**Journal of Animal & Plant Sciences, 2010. Vol. 6, Issue 2: 611- 622.**
*Publication date: 29/03/2010,* http://www.biosciences.elewa.org/JAPS; **ISSN 2071 - 7024**

# Metagenomics and its application in soil microbial community studies: biotechnological prospects

-Review paper-

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**Key words**
Biotechnology, metagenomics, soil ecosystem,

## 1 SUMMARY

**The use of traditional microbiological culturing methods for the study of microbes has had limited success. It has been estimated that 99% of the microbes can not be cultivated easily. Different problems have been faced by researchers during attempts to culture some microbes. Over the past decade, "metagenomics," which is culture-independent genomics analysis of microbes, has been developed to overcome these difficulties. Soil is considered to be a complex environment, which is a major reservoir of microbial genetic diversity. Metagenomic approaches promise increased accessibility of the genetic resources contained in the soil. This paper reviews the current knowledge of culture-independent 'metagenomics' techniques and their application to studies of soil microbial communities**

## 2 INTRODUCTION

The biosphere is dominated by microorganisms (Whitman *et al.,* 1998) that have much practical significance in medicine, engineering and agriculture (Sloan *et al.,* 2006). Due to their significance, genetic and biological diversity of microorganisms is an important area of scientific research (Ghazanfar & Azim, 2009). Biotechnology has become an important information science in the study of genetic and biological diversity of microbial communities. It is acknowledged that biotechnology has a continuous demand for novel genes, enzymes and compounds. Different studies have demonstrated that natural diversity is the best supplier for these novel molecules. This can be explained by the vast richness of soil and other microbial niches (Schmeisser *et al.,* 2007).

The global microbial diversity presents an enormous, largely untapped genetic and biological pool that could be exploited for the recovery of novel genes, biomolecules for metabolic pathways and various valuable products (Cowan, 2000). Despite the obvious importance of microbes, very little is known of their diversity, for example, how many species are present in the environment, and what each individual species does i.e. its ecological function (Singh *et al.,* 2008) Until recently, there

were no appropriate techniques available to answer these important questions due to the limitations encountered in the culturing of microbes. Traditional methods of culturing microorganisms limit analysis to those that grow under laboratory conditions (Hugenholtz *et al.,* 1998; Rondon *et al*., 2000). However, it is widely accepted that up to 99% of the microbes in the environment can not be readily cultivated (Hanada, 2003; Rappe & Giovannoni, 2003; Kamagata & Tamaki, 2005; Sekiguchi, 2006). Thus, most microbes have not been described and assessed for biotechnology. To overcome the difficulties and limitations associated with cultivation techniques, different DNA-based molecular methods have been developed for characterizing microbial species and assemblages, and these have significantly influenced our understanding of microbial diversity and ecology (DeLong, 2005). In general, methods based on 16S rRNA gene analysis provide extensive information about the taxa and species present in an environment. However, these data usually provide only little if any information about the functional role of the different microbes within the community and the genetic information they contain of microbial niches (Streit & Schmitz, 2004). To overcome these difficulties, a new technology has been introduced since the late 1990s, known as 'metagenomics' Metagenomics is a rapidly growing field of research that aims at studying uncultured organisms to understand the true diversity of microbes, their functions, cooperation and evolution, in environments such as soil, water, ancient remains of animals, or the digestive system of animals and humans (Ghazanfar & Azim, 2009). Metagenomics is an emerging field in which the power of genomic analysis (the analysis of the entire DNA in an organism) is applied to entire communities of microbes, bypassing the need to isolate and culture individual microbial species. This review summarizes the molecular methods of culture-independent insight 'metagenomics' and their recent application to studies of soil microbial communities.

## 3 WHAT IS METAGENOMICS?

Coined by Jo Handelsman and others in the University of Wisconsin, Department of Plant Pathology in 1982 (Handelsman, 2004), metagenomics is a new field of research that allows the study of genomes recovered from environmental samples. Metagenomics can be defined as "the application of modern genomics techniques to the study of communities of microbial organisms directly in their natural environments, bypassing the need for isolation and laboratory cultivation of individual species (Chen & Pachter, 2005). Metagenomics is the culture-independent analysis of a mixture of microbial genomes (Schloss *et al.,* 2003; Riesenfeld *et al* 2004; Susannah *et al.,* 2005; Patrick *et al.,* 2005)

The metagenomic methodology has been developed as an effective tool for the discovery of new natural products and microbial functions (He *et al.,* 2007) it can be described as the application of the genomics suit of technologies to uncultivated microorganisms (Gabor *et al.,* 2007; Singh *et al.,* 2008). Initially, non-cultured microflora and ancient DNA investigations were the prime targets of metagenomic studies. However, nowadays the technology is applied in the study of an array of microbial diversities like deep-sea aquatic microflora, soil microbes and gastrointestinal ecosystems of human and animals (Shanks *et al*., 2006; Lu *et al.,* 2007; Ghazanfar & Azim, 2009).

Studies have revealed that only 0.001-0.1% of the total microbes in sea water, 0.25% in freshwater, 0.25% in sediments and only 0.3% of soil microorganisms could be cultivable *in vitro* (Amann *et al*., 1995; Singh *et al.,* 2008). The current metagenomic studies have largely progressed due to the construction of efficient gene cloning vectors like bacterial artificial chromosomes (BACs) or cosmids, (Xu, 2006; Babcock *et al*., 2007) which allow cloning and expression of larger and complex DNA segments or genes and the development of methods for generation and analysis of the data (Singh *et al.,* 2008; Ghazanfar & Azim, 2009).

**3.1 Applications of metagenomics**: Many microorganisms are able to degrade waste products, make new drugs for medical applications, produce environmentally friendly plastics, or even make some of the food we eat (figure 1). By isolating the DNA from these organisms, it provides us with the

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opportunity to optimize these processes and adapt them for use by society. Another valuable application of metagenomics is that it provides the capacity to effectively characterize the genetic diversity present water, soil and rumen source samples regardless of the availability of laboratory culturing techniques. Metagenomics is a new and exciting field of molecular biology that is likely to grow into a standard technique for understanding biological diversity.

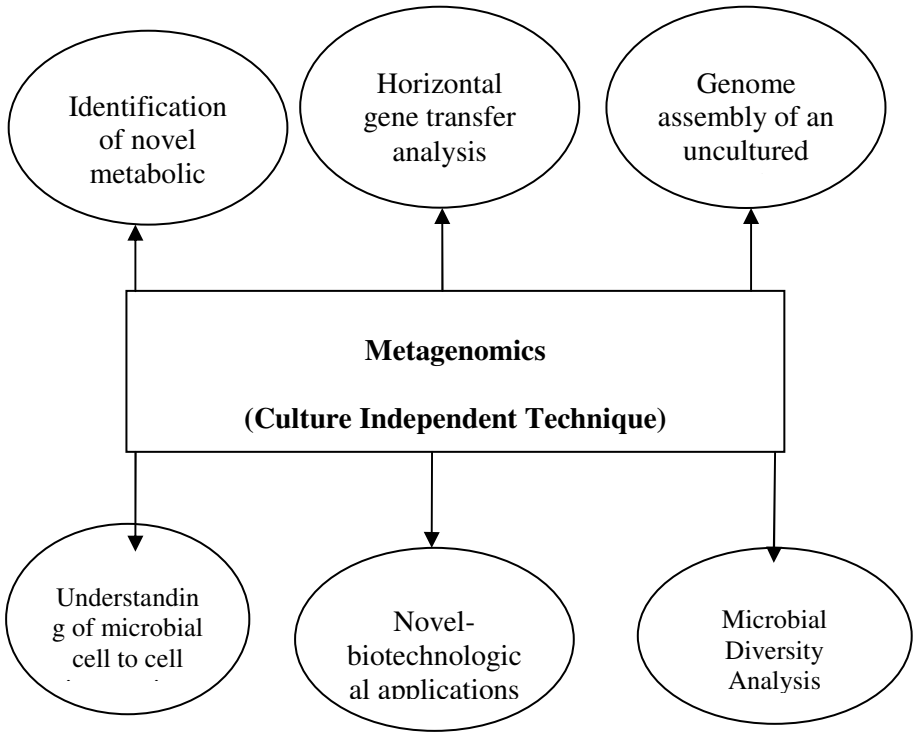


**Figure 1:** Applications of metagenomics.

**3.2 Metagenomics technologies: the process.** Metagenomics is a means of systematically investigating, classifying and manipulating the entire genetic material isolated from environmental samples. (Zeyaullah *et al.*, 2009). The process involves isolating DNA from environmental sources and cloning it into vectors that replicate in cultured organisms. Figure 2 shows a schematic illustration of a typical multi-step metagenomics process which relies on the efficiency of three main steps, i.e.

1. Sampling and nucleic acids extraction
2. Library construction.
3. Analysis of metagenomic libraries

**3.2.1 Sampling and nucleic acids extraction**: In the metagenomics process (Figure 2) the samples could be from any environment, soil or habitat including the GI ecosystem (Ghazanfar & Azim, 2009). Specifically, soil microbial communities are composed of a mixture of archaea, bacteria and protists displaying a diversity of cell wall characteristics and varying in their susceptibility to lysis (Kauffmann *et al.*, 2004). Thus, some special techniques are required for their extraction. Although, various kits are commercially available for DNA isolation from environmental samples, many laboratories have developed their own methods with the aim of optimising extraction and reducing bias caused by unequal lysis of different members of the soil microbial community (Frostegard *et al.*, 1999; Krsek & Wellington, 1999; Miller *et al.*, 1999). There are two types of extraction techniques: (1) direct, in situ, extraction where the cells are lysed in the soil sample and then

the DNA is recovered; and (2) indirect extraction techniques, where the cells are removed from the soil and then lysed for DNA recovery (Schmeisser *et al.,* 2007). Soil is a particularly complex matrix containing many substances, such as humic acids, which can be co-extracted during DNA isolation. Removal of humic acids is essential before the DNA can be processed further. For this purpose, a range of DNA purification techniques has been developed. Sephadex G-200 spin columns have proven to be one of the best ways to remove contaminants from soil DNA (Miller *et al.*, 1999). Recently, a pulse field electrophoresis procedure using a two-phase agarose gel, with one phase containing polyvinylpyrrolidone (PVPP), was developed for removal of humics (Quaiser *et al.,* 2002).

**3.2.2 Construction of a metagenomic library:** DNA isolation and purification is followed by the construction of DNA libraries in suitable cloning vectors and host strains. The classical approach includes the construction of small insert libraries (<10 kb) in a standard sequencing vector and in *Escherichia coli* as a host strain (Henne *et al.,* 1999). However, small insert libraries do not allow detection of large gene clusters or operons. To circumvent this limitation researchers have been employing large insert libraries, such as cosmid DNA libraries (mostly in pWE15 vector of Stratagene) with insert sizes ranging from 25-35 kb (Entcheva *et al.*, 2001) and/or Bacterial Artificial Chromosome (BAC) libraries with insert up to 200 kb (Beja *et al.,* 2000; Rondon *et al.*, 2000).

Additionally, the construction of fosmid with inserts of 40 kb of foreign DNA has been reported (Beja *et al.,* 2002). *E. coli* is still the preferred host for the cloning and expression of any metagenome-derived genes and only very recently have other hosts such as *Streptomyces lividans* been employed to identify genes involved in the biosynthesis of novel antibiotics (Courtois *et al.*, 2003). Metagenomic libraries are also being developed in other Gram-negative hosts by several laboratories, and these will become available soon.

**3.2.3 Analysis of metagenomic libraries:** Two methods are used for the analysis of genetic material of metagenomic library.

**3.2.3.1 Sequence-based metagenomics:** Sequence-based metagenomics provides information on the distribution of functions in a community, linkage of traits, genomic organization and horizontal gene transfer. Approaches typically involve either sequencing of random clones to accumulate vast stores of sequence information or identification of clones based on methods that detect a particular sequence. With both of these approaches, phylogenetic markers are sought on the clone of interest to link cloned sequences with the probable origin of the DNA

**3.2.3.2 Function-based analysis**: Function-based analysis enables identification of new enzymes, antibiotics or other reagents in libraries from diverse environments. Approaches include: (i) heterologous expression, in which clones that express the desired function are identified. An important limitation to heterologous expression is that the domesticated host bacterium must be able to express (transcribe and translate) the genes for the products to be detected; (ii) selections, in which the clone expressing the desired function grows and others do not. Selections provide the most powerful approach to finding rare clones. Examples of selectable characteristics include antibiotic resistance and metal resistance.

A 'functional-anchor approach' involves identifying all of the clones that express a certain function and sequencing them completely to determine the diversity of genomic environments from which that function originates.

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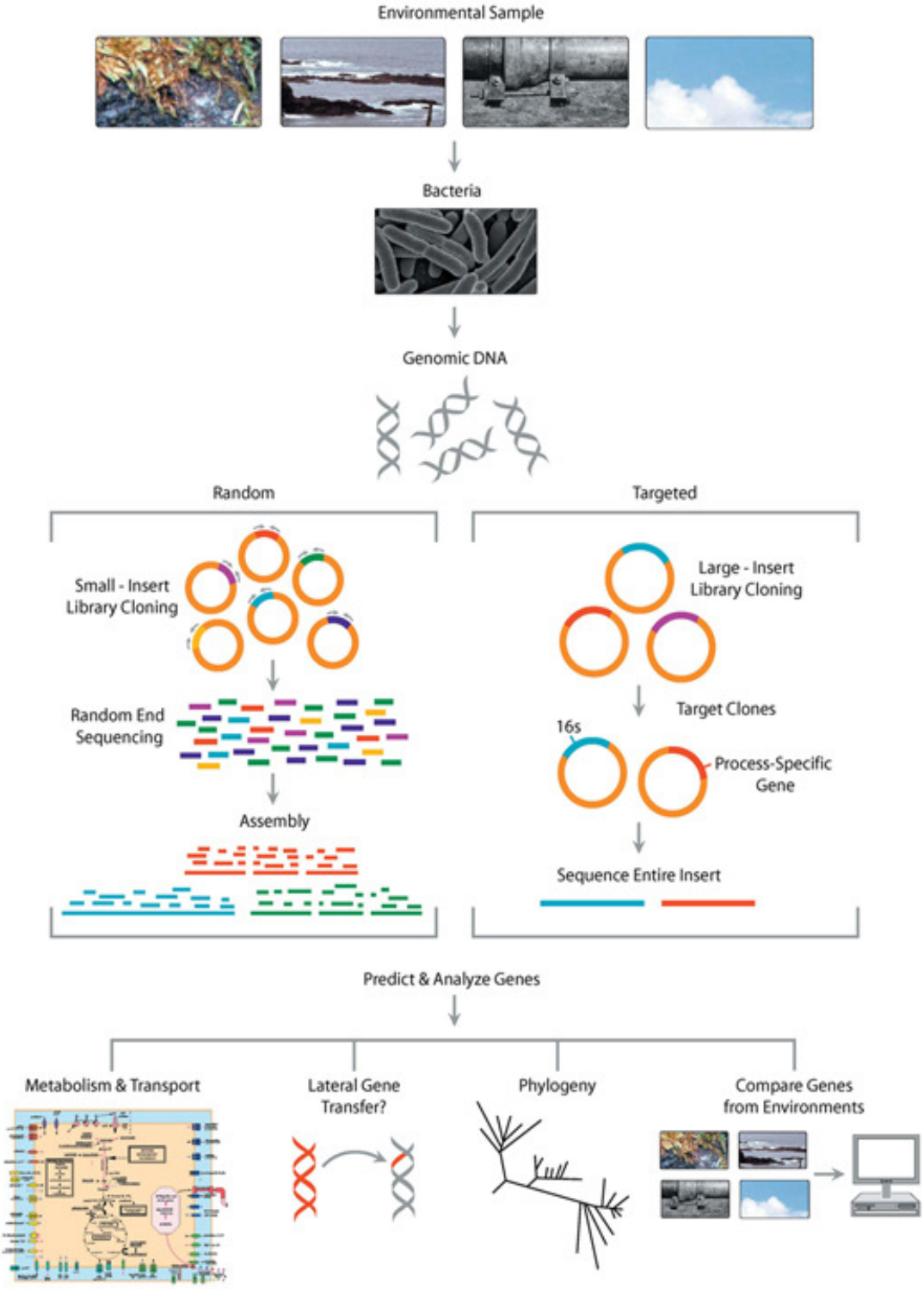


**Figure 2:** Steps involved in a metagenomics.

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## 4 SOIL MICROBIAL COMMUNITIES AND METAGENOMICS

Soil is a complex environment, which is a major reservoir of microbial genetic diversity (Robe *et al.,* 2003). Soil is dominated by the solid phase (Rolf, 2004) and the soil microorganisms are localized in close association with soil particles, e.g. with complexes of clay–organic matter (Foster, 1988). The microorganisms can be found as single cells or microcolonies. Their metabolism and interactions with other organisms and with soil particles is dependent on the conditions at the microhabitat level, which often differ between microhabitats even over very small distances. The microhabitats for soil microorganisms include micropores and the surfaces of soil aggregates of various composition and sizes (Ranjard & Richaume, 2001; Torsvik *et al.*, 2002).

Thus, soil can be regarded as very heterogeneous with respect to conditions for microbial growth and for the distribution of microorganisms and matrix substances. This heterogeneity results in a wide variety of microbial niches and a high diversity of soil microorganisms. The microbial diversity in soils exceeds that of other environments and is far greater than that of eukaryotic organisms. One gram of soil can contain up to 10 billion microorganisms of possibly thousands of different species (Rosello & Amann, 2001). The genetic complexity of microbial soil communities has been estimated by re-association of community DNA. Not taking into account the genomes of rare and unrecovered microorganisms, such analyses have shown that the soil community size is equivalent to 6000–10 000 *Escherichia coli* genomes (Ovreas, 2000; Torsvik *et al.,* 2002).

While analysing the re-association kinetics of the total bacterial DNA in a 30 g soil sample Doolittle (1999) reported that it contained more than 500 000 species. These results illustrate that the genetic diversity of the soil metagenome is a rich and widely unexplored resource for new industrial enzymes and bioactive compounds. Simple calculations of soil microbial diversity place it in the range of between 3,000 and 11,000 genomes per gram of soil with less than 1% being accessible through cultivation techniques (Torsvik & Ovreas, 2002; Torsvik *et al.,* 2002; Curtis & Sloan, 2004). This is probably very similar for many other microbial niches but it is also clear that many other microbial communities are less diverse. Pure culture analysis of soil microorganisms has revealed that they are a rich source of novel therapeutic compounds such as antibiotics (Raaijmakers *et al.*, 1997), anticancer agents (Shen *et al.*, 2001), immunosuppressants (Skoko *et al.,* 2005), as well as a wide range of biotechnologically valuable products (Ullrich *et al.,* 2004; Inoue *et al.,* 2005).

However, the cultivation-dependent approach is limited by the fact that the overwhelming majority of microorganisms present in soil cannot be cultured under laboratory conditions. Soil metagenomics can provide a cultivation-independent assessment of the largely untapped genetic reservoir of soil microbial communities. Culturable microorganisms provide very limited information on soil microbial diversity, because only 0.1-10% of soil microorganisms can be cultured under conventional laboratory conditions. Soil metagenomic methods, comprising isolation of soil DNA and construction and screening of clone libraries, enable researchers to peep at more complete scenario of soil microbial communities, and thus, to better understand their interactions.

**4.1 Application of soil metagenomics:** The development and application of metagenomics has enabled access to the uncultivated soil microbial community, availing a rich source of novel and useful biomolecules. Some examples of application of soil metagenomics are:

**4.1.1 Antibiotics and pharmaceuticals:** Soil metagenomics has the potential to substantially impact on antibiotic production. Two previous studies reported the successful screening of soil metagenomic libraries for indirubin (MacNeil *et al.,* 2001; Lim *et al.,* 2005) while a range of novel antibiotics have been detected in metagenomic libraries (Gillespie *et al.,* 2002; Brady *et al.,* 2004b). A clone found in a soil metagenomic library produces deoxyviolacein and the broad spectrum antibiotic violacein (Brady *et al.,* 2001. During the screening of seven different soil metagenomic libraries, it was reported that these libraries exposed 11 clones producing longchain N-acyltyrosine antibiotics, and analysis of their synthases indicated that ten of them were novel (Brady *et al.*, 2004b). Metagenomic libraries have also been used for isolating natural antibiotic resistance genes. Riesenfeld *et al.* (2004) identified nine aminoglycoside and one tetracycline antibiotics resistance genes from soil.

**4.1.2 Oxidoreductases/dehydrogenases:** A metagenomic study searching for the diversity of bacteria in the environment capable of utilising 4-

hydroxybutyrate found five clones displaying novel 4-hydroxybutyrate dehydrogenase activity (Henne *et al.,* 1999). Alcohol oxidoreductases capable of oxidising short chain polyols are useful biocatalysts in industrial production of chiral hydroxy esters, hydroxy acids, amino acids and alcohols (Knietsch *et al.,* 2003b). In a metagenomic study without enrichment, a total of 24 positive clones were obtained and tested for their substrate specificity. To improve the detection frequency, an enrichment was performed using glycerol or 1, 2-propanediol, after which a further 24 positive clones were detected (Knietsch *et al*., 2003c).

**4.1.3 Amidases:** In a study involving general screening of a soil metagenomic library for biocatalysts, one amidase-positive clone was detected (Voget *et al.,* 2003). Amidases are used in the biosynthesis of *β-lactam* antibiotics. A separate study targetting amidases of the soil metagenome using enrichment detected seven amidase-positive clones, one of which encoded a novel penicillin acylase (Gabor *et al*., 2004b; Gabor & Janssen, 2004).

**4.1.4 Vitamin biosynthesis:** Soil metagenomics has been applied to the search for novel genes encoding the synthesis of vitamins such as biotin (Entcheva *et al*., 2001). Seven cosmids were detected in metagenomic libraries obtained after avidin enrichment of environmental samples and the highest levels of biotin production in this study were detected in a cosmid obtained from forest soil (Entcheva *et al.,* 2001).

**4.1.5 Polysaccharide degrading/modifying enzymes/ amylolytic genes:** Amylases have been the focus of many metagenomic studies with several reports available on the isolation of novel amylolytic enzymes from metagenomic DNA libraries (Richardson *et al*., 2002; Voget *et al.,* 2003; Yun *et al.,* 2004; Ferrer *et al.,* 2005b). Cellulases have numerous applications and biotechnological potential for various industries including chemicals, fuel, food, brewery and wine, animal feed, textile and laundry, pulp and paper and agriculture (Bhat, 2000; Sun & Cheng, 2002; Wong & Saddler, 1992a,; Beauchemin et al., 2001, 2003). Functional screening of a soil metagenomic library for cellulases revealed a total of eight cellulolytic clones, one of which was purified and characterized (Voget *et al.,* 2006). Agarases, the enzymes that can liquify agar, have been identified during the screening a soil metagenomic library, in which a total of 4 agarolytic clones containing 12 agarase genes were identified (Voget *et al*., 2003).

**4.1.6 Lipolytic genes:** Metagenomics has identified a number of novel genes encoding lipolytic enzymes such as esterases and lipases. Esterase EstCE1 was derived from a soil metagenome (Elend *et al*., 2006), and this enzyme displays remarkable characteristics that cannot be related to the original environment from which they were derived. The high level of stability of this enzyme together with its unique substrate specificities make it highly useful for biotechnological applications.

Environmental DNA libraries prepared from three different soil samples were screened for genes conferring lipolytic activity on *Escherichia coli* clones. Screening on triolein agar revealed 1 positive clone out of 730,000 clones, and screening on tributyrin agar revealed 3 positive clones out of 286,000 *E. coli* clones. Substrate specificity analysis revealed that one recombinant strain harbored a lipase and the other three contained esterases. The genes responsible for the lipolytic activity were identified and characterized (Henne *et al*., 2000). Further screening identified genes conferring Na (Li)/H antiporter activity on the antiporter-deficient *Escherichia coli* strain KNabc (Majernik *et al.,* 2001). This powerful selection facilitated the discovery of two novel antiporter proteins in a library of 1,480,000 clones containing DNA isolated from soil.

**Table 1:** Examples of recently identified lipolytic and metagenome-derived biocatalysts together with details on the respective metagenomic libraries and the origin of the samples.

| Authors | Target gene / natural product | Source | Vector used for library construction | Host strain |
|---|---|---|---|---|
| Henne *et al.*, 2000 | Lipase, Esterase | Meadow Soil | pBluescript SK | *E. coli DH5* |
| Rondon *et al.*, 2000 | Antibacterial, Hemolytic activities, Lipase, amylase, nuclease | North American Soil | pBeloBAC11 | *E. coli* DH10B |
| Henne *et al.*, 1999 | 4-hydroxybutyrate dehydrogenase | Soil | pBluescript SK | *E.coil* DH5 alpha |
| Majernik *et a*l., 2001 | H+ antiporters | Soil | pBluescript SK | *E.coil KNabc* |
| Lee *et al.*, 2004 | unique lipolytic activity | Forest Topsoil | fosmid | *E.coil* |
| Voget *et al.*, 2003 | novel biocatalysts | unplanted field Soil | Cosmid | *E.coil* |
| Knietsch *et al.,* 2003 | Alcohol oxidoreductase | meadow, sugar beet field, cropland soil | pBluescript SK | *E.coil* DH5 alpha |
| Knietsch *et al.*, 2003 | Carbonyls formation | Soil | n.r | *E.coil* |
| Knietsch *et al.*, 2003 | Coenzyme B(12)-dependent glycerol and diol dehydratases | Soil | pBluescript SK | *E.coil* DH5 alpha |
| Kim *et al.,* 2006 | esterase | Soil | pCCIFOS | *E.coil EP1300-TI* |
| Li *et al.,* 2005 | Lipase | Soil | pEpiFOS-5 | *E.coil EP1-100* |
| Gabor *et al.,* 2004 | Amidase | Soil and Enrichment culture | pZero-2 | *E.coil TOP10* |
| Yun *et al.*, 2004 | Amylase | Soil | *pUC19* | *E.coil* DH5 alpha |
| Ginollhac *et al.,* 2004 | Polyketide syntase | Soil | Cosmid | *E.coil* |
| Riesenfeld *et al.,* 2004 | aminoglycoside and tetracycline Antibiotic resistance | Soil | *pJN105 pCF430* | DH10B<br>DH5 alpha |
| Brady *et al.,* 2004 b | deoxyviolacein and broad spectrum antibiotic violacein | Soil | Cosmid | *E. coli* |

## 5 CONCLUSION

Metagenomics is a rapidly growing field of research that has had a dramatic effect on the way the microbial world is viewed and studied. By permitting the direct investigation of bacteria, viruses and fungi irrespective of their culturability and taxonomic identities, metagenomics has changed microbiological theory and methods and has also challenged the classical concept of species. This new field of biology has proven to be rich and comprehensive and is making important contributions in many areas including ecology, biodiversity, bioremediation, bioprospection of natural products, and in medicine.

This review has addressed in a coherent manner the diverse and multiple aspects of metagenomics and the multiplicity of the potential applications of the soil microbial communities. Uncultured soil microorganisms comprise the majority of the planet's biological diversity. Microorganisms represent two main domains of life and contain vast biodiversity that is the product of billions of years of evolution. In many environments, scientists are able to grow only less than 1% of all microorganisms under standard laboratory conditions

Therefore, culture-independent methods are essential to understand the genetic diversity, population structure, and ecological roles of the majority of microorganisms. Metagenomics has potential to answer fundamental questions in microbial ecology, especially of soil ecosystems which contain the greatest diversity of microorganisms on earth, with 5,000–10,000 species of microorganism per gram of soil.

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