



Assessment of Physico-Chemical Properties of Sewage Sludge and Unlocking Its Prospect as a Powerhouse of Antibiotic-Resistant Bacteria

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ABSTRACT

Sewage sludge is a reservoir of pharmaceutically active compounds (Antibiotics), antibiotic-resistant genes and antibiotic-resistant bacteria, a potential risk to mankind and the environment. This study unravels the physicochemical aspects of sewage sludge and its valorization using bio-remediation. Assessment of physico-chemical parameters was conclusive of the fact that sludge samples were enriched with inorganic components (Chloride, Phosphate and Sulfate) and organic constituents. Sludge samples were found to be contaminated with heavy metals Cr (12.69 mg.kg⁻¹), As (1.23 mg.kg⁻¹), Se (0.86 mg.kg⁻¹), Hg (1.49 mg.kg⁻¹), and Pb (30.32 mg.kg⁻¹), with non-adherence to prescribed limits (Haryana State Pollution Control Board). Screening for bacterial isolates capable of utilizing Cephalosporin (Cefixime:200mg) as their sole source of carbon and energy was carried out. 5 isolates exhibiting fastidious growth were characterized as *Bacillus* sp., *Escherichia* sp., *Pseudomonas* sp., *Streptococcus* sp. and *Enterobacter* sp. Noteworthy is the fact, isolates exhibited a broad range of tolerance as reflected by Minimum Inhibitory Concentration (MIC). This was carried out by the *Agar dilution method* using cefixime (10-100 mg.L⁻¹). 0.03, 0.05, 0.07, 0.06 and 0.09% w/v were the respective Minimum Inhibitory Concentration (MIC) range. The isolate with maximum tolerance to cefixime was subjected to 16S rDNA sequencing and characterized as *Enterobacter* SCEf1.

INTRODUCTION

Wastewater Treatment Plants (WWTPs), commonly referred to as Sewage Treatment Plants (STPs), are operationally based on a tier and end-of-pipe treatment approach (Sharma 2019). The main objective is resource recovery, mobilization and utilization of biodegradable pollutants. Unwanted components are removed from wastewater treatment as sewage sludge, which accounts for 1-25% of polluted waste material. Sludge from sewage treatment plants is classified as hazardous waste (HW) because it retains 50-80 % of the initial contaminants (Fijalkowski et al. 2017). Sewage waste poses a higher risk to the ecosystem, given the fact that it harbors priority pollutants, including antibiotic-resistant genes as one of the emerging public health challenges (ARGs) (Jin et al. 2014, Soudejani et al. 2019). Contrary to the above-cited fact, aiming to achieve environmental sustainability, sewage sludge has been explored for agricultural improvement. It is reportedly known to contain higher carbon, nitrogen (N), phosphorus (P), potassium (K) and calcium (Ca) reserves (Sharma et al. 2024).

Release of active pharmaceutical ingredients (APIs) (anti-inflammatory agents, analgesics, blood lipid regulators, antidepressants, antiepileptics and antibiotics) has become an inevitable part of current societal challenges. Recent studies uncover their presence in different ecosystems, mainly terrestrial and aquatic ecosystems,

which requires addressing regulatory concerns (Bhambhani & Sharma 2023).

Antibiotic Resistance (AR) is an unavoidable repercussion of unabated use and improper disposal of antibiotics, which is evolving as a major public health concern in the post-pandemic era. At a global level, antibiotic stewardship programs are being implemented at the community level to sensitise people about the challenging times we may face in the times to come. In the last few decades, persistence of numerous antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARG) has been reported, including sewage wastewater (Figueira et al. 2011), with a possible link to environmental transmission. Additionally, unchecked disposal of APIs from hospitals, pharmaceutical industries and aquacultures significantly contributes to contamination of water resources (Sultan et al. 2023). Accelerated effect of sub-inhibitory concentration of antibiotic on the frequency of horizontal transfer of ARGs has been reported (Bruchmann et al. 2015), subsequently, leading to exposure of microbes in wastewater to increasing antibiotic resistance level (Sharma & Sharma 2021, Varela et al. 2014).

Cephalosporin, a semi-synthetic β -lactam broad-spectrum antibiotic, is used for the treatment of upper respiratory tract infections. Anti-bacterial activity, especially against Gram-negative bacteria. It is due to its ability to inhibit cell wall biosynthesis. Owing to its broad-spectrum utility, it contributes 50-70% of total antibiotic consumption globally (Selvi et al. 2015, Klein et al. 2018). Through the route of natural circulation of resources, antibiotics have been found to be contaminating freshwater bodies, which significantly increases the chemical oxygen demand (COD), indicating toxicity. Earlier, traces of antibiotics have been reported in hospital wastewaters (Baranchesme & Munir 2018), WWTP biosolids, surface waters, and ground waters (Zhang et al. 2018), drinking water, sediments and biota (Williams & Kookana, 2018). Conventional treatment practices for remediating the environment from leftover antibiotic traces mainly include various physico-chemical and chemical methods, advanced oxidation (Zhang et al. 2017), photo-transformation (Wang & Lin 2012), electrolysis (Kong et al. 2019), and adsorption (Awwad et al. 2015), being a few of them. Additionally, these treatment methods do hold some disadvantages, such as being expensive, labor-intensive, and may often introduce toxic intermediates, leading to an environmental burden. Stated reasons are persuasive enough for scientific community to explore more feasible, affordable and eco-centric in terms of carbon neutrality means to support the green and blue infrastructure campaign of the United Nations, aiming at waste management (Bhandari et al. 2023).

Bioremediation, by virtue of its ease of applicability, could be devised to combat the growing menace of antibiotic

pollution. With an expanding knowledge of microbial community dynamics in wastewater systems, a potential reservoir harboring antibiotic-resistant bacteria, our study was aimed at screening cephalosporin-resistant bacteria with the potential of antibiotic-degrading efficacy. To the best of our knowledge, we are proposing a novel concept based on exploring microbial diversity of sewage sludge as a bio-prospective tool for addressing antibiotic pollution at an ecological level. Perhaps, recent studies have reported microbial community dynamics of wastes released from healthcare settings (hospitals) for antibiotic removal through biodegradation. We have attempted to validate the concept behind the environmental route of antibiotic transmission by microbiological interventions.

MATERIALS AND METHODS

Chemicals

All the chemicals used in the study were of analytical grade. The laboratory glassware used was washed, rinsed, and pre-sterilized. Cefixime (200mg) was procured from a nearby pharmacy store.

Site Description and Sludge Sampling

Dried and liquid sludge samples (in triplicate) were collected from the Sewage Treatment Plant (STP), University campus. Using a plastic scoop, composite samples were withdrawn from 5 sampling points (Dried sludge beds) and placed in pre-sterile capped glass beakers, transported, and refrigerated for further analysis (Figs. 1a, b) (APHA 2017).

Experimental Plan

The samples were processed for the following scheme of experiments (Fig. 2).

Physico-Chemical Analysis of Sludge Samples

Assessment of pollution indicators in sludge samples governs its adherence/non-adherence to prescribed standards (Sharma et al. 2013). The parameters under consideration could be categorized as *on-site* and *in vitro* parameters. Briefly, on-site parameters included pH, temperature, color and Dissolved Oxygen (D.O.). Off-site parameters were further analyzed by titrimetric and spectrophotometric methods. Heavy metal analysis was conducted by Atomic Absorption Spectrophotometer (AAS) (APHA 2017).

Screening of Antibiotic-Resistant Bacteria by Bio-Enrichment

Bio-enrichment strategy is the one that provides an *advantageous* environment for a specific type of strain to

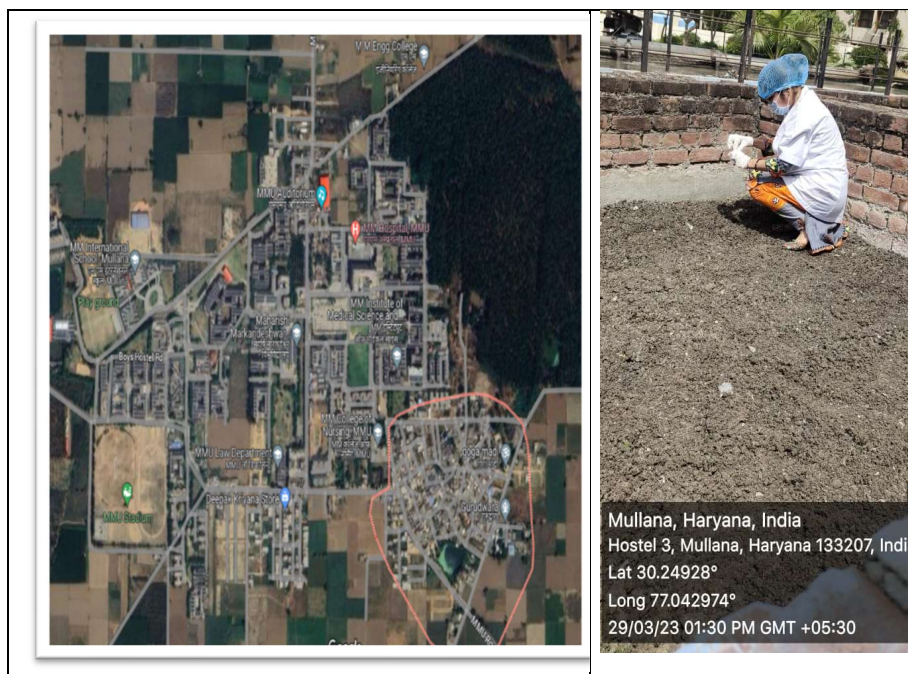


Fig.1(a) and (b): Area under study and sampling site.

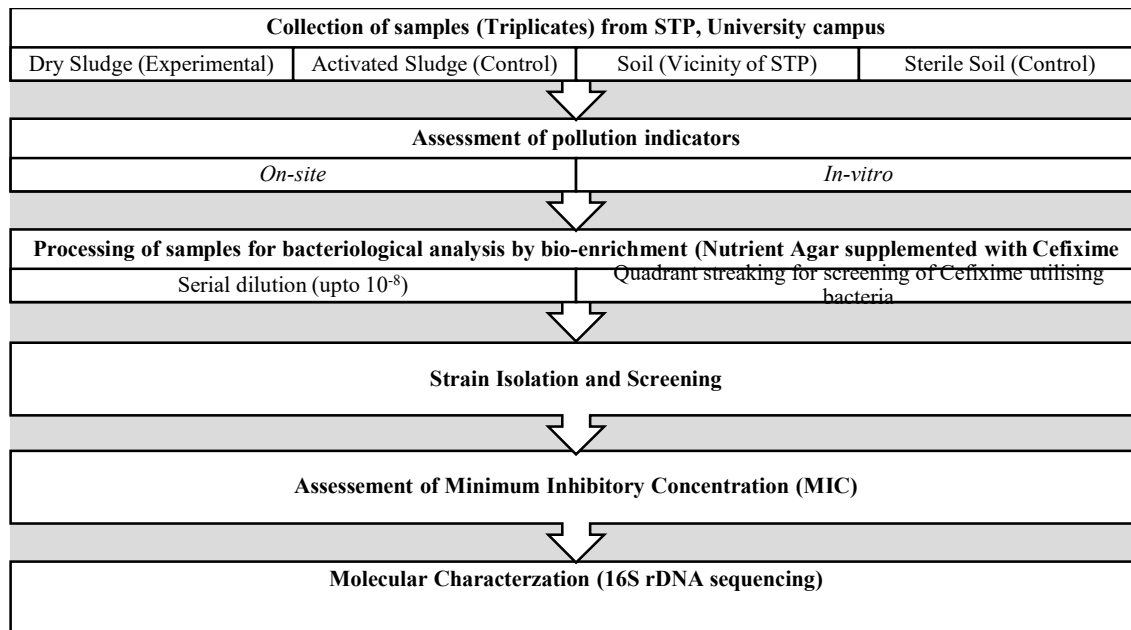


Fig. 2: Layout of experimental setting.

overgrow selectively. This essentially intends to supplement the desired metabolite in culture media, which may exemplify the growth of the desired microbe (Overmann 2013). Considering sludge as a prospective source of antibiotic-resistant bacteria, the samples (dry and liquid) were subjected

to serial dilution (upto- 10^{-10}) to obtain a pure culture. Freshly prepared nutrient agar media amended with 0.1% w/v Cefixime (200 mg), a commonly used antibiotic to treat bacterial infections of the upper respiratory tract, with the following specifications, was used Fig. 3.

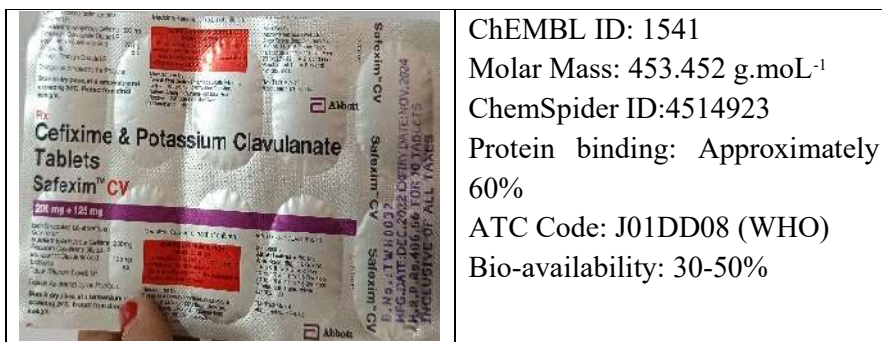


Fig 3: Antibiotic specifications.

From all dilutions, a loopful of vortexed suspension was quadrant streaked and incubated at 37°C for 24-48 hours until fine pinpoint colonies were obtained. Furthermore, those colonies that exhibited confluent growth were processed further on agar slants to maintain a pure culture. Subsequent sub-culturing was conducted, and glycerol stocks were prepared for further use.

Minimum Inhibitory Concentration (MIC)

The bacterial isolate having maximum adaptability was further processed with the agar dilution method for the determination of the concentration of antibiotic at which bacterial growth is inhibited. Cefixime with a tested range of 10-100 mg.L⁻¹ was supplemented to agar plates against 2 controls (Abiotic and biotic), followed by inoculation from the mother culture having a cell density of 1.5×10^8 CFU.mL⁻¹, i.e., O.D. of 0.5 MacFarland standard. All the plates were incubated according to the procedure previously indicated.

Molecular Characterization

A pure culture of an acclimatized bacterial isolate was further processed for 16S rDNA sequencing. The amplicon was processed for the generation of a consensus sequence, which was compared with known sequences present in the NCBI database.

Briefly, genomic DNA was isolated using an in-house Bacterial DNA isolation kit, and the purity and concentration were analyzed using a Denovix DS-11 spectrophotometer. The 16S rDNA region of the isolated DNA was amplified using Emerald Amp GT PCR Master Mix, Catalog number # RR310A. The kit details include Lot ID# AN71012N and expiry of JUL-2025. The amplification of a 1000-1500bp amplicon was obtained using Emerald Amp GT PCR Master Mix. The test amplicon of 1500bp was purified via EXO-SAP purification, and bi-directional cycle sequencing was carried out with forward and reverse primers using the BDT V3.1

Cycle sequencing kit on the ABI 3730 Genetic Analyzer as per standardized sequencing protocol. The sequences obtained by sequencing with forward and reverse primers were assembled using Gene Tool software to generate a consensus sequence, and BLAST analysis was carried out to compare with the sequences available in the NCBI GenBank database. The first ten sequences in the database that showed the highest similarity were selected based on maximum identity score and phylogenetic tree result.

RESULTS AND DISCUSSION

Physico-Chemical Analysis of Sludge Samples

The potential risk of discharging activated sludge to any locality or using it for sustainable practices, including improving agricultural practices or bioremediation processes, is quite high, attributed to pathogenic microflora and other contaminants. Considering above above-stated facts, sewage sludge samples were assessed for different pollution indicators. The findings are expressed as (Mean \pm S.D.) in Table 1. All sludge samples were from brownish to black with moderate to fine porosity.

pH indicates hydrogen ion concentration. All the physico-chemical properties are interdependent variables that evaluate the operational performance of STP. Bacterial diversity, growth, and acid production are the key factors influenced by pH, which in turn influence the heavy metal solubility. It has been reported that acidic medium tends to enhance heavy metal solubility, resulting in vigorous toxicity and vice versa (El-Nahhal et al. 2014) pH of composite samples ranged between 9-10.

EC is an expression of salinity, which was found to be 5.9 ± 0.02 and 0.03 ± 6.7 mS.cm⁻¹ as shown in Table 1. It is likely due to the accumulation of soluble salts in wastewater in various environmental settings. Our justification is supported by (El-Nahhal et al. 2014), who reported an EC of 2.49 ± 0.04 mS.cm⁻¹ in sewage sludge samples.

Furthermore, samples were found to possess excess chloride content (400 ± 0.01 and 300 ± 0.02) due to salty whey and brines (Sharma et al. 2013). Additionally, TDS was found to be 134.3 ± 0.01 and 122.5 ± 0.01 .

The moisture content in respective sludge samples is expressed in terms of Mean \pm S.D. (solid sludge 22.8 ± 0.01 and liquid sludge 20.6 ± 0.02). The result reflects the efficient dewatering of sewage waste by the mechanical unit in the treatment plant (Kocbek et al. 2022).

The application of sewage sludge in soil nourishment is widely known due to the presence of enriching inorganic and organic components (Sharma et al. 2024). According to Sonmez and Bozkurt (2006), sewage sludge, manure, and humic acid significantly affect soil health, metal concentration, and plant growth. Samples were found to have lower nitrate levels, 0.1 ± 0.04 and 0.01 ± 0.06 . Phosphate was found in a moderate range, 0.95 ± 0.05 and 1.09 ± 0.03 mg.L⁻¹, making it suitable to apply in phosphate-deficient soil. Sulfate was found to be 6.6 ± 0.01 and 5.3 ± 0.01 mg.L⁻¹ in solid and liquid sludge, respectively.

Moreover, all samples were found to have higher Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD), attributed to higher organic matter, Total Organic Carbon (TOC) (21 ± 0.01 and 25.9 ± 0.01) (Kale et al. 2010).

In India, sewage wastewater is found to be polluted with various xenobiotic heavy metals, including one of them. According to a study conducted by Pathak et al. (2008), average values of heavy metals in sewage sludge were found to be Cd-41–54, Cr-102–8810, and Pb-91–129 mg.kg⁻¹. Heavy metal analysis of our sludge sample concluded that STP is receiving highly contaminated water from environmental proximities, with a concerning level of all tested heavy metals in solid sewage sludge.

Bio-Enrichment

Nutrient agar plates inoculated with actively growing culture (O.D.₆₆₀ = 0.1) were observed after 24 h of incubation. Diversity analysis of all dilutions is represented in Table 2. Distinct types of bacterial colonies were observed. 5 bacterial

Table 1: Physico-chemical analysis of sewage sludge.

S.No.	Parameters	Sewage Sludge (Solid) (Mean \pm S.D.)	Sewage Sludge (Liquid) Mean \pm S.D. [mg.L ⁻¹]
1.	pH	10	9
2.	BOD [mg.L ⁻¹]	189.2 \pm 0.02	166 \pm 0.01
3.	COD [mg.L ⁻¹]	400 \pm 0.01	500 \pm 0.02
4.	Acidity [mg.L ⁻¹]	150 \pm 0.03	137 \pm 0.02
5.	Alkalinity [mg.L ⁻¹]	75 \pm 0.1	58 \pm 0.01
6.	Chloride [mg.L ⁻¹]	400 \pm 0.01	300 \pm 0.02
7.	Hardness [mg.L ⁻¹]	140.8 \pm 0.03	146.08 \pm 0.01
8.	Nitrate [mg.L ⁻¹]	0.1 \pm 0.04	0.01 \pm 0.06
9.	Phosphate [mg.L ⁻¹]	0.95 \pm 0.05	1.09 \pm 0.03
10.	Solids (Dissolved, Suspended and Total) [mg.L ⁻¹]	67.85 \pm 0.03, 164.8 \pm 0.0, 134.3 \pm 0.01	66.05 \pm 0.03, 144 \pm 0.01, 122.5 \pm 0.01
11.	Phenolic Compounds [mg.L ⁻¹]	50 \pm 0.02	59 \pm 0.01
12.	Electrical Conductivity (EC) [mS.m ⁻¹]	5.9 \pm 0.02	6.7 \pm 0.03
13.	Oil and Grease [mg.L ⁻¹]	3.8 \pm 0.06	3.9 \pm 0.07
14.	Sulfate [mg.L ⁻¹]	6.6 \pm 0.01	5.3 \pm 0.01
15.	Total Organic Carbon (TOC) [mg.L ⁻¹ C]	21 \pm 0.01	25.9 \pm 0.01
16.	Moisture Content, %	22.8 \pm 0.01	20.6 \pm 0.02
Priority Contaminants			
17.	Chromium (Cr)	12.69 [mg.kg ⁻¹]	0.037 [mg.L ⁻¹]
18.	Arsenic (As)	1.23 [mg.kg ⁻¹]	0.018 [mg.L ⁻¹]
19.	Selenium (Se)	0.86 [mg.kg ⁻¹]	0.005 [mg.L ⁻¹]
20.	Cadmium (Cd)	0.95 [mg.kg ⁻¹]	0.005 [mg.L ⁻¹]
21.	Mercury (Hg)	1.49 [mg.kg ⁻¹]	0.004 [mg.L ⁻¹]
22.	Lead (Pb)	30.32 [mg.kg ⁻¹]	[mg.L ⁻¹]

isolates coded as SSS.1, SSS2.1, SSS2.2, LSS2.1, LSS2.1 were found to show grow profoundly due to their adaptability. Biochemical characterization in accordance with Bergey's manual leads us to conclude the isolates were *Bacillus* sp., *Escherichia* sp., *Pseudomonas* sp., *Streptococcus* sp., and *Enterobacter* sp., respectively. Besides, earlier studies highlighting the utility of bacterial species for bioremediation of cephalosporin derivatives have been reported by (Krishnan et al. 2022) (*P. putida* and *P. fluorescens*) and (Wagner et al. 2011) (*Bacteroides* sp. and *Bacillus* sp.).

Minimum Inhibitory Concentration (MIC)

MIC was considered for the lowest concentration of antibiotic where bacterial growth ceased, and a single colony within the streaked area was considered (Owuama 2017). The respective MICs for *Bacillus* sp., *Escherichia* sp., *Pseudomonas* sp., and *Streptococcus* sp. were 0.03, 0.05, 0.07 and 0.06% w/v, respectively; however, in the case of *Enterobacter* sp. It was found that at 0.09% w/v Cefixime concentration, the colony count declines. Hence,

Table 2: Bacteriological and biochemical analysis of sludge samples.

S.No	Code	Microscopic characteristic	Dilution	Growth	Total Plate Count	Gram Staining
1.	SSS1	Monococci	10 ⁰	Confluent	TNTC	Positive
2.		Diplococci, monococci	10 ⁻²	Confluent to moderate		
3.		Pleomorphic	10 ⁻⁴	Moderate	650	
4.		Bacilli	10 ⁻⁶	Isolated colonies	30	Negative
5.		Cocco-bacilli	10 ⁻⁸	Scattered isolated colonies	21	Positive
6.		Staphylococci	10 ⁻¹⁰	Few colonies	8	
7.	SSS2	Cocci	10 ⁰	Lawn/ mat culture	TNTC	
8.		Streptococci	10 ⁻²	Heavy growth		
9.		Streptobacilli	10 ⁻⁴	Moderate	720	
10.		Diphtheroids	10 ⁻⁶	Moderate	680	
11.		Diplococci	10 ⁻⁸ , 10 ⁻¹⁰	Scattered Overlapping colonies	16	
12.	LSS1	Spirillum, Monococci, Diplococci, Bacilli (Mixed Culture)	10 ⁰	Confluent	TNTC	Both Negative and Positive
13.		Cocci	10 ⁻⁴	Clustered colonies	65	Positive
14.		Cocci, Bacilli	10 ⁻⁶	Isolated	34	
15.		Bacilli, Cocci, Diplobacilli, Long Bacilli	10 ⁻⁸	Few colonies	13	Negative
16.	LSS2	Bacilli, Cocci	10 ⁰	Heavy growth	TNTC	
17.		Bacilli, Cocci	10 ⁻²	Moderate	550	
18.		Streptobacilli Long Bacilli	10 ⁻⁶	Heavy growth	700	
19.		Bacilli	10 ⁻⁸	Isolated colonies	45	



Fig. 4: Agar dilution method: *Enterobacter* sp.

Table 3: Comparison for similarity referring NCBI database.

S.No.	Description	Max Score	Total Score	Query coverage	E value	Per ident
1.	Uncultured bacterium clone cz23 16S ribosomal RNA gene. partial sequence	1585	1585	100%	0.0	99.43%
2.	<i>Pantoea agglomerans</i> strain OsEp_A&N_30A14 16S ribosomal RNA	1580	1580			99.31%
3.	<i>Enterobacter hormaechei</i> strain W1 16S ribosomal RNA gene. partial sequence	1580	1580			
4.	<i>Enterobacter ludwigii</i> strain SAK5 16S ribosomal RNA gene. partial sequence	1580	1580			
5.	<i>Pantoea agglomerans</i> strain FZ3SG 16S ribosomal RNA partial	1580	1580			
6.	<i>Gamma proteobacterium</i> BIWA51.gene for 16S ribosomal RNA partial	1580	1580			
7.	<i>Enterobacter rogenkampii</i> K-475-2chromosome, complete genome	1580	12512			
8.	<i>Enterobacter</i> sp. strain AS9 16S ribosomal RNA gene. partial sequence	1580	1580			
9.	<i>Enterobacter</i> sp. strain AS5 16S ribosomal RNA gene. partial sequence	1580	1580			
10.	<i>Enterobacter</i> sp. strain AaIM_Mm9 16S ribosomal RNA gene. complete	1580	1580			

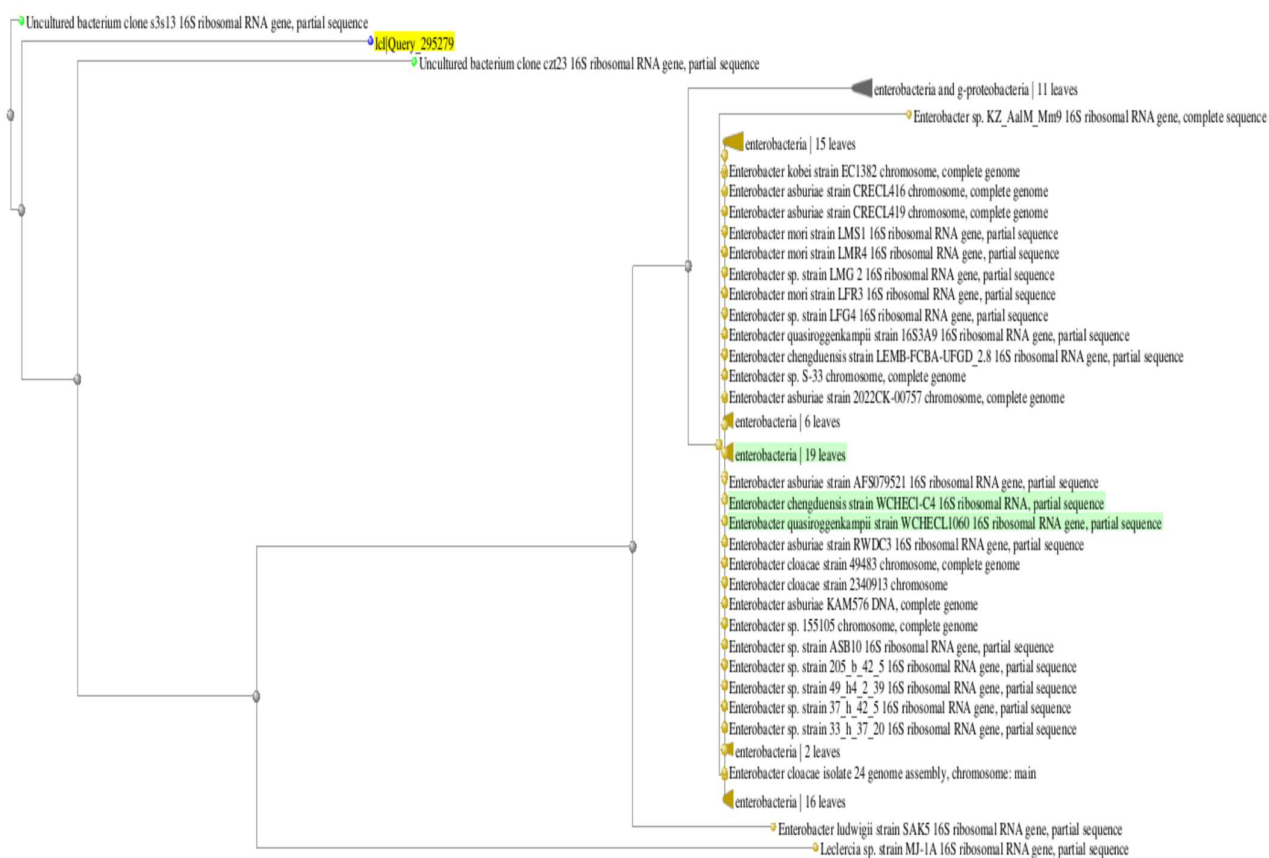


Fig. 5: Phylogenetic Tree.

0.09% w/v Cefixime concentration was considered as MIC. Thus, the results of the agar dilution method pointed towards *Enterobacter* sp. being most tolerant toward higher antibiotic concentration. Fig. 4 represents the agar dilution method of MIC determination.

Molecular Characterization

The 16S rDNA gene was sequenced, revealing a length of approximately 1500 base pairs. The nucleotide sequence was analyzed using BLAST and NCBI tools, followed by

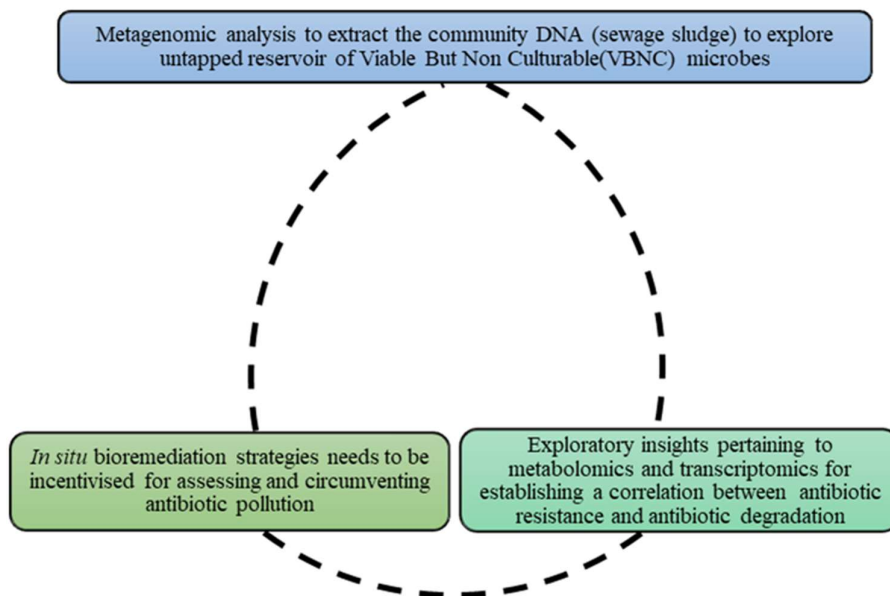


Fig. 6: Interaction between the current scope of study, future projections and practical implications.

phylogenetic analysis. The results indicated that the isolate is a novel strain, designated *Enterobacter* SCef1, showing 99.43% sequence similarity with the *Uncultured bacterium clone cz123* 16S ribosomal RNA gene (partial sequence) (Table 3; Fig. 5).

CONCLUSIONS

Sewage sludge, being a reservoir of various organic as well as inorganic nutrients, is inhabited by a diversity of microbes. Previous studies support the fact that dispersal of antibiotics in various environmental metrics is possibly due to reasonable transmission of pharmaceutically active compounds (antibiotics). This may be the outcome of the unchecked release of hospital effluents contaminated with antibiotics, as surface run-off. Of late, persistence of antibiotics has been a matter of public health concern because of antibiotic resistance. Furthermore, a few bacterial isolates were found to exhibit antibiotic resistance, *Bacillus* sp., *Escherichia* sp., *Pseudomonas* sp., *Streptococcus* sp. and *Enterobacter* SCef1. The acquisition of antibiotic resistance genes by bacteria is closely associated with heavy metal resistance. Certainly, in our findings, heavy metals were also found in traces, encompassing this dual resistance concept. Conventional methods, including the Fenton process and ozonation, are advantageous in attaining significant biomineralization if coupled with the biological processes. This may be carried out by microbial diversity analysis and screening for potential antibiotic-degrading bacterial isolates. This may be upscaled by optimizing the bioprocess

under various process parameters and can be translated *in situ*. Thus, the future of environmental clean-up, mitigation of residual antibiotics and their metabolites relies on the amalgamation of conventional and biological processes, implying white biotechnology. This may necessarily address the emerging public health challenge of antibiotic pollution and resistance henceforth. We believe our study aligns with Sustainable Development Goal 3 (Good Health and Well-being).

FUTURE PROSPECTS

Fig. 6 depicts a nexus between the current scope of study, future projections and practical implications aiming to address an emerging public health challenge of Antimicrobial Resistance (AMR).

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