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METAGENOMIC APPROACH FOR MICROBIAL DIVERSITY IN ENVIRONMENTAL SAMPLES

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ABSTRACT

The metagenomics is called as fourth domain of life which deals with the molecular approach for the culturing of the microbes. The development of metagenomics stemmed from the evidence that as-yet-uncultured microorganisms represent the vast majority of organisms in most environments on earth. These microbes hold the secret for generating renewable biofuels and bioremediation. This report throws light metagenomic approach of soil, water; methodologies involved in it and reported prevalent microbes in the above environmental niche. The importance of the bacteria in drug design, drug delivery and important products obtained from them by using the culturing technique called as *Metagenomics*.

KEYWORDS: Metagenomics, Bio-films, Gel-Electrophoresis, cloning and screening.

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INTRODUCTION

Every part of the world is surrounded by the seemingly enormous and differential microbes. The diversity of microorganisms reflects a staggering array of functions. Microorganisms drive most of the chemical cycles on Earth, process waste and plays crucial role in the maintenance of the food web. During the course of time with the development of microscopy and microbiological techniques the microbes were isolated and manipulated in various industries like bakery, dairy, pharmaceutical and many more for the welfare of humans. The microbes dominate each and every part of the environmental niche, by the development of the culturing technique microbiologists begun to appreciate the true magnitude of diversity within microbial communities. The most dominating species of microbes are bacteria, Archea, fungi and viruses contribute the second class. Out of the total prokaryotes many of the prokaryotes are uncultured, is well established that only approximately 1% of bacteria on Earth can be readily cultivated , so called *'great plate count anomaly'*, based on the observation that microscopic counts are considerably larger than the equivalent total viable counts (28),(1),(12). There are currently estimated to be 61 distinct bacterial phyla, of which 31 have no cultivable representatives (13). The topology of the archaeal phylogenetic tree remains uncertain, but it is clear that the 54 species of Archaea cultured to date represent only a fraction of the total diversity, with 49 lineages mostly uncultured (3). Because the majority of bacteria and Archaea remain uncultivable, the diversity of complex bacterial communities is inevitably underestimated using standard cultivation methods. There are many reasons for the uncultivable nature of the bacteria; some of them are listed below:

- Some bacteria are genuinely resistant to culture in isolation on conventional media. Certain bacteria have fastidious growth requirements including the need for specific nutrients, pH conditions, incubation temperatures or levels of oxygen in the atmosphere investigated the effect of different substrates and culture conditions on the growth of bacteria. (17) The growth of a bacterium is not met by the artificial medium and incubation conditions, or if there is competition for nutrients among mixtures of organisms cultured together.
  Growth may also be inhibited by bacteriocins released from other bacteria in a mixed culture or by antibacterial substances present within the medium (30).
- The bacterial biofilms have more than one organism and they have specific interaction, such interaction enable the biofilms to function as the complex unit and also the presence of the mechanism called as *cross feeding* and *metabolic cooperation.* Many of the complex biofilms produce the circulating regulatory molecules that mediate cellular communication. The term 'bacterial cytokine' was coined by (21).The reasons mentioned above cannot be manipulated and made available to the bacteria to culture them *in-vitro*. To overcome this problem many culturing techniques are suggested, out of which *metagenomic*, is the molecular approach for the culturing of the microbes. *Metagenomics* is the culture-independent genomic analysis of microbial communities. The term is derived from the statistical concept of meta-analysis and genomics (24).

METAGENOMIC APPROACH FOR SOIL

Traditional methods for culturing microorganisms limit analysis to those that grow under laboratory conditions (14), (1). The microbes can be isolated directly and they can be explored or exploited directly or by molecular approach. Cultivation and isolation of microorganisms is the traditional method but, as only 0.1% to 1.0% of the soil bacteria are cultivable using standard cultivation methods(31), (1), (32) the diversity of soil microbial communities has been mainly unexplored so the metagenomic approaches are used .To overcome the problem of the direct approach of the isolation procedure the

molecular basis of isolation of microbes can be used in which the direct isolation of the nucleic acid (mainly DNA) takes place rather than the isolation of the complete organism. Theoretically, the microbial DNA isolated from a soil sample represents the collective DNA of all the indigenous soil microorganisms, and is named the *soil metagenome* (11), (23). Soil DNA is recovered through separation of cells from soil particles followed by cell lysis and DNA recovery, or through direct lysis of cells contained within soil and recovery of DNA. Recovered soil DNA is fragmented ligated into the linearized cloning vector of choice which might be a plasmid, cosmid, fosmid or BAC (bacterial artificial chromosome). Following the introduction of the recombinant vector into a suitable bacterial cloning host, screening strategies can be designed to identify these clones which might contain new and useful genes.

EXTRACTION OF DNA

The DNA extraction from the soil is the most challenging process when we proceed towards metagenomic approach is the interference of the complex soil matrix, which contains compounds like humic acid and also polyphenolic compounds, which are co-purified with the DNA. These compounds are difficult to remove, and it is well known that polyphenols also interfere with enzymatic modifications of the isolated DNA (33). Soil is collected (5).Soil is suspended in of buffer (38), and incubated at 60°C for 2 h with occasional gentle shaking. Soil is sieved to remove particulates larger than 1 cm, and no plant roots were visible in the sample. The suspension is extracted with an equal volume of chloroform, and the DNA in the supernatant is precipitated with isopropanol (6). Precipitated DNA is dissolved in distilled water and electrophoresed in a low-melting-temperature agarose gel. After electrophoresis at 100 V for 1 h, a strip from each side of the gel is cut off and stained to localize DNA. DNA-containing regions are cut from the rest of the gel (unstained) and stored overnight in TE (TRIS-EDTA) plus polyamines. In numerous experiments, this method produced DNA of appropriate size and cleanliness for subsequent cloning.

CLONNING AND SCREENING

The construction of the library is usually the same as we use for normal cloning experiment that is fragmentation of isolated soil DNA by restriction digestion or by mechanical shearing, insertion of the DNA fragment into suitable vector system and transformation into suitable host**.** The construction of the library is simple task, but the clone screening after transformation is the daunting task. Screening of these libraries can be done by functional and sequence-based approaches. Two BAC (bacterial artificial chromosome) libraries are constructed in first method the isolated gel zone containing DNA is digested with Gelase. Preparation of the vector pBeloBAC11, digestion of the insert DNA with *Hin*d III, ligation, and transformation is done (24).White colonies are picked onto plates gridded to be compatible with 96-well microtiter plates. The library is replicated into duplicate sets of 96-well microtiter plates with freezing medium and stored at 280°C (6). In second method, Vector preparation is as described above but included a ligation step followed by gel purification to remove any self-ligated product. The pBeloBAC11 vector is subsequently electroeluted from the gel slice and dialyzed against TE (TRIS-EDTA). Isolated metagenomic DNA is run on a preparative pulsed-field gel, and DNA greater than 50 kb is isolated, electro eluted, and dialyzed against TE (TRIS- EDTA). Following *Hin*dIII digestion, insert DNA is loaded onto a second preparative gel and size-selected to retain DNA of 40 kb or larger. Ligation, transformation, and storage steps are performed as described above. Soil metagenomic libraries may contain only few clones that carry gene of interest or the operon. Hence, screening of the libraries is a daunting task. Thus two types of screening are carried out in screening of the soil metagnomic libraries:

1. FUNCTIONAL SCREENING

The functional screening of the libraries is performed in dual-culture assay. This assay

allow target phytopathogenic organisms to grow out over metagenomic library clones arrayed on Petri dishes. Scoring during and following growth is for irregularities / inhibitions in growth of the target organism. (6), (8), (35). This experimental set-up led to the detection of few positive clones (range up to 48 per library), amounting to < 0.05% of positives for all libraries. Such small no of clone concluded that low numbers can be attributed either to a rare occurrence of target genes/operons in the vectors used or the molecular machinery i.e. vectors are larger than the inserts, thus the molecular or the genetic screening is used. Another approach is screening of soil-based libraries for genes conferring polyol oxido-reductase activity. (15), (16) which is based on the ability of the recombinant *E. coli* strains to form carbonyls from polyols. Another example is the detection of *E. coli* clones with proteolytic activity on agar plates containing skimmed milk (10), (26).

2. GENETIC (MOLECULAR) SCREENING

The libraries obtained in the *Metacontrol* project are also screened using molecular tools, such as hybridization and PCR amplification methods (35). The success in detecting novel operons, such as those involved in polyketide biosynthesis, are supposed to depend on the application of deliberate degeneracy in the probes and primers used(6),(8).In this method the total DNA extracted from the soil are used as a target sequence and the genes of polyketide biosynthesis operon (PKS1) are amplified with degenerate primers. The amplicons obtained are used for the generation of probes that would thus detect the relevant PKS sequences in the library (8), (22), (35). This method developed the positive clone in soil metagenomic libraries. The amplicons are then sequenced to check for redundancies and for known PKS sequences. Then, positive clones are identified using colony hybridization with relevant probes.

METAGENOMIC APPROACH FOR WATER SAMPLES

Earth roughly covers 71% of the water of which only 2.5% is the freshwater and the remaining is the ocean water source. Out of the 2.5% the very less water is access able to us as it is in the ice form. The water has been a shelter for many biotic factors including the giant whales in the ocean to variety of the coral reefs, aquatic plants, algae and the microscopic microbes are cultured in labs. In the “OMIC” era the uncultured microbes are cultured, this approach is known as the METAGENOMICS. Marine environments, including the subsurface are believed to contain a total of approximately 3.67×10^{30} microorganisms (37) and with approximately 71% of the earth's surface of 361 million square kilometers covered by the ocean, this environment represents an enormous pool of potential microbial biodiversity and exploitable biotechnology or "blue biotechnology". Fresh water and the related microbial population in the distribution system constitute one of the most extensively studied oligotrophic systems (18). Both the marine and fresh water, cultivated and uncultivated microbes, the comparative sequence analysis of 16S *rRNA* enables the investigation of phylogenetic relationships among microorganisms. More importantly, *rDNA* also provides an environmental signal since microbial populations constantly fluctuate in response to changing conditions, and *rDNA* is generally a reflection of cell abundance. It is also easier to extract and purify DNA than RNA from natural samples without degradation. Thus, a profile of microbial community rDNAs ought to reflect the character of the surrounding environment (20). Also the innovative enzymes and the products can be recovered by exploiting the microbes. The procedure involves sample collection, DNA isolation, cloning, screening, sequencing by informatics tools.

DNA EXTRACTION

The water samples can be collected from normal tap water, drinking water, water from the sewage tanks, surface and subsurface level from river, marine water ,deep sea sediment , fish microbiota, Antarctic sea water, ice samples ,marine hot springs, Sea Hare eggs, Coastal solfataric vent etc. The samples are sequentially filtered through a 30 mm pore-size filter and the

filtrate was passed through a 1.2 mm carbon filter. Finally, this filtrate is concentrated on an ultrafiltration system and the concentrate is filtered on 0.22 mm pore-size polycarbonate filters and stored at -20°C until further DNA extraction. The DNA is extracted by using a cetyltrimethylammonium bromide (CTAB) buffer DNA isolation technique(27).The water samples are then mixed with DNA extraction buffer (100 mM Tris HCl, 100 mM EDTA, 100 mM Na2HPO4 ,1.5 M NaCl, 1% SDS) for several hours. RNA is degraded with RNase A. The resulting DNA extracts are incubated with protease and Sarcosyl in TE(TRIS-EDTA) buffer overnight. Total genomic DNA is then repeatedly extracted with chloroform-phenol (1:1, vol/vol), washed once with chloroform, and dialyzed against of TE(TRIS-EDTA) buffer at 4°C overnight. Finally, an aliquot of the DNA is analyzed on agarose gel to ensure that the DNA is not degraded.

CLONING AND SCREENING

E.coli DH5α is used as host strain and the plasmid pUC 4K, pUC 18 and pUC 19 are employed as vector for the cloning experiments (27).In case of the river water metagenomic the isolated DNA fragments obtained from river water into broad-host-range expression vectors pUC 4K (plasmid University of California encoding kanamycin resistance), pUC 18 (plasmid university of California lab development no:18) and pUC 19(plasmid university of California lab development no:19) encoding ampicillin resistance. Strains are grown in Luria Bertani (LB) medium containing appropriate concentration of antibiotic kanamycin (30 µg/ml) for pUC 4K and ampicillin (50 µg/ml) for pUC 18 and 19) for plasmid maintenance. The isolated DNA and the expression vector are digested with restriction enzyme E-co RI. The digested DNA fragment is gel purified and ligated with T4 DNA ligase (type of ligase enzyme that catalyzes the formation of a phosphodiester bond between juxtaposed 5'-phosphate and 3'-hydroxyl termini in duplex DNA or RNA). The vector containing the insert is transformed into competent cells of *E.coli* DH5α by CaCl2 method for library Construction (4). *E.coli* transformants are selected on Luria Bertani agar plates containing ampicillin (50µg/ml) for pUC 18 and 19, kanamycin (30 µg/ml) for pUC 4K, 5 bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) (20 mg/ml) and Isopropyl β-D-1 thiogalactopyranoside (IPTG) (24 mg/ml) by blue white selection. To determine the average insert size for each library, the plasmids from colonies are extracted and digested with EcoR I and the fragments are analyzed through agarose gel electrophoresis (25). The screening can be done by using RFLP (restriction fragment length polymorphism) and the functional screening. Full-length inserts are screened by restriction fragment length polymorphism (RFLP) analysis. In the full length screening the PCR amplicons are digested with the restriction enzymes. The digested amplicons are separated on the metaphore agarose gel and is compared manually. The clones with the identical restriction patterns are grouped together into RFLP types. The 16S rRNA genes from one representative of each RFLP type in the first library are completely sequenced. Phylogenetic classification is determined using BLAST and the ARB sequence analysis tool.

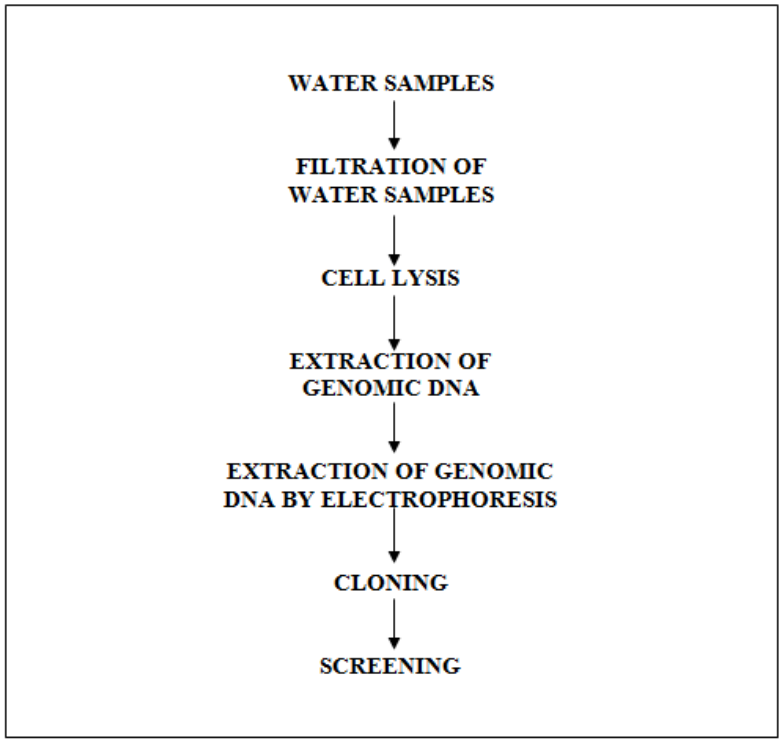

FIGURE 1
A flowchart of METAGENOMIC APPROACH FOR WATER SAMPLES, a general approach

TABLE 1
A tabular representation of the procedure involved in the metagenomic approach of the soil and water samples interfering factors and predominant microorganisms.

Samples	Procedure	Interfering factors	Microorganisms
Soil	Sample collection Sieving Extraction of DNA Library construction Metagenomic library screening	Humic acid Polyphenolic compounds	DNAase-producing organisms Antimicrobial activity of clones. Amylase producing organisms Lipase and hemolytic producing organisms
Water	Sample collection Filtration Extraction of DNA Library construction Metagenomic library screening	DNA of other organisms Particulate matter in water	2,4-D degrading activity. *Actinobacteria* *Alpha-Betaproteobacteria* *Bacteroidetes* *Actinobacteria* *Betaproteobacteria* *Alphaproteobacteria* *Bacteroidetes*

CONCLUSION

Metagenomics is the culture-independent genomic analysis of microbial communities. It is shortly defined as the molecular approach for the analysis of the microbes. It has been reported that only 1% of the microbes are readily cultivated on earth so called *'great plate count anomaly'*. Many microbes which are uncultured from the environmental niche such as hot water springs, biofilms, extreme environment, archea, or some antic viruses through the process called

as metagenomics. Their genomic sequence can be detected and manipulated for the human increasing beneficiaries. The general process of the metagenomics involves sample collection, genomic DNA isolation, cloning, transformation and screening.The soil and water are the cradle of the microbes, which is shared by both cultured and the uncultured bacteria. The metagenomic approach of these vital samples reported that it contains microbes with the DNAase activity, amylase activity, lipase activity, hemolytic activity and other bioactive compounds etc. Similarly in the water metagenomic approaches the different enzyme activity by the bacteria from the different sample is reported. Microbes identified after the screening from soil shows drastic antimicrobial activity for eg. *Streptomyces*, for antagonistic activity against *Bacillus subtilis* 1A72, *Staphylococcus aureus*21, *Enterococcus faecalis* 40, *Escherichia coli* 9, *Pseudomonas aeruginosa* 39, *Fusarium oxysporum* LNPV, *Aspergillus fumigatus* Gasp 4707 and *Neurospora crassa HK*. They exhibited 56% antimicrobial activity against at least *B. subtilis*, 13% against *S. aureus*, 4% against *E. faecalis* and <1% partial inhibition of growth of *Neurospora crassa* mycelium (34). soil from a site near Madison, Wis., that had been characterized previously and found to contain a diverse community of bacteria and archaea(5),(9). The following activities have been identified from metagenomic approach for different soil samples:

(i) DNase-producing clone: The DNase-producing clone SL1-11G4 contains
insert of approximately 25 kb in size. One transposon insertion that abolished activity was located in a potential ORF(Open Reading Frames) with homology to a family of single-strand-specific nucleases typified by S1 nuclease from *Aspergillus oryzae* (19) and including sequences of plant (e.g.,*Hemerocallis*, *Hordeum*, and *Zinnia*), fungal (*Aspergillus* and *Penicillium*), protozoal (*Leishmania*), and bacterial (*Mesorhizobium*)origin(2),(7),(29)

(ii) Antibacterial clone: One clone (SL1-36C7) produced an activity that is inhibitory to *B. subtilis*, weakly active against *Staphylococcus aureus*, and not active against *E. coli.*(36)

(iii) Amylase-producing, lipase-producing, and hemolytic clones: Eight clones that produces amylase activity and two lipase-producing clones from *SL1 and 29 hemolytic clones from *SL2 are reported. Restriction digest analysis of these clones suggests that they result from independent cloning events and are not the result of duplicate clones. The variety of restriction patterns demonstrates the molecular diversity of DNA cloned in the BAC libraries.
Sample taken from any water body including mesotropic lakes, hot water springs, the marine samples, rivers, even the waste water treatment plant etc. The screening results revels that the type of bacteria present basically depends upon the source of the sample, the temperature, the pH of the source, ionic conductivity of the water environment.The microbes isolated from the marine niche specially shows the different enzyme producing activity, similarly the microbes from the irrigating water of the river shows the *tfdAα gene* which has 2, 4-D degrading activity and used in the xenobiotic compound degradation and the isolated microbes can be used in process of bioremediation .The technique of the metagenomics is applied to the samples from the mesotrophic lakes, the dominating microbes are *Actinobacteria Alpha- and Beta proteobacteria and Bacteroidetes Actinobacteria, Betaproteobacteria, Alphaproteobacteria and Bacteroidetes.* The class proteobacteria dominates the hot water springs. The marine metagenomics is dominant niche which can be manipulated as a wide range of novel biocatalysts which meet the need of the current industries, and which are required to improve current and develop new, cleaner, industrial production processes, to reduce energy and raw material consumption, and for the generation of renewable biofuels; marine metagenomics coupled with biotechnology has the potential to contribute to all these pressing needs. *Metagenomics* is called as *fourth domain of life*, and a vast diversity of the bacteria is exploited. This diversity represents a vast

genetic bounty that may be exploited for the discovery of novel genes, entire metabolic pathways and potentially valuable end-products thereof. Cracking the secrets of the various environmental niche the countless microbial communities will reveal solutions to myriad challenges in human health, agriculture and environmental stewardship. The metagenomic processes is used in the production of the novel antibiotics, antiviral drug, etc. also the protein coats of the viruses are used as antigens and specific antibodies are produced for that antigen. The metagenomics has been applied in the commercial production of the industrially important products like vitamins, pigments like photorodhospins etc. The process is also applied in the core fields like environmental remediation, biodefence, forensics and alternative energy. In addition to the practical applications, the knowledge gained through metagenomics it is likely that fundamental biological concepts of genomics, species, evolution, and ecosystem will have effect beyond the specific field of microbiology. *SL1,*SL2-designation of libraries created in METACONROL PROJECT.*

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