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Recent Trends in Characterization of Microbial Diversity from Environment: A Review

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Abstract: Microorganisms find application in all spheres of life: agriculture, food, medicines, brewing and baking industry etc. But, until now only 1 per cent of the microorganisms are characterized. This has been performed through the culture dependent and culture independent methods. The omics based approaches *viz*. metagenomics, metatranscriptomics, and metaproteomics provide better understanding of genes from microbial community and diverse ecosystem. These recent advances leads to development of characterization methods which are rapid, sensitive detect the pico amount of sample and will leads to identification of the microorganisms up to species level. Metagenomics and gene mining tools are the culture independent approach that has implication for finding novel organism with unique metabolic potential and leads to identification of the microbial strains that has potential to be utilized at the industry level. This review is an attempt to describe the recent advances in technology for the microbial diversity analysis from diverse habitats and to understand the microorganisms involved in various microbial processes.

Key words: Microbial diversity. Microbial community, Omics, DNA based analysis, DNA sequencing.

Introduction

Microbial diversity describes richness, complexity and variability of microorganisms isolated from the various natural habitats like marshy soil, field soil, dead insect, termite gut, volcanic eruptions etc. The microbial diversity helps us to find the potential novel microbial strains involved in the process and

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to understand structure and function of ecosystem is the outcome of multiple isolation, characterization and sequencing methods that leads to identification of the microorgansisms up to the species or sub species level. Microorganisms are ubiquitous and have the richest spectrum of molecular and biochemical diversity dictated in their genome. They play a vital role in every aspect of life. They support a variety of essential processes and operations in our present lifestyle. More and more industrial processes are now being designed to exploit their unharnessed potentials. Currently they are being extensively used in dye industries, pharmaceutical industries, food and fermentation industries etc. They are not only associated with the health and welfare of human beings but also cause disease, and also carry out degradation of a variety of substances. The diversity of microorganisms makes their classification and characterization necessary, but unfortunately the presently characterized prokaryotic species represent only about 1 per cent of all bacterial species characterized (Stanley, 2002). Microbes can be used in biotransformation to produce various pharmacologically important compounds at industry level, as they mediate specific reaction and have no environmental hazards.

The soil metagenome based gene mining is a tool that acts as source of future novel microbial products, having industrial significance (Daniel 2004). Tripathi *et al* (2007) studied the implication of microbial diversity for biotechnological and industrial applications. They indicated, that germplasm obtained from rich biodiversity source provides material for the development of vaccines, industrial and pharmaceutical products. The molecular marker based analysis has also implication in crop breeding programme (Kumar and Katiyar, 2007), characterization of heat scab in Wheat (Deswal *et al*., 2003), and biodiversity and food security (Goyal *et al*., 2011).

This article aims to review various techniques that are currently being used to study and elucidate microbial communities active in various natural and synthetic environments (Figure 1). It also covers recent advances from similar research work regarding analysis of microbial diversity. The recent advances in microbial characterization based on advanced DNA sequencing methods like Next Gen sequencing will leads to alpha and beta diversity characterization with community and among microbial community, respectively. More recently, Cocolin *et al*. (2011) reviewed that traditional microbiological methods of microbial characterization dependent on the media requirement that allow their growth, but all microbes of the complex ecosystem do not cultivated. Biotechnology involves, a set techniques used for modification of the living system, products and identification of new microorganism having economic importance (Figure 2). Due to the sequencing of microbial genomes, generated a huge data which will be helpful for the industrial utilization of microbial strains and data mining aid in this research (Kumar and Kumar, 2012), and microbial

genome sequence analysis further facilitated by computational analysis (Sharma et al., 2012).

1. Culture dependent methods

Microbial diversity can be characterised based on culture dependent studies and culture independent studies (Juck, *et al*., 2000). Traditional methods mainly rely upon culture dependent assays which restrict the studies to only those microorganisms that can be cultured successfully in laboratory. Conventionally, these studies are based upon the phenotypic appearances of the microorganisms that are observed after culturing them onto the media plates. Even though there is immense improvement in the field of microbiology, still it is not possible to culture all species in laboratory conditions. This is a major drawback of conventional methods of characterization of microbial strains as they require strains to grow under specific growth conditions (Bakonyi *et al.,* 2003). The biochemical characterization differentiates the *Bacillus thuringiensis* isolates based on their nutritional requirement (Kumar *et al*., 2011), determination of microbial load from locally available food samples (Kumar *et al*., 2011), and bioremediation (Selwal *et al*., 2011)

The traditional approaches like phonetic classification, numerical taxonomy, and G+C content, etc has been used for microbial characterization but the authenticity of the starins can be determined through advance DNA based techniques.

2. Culture independent methods

These methods are based on direct isolation of nucleic acids and proteins from the microbial sample and their further examination through molecular biology based methods. This is one of the most powerful approaches for microbial characterization. The various techniques have been devised which can detect changes at single cell level and the advantage of this approach is that it allows cultivation independent characterization of microbial diversity (Pace *et al*. 1986; Hugenholtz *et al*., 1998; 1999; Amann and Ludwig, 2000; Schloter *et al*., 2000). The ribosomal RNA remains conserved during the course of evolution and helps to detect phylogenetic relationships among the isolated strains (Woese, 1987). Terminal–restriction fragment length polymorphism (t-RFLP) is one such method that allows microbial diversity anlysis. This technique can be used to establish species diversity in activated bioreactor sludge, aquifer sand and termite guts (Liu *et al*.1997).

2.1. Restriction Fragment Length Polymorphism (RFLP)

This technique is based on the possibility of generation of different banding patterns after restriction enzyme digestion of the nucleic acids. The differences in fragment lengths can be visualized through agarose gel electrophoresis. This method can be used for both DNA and RNA and it is generally being used to measure genetic diversity and perform linkage mapping. But the limitation associated with RFLP is that it is time consuming and laborious (Botstein *et al*., 1980).

2.2. Terminal-restriction fragment length polymorphism (t-RFLP)

T-RFLP analysis makes use of three different techniques: RFLP, PCR and electrophoresis and it is fast and semi-quantitative analysis method which makes it one of the important approaches for analysis and comparison of bacterial community structures (Liu *et al*., 1997). T-RFLP is PCR based fingerprinting technique which works on DNA isolation from microbial community and primer labelled with fluorescent dye used for selected amplification of gene of interest. The resulting PCR fragment is digested with one or two restriction enzymes and RFLP pattern analyzed based on peak height of t-RFLP patterns. The Gene Marker® software can be utilized for analysis of diversity data generated through t-RFLP (Hulu and Wang Shang, 2006). This can also be targeted to the ribosomal RNA genes and the PCR products are tagged using fluorescently labelled primers. After PCR, the amplicons are restriction digested and separated using capillary electrophoresis.

2.3. PCR and its variants

Polymerase chain reaction (PCR) is one of the most widely used molecular techniques with good sensitivity and its variants like multiplex PCR and real time PCR provide a means for rapid and convenient assay. Multiplex PCR assays have been developed for the detection of various pathogens (Kong *et al*., 1995; LaGier *et al.,* 2004). In multiplex PCR, multiple sets of primers are used within a single PCR mixture so that amplicons of varying sizes are specific to different DNA sequences are obtained in a single experiment. Annealing temperatures for each of the primer sets must be optimized within a single reaction, and amplicon sizes, i.e., their base pair length, should be different enough to form distinct bands for easy differentiation during gel electrophoresis.The purpose of targeting multiple genes at once is to obtain additional information from a single test which would otherwise require more amounts of reagents and time. It enables both detection and quantification in terms of number of copies generated of one or more specific sequences in a DNA sample during the amplification. Products can be detected using non-specific fluorescent dyes that intercalate with any double-stranded DNA or by sequence-specific DNA probes which consist of oligonucleotides labelled with a

fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target. Quantitative real-time PCR has a very high degree of precision and is commonly used to test the presence of a DNA sequence in a sample and also the number of its copies in the sample. Turton *et al*. (2010) devised a multiplex PCR based on capsular types K1, K2, K5, K54 and K57, two putative virulence factors (*rmpA* and *wcaG*) and the 16S–23S internal transcribed spacer unit for the detection and characterization of *Klebsiella pneumonia*. Odinot *et al* (1995) used a PCR-based DNA RAPD fingerprinting and inter-repeat PCR (IR-PCR) for the characterization of clinical isolates of *Yersinia enterocolitica*.

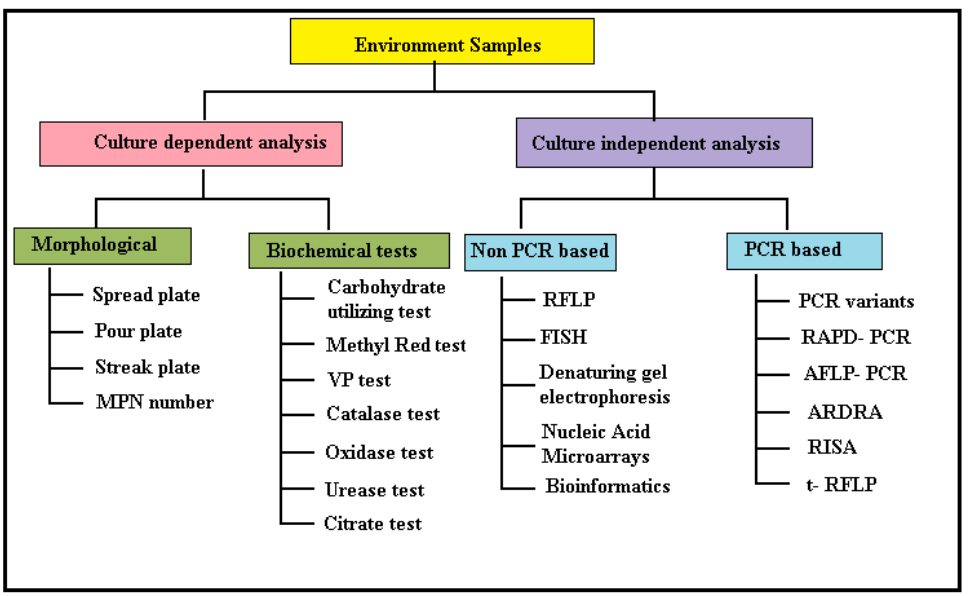

Fig.1 Flow chart showing techniques for microbial characterization

3. PCR based molecular markers

Genetic markers are the DNA sequences with known locations on a chromosome and are used to identify individuals or species. These variations arise due to mutation or alteration in the genomic loci which become integrated into the genome. Genetic marker can range from short DNA sequence, such as a single base-pair change (single nucleotide polymorphism, SNP), to large sequences such as minisatellites.

3.1. Randomly Amplified Polymorphic DNA (RAPD)

This approach is based on using short random sequence oligonucleotides, about 10 bases in length as primers for amplification of total genomic DNA under low annealing temperatures (Williams *et al*., 1990). These random primers bind several priming sites depending upon base complementarities in the template genomic DNA and amplify the DNA. Nucleotide variations between the templates result in the presence or absence of bands because of changes in the priming sites. Crowhurst *et al*., (1995) used RAPD markers to assess the genetic variability of 39 isolates of *Fusarium oxysporum* isolated from *Pterocarpus indicus*. Leal *et al*. (1994) used RAPD markers to characterize twenty isolates of the entomopathogenic fungus *Metarhizium anisopliae* from Australia, Brazil, Finland and the Philippines. Distinct RAPD-PCR profiles were seen for most of these isolates. However, some similarities were seen between isolates from the same countries. Kumar, *et al* (2010) characterized the diversity of 70 *Bacillus thuringiensis* strains isolated from the cotton field soil using RAPD. It was found that these strains varied according to their source of similarity coefficient. But the limitations associated with RAPD is that one requires large number of primers to get reliable data, it is time consuming and non-reproducible.

3.2. Amplified Fragment Length Polymorphism (AFLP)

AFLP markers provide high, resolution, and sensitivity as compared to other methods. In addition, no prior knowledge of the base sequences is needed for amplification (Meudt and Clarke, 2007) making AFLPs extremely beneficial for studying various taxa whose genomic makeup are still unknown. Perrone *et al*. (2006) collected 283 representative strains of *Aspergillus* belonging to Section *Nigri* from the main wine producing European countries and characterized using AFLP markers.

3.3. Fluorescent *in situ* hybridization (FISH)

This technique is useful in enumeration of one particular community from a mixed consortium of microorganisms using fluorescent probes which hybridize with specific sequences and detect that organism (Wagner *et al*., 2003). This provides direct analysis of the nuclear genome of the microorganisms and this technique has been accepted as a preferred method for the microorganisms that are difficult to culture. It can also be used to identify the location of certain genes in the whole genome by first fixing the cells on a glass slide and then hybridizing with suitable probes. These probes are then detected by visualization under a confocal laser scanning microscope. Hence, the location and the presence or absence of the particular sequence can be analyzed in one go. This data can be further used for characterization of microorganisms based on sequence similarity. Wagner *et al*. (1993) used group- specific oligonucleotide probes for the *in-situ* analysis of microbial community structure in activated sludge of sewage plants

and it was found that *Proteobacteria* accounted for 80% of the active flora in the sewage. Daims *et al*. (2001) made use of FISH coupled with microautoradiography for the analysis of *Nitrospira* like nitrite oxidizers in waste water treatment plants. They found considerable differences in the colonies in activated sludge and biofilms.

3.4. 16S rRNA based methods

Sequencing and analysis of 16S rRNA has become an important approach in microbial characterization and seems to be better than all other described approaches. It is envised by different workers that 16S rRNA remains conserved during the course of evolution which makes 16S rRNA techniques a perfect selection for microbial characterization. This approach has been successfully applied to various microorganisms for the quantitative assessment of microbial diversity. A number of variations based on this approach are in use presently, some of them are explained below:

3.5. Amplified ribosomal DNA restriction analysis (ARDRA)

This is also known as 16S-RFLP and involves the PCR amplification of the 16S rRNA gene sequences present in the genome of the microorganisms. These amplicons are then subjected to restriction digestion to yield RFLP fingerprints; universal primers are used for characterization at higher levels and species specific primers are used for subgroup analysis. It is asserted that 97% identity in the 16S rRNA or lower between any two bacteria was dissimilar enough to characterize those bacteria as different species (Hagström *et al*., 2000). The microbial diversity can be described using ARDRA by first generating 16S rRNA gene clone Libraries (Blackall *et al*., 1998). The plasmid inserts from the clones were then grouped by ARDRA and subjected to partial sequencing. Hong *et al*. (2007) isolated about 412 colonies from compost and analyzed for facultative thermophilic characters by traditional colony observation, staining and biochemical tests and ARDRA. Elboutahiri (2009) used various techniques namely, ARDRA, rep-PCR and RAPD for genotypic characterization of 10 isolates of *Sinorhizobium meliloti* and *Rhizobium sullae* from the root nodules of alfalfa and sulla.

3.6. Ribosomal intergenic spacer analysis (RISA)

This is a rapid and effective bacterial community analysis technique. RISA makes use of the diversity and length of the intergenic spacer sequence region that exists between the genes of 16S rRNA and the 23S rRNA in the genome and can effectively distinguish various microbial communities. Being a rapid and effective approach, it can successfully detect shifts due to perturbations in the bacterial community (Fisher and Triplett, 1999). RISA, along with 16S rRNA sequencing, can be used to investigate the bacterial diversity and

community structure (Yu and Mohn, 2001). Ranjard *et al*. (2001) used automated rRNA intergenic spacer analysis (ARISA) to characterize bacterial (B-ARISA) and fungal (F-ARISA) communities from different soil types.

3.7. Denaturing gel electrophoresis

This approach involves denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) to separate various DNA fragments not on the basis of their size but on the basis of sequence dissimilarities (Muyzer *et al*.; 1993; Sakano *et al*.; 2002; Zhang *et al*., 2005). Sequences as small as 200-700 bp can be easily analyzed using this technique. As differing sequences of DNA, from different microbial strains, denature at different denaturant concentrations, individual bands are observed rather than a smear with each band representing a different bacterial population present in the community. TGGE is similar in operation to DGGE with the only difference that heat is used as the tool for denaturing DNA. Double stranded molecules migrate through the gel and the temperature is increased along with. At a certain temperature, the DNA strands start melting leading to a slowdown in migration rates. Since the melting temperature strongly depends on the base sequence, DNA fragments of the same size can also be separated depending on different sequences (Tabatabaei *et al*., 2009). Once separated, the fragments are excised from the gel and are sequenced so that the phylogenetic compositions can be ascertained. DGE is a powerful tool and can be used to study microbial community composition (Muyzer *et al*.,1993), to analyze shifts in microbial population (Ferris and Ward,1997), and to study the evolution of bacterial populations over time (Simpson *et al*., 2000). As the fragments under consideration are small in size, the specificity of the analysis is hampered. Secondly, DGE makes use of only single stranded DNA molecules amplified during PCR which can lead to overestimation of sequence homogeneity (Zhang *et al*., 2005).

4. Recent approaches for microbial diversity characterization from different habitats

4.1. Bioinformatics and Microbial Diversity

A phylogenetic tree or evolutionary tree is a diagram in the form of a tree that depicts evolutionary relationships among various biological species generated on the bases of similarities and differences in their physical/ genetic analysis of the microbial strain. The taxa that have descended from a common ancestor are joined together in the tree. The nodes in the tree are called taxonomic units and internal nodes are generally called hypothetical taxonomic units (HTUs) as they cannot be directly observed. One of the most common tools of bioinformatics used for carrying out phylogenetic analysis is *Clustal W*, which performs multiple sequence alignment. First of all, all the sequences to be aligned are compared to obtain similarity. Based on the similarity matrix, a guide tree relating all the sequences is generated. The final step involves progressive

alignment in which the order of the alignments is determined by the guide tree. Each of the most closely related pairs of sequences in the ultimate branches of the guide tree are aligned to each other and the less related sequences are aligned to build a profile. These profiles are aligned to each other until a full dendrogram is obtained. The program is simple to use, completely menu driven and on-line help is provided (Higgins *et al*., 1992). More recently it has been found that *in silico* comparative genomes of microbial carotenod biosynthesis pathway phylogenetic lineages shown that microorganisms involved are; *proteobacteria*; *firmicutes*; *cyanobacteria* and photosynthetic eukaryotes; and archaea, bacteroidetes and two separate sub-lineages of *Actinobacteria* (Klassen 2010).

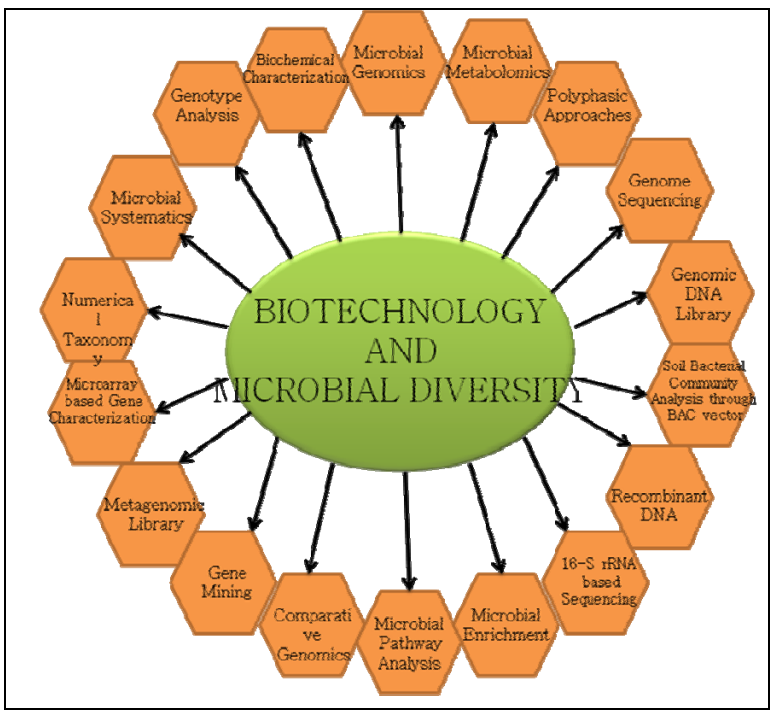

Figure 2. Application of Biotehnology in Microbal Diversity Caracterisation

4.2. Microbial community analysis

The behaviour of the isolated organism from community is different when the organism is exploited for commercial purpose but within the microbial community, the organism behaves differently. There is limited work done in this area. So, we need to explore the metabolic potential of the microorganism when present in microbial community, such as organism isolated from fermenter, bioreactor, methanogenic organisms, organisms involved in degradation of harmful compounds such as pesticides and pharmaceutical compounds in the environment. One of the methods used for analysis of microbial communities is

16S rRNA based profiling. Sequencing and analysis of 16S rRNA can help define identities of microorganisms for community analysis based on phylogenetic tree methodology (Liu *et al*. 2007, 2008; Wang *et al*. 2007). These studies have also gained importance because there has been a drastic increase in rRNA sequence databases (Medini *et al*., 2008). However, it must be kept in mind that such studies can provide a basic insight into community analysis but need to be supported by other evidences to establish complete identities. Metatranscriptomics or environmental transcriptomics is another RNA based technique that works on total mRNA isolated from a microbial community. One of the major difficulties, besides the low abundance of mRNAs, is short half lives of these molecules (Belasco 1993). Proteomic studies have also been applied for microbial community analysis. Similar to metagenomics in the concept, metaproteomic studies are based on isolation and analysis of total protein content of a microbial community from an environmental sample (Marco 2010).

4.3. Metagenomics

This is an emerging area which provides a new insight in the field of microbial diversity analysis. Metagenomic studies revolve around DNA samples isolated from a microbial community as a whole. The first step in performing metagenomic analysis is DNA extraction of microbial community. However, all sources may not be easy to work with during such isolations, such as, for isolating DNA samples from indigenous microbial communities of oceans or vast water bodies (Beja *et al*., 2000). The DNA isolated is then cloned into suitable hosts to generate metagenomic libraries. It is advisable to use high capacity cloning vectors like cosmids to enable larger inserts of about 25-35 kB (Entcheva *et al*., 2001) so that gene clusters and operons can be studied as one natural unit. These clones are then sequenced and studied. Analysis is mostly based upon sequencing of 16S rRNA of the metagenome which can be used for further characterization or comparative studies. Chen *et al*. (2007) collected and analysed the metagenomic DNA from bioreactors treating gold-bearing concentrates. The major populations in the community under consideration were found to be *Acidithiobacillus, Leptospirillum, Sulfobacillus*, *Sphingomonas* and one archeabacteria *Ferroplasma*. They did PCR for sulfur oxygenase reductase (SOR) genes and identified three sor-like genes, namely, sor $_{Fx}$, sor $_{SA}$, and sor $_{SB}$ from the metagenome. This was for the first time that SOR genes were reported in *Acidithiobacillus.* More recently, Suenaga (2012) reviewed and shown that how the targeted metagenomics leads for identification of gene species specific cluster to understand the relationship. It was concluded that targeted metagenomics approach will provide extensive insight into the functional and ecological roles of the ecosystem.

4.4. Sequencing based technologies and microbial diversity

The microbial diversity analysis based on tools of DNA sequencing techniques aids in the study of environmental complexity in terms of ocean structure and geomicrobiology. Advanced sequencing techniques like pyrosequencing and Next Gen sequencing have solved the problems of read length and error bias during sequencing. These methodologies not only reduce the cost and time but also improve the quality of reads of sequencing. Therefore massive parallel sequencings, metagenomics and metaproteomics based approaches will have capability to solve the complex atrocities of the ecosystem and environment to understand; microbial communities and geomicrobiology. Uncultured microorganisms are better characterized through metagenomics and their analysis is facilitated by computer softwares *viz*. MEGAN, SEED, CAMERA, PFAM, RAST server, and Uniprot, etc. They make sequenced DNA analysis easy and help in metagenomic studies for identification of novel genes and enzymes of economic importance. These advancements have a positive impact on the capability to decipher the novel enzymes/ microbial strain from the complex ecosystem and in course of time prove boon to the industrial microbiology for newer sources for novel drugs, and industrial enzymes (Cardenas and Tiedje 2008) Recently Mills *et al*., (2012) described enumeration of microbial cells from seafloor sediment and found out the biological effect of biogeochemical processes.

Conclusions

There are great avenues in the microbial diversity and characterization of new strain with enhanced or new metabolic potential, and production of novel compounds and further these micro-organisms have unique/ enhanced metabolite production capability that can be utilized in the industry. With the advancement in the genetic engineering, metabolisms, metagenomics and genome sequencing techniques, it is possible to find out the ORF/gene responsible for particular metabolite production, novel culture independent techniques & omics based techniques will play a great role in the exploration and utilization of microbes for the exploitation of rich source of microbial biodiversity. There is a need to characterize microbes from natural ecological habitats, animals, plants and other ecosystems. Biotechnology provides numerous tools and techniques in this direction and further screening of these identified micro-organisms for useful purposes and the microbial genomics will take this one more step ahead. Consistent work in this area is required for the characterization of microbial community diversity from the different ecosystem and to develop more rapid and accurate analytical techniques for the detection and analysis of metabolites produced by microbial strains that occupy a unique status among the community. Therefore, community dynamics based on mathematical modelling will leads to solve the present day problem in microbial characterization and to understand structure and function of the ecosystem.

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NAJNOVIJI TRENDOVI U KARAKTERIZACIJI MIKROBIOLOŠKE RAZNOVRSNOSTI U ŽIVOTNOJ SREDINI: PREGLED

-pregledni rad-

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Rezime

Mikroorganizmi se primenjuju u svim sferama života: poljoprivredi, prehrambenoj industriji, medicini, industriji piva, pekarskoj industriji itd. Međutim, do sada je izvršena karakterizacija samo 1 % mikroorganizama, primenom metoda koji zavise od kultivacije ili metoda koji ne zavise od kultivacije mikroorganizama. Pristupi zasnovani na primeni omike, kao što su metagenomika, metatranskriptomika i metaproteomika omogućuju bolje razumevanje gena iz mikrobiološke zajednice i različitih ekosistema. Ova najnovija dostignuća dovode do razvoja metoda karakterizacije koji su brzi i visokog stepena osetljivosti, jer detektuju piko količine uzorka, i vrše identifikaciju mikroorganizama do nivoa vrste. Metagenomika i metode dubinske analize gena (gene mining) predstavljaju pristup nezavistan od kultivacije mikroorganizama koji ima implikacije na pronalaženje novih organizama sa jedinstvenim metaboličkim potencijalom i kojim se vrši identifikacija mikrobnih sojeva koji se mogu koristiti na industrijskom nivou. Ovaj pregled predstavlja pokušaj opisivanja najnovijih pomaka u tehnologiji za analizu raznovrsnosti mikroorganizama iz različitih sredina i razumevanja mikroorganizama koji učestvuju u različitim mikrobiološkim procesima.

Ključne reči: mikrobiološka raznovrsnost, mikrobiološka zajednica, omika, analiza na bazi DNK, sekvenciranje DNK.