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SUMMARY OF THE THESIS

A landfill is a man-made engineered facility used for the disposal of municipal solid wastes (MSW). A landfill is a very heterogeneous ecosystem that consists of both bacterial and archaeal populations whose complex and orchestrated metabolism results in a steady state ecosystem. A number of syntrophic associations are involved between bacterial and archaeal populations that lead to the successful catabolism of organic substrates. Because of the complex nature of the niche very few microbiological and molecular phylogenetic studies of the habitat have been conducted all over the world. Moreover, the conditions such as the amount and type of waste entering a landfill facility and climatic conditions vary tremendously from one site to the other. Therefore studies done in one country cannot be extrapolated to sites located in other countries. As far as I know no thorough study of the microbial diversity of a landfill ecosystem has been ever conducted in India. The type of waste disposed off in landfills in our country varies appreciably from that in the developed countries. With India being an emerging global economy, its ever increasing urban population and changing consumption pattern in society has led to a sharp increase in waste generation. Therefore there is a need to manage the MSW in an environmentally sound manner. This would help in decreasing the number of sites required for waste disposal and will also reduce global warming. However, the proper implementation of a landfill management regime will require a thorough knowledge and understanding of the microbiological processes that lead to waste stabilization. Therefore my attempt in this study has been to unravel the type of microbial populations that are involved in waste degradation in a landfill in Chandigarh using both culture dependent and culture independent approaches. From the analysis of this diversity an attempt was also made to correlate the type of bacteria and archaea present in this ecosystem with the succession of reactions which leads to the formation of the major end product, methane.

Chapter 1 of the thesis presents a very brief background of the topic and the objectives of my study.

Chapter 2 describes the materials and methods used for the work

Chapter 3 deals with the results obtained in my research work. A brief summary is presented here.

For my thesis work, the landfill area was sampled twice in consecutive years (2004-2005) at the start of the summer season. During the first round of sampling, the aim was to isolate facultatively anaerobic bacteria and samples were collected from the surface soil and from a depth of 3ft. For isolation of bacteria the surface soil was plated on TSBA and the 3ft soil was plated on three types of media: plate count agar (PCA), carbohydrate fermenting

medium (CFM) and media containing polymers like xylan, gelatin, starch and cellulose. The latter two media were designed specifically for the isolation of fermentative and polymer degrading bacteria as these were expected to be present in greater frequencies in the ecosystem. A total of 183 strains were isolated and preserved from the two samples. For initial analyses 57 strains were selected and characterized using a combination of biochemical, FAME and partial 16S rRNA gene sequence analyses. Section 3.2 and 3.3 describe the results of diversity of culturable bacteria obtained from this round of sampling.

In the process of characterizing the bacterial strains two strains SK 55^T and SK 18^T (isolated from surface soil) were found to be novel taxa at the level of genus (*Paenisporosarcina* gen. nov.) and species (*Microbacterium* sp.) respectively. A detailed phylogenetic analysis of strain SK 55^T also led to the transfer of an established taxon *Sporosarcina macmurdoensis* into this novel genus *Paenisporosarcina*. Strain SK 55 showed close similarity (98.3 %) to *Sporosarcina macmurdoensis* (Reddy *et al.*, 2003) at the 16S rRNA gene sequence level. Phylogenetic analysis showed that these two organisms are not monophyletic with the species of *Sporosarcina* and form a separate clade. This was also supported by fatty acids and polar lipid analysis in this study. The evidences were strong enough to propose a new genus *Paenisporosarcina* to accommodate the strain SK 55 and *S. macmurdoensis* (IJSEM no: IJS/2008/004994, revised manuscript submitted).

Strain SK 18^T was identified as a novel *Microbacterium* sp. for which the name *Microbacterium immunditiarum* sp. nov. is proposed. More than half of the strains (58.6 %, identified in the surface sample) were found to be members of the genus *Bacillus* with 3 strains clustering within the *B. subtilis* clade. Four isolates SK 36, 47, 50 and 52 were phylogenetically closely related to *B. jeotgali*, *B. selenatarsenatis*, *B. boroniphilus* and *B. subterraneus*. The latter two species are known to use heavy metals as their terminal electron acceptors (Kanso *et al.*, 2002; Yamamura *et al.*, 2007). Few isolates (8 out of 29 analyzed) were identified as members of the phylum *Actinobacteria* distributed in six genera *Actinomadura*, *Dietzia*, *Kocuria*, *Microbacterium*, *Mycobacterium* and *Nocardia*.

Similarly, analyses of the diversity of culturable bacteria of the 3ft sample revealed that the most abundant taxa were members of the genus *Bacillus* with 18 isolates out of a total of 28. Two isolates PCA 20 and CFM 20 were identified as *Rhodococcus* and *Arthrobacter* sp whose members are known to metabolize xenobiotic compounds. In fact, the phylogenetically closest relatives of the former two species i.e., *R. pyridinivorans* and *A. chlorophenolicus* are known to degrade the xenobiotics pyridine and 4-chlorophenol respectively.

Only two Gram-negative taxa SK 65 and PCA 19 both members of the class *Alphaproteobacteria* were detected from a total of 57 isolates analyzed in this part of the

study. Both appear to be novel isolates at species level although a few more properties of the strains need to be checked before proposing them as new taxa.

The 3ft sample was also analyzed using the culture independent approach based on the 16S rRNA gene wherein separate libraries were constructed for the analyses of the bacterial and archaeal populations. A phylogenetic analysis of the bacterial library revealed a very limited diversity with all the clones again falling within the radiation of the division *Firmicutes*. Interestingly, almost all (but one) clones were identified as phylotypes within the *Bacillus* rRNA group 2. A single clone C 28B was identified as a member of the family *Paenibacillaceae*. Although expected to retrieve obligately anaerobic bacteria none of the 28 clones analyzed were found to this group. Moreover, Gram negative taxa were prominent by their absence in these clones. Analyses of the archaeal clonal library revealed that the most abundant population were the methanogens with majority of them identified as members of the genus *Methanosarcina*. Few clones were also identified as phylotypes of the genus *Methanoculleus*. This result was in agreement to previous molecular characterization studies of the archaeal population of the landfill ecosystem (Huang *et al.*, 2002, 2003; Chen *et al.*, 2003a, 2003b; Mori *et al.*, 2003; Uz *et al.*, 2003; Laloui-Carpentier *et al.*, 2006).

In addition to taxonomic studies, isolates of the 3ft sample were also checked for their ability to degrade various polymers such as xylan, protein, gelatin and starch. This was done to assess the polymer degrading potential of the isolated strains as these might play crucial roles in degrading similar large molecules in their natural habitat. Indeed many isolates with the potential to degrade the above mentioned polymers were identified in this part of the study.

In the second round of sampling of the landfill site three samples were collected: soil from a depth of 3ft, 5ft and 5.5ft. The aim of this part of the study was to concentrate both on facultative and obligately anaerobic bacteria and, therefore, proper precautions were taken during processing the samples and/or media for their growth. The results of this part of the study are detailed in section 3.5. Metabolic profiling of the community using Biolog system suggested that the sample from the 3ft depth is more diverse compared to the other two samples. For isolation of culturable bacteria the soil samples were plated on three media, plate count agar (PCA), tryptic soya broth agar (TSBA) and TSBA diluted 100 times (TSBA 100). The nutritionally poor medium TSBA 100 was chosen to isolate oligotrophic bacteria. A total of 260 strains were isolated and preserved from the three samples. For initial analyses 31, 21 and 28 isolates from the 3, 5 and 5.5ft samples respectively were selected and tentatively characterized based on results of partial 16S rRNA gene sequencing, oxidation pattern of 95 carbon substrates using Biolog and fatty acid analysis using MIDI system. A

total of 10 isolates appear to be potential novel taxa at either genus or species level (Tables 3.21, 3.41 & 3.81). Strains 5LF 17TD and 5LF 43TD were identified as members of two potential novel genera. Strains 5.5LF 38TD, 48TD, 3LF 16P, 5.5LF 38T; 3LF40T, 5LF 22P and 5LF 19TDLC were identified as potential novel species within the genera *Bacillus*; *Paenibacillus* and *Stenotrophomonas* respectively.

More than half of the identified strains in the second of sampling (63.7 %) were members of the genus *Bacillus*. This was similar to the results obtained in the first round of sampling where more than a half (56.1 %) of the isolates analyzed were identified as *Bacillus* sp. The next most abundant taxon identified were members of the genus *Paenibacillus* that represented 8.7 % of the total isolates analyzed. A few of the latter species showed close phylogenetic relationship to *P. macerans* and *P. cellulosilyticus* that are known to degrade polysaccharides. These results are not surprising as these organisms are known to harbor polymer degrading activities and are in accordance to previous studies where members of the genus *Bacillus* and *Paenibacillus* were the dominant gram-positive taxa isolated from refuse samples (Pourcher *et al.*, 2001). Four isolates (5 %) were identified as members of the division *Actinobacteria* distributed in four genera *Corynebacterium*, *Microbacterium*, *Micromonospora* and *Rhodococcus*. Around 16.2 % of the isolates were distributed in the genera *Brevibacillus*, *Cohnella*, *Lysinibacillus*, *Ornithinibacillus*, *Streptococcus*, *Staphylococcus*, *Terribacillus* and *Bacillus* rRNA group 2. Only 3 isolates (3.7 %) were identified as members of the Gram-negative lineage. Two of them were members of the genus *Pseudomonas* and one appears to be a novel species within the genus *Stenotrophomonas*. This result was in agreement to that obtained from the first round of samples where only two isolates were found to be Gram-negative. At this point of time it is not possible to explain the reason behind the apparently low representation of Gram-negative taxa in the landfill samples. This may not be attributed to the choice of isolation media because media used (TSB, PCA, TSBA 100 etc.) do support growth of Gram-negative organisms. However, it does appear that they are probably present in low numbers as data from culture independent analysis (as explained below) also failed to retrieve Gram-negative phylotypes in the clonal libraries.

Taxonomic study of culturable landfill isolates has led to taxonomic refinements of certain bacterial groups. Phylogenetic analyses of strain 3LF 22T and DNA-DNA hybridization studies led to the proposal for unification of the genera *Pelagibacillus* and *Terribacillus* and transfer of the species *Pelagibacillus goriensis* to the genus *Terribacillus* as *Terribacillus goriensis* comb nov. sp nov. (IJSEM no: 65579, In press). Strain 3LF 29T was identified as *Bacillus silvestris* (belonging to rRNA group 2 of Ash *et al.*, 1991). Phylogenetic

analysis showed its closeness to *Caryophanon latum*, a non-spore forming member of *Bacillus* rRNA group 2. Based on comparative fatty acids profile and phylogenetic analysis, a new genus *Solibacillus silvestris* comb. nov. has been proposed to accommodate the strain and *Bacillus silvestris* (IJSEM no: IJS/2008/003194, revised manuscript submitted). Moreover, some species like *B. sporothermodurans*, *B. thermoamylovorans*, *B. humi*, *B. barbaricus* and *B. clausii* were repeatedly recovered in landfill samples and formed clades separated from other members of the *Bacillus* sensu stricto in our phylogenetic analyses (Figs 3.16-3.19). Preliminary analyses of strains retrieved in this study that were closely related to the above mentioned taxa, highlighted the distinctiveness of this group of *Bacillus* sp. and therefore they should be reclassified by creating new genus (or genera) or transferring them to other existing genera.

For isolation of obligately anaerobic bacteria from the 3ft, 5ft and 5.5ft landfill samples, three types of media were used: reinforced clostridial agar (RCA) for isolation of fermentative bacteria, anaerobic agar (AA) for isolation of heterotrophic populations and enrichment medium for isolation of sulphate reducing bacteria (SRB). Out of a total of 30 isolates recovered under strict anaerobic conditions, 15 were found to be obligately anaerobic and the other 15 were facultative. In addition 10 SRBs were isolated and preserved from the three landfill samples. Three obligate anaerobes and one sulphate reducer were characterized in the study. The three obligately anaerobic cultures were found to be members of the genus *Clostridium* and the SRB was identified as a novel species within the genus *Desulfotomaculum*.

In culture independent study of the 5.5ft sample, separate archaeal and bacterial clonal libraries were constructed from these three samples. In this study detailed phylogenetic analyses was carried out for clonal libraries of the 5.5ft sample. Analyses of the archaeal library revealed limited diversity with all the phylotypes being members of the two methanogenic genera *Methanoculleus* and *Methanosarcina*. This was similar to the result obtained from the analysis of the 3ft sample in the first round of sampling where all 28 clones could be phylogenetically placed within these two methanogenic genera. However, there were quantitative variations in the proportions of the two genera within the two clonal libraries. Majority of the archaeal clones from the 3ft sample were identified as members of the genus *Methanosarcina* whereas those from the 5.5ft sample were affiliated to *Methanoculleus*. This difference in population composition could be explained by the spatial distribution of the methanogenic substrate acetate across the two depths of the landfill site. Overall observation is that both 3ft and 5.5ft samples were rich in methanogenic populations as the major archaeal group whose metabolism is critical for the steady state operation of a landfill site.

Phylogenetic analyses of bacterial clone sequences from the 5.5ft sample placed them within five bacterial phyla with majority belonging to the phylum *Firmicutes* (~ 66.6 %). Besides *Firmicutes*, other four phyla *Actinobacteria*, *Chloroflexi*, *Gemmatimonadetes* and *Thermotogae* were represented by one clone each. In contrast to the results obtained previously for the 3ft sample, majority of the clones analyzed (61 %) were phylotypes showing close relatedness to sequences retrieved from anaerobic environments like anaerobic digestors, guts of termites, leachates from municipal landfills etc. The most abundant known taxa recovered in the 5.5ft bacterial clone library were phylotypes of the family *Clostridiaceae*. Some of the clone sequences showed close sequence similarity to species within Clostridial clusters III, IV and XIVa whose members are known to be involved in anaerobic degradation of cellulose (Van Dyke & McCarthy, 2002). Some of the clone sequences showed close phylogenetic proximity to syntrophs that are known to provide substrates for methanogenesis and sulfate reduction. Metabolism of this kind fits perfectly well with a landfill ecosystem as these types of bacteria might play an important role in providing substrates for other trophic groups especially from substrates that are not metabolizable by a single culture. Few of the clones (~ 8 %) were members of the *Bacillus* rRNA group 6 that consists of haloalkaliphilic and/or alkalitolerant species (Fig 4.6). This result is consistent with the pH of the 5.5ft sample that alkaline. An overall assessment of the culture independent bacterial diversity of the 5.5ft sample suggested that the composition of the bacterial population within the phylum *Firmicutes* and other lineages was very diverse and complex (Fig 3.22). Almost all the 52 clones analyzed using ARDRA showed a different restriction pattern indicating heterogeneity in their sequences (Fig 3.20). About 45 % clones showed ≤ 85 % sequence similarity with the sequences of culturable bacteria; the closest ones belong to phylum *Firmicutes*. Whether they represent a new lineage at phylum rank is difficult to comment at this point of time. They were, therefore, tentatively placed in the category of Gram-positive taxa (Fig 4.6). The presence of phylotypes phylogenetically closely related to anaerobic hydrolytic bacteria like cellulose degraders is consistent with the chemical composition and overall metabolic scheme of the landfill environment. Of the 41 clones analyzed, only one clone sequence fell within the radiation of the Gram-negative taxa that had no closest cultured representative and was tentatively placed within the phylum *Gemmatimonadates* because this was the closest one (88.9 %). Low abundance of Gram-negative bacteria in culture independent analysis substantiates the findings of culture dependent approach where only 3.7 % belonged to Gram-negative group.

Chapter 4 discusses the results obtained here in relation to the present knowledge in this area and proposes a possible scheme of degradation of substrates in this environment (Fig 4.11).

Chapter 5 highlights the major findings of the work as in the form of conclusions.