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Isolation and molecular identification of *Bacillus safensis* (MK-12.1) exhibiting broad-spectrum antibacterial activity against multi-drug resistant isolates

Abstract

Introduction: The emergence of drug resistance in pathogenic bacteria is a global challenge over the past century. Therefore, the search for new antibacterial agents is essential to tickle the challenge of increasing spread of multidrug-resistant (MDR) bacteria. The *Bacillus spp*. has the potential to produce a wide arsenal of biologically active metabolites.

Objective: The current study was aimed to isolate an antibacterial exhibiting *Bacillus safensis* from a diverse region and characterize their antibacterial metabolites.

Material and methods: *Bacillus safensis* was isolated and screened for antibacterial activities against a set of indicator isolates by standard agar well diffusion assay. The strain MK-12.1 was identified based on 16S rRNA gene sequence supported by biochemical characterization. The molecular nature, size, and toxicity of the antibacterial metabolites produced by strain MK-12.1 were evaluated. Various culturing parameters such as temperature, pH, incubation time, and growth medium were optimized for growth and antibacterial metabolites synthesis.

Results: Among a total of 69 isolated *Bacillus spp*. the strain MK-12.1exhibited promising antibacterial activities against all indicator strains including MDR pathogenic bacteria. The antibacterial metabolites were extracted using ethyl acetate and showed maximum antibacterial activity against *Salmonella typhimurium* while comparatively lower activity was recorded against *Klebsiella pneumoniae*. The minimum inhibitory concentration of extracted antibacterial metabolites was determined and range from 2.34-4.68 mg/ml against ATCC strains and 3.12-6.25 mg/ml against clinical MDR. The antibacterial metabolites were confirmed to be protein in nature, showed no hemolysis on human blood agar, and molecular weight was estimated to be >20kDa. The optimum antibacterial activity and biomass were achieved at pH 8, 30°C after 48 hours in the shaking incubator when cultured in a modified Opt medium.

Conclusion: The present study revealed that the unexplored diverse regions are rich sources of antibacterial metabolites producing *Bacillus spp*. and may produce a novel antibacterial agent that could be used to combat MDR pathogenic bacteria in future.

Keywords: *Bacillus spp*. antibacterial metabolites, partial extraction, molecular identification, optimization

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Introduction

The exploration of new antibacterial agents from natural habitats is becoming more critical due to the fast-growing resistance in human pathogens. In a natural habitat, the soil contains *Streptomyces* and *Bacillus spp*. which are a prolific source of natural bio-active compounds.[1,2] The natural bioactive compounds produced by bacteria are playing an important role to treat infection and seem to be still a good source in the future.[3] The standard research strategies conducted in the past decade, from genome mining via high-throughput screening, did not get any new therapeutic agent. Even new improved natural compounds library for bioavailability did not reveal accurate physicochemical properties of a therapeutic agent. Moreover, the natural bioactive compounds, augmented for ant-bacterial activity, not evaluated for human or animal applications may be associated with low activity and high cytotoxicity. The main drawback of natural bioactive compounds research is, they often demonstrated, already reported bio-active compounds or bioactive compounds producing bacteria. Additionally, drug development costs approximately 1.103 USD and Subsequently, only a small margin is expected for novel antibiotics that could be used against MDR pathogenic bacteria. The new antibiotic will reserve an option and will outcome in rather low sale statistics. Therefore, most of the pharmaceutical industries and research groups avoid working in this field. Currently, a total of about 500 investigators belongs to 50 research groups are active across the globe in the field of antibiotic discovery.[4] The natural environment is a reservoir of numerous potential antimicrobial metabolites producing microbes that can be employed for the synthesis of novel antibiotics. Dumps soil remains the most significant target for bio-prospect screening research as its inhabitants harbor widespread distribution of antimicrobial compounds. These compounds regulate the microflora of the specific biological niche and use particular compounds to communicate and survive in a consortium.[5,6] Several important antibiotics such as vancomycin and kanamycin produced

by *Streptomyces orientalis* and *Saccharopolyspora erythraea* respectively, were isolated from soil samples.[7,8] The bacterial class bacilli described as a bio-resource with a high potential for bioactive compounds synthesis after actinomycetes.[2,9] In the present study, unexplored region of southern Pakistan were investigated. Several antibacterial metabolites producing bacterial isolates were isolated and one isolate MK-12.1 exhibited broad-spectrum antibacterial activity against all indicator strains was selected for further study.

Materials and methods

Soil samples collection and pre-treatment

The soil samples (n=9) were obtained from diverse localities of the Kohat region, Pakistan. The strain MK-12.1 was isolated from a sample collected from waste dump soils (33.5638o N, 71.4656o E). Others localities included mill effluent soil, riverbank soil, rose garden rhizosphere soil, university lawn soil, dry mountain soil, cultivated land soil, stream bank soil, and forest soil. The soil samples were pre-treated for the rational isolation of *Bacillus spp*. using the physicochemical method as previously described.[10] The samples were serially diluted and 200 µl from each dilution was plated on tryptic soy agar (TSA) plates. Total plate count was determined for each sample and culture was purified by repeated sub-culturing.

Screening for antibacterial metabolites synthesis

Preliminarily, all isolated strains were evaluated for antagonistic activity against test bacteria using the cross streaking method.[11] Later on, the cell-free supernatant (CFS) (70µl) from antibacterial metabolites producing isolate culture was evaluated for antibacterial activity against 9 ATCC and 4 Clinical MDR strains (Table 1). The zone of inhibition was measured around the wells in mm and antibiotic streptomycin was used as a positive control.

Table 1 Test ATCC and local clinical MDR strains used in the current study

Test strains	Description
Staphylococcus aureus	ATCC (29213)
Staphylococcus epidermidis	ATCC (12228)
Streptococcus pneumonia	ATCC (6305)
Salmonella typhimurium	ATCC (14028)
Shigella flexneri	ATCC (12022)
Klebsiella pneumoniae	ATCC (13889)
Vibrio cholerae	ATCC (9459)
Pseudomonas aeruginosa	ATCC (27853)
Escherichia coli	ATCC (25922)
Acinetobacter baumannii	Local clinical MDR
Staphylococcus aureus	Local clinical MDR
Escherichia coli	Local clinical MDR
Pseudomonas aeruginosa	Local clinical MDR

Morphological characterization and 16S rRNA gene sequencing

The antibacterial metabolites producing bacterial isolate was initially characterized based on colony morphology, cell morphology, and biochemical testing. Subsequently, the isolate was molecularly identified by 16S rRNA gene sequencing analysis. The gDNA from soil isolate was extracted using the phenol:chloroform method as described previously.[12] The 16S rRNA gene was amplified using 9F and 1510R as a forward and reverse primer, respectively. The PCR was run in a 25µL reaction mixture and the amplified product was visualization as described earlier.[13] The PCR product was purified and sequenced by Sanger sequencing using the commercial services of Macroge (Macrogen Korea Inc.). The 16S rRNA gene sequence was aligned with related sequences freely available at NCBI using ClustalW and the phylogenetic tree was generated using MEGA-X.[14] To infer the evolutionary history of the soil isolate MK-12.1, bootstrap values were calculated on 1000 replicates.[15]

Optimization of growth and antibacterial metabolites production

Culturing parameters such as incubation time, temperature, pH, and the medium was optimized for growth as well as antibacterial metabolites production. To evaluate the effect of temperature and pH, the erlenmeyer flask containing 100 ml medium was inoculated with 1ml standardize inoculum (equal to 1 McFarland) of soil bacterial MK-12.1. The pH of Opt medium and incubation temperature was set to 6, 7, 8, 9, and 25, 30, 37, and 42°C respectively. For optimization of culturing medium a standardized inoculum of 1ml was poured in various media such as Luria Bertani (LB) broth, Tryptic soy broth (TSB), Nutrient broth (NB), Brain heart infusion (BHI) broth, Opt broth[16] and Opt broth with little modification and maintained at 30°C for 48 hours (Table 2). The effect of incubation time and aeration was examined by culturing the antibacterial producing isolate in shaking (120rpm)/static incubator and activity and growth was determined at different time intervals i-e 24, 48, and 72 hours. The antibacterial activity of CFS from MK-12.1 was measured in mm and growth as O.D at 600 nm.

Table 2 Composition of Optimized (Opt) medium (component/ L)

Component	Quantity
Peptone	35 gm
Sucrose	20 gm
Yeast extract	8 gm
Potassium di-hydrogen phosphate	2 gm
Magnesium sulfate	0.045 gm
Trace elements	9 mL

Partial purification of antibacterial metabolites

The antibacterial metabolites producing bacteria *B. safensis* MK-12.1 was cultured in optimized condition. Subsequently, the culture was centrifuged at 6000rpm for 20 minutes. To remove cell debris and other impurities the CFS was passed through a 0.45 pore size membrane. The antibacterial metabolites were extracted from CFS using ethyl acetate (1:1 ethyl acetate and CFS) and the solvent was evaporated using a rotary evaporator. The yellow color dry powder was weighed and dissolved in phosphate buffer solution for further analysis.

Determination of minimum inhibitory concentration (MIC)

The MIC of extracted metabolites was determined against 11 tested bacterial strains using the serial dilution method.[17] The minimum concentration of metabolites that completely restricted the growth of tested bacteria was called MIC. Streptomycin and phosphate buffer solution was used as positive and negative control respectively.

Characterization of antibacterial metabolites

The molecular weight of antibacterial metabolites was determined using the dialysis membrane with a pore size of <20 kDa.[18] To determine the nature of active metabolites the extracted antibacterial

metabolites were mixed with proteinase K and kept for 30 minutes at room temperature. Temperature sensitivity was assessed by heating the antibacterial metabolites from 20 to 121°C for 15 minutes and later on antibacterial activity was examined. Hemolytic activity of antibacterial metabolites was studied on 5% human blood agar plates.

Statistical analysis

The statistical analyses were conducted by calculating standard deviation and variance using MS word excel and ANOVA, respectively.

Results

Plate count and isolation of antibacterial metabolites producing bacteria

Plate count for each soil sample was calculated on TSA plates, which ranged from 4×10^4 to 8×10^5 colony-forming unit (CFU/g). The lowest CFU/g 4×10^4 was determined for soils sample obtained from mill affluent areas. A total of 69 *Bacillus spp*. were purified and 12 were found to be producing antibacterial metabolites. Out of 12 bacterial isolates, 3 antagonize the growth of Gram-negative strains while 9 showed antagonistic activity against at least one Gram-negative and one gram-positive bacterial strain. One isolate MK-12.1 revealed promising activity against all tested bacterial strains (Figure 1). The extracted antibacterial metabolites showed maximum antibacterial activity against *Salmonella typhimurium* while *Klebsiella pneumoniae* presented resistance towards antibacterial metabolites synthesis by *B. safensis* strain MK-12.1 (Figure 2).

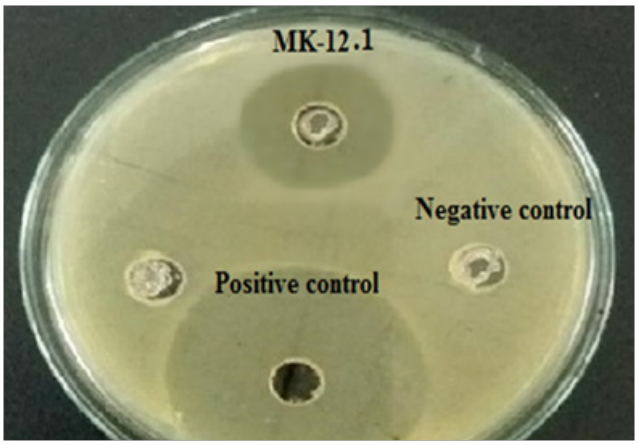

Figure 1 Antibacterial activity of partially purified antibacterial metabolites produced by strain MK-12.1 against *S.aureus*.

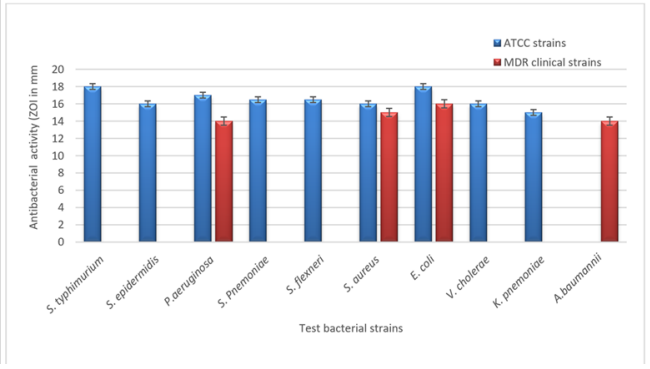

Figure 2 The antibacterial activity of *B. safensis* strain MK-12.1 against indicator ATCC and MDR clinical bacterial strains.

Identification of antibacterial metabolites producing bacteria

The antibacterial metabolites producing bacteria *B. safensis* MK-12.1 was characterized in TSA medium. The cell morphology reveals that MK-12.1 is Gram-positive, spore-forming rod-shaped bacteria. Colony morphology was round, undulant with irregular margin, non-luminescent, and dull-white in color. Biochemical testing showed that MK-12.1 is positive for oxidase, catalase, Voges Proskauer test, and negative for methyl red, indole, DNase, amylase, H_2S, and urea's production. To infer diversity and phylogeny of MK-12.1, the 16S rRNA gene sequence was compared with publically available sequences deposited in the NCBI database (Figure 3). This revealed that the soil isolate belongs to *Bacillus spp*. and identified as *B. safensis* strain MK-12.1. It seems that *Bacillus* is abundantly found in soil and play a key role in the biogeochemical process by producing various type of bioactive metabolites. The abundance of *Bacillus spp*. in soil is probably due to their ability to form a highly resistant endospore that helps them to survive under extreme environments and our pre-treatment of samples favors spore-forming bacteria. The nucleotide sequencing data of MK-12.1 was deposited to the Gene bank database under accession number MN519460. The BLAST results of the 16S rRNA sequence showed a high level of similarity (99.73) with *Bacillus safensis* strain NBRC.

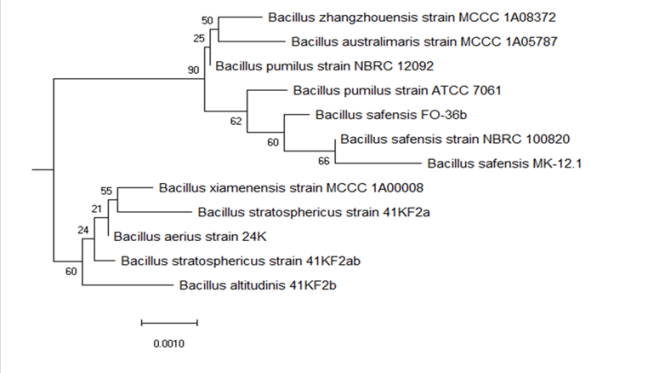

Figure 3 Phylogenetic tree constructed by neighbor-joining method indicating the evolutionary relationship of *B. safensis* strain MK-12.1with other *Bacillus* species.

Optimization for Growth and antibacterial metabolites production

The CFS obtained from *B. safensis* MK-12.1 cultured at 30°C exhibited maximum activity i-e 18 mm followed by 35°C with 17mm zone of inhibition (ZOI) (Figure 4). On the other hand minimum, antibacterial activity was noted when MK-12.1 was grown at 45°C and 25°C indicating that its mesophile in nature and prefers to grow in moderate condition. The present study result revealed that nitrogen-rich media such as modified Opt, TSB, and BHI boost antibacterial metabolites production as compared to other media (Figure 5). Maximum ZOI and biomass was obtained when MK-12.1 was grown in a modified Opt medium (Table 2). The strain MK-12.1 exhibited higher activity at pH 8 as compared to pH 5, 6, 7, and 9. This is probably due to the optimum pH that assists the antibacterial metabolites synthesis. Moreover, maximum growth and antibacterial metabolites production were noted in shaking incubator after 48 hours of incubation that after a decline in ZOI and growth was observed (Figure 6).

Citation: Iqbal S, Qasim M, Begum F, et al. Isolation and molecular identification of *Bacillus safensis* (MK-12.1) exhibiting broad-spectrum antibacterial activity against multi-drug resistant isolates. *J Bacteriol Mycol Open Access.* 2021;9(2):87–92. DOI: 10.15406/jbmoa.2021.09.00302

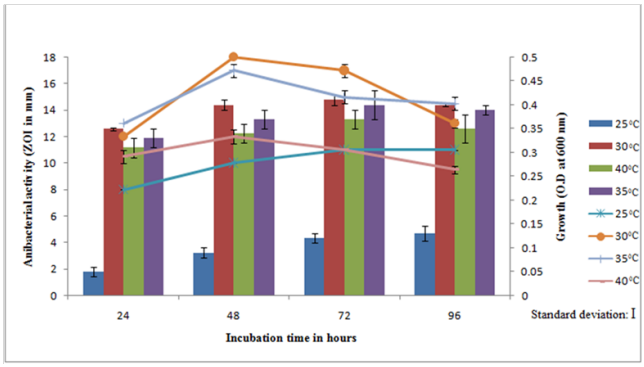

Figure 4 The effect of incubation time and temperature on growth and antibacterial activity of *B. safensis* strain MK-12.1 against indicator strain (*S.aureus*).

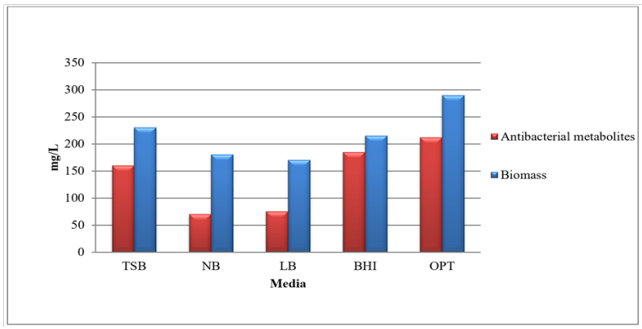

Figure 5 Antibacterial metabolites production by *B. safensis* MK-12.1 grown in various culture media. Trypticase soy broth (TSB), Nutrients broth (NB), Luria Bertani (LB), Brain heart infusion (BHI) and Optimized (Opt) broth medium.

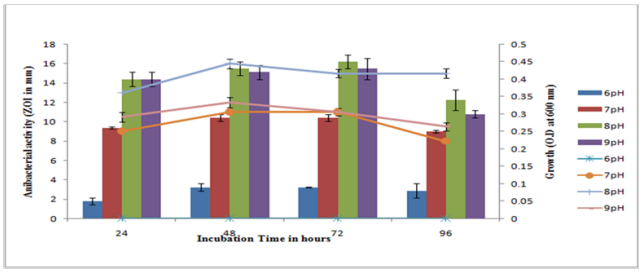

Figure 6 The effect of pH and incubation time on growth and antibacterial activity of *B. safensis* strain MK-12.1 against *S.aureus*.

Partial purification and characterization of antibacterial metabolites

The antibacterial metabolites extracted from *B. safenis* MK-12.1 showed enhance activity against tested bacterial strains as compared to CFS. The antibacterial metabolites lost their antagonistic activity when treated with Proteinase K and high temperature. The susceptibility of metabolites to high temperature and proteinase K results indicates that it is a protein in nature. The molecular weight of bioactive metabolites was estimated to be > 20KDa. Additionally, no hemolytic activity was observed when an antibacterial metabolite was evaluated for hemolysis against human blood.

Determination of MIC

The MIC of extracted antibacterial metabolites was determined and range from 2.34 - 4.68 mg/ml against ATCC strains. While against clinical MDR strains MIC ranged from 3.12 - 6.25 mg/ml (Table 3).

Discussion

The emergence of multi-drug resistance in human pathogen led the scientist to search the novel sources or novel compounds from the existing sources for therapeutic agents to combat the resistant pathogen. The microorganism is a rich source of antimicrobial metabolites and approximately 90% of the current antibiotics in the market are primarily derived from the microorganism.[19] Previously most of the antibiotics are isolated from soil bacteria via antibacterial activity-based screening approaches.[20] Therefore, it can also be expected that soil microorganisms will be the most prominent source of new antibiotics in the future.[21] *Streptomyces* and *Bacilli* are the proven proliferative antimicrobial metabolites producing species.[22] Although many bioactive metabolites have already been isolated from these soil bacteria still, they contain many more unexplored genes for the synthesis of bioactive compounds.[23] Only a few studies have been conducted on bacterial potential for bioactive compounds synthesis on waste dump soil where microorganisms should be capable to deal with relatively diverse and extreme environments. Therefore, the chance for isolation of novel bacteria having highly diverse metabolic capabilities.[24] In the current study, *B. safensis* MK-12.1 was isolated from unexplored waste dump soil Kohat, Pakistan that showed promising antibacterial activity against tested strains. This is in agreement with the fact that *Bacillus* is the predominant soil bacteria to form endospore and antibacterial metabolites which facilitate them to colonize in such a harsh environment.[25]

Table 3 MIC of antibacterial metabolites produced by *B.safensis* MK-12.1 against various test bacteria (mg/mL)

S. no	Test bacteria	Strain no.	MIC	
			MK-12.1 extract (mg/ml)	Streptomycin (+ive control) µg/ml
	Salmonella typhimurium	ATCC 29213	3.12	50
	Staphylococcus epidermidis	ATCC 12228	3.12	25
	Pseudomonas aeruginosa	ATCC 6305	4.68	25
	Streptococcus pneumoniae	ATCC 14028	2.34	25
	Shigella flexneri	ATCC 12022	3.12	25
	Staphylococcus aureus	ATCC 13889	3.12	50
	Escherichia coli	ATCC 9459	2.34	25
	Vibrio cholerae	ATCC 27853	3.12	50
	Klebsiella pneumoniae	ATCC 25922	4.68	50
	Acinetobacter baumannii	Local clinical MDR	6.25	50
	Staphylococcus aureus	Local clinical MDR	3.12	25
	Escherichia coli	Local clinical MDR	3.12	25
	Pseudomonas aeruginosa	Local clinical MDR	3.12	50

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The evolutionary history of MK-12.1 was inferred based on 16S rRNA gene sequence analysis. The soil isolate *B. safensis* MK-12.1 lies in a clade with *B. safensis* NBRC 100820. Previous studies demonstrated that 16S rRNA is an improved approach to identify soil bacteria as compared to conventional phenotypic and biochemical methods.[26] The strain *Bacillus safensis* MK-12.1 showed fascinating bioactivity against all tested ATCC as well as MDR strains. The ATCC strains *Salmonella typhimurium, Shigella flexneri, Staphylococcus aureus, Vibrio cholerae,* and *Staphylococcus epidermidis* was inhibited more efficiently (MICs range from 2.34 to 3.12 mg/ml) as compared to other ATCC strains such as *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (MICs 4.68 mg/ml). These results can be related to another study where antibacterial metabolites from actinomycetes were purified and MIC against various strains was determined.[27] The study to evaluate antibacterial metabolites production usually involved optimum culture medium and condition. Modified Opt medium, pH 8 and 30°C was found to be the optimum medium and condition for growth and antibacterial metabolites production. Previous studies revealed that pH, temperature, carbon, and nitrogen in the medium directly influence the growth and antibacterial metabolites production. These results are in agreement with another report where *lactobacillus* sp. produces the maximum amount of bacitracin at 25-30°C.[28] The partially purified antibacterial metabolites from *B. safensis* MK-12.1 culture lost the antagonistic activity at an elevated temperature and did not show any hemolysis on human blood agar indicate its suitability for further pharmacological study.

Conclusion

In conclusion, the results indicate that the soil of Kohat region is rich in terms of bioactive metabolites producing *Bacillus spp*. from which novel bioactive compounds can be elicited in the future.

Acknowledgements

None.

Conflicts of interest

The authors declare that there is no conflict of interest.

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