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Original Research

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 environment. This study unravels the physicochemical aspects of sewage sludge and its valorisation using bio-remediation). Assessment of physico-chemical parameters were conclusive of the fact that sludge samples were enriched with inorganic components (Chloride, Phosphate and Sulphate) and organic constituents. Sludge samples were found to be contaminated with heavy metals Cr (12.69 mg/Kg), As (1.23 mg/Kg), Se (0.86 mg/Kg), Hg (1.49 mg/Kg) and Pb (30.32mg/Kg), 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 characterised 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 *Agar dilution method* using cefixime (10-100mg/l). 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 characterised as *Enterobacter* SCef1.

1. INTRODUCTION

Waste Water Treatment Plants (WWTPs), commonly referred to as Sewage Treatment Plants (STPs) are operationally based on three tier and end of pipe treatment approach (Sharma 2019). The main objective is resource recovery, mobilisation and utilisation 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 possesses higher risk to ecosystem, given the fact it harbours priority pollutants including antibiotic resistant genes as one of emerging public health challenges (ARGs) (Jin et al. 2014; Soudejani et al. 2019). Contrary to the above cited fact, aiming to achieve environmental sustenance; sewage sludge has been explored for agriculture improvisation. 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, analgesic, blood lipid regulators, antidepressants, antiepileptics and *antibiotics*) have become an inevitable part of current societal challenges. Recent studies uncover their presence in different ecosystems mainly, terrestrial and aquatic ecosystem which requires addressing of 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 post-pandemic era. At a global level, antibiotic stewardship programs are being implemented at community level to sensitise people about the challenging times we may face in times to come. In last few decades, persistence of numerous antibiotic-resistant bacteria (ARB) and antibiotic resistance gene (ARG) have 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 contribute to contamination of water resources (Sultan et al. 2023). Accelerated effect of sub-inhibitory concentration of antibiotic on 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 treatment of upper respiratory tract infections. Anti-bacterial activity especially against Gram- negative bacteria. 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, antibiotic has been found to be contaminating freshwater bodies which significantly increase the *chemical oxygen demand* (COD) indicating toxicity strength. 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 environment from leftover antibiotic traces mainly includes 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 few of them. Additionally, these treatment methods do hold some disadvantages such as expensive, labour-intensive, and may often introduce toxic intermediates leading to *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 United Nations aiming at waste management (Bhandari et al. 2023).

Bioremediation by virtue of its ease of applicability could be devised to combat growing menace of antibiotic pollution. With an expanding knowledge of microbial community dynamics in waste water systems a potential reservoir harbouring antibiotic resistant bacteria, our study was aimed at screening cephalosporin resistant bacteria with a potential of antibiotic degrading efficacy. *To the best of our knowledge, we are proposing a novel concept based at 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 bio-degradation. We have attempted to validate the concept behind environmental route of antibiotic transmission by microbiological interventions.

2. MATERIALS AND METHODS

2.1 Chemicals

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

2.2 Site description and sludge sampling

Dried and liquid sludge samples (in triplicates) were collected from 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 (Figure 1 (a, b)) (APHA 2017).

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

2.3 Experimental plan

The samples were processed for the below mentioned scheme of experiments (Figure 2).

2.3.1 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 categorised as *on-site* and *in vitro* parameters. Briefly, *on-site* parameters included pH, temperature, colour and Dissolved Oxygen (D.O.). *Off-site* parameters were further analysed by titrimetric and spectrophotometric methods. Heavy metal analysis was conducted by Atomic Absorption Spectrophotometer (AAS) (APHA 2017).

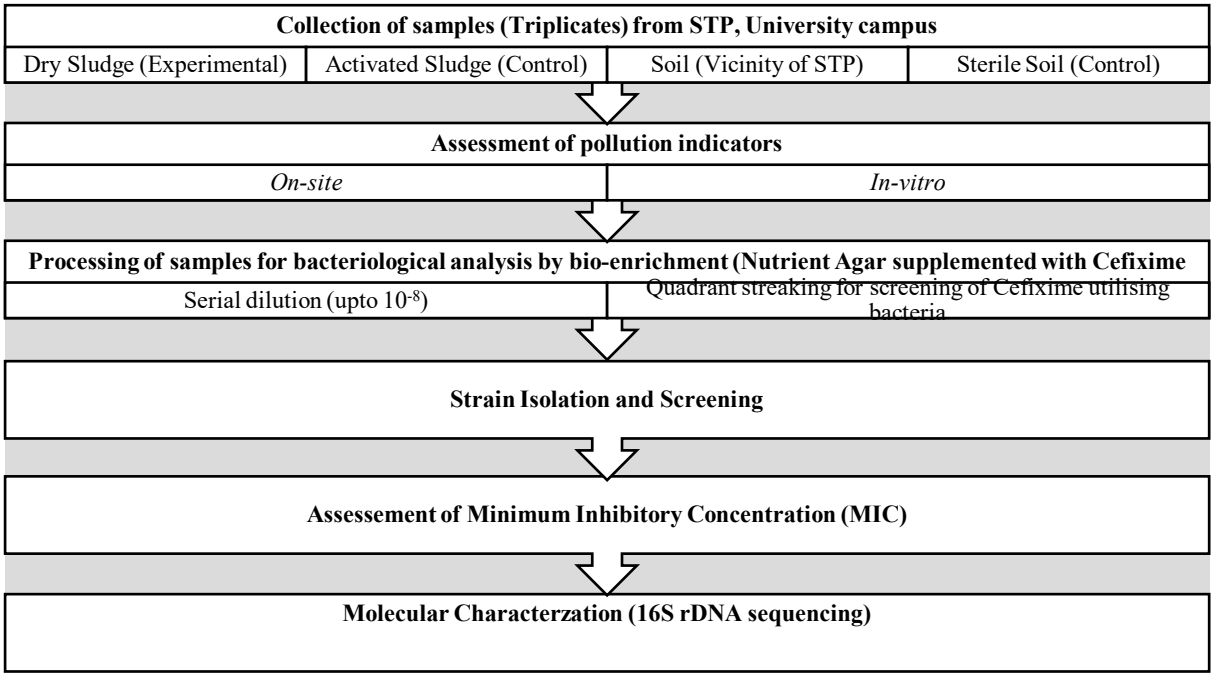

Fig 2: Layout of experimental setting

2.3.2 Screening of antibiotic-resistant bacteria by bio-enrichment

Bio-enrichment strategy is the one which provides an *advantageous* environment for a specific type of strain to 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 upper respiratory tract with the following specifications was used Figure 3.

	ChEMBL ID: 1541 Molar Mass: 453.452g/Mol ChemSpider ID:4514923 Protein binding: Approximately 60% ATC Code: J01DD08 (WHO) Bio-availability: 30-50%

Fig 3: Antibiotic specifications

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

2.3.3 Minimum Inhibitory Concentration (MIC)

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

2.3.4 Molecular Characterization

Pure culture of acclimatised bacterial isolate was further processed for 16 S rDNA sequencing. The amplicon was processed for generation of consensus sequence which was compared with known sequences present in NCBI database.

Briefly, genomic DNA was isolated using in-house Bacterial DNA isolation kit and the purity and concentration was analyzed using Denovix DS-11 spectrophotometer. 16S rDNA region of 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 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 primer and reverse primers using BDT V3.1 Cycle sequencing kit on ABI 3730 Genetic Analyzer as per standardised 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 gen bank database. The first ten sequences in the database that showed highest similarity were selected based on maximum identity score and phylogenic tree result.

3. RESULTS AND DISCUSSION

3.1 Physico-chemical analysis of sludge samples

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

pH indicates hydrogen ion concentration. All the physico-chemical properties are inter-dependent variables which evaluate operational performance of STP. Bacterial diversity, growth and acid production are the key factors influenced by pH which turn influence the heavy metal solubility. It is been reported that acidic medium trends 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 6.7± 0.03 $mS \cdot cm^{-1}$ as shown in Table1. It is likely due to accumulation of soluble salts in wastewater by various environmental settings. Our justification is supported by (El-Nahhal et al. 2014) who reported an EC of is 2.49 ± 0.04 $mS \cdot cm^{-1}$ 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± 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 presence of enriching inorganic and organic components (Sharma et al. 2024). According to (Sonmez & Bozkurt 2006). sewage sludge, manure and humic acid significantly effects the soil health, metal concentration and plant growth. Samples were found to have lower nitrate level; 0.1±0.04 and 0.01±0.06. Phosphate was found in moderate range; 0.95±0.05 and 1.09±0.03 mg/l making it suitable to apply in phosphate deficient soil. Sulphate was found to be 6.6± 0.01 and 5.3± 0.01mg/l in solid and liquid sludge respectively.

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

In India, sewage waste water is found to be polluted with various xenobiotic heavy metals being one of them. According to a 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-91–129mg/kg. Heavy metal analysis of our sludge sample concluded that STP is receiving highly contaminated water from environmental proximities; concerned level of all tested heavy in solid sewage sludge.

Table 1. Physico-chemical analysis of sewage sludge

S.No.	Parameters	Sewage Sludge (Solid) (Mean ± S.D.)	Sewage Sludge (Liquid) Mean ± S.D.) (mg/l)
1	pH	10	9
1	BOD (mg/L)	189.2±0.02	166±0.01

2	COD (mg/L)	400± 0.01	500± 0.02
3	Acidity (mg/L)	150± 0.03	137± 0.02
4	Alkalinity(mg/L)	75± 0.1	58± 0.01
5	Chloride (mg/L)	400± 0.01	300± 0.02
6	Hardness (mg/L)	140.8±0.03	146.08±0.01
7	Nitrate (mg/L)	0.1±0.04	0.01±0.06
8	Phosphate (mg/L)	0.95±0.05	1.09±0.03
9	Solids (Dissolved, Suspended and Total) (mg/L)	67.85±0.03, 164.8±0.0, 134.3±0.01	66.05± 0.03, 144±0.01, 122.5± 0.01
10	Phenolic Compounds	50± 0.02	59± 0.01
11	Electrical Conductivity (EC)(mS/m)	5.9± 0.02	6.7± 0.03
12	Oil and Grease (mg/L)	3.8± 0.06	3.9± 0.07
13	Sulphate (mg/L)	6.6± 0.01	5.3± 0.01
14	Total Organic Carbon (TOC) (mg/L C)	21± 0.01	25.9± 0.01
15	Moisture Content	22.8± 0.01	20.6± 0.02
Priority Contaminants			
16	Chromium (Cr)	12.69 (mg/Kg)	0.037 (mg/l)
17	Arsenic (As)	1.23 (mg/Kg)	0.018 (mg/l)
18	Selenium (Se)	0.86 (mg/Kg)	0.005 (mg/l)
19	Cadmium (Cd)	0.95 (mg/Kg)	0.005 (mg/l)
20	Mercury (Hg)	1.49 (mg/Kg)	0.004 (mg/l)
21	Lead (Pb)	30.32 (mg/Kg)	0.91 (mg/l)

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	

3.2 Bio-enrichment

Nutrient agar plates inoculated with actively growing culture (O.D. $_{660}$=0.1) were observed after 24hrs of incubation. Diversity analysis of all dilution is represented in Table 2. Distinct types of bacterial colonies were observed. 5 bacterial isolates coded as SSS.1, SSS2.1, SSS2.2, LSS2.1, LSS2.1were 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 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) (*Bacteroids* sp. and *Bacillus* sp.)

3.3 Minimum Inhibitory Concentration (MIC)

MIC was considered for the lowest concentration of antibiotic where bacterial growth ceased and single colony within streaked area was considered (Owuama 2017). The respective MIC for *Bacillus* sp., *Escherichia* sp., *Pseudomonas* sp., *Streptococcus* sp. was 0.03, 0.05, 0.07 and 0.06% w/v; respectively. However, in case of *Enterobacter* sp. It was found that at 0.09% w/v Cefixime concentration the colony count declines. Hence, 0.09% w/v Cefixime concentration was considered as MIC. Thus, the results of agar dilution method pointed towards *Enterobacter* sp. being most tolerant toward higher antibiotic concentration. Figure 4 represents agar dilution method of MIC determination.

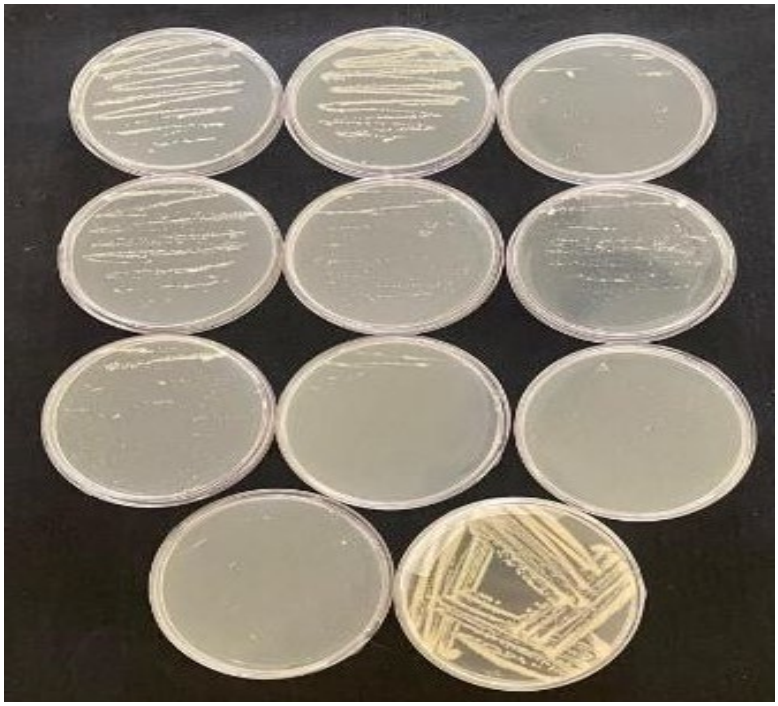

Fig 4: Agar dilution method: *Enterobacter* sp.

3.4 Molecular characterization

The 16S rDNA sequence was sequences and the length of gene was found to be 1500bp. The nucleotide sequence was analysed using BLAST and NCBI software followed by deducing the phylogeny. The results concluded that it's a novel isolate of *Enterobacter* SCef1 showing maximum similarity of 99.43% with Uncultured bacterium clone czt23 16S ribosomal RNA gene. partial sequence (Table 3; Figure 5).

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 czt23 16S ribosomal RNA gene. partial sequence	1585	1585			99.43%
2	*Pantoea agglomerans* strain OsEp_A&N_30A14 16S ribosomal RNA	1580	1580			
3	*Enterobacter hormaechei s*train W1 16S ribosomal RNA gene. partial sequence	1580	1580			
4	*Enterobacter ludwigii* strain SAK5 16S ribosomal RNA gene. partial sequence	1580	1580	100%	0.0	99.31%
5	*Pantoea agglomerans* strain FZ3SG 16S ribosomal RNA partial	1580	1580			
6	*Gamma proteobacterium* BIWA51.gene for 16S ribosomal RNA partial	1580	1580			

7	*Enterobacter roggenkampii* K-475-2chromosome, complete genome	1580	12512			
8	*Enterobacter* sp. strain AS9 16S ribosomal RNA gene. partial sequence	1580	1580			
9	*Enterobacter* sp. strain AS5 16S ribosomal RNA gene. partial sequence	1580	1580			
10	*Enterobacter* sp. strain AaIM_Mm9 16S ribosomal RNA gene. complete	1580	1580			

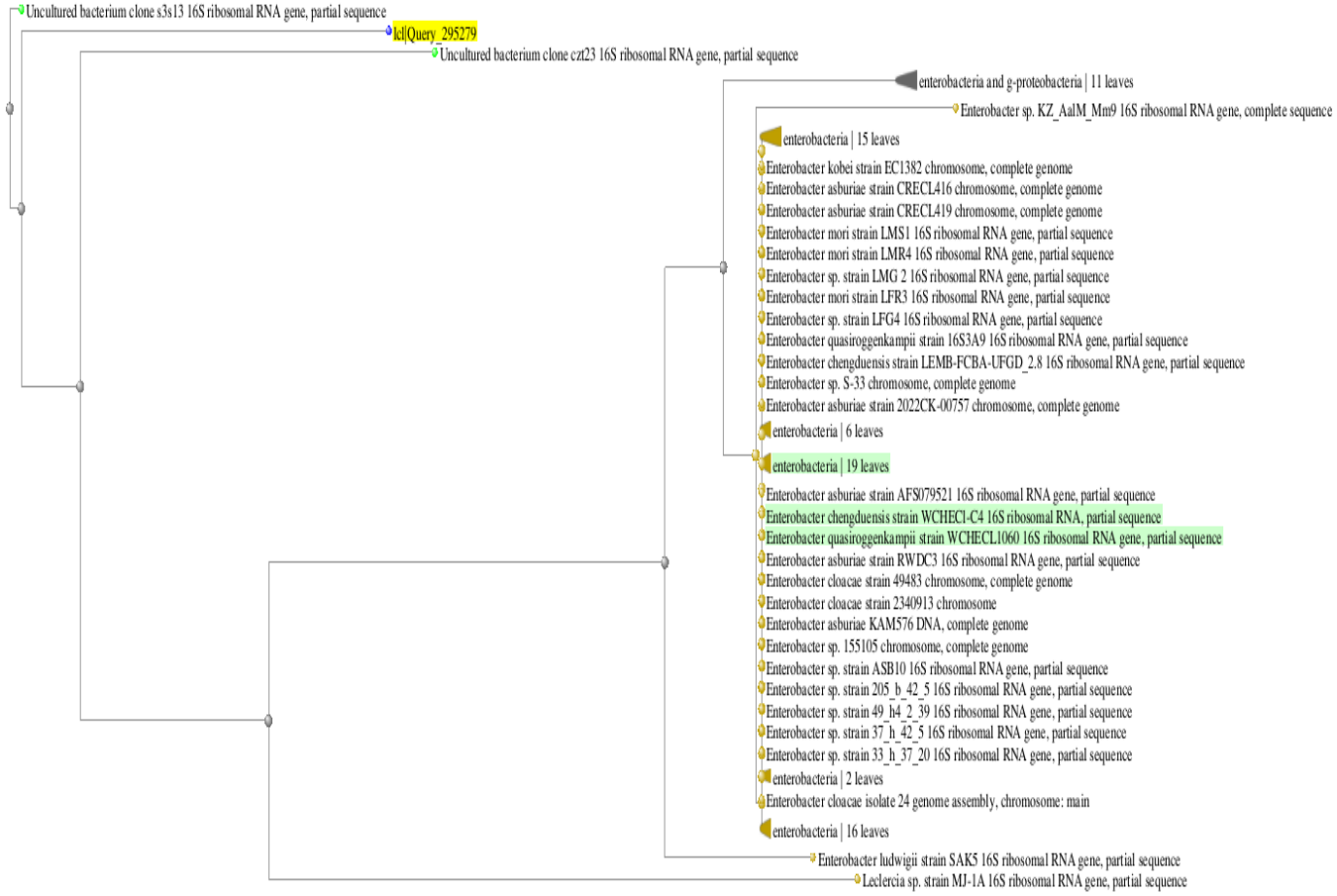

Fig 5: Phylogenetic Tree

4. CONCLUSIONS

Sewage sludge being reservoir of various organic as well as inorganic nutrients is inhabited by diversity of microbes. Previous studies supporting the fact that dispersal of antibiotic in various environmental metrics is possibly due to reasonable transmission of pharmaceutically active compounds (antibiotics). This may be the outcome of 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, few bacterial isolates were found to exhibit antibiotic resistance; *Bacillus* sp., *Escherichia* sp., *Pseudomonas* sp., *Streptococcus* sp. and *Enterobacter*SCef1. 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, comprehending this dual resistance concept. Conventional methods including Fenton process and ozonation are advantageous in attaining significant bio-mineralisation, 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 optimising the bioprocess under various process parameters and can be translated *in situ*. Thus, future of *environmental clean-up*; mitigation of residual antibiotic and their metabolites relies on amalgamation of conventional and biological processes implying white biotechnology. This may necessarily address 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).*

5. FUTURE PROSPECTS

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

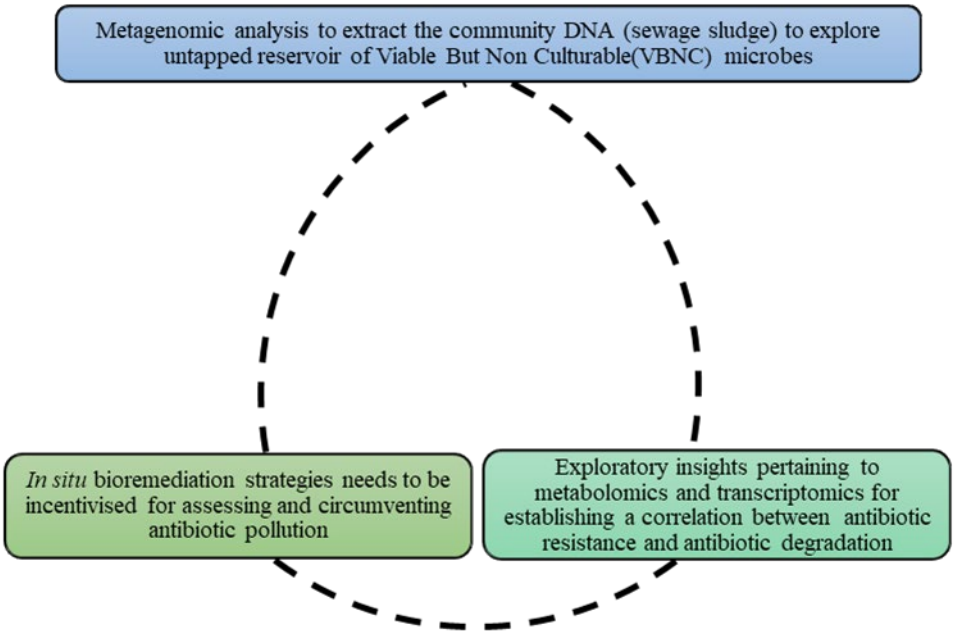

Fig. 6: Interaction between current scope of study, future projections and practical implications

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