Control of Burn Wound Pathogens By Antibacterial Compounds Isolated From Bacillus Thuringiensis

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Abstract- *Bacillus thuringiensisis commonly known for its larvicidal activity is being widely used in controlling disease vector insects. Besides its environmental use Bacillus thuringiensis also produce proteinic compounds such as bacteriocin and chitinolytic enzymes which are responsible for antibacterial activity against human pathogens. Bacillus thuringiensis isolated from soils collected from different areas of Thane district showed antibacterial activity against the clinical pathogens isolated from Burn wound samples procured from local hospitals. Pathogenic bacteria isolated from burn samples were also tested for its antibiotic susceptibility with 16 different broad spectrum antibiotics. Bacillus thringiensis inhibiting maximum clinical pathogens were selected and subject to solvent extraction process, which yielded out the antibacterial compound. This compound was dissolved in DMSO and further used for determining its minimum inhibitory concentration. Organisms than are inhibited by Bacillus thuringiensis belong to thefollowing genus, Pseudomonas, Staphylococcus, Bacillus and Proteus*

Keywords- Bacillus thuringiensis , Pseudomonas, Staphylococcus, Bacillus ,Proteus

I. INTRODUCTION

One of the major cause of morbidity in burned patients, is uncontrolled growth of microorganisms which leads to infection in burns. Burns can be defined as tissue injury caused due to thermal, radiation, chemical or electrical contact resulting in protein denaturation, burn wound edema and loss of intravascular fluid volume. Burns provide a rich breeding platform for pathogens causing infections. 75% of the deaths are due to infections (Vindenes and Bjerknes 1995; Revathi et.al. 1998). In case of burn wounds, the first line of defense is the skin which is destroyed. This helps the microorganisms to invade the host wounds easily. Doctors administer systemic antibiotics and topical agents to control the infections. Due to repeated use of antibiotics, AS burn wound healing of second and third degree burns take prolong hospitalization , organism tend to develop resistance to these antibiotics. Available antibiotics recently used in hospitals and burn care centers are Doripenem and Nitrofuranzone. But still considering the rapid developing resistance to these drugs by pathogens urges a need to develop new antibacterial compounds.

As the organism started building resistance to these drugs, various other sources for controlling these organisms were evaluated by researchers. Antibacterial activity of honey, *Dracaena cinnabari* resin, herbs, plants and phytochemicals on common human pathogens and MDR (Andargar and Belay 2004; Ansari 2016; Borges and Cristina 2016).

Results obtained from these are tremendous, but the quantity required to extract a key component in higher concentration is high. This makes the development of antibacterial compound from plant origin to fall back in the race of developing new antibacterial compounds.

The need to develop a new antibacterial compound and from a alternative source has put a limelight on bioactive compounds from microbial origin itself. A soil borne organism having high commercial value, since decades has proved to be a most reliable larvicidal and has the ability to kill insects is *Bacillus thuringiensis*.

One of the main characteristics shared among *Bacillus* strains is the ability to produce a wide range of antimicrobial compounds active against bacteria and fungi. *Bacillus thuringiensis* produces several metabolites with potential applied uses, in particular, chitinolytic enzymes and bacteriocins. Thus the present study is focused on the antibacterial activity of the compounds secreted in the cell free supernatant of wild type *Bacillus thuringiensis* isolated from soil samples. The Cell free supernatants were studied for its antibacterial activity against the burn wound isolates which were isolated from clinical burn wounds. The cell free supernatant of *Bacillus thuringiensis* were tested against the resistant and sensitive burn wound isolates.

II. MATERIALS AND METHODS

Enrichment & Isolation of Wild type *Bacillus thuringiensis* from soils of different ecological niche

Soil samples were collected from various natural sources like agricultural soil (Badlapur), Non agricultural soil

(Yeoor and SGNP), area near Central Effluent Treatment Plant (Badlapur CETP) and area near Hot water springs (Vajreshwari). The soil samples was taken and collected in a plastic zip lock bag.

Collected soil samples were further processed in sterile T3 broth medium and kept in boiling water bath at 80˚C for 1 hr. And streaked on sterile Nutrient agar plates and incubated at 37˚C for 24 hrs. After incubation the colonies showing characteristics resemblance with the standard *Bacillus thuringiensis* i.e flat omlet type colony were isolated and identified till sub species level by performing biochemicals prescribed in Martin and Travers (1989).The BT were confirmed by crystal spores staining with 0.25% coomassie brilliant blue solution .Crystal staining is also a confirmatory test for identifying *Bacillus thuringiensis*.

Enrichment, isolation and identification of burn wound sepsis causing isolates from burn wound samples

Burn wound samples were collected from Central Hospital, (Ulhasnagar) and Masina Hospital, (Byculla) in sterile transport medium 'RINGERS' solution.

Burn wound clinical samples were then processed after enrichement & inoculation on Nutrient agar Plate, Mac Conkey's agar plate and Super Imposed blood agar plate and were incubated at 37°C for 24 hrs. The isolates were identified by Gram staining & Biochemical studies using Bergey's Manual of determinative bacteriology. Biochemicals used for the study were Esculine hydrolysis , Salicin utilization , Lecithin hydrolysis , Sucrose utilization , Starch hydrolysis and Urease activity.

Antibiotic susceptibility of the clinical isolates

Isolated clinical organisms from burn wound samples were subjected to Antibiotic susceptibility testing for screening of multiple drug resistance in organisms by Bauers method (Bauer A.W. 1966).

Determination of antibacterial activity of wild type *Bacillus thuringiensis* against burn wound pathogens:-

Clinical pathogens isolated from burn wound samples were bulk seeded in nutrient agar butts and poured in sterile plate. 24 hr old *Bacillus thuringiensis* growth was spot inoculated on the seeded plates and incubated at 37˚C for 24 hrs. After incubation, zone of clearance was observed concluding the antibacterial activity of *Bacillus thuringiensis.*(Balouiri2016)

Study and extraction of the antibacterial compounds from wild type *Bacillus thuringiensis* and their antibacterial activity against multi drug resistant clinical pathogens

Wild type*Bacillus thuringiensis* showing zone of inhibition were grown in bulk by inoculating in 2000 ml of nutrient broth for 24 hr at 37˚C under shaking condition at 200 rpm. After incubation the cell free supernatant was extracted by subjecting the cultured broth to centrifugation and then extracted in 99.9 % pure chloroform (Bharti, et.al 2012). The intermediate layer between aqueous and solvent layer was collected in a beaker and kept in oven at 55˚C till all the chloroform is evaporated. The completely dried extracted compound was dissolved in DMSO and used for its antibacterial activity by performing micro titter assay in 96 well micro titter plate. With the help of 8 tipped multichannel micro pipette 160 µl of sterile nutrient broth was added to the wells. 20µl of liquid culture and 20µl of antibacterial compound was inoculated in the wells containing nutrient broth. The plate was covered with paraffin tape and incubated at 37˚C for 24 hrs. After incubation next day 50 µl of 2% TTC solution was added and again incubated in dark for 2 hrs. The one showing antibacterial activity showed no color change of the medium. The well in which the cells were still live showed red coloration of the medium. Further the compound was subjected to determination of its minimum inhibitory concentration.

Determination of minimum inhibitory concentration of the extracellular antibacterial compound:-

The effective compounds showing antibacterial effect were then further studied for its MIC. The assay for MIC was performed in 96 well micro titter plates. All materials required for this assay were UV sterilized in laminar air flow. With the help of single tipped multi channel micro pipette 100µl of nutrient broth inoculated with test culture and incubated for 2hrs was added in all wells except the first well. In the first well 200µl (1%) of undiluted antibacterial compound was added. From first well 100µl was pippeted out in the second well and from second well 100µl was pippeted out in third well, so on the serial dilutions were performed till twelfth well. Covered with parafilm tape and incubated at 37˚C for 24 hrs. After 24 hrs the plate was re-incubated in dark by adding 2% TTC solution. The well in which there was not enough concentration of compound to inhibit the organisms showed red coloration. Last well in which there was very low concentration showed dark red coloration, the shade of the red color got faint as the concentration was increased. And as the optimum concentration was achieved were the organisms were inhibited completely and showed no color from that well.In the first wells there was undiluted antibacterial compound.

This assay was repeated twice more to check the viability of the antibacterial compound.

The plates were then further read on ELISA plate reader. Before that corrected OD of the plate was adjusted by subtracting the OD of test antibacterial compound and nutrient broth of each concentration, used in MIC assay. (Balouiri2016).

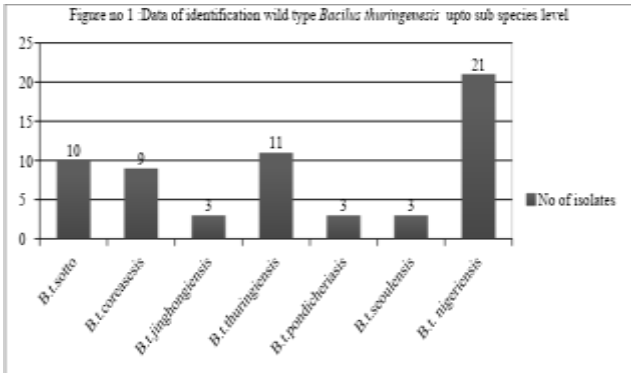
III. RESULTS AND DISCUSSION

Collection of soil samples and Isolation of *Bacillus thuringiensis*:-

85 soil samples were collected from natural sources like agricultural soil, non-agricultural soil, industrial soil and hot water springs area of Maharashtra province. 60 *Bacillus thuringiensis* were isolated from these samples. Agricultural soil yielded 30 *Bacillus thuringiensis* compared to 24 in non-agricultural soils, while soils from polluted area and near hot water springs showed 2 and 4 Bacillus *thuringiensis* respectively.

Identification of *Bacillus thuringiensis* isolated from soil samples

Gram staining of the obtained 60 *Bacillus thuringiensis* was performed. All 60 isolates showed similar characteristics as that of *Bacillus thuringiensis*, i.e Gram positive rods. These 60 *Bacillus thuringiensis* were then further identified till subspecies level by using biochemical methods. The obtained *Bacillus thuringiensis* results were compared with the result obtained by Travers 1989 and the subspecies of *Bacillus thuringiensis* were confirmed. From isolated 60 *Bacillus thuringiensis* it was found that these *Bacillus thuringienisis* belong to 7 different types of subspecies. 10 belong to *Bacillus thuringiensissotto*, 9 *Bacillus thuringiensis* to*coreanesis*, 3 to *Bacillus thuringiensisjinghongiensis* , 11 to *Bacillus thuringiensisthuringiensis*, 3 to *Bacillus thuringiensispondicheriasis*, 3 to *Bacillus thuringiensisseoulensis* and 21 to *Bacillus thuringiensisnigerienisis*. The results are presented in figure no 1



The *Bacillus thuringiensis* isolates were confirmed by parasporalstaining by Coomassie brilliant blue which bind to the crystals and the crystals appear blue while the spore are bind with saffranin stain and they appear pink in colour.

Collection of burn wound clinical samples and isolation of microorganisms

Twenty three samples of burn wound were collected. These samples were then subjected to standard isolation and identification method. From the 23 samples, forty five organisms were isolated. These isolates were spotted on all the selective and differential media selected and the result were observed after 24 hrs. The observed result showed utilization of various substrates and sugars confirmed presence of bacterial pathogens in clinical samples. These obtained isolates were further subjected to Grams staining, of the 45 isolates; 33 were Gram negative rods, 8 were Gram positive rods and 4 were found to be Gram positive cocci. The isolates were then transferred to Nutrient agar slants and stored at 10°C for further use. The results are presented in Table no 1

Table no. 1 :- Gram character of Pathogens isolated from Clinical samples

Gram Character	Gram Negative Rods	Gram Positive Rods	Gram Positive Cocci
Samples/Isolates Burn wound	Isolate No.	Isolate No.	Isolate No.
	BW 1,BW 2(1),BW 2 (2) BW 3 (1),BW 3 (2),BW 3 (3) BW 4,BW 5 (2),BW 6 (1) BW 6 (2),BW 7,BW 8 (1) BW 8 (2),BW 10 (1),BW 10 (2)	BW 9 (1) ,BW 9 (2) BW 12 (3),BW 13 (2) (SF),BW 17 (1) BW 18 (4),BW 20 (1) (SF),BW	BW 5 (1) BtW 12 (1) BW 16 (1) BW 20 (2)

	BW 11 (1),BW 11 (2),BW 11 (3),BW 12 (2),BW 12 (4),BW 13 (1),BW 15,BW 16 (2),BW 17 (2),BW 18 (1),BW 18 (2) BW 18 (3),BW 19 (1),BW 19 (2),BW 19 (3),BW 21 (1) BW 21 (2),BW 23	22	

Key :- SF- Spore former

Based on Gram character the organism were subjected to Antibiotic susceptibility test. The isolates were to be screened for its antibiotic sensitivity by Kirby Bauer's method. The NCCLS standards were referred for this method. Based on the ability of the antibiotic to inhibit Gram negative as well as Gram positive organism, which shows broad spectrum activity the antibiotics were selected. Total of 16 broad spectrum antibiotics were selected to determine drug resistance. Organisms showing resistance to 8 or more than 8 antibiotics from chosen 16 were considered as drug resistant strains. From the biochemical test performed, it was observed that forty five isolated clinical pathogens showed presence of 15 *Pseudomonas aeruginosa*, 5 each of *Proteus vulgaris, Corneybacteirum xerosis* and *Staphylococcus aureus*, 4 *Pseudomonas fluorescencs*, 3 *Proteus penerii*, 2 each of *Salmonella enterica* and *Corneybacterium kutsceri*, 1 each of *Bacillus pasturii, Bacillus sphaericus, Klebsiella pneumonia* and *Klebshiella oxytoca*.

Out of the 45 organisms isolated from the clinical samples, 16 were found to be resistant to the broad spectrum antibiotics. The 16 isolates were identified and they belonged to the following genus and species. 2 isolates were *Corneybacterium xerosis*, 3 were of *Staphylococcus aureus*, 2 of *Proteus vulgaris*, 7 of *Pseudomonas aeruginosa* and 1 each of *Pseudomonas fluorescens* and *Proteus penneri*. Results are represented in table no. 2 and 3 below.

A comparative study of resistant and sensitive isolates obtained from clinical samples has been put forward in figure no 2.

From all the organisms which showed drug resistance to minimum 8 antibiotics, some organisms showed drug resistance to more than 14-15 antibiotics. These organisms belong to *Corneybacterium xerosis, proteus penneri* and *Pseudomonas aeruginosa.*

Table no 2: Results of antibiotic sensitivity and identification of Gram positive burn wound isolates

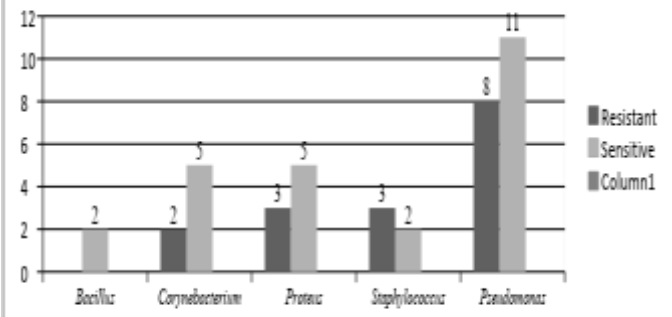
Isolate No	Identified Organism	**Antibiotic sensitivity**	**Isolate No**	**Identified Organism**	**Antibiotic sensitivity**
Gram positive Cocci			**Gram positive rods (Non spore forming rods)**		
BW 5 (1)	*Staphylococcus aureus*	Sensitive	BW 2 (1)	*Corneybacteriumkutsceri*	Sensitive
BW 12 (1)	*Staphylococcus aureus*	Sensitive	BW 9 (1)	*Corneybacteriumxerosis*	Sensitive
BW 16 (1)	*Staphylococcus aureus*	Resistant	BW 9 (2)	*Corneybacteriumkutsceri*	Sensitive
BW 18 (1)	*Staphylococcus aureus*	Resistant	BW 12 (3)	*Corneybacteriumxerosis*	Sensitive
BW 20 (2)	*Staphylococcus aureus*	Resistant	BW 17 (1)	*Corneybacteriumxerosis*	Resistant
Gram positive spore forming organisms			BW 18 (4)	*Corneybacteriumxerosis*	Resistant
BW 13 (2)	*Bacillus pasturii*	Sensitive	BW 22	*Corneybacteriumxerosis*	Sensitive
BW 20 (1)	*Bacillus sphaericus*	Sensitive			

Table no. 3 Results of antibiotic sensitivity and identification of Gram negative burn wound isolates

Isolate No	Identified Organism	**Antibiotic Sensitivity**	**Isolate No**	Identified Organism	**Antibiotic sensitivity**
Gram negative rods			**Gram negative rods**		
BW 1	*Proteus penneri*	Sensitive	BW 11(2)	*Klebsiella pneumonia*	Resistant
BW 2 (2)	*Pseudomonas aeruginosa*	Sensitive	BW 11 (3)	*Pseudomonas aeruginosa*	Resistant

BW 3 (1)	*Proteus vulgaris*	Resistant	BW 12 (2)	*Pseudomonas aeruginosa*	Sensitive
BW 3 (2)	*Pseudomonas aeruginosa*	Sensitive	BW 12 (4)	*Pseudomonas fluorescens*	Resistant
BW 3 (3)	*Proteus penneri*	Sensitive	BW 13 (1)	*Salmonella enterica*	Resistant
BW 4	*Proteus vulgaris*	Sensitive	BW 15	*Klebsiella oxytoca*	Sensitive
BW 5(2)	*Pseudomonas aeruginosa*	Resistant	BW 16 (2)	*Proteus vulgaris*	Sensitive
BW 6 (1)	*Proteus vulgaris*	Resistant	BW 17 (2)	*Pseudomonas aeruginosa*	Sensitive
BW 6(2)	*Pseudomonas aeruginosa*	Sensitive	BW 18 (2)	*Pseudomonas aeruginosa*	Resistant
BW 7	*Pseudomonas aeruginosa*	Resistant	BW 18 (3)	*Pseudomonas fluorescens*	Sensitive
BW 8 (1)	*Proteus vulgaris*	Sensitive	BW 19 (1)	*Pseudomonas aeruginosa*	Resistant
BW 8 (2)	*Pseudomonas aeruginosa*	Sensitive	BW 19 (2)	*Pseudomonas fluorescens*	Sensitive
BW 10(1)	*Pseudomonas aeruginosa*	Sensitive	BW 19 (3)	*Pseudomonas aeruginosa*	Sensitive
BW 10(2)	*Pseudomonas fluorescens*	Sensitive	BW 21 (1)	*Proteus penneri*	Resistant
BW 11 (1)	*Salmonella enterica*	Sensitive	BW 21 (2)	*Pseudomonas aeruginosa*	Resistant
			BW 23	*Pseudomonas aeruginosa*	Resistant

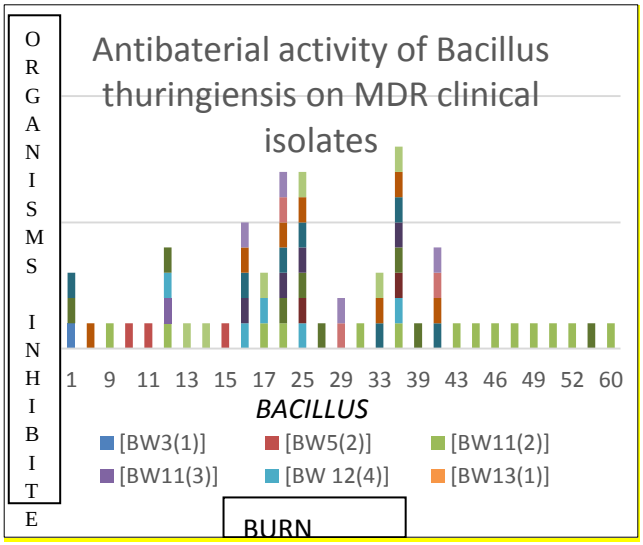
Figure no 2 : Comparative data of resistant and sensitive strains obtained from burn wound samples



Further study was carried out to determine antibacterial activity of the 60 wild type *Bacillus thuringiensis* againstthe MDR pathogens. From the obtained results it was found that wild type *Bacillus thuringiensis* shows inhibition of clinical isolates. Multi drug resistant clinical isolates belonging to the following genus *Pseudomonas aeruginosa*, *Pseudomonas fluorescence*, *Proteus vulgaris*, *Proteus penneri* and *Klebsiella pneumonia* were inhibited by *Bacillus thuringiensis.*

From this sixty wild type *Bacillus thuringiensis*, some of them showed inhibitory activity against maximum number of burn wound pathogens .Results showed that 3 *Bacillus thuringiensis sotto* inhibited 8 isolates, Bacillus *thuringiensisnigeriensis* and *Bacillus thuringiensis coreanesis* inhibited 7 organisms each. Results are interpreted in following fig no. 3

Fig no. 3 Data of *Bacillus thuringiensis* inhibiting multidrug resistant strains isolated from clinical samples.



Bacillus thuringiensis inhibiting most number of organisms isolated from burn wound clinical pathogens i.e *Bacillus thuringiensis sotto* was selected for further processing of, extracting antibacterial compound by using CCL3 solvent extraction method and tested again on MDR strains which showed similar results.

After extraction of the extracellular antibacterial compound, minimum inhibitory concentration (MIC) of the compound was carried out in micro-titer plate. To determine MIC *Bacillus thuringiensis sotto* was tested against burn wound isolate BW18(4) identified as *Corneybacterium xerosis.* Concentration of the extracted antibacterial compound was 1 % and the MIC of the compound was determined to be between 1 % and 0.5 %. Results are presented in table no.4.

Table no. 4 Descriptive analysis:- Percentage inhibition of *Corneybacterium xerosis*growth by extract from *Bacillus thuringiensis sotto.*

PERCENTAGE INHIBITION OF GROWTH	
Wild type burn wound clinical isolate	
Extract (PPM)	*Corneybacterium xerosis*
9	-
19	2.94±1.11
39	4.79±3.01
78	6.88±1.46
156	12.53±1.26
312	20.85±1.58
625	26.24±1.89
1250	48.17±3.599
2500	88.83±0.73
5000	94.62±1.70
10000	100±ND

Key :- Results are represented as mean ± standard deviation of percentage inhibition of growth.
Confidence interval = 99 %.
*= significance value is less than 0.01
Highlighted value is minimum inhibitory concentrations values;
ND- Not detected.

In the above table minimum inhibitory concentration of *Bacillus thuringinesis* for burn wound pathogen, *Corneybacterium xerosis* is 10000 ppm.

IV. CONCLUSION

Sixty *Bacillus thuringiensis* isolates were isolated from 85 soil samples collected from different ecological niche. The identification of the isolated *Bacillus thuringinesis* was carried out upto the subspecies level. Forty five pathogenic bacteria were isolated from clinical burn wound samples collected from burn wound samples were identified by biochemical tests. The antibiotic susceptibility of the clinical isolates against sixteen different broad spectrum antibiotics should that ,sixteen isolates out of the forty five pathogens were found to be resistant to broad spectrum antibiotics. The isolated *Bacillus thuringiensis* were tested against these forty six burn wound isolates, for its antibacterial activity. Three *B. thuringiensis* belonging to subspecies of *Bacillus thuringiensis sotto*, *Bacillus thuringiensis nigeriensis* and *Bacillus thuringiensis coreanesis* were found to inhibit maximum multi drug resistant strains isolates from burn wound samples. These three *B. thuringiensis* were then subjected to solvent extraction of antibacterial compound from cell free supernatant. The extracted compound was also tested on multidrug resistant strain, *Corneybacterium xerosis* which showed inhibition. Further minimum inhibitory concentration of extracted antibacterial compound was determined which resulted to be 10000 ppm.Thus the extracted antibacterial com[pond showed promising results in control of burn wound sepsis causing organisms

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