Study on the presence of bacterial isolates with the potential to produce antimicrobial peptides in soil samples in and around Mysore city, India

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Abstract: **The present study aims to screen soil samples collected from market regions in and around Mysore district, India, for the presence of antimicrobial peptides-producing bacteria. Totally 30 samples were subjected to isolation using the standard serial dilution technique. Morphological characterization of the isolated presumptive cultures by Gram's staining method confirmed the presence of both Gram-positive and Gram-negative bacteria in the samples screened, the former being more in number. Primary screening of the isolates using the perpendicular streaking method resulted in an encounter of four isolates with antibacterial activity *viz.* AGS7, AKS9, AZS12, and BPS26. Among them, processed culture supernatant of AZS12 strain demonstrated broad-spectrum antibacterial activity against clinically important bacterial pathogens namely *Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans,* and *Salmonella enterica ser. paratyphi*. A maximum zone of inhibition of 22 mm was recorded against *K. pneumoniae* and *Streptococcus mutans* at a 50 µl concentration of cell-free culture supernatant of AZS12. This secondary screening validated that AZS12 was an antimicrobial peptide-producing bacterial isolate. Biochemical characterization using a panel of tests indicated that AZS12 belonged to the genus of *Bacillus* and 16S rRNA gene sequence analysis revealed that the isolate was a strain of *B. siamensis*. The sequence was deposited in GenBank under the accession number ON197155. Further, phylogenetic analysis of the obtained 16S rRNA sequence with the other members of *Bacillus* spp. indicated that AZS12 was a novel strain of *B. siamensis*. Finally, the culture was deposited in National Centre for Cell Science (NCCS).**

Keywords: *Bacillus siamensis, antimicrobial peptides, soil bacteria, market, antagonism, human pathogens*

I. INTRODUCTION:

The use of antibiotics in the treatment of infectious diseases was one of the revolutionary applications in the history of medicine during the 20th century [1]. This approach enabled the management of many difficult-to-cure diseases and thereby reduced the mortality rate caused by several infections [2]. Besides its application in the therapy of infections, it paved the way for several medicinal advancements, including modern medical processes, organ transplants, surgeries, and even cancer treatment. Today, the application of antibiotics is not only limited to human use, but contrarily is used in fields including veterinary, agriculture, poultry, cattle, and swine breeding [3]. However, extensive misuse of these chemical compounds (isolated from microbes and chemically synthesized) has resulted in the emergence of antimicrobial resistance, thus making disease management a challenge in all fields of application [3, 4]. Control of antimicrobial resistance has become a menace due to the improper management of drug usage from end to end, resulting in the rapid spread of resistance and the emergence of multidrug resistance among microbes [3, 5]. New antimicrobial agents are continuously being discovered and characterized to address the challenges associated with the emergence of antimicrobial resistance. Howbeit, the discovery of promising antibiotics is a difficult task [6], and the pipeline of new antibiotics is diminishing [3].

The scientific community strongly recommends natural products with antimicrobial properties as apt alternatives for future antibiotics. In this line, advancements in the discovery of novel peptides with antimicrobial properties as a potential alternative to conventional antibiotics are under progress. These antimicrobial peptides (AMPs) have demonstrated several advantages over conventional antibiotics because they overcome bacterial resistance and possess broad-spectrum antimicrobial activities not limited to bacteria, fungi, viruses, and even cancer [7, 8, 9]. They have the potential to affect biofilm-producing microbes [10, 11]. Also, they are required in lower concentrations, unlike the conventional antibiotics, exhibit higher microbicidal activity, and the mutation-selection window for a given antimicrobial-pathogen combination is narrower. The narrow mutation-selection window of AMPs is more likely to reduce the possibility of development of resistance to them [12, 13].

Antimicrobial peptides are highly conserved small molecules composed of amino acids ranging from 10-50 in number. Compared to conventional antibiotics, AMPs are less toxic, positively modulate the host immune system, and for this reason, they are also called host defense peptides. The major natural sources of AMPs include microbes, insects, nematodes, amphibians, plants, and mammals [9, 14]. At the time of this submission, a total of 3425 AMPs with a vast range of activities have been enlisted in the Antimicrobial Peptide Database, among which the animals are found to be the major source (3425 of AMPs) followed by microbes (423 AMPs). These AMPs include antibacterial, antiviral, antifungal, anti-parasitic, antibiofilm, anti-endotoxin, anti-toxin, anti-drug resistant, anti-cancer, anti-diabetic, anti-inflammatory, antioxidant, spermicidal, insecticidal, and wound healing peptides. Among microbes, a higher number of AMPs (385) are isolated from bacteria (https://aps.unmc.edu/).

AMPs establish antimicrobial properties by inhibiting processes of gene expression, protein synthesis, cell wall synthesis, or delocalization of cell surface proteins in the target host cell [15]. They are reported to possess multitargeted action, making them effective against multidrug-resistant pathogens [16]. To date, thousands of AMPs are reported and classified based on structure, amino acid-rich species, source, charge, and activity [17]. Yet, with the growing cases of antimicrobial resistance and the diminishing discovery of antibiotics, the exploration for sources to isolate and characterize of novel AMPs continues.

Our laboratory majorly focuses on the isolation and characterization of microbes with a prime intention of identifying novel strains producing broad-spectrum antibacterial AMPs. Among the natural resources for the isolation of beneficial microorganisms, the soil is a promising and diverse source of bacteria capable of producing antimicrobial metabolites against co-existing microbes to survive the competition for existence. Besides, environmental factors such as climate, moisture, pH, and temperature influence the physiology and metabolism of soil microbes and thus have an indirect effect on their ability to synthesize antibiotic compounds. Antibiotics used in food production sectors for growth promotion end up reaching the soil affecting the soil microbiome by giving rise to antibiotic-resistant microorganisms. In such an ecosystem, certain microorganisms can evolve to produce antimicrobials against these drug-resistant microbes to survive the competition for existence. Our group was interested in evaluating this theory. With this background, in this study, we attempted the isolation and characterization of antimicrobial peptide-producing bacteria from soil collected from markets dedicated to storing and selling produce, including vegetables, fruits, and flesh, in and around Mysore, India.

II. MATERIALS AND METHODS:

Study location:

The soil samples were collected from the Mysore district, situated in the southern part of Karnataka, India. It is located between latitude 11º45’ to 12º40’N and longitude 75º57’ to 77º15’ E. The district lies on the land of the southern Deccan plateau, and the temperature here varies from 15 ºC during winters to 35 ºC during summers. Types of soils found in Mysore include red, loamy, clay, lateritic, alluvial, colluvial, deep black, and brown soil.

Soil Sampling:

The soil samples were collected from 30 locations in and around Mysore, Karnataka, including vegetables, fruits, and flesh markets. Briefly, each collection site was dug from a 10-15 cm depth and approximately 30 g of the soil was collected into sterile glass bottles using sterile spatulas. The rock sediments, plastic wastes and other debris were removed from the soil samples during collection. The soil samples collected were transported to the laboratory and stored at 4 ºC until future use.

Isolation of Bacterial Strains and Growth Conditions:

For isolation, 5 g of soil sample was aliquoted into a sterile glass bottle and 30 ml of sterile distilled water was aseptically added. The soil suspension was vigorously shaken for 1 min and allowed to settle for 5-10 min. One milliliter of the resultant supernatant was subjected to serial dilution with sterile saline and 100 µl from 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions of each soil sample were used to prepare nutrient agar (HiMedia, India) pour plates. The plates were incubated at 37 ºC for 7 days. Following incubation, colonies that inhibited the growth of surrounding colonies resulting in clear zones were picked up, purified by subculturing by streaking on nutrient agar plates and cryopreserved at -20 ºC in nutrient broth (HiMedia, India) containing 15% glycerol for future use.

Morphological Characterization of Isolated Bacteria Strains:

Morphological characterization of the purified isolates was carried out using Gram’s staining method. Briefly, each purified isolate was streaked on nutrient agar and incubated at 37 ºC for 24 h to obtain isolated colonies. One colony of each isolate was then subcultured separately in nutrient broth at 37 ºC for 24 h and further subjected to Gram’s staining procedure as described elsewhere [18].

Primary Screening: Antimicrobial Activity:

Two to three bacteria strains isolated from each sample with inhibitory activity against the surrounding colonies were selected and screened for antimicrobial activity against seven bacterial pathogens. The selected bacterial pathogens included *Klebsiella pneumoniae* (MTCC 4032), *Pseudomonas aeruginosa* (MTCC 2453), *Proteus mirabilis* (MTCC 1429), *Staphylococcus aureus* (MTCC 96), *Staphylococcus epidermidis* (MTCC 435), *Streptococcus mutans* (MTCC 497), and *Salmonella enterica ser. paratyphi* (MTCC 735). The antibacterial activity of the isolates was examined using the perpendicular streaking method as described elsewhere [3]. Bacterial inhibitory property against at least one indicator strain was demonstrated by all 4 isolates. Among the 4, one strain designated AZS12 demonstrated antibacterial activity against all seven bacterial pathogenic strains by inhibiting their growth. The experiment was repeated at least twice for the reproducibility of the results.

Secondary Screening: Antimicrobial Peptide (AMP) Mediated Activity:

The four bacterial isolates that demonstrated antimicrobial activity against the bacterial pathogens of clinical importance during primary screening were further screened for absolute confirmation of the production of antimicrobial peptides. In order to achieve this, each isolate was cultivated in nutrient broth by incubating at 37 ºC for 48 h and cell-free culture supernatant filtrate was obtained. This filtrate was examined for its stability upon protease digestion by mixing it with papain (1mg/ml dissolved in 100 mM phosphate buffer, pH 6.0) in a 1:1 ratio and then incubating at 37 ºC for 2 h. The buffer alone was used as a negative control. After incubation, 50 µl of each filtrate was subjected to agar disk diffusion assay [19] against all the seven bacterial pathogens by incubating the plates at 37 ºC for 48 h and observed for the zone of inhibition. In this step, the processed culture supernatant of only one strain i.e., AZS12 demonstrated inhibitory activity against the growth of the bacterial pathogens, indicating the production of antimicrobial peptides. The experiment was repeated at least twice for the reproducibility of the results.

Biochemical Characterization of AMP Producing Strain, AZS12:

The biochemical characteristics of the strain AZS12 were identified by performing biochemical tests, including IMViC, citrate utilization, gelatin liquefaction, hydrogen sulfide, nitrate reduction, urease, catalase, oxidase triple sugar iron, hydrolysis of starch, lipid and casein, cellulose degradation, and fermentation of glucose, lactose, sucrose, and mannitol were examined for further characterization. The experiment was repeated at least twice for the reproducibility of the results.

Extraction of Genomic DNA from AMP Producing Strain, AZS12

Whole genomic DNA extraction from the bacterial culture of the AZS12 strain was performed according to the published protocol [20], with some modifications. Briefly, bacterial cells were grown in nutrient broth at 37 ºC shaking incubator. One milliliter overnight culture was centrifuged at 6,000 rpm for 5 min in sterile 1.5 ml eppendorf tubes. The pellet was resuspended in 400 µl of 1x TE buffer. To the mixture, lysozyme was added to a final concentration of 10 µg/ml followed by incubation at 37 ºC for 30 min. To the suspension, 50 µl of 10% SDS and 10 µl of proteinase K (10 mg/ml) solutions were added, mixed gently, and incubated at 55 ºC for 1 h with intermittent mixing. A mixture of phenol: chloroform: isoamyl alcohol in the ratio 25:24:1 was added, mixed and centrifuged at 12,000 rpm for 5 min. The upper aqueous phase was removed and transferred into a fresh eppendorf tube without disturbing the protein interface. To this, 50 µl of 5 M NaCl and an equal volume of absolute ethanol were added, gently mixed and incubated at -20 ºC overnight. The DNA precipitate was pelleted by centrifugation at 12,000 rpm at 4 ºC for 10 min. The supernatant was discarded carefully without losing the pellet. Later the DNA pellet was washed in 70% ice-cold ethanol followed by centrifugation at 12,000 rpm for 5 min. The supernatant was discarded and residual alcohol was removed by air drying. Thus obtained DNA pellet was resuspended in 100 µl of Tris buffer/nuclease-free water and incubated at 55 ºC for 20 min. The concentration of extracted DNA was measured using a NanoDrop spectrophotometer (Thermo Scientific) and stored at -20 ºC for further use.

Molecular Identification of AMP Producing Strain, AZS12:

The molecular identification of the bacterial strain AZS12 was performed by sequence analysis of its 16S rRNA gene. To achieve this, PCR amplification of the 16S rRNA gene from the extracted genomic DNA of the strain was performed using the universal primers 16F27 (5'-CCA GAG TTT GAT CMT GGC TCA G-3') and 16R1492 (5'-TAC GGY TAC CTT GTT ACG ACT T-3') [21]. PCR amplification conditions included: initial denaturation at 94 ºC for 5 min, followed by 30 cycles of 94 ºC for 1 min, 55 ºC for 30 s, and 72 ºC for 1 min 30 s, with a final 10 min extension at 72 ºC. Approximately 5 µl of each PCR product was visualized by agarose gel (2% w/v) electrophoresis. Further, the amplified 16S rRNA gene PCR product was purified by PEG-NaCl precipitation and directly sequenced on an ABI® 3730XL automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA) as per the manufacturer's instructions. Essentially, sequencing was carried out from both ends using additional internal primers to read each position at least twice. Assembly was carried out using the Lasergene package followed by identification using the EzBioCloud database [22]. The 16S rRNA gene sequence thus obtained was submitted to GenBank under the accession number ON197155.

Phylogenetic Analysis:

The 16S rRNA gene sequences of closely related strains were retrieved from the NCBI database (www.ncbi.nlm.nih.gov) and aligned using the CLUSTAL_W program of MEGA version 5.0 [23]. The alignment was corrected manually using BioEdit software v7.2.5 [24]. Upon calculating the pair-wise evolutionary distances [25], a neighbor-joining phylogenetic tree was constructed using the FigTree v.1.4.2 software [http://tree.bio.ed.ac.uk/software/figtree/].

III. RESULTS

Isolation of microorganisms exhibiting antimicrobial activity:

Nineteen out of the 30 soil samples screened exhibited growth inhibitory activity on the surrounding colonies at the dilutions of either 10^{-2} or 10^{-3}. A total of 49 isolates from these 19 samples (2-3 isolates from each sample) were selected and sub-cultured to obtain pure isolates for further screening.

Morphological characterization:

Morphological characterization of the 49 selected isolates was done using Gram's staining method, and the isolates included both Gram-positive and Gram-negative bacteria. Morphologically, these Gram-positive and Gram-negative isolates included bacilli, cocci, long-chain bacilli and cocci-like structures under microscopic observation.

Primary screening of the isolates:

All the selected 49 isolates with growth inhibitory properties were subjected to primary screening of antibacterial activity against seven bacterial pathogens of clinical importance, namely *Klebsiella pneumoniae, Pseudomonas aeruginosa, Proteus mirabilis, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus mutans* and *Salmonella enterica ser. paratyphi* using the perpendicular streaking method. In this test, 4 of the 49 isolates, AGS7, AKS9, AZS12, and BPS26, showed inhibitory activity against at least one pathogen, and AZS12 demonstrated antibacterial activity against all seven pathogenic strains by inhibiting their growth.

Secondary screening of the isolates: Confirmation of production of antimicrobial peptides (AMPs):

The four bacterial isolates from the primary screening step, i.e., AGS7, AKS9, AZS12, and BPS26, were subjected to secondary screening of evaluating the production of antimicrobial peptides by processing their culture supernatant as described in the materials and methods section. The results demonstrated that the culture supernatant of isolates AGS7, AKS9, and BPS26 had no inhibitory activity against any of the tested seven bacterial pathogens. Only the culture supernatant of the AZS12 strain exhibited significant inhibitory activity against the growth of all bacterial pathogens tested, indicating the potential of the strain to produce antimicrobial peptides. At 50 µl of the culture supernatant filtrate, the AZS12 strain showed zone of inhibition measuring 22 mm, 17 mm, 18 mm, 14 mm, 18 mm, 22 mm, and 16 mm against *K. pneumoniae, P. aeruginosa, P. mirabilis, S. aureus, S. epidermidis, S. mutans* and *S. eneterica ser. paratyphi* respectively (Table 3).

Characterization and identification of AMPs-producing bacterial strain:

Morphological observation of the antimicrobial peptide-producing AZS12 isolate was Gram-positive, rod-shaped, and the colonies revealed round, small, pin-pointed, creamy, and raised features. Further, the isolate was subjected to biochemical characterization, including IMViC, citrate utilization, gelatin liquefaction, hydrogen sulfide, nitrate reduction, urease, catalase, oxidase triple sugar iron, hydrolysis of starch, lipid and casein, cellulose degradation, fermentation of glucose, lactose, sucrose, and mannitol. The results are tabulated in table 4. Both morphological and biochemical properties of the isolate indicated that the isolate

belonged to the *Bacillus* genus. Molecular identification using the 16S rRNA gene sequence of the isolate (Table 5) was in agreement with the phenotypic identification, as the BLAST analysis of nucleotide sequence revealed a significant identity (99.93%) of AZS12 strain with *Bacillus siamensis* species. The sequence was deposited in GenBank under the accession number ON197155. Further, the neighboring joining phylogenetic tree constructed with 16S rRNA gene sequences of other *Bacillus* species also confirmed that the strain AZS12 belongs to the *Bacillus* genus. The strain formed a distinct cluster along with *Bacillus siamensis* (Fig. 1) with a high bootstrap value. Based on these results, strain AZS12 was assigned as *Bacillus siamensis* and was deposited in the NCCS under accession number MCC 5166.

IV. DISCUSSION:

AMPs are one of the promising alternatives to antibiotics as they have existed for millions of years in different life forms in nature with no or limited resistance development against their target host [13]. They are effective against a broad array of infectious agents, including bacteria, fungi, parasites, viruses, and even tumor cells [26]. Since our group was interested in exploring novel bacteria with the potential to produce antibacterial peptide molecules, we attempted to isolate bacteria from natural sources, wherein evident competition for survival exists to protect themselves from other microbes.

Soil has been the major source of novel microbes with unique properties that can be exploited in the healthcare sector not only to control and treat several diseases caused by bacteria, parasites, and helminths but to lower cholesterol and suppress the immune system [27]. Therefore, soil and its location harbor a unique niche of microbes and can be explored for novel antimicrobial compounds. Also, in an attempt to respond the increasing population, urbanization and thereby raising food demand, food producers are turning towards faster and cheaper methods of food production. Antibiotic usage is one of the options employed by food producers to promote growth and prevent infections and loss of the final produce of food. They are administered to animals in sub-therapeutic doses and added to their feeds, leading to bioaccumulation [28]. Upon reaching the market for consumption by end users, these bioaccumulated antibiotics end up in the surrounding environment, including soil and water. This indirect introduction of antibiotics to the niche of microbes may contribute to the development of antimicrobial-resistant bacteria and bacteria capable of killing these bacteria by producing antimicrobial compounds such as bacteriocins and antimicrobial peptides. With this background, we selected vegetables, fruits, and flesh markets in and around Mysore district, India, as a potential source of antimicrobial peptides producing novel bacterial isolates.

The isolation of diverse bacterial isolates from the collected samples indicated the heterogeneity of the soil in different ecological niches of the market regions. This diversity of isolates was supported by their morphological characterization using the conventional method of Gram's staining. This method indicated the bacteria isolates included both Gram-positive and Gram-negative strains, and the former kind dominated the latter in numbers. These results correlated with previous studies described elsewhere [3, 29]. Both Gram-positive and Gram-negative isolates included bacilli, cocci, long-chain bacilli and cocci-like bacteria.

The presumptive isolates were screened for antimicrobial activity through primary screening using the perpendicular streaking method, followed by secondary screening for antimicrobial peptide-mediated activity by agar well diffusion method. The approach of subjecting the isolates to primary and secondary screening for evaluation of antibacterial activity was in agreement with previous publications [3, 29, 30]. In current study, primary screening was employed to select bacterial isolates with antibacterial activity against clinically important bacteria pathogens namely *K. pneumoniae, P. aeruginosa, P. mirabilis, S. aureus, S. epidermidis, S. mutans* and *S. eneterica ser. paratyphi*, irrespective of their antibacterial property(s). At this screening step, among the 49 isolates tested, 4 isolates (AGS7, AKS9, AZS12, and BPS26) demonstrated antibacterial activity against at least one of the bacterial pathogens. Among these 4 isolates, AGS7 and AKS9 were isolated from vegetable market samples, AZS12 was from fruits market samples and BPS26 was from flesh market samples.

Further, in the secondary screening step, bacterial culture filtrate of all former mentioned 4 isolates were processed and used in an agar well diffusion assay to select bacteria specifically with antibacterial activity mediated by antimicrobial peptides. The culture filtrate was processed by examining its stability upon protease digestion. Antimicrobial activity of the culture filtrate of only one isolate i.e., AZS12, upon protease digestion, confirmed that the antimicrobial compound produced was a peptide(s). This result indicated that the other three isolates (AGS7, AKS9, and BPS26) had different mechanism(s) of antimicrobial activity other than AMP-mediated activity.

The biochemical characterization and 16S rRNA gene sequencing of the AMP-producing isolate AZS12 was carried out for its specific identification. Results obtained by conducting a battery of biochemical tests confirmed that the AZS12 strain belonged to the genus of *Bacillus* and the 16S rRNA gene sequence analysis revealed that it was a strain of *Bacillus siamensis*. Further phylogenetic analysis in comparison with the members of the *Bacillus* genus also confirmed that AZS12 was a novel strain of *B. siamensis*. As supported by many studies, *Bacillus* species are the most predominant bacteria present in the soil [3, 31, 32, 33].

Strains of *Bacillus siamensis* isolated from diverse natural sources have been reported to possess antibacterial activity against both Gram-positive and Gram-negative bacterial pathogens including aquatic, foodborne, human, and plant pathogens [34, 35, 36, 37, 38]. Our experimental results are confirmatory of these reported findings. Also, members of the *Bacillus* genus are known to produce peptides with broad-spectrum antimicrobial activity against pathogenic microbes, including bacteria and fungi, though the precise mechanism of action of these peptides is unclear [39]. Some strains of *Bacillus siamensis* are known to produce cyclic lipopeptide molecules with antibacterial and antibiofilm activity against both Gram-positive and Gram-negative bacteria [37, 40]. In our study, processed cell-free culture supernatant of the novel strain of *B. siamensis* that we have isolated and characterized did demonstrate a zone of inhibition against both Gram-positive and Gram-negative bacteria. The said culture supernatant was stable against protease digestion in turn supporting that this novel isolate produced stable peptide molecule(s) with broad-spectrum antibacterial activity as secondary metabolites. Further detailed characterization of the culture supernatant by employing standard and accurate techniques would enable us to identify this antimicrobial peptide(s) and its mechanism of action against pathogenic bacteria. Besides, these peptide molecules can further be examined for possible antibiofilm, antifungal, anti-viral, and anti-cancer activities.

V. CONCLUSION:

In conclusion, the results of this study strongly support that the novel strain of *B. siamensis* that we have isolated from a unique niche of microbes has the potential to be explored as a source of novel and alternative drug(s) for use in the treatment of bacterial infectious diseases.

Figures and Tables

Table 1 Dilutions of soil samples showing colonies with antagonistic activity

Soil sample	Dilution	Colonies showing antagonism
S3	10^{-2}	AAS3, ABS3
S4	10^{-3}	ACS4, ADS4. AES5
S7	10^{-3}	AFS7, AGS7
S8	10^{-3}	AHS8, AIS8
S9	10^{-3}	AJS9, AKS9
S10	10^{-2}	ALS10, AMS10, ANS10
S12	10^{-3}	AOS12, APS12, AZS12
S13	10^{-2}	AQS13, ARS13, ASS13
S15	10^{-2}	ATS15, AUS15
S17	10^{-3}	AVS17, AWS17, AXS17
S19	10^{-2}	AYS19, BAS19, BBS19
S20	10^{-3}	BCS20, BDS20, BES20
S21	10^{-2}	BFS21, BGS21
S22	10^{-2}	BHS22, BIS22
S24	10^{-2}	BJS24, BKS24, BLS24
S25	10^{-3}	BMS25, BNS25, BOS25
S26	10^{-2}	BPS26, BQS26
S28	10^{-3}	BRS28, BSS28, BTS28
S30	10^{-3}	BUS30, BVS30, BWS30

Table 2 Antimicrobial activity of the bacterial isolates of soil samples from market regions against clinically important bacterial pathogens

Isolates (Sample ID)	*K. pneumoniae*	*P. aeruginosa*	*P. mirabilis*	*S. aureus*	*S. epidermidis*	*Streptococcus mutans*	*S. enterica ser. paratyphi*
AGS7 (S7)	+	-	-	+	-	-	-
AKS9 (S9)	+	+	+	-	-	-	-
AZS12 (S12)	+	+	+	+	+	+	+
BPS26 (S26)	-	-	-	+	+	-	-

Table 3 Antibacterial activity of AZS12 culture supernatant against test bacterial pathogens

Pathogens	Zone of inhibition (mm) at 50 µl concentration of AZS12 culture filtrate
Klebsiella pneumoniae MTCC 4032	22
Pseudomonas aeruginosa MTCC 2453	17
Proteus mirabilis MTCC 1429	18
Salmonella enterica ser. paratyphi MTCC 735	16
Staphylococcus aureus MTCC 96	14
Staphylococcus epidermidis MTCC 435	18
Streptococcus mutans MTCC 497	22

Table 4 Biochemical characteristics of the soil isolated bacterial strain, AZS12

Biochemical Tests	Results
Indole test	Negative
Methyl red test	Negative
Voges Proskauer	Negative
Citrate utilization test	Positive
Gelatin liquefaction test	Positive
Hydrogen sulfide test	Negative
Nitrate reduction test	Negative
Urease test	Negative

Triple sugar iron test (TSI)	Positive
Glucose Fermentation	Positive
Lactose Fermentation	Positive
Sucrose Fermentation	Positive
Mannitol Fermentation	Positive
Starch Hydrolysis	Positive
Lipid Hydrolysis	Positive
Casein Hydrolysis	Positive
Cellulose Degradation Test	Positive
Catalase Test	Negative
Oxidase Test	Negative

Table 5 16S rRNA gene sequence of AZS12 strain of bacterial isolate

Isolate	Nucleotide sequence of 16S rRNA gene (1380 bases)	Identification
AZS12	GGCTCAGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCG AGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGT GAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGG AAACCGGGGCTAATACCGGATGGTTGTCTGAACCGCATGGTTCAGA CATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGC ATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTA GCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCC CAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACG AAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGAT CGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGG CGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTG CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTA TTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAA GCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGA GTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGT AGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGT AACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGA TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGG GGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCT GGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGG CCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAA GAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGAC GTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAG CTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT TGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCG GTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCC CTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGG CAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGT TCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAG TAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGC	***Bacillus siamensis***

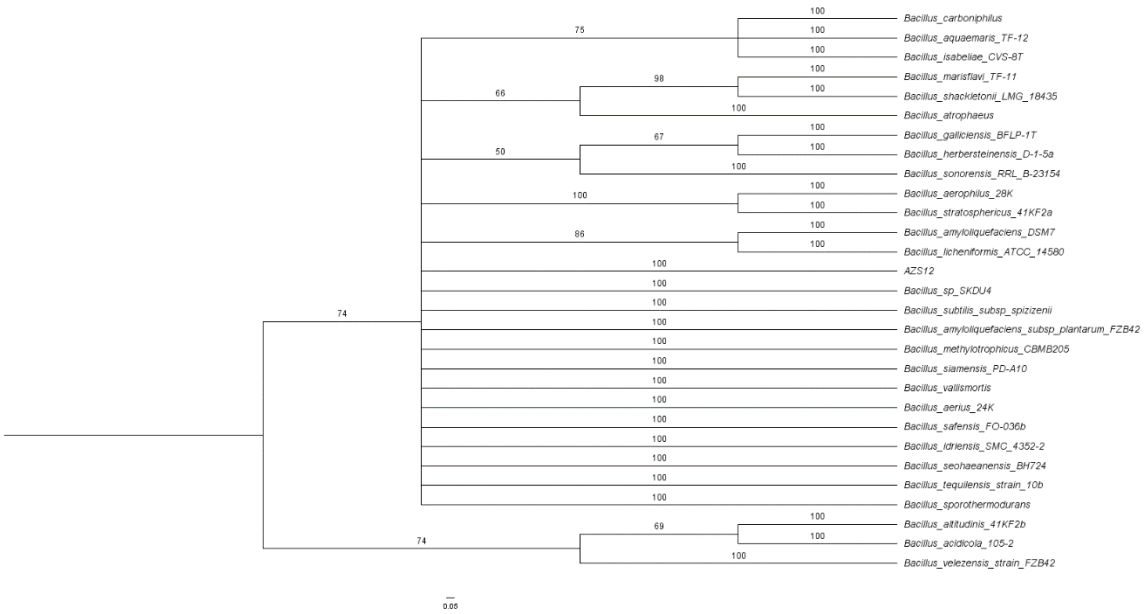

Figure 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences, showing the phylogenetic relationship between

strain AZS12 and other members of the genus *Bacillus*. The branching pattern was generated by the neighbour-joining method.

Bootstrap values (expressed as percentages of 1000 replications) 50% are shown at the branch points. Bar, 0.05 substitutions per nucleotide position

Author contribution NLD designed the research. SS performed the experiments, analyzed the data, and prepared the figure. NLD and SS wrote the manuscript.

Declarations

Competing interests The authors declare no competing interests.

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