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Isolation, Identification and Screening of *Bacillus* species with Antimicrobial Activity from Different Soil Samples of Kathmandu Valley

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Abstract

Bacillus species are one of the predominant soil bacteria that are able to produce essential secondary metabolites that have antagonistic effects on other microorganisms. They are Gram-positive, endospore-forming, chemoheterotrophic, aerobic or facultative anaerobic rods usually consisting of peritrichous flagella for motility. The major aim of this study was to isolate the antimicrobials producing *Bacillus* spp. from soil samples of different parts of the Kathmandu Valley, identify them and to assess their antimicrobial activity against different pathogenic bacteria. The test organisms used were *Staphylococcus aureus* (ATCC 25923), *E. coli* (ATCC 25922), *Pseudomonas* spp., *Salmonella* spp., methicillin-resistant *Staphylococcus aureus* (MRSA) and extended spectrum beta-lactamase (ESBL) producing *E. coli*. Twenty four isolates from 9 soil samples identified as *Bacillus* spp. showed the zone of inhibition around their growth on Nutrient agar during isolation. These 24 isolates were chosen for primary screening of production of antimicrobial by perpendicular streaking method using four test organisms. . Of these 24 isolates, six isolates showing a significant zone of inhibition (≥1mm) against two or more test organisms from the primary screening were chosen for secondary screening which was further tested with six test organisms including ESBL *E.coli* and MRSA. They were further characterized through different physiological and biochemical tests. All 6 isolates showed inhibitory action against MRSA and the largest zone of inhibition (30mm) was shown by isolate U6. Isolate U3 was found to have broad spectrum antimicrobial activity with inhibitory effect against gram negative organisms- *Pseudomonas* and *Salmonella* and gram positive organism *S. aureus* (ATCC 25923). Isolate U5 showed a zone of inhibition of about 25mm against *S. aureus* which was comparable to that of erythromycin. Hence, this study determines the soil in Kathmandu Valley as a potential source of antimicrobial producing *Bacillus* spp. and recommends isolation and further characterization of *Bacillus* isolates as a possible source of novel drug to combat with the emergence of multidrug resistant strains.

Keywords: Antimicrobials, Kathmandu, *Bacillus spp., Staphylococcus aureus,* MRSA

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Introduction

Members of the *Bacillus* genus are the predominant soil bacteria with resistant-endospore forming ability and play a major role in organic matter decomposition, bio-transformation, biogas production and nitrogen fixation by associating with the plant roots which ultimately promote the growth of the plants [1,2]. They exhibit a wide range of physiological abilities and produce several metabolites with antagonistic effects as a strategy to survive, eliminate competition with other existing organisms and colonize their natural habitat. Most of the *Bacillus* species accompany actinomycetes and other antibiotic producers in the ecosystem. Therefore, they might have acquired resistance to antibiotics from such sources [3,4]. They produce a wide range of antimicrobial compounds that have different chemical structures and stability through a broad range of pH and temperature and are resistant to enzyme treatments to some extent, therefore, can be used as antibacterial, antifungal and antiviral agents [5]. A *Bacillus* strain is singly capable to produce different antimicrobial compounds and each compound can be active only against the same or closely related species i.e. other gram positive bacteria or may have broad spectrum activity, for example, bacteriocins usually show action against closely related bacteria [4, 6]. Most of the species from the genus *Bacillus* are considered as safe microorganisms and they are easier to handle in the lab with a low incubation period i.e. only 24-48hrs thereby, making *Bacillus* spp. a preferable microorganism to investigate antimicrobial properties [6].

The multidrug resistant pathogens are emerging at an alarming rate, and this situation can be linked to inappropriate usage and shortage on the part of the manufacturers causing a steady decline of efficient antibiotics. Moreover, it has created a consequential issue in the treatment of infectious disease, so, the exploration on this topic is yet important for the discovery of novel antibiotics with new metabolites from thus far unscathed

habitats of potential sources that help in controlling the problem [7].

Kathmandu Valley lies in an alluvial plain where melting snow of four different mountain ranges in the Himalayas feeds rivers and streams that flow down from the mountain. Based on the variation in different locations of the valley, soil type and their composition, there is a possibility of different diversified habitat. Therefore, the quest of discovering novel microflora and finding antimicrobial producing *Bacillus* spp. can be fulfilled [8]. Thus, the project will help in analysis of distribution and antimicrobial activities of *Bacillus* spp. collected from the sampling sites.

Materials and Methods

Sample collection

Nine soil samples were collected from different areas of the Kathmandu Valley (organically cultivated fields, rhizospheric area and river banks) which were processed during the study. The samples were collected from the depth of 10-12.5 cm in the sterile polythene bags and taken to the laboratory. The sample collection sites were Sundarijal, Balaju, Bhaktapur, Sitapaila and Dillibazar and the samples were designated as S1, S2, S3, S4, S5, S6, S7, S8 and S9.

The samples S1 and S8 were from Bhaktapur (Nagarkot Latitude: 27°4254°N, Longitude: 85°3114°E and 27°4015.7°N, 85°2621.3°E), S2 from Dillibazar (27.7054° N, 85.3267° E), S3 from Sundarijal (27.7909° N, 85.4272° E),S4 from Shivapuri (27.8129° N, 85.3859° E) ,S5 from Gokarneshwor (27° 44' 2.688" N 85° 22' 55.38" E), S6 and S7 from Balaju (27.73192° N, 85.29945° E and 27.7309° N, 85.2955° E) and S9 from Sitapaila (27.7170° N, 85.2735° E).

Isolation and screening of antimicrobial producing species

The bacteria were isolated using a spread plate technique after heat treatment of dilutions (10^{-4} and 10^{-5}) for 10 minutes in a water bath at 80°C [9]. The zone of inhibition producing colonies were observed and selected preliminarily on Nutrient Agar. Initially, the isolated colonies were identified by Gram staining and spore staining and then sub-cultured.

Primary screening was done by 'perpendicular streaking method' in which a vertical line of isolates was streaked on Nutrient Agar (with 2% agar to prevent spreading colonies of *Bacillus* spp.) and incubated at 37°C for 24 hrs. The test organisms are S. aureus, E. coli, Pseudomonas spp. and Salmonella spp. (standardized with 0.5 McFarland, with cell suspension 108 CFU/ml) were then streaked perpendicular to the line of growth of isolate. These plates were then incubated overnight at 37°C .The zone of inhibition was measured respectively after the complete incubation [10].

Characterization of antimicrobial producing isolates

Characterization of *Bacillus* spp. was done on the basis of morphological properties, biochemical tests and growth at a temperature at 55°C and salt concentration of 6.5% NaCl according to Bergey's Manual of Determinative Bacteriology [11].

Production of crude extract

Six isolates with high antimicrobial activity through primary screening were selected and inoculated a loopful of sample in about 25 ml of nutrient broth (NB) in a conical flask and incubated for 48 hrs at 37°C in the shaker incubator. The broth culture was then transferred in tubes and centrifuged at 2012 g for 20 min. After the centrifugation, the pellets were discarded and the supernatant was mixed with ethyl acetate (1:1 ratio), collected in screw cap tubes using sterile dropper and then centrifuged at 6000 rpm for 20 min. The upper layer was collected and transferred to vials. This extract was labeled as crude extract [12].

Agar well diffusion method

Six test organisms, MRSA, ESBL *E. coli* (Available at the research laboratory of St. Xavier's College, Maitighar, Kathmandu, Nepal) and four same test organism as used in the primary screening and were taken and swabbed on Muller Hinton agar plates after standardization with 0.5 McFarland solution. On the agar plate, 5 wells of 8 mm diameter using sterile cork borer and 150 µl of crude extract was poured in each well. They were then incubated at 37°C without inverting for 24 hrs. The ethyl acetate was used as negative control and antibiotic discs erythromycin (15 mcg), nitrofurantoin (300 mcg), vancomycin (30 mcg) and gentamicin (30 mcg) (HiMedia Laboratories) were used as positive control. [12]

Results

Isolation and identification of the *Bacillus* spp.

From the 9 soil samples collected from different parts of the Kathmandu Valley, 24 isolates that produced a zone of inhibition around their growth on the isolation media i.e. Nutrient agar selected for identification and screening. The zone of inhibition around them is suggestive of antimicrobial(s) produced by them that diffuse through the media and inhibit growth of other microorganisms around them. These isolates were identified as *Bacillus* spp. on the basis of gram staining (gram positive rods) and spore staining (sporulating).

Table 1. Zone of inhibition demonstrated by antimicrobial metabolite producing isolates against test organisms

Sample	Isolate Number	Name of the isolate	*S. aureus*	*E. coli*	*Pseudomonas* spp.	*Salmonella* spp.
S2	5	U1	1 mm	-	-	6 mm
S3	1	U2	22 mm	1 mm	-	8 mm
S4	1	U3	11 mm	-	-	-
S5	3	U4	1 mm	-	1 mm	2 mm
S6	1	U5	6 mm	-	-	9 mm
S1	2	U6	15 mm	-	-	-
S3	1	U7	5 mm	-	-	-
S6	2	U8	5 mm	-	-	-

- : not inhibited; U1-8: *Bacillus* spp. isolates

Table 2. Characterization of the isolates based on morphology and different biochemical tests

Characteristics	U1	U2	U3	U4	U5	U6
Spore	Spore-former	Spore-former	Spore-former	Non Spore-former	Spore-former	Spore-former
Motility	Motile	Motile	Non-motile	Non-motile	Motile	Motile
Cell Diameter	>1mm	<1mm	>1mm	<1mm	<1mm	<1mm
Catalase	+	+	+	+	+	+
MR	+	+	+	+	-	-
VP	-	+	-	-	+	+
Citrate	-	+	+	-	+	+
Starch hydrolysis	+	+	-	-	+	+
Nitrate reduction	-	-	-	-	+	+
Acid from glucose	+	-	-	-	-	+
Acid from mannitol	-	+	+	-	+	-
Acid from arabinose	-	-	-	-	-	-
Growth at 55°C	-	-	-	-	-	-
Growth in 6.5%NaCl	+	+	+	-	+	+
Identified as:	*Bacillus* spp.					

+ : positive, - : negative

Primary screening of the antimicrobial producing isolates

The 24 isolates of *Bacillus* spp. were chosen for primary screening to detect their ability to produce antimicrobial substances by perpendicular streaking method. All of the isolates were found to show the zone of inhibition around their growth in primary screening (perpendicular streaking method) against four test organisms viz. S. aureus, E. coli, Pseudomonas spp. and Salmonella s pp. Of these 24 isolates, eight isolates produced a significant zone of inhibition (≥1mm) against any one of the test organisms (**Table1**).

Characterization of antimicrobial producing isolates

Among these eight isolates six isolates showing a significant zone of inhibition (≥1mm) against two or more test organisms during primary screening were chosen for secondary screening and were further characterized through different morphological, physiological and biochemical tests (**Table 2**).

Secondary Screening of the antimicrobial producing isolates

The six isolates showing a significant zone of inhibition (≥1mm) against two or more test organisms during

Table 3. Zone of inhibition demonstrated by crude antimicrobial metabolite extract against test organisms

	Test organisms					
Isolates	MRSA	*S. aureus*	ESBL producing *E. coli*	*E. coli*	*Pseudomonas* spp.	*Salmonella* spp.
U1	11 mm	12 mm	-	-	-	20 mm
U2	12 mm	12 mm	-	-	-	15 mm
U3	12 mm	12 mm	-	-	15 mm	15 mm
U4	20 mm	15 mm	-	-	-	-
U5	20 mm	25 mm	-	-	-	-
U6	30 mm	-	-	-	-	18 mm

- : not inhibited

primary screening were chosen for secondary screening by agar well diffusion method and tested against test organisms including MRSA and ESBL producing E. coli as tabulated in **Table 3**.

The results obtained in this study showed that the isolates U3, U5 and U6 have the potential for producing antimicrobial substances inhibiting the Gram positive. Similarly, U1, U2 and U3 have inhibited Gram negatives *Salmonella* spp. as well. The most prominent finding of the study was isolate U5, which produced a zone of inhibition of about 25 mm against *Staphylococcus aureus* (ATCC 25923) which was comparable to the zone of inhibition produced by erythromycin against the organism.

Discussion

The study was carried out to isolate and identify antimicrobial metabolites producing *Bacillus* spp. from various sites of Kathmandu valley in Nepal. The initial selection of the antimicrobial producing strains was based on the clear zone around their colony shown by different bacterial colonies in primary culture on NA plates (**Figure 1)**. In a similar study done by Rai et al, the colonies with a clear halo zone were considered as antibiotic producers. The competition for the growth of a type of organism present in the sample was inhibited by the other organisms marking a boundary where no organism grew giving rise to the halo zone [12].

The identification tests performed as per Bergey's Manual of Determinative Bacteriology showed that the isolates U1, U3 and U4 were close to those of *Bacillus macquariensis, B. sphaericus* and *B. inosolitus* respectively whereas the results of isolates U2, U5 and U6 were close to that of *B. subtilis* (**Figure 2)**. NB used as a culture medium during production of crude extract in this study supported the production of antimicrobial compounds which was observed in the form of a zone of inhibition against the test organisms (**Figure 3)**. In contrast, a study showed that culture of *B. subtilis* B38 strain in NB resulted in sufficient growth, but did not exhibit any antibacterial activity [6]. Extract from trypticase soy broth (TSB) showed better results than NB in another study [13]. Although majority of the antimicrobial compounds do not require metal ions to perform their biological activities, there are some compounds that need metal ions to maintain their structures and physiological roles properly which may not be provided by nutrient broth and the difference in inhibitory effect of the extracts might have differed due to the type of the compound produced [14].

Figure 1. Isolates with antimicrobial activity on Nutrient agar.

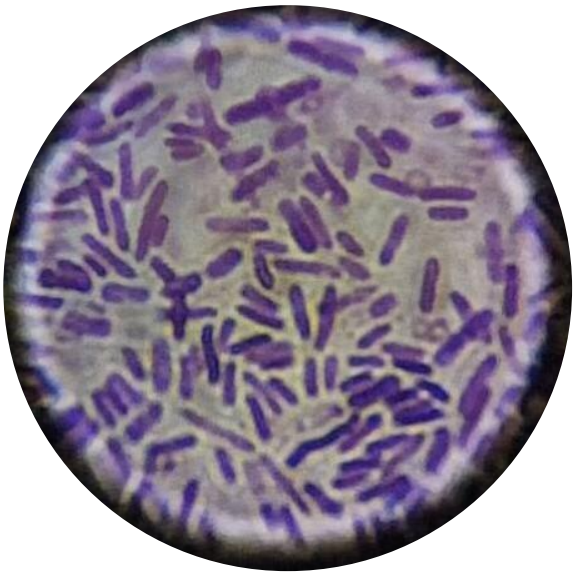

Figure 2. Grams staining under 100x

Figure 3. Agar-well diffusion method with test organism *S. aureus* (Central well- Negative control).

Further, extracts of all 6 isolates demonstrated a zone of inhibition against MRSA with the highest zone of inhibition of 30 mm being shown by the isolate U6. It can be inferred that the antimicrobials produced by the isolates have greater potency as a novel remedy for the alarming rate of antibiotic resistance and in particular, the methicillin resistant *Staphylococcus aureus*.

The extract from the isolates showed more inhibition against Gram positive bacteria than Gram negative bacteria, however, *E. coli* and ESBL producing *E. coli* were not inhibited by any of them. In a similar study, 63.63% colonies demonstrated inhibitory effects against gram positive bacteria and 27.27% colonies were inhibitory to gram negative bacteria. 9.09% of the colonies had a broad spectrum activity [12]. It has been reported that antimicrobial properties of genus *Bacillus* show greater inhibition to the gram positive bacteria than gram negative bacteria in comparison. The resistance of Gram-negative test strains to certain antimicrobial metabolites is due to the barrier created for the hydrophobic compounds because of the presence of outer lipopolysaccharide layer which is less permeable. It is also suggested that the produced antimicrobial substances produced by a Gram-positive bacterium are restricted to other gram-positive bacteria [15,16]. In contrast, the antimicrobials produced by *B. subtilis* MIR 15 in a study have shown inhibitory action mostly against gram-negative bacteria including *E. coli* and *P. aeruginosa* [4]. Among all 6 isolates, crude obtained from isolate U3 was found to have broad spectrum activity with inhibitory effect even against gram negative organisms like *Pseudomonas* spp. and *Salmonella* spp. One of the important findings that this isolate was active against *Pseudomonas* spp. is of great importance as Gram negative bacterium such as *Pseudomonas* spp. is usually resistant to a wide range of antibiotics [17].

The extracellular secretion of antimicrobial metabolites in soluble form are significant from an industrial point of view as disruption of the bacterial cells and solubilization processes can be avoided during downstream processing. The reason that we could not characterize *Bacillus* spp. was due to unavailability of molecular tools (detection of marker genes) in the routine laboratory of St. Xavier's College, Nepal and limited capital (non-funded) for outsourcing.

The increase in antibiotic resistance has been attributed to inappropriate use, inadequacies on the part of the manufacturers and leads to the steady decline of effective antibiotics annually worldwide. Antibiotic resistance is present in every country. The patients with drug resistant infections consuming more healthcare resources are high risk to clinical outcome and eventually death than the patients with nonresistant infections. This situation is a serious challenge to drug manufacturers, public health practitioners worldwide [18].

Reducing the spread of resistant pathogens and the rate of evolution of resistance is complex. Antibiotic resistance is forcing scientists to search for these antibiotic-producing bacteria in hopes of finding new ways of killing pathogens. Therefore, this study is an attempt to identify *Bacillus* species with potential of antibiotic production that could be used to stem the scourge of drug resistance which suggest that the soil of Kathmandu valley is inhabited by different antimicrobials producing *Bacillus* spp and its proper study may create a way towards the development of novel antibiotics.

Conclusion

This study demonstrated that the isolated strains of antimicrobial producing *Bacillus* spp. can be used as a potent source of antibiotics as isolates U3, U5 and U6 showed an appreciable zone of inhibition against the test organisms especially with the Gram positive ones including MRSA, than the gram negative ones. However, isolates U1, U2 and U3 were effective antimicrobials for Gram negative organisms such as *Salmonella* spp. and *Pseudomonas* spp.

Authors' contributions

AT designed and conceptualized the project. AT, AB, AS MS and PS carried out the experiments, lab works and participated in the data analysis. SP helped to design the study, amended the methodology, managed necessary arrangements during laboratory investigations and supervised the complete study. All authors have read, edited and approved the final manuscript.

Competing interests

No competing interests were disclosed.

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Ethical approval and consent

Humans or human samples were not used in this study. So, ethical approval from concerned authority was not needed.

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