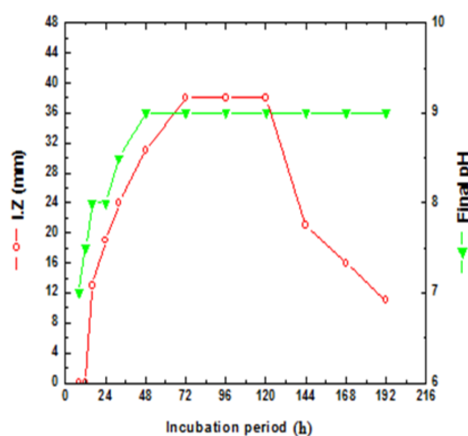

Fig. 5: Effect of incubation period of the optimized fermentation medium on the antimicrobial production of B. licheniformis NRC-18 against S. aureus

Discussion:

The need for antibiotics and antimicrobials continues to be a major challenge as infectious diseases affect millions of people worldwide. Further, antimicrobial resistance is a growing concern as the number of resistant bacteria and the geographic distribution of these organisms are both rising. Marine-derived microorganisms have proven to be a useful source of such natural products and progress has recently been reviewed. Accordingly, the present research work was carried out to isolate marine microorganisms from both Mediterranean and Red sea, Egypt that have antimicrobial activity against pathogenic test strains, and characterization of one of the best isolates both phenotypically and genotypically and finally, optimization study was done of the environmental and nutritional conditions.

From the screening study, it was observed that the majority of the marine microorganisms were able to produce the antimicrobial secondary metabolites only on solid media (primary screening) and their inhibitory activity were not expressed in the culture filtrate of the fermentation media (secondary screening), as the inhibitory compounds did not diffuse into the aqueous environment . This observation was in accordance with that reported by Rosenfield and Zobell, 1947 and later by Burkholder *et al.*, 1966. They concluded that the inhibitory compounds remain closely bound to the outer cell surface and secreted into the solid medium. Lemos *et al.*, 1985 concluded that if the antibiotics remained bound to the cells, they can be excreted slowly and continually to the environment, preventing colonization of the adjacent space by competitors. A rapid release of the antibiotic bacteria probably would not provide them any competitive advantage because it would be immediately washed away by the seawater.

The selected marine bacterial isolate showed antimicrobial activity against gram positive bacteria such as *Staphylococcus aureus* and gram negative bacteria such as *Escherichia coli and Pseudomonas aeruginosa*. Based on morphology, biochemical (API 50CHB) and gene sequencing, the isolate was identified as *Bacillus licheniformis* (99% similarity by 16S rRNA gene sequencing). However, this marine bacilli was isolated from Mediterranean seawaer and it was nominated as *B. licheniformis* NRC-18.

Optimzation study, in the present study, showed that maximum inhibition of 24 mm was observed by *B.licheniformis* NRC18 against *Staphylococcus aureus* at 180 rpm. Early study by Loptanev *et al.*, 1973 showed that the increasing of aeration speed results in increasing of alkaline protease production by *Bacillus polymyxa* from 75 to 200 r.p.m.at pH 7.0. Kumar and Takagi, 1999 reported that *Bacillus cereus* strain 146 showed maximum protease activity at 170 rpm agitation speed after 48 h of incubation. Beg *et al.*, 2003 concluded that at this speed, aeration of the culture medium was increased, and this could lead to sufficient supply of dissolved oxygen in the media. Nutrient uptake by bacteria also will be increased.

Changes in external pH affect many cellular processes such as the regulation of the biosynthesis of secondary metabolites (Solé *et al.,* 1997). The effect of pH was studied by adjusting initial pH (3,5,7 and 9). The highest I.Z. was obtained by initial pH 7.0. Similar results were obtained by Chang *et al.*, 1991 and Yousaf, 1997 who reported that the optimum for bacitracin yield from *B. licheniformis* was obtained with initial pH of 7.0.

The antimicrobial biosynthesis was strongly affected by different incubation temperatures. The results showed that inhibition zone increased with the increase of temperature reaching its maximum value of 23-24 mm at temperatures ranging from 30- 35°C.

Early studies by Berdy, 1974 have shown that maximum titers of bacitracin were obtained at incubation temperature of 37°C. Also, Awais *et al*., 2007 reported that maximum inhibition was observed by *Bacillus subtilis* and *Bacillus pumilus* strains at 30ºC against *S. aureus* and *M. luteus*.

The results showed that 0.5 % (v/v) inoculum sizes gave the maximum antimicrobial production. Further increase in inoculum size resulted in increase in the cell growth of *B.licheniformis* but with decrease in the antimicrobial production. El-Safey and Abdul-Raouf, 2004 reported that the optimum inoculum size for highest protease production by *Bacillus subtilis* was 1.0 ml^{-1} inoculum volume.

On studying the effect of different carbon source, the results indicated that glucose affected the antimicrobial substance production of *Bacillus licheniformis* NRC-18 reaching the highest antimicrobial activity at 1% w/v since the inhibition zone reached 33 mm. However, at the end of incubation period; the final pH value was directed towards alkalinity (pH 9). This result was in accordance with Demain, 1972 who pointed out that many fermentation require glucose as the carbon source, and that production may be controlled by catabolite repression. In *Bacillus subtilis,* bacitracin produced an initial pH fall, but the production of peptide induced alkaline pH. Also, Awais *et al.*, 2007 studied the effect of glucose concentration on the production of antibiotic and the results showed that *B. subtilis* produced a maximum zone of inhibition in 1% glucose.

Nitrogen source was also an important factor for the biosynthesis of antibiotic. It was found that the inorganic nitrogen sources weakly supported both antimicrobial production and cell growth. On the other hand, the addition of some complex nitrogen sources resulted in a significant increase in antimicrobial production. However, Soybean showed its superiority for the antimicrobial biosynthesis over the other investigated nitrogen sources. On the other hand, although peptone and tryptone gave the same I. Z. but there is a wide variation in the biomass production of a value 0.75 and 3.0 A° respectively. These results were in agreement with those reported by Pavl and Banerjee, 1983 who concluded that the organic nitrogen sources produce fair amounts of antibiotic showing very little variation in quantity but the biomass production showed a wide variability. The antimicrobial production increased with the increase of soybean concentration till the highest zone of inhibition of 38 mm which is corresponding to 1 g%. Further increase of soybean concentration resulted in no change in the antimicrobial activity.

It was indicated that the maximum antimicrobial production was obtained after 72 hours, in which the inhibition zone reached 38 mm. Awais *et al.*, 2008 reported that *B.pumilus* produced maximum inhibition against *S. aureus* at 48 and 72 h.

REFERENCES

Altschul, S.F., T.L. Madden, A.A. Schäffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman, 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res.: 25: 3389-3402.

Awais, M., A.A. Shah, Abdul Hameed and F. Hasan, 2007. Isolation, identification and optimization of bacitracin produced by Bacillus sp. Pak. J. Bot., 39(4): 1303-1312.

Awais, M., A. Pervez, S. Qayyum and M. Saleem, 2008. Effects of glucose, incubation period and pH on the production of peptide antibiotics by Bacillus pumilus. Afri. J. Microbiol.Res., 2: 114-119.

Beg, Q.K., V. Sahai and R. Gupta, 2003. Statistical media optimization and alkaline protease production from Bacillus mojavensis in bioreactor. Process Biochem., 39: 203-209.

Berdy, J., 1974. Recent developments of antibiotic research and classification of antibiotics according to chemical structure. Adv. Appl. Microbiol., 18: 309-406.

Burkholder, P. R.; Pfister, R. M. and Leitz, F. H. (1966): Production of a pyrrole antibiotic by a marine bacterium. Appl. Microbiol.: 14, (4), 649-653.

Chang, S.C., Y.H. Wei, D.L. Wei, Y.Y. Chen and S.C. Jong, 1991. Factors affecting the production of eremofortin C and PR toxin in Penicillium roqueforti. Appl. Environ. Microbiol., 57: 2581-2585.

Cladera-Olivera, F., G.R. Caron and A. Brandelli, 2004. Bacteriocin-like peptide production by Bacillus licheniformis strain P40. Lett., Appl. Microbial., 38: 251.

Demain, A.L., 1972. Cellular and environmental factors affecting the synthesis and excretion of metabolites. J. Appl. Chem. Biotechnol., 22: 345-362.

El-Safey, E.M. and U.M. Abdul-Raouf, 2004. Production, purification and characterization of protease Enzyme from Bacillus Subtilis. International Conferences for Development and the Environment in the Arab World, Assiut Univ., 23-25: 14.

Jaruchoktaweechai, C., K. Suwanborirux, S. Anasupawatt, P. Kittakoop and P. Menasveta 2000. New macrolactins from a marine Bacillus sp. Sc026. J. Nat. prod., 63: 984-986.

Kumar, C.G. and H. Takagi, 1999. Research review paper: microbial alkaline proteases: from a bioindustrial viewpoint. Biotechnol. Adv., 17: 561-594.

Lane, D.J., 1991. 16S/ 23S sequencing. In Nucleic Acid Techniques in Bacterial Systematics: pp. 115- 175. Edited by E. Stackebrandt and M. Goodfellow, Chichester: Wiley.

Lemos, M.L., A.E. Toranzo and L.J. Barjia, 1985. Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds. Microb. Ecol., 11: 149-163.

Loptanev, S.V., G.I. Kleiner and E.S. Bylinkina, 1973. Effect of aeration and agitation on the biosynthesis of nystatin. Antibiotiki., 18: 608-613.

Mullis, K.B. and F.A. Faloona, 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol., 155: 335-350.

Nagai, K., K. Kamigiri, N. Arao, K. Suzumura, Y. Kawano, M. Yamaoka, H. Zhang, M. Watanabe and K. Suzuki, 2003. YM- 266183 and YM- 266184, a novel thiopeptide antibiotics produced by Bacillus cereus isolated from a marine sponge. J. Antibioti., 56(2): 123- 128.

Oppenheimer, C.H. and C.E. ZoBell, 1952. The growth and viability of sixty-three species of marine bacteria as influenced by hydrostatic pressure. J. Mar. Res., 11: 10-18.

Pavl, A.K. and A.K. Banerjee, 1983. Determination of optimum conditions for antibiotic production by Streptomyces galbus. Folia Microbiol., 28: 397-405.

Pridham, T.G., P. Anderson, C. Foley, L.A. Lindenfelser, C.W. Hesseltine and R.G. Benedict, 1956. A selection of media for maintenance and taxonomic study of Streptomyces. Antibiot. Ann., 57: 947-953.

Rosenfeld, W.D. and C.E. Zobell, 1947. Antibiotic production by marine microorganisms. J. Bacteriol., 54: 393-398.

Saitou, N. and M. Nei, 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution. 4: 406-425.

Solé, M., A. Francia, N. Rius and J.G. Lorén, 1997. The role of pH in the “glucose effect” on prodigiosin production by non-proliferating cells of Serratia marcescens. Lett. Appl. Microbiol., 25: 81-84. 1.6 MB.

Tamura, K., M. Nei and S. Kumar, 2004. Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA): 101: 11030-11035.

Tamura, K., J. Dudley, M. Nei and S. Kumar, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution., 24: 1596-1599.

Yousaf, M., 1997. Studies on the cultural conditions for the production of antibiotic bacitracin by B. licheniformis. PhD Thesis, Islamia University, Bahawalpur.