Original Article

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Characterization and Activity of Antimicrobial Polypeptide of Bacillus Spp from Wastelands of Kathmandu Valley

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ABSTRACT

Background: *Bacillus* genus have been found to produce antimicrobial polypeptides that have a remarkable capability to combat the current increasing problem of antibiotic resistance. The aim of this cross sectional study was to determine the proportion and pattern of distribution of antibiotic producing and non-producing species of Bacillus in soil and also to analyse the activity of the antibiotic producers.

Methods: A total of 40 soil samples were collected from the wastelands of Kathmandu Valley and 121 isolates were studied from them. They were identified according to Bergey's Manual. The antibiotic were extracted and the activity was analysed by agar- well diffusion method against the test organisms *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853.

Result: *Bacillus subtilis* species were found to be in highest number (17.3%) and *Bacillus macerans* (0.82%) in lowest number. 18.33% of *Bacillus* isolates were found to produce antibiotics. The highest number of antibiotic producing isolate was *B. subtilis* (40.09%) and the least was *B. circulans* (4.54%). The number of antibiotic producing bacteria were not found to be affected by moisture content of soil and pH change. Among the antibiotic producers, 63.63% produced antibiotic against Gram positive bacteria, 27.27% against Gram negative bacteria and *B. alvei* (9.09%) had a broad spectrum activity. Some colonies were even found to produce antibiotic with a greater potential than the conventional antibiotics.

Conclusion: Based on property of soil, moist-neutral soil had the highest number of bacilli isolates and dry-acidic and moist-alkaline had least number of isolates.

Keywords: *Bacillus, Characterization, Crude Antibiotic, Antimicrobial Activity, Kathmandu*

Introduction

The current leading problem of the world is that the emergence of drug resistant strains of pathogens is more rapid than the rate of discovery of new drugs and antibiotics (1). In this regard, Anti-Microbial Polypeptides (AMPs), which are ubiquitous gene-encoded natural antibiotics, provide a promising alternative for a new generation of antibiotics (2, 3, 4).

Conventional antibiotics generally target metabolic enzymes that may selectively develop resistance, whereas AMPs kill microbes primarily through the generation of membrane pores, thus making it inherently more difficult for the organisms to develop resistance (5). Since AMPs are a natural part of the antimicrobial defense system, the possibility of developing pathogen resistance or unwanted side effects is less likely than with chemically synthesised conventional antibiotics. Moreover, compared with conventional antibiotics, which are mostly active against bacteria or fungi, AMPs are considered to be antibacterial, antifungal, and antiviral drugs that could be used in the treatment of infectious diseases and parasitic infections and may also be suitable for the treatment of cancer and HIV infection (2, 3, 6, 7).

The genus *Bacillus* is capable of producing a large number of AMPs and is therefore viewed as a promising starting point in the search for new inhibitory substances (8, 9). Several studies have shown that members of the genus *Bacillus* produce a wide arsenal of antimicrobial substances, including ribosomally and nonribosomally synthesized lipopeptides, bacteriocins, and other kinds of peptides (10, 11, 12).

The first class of AMPs comprises ribosomally synthesized peptides, including bacteriocins whereas the second class comprises small microbial peptides synthesized enzymatically by non-ribosomal pathways (13). The Bacteriocins and Bacteriocin-like inhibitory substances (BLIS) contain lanthionine and/or methyllanthionine residues employed to form a ring through intramolecular post-translational modifications like subtilin, a 32-aminoacid pentacyclic lantibiotic (3320 Da) produced by *B. subtilis* ATCC 6633. Mersacidin (1825 Da) is a lantibiotic, produced by *Bacillus* spp. strain HIL and includes globular and uncharged lantibiotics (14). Non-Ribosomal Bio-Peptides include cyclic lipopeptidides (iturin group) and macrolactones (surfactins, fengycins and plipastatins) (15).

Iturin A, bacillomycins and mycosubtilin form channels in bacterial cell membrane. Mycosubtilin alters the permeability of the plasma membrane, releasing nucleotides and proteins. Among small peptides secreted by *B. subtilis*, bacilysin contains an N- terminal alanine residue and L-anticapsin. The release of L-anticapsin irreversibly inhibits glucosamine synthase, involved in the synthesis of nucleotides, amino acids and coenzymes and resulting in the lysis of microbial cells such as *S. aureus* and *Candida albicans* (16). The non-ribosomal dodecapeptide bacitracin (1486 Da), released by some *B. licheniformis* and *B. subtilis* strains, proved to be an inhibitor of cell wall biosynthesis of Gram positive bacteria (17).

Materials and Methods

Materials and Reagents: The media used for culture were Nutrient Agar, Nutrient Broth and Mueller Hinton Agar. For the biochemical characterisation of the *Bacillus* genus, the biochemical media used were Simmon citrate media, Hugh and Leifson, Carbohydrate fermentation media (Glucose, Amylase, Mannitol and Arabinose), 6.5% NaCl media and Gelatin Agar. The reagents used included Gram staining reagent, Spore staining reagent, Catalase reagent, Methyl Red, Voges-Proskauer reagent (Barrit's reagent) and $HgCl_2$ solution.

Isolation of Bacteria: Soil sample was collected from about 10cm below the surface of the earth. 1g of soil sample was added to 10ml of water, the contents of the tube were vortexed for 5 minutes and serial dilution was made up to 10^{-6} .From each dilution, 0.1ml was poured on NA and then spread on Nutrient Agar and incubated overnight at 28°C.The colonies forming a clear halo zone around them were taken as antibiotic producers. Other colonies of *Bacillus* were also identified by study of colony morphology followed by Gram Staining and Spore Staining. They were identified according to Bergey's Manual of Determinative Bacteriology.

Extraction of antibiotic by Tube Culture Method: All the colonies suspected to produce antibiotics were cultured in Nutrient Broth for about 3 days. The cultures tubes were checked every day for turbidity or visual change with periodic shaking of the tubes in between. When sufficient growth indicated by the formation of thick pellicle was observed, the tubes were centrifuged at 6000 rpm for 20 minutes. The pellet was discarded and to the supernatant, equal volume of ethyl acetate was added and again centrifuged at 6000 rpm for 20 minutes. The lower layer of liquid obtained was the crude antibiotic. The antibacterial activity of the obtained raw extract was evaluated using agar-well disc diffusion method.

Agar-well Diffusion Method for Antimicrobial Activity Testing: A standard inoculum of *Staphylococcus aureus* (ATCC 25923*), Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) (with turbidity as 0.5 Mc Farland solution) was swabbed onto the surface of a Mueller Hinton Agar (MHA) plate. Wells were bored in the agar, where 100µl of antibiotic was allowed to diffuse in the well and allowed to diffuse. A well with 100µl of ethyl acetate was kept as negative control. After overnight incubation, the diameter of zone of inhibition was measured for each crude antibiotic diffused in the well.

Disc Diffusion Method for Antimicrobial Susceptibility Testing: A standard inoculum of bacteria was swabbed onto the surface of a Mueller Hinton Agar (MHA) plate. Antibiotic discs were placed on the agar. After overnight incubation, the diameter of zone of inhibition was measured for each antibiotic disc.

Result

A Total of 40 soil samples were collected from wastelands of different locations during the study period, from which 121 bacterial colonies were isolated and studied further. 11.5%, 40.49%, 33.8% and 14.04% of the isolates belonged to Group I, II, III and IV Bacilli respectively. Group V and VI Bacilli were not isolated. Moist-neutral soil had the highest number of bacilli isolates (23.14%) and dry-acidic and moist-alkaline had equal number of isolates (9.09%).

Out of the total 121 colonies studied, 22 (18.33%) of *Bacillus* isolates were found to produce antibiotics. Majority of the antibiotic producers belonged to the Group II Bacilli (59.09%) and none of the isolate from Group IV bacilli produced antibiotic. The highest number of antibiotic producing isolate was *B. subtilis* (40.09%) and the least number of antibiotic producing isolate belonged to the species *B. circulans* (4.54%).

Among the 22 antibiotic producer isolates, 77.71% were from dry soil and 22.29% were from moist soil. Among the antibiotic producers *B. licheniformis* (18.18%) and *B. alvei* (9.09%) were only found in dry soil. Similarly, the antibiotic producing colony of *B. circulans* (4.54%) was only found in moist soil. The highest number of antibiotic producers were found in basic soil (45.45%) whereas the least were found in acidic soil (13.6%). *B. subtilis* was the only antibiotic producing species isolated from acidic soil.

The highest zone of inhibition was by U2 against *E. coli* (22mm) and the lowest activity was by 2M2 against *S. aureus* (4mm)**.** While studying the antimicrobial activity of the 22 antibiotic producing isolates, it was found that 63.63% colonies produced antibiotic against Gram positive bacteria 27.27% against Gram negative bacteria and 9.09% colonies had a broad spectrum activity.

Table 1: Number wise distribution according to soil moisture and pH.

Bacilli Group		Dry Soil			Moist Soil			Total
		A	N	Al	A	N	Al	
Group I	N	1	3	4	1	4	1	14
	%	0.82	2.47	3.3	0.82	3.3	0.82	11.57
Group II	N	6	8	14	7	9	5	49
	%	4.95	6.61	11.5	5.78	7.43	4.13	40.49
Group III	N	2	12	9	5	10	3	41
	%	1.65	9.91	7.43	4.13	8.26	2.47	33.88
Group IV	N	2	1	0	7	5	2	17
	%	1.65	0.82	0	5.78	4.13	1.65	14.04
Total	**N**	**11**	**24**	**27**	**20**	**28**	**11**	**121**
	%	**9.09**	**19.83**	**22.31**	**16.52**	**23.14**	**9.09**	**100**

(A= Acidic soil; N= Neutral soil; Al= Alkaline soil; N=Number of isolates)

Table 2: Isolation pattern of antibiotic producers.

S.N	Antibiotic Producer	Number	% N=22
	Group I	5	27.7
1	B. polymyxa	3	13.63
2	B. alvei	2	9.09
	Group II	13	59.09
3	B. subtilis	9	40.09
4	B. licheniformis	4	18.18
	Group III	4	18.18
5	B. brevis	3	13.63
6	B. circulans	1	4.54
	Group IV	0	0
	Total	**22**	**100**

Table 3: Zone of inhibition by the antibiotic producing isolates.

S.N	Antibiotic Producer	Diameter of Zone of Inhibition (in mm)		
		S. aureus	E. coli	P. Aeruginosa
1	*B. subtilis* (B1)	11		
2	*B. subtilis* (B4)	9		
3	*B. polymyxa* (C1)			9
4	*B. polymyxa* (D2)		15	
5	*B. subtilis* (E7)	9		
6	*B. brevis* (H1)		10	
7	*B. licheniformis* (H6)			
8	*B. subtilis* (J1)	15		
9	*B. alvei* (J2)	15	5	
10	*B. circulans* (L2)	5		
11	*B. licheniformis* (O5)	12		
12	*B. subtilis* (R1)	12		

S.N	Antibiotic Producer	Diameter of Zone of Inhibition (in mm)		
		S. aureus	E. coli	P. Aeruginosa
13	*B. polymyxa* (U2)		22	14
14	*B. alvei* (U3)	20	15	
15	*B. brevis* (U4)		15	20
16	*B. subtilis* (V2)	7		
17	*B. licheniformis* (W1)	8		
18	*B. licheniformis* (X2)	15		
19	*B. brevis* (Y2)		14	18
20	*B. subtilis* (Z1)	14		
21	*B. subtilis* (2I2)	10		
22	*B. subtilis* (2M2)	4		

Table 4: Classification of the antibiotic producers based on their antimicrobial activity.

Antibiotic Producer	Antimicrobial Activity					
	Gram Positive Inhibitor		Gram Negative Inhibitor		Broad Spectrum Inhibitor	
	Number	%	Number	%	Number	%
Group I						
B. polymyxa			3	13.63		
B. alvei					2	9.09
Group II						
B. subtilis	9	40.90				
B. licheniformis	4	18.18				
Group III						
B. brevis			3	13.63		
B. circulans	1	4.54				
Total	**14**	**63.63**	**6**	**27.27**	**2**	**9.09**

Table 5: Antimicrobial susceptibility testing of pathogenic test strains.

S.N	Antibiotic	Zone of Inhibition (mm)	Remarkable Zone of inhibition with extracted crude antibiotics (mm)
	Staphylococcus aureus		
1	Penicillin	20	20 (U2)
2	Amikacin	22	15 (J1)
3	Bacitracin	11	15 (J2)
4	Erythromycin	23	15 (X2)
5	Methicillin	25	
	Escherichia coli		
1	Ampicillin	15	22 (U2)
2	Nalidixic Acid	26	15 (D2)
3	Co-trimoxazole	25	15 (U3)
4	Ceftazidime	11	15 (U4)
5	Imipenem	30	
	Pseudomonas aeruginosa		
1	Gentamicin	20	20 (U4)
2	Ampicillin	0	18 (D2)
3	Ciprofloxacin	26	
4	Cefazidine	0	
5	Co-trimoxazole	15	

Fig. 1: Antibiotic producing colony isolated on nutrient agar.

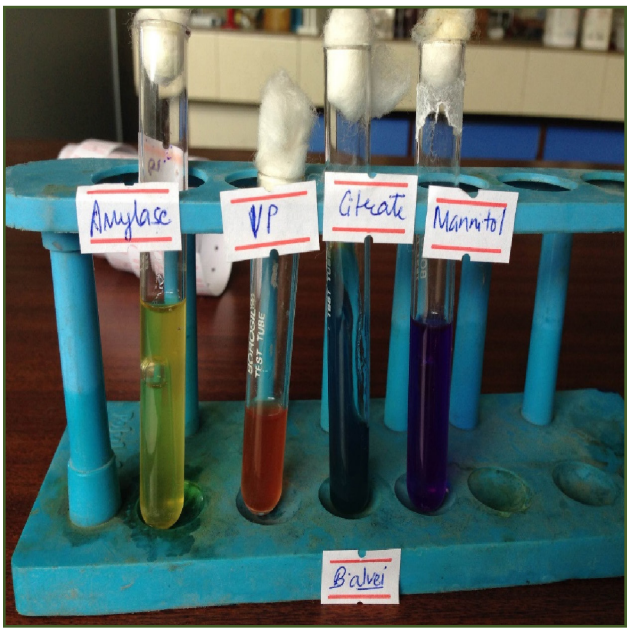

Fig. 2: Biochemical tests of Bacillus alvei (Amylase +ve, VP +ve, Citrate –ve, Mannitol –ve).

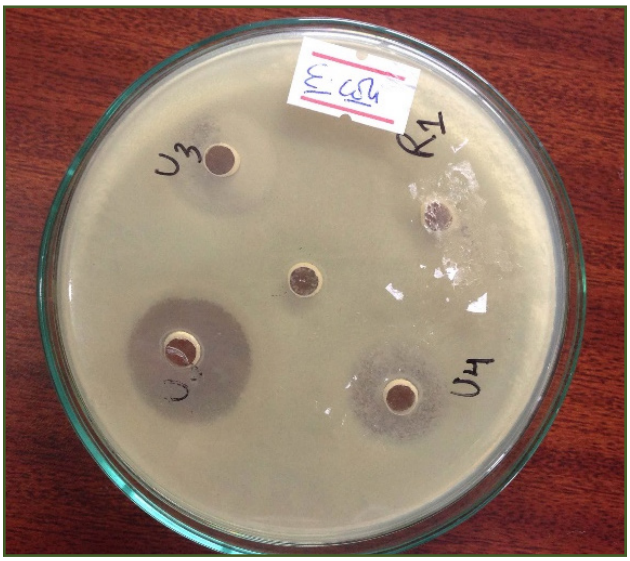

Fig. 3: Agar-well diffusion method for testing antimicrobial activity (Central well- Negative control with ethyl acetate).

Discussion

The present study was conducted in the Microbiology laboratory of St. Xavier's College from February 2016 to June 2016. A total of 121 bacterial colonies were isolated from those samples and studied further.

11.5% colonies belonged to Group I Bacilli. However, the result of the study is different from a similar study carried out by Adamu et al (18), where they found that only 5.7% of the isolates belonged to Group I Bacilli. 40.49% colonies belonged to Group II Bacilli. Similar results were observed in the experiments carried out as 30.8% belonging to Group II Bacilli (18).

The Group III and IV are all strict aerobes and they have similar growth requirement (19). In this regard, the result of this study is contrasting as in this study 33.8% colonies belonged to Group III Bacilli and only 14.04% colonies belonged to Group IV Bacilli. The high frequency of Group III Bacilli is also different from a similar research where

5.7% of the total isolates belonged to Group III (18). The Group V and VI comprise of thermophilic and acidophilic thermophiles respectively (19). Hence, the present study could not isolate these bacteria.

Out of the different species isolated, *B. subtilis* species were found to be in highest number i.e 17.3% and the lowest number of species isolated was *B. macerans* (0.82%). In a similar experiment by Adamu et al (18), they identified only 3.8% of the isolates as *B. subtilis*. In their study, the species with the highest frequency among the isolated ones were *B. lichenifomis* (13.5%) and *B. mycoides* (13.5%).

In this study *B. lichenifomis* (9.09%) and *B. mycoides* (9.91%) were isolated in approximately equal proportion. This result is similar to the study by Adamu et al (18) where it was found that the isolates of *B. lichenifomis* (13.5%) and *B. mycoides* (13.5%) to be in equal proportion.

Out of 121 bacterial isolates, 62 (51.18%) were from dry soil and 59 (48.82%) were from moist soil. Among them the predominant species of dry soil was found to be *B. subtilis* (9.09%) and *B. brevis* (9.09%) and that of moist soil was *B. subtilis* (8.21%). The high number of these species of *Bacillus* isolated in this study matches with the feasibility of their isolation based on their growth requirement as presented by the study of Slepecky and Hemphill (20) which defines that *B. subtilis* group are non-fastidiuos among the other group of bacilli.

Out of the total isolates, the species found uniquely found in acidic soil was *B. marinus,* in neutral soil was *B. subtilis* and that of moist soil was *B. circulans*. The isolation of *B. marinus* in high number in acidic soil and *B. subtilis* together with *B. lichenifoemis* was also reported by Slepecky and Hemphill (20).

Out of the total 121 colonies studied, 22 (18.33%) of *Bacillus* isolates were found to produce antibiotics. The highest number of antibiotic producing isolate was *B. subtilis* (40.09%) and the least number of antibiotic producing isolate belonged to the species *B. circulans* (4.54%). This result is contrasting to a study carried out in soil of marine environment where antibiotic producers of genus *Bacillus* were all identified as *B. subtilis* (21). The highest number of antibiotic producers were found in basic soil (45.45%) whereas the least were found in acidic soil (13.6%). *B. subtilis* was the only antibiotic producing species isolated from acidic soil.

In the present study, 11.5% of the antibiotic producers produced antibiotic against Gram positive bacteria. In a study similar like this where the activity of the antibiotic producers was only tested against Gram positive bacteria, 5.88% showed antibacterial activity against Gram-positive bacteria, and also had good activity against MRSA (21). (Darabpour et al 2012). The frequency of the Gram positive inhibitors is higher in this study compared to the study of Darabpour et al (21). The largest zone of inhibition was obtained as 15 mm by *B. subtilis* (J2). In a similar study carried out by Darabapour et al (21), they isolated *B. subtilis* with a zone of inhibition 23 mm against MRSA.

The present study found that 27.27% of the isolates produced antibiotic against Gram negative bacteria. Among them, the majority were *B. brevis* and *B. polymyxa*. Shandhya et al (22) performed a similar study where *Bacillus* strains were observed to inhibit growth of Gram negative test organisms such as *E.coli, Klebsiella* and *Pseudomonas*.

9.09% colonies had a broad spectrum activity. Abdulkadir and Waliyu (23) and Darabapour et al (21) also found *Bacillus alvei* inhibited the growth of both Gram positive and Gram negative bacteria and they were indicated as broad spectrum antibiotic producer.

The specimen of *Staphylococcus aureus* ATCC 25923 used in this research was also tested against Penicillin, Amikacin, Bacitracin, Erythromycin and Methicillin together with the crude antibiotic extracted in this study. It was found that a colony of *B. alvei* (U3) gave a zone of inhibition 20 mm which was higher than the zone of inhibition by Bacitracin (11 mm) and equal to Penicllin (20mm).

The *Escherichia coli* ATCC 25922 used was also tested with Ampicillin, Nalidixic acid, Co-trimoxazole, Ceftazimide, and Imipinem. A colony of *B. polymyxa* (U2) isolated in this study gave a zone of inhibition 22mm which was higher than the zone of inhibition by Ceftazimide (11 mm) and Ampicillin (15mm)

Similary, for *Pseudomonas aeruginosa* ATCC 27853, Gentamicin, Ciprofloxacin, Ampicillin, Ceftazidine and Co-trimoxazole were used. It was found that the *Pseudomonas aeruginosa* was Resistant to Ampicillin and Cefazidine whereas the zone of inhibition by Co-trimoxazole and Gentamicin were only 15 mm and 20 mm respectively. But, an isolate of *B. brevis* (U4) from this study gave a zone of inhibition 20mm for this test strain.

The antibiotics extracted from the *Bacillus* species in this study were only in a crude form. However, they gave an appreciable zone of inhibition and some even gave a zone of inhibition higher than the standard antibiotics used. This shows that these isolated strains produced antibiotics with a greater potential than the tested standard antibiotics. The standard antibiotics used in this study are the commonly used ones. Thus, we can say that some of these isolated strains have ability to produce antibiotics with a greater potential than the commonly used antibioitcs.

Conclusion

Based on property of soil, moist-neutral soil had the highest number of bacilli isolates and dry-acidic and moist-alkaline had least number of isolates. Out of the different species isolated, *Bacillus subtilis* species were found to be in highest number and the lowest number of species isolated was *Bacillus macerans*. Among them the predominant species of dry soil was *B. subtilis* and *B. brevis* and that of moist soil was *B. subtilis*. Among the species found uniquely in acidic soil was *B. marinus* in neutral soil was *B. subtilis* and that in alkaline soil was *B. circulans*. Out of the total colonies studied, 18.33% of *Bacillus* isolates were found to produce antibiotics. Majority of the antibiotic producers belonged to the Group II Bacilli and none of the isolate from Group IV bacilli produced antibiotic. The highest number of antibiotic producing isolate was *B. subtilis* and the least number of antibiotic producing isolate belonged to the species *B. circulans*. Among the antibiotic producers 77.71% were from dry soil and 22.29% were from moist soil. Also, the highest number of antibiotic producers were found in basic soil whereas the least were found in acidic soil. Furthermore, among the antibiotic producers, 63.63% produced antibiotic against Gram positive bacteria, 27.27% against Gram negative bacteria and 9.09% had a broad spectrum activity. Some colonies were even found to produce antibiotic with a greater potential than the commonly used antibiotics.

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