Exploring and Characterizing Cultivable Bacterial Diversity from Dumping Ground in Ambernath, India

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Abstract- *Dumping grounds are potent site for growth and multiplication of extremophiles especially thermophile due to varying composition of carbon and nitrogen source and also high temperature which favours growth. The dumping site selected for isolation in this study contains Industrial and domestic wastes. Physicochemical characterisation of all these isolates was carried out from DSSTL Thane. In the present study, total 08 isolates with different morphotypes were obtained which were further characterised for pH, temperature and salt tolerance. Among the eight isolates, isolate CM-55 showed growth up to pH 9, Salt 4 % and able to tolerate temperature up to 950C. Isolate CM-55 was further characterized using 16SrRNA gene sequencing. Its taxonomic classification was revealed to beclosest taxonomic neighbour of Bacillus halotoleransATCC 25096(T) with 99.84% sequence similarity.*

Keywords- Dumping ground;Bacterial diversity; 16S rRNA gene sequencing.

I. INTRODUCTION

Dumping ground is a site where different wastes such as domestic waste, industrial waste, and commercial waste are disposed. When the waste is deposited on land, the microorganisms from soil start decomposing and they colonize on garbage, carrying out the degradation and transformation of degradable (organic) materials in waste[Staineret al., 1989]. These microorganisms use waste components as nutrients. Nowadays, disposal of waste is a problem that is growing with human population and civilization.

In the present study, the dumping ground of Ambernath is selected for this study. It is full beyond its capacity, under unorganized conditions. Studies have shown that soil and ground water ecosystems can be polluted due to poorly designed waste disposal facilities[Amadiet al., 2012]. Waste is dumped in open and burnt. Smoke coming from there poses public health risk. Garbage collectors collect all the waste from the markets, housing societies, hotels etc. and collect them and transfer them to dumping grounds. Nearly 95% of the waste generated in the city is disposed off in this manner. The waste at the dumping ground is covered with debris and spread evenly in layers. The organic waste undergoes natural decomposition and generates a fluid, which is known a leachate, and is very harmful to the ecosystem, if not treated properly. The leachate penetrates the soil and, if not prevented, pollutes the ground water. Also, flies, mosquitoes and many other pests breed on the waste and unless properly maintained, the dumps are a public health hazard. Hence waste management is an important step [Bilgiliet al., 2007].

Industrial as well as domestic wastes are dumped in Ambernath dumping ground. The management of household, commercial and industrial waste over there is problem that is increasing with population.Due to disposal of industrial waste including chemicals, heavy metals and repeated burning of wastes etc. temperature of soil is high (ranging from 30o C to 50oC). It is also observed that pH of soil is alkaline (around 8). Thus such conditions in the dumping ground are extreme favouring the growth of extremophiles such as thermophiles and alkalophiles /alkalotolerant organisms. Using these organisms with an ability to grow in such extreme conditions and also decompose the waste possess high potential. Also, microorganisms can carry out much of the natural degradation that slowly remediates pollutants. There are number of specific mechanisms involved which include biodegradation often through the biosynthesis of enzyme, induced precipitation, bioregeneration of granular activated carbon[Nath K andBhakhar MS, 2010]. Usually, soil of dumping site has high content of heavy metals, minerals, and salt etc.[J. O. Azeezet al., 2011]. Thus, heavy metal degrading organisms can be explored and the can be used for bioremediation purpose. Also, all types of these extremophiles can be tested for production of various enzymes such as amylase, cellulase, pectinase, lipases which are thermostable and can be used in industry as option to polluting chemicals used for same purpose.

Thus, this study was aimed to isolate bacterial diversity which can tolerate extreme conditionsand can be

used for bioremediation as well as for industrial purpose. Here, in this study, total of 08 samples were collected from Ambernath dumping ground in different sampling area with different depths so that there is high probability of getting variety of organisms from soil. Bacteria isolated from this study can be further tested for their application in waste management and enzymes produced by them can be tested for industrial application.

II. MATERIAL AND METHODS

Sample collection

10 Different soil samples were collected from dumping ground of Ambernath which were taken from different sampling sites. All the samples were processed (air dried, crushed, passed through 2mm sieve) and refrigerated at 4^{o} C till it was used for isolation.

Physicochemical analysis of soil samples

Different physiochemical parameters were analysed for all the soil samples such as pH, Organic carbon, Phosphorous, Potassium and Sodium content of soil.Methods used to determine above characters were standard methods used in government soil testing lab (DSSTL, Thane).

Bacterial isolation from soil samples

For isolation of bacteria, 1gm of each soil sample was dissolved in sterile saline and serially diluted. Considering the high turbidity in lower dilutions, only 10^{-3} and 10^{-5} dilutions of each sample were plated on sterile Nutrient agar plates using spread plate method. All the plates were incubated at 37^{o}C for 48 hours. After incubation, all the plates were observed, isolated colonies were selected and sub-cultured to get pure culture.

Characterization of bacterial isolates

Morphological characterization of the isolates was carried out by Gram staining and motility. Also different biochemical tests like IMViC, citrate utilization, sodium thioglycolate, Urease, catalase, Nitratase, TSI and sugar utilization test were performed.All the tests were performed in triplicates using standards methods to confirm the obtained results and reduce error. Also biochemical tests of *Staphylococcus aureus* and *Escherichia coli* were performed to detect errors during media preparation (controls).

Hemolytic activity test

All isolates were spot inoculated on sterile Super Imposed Blood Agar (SIBA) plates. SIBA plates consisted of a layer of blood agar overlaid on sterile NA. Plates were incubated at 37^{o}C for 24 hours. After incubation haemolytic pattern was observed.

Effect of pH on growth

Pure culture of each isolate was inoculated in sterile nutrient broth of different pH ranging from 7 to 11 in different test tubes. Test tubes were incubated at 37^{o}C for 24 hours. After incubation all test tubes were observed. Turbidity observed in the test tube were compared with the control tube respectively.

Effect of temperature on growth

All the isolates showed temperature tolerances up to 70^{o}C therefore were subjected to different temperatures ranging from 80^{o}C to 100^{o}C for 10 minutes in test tube and then incubated at 37^{o}C for 24 hours. After incubation all test tubes were observed for turbidity and results were noted.

Effect of salt concentration on growth

Sterile test tubes containing nutrient broth of different salt concentration ranging from 1% to 6% were prepared. Each isolate was inoculated in these test tubes and incubated at 37^{o}C for 24 hours. After incubation each test tube is observed for turbidity.

Thermophilic activity

All the isolates were streaked on sterile nutrient agar plates and incubated at 55^{o} C for 48 hours to check the thermophilic nature of the isolates.Further the production of amylase enzyme was checked for the isolates growing at high temperature.

Amylase activity

CM55 pure culture was spot inoculated on 2 sterile starch agar plates. 1 plate was incubated at 37^{o}C and other was incubated at 55^{o}C for 24 hours to detect amylase enzyme production and also the effect of high temperature on enzyme production. After incubation iodine was added to the plate to observe zone of starch hydrolyses.

Taxonomic classification based on 16S rRNA gene sequencing

The isolate CM55 was purified by re-streaking of isolated single colony and was preserved in glycerol stocks in deep freezers (-80°C). Simultaneously, bacterial isolatewas identified using 16S rRNA gene sequencing using universal bacteria specific primers(27F: 5'GAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-TACGGYTACCTTGTTACGA-3')as described in previous reports (Prakash et al., 2014). Sequencing was carried out using 96 capillary DNA Analyzer3730XL (Applied BioSystems, USA) as described in previous reports.The sequences obtained were quality checked, trimmed and assembled with DNASTAR SeqMan Pro v10 andlater identified using EzTaxon database. Additionally, phylogenetic analysis of these sequences was carried out as described by Prakash et al. (2014).

III. RESULTS

Sampling and physicochemical analysis of soil samples

In total, 8 soil samples were collected from dumping ground from different sampling sites. The pH of the soil ranged from 7.2-8 indicating slightly alkaline nature of the soil. The soil health analysis carried out to understand the chemical nature of the soil revealed the extremely high concentrations of Organic carbon, Potassium and Phosphorus the standard range (See Figure 1A-1C). While, the concentration of Sodium for few samples was within the standard range while for other samples it was higher than the standard range (see Figure 1D).The results of physicochemical analysis indicate that all the parameters tested were observed to exceed normal standard values. Mean pH of soil is moderately alkaline (7.75). Mean organic content of dumping ground soil is 3.64% where the normal organic carbon content is between 0.40 and 0.60%. Mean phosphorous content of the dumping ground soil observed is 137.65Kg/ha. Mean concentration of potassium in soil is observed as 4857.44kg/ha which is very very high from normal standard value 150-200kg/ha. Mean concentration of sodium in the dumping ground soil is 14.22meq % which is between the normal range 5-15meq%.Thus, this analysis revealed that the soil samples collected from the dumping ground is of alkaline nature with extremely high concentrations of the above mentioned chemical elements.

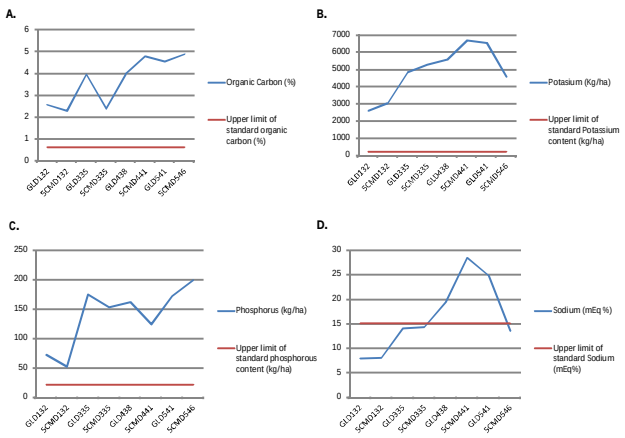

Figure 1.Chemical analysis of the soil samples. The illustration describes the concentration of the particular chemical component across different samples along with the upper limit of the standard range as per the guidelines. (A) Organic carbon content (B) Potassium content (C) Phosphorus content (D) Sodium content

Bacterial isolation and characterization

After spreading the dilutions on media plates from the dilutions 10^{-3} and 10^{-5}, in total, 9 different morphotypes (see Table 1 and Table 2) were further purified and subjected for characterization. Gram staining, motility, haemolytic activity and biochemical tests were carried out to characterize all the isolates.

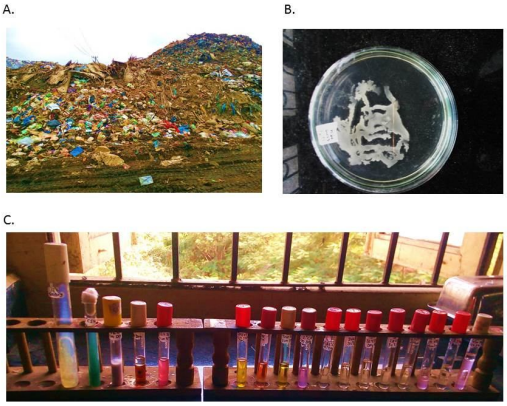

Figure 2.The figure describes the details about sampling site and the characterization of isolates. (A) The dumping ground location from where the soil samples were collected. (B) Motility test result for the isolate CM55 ($_{5CM}D3^{35}$ isolate). (C) IMViC test results observed for the isolate CM55($_{5CM}D3^{35}$ isolate).

It was observed that majority of the isolates were Gram positive cocci, while few were observed to be Gram positive rods. Motility test revealed that out of 9 isolates, 5 were non-motile while the rest were motile organisms (see

Table 2). The results of the biochemical characterization carried out are shown in table 1.It was observed that all the isolates were catalase producers, while most of the isolates were observed to be nitrate reducing bacteria (see Table 1.)

Biochemical	**SAMPLES**								
	$_{GL}D1^{32}$ (white colony)	***$_{5CM}D1^{32}$ (white mucoid colony)***	***$_{5CM}D1^{32}$ (colourless butyrous colony)***	***$_{5CM}D3^{35}$ (off white colony)***	***$_{5CM}D3^{35}$ (yellow colony)***	***$_{GL}D4^{38}$ (orange colony)***	***$_{5cm}D4^{41}$ (light orange colony)***	***$_{GL}D5^{41}$ (off white colony)***	***$_{5cm}D5^{46}$ (0ff white colony)***
IMViC tests:									
1)Indole test	-	-	-	-	-	-	-	-	-
2)Methyl red test	+	+	+	+	+	+	+	+	+
3)Voges-Proskauer test	-	-	-	-	-	-	-	-	-
4)Citrate utilization test	-	-	-	-	-	+	-	-	-
Catalase test	+	+	+	+	+	+	+	+	+
Sodium thioglycolate broth	-	-	-	-	-	-	-	-	-
Nitrate reduction test	+	+	+	+	+	+	+	-	
Urease test	-			-					
Litmus test	+	-	+	+	-	+	-	-	-

Table 1.The table describes the biochemical characteristics such as IMViC test, Catalase test, Nitrate reduction test etc. of all the isolates.

Along with these biochemical tests, haemolytic activity of the isolates was also tested, which helps in characterizing any *streptococcal* species and also as to confirm their non-pathogenicity for their further potential use in the field for bio-degradation. It was observed amongst all the isolates only one isolate ($_{5CM}D35^{35}$ off white colony) exhibited alpha-haemolytic activity (see Table 2).

Effect of various environmental factors

Effects of different growth parameters such as pH, temperature, salt concentration on growth of isolates were studied. The effect of pH was studied for isolates from pH range 7 to 11. It was observed that majority of the isolates were able to grow at the pH 10, while the isolate CM55 ($_{5CM}D3^{35}$)was able to grow at the extreme pH of 11, indicating its alkalophilic nature (see Table 2). Further, isolate $_{GL}D4^{38}$ (orange) was observed to withstand salt concentration as high as 6%, indicating its ability to tolerate high salt concentration; while very few of other isolates could withstand the salt concentration of 3% and above (see Table 2). The effect of temperature, thermo-tolerance ability of all the isolates was checked using the Thermal DeathPoint (TDP) method. It was observed that almost all isolates were able to tolerate the high temperatures up to 100°C (see Table 2).

Further, all the isolates were streaked on nutrient agar plates and incubated at 55^{o}C for 48 hours to check their thermophilic nature. It was observed thatthe isolate designated as CM55 was observed to be able to grow at 55^{o}C.This isolate was selected for further characterization using molecular techniques.

Table 2.The table above illustrates the cultural characteristics such as gram characteristics and motility test of all the isolates. Also the observations on the temperature tolerance, growth at various pH and salt concentration for all the isolates are described in the table.

Isolates ↓	Gram character	Motility	Haemolytic activity	Temperature tolerance(TDP)				pH Tolerance					Salt Tolerance					
				80ºC	90ºC	95ºC	100ºC	7	8	9	10	11	1%	2%	3%	4%	5%	6%
GL$D1^{32}$(white colonies)	Gram positive cocci	Non motile	γ-hemolysis	+	+	+	+	+	+	+	-	-	+	+	-	-	-	-
5cm$D1^{32}$(white mucoidal colonies)	Gram positive cocci	Motile	γ-hemolysis	+	+	+	-	+	+	+	-	-	+	+	+	+	-	-
5cm$D1^{32}$(white butyrous colonies)	Gram positive cocci	Motile	γ-hemolysis	+	+	+	-	+	+	+	-	-	+	+	+	-	-	-
5cm$D3^{35}$(white colonies)	Gram positive rods	Motile	α-hemolysis	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-
5cm$D3^{35}$(yellow colonies) isolate CM55	Gram positive rods	Non motile	γ-hemolysis	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-
GL$D4^{38}$(orange colonies)	Gram positive cocci	Non motile	γ-hemolysis	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+
5cm$D4^{41}$(light orange colonies)	Gram positive cocci	Non motile	γ-hemolysis	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
GL$D5^{41}$(white colonies)	Gram positive cocci	Non motile	γ-hemolysis	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
5cm$D5^{46}$(white colonies)	Gram positive cocci	Motile	γ-hemolysis	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-

Amylase activity

In plate, incubated at 55°C zone of starch hydrolyses is of 26.5mm and in a plate incubated at 37°C zone observed was of 24.5mm.This suggests that enzyme production was observed to be more effective at 55°C than at 37°C.

Phylogenetic analysis of isolate CM55

The 16S rRNA gene based sequencing yielded a 1250 bp long amplicon sequence which was further used for taxonomic classification using EzTaxon database. The sequence similarity based analysis using EzTaxon database should that *Bacillus halotolerans*ATCC 25096(T) is the closest taxonomic neighbour of isolate CM55 with 99.84% sequence similarity.Further the phylogenetic analysis carried out using Neighbour Joining (NJ) and Maximum Likelihood (ML) method showed that the isolate CM55 shared the taxonomic clade with *Bacillus subtilis subsp. spizizenii* NRRL B-23049 (T).Both the phylogenetic methods employed (See figure 1 and figure 2) clearly demonstrate the phylogenetic relatedness of the isolate CM55 with *Bacillus subtilis subsp. spizizenii*.

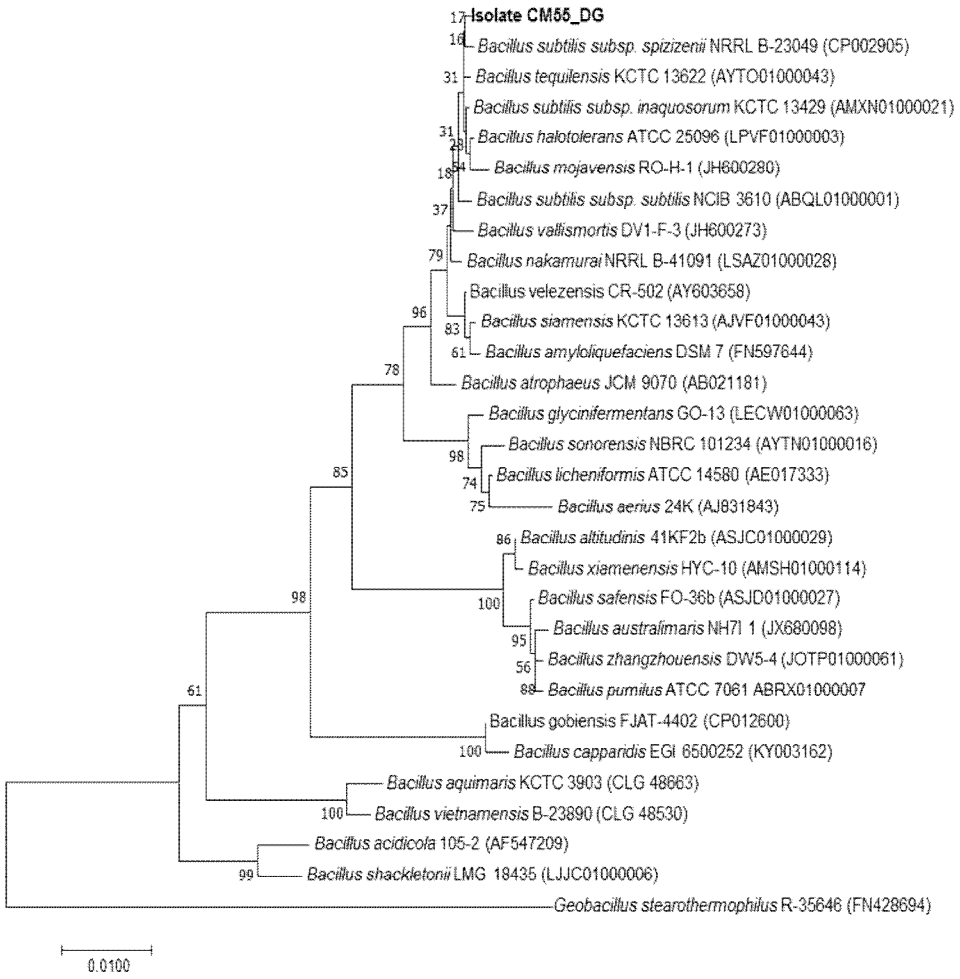

Figure 3.**Evolutionary relationships of taxa**.The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 0.24031135 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura 3-parameter method [3] and are in the units of the number of base substitutions per site. The rate variation among sites was modelled with a gamma distribution (shape parameter = 1). The analysis involved 30 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1244 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [4].

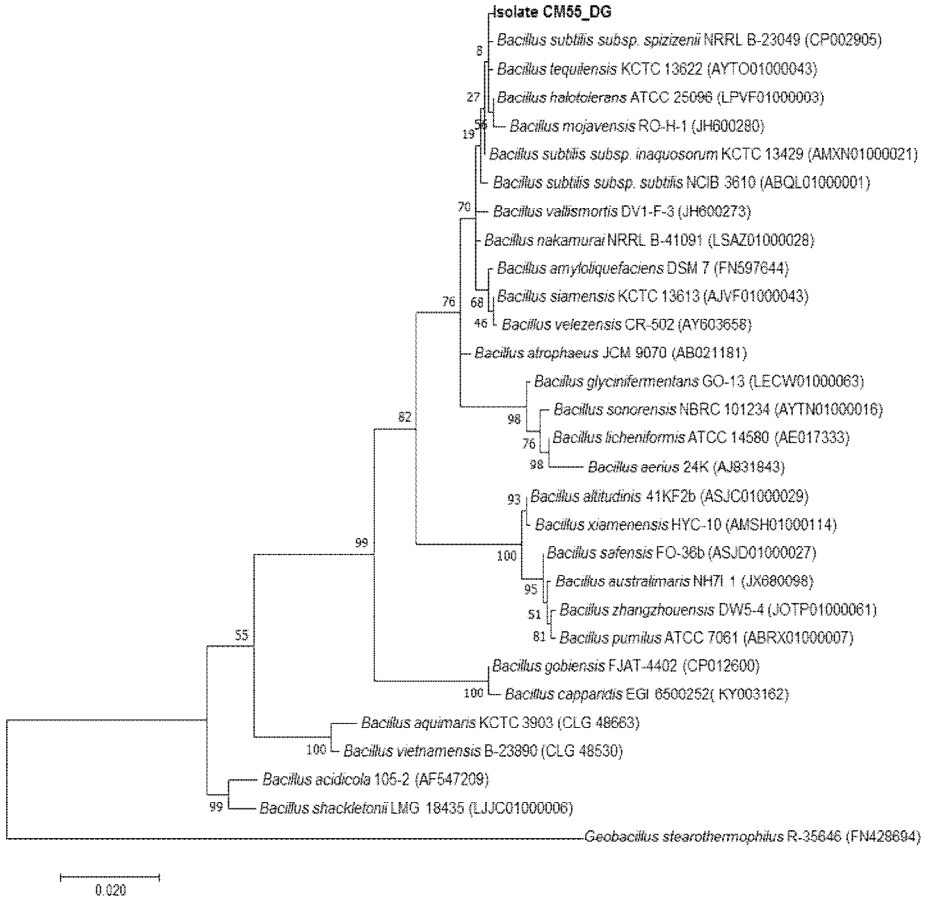

Figure 4..Molecular Phylogenetic analysis by Maximum Likelihood method.The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura 3-parameter model [1]. The tree with the highest log likelihood (-3364.91) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.5138)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 66.21% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 30 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1229 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [2].

IV. DISCUSSION

Our investigation on the soil chemical analysisshowed that the soil samples collected from the dumping ground harbour extreme conditions such as high salt concentration etc. due to large amount of chemical waste disposal and improper waste management. There is need of quick action for waste management, because these conditions are creating hazard to environment as well as to the public health.

In the current microbial analysis of soil total 9 different bacterial isolates were obtained. 3 of them are pigment producing bacteria. The bacterial isolate designated as CM55 was identified by 16s rRNA and after phylogenetic analysis it was found to be Bacillus subtilis subsp. spizizenii.This isolate is able to grow at 55°C as well as at 37°C. Also it produces thermostable amylase enzyme. It was observed that amount of enzyme produced is high at 55°C than at 37°C. This thermostable amylase enzyme can be purified further and checked for its industrial application. There are many potential and widely used applications of this enzyme on the industrial front of Paper industry, Detergent industry and in textile industries for desizing of textiles. Also, Cellular components of thermophilic organisms (enzymes, proteins and nucleic acids) are also thermostable. Apart from high temperature they are also known to withstand denaturants of extremely acidic and alkaline conditions. Thermostable enzymes are highly specific and thus have considerable potential for many industrial applications. The use of such

enzymes in maximising reactions accomplished in the food and paper industry, detergents, drugs, toxic wastes removal and drilling for oil is being studied extensively.

Among the other isolates, an isolate designated as 5cmD^{33}5(yellow) is an alkalophile and able to grow at pH 9. It can also withstand temperature of 100º C.This isolate can be further tested for production of enzyme which can tolerate alkaline conditions. GLD4^{38} (orange) can grow at 6% NaCl concentration. It can be even checked for growth at concentrations higher than 6%. Isolates5cmD3^{35}(white colonies), GLD4^{38}(orange colonies), GLD5^{41}(white colonies), 5cmD5^{46}(white colonies) are able to withstand temperature of 100°C;these isolates can also be further discovered for thermo-tolerant enzymes. Moreover, the ability of these isolates to grow at such high temperatures can be attributed to their thermos-stable cellular components.

All these isolates can be further tested for their capacity of waste degradation as they are able to grow at extreme conditions. In addition, enzyme produced by these extremophiles can be used for different applications. Also the ability of these organisms to degrade the waste can be tested, as they possess potential to grow in the extreme conditions.

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