Microbial community dynamics and their relationships with organic and metal pollutants of sugarcane molasses-based distillery wastewater sludge

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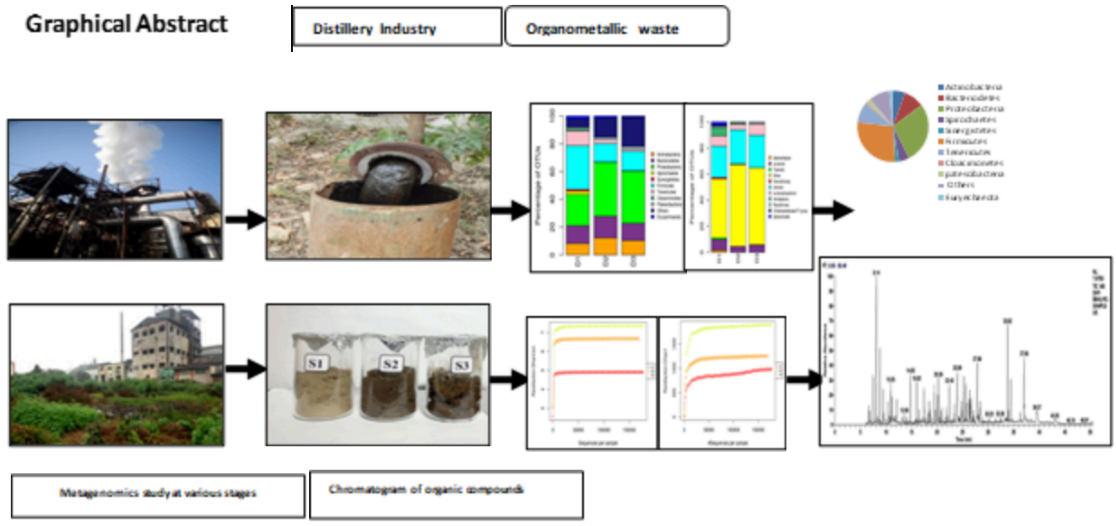

Abstract

Distillery sludge is a major source of aquatic pollution, but little is known about their microbial community and their association with the organic and metal pollutants. Sugarcane molasses-based distillery is an important industry in India, although the waste is usually treated prior to disposal, the treatment is often inadequate. The adverse effects of the organic and metal pollutants in sugarcane molasses-based distillery sludge on the microbial biodiversity and abundance in the disposal site have not been elucidated. This study aims to address this gap of knowledge. Samples were collected from the discharge point, 1 and 2 km downstream (D1, D2, and D3, respectively) of a sugarcane distillery in Uttar Pradesh, India, and their physico-chemical properties characterised. Using QIIME, taxonomic assignment for the V3 and V4 hypervariable regions of 16S rRNA was performed. The phyla Proteobacteria (28-39%), Firmicutes (20-28%), Bacteriodetes (9-10%), Actinobacteria (5-10%), Tenericutes (1-9%) and Patescibacteria (2%) were the predominant bacteria in all three sites. Euryechaeota, were detected in sites D1 and D2 (1-2%) but absent in D3. Spirochaetes (5%), Sinergistetes (2%) and Cloacimonetes (1%) were only detected in samples from site D1. Shannon, Simpson, Chao1, and Observed-species indices indicated that site D1 (10.18, 0.0013, 36706.55 and 45653.84, respectively) has higher bacterial diversity and richness than D2 (6.66, 0.0001, 25987.71 and 49655.89, respectively) and D3 (8.31, 0.002, 30345.53 and 30654.88, respectively), suggesting the organic and metal pollutants provided the stressors to favour the survival of microbial community that can biodegrade and detoxify them in the distillery sludge. This study confirmed that the treatment of the distillery waste was not sufficiently effective and provided new metagenomic information on its impact on the surrounding microbial community. It also offered new insights into potential bioremediation candidates.

Keywords: - Bacterial community; distillery sludge; organic and metal compounds; metagenomics, diversity indices

1. Introduction

The distillery industry is a key economic actor in developing countries, including India (Annan et al., 2013; Shammi et al., 2016; Tripathi et al., 2021a). However, distilleries are also major sources of environmental pollution due to the presence of toxic organic and inorganic compounds in the discharged wastewater (Kumar and Chandra, 2020; Jiang et al., 2019; Tripathi et al., 2021b). According to the All-India Distiller's Association (AIDA), there are approximately 397 sugarcane molasses-based distilleries in India and an average 3.5×10^{16} L of raw effluent is discharged into the receiving water per annum (AIDA, 2016). These wastewater and sludge have highly complex chemical compositions, they can contain up to 55-60% of organic compounds generated by the fermentation process (Valentino et al., 2019; Leng et al., 2020). In addition, Amino acids, fatty acids, pentadecanoic acids, organic compounds, amino carbonyl compounds, aldehyde-amines, metal and non-metal ions and elements, phenolic compounds, fungicides, endocrine disruptors (EDCs) have also been detected in distillery wastewater (Caizán-Juanarena, et al., 2020; Chowdhary et al., 2018; Canle et al., 2017; Campanale et. al., 2020; Varjani and Sudha, 2020). These compounds are harmful to human health and the environment.

Distillery sludge contains a large number of residual organic and inorganic contaminants, but relatively few carbon and nitrogen nutrient resources (Rashmi et al., 2020). Sludge-dwelling microorganisms could mineralize organic compounds and utilise them as a source of energy for their metabolic processes (Srivastava et al., 2020). As a result, these endogenous bacterial species inhabiting the distillery sludge disposal sites could be developed and integrated into a long-term waste management strategy (Duncan et al., 2017). Though several of these microorganisms have been reported for bioremediation and detoxification of the residual organic pollutants (Chandra and Kumar, 2017; Chandra et al., 2018a; Tripathi et al., 2021c, d), the impact of the diverse forms of pollutants on the bacterial communities are still

unknown. Studies have been carried out to examine the diversity of microorganisms associated with the industrial production processes of sugarcane-ethanol (Costa et al., 2015) and Hong Qu glutinous rice wine (Liang et al., 2020), and the rhizospheric bacterial communities associated with *Saccharum arundinaceum* grown on distillery sludge (Kumar and Chandra, 2020). To our knowledge the microbial diversity in sugarcane distillery sludge has yet to be elucidated.

The aim of this research is to address the knowledge gap of the impact of residual organic and metal pollutants in sugarcane molasses-based distillery sludge on the microbial biodiversity and abundance using 16S rRNA-based next-generation sequencing (NGS), as well as their modes of actions and survival attributes. This is the first metagenomic analysis on microbial communities and their relationships with organic and metal compounds in a polluted sugarcane molasses-based distillery sludge site. The results provide essential knowledge and new insight on the bacterial populations that can facilitate sustainable ecological restoration and bioremediation of distillery sludge sites.

2. Material Method:

2.1 Site description and sampling

Sludge samples in triplicates were collected from three discharged effluents sites at M/s Unnao Distillers, Unnao, Uttar Pradesh, India (26°32'N, 80°30'E). Site D1 was near the discharge of industrial effluent, site D2 was one km away from the industry and mixing point of industrial effluent and sewage wastewater, and the site D3 was 2 km away from the industry shown in Fig 1. The collected sludge samples were immediately transferred to the polyethylene sterilized bags and transported under the ice-cold condition (~ 4 °C) for laboratory analysis (Chandra et al., 2018c).

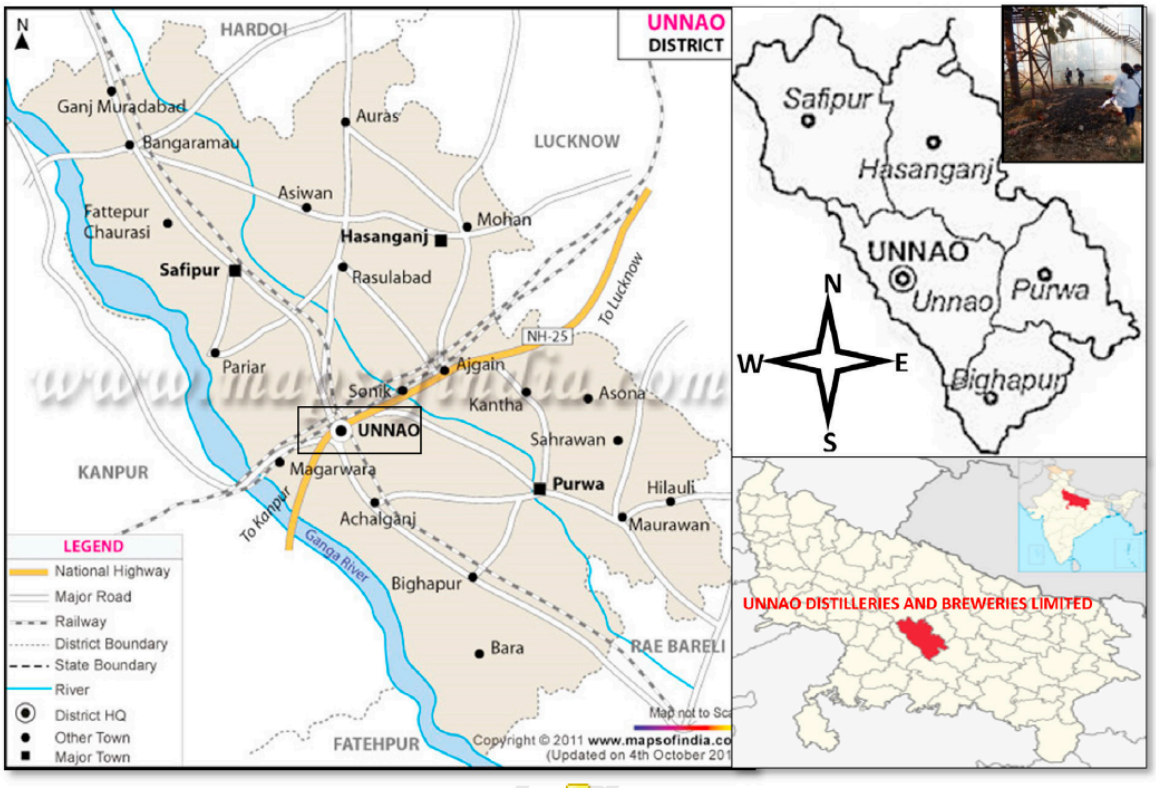

Fig. 1. Map of location of study sites located in Unnao near Kanpur, Uttar Pradesh, India

2.2 Physico-chemical characterization of collected samples

The distillery industry sludge sample was air-dried and crushed into powder form. Orion pH meter (Model-960, Thermo Scientific, FL, USA) and Orion conductivity meter (Model-A322, Thermo Scientific, FL, USA) were used to measure the pH and electrical conductivity (EC) of sludge samples, respectively; the colorimetric method was used for phosphate measurement measured with the selective ion electrode of (Thermo Orion Model, 960) (Tripathi et al., 2021a, e). The total concentration of sodium (Na^+), chloride (Cl^-), and sulfate (SO_4^{2-}) were measured according to Tripathi et al (2021c). Phenol concentrations present in the sludge sample were determined based on the method described by Kumar and Chandra (2020). The metal (Cd, Cu, Mn, Cr, Fe, Ni, and Zn) concentrations in the sludge sample were determined after sample digestion with nitric, perchloric, and hydrofluoric acid according to the procedure by (Tripathi et al., 2021b; Lee et al., 2013; Govarthanan et al.,

2013), followed by flame atomic absorption spectrophotometry (AAS, ZEEnit 700, and Analytic Jena, Germany) as previously described (Chandra et al., 2017).

2.3 Detection and characterization of various organic Pollutants

The GC-MS analysis was carried out to identify the organic pollutants in all the sludge samples (D1, D2, and D3). Ethyl acetate as a solvent was used to extract the contaminants from distillery sludge three times and derivatized using Bis-trifluoroacetamide (BSTFA) and pyridine as per method described earlier (Tripathi et al., 2021a &b) and analyzed by GC–MS (TRACE GC Ultra Gas Chromatographs, Thermo Fisher Scientific, USA) with a TriPlus auto sampler coupled to TSQ Quantum XLS triple quadrupole mass spectrometer (Thermo Scientific, USA). Organic compound separation was carried out in a DB-5MS capillary column (No. 2713S18) by running with helium as the carrier gas at a flow rate of 1.1 mL min^{-1}. The GC oven conditions were as follows: an initial temperature of 65 °C (hold time: 2 min), increased to 230 °C (flow rate of 6 °C min^{-1}) and finally increased up to 290 °C (hold time: 20 min; flow rate of 10 °C min^{-1}) (Tripathi et al., 2021a & b). An aliquot (1.0 µL) of the derivatized sample was injected in the GC column in the splitless mode. The injector temperature was 250 °C while the mass detector was operated at 300 °C. The MS was operated in full-scan mode (45–800 m z^{-1}) at electron energy of 70 eV with a solvent delay of 7 min. The organic compounds were detected and identified through spectral matching of mass spectra obtained at different retention times with mass spectra of standard compounds mentioned in National Institute of Standard and Technology (version 1.0.0.12, NIST, USA) mass spectra library existing with GC–MS.

2.4 Bacterial community's characterization of sludge

The pico green Victor 3 fluorometry procedure was used to determine the amount of template DNA. A Nano Drop (Thermal Scientific, US) was used to quantify the DNA samples. A 1% agarose gel was used to verify the DNA consistency. V3-V4 PCR was set up using V3-

V4 Forward and Reverse Primer and the PCR product was loaded on an agarose gel to screen for positive amplification using the 16S amplicon as a template (around 460 bp). Positive V3-V4 amplification was observed in all D1, D2, and D3 samples, with a band size of 460 bp. The size distribution of the prototype was tested using an Agilent Technologies 2100 Bioanalyzer and a 1000 chip of DNA for the verification of the size of an enriched PCR fragment. On Illumina sequencing platforms, optimal cluster densities were created across every lane of every flow cell to achieve the highest data quality and accurate quantitation of the template DNA library. Further, the quantification of the prepared library was performed by qPCR as per the Illumina qPCR Quantification Protocol Guide. Roche's rapid library standard quantification was utilized for standard curve calibration and calculation of the concentration of the library sample.

2.5 Bioinformatics analysis

The raw PCR sequences were merged; the barcodes and primers were removed via a trimmed and denoised process. It was clustered based on sequence similarities (97%) into the operational-taxonomic units (OTUs) using the UCLUST program (version 1.2.22q). The OTUs with phylogenetic relation were analyzed and ribosomal database project (RDP) classifier (version 2.2) taxonomic assignment was performed against the SILVA OTUs database (version 123) with a confidence threshold of 70% (Bokulich et al., 2013). Differences in the diversity among the samples were calculated using Shannon, Chao1, and Observed-species indices. The Shannon metric evaluated the observed OTU abundance and evenness, and species richness in the samples D1, D2, and D3 and Chao1 metric estimation showed the presence of species richness in the sampling site of the environment (Wang et al., 2019). Chao1, based on Shannon, and Observed-species indices, rarefactions curves were prepared via QIIME (version 1.7.0) and displayed by R software (version 2.15.3).

2.6 Statistical data analysis

All the tests were carried out in triplicate. The standard deviation of physico-chemical parameters of D1, D2, and D3 was calculated using the IBM SPSS software package (version 22.0; SPSS Inc., USA). The data was provided as a mean ± SD value with statistically relevant variations $p < 0.05$ using Student's t-test.

3. Result and discussion

3.1 Physio-chemical properties of the sludge samples

The effluent sludge from the distillery was turbid, dark brown in color and noisome. The dark brown color could be attributed to the melanoidin polymers (Tripathi et al., 2021c; Sharma et al., 2021a). The dark colour of the effluent decreased the opacity of water and thus reduced the photosynthetic behaviour of aquatic plants (Tripathi et al., 2021c; Chandra et al., 2018b). The physico-chemical properties of the sludges are listed in Table 1. The pH of the samples in sites D1, D2, and D3 were 8.29 ± 0.10, 7.29 ± 0.19, and 8.01 ± 0.12, respectively. The pH reduction in D2 probably resulting from the mixing of domestic wastewater before returning to around pH8 in site D3. A high EC level in all the effluent indicated the presence of different cations and anions i.e., sodium (Na^+), chloride (Cl^-), sulfate (SO_4^{2-}), and phosphate (PO_4^{3-}) that were essential to the microbial community (Aravinthan et al., 2015; Govarthanan et al., 2014; Selvam et al., 2017; Govarthanan et al., 2016). The level of cations and anion decreased continuously from D1, D2, and D3 as a result of mixing of different domestic effluent with distillery effluent. High concentrations of heavy metals (manganese (Mn), iron (Fe), nickel (Ni), zinc (Zn), copper (Cu), cadmium (Cd), and chromium (Cr) were detected in the sludge due to the corrosion of equipment from the manufacturing process. Heavy metals can cause serious health hazards via the food chain; this is particularly critical in developing countries due to a lack of proper equipment for irrigation and effluents have been utilized directly for agricultural purposes. In this way, heavy metals accumulate in the crops and are

transferred to human beings via food consumption (Srivastava et al., 2020; Defew et al., 2005; Tripathi et al., d & e).

The bacterial diversity and abundance were affected by the amount of water in the sludge, as well as the amount of organic matter and total nitrogen. Notably, the presence of various pollutants could affect composition of the bacterial community in the distillery sludge. For example, it was reported that the bacterial communities and fungi isolated from distillery sludge could biodegrade melanoidin (Sharma et al., 2021b; Sharma et al., 2020).

Table 1. The physico-chemical characteristics of distillery sludge collected from dumping site located (D1) in premises of Unnao Distilleries & Breweries Limited, 1 km (D2) and 2 km (D3) away downstream.

S. No.	Parameters	Distillery Sludge sample (D1)	Distillery Sludge sample (D2)	Distillery Sludge sample (D3)	Permissible Limit, USEPA (2002)
1.	pH	8.29 ± 0.10	7.29 ± 0.19*	8.01 ± 0.12**	-
2.	EC	5.7 ± 0.00	6.8 ± 0.00**	7.9 ± 0.00*	-
3.	CEC	68.19 ± 0.34	58.19 ± 0.98**	72.19 ± 0.34**	-
4.	TOC	15.25 ± 0.19	13.25 ± 0.23***	19.54 ± 0.78**	-
5.	TKN	5.65 ± 0.08	4.65 ± 0.02**	7.43 ± 0.23*	-
6.	Ammonical Nitrogen	18.25 ± 0.89	16.34 ± 0.67**	20.89 ± 0.98**	1
7.	Total oxygen	35.35 ± 0.09	38.35 ± 0.02	40.65 ± 0.01**	-
8.	Sodium (Na^+)	35.15 ± 4.32	40.15 ± 7.32	31.30 ± 3.32	200
9.	Chloride (Cl^-)	798.25 ± 15.12	734.23 ± 11.12*	676.43 ± 89.76*	1500
10.	Sulfate (SO_4^{2-})	165.08 ± 8.43	134.10 ± 6.43	199.09 ± 9.49	-
11.	Phenol	450.14 ± 1.22	398.14 ± 1.34	399.1423 ± 7.00	-
12.	Phosphate (PO_4^{3-})	1267.23 ± 9.70	1190.23 ± 7.70	1150.34 ±2.90	-
Heavy metals					
a)	Iron (Fe)	1678 ± 45.28	1598 ± 34.54	1502 ± 32.45**	2.0
b)	Zinc (Zn)	88.88 ± 1.87	56.78 ± 3.87	95.23 ± 2.65*	2.0
c)	Copper (Cu)	71.56 ± 0.99	76.76 ± 1.99***	75.43 ± 4.77***	0.5
d)	Chromium (Cr)	20.628 ± 0.00	18.435 ± 2.00**	19.435 ± 1.00**	0.05
e)	Cadmium (Cd)	2.011 ± 0.00	1.013 ± 2.00***	2.009 ± 1.00*	0.01
f)	Manganese (Mn)	99.20 ± 0.19	88.18 ± 9.34	92.13 ± 0.21*	0.20
g)	Nickel (Ni)	13.123 ± 0.09	15.321 ± 0.09	11.311 ± 0.05**	0.1

All values are mean of three replicate ± SD and presented in mg kg^{-1} except electrical conductivity (µS cm^{-1}), TOC (%), TKN (%), total hydrogen (%), and total oxygen (%); CEC (Cmol), *EC* electric conductivity, *CEC* cation exchange capacity, *TOC* total organic carbon, *TKN* total Kjeldahl nitrogen. Student's *t* test: *non-significant ($p < 0.05$), **less significant $p < 0.01$, ***highly significant ($p < 0.001$)

3.2 GC-MS analysis for detected organic pollutants

Organic toxic compounds are released in many stages of industrial alcohol production, i.e., acid digestion, fermentation, and methanogenesis. Several studies have identified similar toxic compounds from the distillery industries sludge (Bhargava and Chandra, 2009; Kumar

and Chandra, 2020). Distillery sludge from sites D1, D2 and D3 appeared to have a distinct set of compounds. The major peaks detected from the sludge samples are presented in Fig. 2, and the compounds are listed in Table 2. The majority of the compounds have not been reported in distillery sludge before. Many of the organic compounds found in sludge samples are hazardous, the toxicity of each compound is briefly described in Table 2 (Mukherjee et al., 2017; Ngo et al., 2020; Ojuederie et al., 2017). The volatile nature of some of the organic compounds in the samples caused harmful health and environmental effects (Tripathi et al., 2021a & b; Igiri et al., 2018; Selvi et al., 2019). Several compounds were found in all samples, such as (3-methoxyphenyl) trimethylstannane, Lucenin 2 and compactone. These recalcitrant compounds were released during the multistage fermentation of molasses melanoidin in alcohol production, but they could not be biodegraded and detoxified by bacterial communities. In contrast, other organic compounds were only detected in D2 and D3, probably as metabolic by-products resulting from the degradation and detoxification processes by microorganisms and also the recombination of ionic bonding of pollutants during effluent run flow and mixing of domestic water (Ameen et al., 2020; Govarthanan et al., 2018).

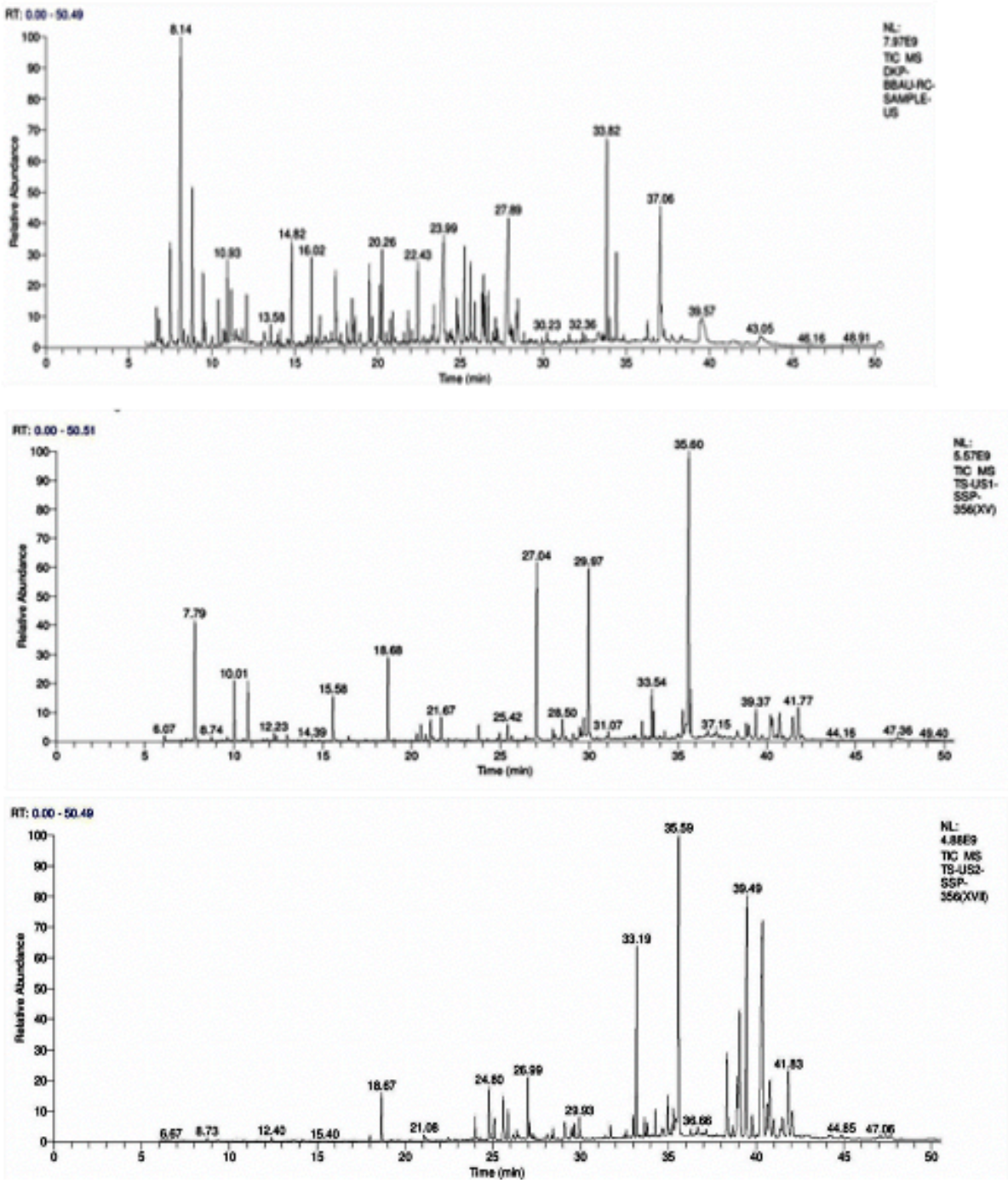

Fig. 2. The GC-MS chromatogram of distillery industry sludge from sites D1 (top) at the discharge site, D2 (middle) at 1 km away, and D3 (bottom) at 2 km away.

Table 2.Organic compounds identified in distillery waste sludge collected from dumping site located (D1) in premises of Unnao Distilleries & Breweries Limited, 1km (D2) and 2 km (D3) away downstream and their toxicity profile.

S.No.	RT	Compound	D1	D2	D3	Toxic Properties
1	6.04	Arabinitol, pentaacetate (CAS)	+	-	-	Headache, dizziness,
2	7.79	Hexanoic acid, trimethylsilyl ester (CAS)	-	+	-	Tiredness, nausea and vomiting
3	8.14	Pantolactone	+	-	-	Asthma, Carcinogen
4	8.73	E-2-(4'-Tolyl)-1-(phenylsulfonyl)ethene	-	-	+	diarrhea and abdominal pain
5	10.01	HEPTANOIC ACID TMS	-	+	-	Very toxic to aquatic life
6	10.93	(8S)-8-Hydroxypatchoulol	+	-	-	Asthma, Carcinogen
7	12.23	Octanoic acid, trimethylsilyl ester	-	+	-	nausea, vomiting
8	12.40	Kadsulignan D	-	-	+	Headache, nausea, vomiting, diarrhea
9	13.58	Benzoic acid trimethylsilyl ester	+	-	-	Eye and skin irritation
10	14.39	Nonanoic acid, trimethylsilyl ester	-	+	-	Eye and skin irritation
11	14.82	2-(1-Hexynyl)benzeonitrile	+	-	-	Central nervous system (CNS) and cardiac
12	15.40	Lupane-3á,12à,28-triol	-	-	+	dizziness, nausea and vomiting
13	15.58	Benzenepropanoic acid, trimethylsilyl ester	-	+	-	Harmful in contact with skin
14	16.02	Nonanoic acid, trimethylsilyl ester	+	-	-	Acute exposure to hexadecane causes irritation,
15	18.36	(3-Methoxyphenyl)trimethylstannane	+	+	+	Asthma, Carcinogen
16	18.68	Decanoic acid, trimethylsilyl ester (CAS)	-	+	-	Serious eye damage/eye irritation
17	20.26	1-Ethoxy-4-hexyl-5-(TMS)-1,4-pentadiene	+	-	-	Vision loss, Movement disorders
18	21.08	Dodecanoic acid, trimethylsilyl ester (CAS)	-	-	+	Degenerative bone disease,
19	21.67	n-Tridecanoic acid, trimethylsilyl ester	-	+	-	epigastria pain
20	23.99	Benzeneacetic acid	+	-	-	kidney and proximal tubule cells
21	24.80	Phthalic acid	-	-	+	Endocrine disruption,
22	25.42	n-Pentadecanoic acid, trimethylsilyl ester	-	+	-	Inflammation and irritation
23	26.99	Hexadecanoic acid, trimethylsilyl ester	-	-	+	Cough. Sore throat.
24	27.04	Sclerodin	-	+	-	Skin & Eye Redness.
25	27.89	á,á,c-triphenyl-benzenepropanol	+	-	-	Movement disorders,Prognosis
26	29.81	Lucenin 2	+	+	+	Difficulty in Breathing
27	29.93	Octadecanoic acid, trimethylsilyl ester	-	-	+	Endocrine disrupting
28	29.94	Hexadecanoic acid, butyl ester	-	+	-	Endocrine disrupting
29	29.97	9,12-Octadecadienoic acid (Z,Z)TMS	-	+	-	Bloody vomiting
30	30.23	3-(4-Ethoxybenzoyl)acrylic acid	+	-	-	dilate the blood vessels
31	32.36	Octadecanoic acid, butyl ester	+	-	-	Endocrine disruption
32	33.19	Dehydrobenzo[20]annulene	-	-	+	Acute toxicity
33	34.06	Dimethoxy[tri(trimethylsilyl)methyl]silane	-	+	-	Acute renal failure
34	34.39	COMPACTONE	+	+	+	Carcinogenic, allergy reaction
35	35.59	5,10-dihydrobenzo[b][1,8]naphthyridine	-	+	+	Hypertension, Stress
36	37.06	cis-Thujan-3-one 2,4-dinitrophenylhydrazone	+	-	-	Acute toxicity
37	39.37	Ethyl2-phenyl-5-bromo-5-(2'-bromoethyl)-selenazoline-4-carboxylate	-	+	-	Osteoporosis, urinary stones,
38	39.49	á-Sitosterol trimethylsilyl ether	-	-	+	Gastrointestinal
39	39.57	Pregnan-18-oic acid, 3,9,11,20-tetrol,3,11-diacetate, 18,20-lactone	+	-	-	Lungs, thorax difficulties
40	41.83	Silane, trimethyl[[(3á,5à)-stigmastan-3-yl]oxy]-	-	-	+	Lung cancer
41	43.05	3á-Chloro-5-cholestene	+	-	-	Hematological, Respiratory
42	44.85	7,22-Ergostadienone	-	+	-	Diarrhoea, Fever, Vomiting

RT- Retention time (in minutes), **C**-control, + present, − absent, **TMS**-trimethylsilyl

3.3 Diversity analysis and percentage abundance of the sludge samples

The structure of bacterial groups in the samples was analysed using NGS technology to gain a new insight into the bacterial diversity. The V3 and V4 hypervariable regions (16S rRNA) were selected for the taxonomic assignment using QIIME to examine, compare, and

determine the composition of bacterial communities in contaminated sites, D1, D2, and D3. In this study, 240376; 288604, and 333147 raw reads were collected from the D1, D2, and D3 samples, respectively. The lengths of sequences were 250 bp, and GC contents were 50.44, and 36.67%, respectively in D1; 250 bp, and GC contents were 51.99, and 36.27%, respectively in D2; and 250 bp, and GC contents were 52.59, and 36.28%, respectively in D3. The Operational Taxonomic Units (OTU) and taxonomy classification were performed using the pre-processed consensus V3-V4 sequences. Pre-processed reads from all samples were pooled and clustered into OTUs based on their sequence similarity using Uclust program (similarity cuto. = 0.97) available in the QIIME software. A total of 13033 OTUs were identified from 544497 reads. From 13033 total OTUs, 10054 OTUs with less than 5 reads were removed and 2979 OTUs were selected for further analysis. In D1, D2, and D3, estimates of alpha diversity indices revealed a higher phylogenetic diversity and evenness. The rarefaction based on Mothur v.1.21.1 was conducted (Fig. 3) to reveal the diversity indices in D1, D2, and D3, including ACE, Chao1, Shannon, and Simpson (Table 3). The Shannon diversity indices and Chao1 species richness were calculated using rarefaction sampling for the estimation of alpha and beta diversity of D1, D2, and D3 sludge samples (Fig. 4). The Shannon and Simpson diversity indices provided inference on the community composition and relative abundance of different species. Both indices suggested higher species richness in samples from site D1than D2. Both ACE and Chao-1 provided indication of species richness in terms of abundance-based coverage, Chao-1 gave more weight to low abundance species. Our data showed again D1 had higher species richness even though these were not in high abundance. This could be explained by the pollution parameters, the bacterial diversity and structure of D1, D2, and D3 microbial communities. Site D3, which was furthest away from the source (2 km downstream) and experience highest mixing and dilution had the lowest microbial diversity and richness.

Table 3. Diversity indices of in distillery waste sludge samples collected from dumping site located (D1) in premises of Unnao Distilleries & Breweries Limited, 1 km (D2) and 2 km (D3) away downstream.

Sample	Ace	Chao 1	Simpson	Shannon
D1	45653.847317	36706.548099	0.001298	10.181837
D2	49655.893298	25987.712398	0.0000987	6.655499
D3	30654.883098	30345.534321	0.002098	8.311326

This is the first report of the full-scale metagenomics analysis of sugarcane molasses-based distillery effluent discharge, and the study of coordination between pollutants in the contaminated site and bacterial communities. The phyla Proteobacteria (28-39%), Firmicutes (20-28%), Bacteriodetes (9-10%), Actinobacteria (5-10%), Tenericutes (1-9%) and Patescibacteria (2%) were the predominant bacteria in all three sites (Fig. 4). Euryechaeota, were detected in sites D1 and D2 (1-2%) but absent in D3. Spirochaetes (5%), Sinergistetes (2%) and Cloacimonetes (1%) were only detected in samples from site D1. The phyla Firmicutes, Cloacimonetes, Actinobacteria, Spirochaetes, Sinergistetes and unclassified bacteria had also been reported in the pit mud of a Luzhou-flavour liquor distillery in China (Liang et al., 2016). Our findings indicated the significant variance of the relative abundance of the bacterial phyla of the samples from the three sites, suggesting the importance of biogeochemical measurements with integrating metagenomic analysis for the study of ecology. Differences in the microbial diversity in these sites could be attributed to the physico-chemical parameters of sludge, contaminant loads, chemical compounds or environmental conditions (Chandra et al., 2018a; Tripathi et al., 2021a). At the origin of the discharge (site D1) where levels of organic and metal pollutants were high, and site D2 that was one km way with mixing with domestic wastewater, a greater bacterial diversity were observed than in site D3 which was furthest away from the distillery site. The bacteria in site D1 could be potential candidates for the detoxification and biodegradation of the persistent organic and metal pollutants in distillery industry sludge.

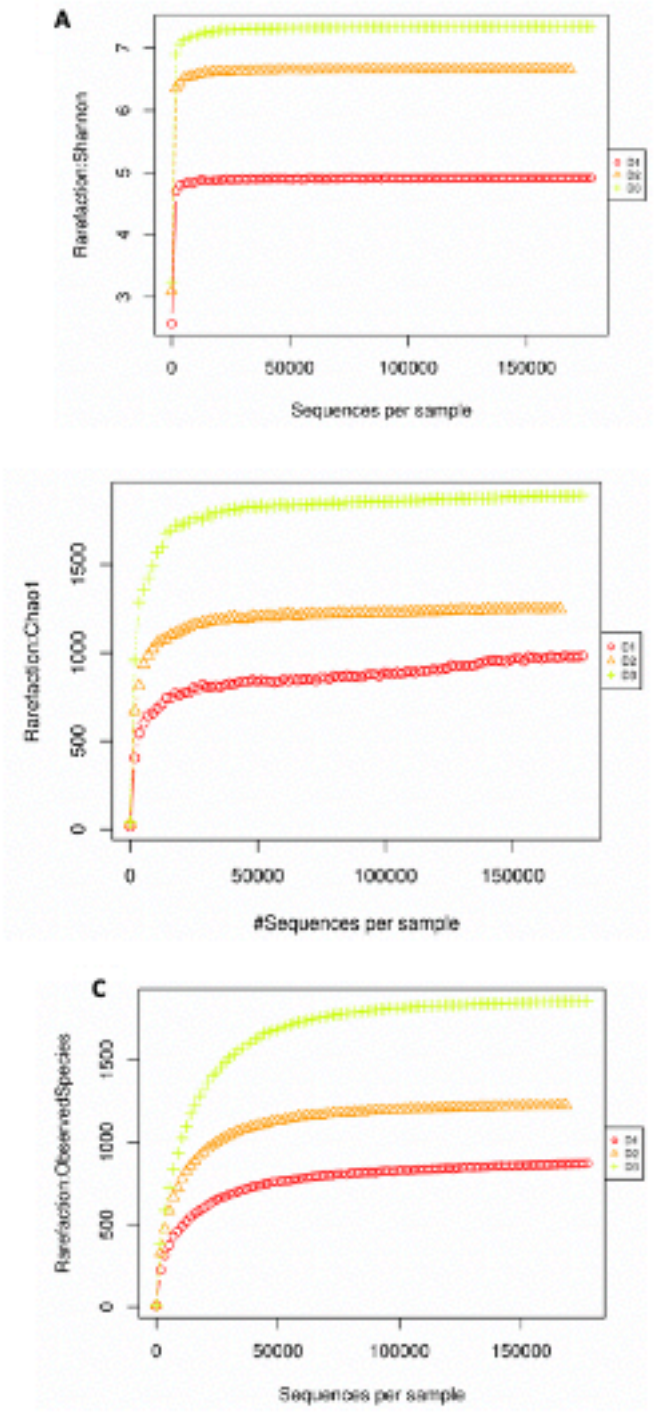

Fig.3 (A). Shannon curve obtained for the samples **(B).** Chao1 curve obtained for the samples **(C).** Observed species curve obtained for the samples

At phylum level, Proteobacteria comprises the highest relative abundance of bacteria in all three sites but was noticeably lower in site D1 (28%) (Fig. 4). Although members of

Proteobacteria could bioremediate hazardous organic and metal pollutants generated during alcohol production (Ahmed et al., 2018), many were not able to tolerate the high stress in the presence of refractory organic contaminants present in site D1. The pollutants were diluted further along the watercourse in sites D2 and D3 site (Table 2) and the proportion of proteobacteria increased to 39% and 36%, respectively. Similarly, reduction in the abundance of Actinobacteria was observed in site D1. Although members of Actinobacteria had the ability to degrade a wide range of hydrocarbons, pesticides, and aliphatic and aromatic compounds, the high concentration of the organic and metal compounds present in the sludge in site D1 affected their survival adversely. In contrast, an increase of relative abundance in both the phyla Firmicutes and Tenericutes was observed in site D1. The percentage of Firmicutes and Tenericutes in samples obtained from site D1 was found to be approximately1.4 times and 3 times higher than the other sites, respectively (Fig. 4). The higher percentage reflected the larger number of organic and metal compounds detected in site D1 (Table 2) and the ability of bacteria to metabolise these organic pollutants as carbon and nitrogen sources (Sharmin et al., 2013; Womersley, 2006). Members of the phylum Firmicutes have a broad range of applications, such as in the anaerobic treatment process of sludge to degrade and detoxify a vast range of substrate present in the distillery sludge contaminated environment. The production of volatile fatty acids by members of Firmicutes could be utilised by other microbial groups. The lignocellulosic materials in sugarcane bagasse and sugarcane molasses offered an abundant source of organic and inorganic materials, Firmicutes could utilize these sugar and organic compounds as a sole carbon and nitrogen source (Kumar and Chandra, 2020). Previous studies also observed Firmicutes as the dominant phylum in the bioremediation of sugarcane distillery waste (Sharmin et al., 2013) and wastewater (Deval et al., 2017).

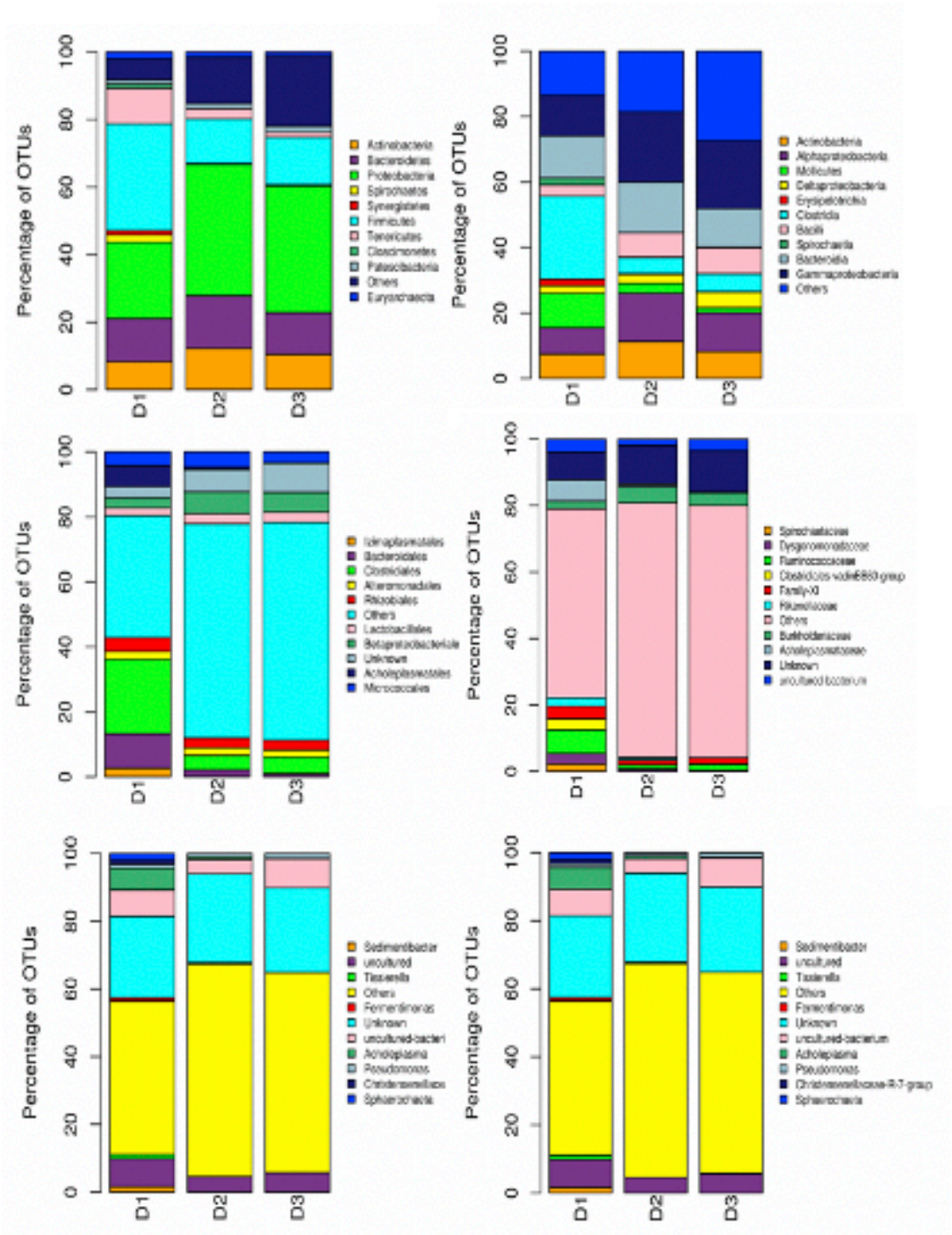

Fig. 4 Classification and percentage relative abundance in distillery waste sludge collected from dumping site located (D1) in premises of Unnao Distilleries & Breweries Limited, 1 km (D2) and 2 km (D3) away downstream. Top left = phylum level; Top right = Class level; Middle left = Order level; Middle right = Order level; Bottom left = Family level; Bottom right = Genus level.

Other significant difference in diversity between the three sites include the detection of Spirochaetes, Sinergistetes and Cloacimonetes in site D1 only and Euryechaeota was highest in site D1 but none was detected in D3. Sinergistetes was a group of filamentous bacteria

associated with bulking and foaming events in the distillery sludge due to the presence of organic pollutants (McIlroy et al., 2016). Cloacimonetes had been associated with distillery sludge (Liang et al., 2016) and Euryechaeota was a phylum of archaea that can tolerate extreme environment. These bacteria were well-suited to survive in the hostile environment of the distillery sludge but could be outcompeted by bacteria of other phyla when the sludge samples were mixed and diluted further downstream in sites D2 and D3. The phylum Bacteriodetes appeared to be evenly distributed in all three sites. They were well known for the degradation of persistent organic pollutants present in distillery waste due to their hydrolytic capacities (Liang et al., 2017).

The metagenomic analysis in class level provided new insight into the diversity of the bacterial community. Both Alphprobacteria and Gammaprobacteria belonged to the Proteobacteria phylum and showed a reduction in abundance observed in site D1 compared to sites D2 and D3. Interestingly, Deltaprobacteria was significantly higher in site D1(Supplementary materials), probably reflecting the predominant sulfur-reducing and iron-reducing members of the group that were able to thrive on distillery sludge containing high sulfate and iron content (Table 1). Interestingly, Erysipelotrichia and Spirochaetia were the only two classes of bacteria observed in site D1, the former belonged to the phylum Firmicutes and the latter, phylum Spirochetes. The results again showed the greater biodiversity in the sludge samples closest to the discharge site. Clostridia was the most abundant class in site D1, this class of bacteria could ferment plant polysaccharides and were widely distributed in the environment (Boutard et al., 2014).

Many bacteria were not identifiable to the Order level; the majority of the bacteria were listed as ‘Others’. Notable observation was Betaproteobacteria which was unique to site D1. According to Fazi et al (2005), Betaproteobacteria was associated with the substrates' rich organic carbon in the aquatic environment, their findings were consistent with the results in

this study, as the high organic substrate content and nutrient status of the refractory organic contaminants were highest in distillery sludge D1. Clostridiales and Alostridiales both showed higher relative abundance in site D1 but showed significant reduction in Acholeplasmatale. It was more challenging to interpret the genomic data in family, genus and species levels as the majority of the data were either classified as Others, Unknown or uncultured bacteria (Fig. 4 and Supplementary materials). However, the general pattern continued to show that there was higher biodiversity in the bacterial community in samples collected from D1 than D2 and D3.

Metagenomics analysis of the sludge samples suggested the organic and metal pollutants have a reinforcing relation with the bacterial diversity. Bacterial communities played a crucial role in the bioremediation of refractory organic pollutants in contaminated site (Chandra et al., 2018a; Zuo et al., 2021). The pollutants of sludge could provide the appropriate ecological niche for the growth and development of the bacterial community. This community supports bioremediation and detoxification of organic and inorganic chemical and chlorinated compounds present in the sludge from the distillery effluent during alcohol production.

4. Conclusion

The complex physico-chemical characteristics of distillery sludge has significant impact on the diversity and relative abundance of the bacterial community. This is the first report of bacterial communities in the distillery sludge and their relationships with organic and metal pollutants. The major phyla detected in this study were: Firmicutes, Proteobacteria, Bacteriodetes, Actinobacteria, Euryechaeota, Tenericutes and Patescibacteria. However, at the site of discharge (D1), where the levels of organic and metal pollutants were the highest, it has higher species richness but low percentage abundance. Spirochaetes, Sinergistetes and Cloacimonetes were unique to the discharge site D1. These bacteria were able to tolerate extreme condition and survived in the hostile environment of the distillery sludge. When the environmental pressure was reduced through mixing and dilution of the receiving water, the competitive

advantage was removed and was reflected in reduced bacterial diversity. Many of the compounds identified in the sludge samples are hazardous to the environment and human health, the treatment process was not sufficiently effective to reduce the level of organic and metal pollutants to an acceptable level, additional steps were required to improve the treatment of the distillery discharge. Bacteria detected in the most pollution site, D1, could be potential candidates to remediate the organic and metal pollutants present in distillery sludge and should be further explored as part of the solution to improve the chemical quality of the discharge.

Credit

ST- conceptualization, data curation, investigation, writing -original draft; DP –formal data analysis, writing – review and revision; RC – funding acquisition, supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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