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EVALUATION OF ANTIMICROBIAL PROPERTIES OF *BACILLUS SUBTILIS* AND *BACILLUS MEGATERIUM* INHABITING MARINE ENVIRONMENTS

[1]Soni S, [2]Ali AMF

[1,2]School of Life Sciences, Amity University, Lucknow, UP, India.

***Corresponding Author:** Sagar Soni

Email ID: sagaeson1@gmail.com

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ABSTRACT

In the present investigation, 14 bacterial strains (MJSS01, MJSS02, MJSS03, MJSS04, MJSS05, MJSS06, MJSS07, NJSS08, MJSS09, MJSS10, MJSS11, MJSS12, MJSS13 and MJSS14) found to be positive during primary screening (crowded plate technique) were subjected to secondary screening and isolate MJSS07 and MJSS14 showing maximum antimicrobial activity was selected for further studies. Strain improvement was performed by U.V.(ultra violet) and EtBr treatment.6μg/ml EtBr was found to be enhanced animicrobial activity and culture was maintained for further studies. The selected isolate was identified as *Bacillus subtilis* and *Bacillus megaterium* on the basis of bergey's manual. During the study of physiochemical factor of bacterial isolate MJSS07 & MJSS14 the maximum growth was observed at a temperature 37° C and a pH of 7.0.

Key words: Bacterial activity, Antibiogram analysis, Agar well diffusion, Solvent extraction

INTRODUCTION

Marine ecosystems represent 95% of the biosphere and coastal regions are particularly promising, because of the rightly adapted species found in these harsh environments. Each of these classes of marine bio-products has a potential multi-billion-dollar market value. So far, more than 10,000 bioactive molecules have been discovered form marine sources with hundreds of new compound still being discovered every year. Thousands of marine organisms are known to contains antibiotic substance and less than 1% have been examined for their pharmaceutical activity. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from microorganisms, many based on their use in traditional medicine. A number of biologically active compounds with varying degrees of action, such as antitumour, anticancer, antimicrotubule, antiproliferative, cytotoxic, photoprotective, as well as antibiotic and antifouling properties, have so far been isolated from marine sources. Some of these bioactive secondary metabolites of marine origin with strong antibacterial, antifungal and antiviral activities, are currently in intense use as antibiotics and may be effective against infectious diseases such as human immunodeficiency virus (HIV) and conditions of multiple bacterial infections (penicillin, cephalosporins, streptomycin and vancomycin). Marine organisms are under persistent threat of infection by resident pathogenic microbes including bacteria, and in response they have engineered complex organic compounds with antibacterial activity from a diverse set of biological precursors. The word *Antibiotic* is derived from Greek word *antibiosis* which literally means against life. The term antibiotic appeared as early as 1928 in the French microbiological literature as **[1,2].** However it was in 1942, Selman & Waksman who defined ANTIBIOTICS as "a chemical substance derived from microorganisms , which has the capacity of inhabiting growth and or even destroying other organisms in dilute solutions".

The term antimicrobial include all the agents that act against all types of microorganisms such as Bacteria (Antibacterial), Viruses (Antiviral), Fungi (Antifungal) & Protozoans (Antiprotozoal). Antibacterial are the largest & most widely known and studied class of Antimicrobials **[3].**

History of antibiotics begins with the observation of Pasteur & Joubert in 1877, who discovered that one type of bacterium could prevent the growth of another. They didn't know at that time that the reason one bacteria failed to grow was that the other bacterium was producing an antibiotic **[4,5,6]**.

Antibiotics play an important role in combating human, animal and plant diseases. The discovery of antibiotics paved the way for better health for millions around the world.

Antibiotic is one of the most important commercially exploited secondary metabolites produced by bacteria, fungi, viruses and Protozoans and employed in a wide range. Most of the antibiotics used today are from the bacteria. Bacteria are easy to isolate, culture, maintain and to improve their strain **[7,8,9]**.

Classification:

Antibiotics are classified in different ways. Antimicrobials can be classified on the basis of microorganisms they act upon such as Antibacterial (bacteria), Antiviral (viruses), Antifungal (fungus) and Antiprotozoans (protozoan) **[10,11]**.

Antibiotics can be narrow spectrum or broad spectrum on the basis of their mode of action. Broad spectrum antibiotics are those which act against more than one or multiple organisms while as Narrow spectrum act against only one **[12,13,14,15]**.

MATERIALS AND METHODOLOGY

Collection of soil sample:

Soil sample was collected in a pre-sterilized Petri plate and sterile spatula from 3 locations i.e, cattle shed, eutrophic lake and garbage waste (non-biodegradable) **[16,17]**.

Isolation of bacteria from soil samples:

The bacteria were isolated from soil sample by serial dilution in 0.85% NaCl solutions and spreading on sterilized nutrient agar media. Then the pure culture plates were prepared by selecting the cultures on the basis of their morphological characteristics **[18,19]**.

Screening of cultures for their antimicrobial activity:

The pure culture broths were prepared and then the antibacterial activity was analysed against gram positive and gram negative strains. *Escheriachia coli, Pseudomonas aeruginosa, Staphylococcus aureus.* The tests were carried out by using agar well diffusion method **[3]**.

Strain identification of isolates:

For identifying the strain numbers of biochemical tests were carried out by using Bergy's manual **[20]**.

Study of growth parameters of isolates:

The growth kinetic of the culture was performed after inoculating it in sterilized broth medium and the absorbance were taken at 620 nm in spectrophotometer at constant time intervals **[19]**.

Production and purification of antimicrobial component:

The productions of antibiotic were carried out by using shake flask fermentation method and then the purification was performed by using solvent extraction method.

Further the antimicrobial component were analyzed for their antimicrobial activity by using agar well diffusion assay **[20]**.

RESULTS

Collection of soil sample:

Three soil samples were collected from different places within Mumbai:-

Table 1: Table showing places of sample collection.

The marine samples (water and soil sediments) were collected from three sites in Mumbai:

Serial number	Location	Types of sample
1.	Juhu Beach, Mumbai, Maharashtra	Water sample
2.	Sagar Vihar, Mumbai, Maharashtra	Soil sample
3.	Marine Drive, Mumbai, Maharashtra	Water sample

Isolation of bacteria from marine sediments and waters:

Microorganism were isolated from sediments and water sample by serial dilution method and mixed culture were obtained by spreading on NAM as shown in fig. The colonies from these cultures plate were differentiated based on colony morphology studies and were named as MJSS01,MJSS02,MJSS03,MJSS04,MJSS05,MJSS06,MJSS07,MJSS08,MJSS09,MJSS10,MJSS11, MJSS12,MJSS13,MJSS14

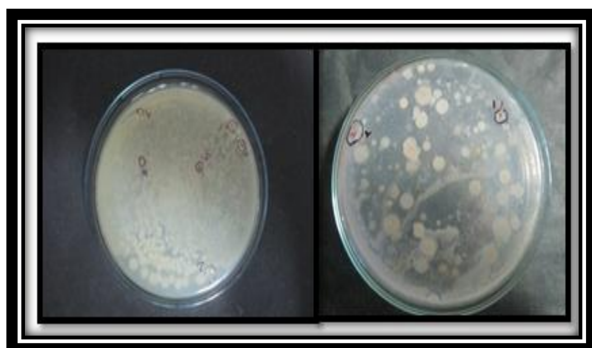
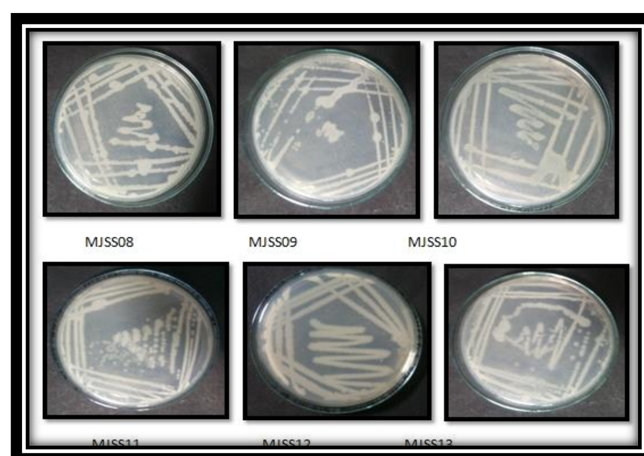


Figure 2: Primary Screening of Microorganisms from Sample

Purification of mixed cultures of bacteria

These mixed cultures were further purified by Discontinuous Quadrant Loop Streaking to obtain pure cultures of bacteria.



Antibiotic sensitivity test of crude secondary metabolites

Isolate **MJSS01, MJSS02, MJSS03, MJSS03, MJSS04, MJSS05, MJSS06, MJSS07,(SEDIMENT) MJSS08, MJSS09, MJSS10, MJSS11, MJSS12, MJSS13, MJSS14 (WATER)** were screened by agar well diffusion method against several pathogens such as *Echerichia coli, staphylococcus aureus, pseudomonas aeruginosa.*

The result were obtained after 24 hrs. The inhibitory zone or zone of clearance was seen around the well which was measured in mm. MJSS01 shows the maximum ZOI i.e. 17.5mm against the *S. aureus,* MJSS02 shows maximum ZOI i.e. 14mm against *E. coli*. And 16mm aganist the *P. aeruginosa*, MJSS03 shows the ZOI i.e. 16.5mm against the *S. aureus,* MJSS04 shows the ZOI i.e. 15.5mm against *E. coli*, 17mm against the *S.aureus*, MJSS05 shows the ZOI i.e. 23mm against *E. coli*, 16.5mm against *S.aureus*, 16.5mm against the *P. aeruginosa*, MJSS06 shows the ZOI i.e. 18.5mm against the *E. coli*, 16.. against *S. sureus* and 15.5mm against the *P. aeruginosa*, MJSS07 shows The ZOI i.e. 24.5mm against *E. coli* and 29.5mm against the *S. aureus*, MJSS08 shows the ZOI i.e. 15.2mm against the *S.aureus,* MJSS09 shows the ZOI i.e 13mm against the *E. coli* and 15mm against *S. aureus*, MJSS10 shows the ZOI i.e. 12mm against *E. coli* and 16mm against *S.aureus*, MJSM11 shows the ZOI i.e. 15.5mm against *E. coli* and 19mm against *S. aureus*, MJSS12 shows the ZOI i.e. 21mm *E. coli*, 17mm against *S.aureus* and 15mm against *P. aeruginosa*, MJSS13 shows the ZOI i.e. 17mm against *E. coli*, 16mm against *S. aureus* and 15mm against *P. aeruginosa*, MJSS14 shows the ZOI i.e. 25mm against *E.coli* and 28.8mm against *S. aureus*.

TABLE-2: antibiogram of mjss01, mjss02 and mjss03 against the bacterial pathogens (in mm)

(SOIL SAMPLE):

S. No.	Pathogens	Zone of inhibition of MJSS01	Zone of inhibition of MJSS02	Zone of inhibition of MJSS03
1	*E. coli*	0.00	14.0	0.00
2	*S. aureus*	17.5	16.0	16.5
3	*P.aeruginosa*	0.00	0.00	0.00

TABLE-3: antibiogram of mjss04, mjss05 and mjss06 against various pathogens (in mm):

S. No.	Pathogen	Zone of inhibition of MJSS04	Zone of inhibition of MJSS05	Zone of inhibition of MJSS06
1	*E.coli*	15.5	23.0	18.5
2	*S.aureus*	17.0	16.5	16.0
3	*P.aeruginosa*	0.00	16.5	15.5

TABLE-4: antibiogram of mjss07 against various pathogens (in mm):

S. No.	Pathogen	Zone of inhibition of MJSS07
1	*E.coli*	24.5
2	*S.aureus*	29.5
3	*P.aeruginosa*	0.00

TABLE-5: antibiogram of mjss08, mjss09 and mjss10

(WATER SAMPLE):

S.No.	PATHOGEN	Zone of inhibition of MJSS08	Zone of inhibition of MJSS09	Zone of inhibition of MJSS10
1	*E. coli*	12	13	12
2	*S. aureus*	21	12	13
3	*P. aeruginosa*	15	14	14

TABLE-6: antibiogram of MJSS11, MJSS12 AND MJSS13:

S. No.	PATHOGENS	Zone of inhibition of MJSS11	Zone of inhibition of MJSS12	Zone of inhibition of MJSS13
1	*E. coli*	15.5mm	15mm	14mm
2	*S. aureus*	---	13mm	14mm
3	*P. aeruginosa*	16mm	19mm	17mm

TABLE-7: antibiogram of MJSS14:

S. No.	PATHOGENS	Zone of inhibition of MJSS13
1	*E. coli*	25
2	*S. aureus*	28
3	*P. aeruginosa*	----

Identification of MJSS07 & MJSS14

The selected culture **MJSS07** & **MJSS14** was identified by Bergey's Manual. Various Biochemical tests were performed.

Table 8: Table showing staining & biochemical tests of MJSS07

S. No	Test	Result
1.	Gram Staining	Positive (*Bacillus*)
2.	Endospore test	Positive
3.	Catalase test	Positive
4.	Mannitol test	Positive
5.	VP- test	Negative

Table 9: Table showing staining & biochemical tests of MJSS14

S. No	Test	Result
1.	Gram Staining	Positive (*Bacillus*)
2.	Endospore test	Positive
3.	Catalase test	Positive
4.	Mannitol test	Positive
5.	VP- test	Positive

Assesment of antimicrobial production of isolate mjss07 & mjss14 during fermentation

The production of antimicrobial components from bacterial culture MJSS07 & MJSS14 during 1st & 2nd day of fermentation was assessed after fermentation by Antibiogram analysis test against various pathogens.

FERMENTATION:-

TABLE 10: Table showing ZOI of day 1 fermentation.

S. No.	PATHOGEN	SOIL SAMPLE	WATER SAMPLE
1	*E. coli*	0.00	23mm
2	*S. aureus*	22mm	21.5mm
3	*P. aeruginosa*	24mm	14mm

TABLE 11: Table showing ZOI of day 2 fermentation.

S. No.	PATHOGEN	SOIL SAMPLE	WATER SAMPLE
1	*E. coli*	23mm	20.5mm
2	*S. aureus*	21.5mm	17.5mm
3	*P. aeruginosa*	14mm	17.5mm

Purification of extracellular antimicrobial component from mjss07 & mjss14 by solvent extraction method

The purification of extracellular antimicrobial component was done by using polar solvents (Acetone, Ethanol & Methanol) and non polar solvents (Chloroform & Ethyl Acetate). The amount of antimicrobial component left after drying was measured as shown below and was dissolved in 2X volume of DMSO (a cryopreservant) to make the concentration of metabolites as 500 mg/ml.

Table 12: Table showing weight of Antimicrobial components purified by different solvents.

S. No	Solvent	Wt. of Empty Bowl (gm)	Wt. of Bowl After Drying (gm)	Wt. of Metabolite (gm)	Amount of DMSO added (µl)	Conc. Of Metabolite (mg/ml)
1.	Chloroform	39.704	40.163	0.459	920	500
2.	Ethyl Acetate	49.662	49.676	0.014	112	500
3.	Acetone	39.98	40.386	0.406	656	500
4.	Methanol	37.324	37.430	0.106	210	500
5.	Ethanol	39.12	39.220	0.1	200	500

Antibiogram analysis of extracellular antimicrobial component purified by solvent extraction method

Antimicrobial activity of extracellular antimicrobial component purified by solvent extraction method from MJSS07 & MJSS14 was observed against various pathogens. All the solvent extracts were used in a concentration of 100mg/ml.

Antibiogram test of purified extracts mjss07 (soil sample) by ethanol, methanol & chloroform

Table 13: Table showing Antibiogram Test of purified extracts MJSS07 by Ethanol, Methanol & Chloroform with Tetracycline and DMSO.

S. No	Pathogens	Zone of inhibition of MJSS 07 by Ethanol (mm)	Zone of inhibition of MJSS 07 by Methanol (mm)	Zone of inhibition of MJSS 07 by DMSO (mm)	Zone of inhibition of MJSS07 by Chloroform (mm)	Zone of inhibition of MJSS07 by Tetracycline (mm)
1.	*E. coli*	18.5	25	16.5	-	45
2.	*S. aureus*	13.5	-	-	18.5	35.5
3.	*P. aeruginosa*	-	-	-	-	33

Antibiogram test of purified extracts mjss07 (soil sample) by ethyl acetate & acetone

Table 14: Table showing Antibiogram Test of purified extracts MJSS07 by Ethyl Acetate & Acetone

S. No.	Pathogens	Zone of inhibition of MJSS14 by Acetone (mm)	Zone of inhibition of MJSS14 by Ethyl Acetate (mm)	Zone of inhibition of MJSS14 by DMSO (mm)	Zone of inhibition of MJSS14 by Tetracycline (mm)
1.	*E. coli*	22.5	15	35	45
2.	*S. aureus*	15	-	-	35
3.	*P. aeruginosa*	-	-	-	33.5

Antibiogram test of purified extracts mjss14 (water sample) by ethanol, methanol & chloroform

Table 15: Table showing antibiogram Test of purified extracts MJSS07 by Ethanol, Methanol & Chloroform with Tetracycline and DMSO.

Pathogens	Zone of inhibition of MJSS 14 by Ethanol (mm)	Zone of inhibition of MJSS 14 by Methanol (mm)	Zone of inhibition of MJSS 14 by DMSO (mm)	Zone of inhibition of MJSS14 by Chloroform (mm)	Zone of inhibition of MJSS14 by Tetracycline (mm)
E. coli	22mm	0.00	21mm	0.00	41mm
S. aureus	15mm	15mm	0.00	0.00	39mm
P. aeruginosa	16mm	0.00	0.00	0.00	33mm

DISCUSSION

The present study was carried out to isolate & identify the marine soil bacteria and marine water bacteria and to study their ability for production of antimicrobial metabolites active against pathogens. Soil and water samples were collected from different locations within Mumbai. Isolation of bacteria was done by serial dilution method on Nutrient Agar Media and further purification was done by discontinuous quadrant streaking as was earlier done by **Jafferey, *et al.,* 2008, Awias, M. *et al., 2007,* Jamil B*. et al*; 2007.**

Primary screening of bacteria was done by Crowded Plate Technique. The plates were incubated for 3-4 days at 37°C and clear zones of inhibition were observed in plates. The same was done by **Awais, M. *et al., 2007,* Palanivel, P*. et al.,* 2012.** Secondary screening of bacterial isolates found positive in primary screening was done by performing antibiogram analysis of crude metabolites by Agar Well Diffusion Method of **Kerby Buer** against pathogens like *S. aereus, E. coli & P. aeruginosa.* The same was done by **Awais, M. *et al.,* 2007, Fitri, L. *et al*., 2010.**

Identification of bacterial isolate selected for further production & purification of antimicrobial component was done by Bergey's Manual. After performing various Staining & Biochemical tests, the isolate was found to be *Bacillus cereus* (**Awais, M. *et al., 2007)***

Production of antimicrobial metabolites in production media was done by Submerged Fermentation. **Awais, M. *et al., 2007***, **Jamil B*.* *et al*., 2007.** The production media (**Khan, *et al.,* 2012)** was optimized for pH, carbon & nitrogen sources and their concentration to get the maximum production of antimicrobial metabolites from isolate **MJSS07 & MJSS14**.

Crude antimicrobial component obtained from isolate **MJSS07 & MJSS14** was purified by solvent extraction method using various polar and non polar solvents. Solvent extraction method was earlier used by **Barke and Seipke, *et al.,* 2010.** Nearly all the solvents used were observed with antimicrobial activity against pathogens, best being the **Methanol** extract of isolate **MJSS07 and Acetone** extract of isolate **MJSS14** .Earlier works reported the extracts with a zone of inhibition of 15mm against *S. aereus* (**Abdulkadir, M., *et al.,* 2012),** 12mm against *E. Coli* (**Fitri, L. *et al*., 2010,)** and 14mm against *P. Aeruginosa* **(Moshafi, M. H., *et al*., 2011).**

The purified extract metabolites have the activity against fungal or not was done by antifungal antibiogram against the fungal species i.s*. Candida albicans* and ethanol and acetone of MJSS07 gave the best zone of inhibition(19mm) as well as ethyl acetate of MJSS14 gave the best zone of inhibition(22.5).

CONCLUSIONS

The bacterial isolate **MJSS07 & MJSS14** identified as *Bacillus subtilis and Bacillus megaterium* a potent and rich source of antimicrobial metabolites active against various pathogens such as *Pseudomonas aeruginosa, Staphylococcus aereus* and *Escherichia coli.* As well as have the antifungal activity against the fungal species i.s *Candida albicans.*

The extraction and purification of antimicrobial component from isolate **MJSS07 & MJSS14** was done by solvent extraction as well as salt precipitation methods. The extraction by salt precipitation method was a potent and rare method. The clear zones of inhibition were observed during antibiogram analysis of extracellular antimicrobial component purified by solvent extraction method against *Pseudomonas aeruginosa*, *Staphylococcus aereus* and *Escherichia coli.* We concluded that there are antimicrobial metabolites soluble in solvents used.

FUTURE PROSPECTS

The bacterial isolate **MJSS07 & MJSS14** can be further identified by 16s rRNA typing. The further purification and isolation of antimicrobial components could be done by sophisticated techniques including HPLC and various chromatographic techniques.

Production of antimicrobial components from **MJSS07 & MJSS14** can be enhanced by further optimization of physiochemical parameters including media, incubation time, pH etc.

The antimicrobial components obtained from **MJSS07 & MJSS14** found active against various pathogens can be used in combination with pre-existing antibiotics in order to enhance the activity.

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