GMR
The Original

IDENTIFICATION OF NOVEL BACTERIAL STRAINS FOR ENHANCED HUMAN WASTE DEGRADATION

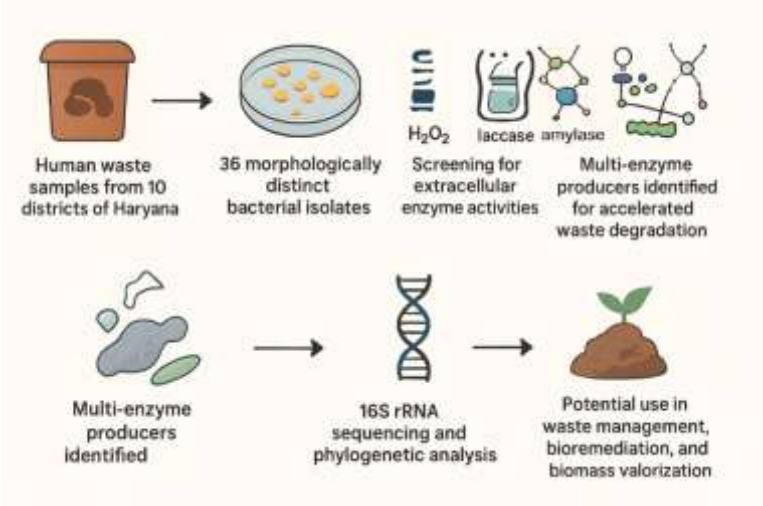
Neelanshi Trivedi[1*], Maya Datt Joshi[2], Nishat Passricha[3]

[1,2,3] GLA University, Mathura, U.P., India
*Corresponding Author: Neelanshi Trivedi, trivedineelanshi@gmail.com

ABSTRACT
Human excrement is a highly rich source of heterogeneous microbial flora that can be used in organic waste breakdown and environmental biotechnology. The objective of this study was to isolate, identify and test the extracellular enzymatic potential and consortium compatibility of bacterial strains from ten districts of Haryana, India on human fecal samples. Overall, 36 morphologically distinct bacterial isolates were isolated, with significant spatial variation in abundance and diversity. The number of isolates obtained from different localities of Jind district were maximum (7) followed by Rewari, Mahendargarh, Rohtak and Faridabad with 4 isolates each respectively, and the lowest number of isolates were found in Nuh and Kurukshetra with 2 isolates each respectively, indicating the effect of physicochemical conditions, organic load and sanitation level on the distribution of microorganisms in the area. There was a high level of functional differentiation among isolates as detected by enzymatic screening. The bacteria Bacillus cereus (NT-55) and Microbacterium paraoxydans (NT-11) were found to be effective multi-enzyme-producing bacteria, with a high level of peroxidase, laccase and amylase activities. Escherichia coli and Shigella sonnei had less peroxidase activity, with Klebsiella and Enterococcus exhibiting prominent enzyme activity. The use of molecular identification techniques (16S rRNA sequencing) revealed wide phylogenetic diversity among the bacteria, including a branch of Microbacterium paraoxydans (NT-11) within the phylum Actinobacteria that might indicate potential novelty. Compatibility assays performed with the consortium showed good synergism among the selected isolates of Bacillus sp., Enterobacter sp. and Acinetobacter sp. and there were no antagonistic reactions observed. This stable consortium of multiple enzymes had a cooperative growth, suggesting that it is able to degrade complex organic matter rapidly. The results underscore the promise of bacteria isolated from human excreta for creating new microbial communities that can speed up the degradation processes and aid waste management and environmental cleanup in a sustainable manner.

KEYWORDS: Bacterial diversity; Bioremediation; Extracellular enzymes; Human waste; Organic waste degradation.

GRAPHICAL ABSTRACT:



INTRODUCTION

Waste management is a basic public health and environmental problem that has plagued humanity for thousands of years and is still poorly managed in many regions of the world. Worldwide, there are an estimated 3.6 billion people who still do not have access to safely managed sanitation facilities (World Health Organization (WHO) and UNICEF, 2023) and an estimated 494 million people who practice open defecation. The impact of the lack of sanitation goes beyond personal health, affecting the transmission of infectious diseases, water quality and

resource contamination, and greenhouse gas emissions. The need to utilize efficient and sustainable solutions to manage human waste is now great, particularly in low- and middle-income countries, due to increasing population density and the growth of urbanization (Mara et al., 2010; Rai et al., 2024). Although septic tanks, sewerage systems, and pit latrines are common sanitation solutions, they are generally quite limiting. In many parts of developing areas, centralized sewage infrastructure is costly, energy intensive and needs extensive maintenance (Tilley et al., 2014). Although less expensive and less complicated to build, septic tanks and pit latrines do have their problems with degradation of organic material, sludge build up, and leakage of pathogens and contaminants into surrounding soil and groundwater (Gyimah et al., 2024). Moreover, these traditional systems do not focus on the resource recovery potential of human waste, including the production of biogas, nutrient recovery and the conversion of organic matter to soil conditioners (Gashaw., 2014). To overcome these limitations, novel solutions are needed that extend beyond traditional engineering solutions and can leverage the potential of microbial biotechnology. Microorganisms are in the middle of the natural decay process of organic wastes and excreta. The complex organic compounds can be broken down into simple ones by anaerobic and aerobic microbial communities, which helps in reducing the amount of waste and eliminates harmful pathogens (Nemet et al., 2021; Rastogi et al., 2020). The microbial community which is routinely used in sanitation systems, however, may be suboptimal when conditions are uncontrolled or suboptimal. For example, waste decomposition rates in septic systems are generally slow, often requiring regular desludging and raising expenses for households and municipalities (Niwagaba et al., 2025; Mehta et al., 2019). Furthermore, the microbial diversity of conventional systems is not necessarily the best suited to break down certain parts of human waste, such as the indigestible plant fiber used in plant-based foods or the bulk of lipids from meat and fish protein-rich foods.

The discovery of new microbes that can break down wastes better is an exciting area for sanitation studies. The new technologies of metagenomics, microbial ecology and synthetic biology are helping to identify new strains of bacteria and fungi that have never been discovered before, with different metabolic pathways that can speed up the degradation of organic matter (Behera et al., 2020; Gupta et al., 2021). For instance, recent research identified specialized microorganisms that are able to produce cellulolytic and ligninolytic enzymes that accelerate the decomposition of plant fiber commonly found in feces (Borker et al., 2024). Some others have pointed out methanogenic archaea that enhance biogas production in anaerobic digestion (AD) (Kong et al., 2019; Sequeda et al., 2023). The findings indicate that the search for microbial diversity beyond the scope of the traditional microbial consortia may yield new avenues for enhancing the efficiency, sustainability and scalability of human waste treatment systems. Novel microbes may be found beyond the laboratory environment. Extremophilic microorganisms are found in extreme environments, such as hot springs, salt marshes, and deep-sea sediments, and have been discovered with unique and interesting enzymatic and metabolic properties (Aanniz et al., 2015; Benammar et al., 2020). The extremophiles can work under extreme conditions of temperature, salinity and pH and thus are suitable candidates for use in various waste treatment scenarios. For instance, in high-temperature composting toilets, thermophilic bacteria may be able to help break down waste more quickly and in coastal sanitation systems, where salinity levels hinder the growth of normal microorganisms, halophilic bacteria might prove beneficial. The versatility of these microbes supports the promise of microbial discovery to solve context-specific sanitation problems. Microbial innovation for sanitation is not just about the environment, it also plays a role in global sustainability targets. Universal access to clean water and sanitation by 2030, through integrated, efficient, equitable, and resilient strategies is key to the United Nations Sustainable Development Goal 6 (SDG 6). It is not only the environmental burden of untreated excreta that can be lowered but also valuable resources can be recovered by using novel microbes to accelerate the waste degradation process. Microbial activity can produce biogas, which can go into renewable energy production; nutrient recovery (as nitrogen, phosphorus, potassium) can help with agricultural productivity and decrease dependency on synthetic fertilizers (Hunter et al., 2020; Fang et al., 2019). These circular economy solutions are aligned with the global strategies to sustainable development, climate change mitigation and food security. Although the future looks bright, there are several issues that need to be resolved for novel microbes to be widely adopted for sanitation. First, the safety and stability of engineered or newly discovered microbial strains need to be assured to prevent any possible unintended ecological or public health hazards (Sharma et al., 2022). Second, socio-economic and cultural issues limit access to new sanitation technologies, especially in resource constrained areas where costs and convenience are critical (Jenkins and Curtis., 2005). Last but not least, there is a need for interdisciplinary cooperation to join microbial sciences, environmental engineering and public health for the design of robust systems which are technically viable, socially acceptable and economically viable. Considering these complexities, this paper seeks to underscore the unique possibilities of finding new microbes to degrade human waste more quickly, and the importance of microbial diversity in solving the global sanitation crisis. This study aims to build upon previous studies of microbial waste degradation, discuss new innovations in microbial discovery and the implications for sustainable sanitation to help develop new solutions that work for human health, environmental health and resource recovery. Finally, future selection and use of new microbial strains could revolutionize the way societies view and think about managing human excrement. Human excreta have been considered as a sanitation issue only and not as a resource, until treated with proper microbial tools. The paradigm shift is not only a solution to the sanitation challenges at hand, but it also represents a more sustainable view of waste as a resource for energy, nutrients and ecological regeneration. The potential of these novel microbes in this context is an exciting and

necessary area of research to address global population growth and environmental pressures for a healthier and more sustainable future.

MATERIALS AND METHODS

In an attempt to represent in broad terms the diversity of micro flora, ten districts of Haryana, India have been selected for collection of human waste samples. The selection of these districts was designed to get the variation in temperature ranges and environment, as the microbrial population may adjust to the local climates; these variations may affect the enzyme activity and degradation capacity of the microbrial population. The human feces at each location were aseptically collected in sterile containers in approximately 500 g of fresh feces. Microbial integrity was ensured with the use of insulated boxes containing ice for transport and samples were processed within 24 hours of collection.

Isolation and purification of microbes

Samples were serially diluted (10^{-1} to 10^{-6}) and spread plated on various selective media. Bacterial isolation was done on nutrient agar (NA) and fungal isolation on potato dextrose agar (PDA) with chloramphenicol. Plates were incubated for 24-72 hours depending on the anticipated growth of microorganisms at 30 ± 2°C. Different colonies with different morphology, pigmentation or growth pattern were selected. Each isolate was sub-cultured several times by quadrant streaking to ensure it was uniform in colony type for purity. Pure bacterial isolates were kept on nutrient agar slants at 4°C and fungal isolates on PDA slants at 4°C. Glycerol stocks (20% v/v) were made and stored at -80°C (Atlas, 2010) for long-term preservation. In this process a wide variety of microbial library consisting of bacteria and fungi, which may be associated with waste degradation, was obtained.

Selection of microbes based on degradation potential

Screening for degrading enzymes

Degradation of organic wastes by microorganisms is mainly via their enzymatic system. Purified isolates were used to evaluate the production of the three main enzymes active in lignin degradation, Laccase, which can degrade lignin, phenolic compounds and other aromatic pollutants (Shraddha et al., 2011) in order to find efficient degraders. Guaiacol-agar plates were used to screen, with positive strains exhibiting reddish-brown areas surrounding colonies because of the oxidation of guaiacol. Peroxidas are enzymes that oxidize phenols, cresols, pesticides and biphenyl compounds (Hammel and Tardone 1997). The activity of peroxidase was measured by benzidine/H_2O_2 as a substrate, positive strains produced blue colored halos. Cellulose is present in sewage plants and is important there because of the presence of an enzyme complex (Cellulose) which decomposes cellulose to glucose (Lynd et al., 2002). Carboxymethyl cellulose (CMC) Agar plates were used for screening and stained with Congo red staining. The production of cellulase was indicated by hydrolysis zones.

Molecular identification of selected strains.

The extraction and amplification of DNA.

Isolates which showed good growth were further identified by molecular typing to determine the taxonomic identity. Cetyltrimethylammonium bromide (CTAB) method (Sambrook and Russell, 2001) was used for genomic DNA extraction. The purity and concentration of DNA was checked by spectrophotometry (260/280 ratio) and agarose gel electrophoresis. The primers used for 16S rRNAgene amplification of the bacterial isolates were 27F (5′-AGAGTTTGATC[A/C]TGGCTCAG-3′) and 1492R (5′-TACGG[C/T]TACCTTGTTACGACTT-3′). PCR reactions were carried out in 50 µL reactions with DNA template, primers, dNTPs, buffer, $MgCl_2$ and Taq polymerase. The cycling protocol consisted of denaturation for 5 minutes at 95°C, then 30 cycles (denaturation for 30 s at 94°C; annealing for 30 s at 55°C; extension for 1 minute at 72°C), and a final extension for 10 minutes at 72°C.

Sequence analysis

Bidirectional sequencing and purification of PCR products were performed. Sequences were translated into FASTA format and screened for chimeric sequences with Chimera-CHECK 2.7 (Ribosomal Database Project II). A BLASTn algorithm (Altschul et al., 1997) was used to search the verified sequences against the following databases: GenBank, EMBL and DDBJ. To establish the evolutionary relationships, phylogenetic trees were drawn with the MEGA software. Species level identification was facilitated by a sequence similarity of ≥98% with reference strains.

The development of microbial consortia.

Strain compatibility testing

Selected bacterial and fungal isolates were used to prepare microbial consortia to gain an efficient degradation of the waste. The first compatibility tests were carried out by co-culturing the strains on nutrient agar and PDA plates. The same consortium comprised strains that did not exhibit antagonistic activity (inhibition zones or growth suppression).

This is a consortium design and rationale.

Consortia design and rationale

Decomposition of organic matter and nitrogen transformations were mainly due to bacterial isolates. Lignocellulose degradation and detoxification of phenolic compounds were made possible by fungal isolates. Different enzymes and metabolic pathways complemented each other in a synergistic interaction, which was realized by this synthetic ecology approach (Zhang and Zhang 2022; Cao et al., 2022).

Pilot-scale testing

The developed consortia were tested in batch reactors containing fresh human waste. Reactors were maintained under controlled temperature (30 ± 2°C) and moisture conditions. Periodic sampling (day 0, 10, 20, and 30) was performed to measure organic carbon, nitrogen, and phosphorus levels, thereby assessing degradation kinetics.

RESULTS AND DISCUSSION

A total of 36 morphologically distinct bacterial strains were purified from ten districts of Haryana (Table 1). The number of isolates varied across locations, with the highest diversity recorded in Jind (7 isolates), followed by Rewari, Mahendargarh, Rohtak, and Faridabad (4 each). In contrast, Kurukshetra and Nuh yielded only two isolates each (Table -1). Such geographical variability in microbial abundance may be attributed to differences in local sanitation practices, organic load, pH and temperature of the waste samples, as reported in earlier studies (Vijayan *et al*., 2023; Gupta *et al*., 2021).

Table 1: A total of 36 distinct microbial cultures purified from 10 collected samples.

S.No.	District	Strain purified
1.	Karnal	3
2.	Rewari	4
3.	Mahendargarh	4
4.	Kurukshetra	2
5.	Rohtak	4
6.	Sirsa	3
7.	Hisar	3
8.	Jind	7
9.	Faridabad	4
10.	Nuh	2

Interestingly, Jind district not only contributed the highest number of isolates but also showed greater colony type diversity, suggesting that environmental heterogeneity favors microbial richness. Similar observations have been reported in septic and composting environments, where higher nutrient loads and moisture gradients enhance bacterial growth and diversification (Major *et al*., 2020). The relatively lower recovery from Nuh and Kurukshetra may reflect reduced microbial activity due to physicochemical constraints such as lower organic matter input or unfavorable anaerobic conditions. These findings highlight the spatial variation in waste-degrading microbial communities and the importance of regional screening for identifying novel strains with high degradative potential.

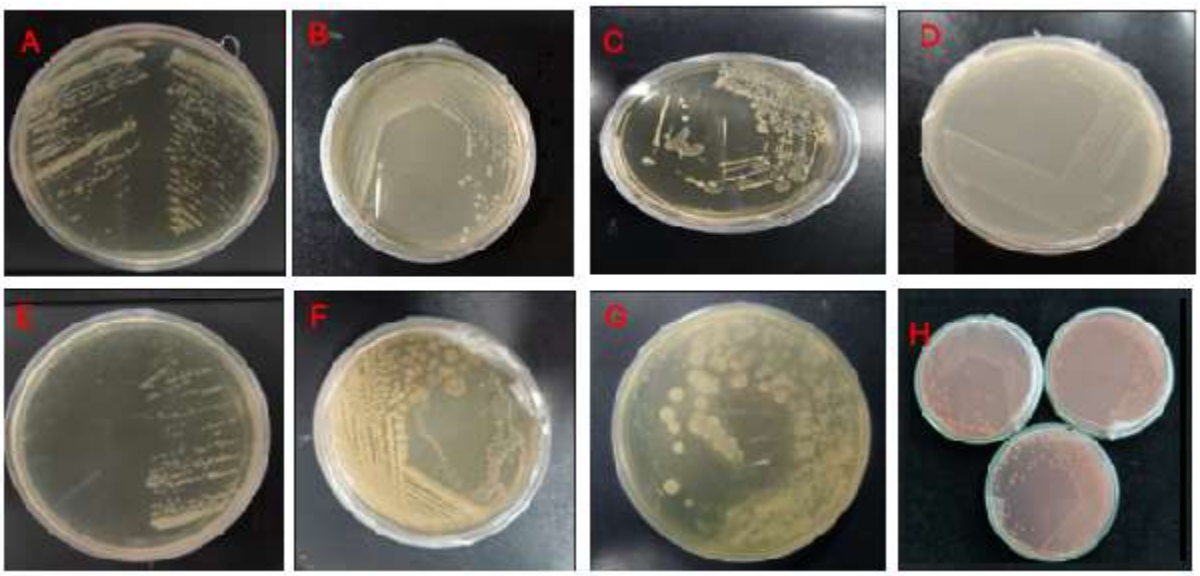


Figure 1 (A-H) Purified microbial strains isolated from collected samples.

The 36 purified strains (Figure 1) will be further screened for their ability to degrade human waste efficiently through enzymatic assays (cellulase, protease, lipase, amylase). Early screening already indicates promising candidates that can accelerate organic matter breakdown compared to conventional isolates, which could be valuable for bioaugmentation in septic tanks and biogas systems.

Enzymatic screening of isolates

All 36 purified strains were screened for their ability to produce key degrading enzymes laccase, peroxidase, and cellulase that play central roles in the breakdown of complex organic matter in human waste. Laccase production was tested using α-naphthol, while peroxidase activity was detected through the H_2O_2–TMB assay. Cellulase activity was confirmed by a clear zone on CMC plates after Gram's iodine staining.

Table 2: List of bacterial isolates showing positive activity for all three enzymes

Sample Name	Organism	Enzyme Profile
NT-5	*Enterobacter sp*.	Weak laccase; moderate amylase; low cellulase (≤8 mm zone)
NT-55	*Bacillus sp*.	Strong laccase; highest amylase activity; weak cellulase
NT-19	*Enterococcus* sp.	Higher peroxidase; slightly better cellulase; weak amylase
NT-66	Escherichia coli	Weak in most assays; minimal amylase; weak cellulase
NT-41	*Klebsiella* sp.	Higher peroxidase; minimal laccase; weak cellulase
NT-6	*Acinetobacter sp*.	Strong laccase producer; weak cellulase; moderate amylase

Out of the 36 isolates, 6 strains tested positive for at least one degrading enzyme (Table 2). Among the isolates screened, *Klebsiella* sp. and *Enterococcus* sp. exhibited comparatively higher peroxidase activity, suggesting their active involvement in the oxidative breakdown of aromatic and phenolic compounds often present in complex organic waste. Such activity is consistent with earlier reports where peroxidase-producing *Enterococcus* strains contributed to lignin degradation and oxidative stress mitigation in waste environments (Gałązka *et al*., 2025).

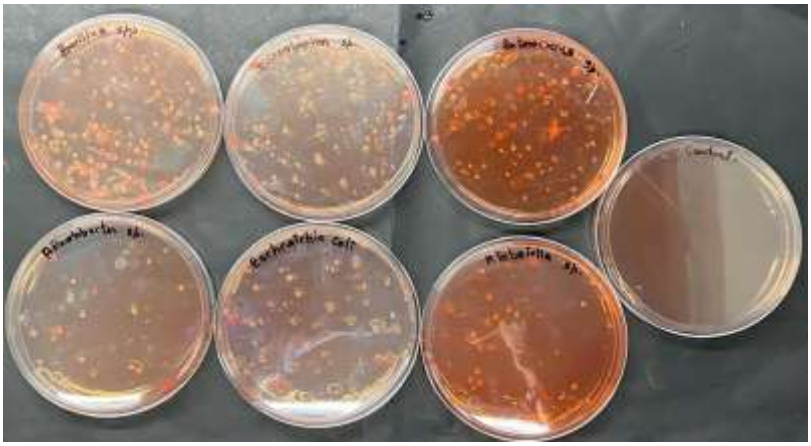


Figure 2. Enzymatic screening of Enterobacter, Bacillus, *Enterococcus, Escherichia coli* (*E. coli)*, *Klebsiella* and Acinetobacter isolates, shown alongside positive and negative controls

In the cellulase assay, all six isolates demonstrated weak activity, producing hydrolysis zones
In the cellulase assay, all six isolates showed low activity with hydrolysis zones of ≤8 mm, indicating that the common basic secretion of cellulase under the given conditions (Figure 2). Of these, the Enterococcus sp. was slightly more active than the other species. These results, although not outstanding, are significant because cellulase activity plays a role in the degradation of the fecal lignocellulosic residues. In the same fashion, previous studies have also found low but consistent levels of cellulase activity in enteric bacteria, indicating that these bacteria are also playing a secondary role in the synergistic microbial consortium (Jenkins and Curtis, 2005; Gupta et al., 2021). On the other hand, Bacillus sp. and Acinetobacter sp. were identified as strong laccase producers and also possessed good bioremediation potential. Laccase enzymes have been extensively investigated for their ability to decolor dyes, degrade phenolics, and humification (Yang et al., 2023; Christopher et al., 2014). In particular, strains of Bacillus are known to produce thermostable and extracellular laccases, which makes them attractive for applications in wastewater and composting. Amylase activity was used as an additional means of differentiation of the isolates. Bacillus sp. showed maximum amylolytic potential, which matched with its industrial uses in starch hydrolysis and enzyme biotechnology (Guta et al., 2024). The amylase activity of E. coli and Enterococcus sp. was weaker, suggesting more specific ecological functions than contributing to bulk starch degradation. E. coli is not a usual enzyme producing microorganism, but the existence of E. coli in faecal samples confirms its role as a helper microorganism in microbial consortiums for anaerobic digestion (Liang et al., 2020; Luo et al., 2020).
The enzyme screening shows in general that human fecal bacteria have diverse functions. The multi-enzyme activities of Bacillus and Acinetobacter (strong laccase and amylase activities) and Klebsiella and Enterococcus (peroxidase activity) indicate their potential as multi-enzyme producers for waste treatment applications. While weaker enzyme producers such as E. coli can be secondary contributors, instead of primary degraders. Multi-functional recovery of the isolates in this study corroborates previous reports on the biotechnological potential of Bacillus, Klebsiella and Acinetobacter in biomass valorization and pollutant removal (Gupta et al., 2021; Yang et al., 2023). Overall, the results indicate that selected strains of Bacillus and Acinetobacter species should be further optimized by quantitative enzyme assays, growth kinetics and pilot-scale tests of waste degradation.

Molecular identification of bacterial isolates
Genomic DNA of the enzyme positive isolates selected was isolated as per the Hi Media kit (Cat. The universal primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTTA) were used for 16S rRNA gene amplification (No. MB505). The amplified products were sequenced and compared to the available 16S rRNA sequences in the NCBI GenBank database by using BLASTn tool. Isolation of bacteria was based on the percentage sequence similarity and phylogenetic relationships were additionally confirmed by multiple sequence alignment in the Clustal W. The isolates identified were Agrobacterium tumefaciens (NT-2), Enterobacter cloacae (NT-5), Microbacterium paraoxydans (NT-11), Enterococcus faecalis (NT-19), Shigella sonnei (NT-33), Klebsiella oxytoca (NT-38), Klebsiella pneumoniae (NT-41), Escherichia coli (NT-66, NT-68) and Bacillus cereus (NT-55) (Figure-4).

BRIN1: Agrobacterium tumefaciens_ PQ460277

(A) BRIN2: Enterobacter cloacae_PQ460278

(B) BRIN3: Microbacterium paraoxydans_ PQ460279

Figure 3: (A- C) Isolation and 16S rRNA identification of BRIN1 (*Agrobacterium tumefaciens*, PQ460277), BRIN2 (*Enterobacter cloacae*, PQ460278) and BRIN3 (*Microbacterium paraoxydans,* PQ460279) showing distinct colony morphology and BLAST confirmation.

Table 3: Accession numbers of molecularly characterized bacterial isolates

Isolate Code	**Closest Match (NCBI BLAST)**	**Accession No.**	**% Similarity**	**Phylum**
NT-2	*Agrobacterium tumefaciens*	PQ460277	99.2%	Proteobacteria
NT-5	*Enterobacter cloacae*	PQ460278	98.7%	Proteobacteria
NT-11	*Microbacterium paraoxydans*	PQ460279	97.5%	*Actinobacteria*
NT-19	*Enterococcus faecalis*	PQ460280	99.5%	Firmicutes
NT-33	*Shigella sonnei*	PQ460281	99.0%	Proteobacteria
NT-38	*Klebsiella oxytoca*	PQ460282	98.9%	Proteobacteria

Isolate Code	Closest Match (NCBI BLAST)	Accession No.	% Similarity	Phylum
NT-41	*Klebsiella pneumoniae*	PQ460283	99.3%	Proteobacteria
NT-55	*Bacillus cereus*	PQ460286	98.8%	Firmicutes
NT-66	*Escherichia coli*	PQ460284	99.1%	Proteobacteria
NT-68	*Escherichia coli*	PQ460285	99.0%	Proteobacteria

The presence of a diverse population of bacteria with waste-degrading potential was confirmed through molecular identification of enzyme-positive isolates. The isolates were found in four major phyla, demonstrating the wide ecological role played by various bacterial lineages for complex substrates degradation. The high similarity values (>98%) for most isolates suggest close relationships to known species (Table-3). Considering that <97% similarity may suggest the likelihood of a new species (Stackebrandt and Goebel, 1994), however, Microbacterium paraoxydans (97.5% similarity) may be a novel or less characterized strain. The results broaden the microbial spectrum of waste degradation in humans. Bacillus cereus (NT-55) and Microbacterium paraoxydans (NT-11) had the most favorable profile of isolates because they exhibited high enzyme activity and low pathogenic risk. On the other hand, isolates like Shigella sonnei and Escherichia coli are known as opportunistic pathogens and are enzymatically active, therefore causing biosafety risks for direct application in field sites. Alternatively, these strains may also be useful for understanding enzyme mechanisms, but are not ideal for use in sanitation systems for bioaugmentation. The presence of Bacillus, Klebsiella, and Enterobacter spp. which were clustered with strains reported as biodegradative further confirms their potential in accelerated degradation of organic wastes. This is similar to the findings of other studies involving microbial community from composting and septic systems where the lignocellulolytic and oxidative enzyme producers were found to play an important role in organic matter degradation (Mello et al., 2017; Hanc et al., 2022).

In total, the molecular identification step verified that the enzyme-positive strains are both known degraders and also candidates that might be new strains, and thus a good base for a futA phylogenetic tree was built up, using the Neighbor-Joining method in the software MEGA X, based on 16S rRNA sequences of the enzyme-positive isolates and closely related reference sequences that were obtained from GenBank. Boostrap analysis was used with 1000 replications to evaluate the robustness of the tree topology.

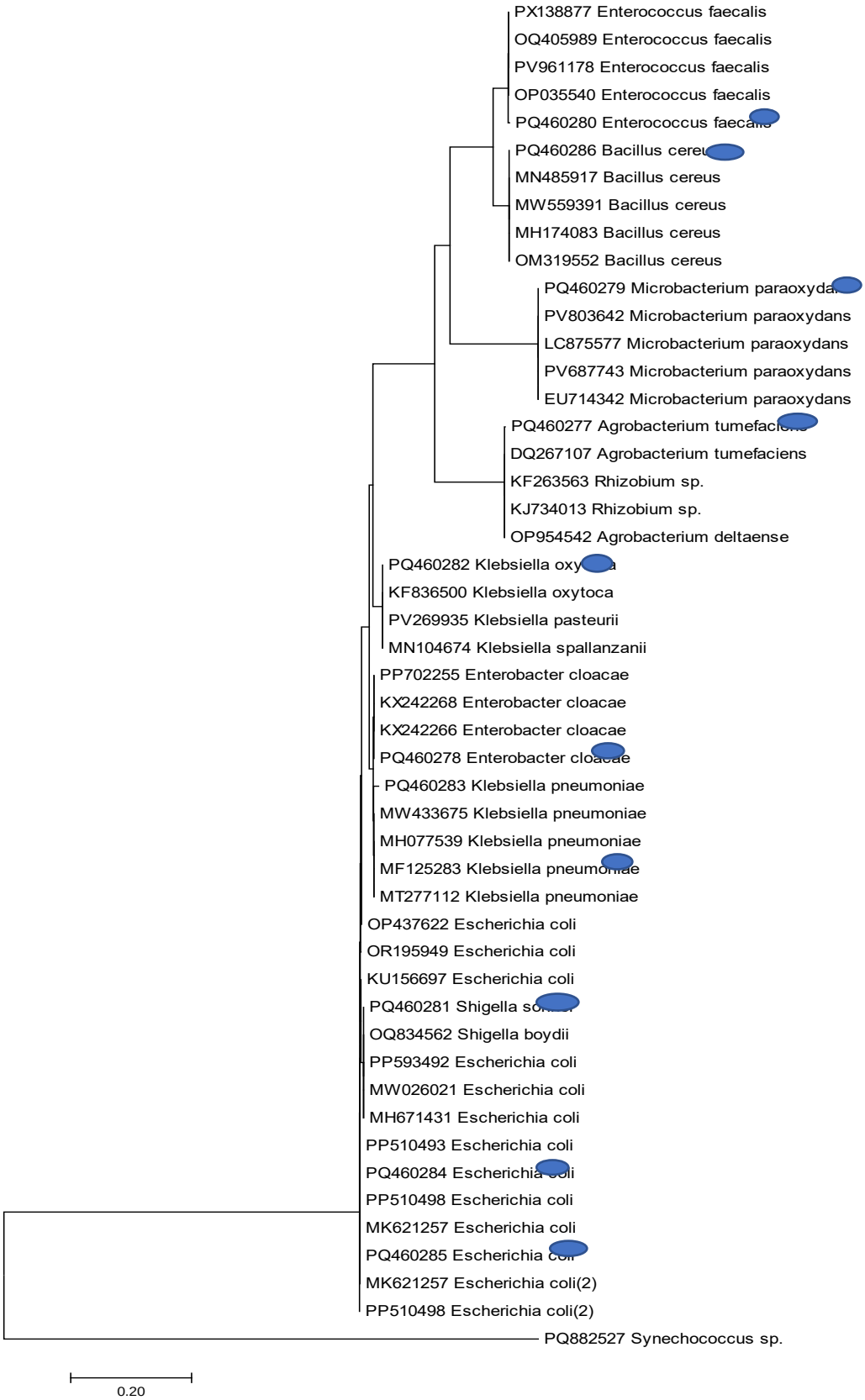


Figure 4: Phylogenetic tree based on the 16S rRNA of Enzyme Positive isolates

The phylogenic tree revealed clear clustering of the isolates with their respective genera. For example, isolates NT-38 and NT-41 were grouped very closely with Klebsiella oxytoca and Klebsiella pneumoniae reference strains and NT-5 was grouped with Enterobacter cloacae. In the same way, within the Firmicutes there were two distinct clades: NT-55 (Bacillus cereus) and NT-19 (Enterococcus faecalis). Interestingly, NT-11 (Microbacterium paraoxydans) was a separate branch, but was still within the cluster of Actinobacteria, indicating possible novelty or strain level divergence. Of particular interest, isolate NT-11 (Microbacterium paraoxydans) was located on a

relatively distant branch of the Actinobacteria cluster, and had a bootstrap support value above 80 percent, which indicates the possibility of a new strain with new enzymatic potential. Branching patterns have been used before to suggest novel microbes that can degrade wastes (Stackebrandt and Goebel, 1994). Phylogenetic analysis concurred with the molecular identification result in the majority of cases, and further indicated that human waste-degrading bacteria have diverse evolutionary origins, which further supports their potential to support a bioaugmentation strategy.

Compatibility and consortium development

Six bacterial isolates Enterobacter cloacae (NT-5), Bacillus cereus (NT-7), Klebsiella pneumoniae (NT-9), Acinetobacter calcoaceticus (NT-11), Enterococcus faecalis (NT-2) and Escherichia coli (NT-3) were tested for compatibility on nutrient agar plates. The goal was to investigate the possibility of the coexistence and to find the combination of strains that can co-metabolize various organic components synergically. Six consortium plates (I–VI) were made, each consisting of different combinations of isolates in pairwise streak patterns (Figure: 5). Plates were grown at 37°C for 48 h, and the growth interaction was determined by observing the growth zone at the intersection of the plates.

Figure 5: Compatibility assay of selected bacterial isolates (Plates I–VI) *Escherichia coli*

(A), *Enterococcus sp.* (B), *Enterobacter sp.* (C), *Bacillus sp.* (D), *Klebsiella sp.* (E) and *Acinetobacter sp.* (F) showing pairwise and multi-strain interactions. Uniform growth without inhibition zones indicates synergistic and compatible behavior among isolates, supporting their suitability for developing a stable microbial consortium for enhanced human waste degradation.

No antagonistic interaction or inhibition zone was observed between the isolates in all the six sets. The morphology and pigmentation of the colonies of each culture type were different, indicating that they are compatible and able to coexist in a mixed culture environment. The absence of lytic zones at the intersections of the streaks shows that there is no negative compatibility among the isolates and thus facilitates the formation of a stable consortium. The absence of growth of the control plates confirmed culture sterility and thus biologically induced interactions were observed. The morphology of the colonies was observed, and significant differences were noted: Bacillus cereus colonies were large and irregular, opaque and had rough margins; Enterobacter cloacae and Klebsiella pneumoniae had smooth, creamy colonies; Acinetobacter calcoaceticus had small, glistening colonies and slight yellow pigmentation; Enterococcus faecalis and E. coli had circular, convex colonies and off-white coloration. This phenotypic difference is thought to be a result of their different enzyme functions and ecological niches in a cooperative system.

Formation of a stable and functionally diverse consortium

Four (Bacillus cereus, Enterobacter cloacae, Klebsiella pneumoniae and Acinetobacter calcoaceticus) were selected on the basis of compatibility and previous enzyme profiling (laccase, protease, lipase, amylase, cellulase) and the selected isolates were grouped into Consortium (C + D + E + F). This combination was chosen to combine complementary degradative functions: Bacillus cereus was found to have high extracellular protease and cellulase activity, which help in the degradation of protein and polysaccharide. Strong activities of oxidative enzymes (laccase, lipase) required for the degradation of lignin-like and phenolic compounds were shown by Enterobacter cloacae and Acinetobacter calcoaceticus. EPS (exopolysaccharide) production was the role played by Klebsiella pneumoniae for greater cell aggregation and stability of the biofilm matrix. The consortium's enzymatic balance is optimal for efficient degradation of a variety of waste compounds, including proteins, carbohydrates, fats, and aromatic residues, which are all major waste fractions of humans. The synergistic interaction of enzyme activities is suggested by the cooperative coexistence where metabolites produced by one strain are used as a substrate by another and the mineralization process is enhanced. The consortium compatibility results indicate that a cooperation between microbial communities has been established. Microbial communities working together are often more efficient in their metabolism than single strains because each species can signal to the other species and share resources (Cao et al., 2022). The present results corroborate previous studies that emphasize Bacillus as a keystone degrader in multi-complex waste materials due to its wide range of extracellular enzyme system (Singh et al., 2021). Enterobacter and Acinetobacter also release the enzyme laccase and lipase which promote oxidative degradation of the phenolic and lipidic fractions (Ren et al., 2019; Cai et al., 2021). Klebsiella, in turn, helps by forming polysaccharide-based biofilms that shield microbial communities from environmental stressors and enhance nutrient retention (Guerra et al., 2022). Stability of mixed consortia in nutrient-variable environments, like human waste, is based on such functional complementarity.

Potential synergistic processes in human feces decomposition

The developed consortium contains several metabolic pathways: Hydrolytic degradation: Bacillus breaks down proteins, starch, and cellulose into simpler monomers. Oxidative degradation: Enterobacter and Acinetobacter use multicopper oxidases and lipases to oxidize phenolic, lignin-like and lipid-rich compounds. Biofilm formation and stabilization: Klebsiella produces EPS that helps in forming stable biofilms and attachment of substrates. These interactions promote both carbon and nitrogen mineralization, contributing to faster degradation of COD and BOD during degradation. The synergistic microbial networks are found to be more efficient than monocultures in waste treatment systems because of metabolic cooperation and enzyme complementarity (Mukherjee et al., 2023).

The selected consortium of Bacillus cereus+Enterobacter cloacae+Acinetobacter calcoaceticus+Klebsiella pneumoniae is a new consortium formulation that performs fast degradation of human waste. The combination is not reported in previous studies, although each strain has been reported for biodegrading the organic substrates. Complementary enzymatic potential and compatibility of the consortium makes it a unique biocatalytic system, suitable for septic tanks, bio-toilets and decentralized sanitation systems. Consortiums have two benefits: effective biodegradation and nutrient recovery without odor and sludge production. It will be further confirmed by molecular validation (16S rRNA or whole genome sequencing) and kinetic trials in the actual waste matrices for its novelty and performance efficiency. Metagenomic or qPCR monitoring should be used to quantify degradation kinetics (COD, BOD, TOC reduction), enzyme activity rates and microbial persistence for further research. To get a long-term view of stability and efficiency, it will be necessary to pilot scale the process in anaerobic digesters or composting systems. Also, before field deployment, there will be a need for biosafety screening (pathogenicity and antibiotic resistance gene profiling). The Consortium, if developed, can be suggested as a novel microbial formulation for eco-friendly waste bioprocessing applications with high performance.

CONCLUSION

In this study, the bacterial diversity and enzyme potential in human excreta from ten districts of Haryana, India have been evaluated. A total of 36 morphologically distinct bacterial isolates were recovered, showing a high spatial variability in abundance and diversity of microorganisms. The number of isolates and the diversity of the colonies were found to be highest in Jind which indicated the importance of environmental heterogeneity and organic load on the microbial richness with local sanitation practices. However, lower nutrient availability or undesirable physicochemical conditions could restrict the microbial growth and diversity in some other areas such as Nuh and Kurukshetra, which provided fewer microbes. The findings highlight the need for regional screening to look for new strains for possible waste management applications. The isolates were also examined functionally using enzymatic screening and showed a high degree of functional diversity. Bacillus cereus (NT-55) and Microbacterium paraoxydans (NT-11) were found to be the most promising in terms of production of multi-enzymes with significant activities of peroxidase, laccase and amylase. These profiles show their potential for efficient degradation of complex organic substrates, such as lignocellulosic material, and their applicability in bioaugmentation strategies, composting and biomass valorization. Klebsiella and Enterococcus isolates showed high peroxidase activity which indicates their important role in the oxidation of aromatic and phenolic compounds. Escherichia coli and Shigella sonnei had measurable enzymatic activity but have biosafety issues for direct field use and can be used in controlled consortia for enzymatic processes.The enzyme-positive isolates were all

phylogenetically diverse, as confirmed by molecular identification using 16S rRNA gene sequencing. The sequence similarity to the known species was high (98% and higher) for most of the strains, confirming the taxonomic identity and, for Microbacterium paraoxydans (NT-11), a branch of its own within the Actinobacteria cluster indicated a possible novelty or strain-level divergence. Phylogenetic analysis also revealed that isolates of Bacillus, Klebsiella and Enterobacter grouped very closely with already described biodegradative strains thus confirming their potential for decomposition of organic wastes at a faster rate. A series of consortium development experiments were then carried out to assess inter-strain compatibility and synergistic growth potential, based on the above results. All the isolates E. coli, Enterococcus sp., Enterobacter sp., Bacillus sp., Klebsiella sp. and Acinetobacter sp. showed good mutual compatibility without any inhibitory interaction in the combination plates. There was no antagonism at the intersection areas, and their colonies grew uniformly and together, proving that they were cooperating in their metabolism. Specifically, the growth uniformity and stability of pigmentation of the Bacillus–Acinetobacter combination were best, indicating better mutualism and cross-feeding effects during the degradation of organic matter. These cooperative microbial relationships suggest the development of a structurally stable, highly enzymatically competent microbial community that can break down proteins, polysaccharides, lipids and lignin-like compounds simultaneously in human excrement. This synergism contributes to the increase of the enzymatic turnover, and of the decomposition rate of the organic matter, when compared to the single strains, and makes this multi-species composting mix novel in the field of waste biodegradation. Based on the results of this study, it can be concluded that human excreta is a good source of enzymatically active bacteria for different ecological and biotechnological purposes. The multi-enzyme producing isolates such as Bacillus cereus, Microbacterium paraoxydans, Acinetobacter sp., and Enterobacter sp. are good candidates for the development of microbial consortia for boosting the waste degradation rates and valorizing the organic residues. Optimization of growth and enzyme production conditions, quantitative degradation kinetics, evaluation of biosafety aspects, and pilot-scale trials to evaluate the practical applicability of these strains in sanitation systems, composting and bioremediation should be done in future studies. The use of these microbial consortia in waste management systems could play a crucial role in sustainable environmental management, accelerated degradation of human waste, and circular bioeconomy solutions.

REFERENCES

1. Aanniz T, Ouadghiri M, Melloul M, Swings J et al. 2015. Thermophilic bacteria in Moroccan hot springs, salt marshes and desert soils. Braz J Microbiol. 46(2) pp443-53.
2. Altschul S F, Madden T L, Schäffer A A, Zhang J et al. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25(17) pp3389–3402.
3. Atlas R M. 2010. Handbook of microbiological media (4th ed.). CRC Press.
4. Behera B K, Chakraborty H J, Patra B, Rout A K et al. 2020. Metagenomic Analysis Reveals Bacterial and Fungal Diversity and Their Bioremediation Potential From Sediments of River Ganga and Yamuna in India. Front Microbiol. 11.
5. Benammar L, İnan Bektaş K, Menasria T, Beldüz A O et al. 2020. Diversity and enzymatic potential of thermophilic bacteria associated with terrestrial hot springs in Algeria. Braz J Microbiol. 51(4)pp1987-2007.
6. Borker S S, Thakur A, Pandey K K, Sharma P et al. 2024. Nutrient recycling of source-separated human faeces using biochar immobilized indigenous psychrotrophic bacteria for sustaining the agroecosystems of north-western Himalaya. Appl Biol Chem. 67(37).
7. Cai Y, Wang R, Rao P, Wu B et al. 2021. Bioremediation of Petroleum Hydrocarbons Using Acinetobacter sp. SCYY-5 Isolated from Contaminated Oil Sludge: Strategy and Effectiveness Study. Int J Environ Res Public Health. 18(2)pp819.
8. Cao Z, Yan W, Ding M and Yuan Y. 2022. Construction of microbial consortia for micobial degradation of complex organic. Front Bioeng Biotechnol. 10.
9. Chhetri R B, Silwal P, Jyapu P and Maharjan Y. 2022. Biodegradation of Organic Waste Using Bacillus species Isolated From Soil. Int J Appl Sci Biotechnol. 10(2) pp104-111.
10. Christopher L P, Yao B and Ji Y. 2014. Lignn biodegradation with laccase- mediator systems. Froniters in Energy Research.2 pp 12.
11. Fang C, Huang R, Dykstra C M, Jiang R et al. 2019. Energy and Nutrient Recovery from Sewage Sludge and Manure via Anaerobic Digestion with Hydrothermal Pretreatment. Environ Sci Technol. 54(2) pp1147–1156.
12. Gałązka A, Jankiewicz U and Orzechowski S. 2025. The Role of Ligninolytic Enzymes in Sustainable Agriculture: Applications and Challenges. Agronomy. 15(2) pp451.
13. Gashaw A. 2014. Anaerobic Co-Digestion of Biodegradable Municipal Solid Waste with Human Excreta for Biogas Production: A Review. Am J Appl Chem. 2(4)pp55-62.
14. Guerra M E S, Destro G, Vieira B, Lima A S et al. 2022. Klebsiella pneumoniae Biofilms and Their Role in Disease Pathogenesis. Front Cell Infect Microbiol. 12.
15. Gupta P, Samant K and Sahu A. 2021. Metagenomic insights into novel microbial communities for waste degradation. J Environ Manage. 284.
16. Guta M, Abebe G, Bacha K and Cools P. 2024. Screening and characterization of thermostable enzyme-producing bacteria from selected hot springs of Ethiopia. Microbiol Spectr. 12(3).

17. Gyimah R, Lebu S, Owusu-Frimpong I, Semiyaga S et al. 2024. Effluents from septic systems and impact on groundwater contamination: a systematic review. Environ Sci Pollut Res Int. 31(54) pp62655-62675.
18. Hammel K E and Tardone P J. 1997. The Oxidative 4--Dechlorination of Polychlorinated Phenols is Catalysed bt extracellolar Fungal Lignin Peroxidase. Biochemistry. 27 pp 6563-6568.
19. Hanc A, Dume B and Hrebeckova H. 2022. Differences of Enzymatic Activity During Composting and Vermicomposting of Sewage Sludge Mixed With Straw Pellet. Front Microbiol (Sec. Microbiotechnology). 12.
20. Hunter B and Deshusses M A. 2020. Resources recovery from high-strength human waste anaerobic digestate using simple nitrification and denitrification filters. Sci Total Environ. 712.
21. Jaiswal D K, Verma J P, Belwal T, Pereira A P D A et al. 2023. Editorial: Microbial co-cultures: a new era of synthetic biology and metabolic engineering. Front Microbiol. 14.
22. Jenkins M W and Curtis V. 2005. Achieving the 'good life': why some people want latrines in rural Benin. Soc Sci Med. 61(11) pp2446-2459.
23. Kong D, Zhang K, Liang J, Gao W et al. 2019. Methanogenic community during the anaerobic digestion of different substrates and organic loading rates. MicrobiologyOpen. 8(5).
24. Liang Z, Xu G, Shi J, Yu S et al. 2020. Sludge digestibility and functionally active microorganisms in methanogenic sludge digesters revealed by E. coli-fed digestion and microbial source tracking. Environ Res. 193.
25. Luo K, Xie X, Yang Q, Chen F et al. 2020. Multi-hydrolytic enzyme accumulation and microbial community structure of anaerobic co-digestion of food waste and waste-activated sludge. Environ Technol. 41(4) pp478-487.
26. Lynd L R, Weimer P J, van Zyl W H and Pretorius I S. 2002. Microbial cellulose utilization: Fundamentals and biotechnology. Microbiol Mol Biol Rev. 66(3).
27. Major N, Schierstaedt J, Jechalke S, Nesme J et al. 2020. Composted Sewage Sludge Influences the Microbiome and Persistence of Human Pathogens in Soil. Microorganisms.8(7).
28. Mara D, Lane J, Scott B and Trouba D. 2010. Sanitation and Health. PLoS Med. 7(11).
29. Mehta M, Mehta D and Yadav U. 2019. Citywide Inclusive Sanitation Through Scheduled Desludging Services: Emerging Experience From India. Front Environ Sci.
30. Mello B L, Alessi A M, Riaño-Pachón D M, deAzevedo E R et al. 2017. Targeted metatranscriptomics of compost-derived consortia reveals a GH11 exerting an unusual exo-1,4-β-xylanase activity. Biotechnol Biofuels. 10 (254).
31. Nemet F, Perić K and Lončarić Z. 2021. Microbiological activities in the composting process : A review. Columella Journal of Agricultural and Environmental Sciences. 8(2) pp 41-53.
32. Niwagaba C B, Batte A, Majara D, Katukiza A Y et al. 2025. Faecal sludge accumulation and characterization in informal settlements of Kampala: insights for facilities design. Discover Sustainability. 6(444).
33. Rai S, Gurung A, Sharma H B, Ranjan V P et al. 2024. Sustainable solid waste management challenges in hill cities of developing Countries: Insights from eastern Himalayan smart cities of Sikkim, India. Waste Management Bulletin. 2 (2) pp1-18.
34. Rastogi M, Nandal M and Khosla B. 2020. Microbes as vital additives for solid waste composting. Heliyon. 6(2).
35. Ren L, Men L, Zhang Z, Guan F et al. 2019. Biodegradation of Polyethylene by Enterobacter sp. D1 from the Guts of Wax Moth Galleria mellonella. Int J Environ Res Public Health. 16(11).
36. Sambrook J and Russell D W. 2001. Molecular cloning: A laboratory manual (3rd ed.). Cold Spring Harbor Laboratory Press.
37. Sequeda Barros R, Durán Contreras M, Romani Morris F, Vanegas Chamorro M et al. 2023. Evaluation of the methanogenic potential of anaerobic digestion of agro-industrial wastes. Heliyon. 9(3).
38. Sharma P, Parakh S K, Singh S P, Parra-Saldívar R et al. 2022. A critical review on microbes-based treatment strategies for mitigation of toxic pollutants. Sci Total Environ. 834.
39. Shraddha, Shekher R, Sehgal S, Kamthania M et al. 2011. Laccase: microbial sources, production, purification, and potential biotechnological applications. Enzyme Res.
40. Stackebrandt E and Goebel B M. 1994. Taxonomic note: A place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol. 44(4) pp846–849.
41. Tilley E, Ulrich L, Luethi C, Reymond P et al. 2014. Compendium of Sanitation Systems and Technologies. 2nd Revised Edition, Swiss Federal Institute of Aquatic Science and Technology (Eawag), Duebendorf.
42. Vijayan J, Nathan V K, Ammini P and Ammanamveetil A M H. 2023. Bacterial diversity in the aquatic system in India based on metagenome analysis-a critical review. Environ Sci Pollut Res Int. 30(11)pp28383-28406.
43. WHO and UNICEF. 2023. Progress on household drinking water, sanitation and hygiene 2000–2022: Special focus on gender. World Health Organization.
44. Yang T, Hu Q, Liu Y, Xu R et al. 2023. Biochemical characteristics and potential application of a thermostable starch branching enzyme from Bacillus licheniformis. AMB Expr. 13(8).
45. Zhang T and Zhang H. 2022. Microbial Consortia Are Needed to Degrade Soil Pollutants. Microorganisms. 10(2) pp261.