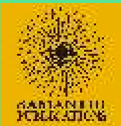

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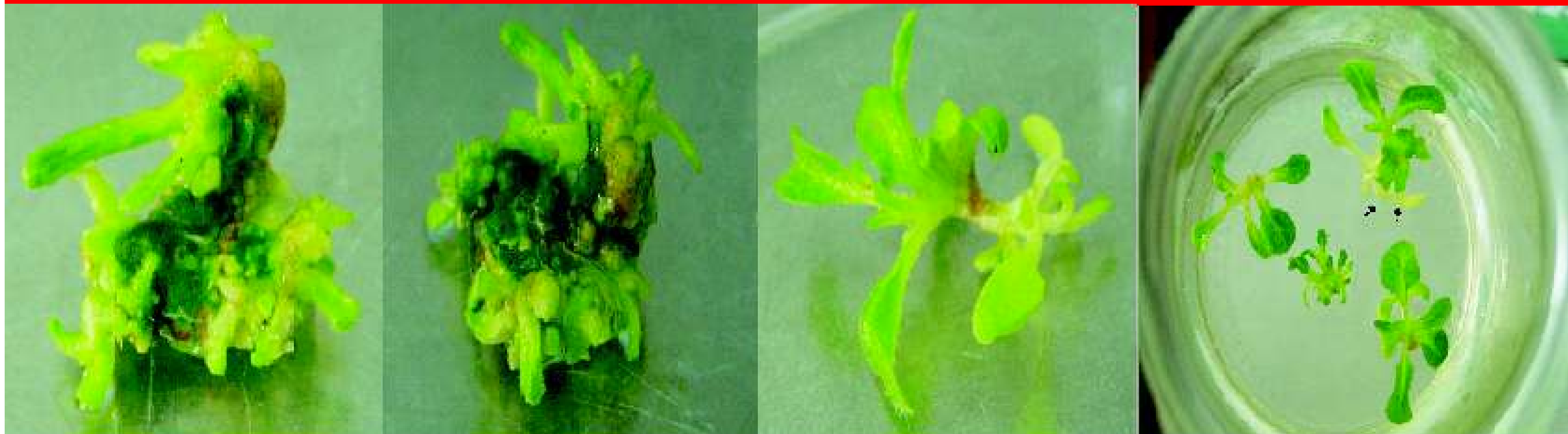

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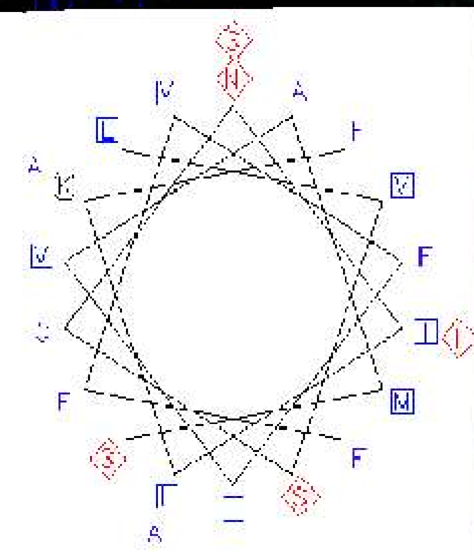
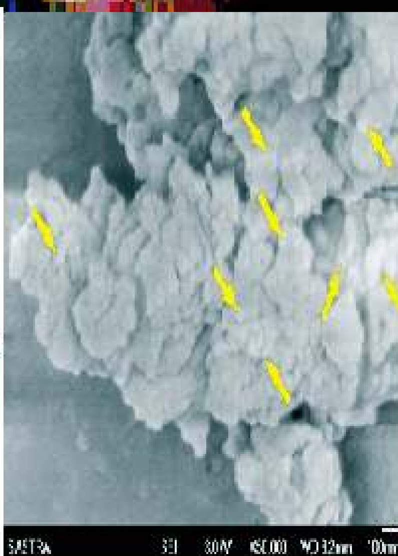
In vitro propagation of *Jatropha curcas*

Genetic transformation studies of *Nicotiana tobaccum*

Parthenium Vermicompost for *Abelmoschus esculentus* (l) Moench

Antibiogram analysis of *Bacillus megaterium* antimicrobials





Research Article

A Study on Isolation, Purification and Antibiogram Analysis of Antimicrobials Extracted from *Bacillus megaterium*

Jahir Alam Khan[1] and Mariya Tabassum[2*]

1. R&D Division, MRD LifeSciences (P) Ltd, Lucknow, Uttar Pradesh, India

2. School of Biotechnology, SHIATS, Allahabad, Uttar Pradesh, India

Abstract

In the present study, six Bacillus strains were selected from road side (Vibhuti Khand, Gomtinagar, Lucknow) for isolation of antimicrobial metabolite producing microorganisms. Among the six isolated strains of Bacillus (AM1, AM2, AM3, AM4, AM5 and AM6) isolate AM1 was found to be most effective in secondary screening tested against three pathogens namely *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli.* The best inhibition zone (24.5mm) was observed against *S. aureus.* In course of identification, the strain was identified as *Bacillus megaterium.* This antimicrobial was further produced in submerged fermentation for 4 days in production media (glucose 10 g, Beef extract 8 g, Yeast extract 5 g, distilled water 1000 ml). The antimicrobial component so obtained was purified for extracellular and intracellular antimicrobial component by salt and solvent extraction procedures respectively. Agar well diffusion was performed to evaluate the antimicrobial activity. When the activity of crude was compared, it was observed that it increased from 20.5mm to 24 mm and 24.5 mm for extracellular and intracellular respectively. Minimum inhibitory concentration for *Staphylococcus aureus* was found to be 1.95 µg/ml for extracellular and intracellular antimicrobial components respectively. Optimum conditions for the production of antimicrobials by selected strain was 72 -96 hrs, pH 7 and temperature 37°C.

Key words: *Bacillus megaterium*, Antimicrobials, MIC, Extracellular extracts, Intracellular extracts, Dialysis.

Introduction

Antimicrobial, in one form or another, have been in use for centuries. The 'anti-microbial' literally means the compound which kills microbes or inhibit the growth of microbes. It can be purified from microbial fermentation and modified chemically or enzymatically for either chemical use or for fundamental studies. The antimicrobial are widely distributed in the nature where they play an important role in regulating the microbial population of soil, water, sewage and compost (Awais *et al.,* 2007). The history of antimicrobials begins with the observations of Pasteur and Joubert, who discovered that one type of bacteria could prevent the growth of another. They did not know at that time that the reason one bacterium failed to grow was that the other bacterium was producing an antibiotic.Technically, antibiotics are only those substances that are produced by one microorganism that kill, or prevent the growth, of another microorganism. Of course, in today's common usage, the term antibiotic is used to refer to almost any drug that attempts to rid your body of a bacterial infection. Antimicrobials include not just antibiotics, but synthetically formed compounds as well.Several antimicrobial compounds are produced by the genus *Bacillus* have been reported. *Bacillus* is an interesting genus to be investigated for antimicrobial activity because *Bacillus species* produces a large number of peptides with biological activities. Eg. cerecin 7 produced by *Bacillus* cereus, tochicin, subpeptin JM4-A and subpeptin JM4-B produced by *Bacillus subtilis* JM4. In this study, we report the discovery and preliminary antimicrobial activity of substance produced by Bacillus species which is isolated from soil.

Materials and Methods

The *Bacillus* strains were isolated from soil sample collected from Roadside (Vibhuti Khand, Gomtinagar, Lucknow) using serial dilution spread plate technique on nutrient agar plates., where 0.5 gm of soil sample was taken with 5 ml of sterile saline, series of dilution of the suspension from 10^{-1} to 10^{-5} were done. 50 µl of the soil suspension were pipetted and spreaded onto solidified nutrient agar plates. All the plates were incubated at 37°C for 2-3 days. The culture plates were observed for any zone of inhibition around any of the growing colonies. Secondary screening/Antimicrobial activity assay of the cultures found to be positive in primary screening was performed by agar-well diffusion method against *P. aeruginosa, S. aureus* and *E. coli* (Muhammad, *et al.*, 2009). A 75 µl aliquot of culture supernatant filterate was loaded in well in agar plates previously spread with selected pathogens. The plates were incubated at 37°C for 24 hrs. Activity was observed as it gave a zone of inhibition around itself (Lisboa, 2006).

For identification various staining techniques and biochemical tests were performed according to Bergey's Manual namely, Gram staining, Endospore staining, Catalase test, Mannitol fermentation test and VP test. For studying the growth curve of the culture positive in secondary screening 100 ml of NB was prepared in a 250 ml flask and autoclaved.1000µl of inoculum of positive isolate was added to the flask and incubated in shaker120 rpm for 24 hrs. After that O.D. was read at 600 nm everyday till decline phase was not reached; for the effect of pH four flasks were taken and to each 20 ml of NB was added which were maintained at pH 5, 7, 9

and 11. The isolate positive in secondary screening was inoculated in each flasks and were incubated at 37°C for 24 hrs at 120 rpm. After 24 hrs of incubation OD was read at 600 nm. For the effect of temperature culture positive in secondary Screening was streaked on sterile NA plates, the plates were incubated at different temperature i.e.16°C, 20°C,

**Corresponding Author*

E-Mail: jahir_84@rediffmail.com

S. No.	Sample	*S. aureus*	*P. aeruginosa*	*E. coli*
1	AM1	20.5	15.5	12.0
2	AM2	12.0	12.0	_
3	Tetracycline	19.5	28.5	19.0

Table 1. Antibiogram of AM1 and AM2 against various pathogens

S. No.	Sample	*S. aureus*	*P. aeruginosa*	*E. coli*
1	AM3	17.5	19.5	11.5
2	AM4	_	16.5	12.5
3	Tetracycline	16.5	30.1	21.5

Table 2. Antibiogram of AM3 and AM4 against various pathogens

Sample	*S. aureus*	*P. aeruginosa*	*E. coli*
AM5	18.5	11.5	11.0
AM6	10.5	19.5	14.0
Tetracycline	18.5	29.0	20.5

Table 3. Antibiogram of AM5 and AM6 against various pathogens

	Test	Result
	Gram Staining	+ve, Streptobacillus
	Endospore Staining	+ve
	Catalase Test	+ve
	Mannitol Test	+ve
	VP Test	-ve

Table 4. Staining and Biochemical Activities of AM1

BSA (0.2 mg/ml)	Distilled water (ml)	Concentration of BSA (mg/ml)	Bradford's reagent (ml)		OD at 595 (nm) 0.00
0.00	0.5	0.00	2.5	Incubated for 10 (min)	0.17
0.05	0.45	0.02	2.5		0.28
0.1	0.4	0.04	2.5		0.36
0.15	0.35	0.06	2.5		0.45
0.2	0.3	0.08	2.5		0.49
0.25	0.25	0.1	2.5		0.57
0.3	0.2	0.12	2.5		0.60
0.35	0.15	0.14	2.5		0.62
0.4	0.1	0.16	2.5		0.71
0.45	0.05	0.18	2.5		0.75
0.5	0.00	0.20	2.5		

Table 5. Estimation of protein by Bradford method

37°C and 50°C. After 24 hrs of incubation, growth was observed. Inoculum for inoculating the production media was prepared in NB. 10 ml of media was prepared in 50 ml flask and autoclaved at 121°C & 15 lbs pressure for 15 min. Antimicrobial producing Bacillus species positive in secondary screening was inoculated by using sterilized loop in the above flask & incubated again at 37°C for 24 hrs in orbital shaker at 120 rpm (Muhammad, *et al.*, 2009). roduction media containing 10 g glucose, 8 g beef extract and 5 g yeast extract extract in 1 litre (Li jing *et al.,* 2009) Production media containing (in g-l) 10 glucose, 8 beef extract and 5 yeast extract pH - 7 (Jeffrey, 2008) was prepared and inoculated with 500µl of inoculums prepared earlier. Flask was incubated at 37°C in a shaker incubator at 120 rpm for four days. The extracellular antimicrobial (proteinaceous) component was recovered from culture supernatant by precipitation using ammonium sulphate upto 70%

saturation followed by centrifugation at 10,000 rpm using at refrigerated centrifuge at 4°C for 15 min (Vijayalakshmi,*et al.,* 2010).This crude antimicrobial was dialyzed and dried and resuspended in 0.02M sodium phosphate buffer (pH 7) (Lisboa, *et al*., 2006).The antimicrobial activity was detected by agar well diffusion. For intracellular antimicrobial component (non proteinaceous) solvent precipitation procedure was used wherein the pellet was resuspended in suspension buffer (TE buffer pH 8), 10% SDS & proteinase K and incubated at 37°C for 1 hr. It was mixed by inversion for 1 hr after adding methanol followed by centrifugation at 10,000 rpm for 10 min at 4°C. Supernatant was air dried and dissolved in 0.02M sodium phosphate buffer (pH-7) (Lisboa *et al.,* 2006). The antimicrobial activity was detected by agar well diffusion method. Protein was estimated by Bradford's method using BSA as standard.

In order to determine the MIC of extracellular as well as intracellular extracts three ml nutrient broth was taken in 12 sterile test tubes. 200 µl

S. aureus *P. aeruginosa* *E. coli*

Figure 1. Showing agar well diffusion of AM1, AM2 against various pathogens

S. aureus *P. aeruginosa* *E. coli*

Figure 2. Showing agar well diffusion of AM3, AM4 against various pathogens

S. aureus *P. aeruginosa* *E. coli*

Figure 3. Showing agar well diffusion of AM5, AM6 against various pathogens

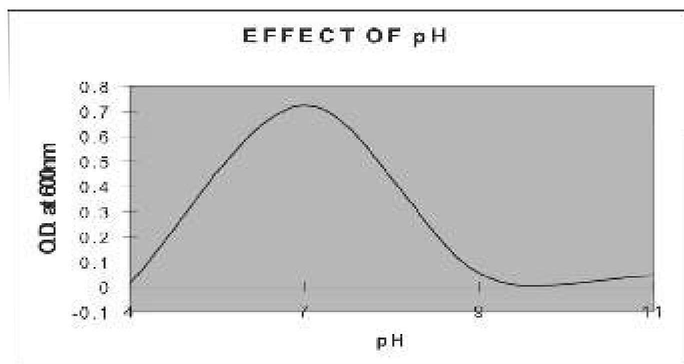


Figure 4. Effect of pH: Estimation of Protein in Antimicrobial Component (standard graph)

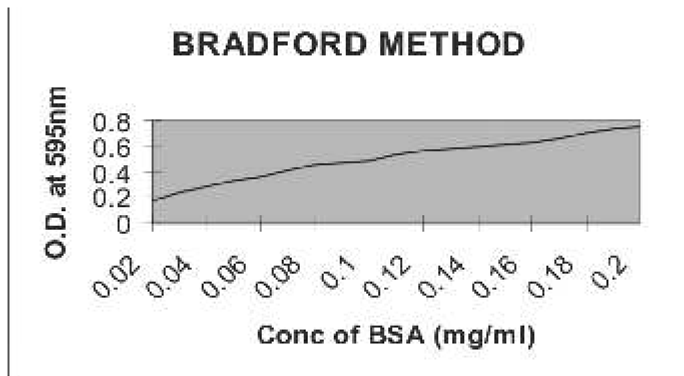


Figure 5. Estimation of protein (standard graphical representation)

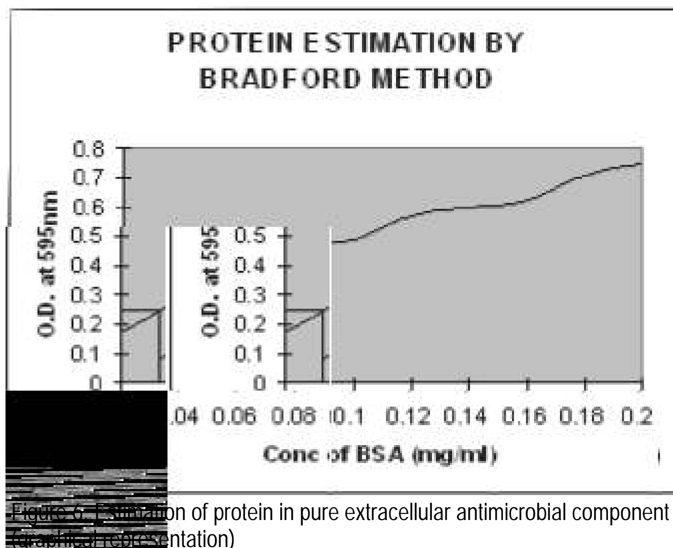
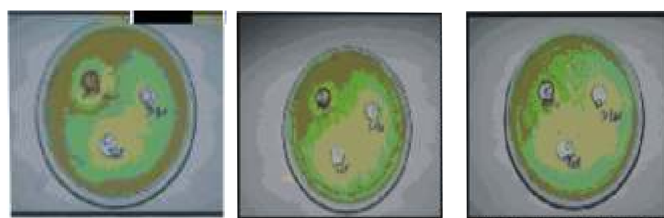


Figure 6. Estimation of protein in pure extracellular antimicrobial component (graphical representation)



S. aureus *P. aeruginosa* *E. coli*

Figure 7. Antibiogram of extracellular antimicrobial component against various pathogens

S. aureus *P. aeruginosa* *E. coli*

Figure 8. Antibiogram of intracellular antimicrobial component against various pathogens

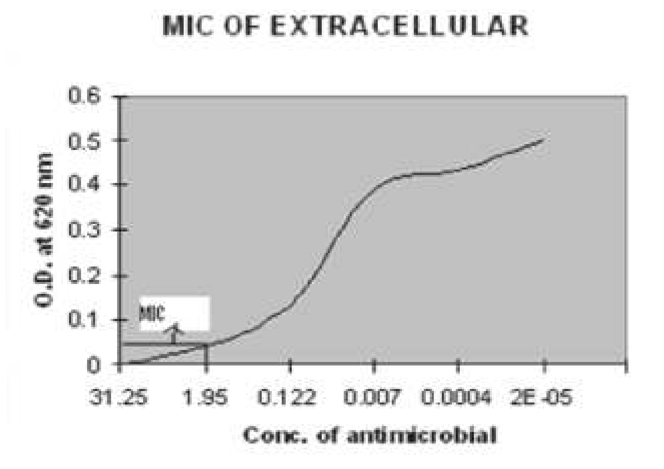


Figure 9. MIC for extracellular antimicrobial component (Graphical representation)

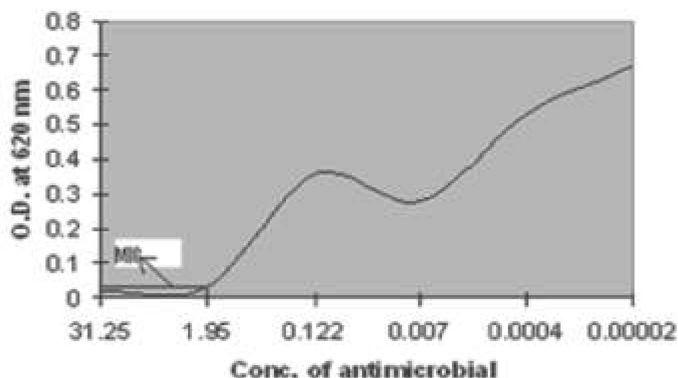


Figure 10. MIC for intracellular antimicrobial component

Results and Discussion

Out of 6 *Bacillus species* subjected for secondary screening process, only 1 isolate (AM1) was selected because it shows the best antimicrobial activity against the selected pathogens of the current study Tables (1-3) & Figs. (1-3) show the results of the secondary screening for all the three isolates subjected to secondary screening.The strain (AM1) was gram positive, *S. bacillus* and identified as *B. megaterium* on the basis of their morphological, staining and biochemical characteristics according to the key of bergey's manual given in the book of Aneja (2003). The results of staining and biochemical activities are presented in Table (4).

After the study of growth parameters it was concluded that the culture reaches stationary phase between 3-4 days, 37°C was found to be optimum temperature and the isolate did not grow below 25°C & above 45°C. The isolate failed to grow at acidic pH but grow well at pH 7 (Fig.

S.No.	Purified Protein (in ml)	Distilled water	Bradford reagent (in ml)		O.D. at 595 nm	Conc of Protein (mg/ml)
Blank	-	1	2.5	Incubated for 10 mins	0.0	0.0
Protein (extracellular)	0.1	0.9	2.5		0.24	0.068
Protein (intracellular)	0.1	0.9	2.5		0.0	0.0

Table 6. Protein estimation by Bradford method

S. No	Sample	*S. aureus*	*P. aeruginosa*	*E. coli*
1	AM1(extracellular)	24.0	18.5	_
2	Tetracycline	19.0	24.5	21.0
3	Distilled Water	_	_	_

Table 7. Antibiogram of extracellular antimicrobial component against various pathogens

S. No	Sample	*S. aureus*	*P. aeruginosa*	*E.coli*
1	AM1	22.5	22.0	21.5
2	Tetracycline	18.5	18.0	17.0
3	Distilled Water	_	_	_

Table 8. Antibiogram of intracellular antimicrobial component against various pathogens

S. No.	Concentration of Extracellular Antimicrobials(µg/ml)	OD (in nm)
1	31.25	0.00
2	1.95	0.04
3	0.122	0.13
4	0.007	0.39
5	0.0004	0.43
6	0.00002	0.5

Table 9. MIC for extracellular antimicrobial component

of antimicrobial component was added to 1st test tube and was serially diluted to 5 test tubes and taken as blank. Again 200 µl of antimicrobial component was added to 1st test tube and was serially diluted to 5 test tubes. 20 µl of *S. aureus* was added to every 6 test tubes and kept in shaker at 37°C for 24 hr. After 24 hr O.D. was read at 600 nm (Peter, *et al.*, 2004).

S. No.	Concentration of Intracellular Antimicrobials(µg/ml)	OD (in nm)
1	31.25	0.02
2	1.95	0.03
3	.122	0.36
4	0.007	0.28
5	0.0004	0.53
6	0.00002	0.67

Table 10. MIC for intracellular antimicrobial component

4).The antimicrobial components (extracellular and intracellular) were evaluated for presence of protein estimation, by Bradford's assay. The Table (5) and Figure (5) below show the standard graph for the protein estimation.

Concentration of protein in purified antimicrobials was calculated by reacting the antimicrobials with bradford's reagent & comparing the resultant O.D

with standard graph. Table (6) and Figure (6) shows the concentration of protein in purified antimicrobials was 0.068 mg/ml and 0.0 mg/ml.

The antimicrobial component (extracellular & intracellular) was tested for its antimicrobial activity against *P. aeruginosa, S. aureus* and *E. coli.* The results are shown in Tables (7-8) and Figs. (7-8). Best Inhibitory activity was observed against *S. aureus* giving a zone of inhibition of 24 mm for extracellular antimicrobial component and 22.5 mm for intracellular antimicrobial component. Earlier the zone of inhibition for crude antimicrobial component was found to be 20 mm against *S. aureus*.

Minimum inhibitory concentration for extracellular and intracellular antimicrobial component was determined by dilution of antimicrobial in NB tubes. Table (9) & Figure (9) show that the MIC of extracellular antimicrobial is 1.95 µmg/ml against *S. aureus.* Table (10) & Figure (10) show that the MIC of intracellular antimicrobial is 1.95 µmg/ml against *S. aureus.*

Bacterial species were isolated and purified from the soil sample collected from road side by serial dilution agar plate method on nutrient agar media as done earlier by Jeffrey (2008). Primary screening of the obtained Bacterial species was carried out by crowded plate technique by incubating the crowded plates for several days and observing for the zone of inhibition on the plates similar method has been followed by Awais *et al.* (2007).

Secondary screening of the Bacterial species found to be positive in primary screening was done by agar well diffusion method of Kirby Buer against the standard test organism *P. aeruginosa, Bacillus, S. aureus* & *E.coli* as done previously by Manjula *et al.* (2009) Anansiriwattana *et al.*(2006). Production of antimicrobial components was done on NYD media, a production media for the production of antimicrobials optimized by Jeffrey (2008). Purification of extracellular antimicrobial extracts was carried out by salt precipitation procdedure upto 70% saturation as done earlier by Lisboa *et al.* (2006) Intracellular antimicrobial extracts were purified by solvent extraction procedures being followed earlier by Lisboa *et al.* (2006). Antibiogram analysis of the purified antimicrobial

extracts was performed by agar well diffusion method and zone of inhibition was found out to be 24 mm in extracellular and 22.5mm in intracellular extract respectively against *S. aureus* earlier by Muhammad *et al.* (2009) 14.5 mm against pathogens (Preecha, *et al*. 2010), 9.3 mm against *Bacillus cereus* ATCC 9634 (Lisboa *et al.,* 2006) 10.0 mm by *B. subtilis* against *Streptomycetes* species (Jeffrey, 2008) 19 mm by *B. subtilis* against *S. aureus* (Awais *et al.,* 2007).

Conclusion

Finally based on the present study it can be concluded that bacterial species *B. megaterium* being purified and identified is a potent source of antimicrobial, which are effective against various pathogens. The salt precipitation method used here for the purification of extracellular antimicrobials is a good and potent method there are very less reports on the antimicrobial extraction from *Bacillus Sp.*, or it can be said, the present study is one of the few reports in the literature wherein salt precipitation method is being used for extraction of antimicrobial components.Giving an indication that extracellular antimicrobial purified here is proteinaceous in nature (confirmed by protein estimation by Bradford's method of protein estimation).We applied the salt precipitation method for purification of intracellular antimicrobials, but surprisingly we did not get any results in antibiogram analysis, showing the intracellular antimicrobial is non-proteinaceous in nature, but we got good results when the intracellular antimicrobials were purified by solvent precipitation method (methanol), we got good results in antibiogram analysis, giving an indication that, there are some secondary metabolites, soluble in the solvent used here.

Future work of the present study included further purification of the antimicrobial components by sophisticated techniques for purification including HPLC.

After further purification the antimicrobial effect can be further enhanced by using various cations such as Ca^{2+}, $Mg^{2+,}$ Zn^{2+}, Pb^{2+}, Hg^{2+}, etc. And also the antimicrobial purified here can be used in combination of preexisting antibiotics, in order to enhance their effects.

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References

Anansiriwattana, W., Tanasupawat, S., Amnuoypol, S and Suwanborirux, K., 2006. Identification and antimicrobial activities of actinomycetes from soils in Samed Island, and geldanamycin from strain PC4 – 3. *Thail. J. Pharm. Sci.* **30**: 49-56.

Aneja, K.R., 2003. *Experiments in Microbiology, Plant Pathology and Biotechnology* (4th Ed.). New Age International (P). Ltd., Publishers: New Delhi.

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

Awais, M., Shah, A.A., Hameed, A. and Hasan, F. 2007. Isolation, Identification and Optimization of Bacitracin produced by *Bacillus Sp. Pak. J. Bot.* **39(4)**: 1303-1312.

Jeffrey, L.S.H. 2008. Isolation, characterization and identification of actinomycetes from agriculture soils at Semongok, Sarawak. *Afr. J. Biotechnol.* 7(20): 3697-3702.

Manjula, C., Rajaguru, P., Muthuselvam, M., 2009. Screening for Antibiotic Sensitivity of Free and Immobilized Actinomycetes Isolated From India. *Adv. Biol. Res.* **3(3-4)**:84-88.

Muhammad, S.A., Ahmad, S. and Hameed, A. 2009. Antibiotic Production By Thermophilic *Bacillus* specie AT -4. *Pak. J. Pharm. Sci.* **22(3)**:339-345.

Lisboa, M.P., Bonatto, L.D., Bizani, D., Henriques, A. P. J and Brandelli, A., 2006. Characterization of a bacteriocin-like substance produced by Bacillus amyloliquefaciens isolated from the Brazilian Atlantic forest. *Int. Microbiol.* **9**:111-118.

Preecha, C., Michael, J. Sadowsky and Prathuangwong, S. 2010. Lipopeptide Surfactin Produced by *Bacillus amyloliquefaciens* KPS46 is required for biocontrol efficacy against Xanthomonas axonopodis pv. Glycines Kasetsart *J. Nat. Sci.* **44(1)**: 84 – 99.

Peter, C.B., Turnbull, Nicky, M., Sirianni, Carlos, T.L., Marian, N., Felicia, N, Leonard, P.F., 2004. MICs of Selected Antibiotics for *Bacillus anthracis, Bacillus cereus*, *Bacillus thringiensis* and *Bacillus mycoides* from a Range of Clinical and Environmental Sources as Determined by the Etest. *J. Clin. Microbiol.* **42(8)**:3626-3634.

Vijayalakshmi, K. and Rajakumar, S. 2010. Antimicrobial protein production by Bacillus amyloliquefaciens MBL27: An application of statistical optimization technique. *Afr. J. Microbiol. Res*. **4(22):** 2388-2396.

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