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EVALUATION OFANTIMICROBIAL PROPERTIES OF *Bacillus megaterium* INHABITING MARINE ENVIRONMENTS

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ABSTRACT

Antimicrobial metabolites are produced as secondary metabolite by microorganism, such compounds are widely distributed in nature, they play an important role in regulating the microbial population of soil, water and sewage. In the present investigation, 7 bacterial strains isolates from marine sediment, collected from the **SAGAR VIHAR, SHANPADA MUMBAI (MAHARASTRA)** found during primary screening (crowded plate technique) in which seven bacterial strain was found to be effective namely as MJSS 01 to MJSS 07 and **MJSS 07** was selected for further strain improvement because its found most effective strain in secondary screening against the tested pathogens namely ***Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus***. Strain improvement done by U.V. treatment and EtBr treatment of selected strain. Isolate was identified as ***Becillus megaterium*** on the basis of Bergey's manual. During the study of physiochemical factor of bacterial isolate MJSS 07 the maximum growth was observed at a temperature 37^{o} C and a pH of 7.0. The production media was optimized for best Nitrogen, Carbon and Metal ions sources. A production of crude antibacterial metabolite was carried out by submerged fermentation and crude extracellular metabolites obtained after centrifugation was purified by solvent extraction method. Metabolites extracted by ACETONE gave the best zone of Inhibition 22.5 mm for *E.coli* respectively. Later on Dialysis was performed to check whether the antimicrobial extracted is proteinacious in nature or not and after that MIC was also done to check its potency.

The purified extract metabolites have the activity against fungal or not was done by antifungal antibiogram against the fungal species i.e. *Candida albicans* and ethanol and acetone of MJSS 07 gave the best zone of inhibition 19mm.

Keywords: Bacterial activity, Antibiogram analysis, agar well diffusion, solvent extraction, MIC, Antifungal activity.

INTRODUCTION

The ocean covers 70% of the Earth's surface and contains about 97% of the Earth's water. Around 50-80% of all life on earth is found beneath the ocean surface. Approximately, less than 10% of the marine species has been identified, and the rests are yet to be explored. In the recent years, there is an immense interest among the researchers in tapping the potential of marine microorganisms for their novel secondary metabolites**. Due to the complexity and dynamic system in ocean, the marine microorganisms have developed unique metabolic and physiologic capabilities that ensure survival in extreme variations in pressure, salinity and temperature.** It also offers the potential in the production of metabolites, which may be different from terrestrial microorganisms . A main characteristic feature of marine bacteria is that a large proportion of them are pigmented. It has been reported earlier that some pigments produced by microorganisms have the ability to prevent the growth of other bacteria.

Antibiotics:

Antibiotics as representatives of biologically active substances from microorganisms: Antibiotics are defined as microbial products that inhibit growth of other microorganisms. After the antibiotic effect of penicillin had been observed by Fleming, a number of other antibiotics were discovered. **The main producers are soil microorganisms.**

Role of antibiotics in producing microorganisms:

Antibiotics are the typical secondary metabolites produced by microorganisms. Secondary metabolites are meant products of microorganisms which are not essential for basic metabolic processes such as reproduction and growth. On the other hand, in the case of many secondary compounds, pieces of evidence of their role in the metabolism of the producer have been brought. These compounds often function as

the so-called signal molecules, used to control the producer's metabolism. One of the functions attributed to antibiotics is a suppression of the competing microorganisms in the environment. Thus the antibiotic-producing microorganisms have an advantage in competing for nutrients with the other microorganisms but antibiotic activity is only one from many other biological activities of secondary microbial products. However, the function of antibiotics in the environment can be observed only with difficulty.

Producers of antibiotics and other biologically active compounds from terrestrial microorganisms:

The majority of the known antibiotics are produced by actinomycetes, fungi and by moulds. With an increasing spectrum of efficiency of microbial metabolites, new, non-traditional sources of such compounds have been looked for. **Tropical soils have an enormous biodiversity and they are a rich source of new antibiotics.** Various species of microorganisms have been checked including the microorganisms living under extreme conditions (high and low temperatures, etc.), sea living microorganisms, and higher marine organisms. Marine microorganisms live in a quite different environment from their terrestrial counterparts and would thus be expected to have a different metabolic pathways and to produce compounds, which posses unique structures and activities. Microorganisms have been the source of many valuable compounds in medicine, industry and agriculture; most are derived from terrestrial habitats. After intensive studies on terrestrial microorganisms, consequent attentions have been focused on other ecosystems such as the sea. Marine microorganisms, including bacteria and fungi, are of considerable importance as promising new sources of a huge number of biologically active products. **Some of these marine species live in a stressful habitat, under cold, lightless and high pressure conditions. These factors have resulted in the development of unique metabolisms, which provide the opportunity to produce metabolites that differ from the terrestrial ones.** Thus, sea microorganisms offer a wonderful resource for the discovery of new compounds with interesting biological activities, including antimicrobial and antiviral properties. Up until now, only a small number of microorganisms have been investigated for bioactive metabolites, yet a huge number of active substances have been isolated, some of which feature unique structural skeletons.

The enterprise of screening microbial metabolite for new leads, first exploited by antibiotic researchers and today expanded to virtually all fields of therapeutic interest, has proven successful and will continue as an important avenue to new drug discovery. Hence the present investigation was undertaken to isolate the antagonistic bacteria present in marine environments of coast of Mumbai from **Sagar Vihar, Shanpada, Mumbai, Maharastra** and to examine their inhibitory action against various selected pathogens.

MATERIALS AND METHODS:

SAMPLE COLLECTION:

The sample (sediments) were collected from the SAGAR VIHAR SHANAPADA, MUMBAI (MAHARASTRA). The sample will be collected in sterile glass bottle and soil in zip lock bags and stored at 4°C. The marine water sample will be stored under sterile conditions for preventing the bacterial cross contamination until use.

ISOLATION AND PURIFICATION OF BACETRIAL SPECIES:

The bacterial species isolated from sediments sample by **CROWEDED PLATE TECHNIQUE,** performed by serial dilution, spread on MARINE AGAR MEDIA plate and incubate at 37ºC for 72 hours for found more populated colonies. Mixed colonies obtained after incubation by quadrant streaking manner on MARINE AGAR MEDIA to find the pure bacterial strain.

SCREENING OF THE OBTAINED CULTURE:

The purified culture were screened for antimicrobial component production by inoculating them in to production media (Dextrose, beef extract, yeast extract, NaCl, MgSO4. pH 7). The antimicrobial component were extracted by centrifuging the fermented broth at 10000 rpm for 10 min and supernatant was collected and used for antibiogram analysis. For antibiogram analysis, the well agar diffusion method was used in the plates spreaded with bacterial (*Escherichia coli* ,*Pseudomonas aeruginosa* and *Staphylococcus aureus*) wells were dug by the help of sterile cork borer and loaded with the 50 µl of crude metabolite.

STRAIN IMPROVEMENT OF SELECTED CULTURE:

The selected strain treated by the **U.V. radiation** to produce the mutation after that performed the **EtBr treatment** to produce some sudden changes in their genome which may be beneficial or harm full for the production of antimicrobial component. The confirmation of mutation of culture done

antibiogram analysis against different pathogens.

IDENTIFICATION AND CHARACTERIZATION OF POTENT BACTRIAL STRAIN:

For the identification of selected culture which are using to further research work, done by GRAM STAINNING, ENDOSPORE STAINING, MANITOL FERMENTATION TEST, VP TEST, and CATALASE TEST.

GROWTH PARAMETERS OF ISOLATED BACTERIAL STRAIN:

Growth curve:

For growth curve, the isolated culture was inoculated in 100 ml of sterile nutrient broth and incubated at 28 °C/ 120 rpm in shaker incubator. OD was read at 600 nm after every 24 hours against uninoculated media

Effect of temperature:

For observing the effect of temperature, The selected strain streak on the marine agar media and incubate at different temperature randomly 20ºC, 28ºC (RT), 37ºC and 50ºC.

Effect of pH:

For observing the effect of pH, the selected strain inoculated in to four test tubes which having the nutrient broth having different pH 5, pH 7, pH 9, and pH 11, than incubate at 120rpm for 24hours.After incubation read the O.D. at 600 nm.

MEDIA OPTIMIZATION:

According to requirements of the selected culture for the better production of secondary metabolites, provided the best sources for the media optimization which are as follows:

NITROGEN SOURCES:

For the better production of antimicrobial component, we provided the different nitrogen sources in same concentration (1.3% in 100ml) which know as production media (Beef+ yeast) as well as provided the modified media which are as follows;MM1(Beef extract), MM2(Yeast extract), MM3(Peptone), MM4(Ammonium sulphate).

We also used the nitrogen source (Beef) which had the best ZOI in previous antibiogram analysis, in different concentration (0.5%, 0.75%, 1.0%, and 1.5%).

To get more activity of antimicrobial component against the pathogens, we used two nitrogen sources in combination in different concentration (Beef+ peptone).

CARBON SOURCES:

To checked the carbon activity of antimicrobial component, we used the normal production media which have the glucose as carbon source at the as well as we changed the carbon sources in place of

Glucose at the same concentration (1% in 100ml) i.e. Maltose, Sucrose, Lactose. These changed media known as modified media.

METAL IONS:

Basically in production media Magnesium sulphate was commonly used, bt we changed the composition of production media, we used the 4 another metal ions in place of $MgSO_4$ namely as $CaSO_4$, $CuSO_4$, $FeSO_4$, $PbSO_4$ at the same concentration (0.1% in 100 ml).

The best Metal ion ($CuSO_4$) which produce the best antimicrobial activity in to production media, previously done antibiogram analysis, used at the different concentration (0.1%, 0.5%, .75%, 1.25%)

EXTRATION AND PURIFICATION OF ANTIMICROBIAL CRUDE BY SHAKE FLASK FERMENTATION:

Submerged Fermentation: Also known as Shake Flask Fermentation is a process in which the organisms are grown in liquid medium which is vigorously aerated & agitated in fermentor. In Batch Fermentation, organisms is grown in known amount of culture medium for a defined period of time and then the cell mass is separated from the liquid before further processing.

The flask containing best sources (Beef+ Peptone 1.3%, Lactose 1.0%, Copper sulphate 0.1%, NaCl 0.5%, pH 7) of media which optimized in media optimization, by shake flask fermentation. Inoculated broth incubate at 120rpm for 4 days as stationary phase was reached after 4th day of incubation during growth kinetics study.

The purification of the extracted crude done by **solvent extraction method.** Crude antimicrobial components were extracted from the fermented broth by centrifuging the broth at 10000 rpm for 10 minutes at 4ºC. Crude antimicrobial metabolite was subjected to purification by solvent extraction procedure by various solvents namely Chloroform, Ethyl Acetate, Acetone, 70% ethanol and 80% methanol. 500 µl of the antimicrobial metabolite was taken in eppendorf tubes and 500 µl of the respective solvent was added. Gentle mixing was done for 1 hour after the proper mixing of the solvent and extracts , ethyl acetate, and chloroform spun at 10000rpm for 10 min and collected the upper layer of the ethyl acetate as well as collected the lower layer of the chloroform in to the weighted bowl, the bowls kept at 50ºC for drying the solvent. The bowls were again weighted after drying the solvent and the amount of the metabolites extracted was calculated with empty bowl weight and add the double volume of DMSO and acetone, 70% ethanol

and 80% methanol (mixed) collected in the weighted bowl containing dissolved metabolites were collected in a weighed bowls and scratched metabolites properly and transfer in to eppendorf tubes, stored at 4ºC

Antibiogram Analysis of Purified Metabolites:

Purified metabolites were assessed for their antimicrobial properties by agar well diffusion method as explained earlier, difference being the control that was sterile DMSO here.

CHARACTERIZATION OF PURIFIED EXTRACT:

A) Effect of temp.:

Purified metabolites were kept on different temperature randomly at 4ºC, 28ºC, 37ºC, and 70ºC.

Than performed the antibiogram analysis to know the result of best metabolites which can be shows the best ZOI against the pathogens.

B) Purification of antimicrobial component from MJSS 07 by effect of metal ion on antimicrobial properties:

The selected purified extract (Acetone) was grown with different metal ion concentration of $MgSO_4$ & $CaSO_4$ on solid media plates to check the effect of metal ion concentration on anti-bacterial properties of the growth.

EVALUATION AND DETERMINATION OF MIC OF SELECTED ISOLATES:

MIC was done to know the lower concentration of the purified antimicrobial metabolites for i.s how much concentration is need to inhibit growth of the pathogens.

ANTIFUNGAL ACTIVITY OF PUURIFIED EXTRACTED SECONDARY METABOLITES AGAINST FUNAL SPECIES:

The purified extracted metabolites had antifungal activity or not was done by antifungal antibiogram against the fungal species i.s. *Candida albicans.*

RESULTS AND DISCUSSION:

ISOLATION AND PURIFICATION OF BACTERIAL SPECIES:

Seven different isolates were selected from mixed culture plates obtained after serial dilution, differentiated on the basis of colony morphology and named as MJSS 01, MJSS 02, MJSS 03, MJSS 04, MJSS 05, MJSS 06, and MJSS 07. All the seven were streaked on MARINE AGAR MEDIA plates by quadrant streaking method.

SCREENIN OF PURIFIED BACTERIAL STRAIN ISOLATE FOR

PRODUCTION OF ANTIMICROBIAL COMPONENT:

All the seven isolates were screened for production of antimicrobial components by agar well diffusion method, result of the same can be seen below in **Table 1.** The isolate MJSS 07 showing maximum zone of inhibition was stained with gram's staining procedure and was found to be gram positive rod shape in chain.

STRAIN IMPROVEMENT OF SELECTED CULTURE:

Strain improvement of selected culture done by UV radiation and EtBr (6mg/ml) treatment to cause the mutation in their genome and found to be some beneficial character. MJSS07 found more impressive culture which grow after the mutation.

IDENTIFICATION AND CHARACTERIZATION OF SELECTED STRAIN:

All the test which performed for identification was positive but VP test was found to be negative. The purified strain identified as ***Bacillus megaterium***.

STUDY OF GROWTH PARAMETERS OF MJSS 07:

Growth parameters were studied in order to have an idea of the optimum temperature; pH and growth curve of the isolate so that the same culture conditions could be provided during production of the metabolite.

1. **Growth curve-**

Figure 1 below shows the growth curve of the isolate MJSS 07, it can be seen that the stationary phase was reached between 2^{nd} to 3^{rd} day after incubation.

2. **Effect of temp. on growth-**

For studying the best suitable temp. for the growth of the isolate, it was streaked on marine agar media plate and growth was quantified based on growth in the plate. **Table 2** below shows the result of the same.

3. **Effect of pH on growth-**

Optimum pH for the growth of MJSS 07 was determined and it was found that the isolate grows maximally at pH 7, thus the production media was maintained at the same pH. **Figure 2** below shows result of effect of pH.

EFFECT OF OPTIMIZED BEST PRODUCTION MEDIA SOURCES:

Combination of nitrogen sources (peptone +beef) of 1.3%, 1% of lactose as Corban source and .1% of metal ion i.s. $CuSO_4$ found the best sources as production media for production of antimicrobial component maximally. **Table 3** below shows result of antibiogram.

Antibiogram analysis of purified metabolites:

Antimicrobial component purified by solvent extraction procedure were assessed for their antimicrobial properties by agar well diffusion method, result of the same can be seen in **Table 4** below.

CHARACTERIZATION OF PURIFIED EXTRACTS:

A) Effect of temp.-

The selected purified extract (Acetone) was grown at different temperature range on solid media plates to check the effect of temperature on the growth. The maximum growth was found at 4ºC & 37ºC. The results are shown below in **Table 5**.

B) Effect of metal ion on antimicrobial properties-

The purified extract gave the best zone of inhibition with metal ion i.s. $MgSO_4$ at 0.5% concentration which result shown in **Table 6** and with the $CaSO_4$ the extract gave no zone of inhibition.

EVALUATION AND DETERMINATION OF MIC OF SELECTED ISOLATES:

The lower concentration of antimicrobial component found after MIC test which result shown in **Table 7** below.

ANTIFUNGAL ACTIVITY OF PURIFIED EXTRACTED METABOLITES AGAINST FUNGAL SPECIES:

Acetone and ethanol purified extracted metabolites gave the best zone of inhibition against to fungal species i.s. 19 mm in comparison to flucan. The result shown in **Table 8** below.

Table 1: Screening of antimicrobial metabolites for antimicrobial properties-

PATHOGENS / SAMPLES	*E. coli*	*S. aureus*	*P.aeruginosa*
	ZON E OF INHIBITION (In mm)		
MJSS 01	00	17.5	00
MJSS 02	14	16	00
MJSS 03	00	16.5	15
MJSS 04	15.5	17	00
MJSS 05	23	16.5	16.5
MJSS 06	16	16	15.5
MJSS 07	24.5	29.5	00

Table 2: Effect of temp. on MJSS 07-

S. No.	Temperature	Growth	Remarks
1.	20ºC	No Growth	-
2.	R.T. (32ºC)	Growth Observed	++
3.	37ºC	Growth Observed	+++
4.	50ºC	No Growth	-

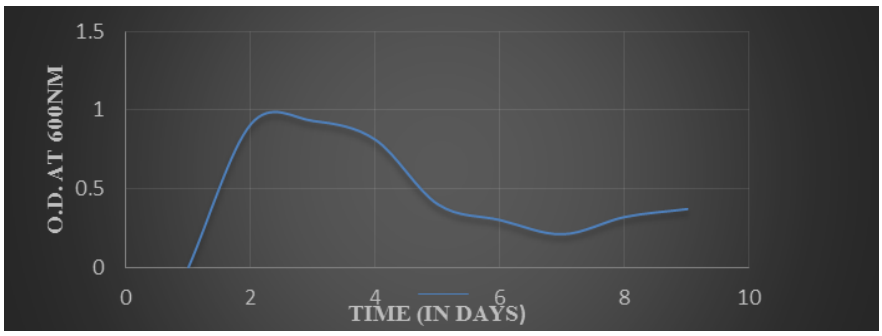

Fig 1: Graphical presentation of growth curve of soil sample-

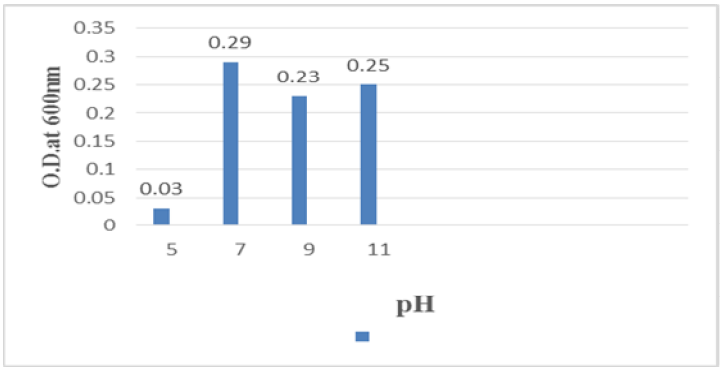

Fig 2: Effect of pH on MJSS 07-

Table 3: optimized best production media sources-

PATHOGENS SOURCES	*E. coli*	*S. aureus*	*P. aeruginosa*
	ZON E OF INHIBITION (In mm)		
N_2 (BEEF, 1.3%)	30	28	39
Combination of N_2 (1.3%) (peptone +beef)(20+80%)	15	14	14.5
Corban (lactose, 1%)	39	32	29
Metal ion ($CuSO_4$, 1%)	20	15	20
Metal ion diff. $conc^n$(.5%)	26	00	24

Table 4: Antibiogram Analysis of Purified Antimicrobial Metabolites Against Bacterial Pathogens-

PATHOGENS SAMPLES	*E. coli*	*S. aureus*	*P. aeruginosa*
	ZON E OF INHIBITION (In mm)		
ETHANOL	18.5	13.5	00
METHANOL	25	00	00
ACETONE	22.5	15	00
CHOLOROFORM	00	18.5	00
ETHYL ACETATE	15	00	00
TETRACYCLINE	45	35.5	33
DMSO	16.5	00	00

Table 5: Effect of temp. on purified metabolites-

Pathogens	ZOI at 4ºC	ZOI at R.T.	ZOI at 37ºC	ZOI at 70 ºC
E. coli	---	---	---	---
S. aureus	23.5mm	21.5mm	---	---
P. aeruginosa	18mm	19mm	19.5mm	---

Table 6: Effect of metal ion on antimicrobial properties-

PATHOGENS SAMPLES	*E.coli*	*S. aureus*	*P. aeruginosa*
	ZON E OF INHIBITION (In mm)		
Metal ion ($MgSO_4$)	00	00	00
Purified extract	00	00	00
Extract + $MgSO_4$ (0.1%)	00	15.5	00
Extract +$MgSO_4$ (0.5%)	16	14	00
Extract + $MgSO_4$ (1%)	00	00	10

Table 7: Table showing O.D. OF MJSS07 in MIC test

S. No.	CONCENTRATION in mg/ml ($C_1V_1=C_2V_2$)	O.D.
1	71.42	0.07
2	10.20	0.59
3	1.45	0.37
4	0.20	0.48
5	0.028	0.65
6	0.004	0.54

Table 8: Showing The ZOI OF Extracted Metabolites Against Fungal Species *Candida albicans*-

METABOLITES	ZONE OF INHBITION BY MJSS07
DMSO	0.00
FLUCAN	18.0mm
ACETONE	19mm
METHANOL	17mm
ETHANOL	19mm
CHLOROFORM	18.5mm
ETHYL ACETATE	16mm

DISCUSSION:

The present study was carried out to isolate & identify the marine soil bacteria and to study their ability for production of antimicrobial metabolites active against pathogens. Soil 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, metal ions and their concentration to get the maximum production of antimicrobial metabolites from isolate **MJSS 07**.

Crude antimicrobial component obtained from isolate **MJSS 07** 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. 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).**

CONCLUSION:

The bacterial isolate was identified as *Bacillus megaterium* a potent and rich source of antimicrobial metabolites active against various pathogens such as *Pseudomonas aeruginosa, Staphylococcus aereus* and *Escherichia coli.*

The extraction and purification of antimicrobial component from isolate **MJSS 07** was done by solvent extracts. The clear zones of inhibition were observed during antibiogram analysis of extracellular antimicrobial component purified by solvent extraction method against *Pseudomonas aeruginosa* and *Staphylococcus aereus* as well as against the fungal species i.s. *Candida albicans*. We concluded that there are some antimicrobial metabolites soluble in solvents used.

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