METAGENOMICS: UNRAVELLING THE UNCULTURED MICROBIAL COMMUNITY

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ABSTRACT

The discovery of the traditional culturing techniques for microorganisms in the laboratory has remained a classical landmark attained in the field of microbiology and sustained as the earliest methods used in the study of microbial physiology, functions and diversity by microbiologists. However, many studies now evidently portrayed these traditional culturing methods significantly unreflective of the uncultured microbial population. Thus, implying these historic microbiological techniques failing to capture the full spectrum of microbial diversity in an environment. Providentially, three decades ago, metagenomics has opened new avenues of research by enabling unprecedented analyses of genome heterogeneity and evolution in environmental contexts and providing access to far more microbial diversity than previously analysed. This new concept in microbial investigation has thrived in establishing the identity of microorganisms in diverse environments and allow a reasonable estimate of their functionality without the need for their cultivation. Metagenomics currently merged with the next-generation sequencing enabled the unravelling and functional exploitation of the uncultured microbial world, and conceivably transformed the archaic perception of microbial ecologists. Therefore, this review reflects on the traditional cultivation technique and the milestone attained since the dawn of metagenomics, with furtherance to the in-depth applications of the new age metagenomics approach in contemporary microbial studies, its challenges and future outlook.

Keywords: Metagenomics, Next-generation sequencing, Traditional culturing, Uncultured microbial world, Function-driven metagenomics.

INTRODUCTION

Before the advent of molecular approaches, the traditional culture method was a norm to study and evaluate microbial physiology, genetics and diversity in different environments. This earliest microbiological approach has profited the scientific world in terms of microbial morphological identification, characterisation and genetic expression principally relying on its unique capability of physical isolation of microorganisms from different environments. Consequently, microbiologists became attracted to the strength and precision of the study of microorganisms in pure culture. This milestone in the field of microbiology became an essential prelude to characterization of microbes in the laboratory (Pace *et al.*, 1986), and made scientists presume lots have been discovered in the world of microorganisms until almost three decades ago when it was discovered that only 1% or less of microbial

community have been studied via culture approach (Torsvik *et al.,* 1990; Torsvik and Ovreas, 2002; Kirk *et al.,* 2004), - this now showcase the limitation of the cultivation approach denoting vast majority of microbial population are practicably unculturable in the laboratory (Torsvik *et al.,* 1990).

Nonetheless, Simu and Hagstrom (2004), stated the various reasons bacteria may be recalcitrant to culturing which include lack of necessary nutrients, symbionts, incorrect combinations of temperature, pressure, or atmospheric gas composition, excess inhibitory compounds, accumulation of toxic waste products from their metabolism, and intrinsically slow growth rate or rapid dispersion from colonies. With these limitless constraints and the deficient knowledge on the diverse appropriate culturing conditions of microbes in their natural habitat, microbiologists are still much distant from accessing the uncultured majority via the traditional cultivation methods.

Staley and Konopka (1985) reported that one of the indicators that cultured microorganisms did not represent much of the microbial world was the oft-observed "great plate count anomaly", and Handelsman (2004) highlighted this discrepancy is between the sizes of populations estimated by dilution plating and by microscopy; then the big unearthing that only 0.1 to 1% of bacteria in soil are readily culturable on common media under standard conditions (Torsvik *et al.,* 1990; Torsvik and Ovreas, 2002) - these and other significant studies (e.g., Colwell *et al.,* 1996; Colwell and Grimes 2000) suggested that culturing techniques has failed to capture the full spectrum of microbial diversity. An important study by Torsvik *et al.,* (1990) produced the first work on soil bacteria diversity which demonstrated with DNA-DNA reassociation techniques that the complexity of the bacterial DNA in the soil was at least 100-fold greater than could be accounted for by culturing methods, and this work suggested that the diversity of the uncultured microbial world exceeded previous estimates.

In a bid to solve the problem of the uncultured world of microorganisms, microbiologist resort to metagenomics, thus expanding the study of unculturable microbe beyond identification, ecology and diversity, currently focusing on the functionality of these uncultured population. This article reiterates the earliest glory of the traditional cultivation approach in the identification, characterisation and accessing the physiology of microbial world, while portraying its huge limitation and gap in unravelling the genuine microbial diversity in the environment. Based on the subject, this review discusses the metagenomics as a suited alternative in terms of picturing true microbial diversity in most environments or samples - approach instinctive due to recent advancement in the combined fields of microbiology, molecular biology and biotechnology.

METAGENOMICS: A CULTURE-INDEPENDENT APPROACH FOR ACCESSING THE UNCULTURED POPULATION

Metagenomics has opened new avenues of research by enabling unprecedented analyses of genome heterogeneity and evolution in environmental contexts and providing access to far more microbial diversity than previously viewed in the petri

dish (Handelsman, 2004). As realised, the probable magnitude of the soil metagenome, encompassing the collective genomes of microbes in the soil, requires large-scale approaches for analysis, this is inaccessible via the old-style cultivation methods thus demands culture-independent approaches (Rondon *et al.,* 2000). The advent of metagenomic technology has now made it possible to make a more comprehensive assessment of the scope of the microbial biodiversity present in the environment or sample, also to determine some of the potential geochemical functions of these microbial communities (Delmont *et al.,* 2011; Eisen, 2007; Handelsman *et al.,* 1998). This new concept in microbial science has now opened the mind of the scientific community concerning the astonishingly large catalogue of biochemical functions in nature be harnessed (Luana *et al.,* 2018).

Aside from accessing the genuine microbial diversity, molecular approaches that involve direct evaluation of genetic material from an environment (metagenomics) and other -omics methods such as proteomics or transcriptomics, are extremely valuable in assessing the biodegradation potential in environmental samples (Chakraborty *et al.,* 2012; Lovley, 2003; Zhai and Ma, 2012). In a factual sense, these approaches also provide an avenue for a solid hypothesis regarding the protagonist of the degradation process and to predict the likely degradation pathway (Dojka *et al.,* 1998).

Metagenomics, an approach based on analyses of environmental gene libraries has broadened the scope by enabling researchers to study uncultivable bacteria as well, this method permits to link potential functions to specific microorganisms in a habitat (Rondon *et al.,* 2000; Scmeisser *et al.,* 2007), but only in a predictable stance. In this case, interest in establishing the identity of microorganisms in the diverse environment and allow a reasonable estimate of their functionality without the need for their cultivation has manifested a recent rapid increase in the use of metagenomics approach. Thus, metagenomic approach has broadly established the new basis to gain access to the physiology and genetics of uncultured organisms, this genomic analysis of a population of microorganisms has emerged as a powerful centrepiece. Direct isolation of genomic DNA from an environment circumvents limitations of culturing the organisms under study; and cloning of it into a cultured organism captures it for study and preservation (Handelsman, 2004). Consequently, advances have been derived from sequence-based and functional analysis in samples from water, soil and associated with eukaryotic hosts (Handelsman, 2004).

One of the earliest breakthroughs in metagenomics was attained in 1991 when the research team of Schmidt generated the first metagenomic library using DNA from marine picoplankton (Schmidt *et al.,* 1991), then the subsequent construction of metagenomic libraries from an enriched consortium sampled from cellulose digesters to mine genes encoding cellulases by Healy *et al.,* (1995). In 1998, the term "metagenome" evolved when Handelsman *et al.,* (1998) described the mining for novel chemical compounds from uncultured microorganisms, which comprises more than 99% of the microbial diversity (Sleator *et al.,* 2008) thus proposing the emergence of a new frontier in science. However, the term "metagenomic libraries" was coined in

the year 2000 by Rondon *et al.*, (2000) who generated libraries in the Bacterial Artificial Chromosome (BACs) using DNA from soil samples. Importantly, the metagenomic study of the Sargasso Sea has further allowed the identification of 148 previously unknown rRNA bacterial phylotypes. Besides, this shotgun sequencing of the Sargasso Sea led to the discovery of almost 70 thousand novel genes among the roughly 1.2 million genes by open reading frame (ORF) identification and alignment of the putative protein products (Venter *et al.,* 2004). This Sargasso study became one of the most widely used illustrative instances of how metagenomics is a feasible way to accumulate genomic knowledge (Luana *et al.,* 2018). All these mentioned, and many earliest metagenomic studies put-together caved the historical basis of this crucial metagenomic approach.

Sequenced-based Metagenomics

The sequenced-based metagenomics is purposely the sequence-driven metagenomic approach, where isolated DNA from an environmental sample is cloned into a suitable vector (a plasmid that can be stably maintained in an organism) and transformed into a host bacterium; importantly, these clones are further sequenced randomly to identify and provide genetic information of microorganisms in the environmental samples (Tyson *et al.*, 2004; Venter *et al.,* 2004). Sequence analysis guided by the identification of phylogenetic markers is a powerful approach initially proposed by the DeLong group, which produced the first genomic sequence linked to a 16S rRNA gene of an uncultured archaeon (Stein *et al.*, 1996).

Recent studies have shown that the characterization of the microbial diversity through the sequencing of 16S rRNA gene is probably the most used metagenomic approach (Chakraborty *et al.*, 2012; Lovley, 2003) because the help of next-generation sequencing (e.g., 454 Pyrosequencing and Illumina) has made it possible to obtain an almost complete picture of the composition of a microbial community, and further phylogenetic information is then used to infer the functionality of the microbial community and to hypothesize about the degradation pathways used in the breakdown process (Dojka *et al.*, 1998).

The key disparity of this molecular method with the traditional culturing method is the fact that entire clades emerge that have never been cultivated in pure culture, this resort back to the reasonable claim about the cultivation approach being unreflective of the uncultured population thus the information about their metabolic pathways is incomplete or probably non-existent. Moreover, phylogenetic similarity does not necessarily mean shared physiology or metabolic capacity (Lovley, 2003).

Sequenced-based metagenome analysis can involve complete sequencing of clones containing phylogenetic anchors that indicate the taxonomic group that is the probable source of the DNA fragment; Otherwise, random sequencing can be conducted to identify gene of interest then phylogenetic anchors can be sought in the flanking DNA to provide a link of phylogeny with the functional gene (Handelsman, 2004). The next-generation sequencing technology (e.g., Illumina) are mostly exploited to sequence full metagenomes, an approach that provides a complete picture of the functions and metabolic potential of the microbial

community (Zhai and Ma, 2012; Schloss and Handelsman, 2005). Other metagenomics studies (e.g., Iwai *et al.*, (2009)) employed the gene target metagenomics, where functional gene analysis was performed by applying a PCR-based approach together with a sequencing technology (pyrosequencing). This approach may provide an in-depth insight into the target functional gene analysis coupled with providing quantitative and genotypic insight of the target genes of interest. This sequenced-typed metagenomic approach is now commonly used to acquire an in-depth insight into the biodegradation potential and the main pathways involved in the degradation process as related in studies by Mason *et al.*, (2005) and Yergeau *et al.*, (2012).

Function-driven Metagenomics

Function-driven metagenomics analysis is a non-sequenced-based metagenomics that involves isolation of DNA from an environmental sample, then cloning the DNA into a suitable vector and transforming the clones into a host bacterium, and further screening of the resulting transformants (Handelsman, 2003); these clones can be screened for phylogenetic markers such as 16S rRNA or other conserved genes by hybridization or multiplex PCR (e.g. Stein *et al.*, 1996) or expression of specific traits, such as enzyme activity or antibiotic production (Courtois *et al.*, 2003; Schloss and Handelsman, 2003). Therefore, this functional metagenomics objectively identifies genes that code for a function of interest (Luana *et al.*, 2018), which involves the generation of expression libraries with thousands of metagenomic clones followed by activity-based screenings (Guazzaroni *et al.*, 2015).

The idea of cloning DNA directly from environmental samples was first proposed by Pace *et al.*, (1986) and in 1991, the first such cloning in a phage vector was reported by Schmidt *et al.*, (1991) and this led to the next advancement which was the construction of a metagenomic library with DNA derived from a mixture of organisms enriched on dried grasses in the laboratory (Healy *et al.*, 1995) as previously mentioned. The greatest strength of the functional-driven metagenomics is the fact is that it does not require the genes of interest be recognizable by sequence analysis, this makes it the only non-sequencing approach to metagenomics that has the potential to identify entirely new classes of genes for new or known functions. However, the significant limitation is that many genes, will not be expressed in any particular host bacterium selected for cloning, these genes are required to be expressed in *Escherichia coli* or another domesticated bacterium to be detected (Handelsman, 2004). This functional analysis approach has been applied to identify several degradative enzymes (Henne *et al.*, 2000), novel antibiotics (Courtois *et al.*, 2003; Gillespie *et al.*, 2002), and antibiotic resistance genes (Riesenfeld *et al.*, 2004).

Handelsman, (2004) described this Heterologous gene expression functional-driven approach as a powerful yet challenging approach to metagenomic analysis employed to identify clones that express a function, of which success requires faithful transcription and translation of the gene or genes of interest and secretion of the gene product if the screen or assay requires it to be

extracellular (such as purification and characterization of enzyme of interest). The diversity of the organisms whose DNA has been successfully expressed in *E. coli* is astonishing (e.g. Cha´vez *et al.,* 1995), nonetheless heterologous expression remains a barrier to obtaining the optimal information from functional metagenomics analyses. Rondon *et al.,* (2000) reported cloning-based approach provides a route to study the phylogenetic, physical, and functional properties of the metagenome, and, complements existing approaches to exploit the genetic diversity of non-cultured microbes.

CURRENT APPLICATIONS OF METAGENOMICS

Handelsman, (2004) specified that microbiology has long relied on assorted methods for analysis, among is metagenomics which can provide the tools to balance the abundance of knowledge attained from the culturing technique plus a broader understanding of most of the microbial life. The current application of metagenomics has streamed passed initial motive of accessing the uncultured microbial communities via diversity study, as this is now applied as a powerful approach in most microbial ecological, genetics and degradation studies.

In reiteration, the metagenomics has surpassed the cultivation technique in capturing genuine microbial diversity population, but its constraint must be acknowledged in identifying the low abundance species and relating their most focused metabolic and degradative roles. This has now fruitfully inspired its application in combination with other cultivation-independent techniques such as stable isotope probing, meta-transcriptomics to practically mitigate these constraints as mostly prioritised in combined approach studies. A key instance is a study of Uhlík *et al.,* (2008) which demonstrated the coupling of DNA Stable-isotope probing (DNA-SIP) with a metagenomic approach to eliminates the weakness of these approaches, as a result, stated that complete operons can be identified combining DNA-SIP with metagenomics analysis. Therefore, with this present-day wider application of the metagenomic approach, a large amount of data has been generated while impacting dissimilar areas of high applicability in our world today such as microbial gene mining; thus, we can only imagine the limitless outreach of metagenomic studies in our future research world.

FUTURE PERSPECTIVE

At the inception of the metagenomics, the application of the first sequencing technology termed Sanger sequencing provided huge progress in the journey of metagenomic studies (Gillespie *et al.,* 2002; Uchiyawa *et al.,* 2005). However, Sanger surfaced with its limitations regardless of its then achievements. Providentially, the advent of Next-Generation Sequencing (NGS) technologies - capable of sequencing millions of DNA fragments simultaneously has greatly reinforced this crucial field (Sunagawa *et al.,* 2015; Oulas, *et al.,* 2015). Consequently, the prospect of metagenomics may not do without the next-generation sequencing (NGS) technology, as these have boosted the panorama of metagenomics as perceived in many recent ground-breaking metagenomic studies. Luana *et al.,* (2018) also agreed that many

of the advances in metagenomics can be credited to novel sequencing approaches and the development of new computational methodologies to analyse the generated data.

The NGS is being exploited to sequence full metagenomes, consequently, providing a broad picture of the functions and metabolic potential of the microbial community studied (Zhai and Ma, 2012; Schloss and Handelsman, 2005). The development of this high throughput sequencing platforms in the 1990s has enabled the production of thousands to millions of DNA reads at a lower cost compared to Sanger's and this has hugely impacted and transformed the field of microbial ecology in terms metagenomics research outputs (Turnbaugh *et al.,* 2009). Furthermore, applying the Illumina sequencing with the metagenomic approach is commonly used to acquire an in-depth insight into the biodegradation potential and pathways involved in the degradation process (Mason *et al.,* 2012; Yergeau *et al.*, 2012). Nevertheless, the detection of unknown genes is a key issue limiting its full exploitation (Schloss and Handelsman, 2005). Regardless, the NGS has unravelled huge, significant and extremely informative data useful today for ecological studies, inferences and as references in several databases. Even though processing and analysis of sequencing data are mostly cumbersome and tedious, especially in the terms of gene assembly often requiring certain bioinformatics expertise. Sharpton, (2014) stated that the possibility of gene assembly with the shotgun metagenome sequencing which enables characterization of the functionality of microbes have further appraised the shotgun type of Illumina sequencing technique although the process of gene assembly is mostly regarded much tasking and time-consuming. On this ground, the prospective application of the NGS for metagenomics has not been fully exploited, the synergy could still discover untold relevant benefits of metagenomics and other advanced microbiological techniques while complimenting and resolving individual experimental limitations.

The distant prospect of metagenomics may be only predictable following the trend of advancement in sequencing technology, thus until current issues with the processing and analysis of the larger metagenome data generated is resolved, scientist in the field of metagenomics would not relent until a novel short-cut is discovered on how to access the biological components of sequenced metagenome without the tedious but much-needed bioinformatics analysis. Fortunately, scientists are optimistic this resolve may come sooner than expected – a case of path towards this novelty may have started as seen with Luana *et al.,* (2018) who proposed the construction of novel synthetic circuits that can detect enzymatic activities or target gene output present in the cloned metagenomic fragment aimed towards improving the screening efficiency of metagenomic libraries. Alternatively, researchers would have to solve the puzzle of unravelling microbial communities in complex metagenome (without employing the functional or sequencing route) from the source by proposing a novelty approach.

CONCLUSION

Though the traditional cultivation method has its merits largely enjoyed by microbiologist before the advent of metagenomics. The most unique feature is

the aptitude to practically isolate various microorganisms on a petri dish and the appreciation of their expressed genotypic features has been phenomenal, and still cherished by an arty microbiologist. Yet this has been factually tagged and proved unreflective of the actual microbial population and termed unrepresentative of genuine microbial diversity in most environment. Gladly, the advancement in microbiology has allowed researchers to further explore life diversity improving phylogenetic, genomic, and ecological notions previously established with the traditional cultivation approach. This metagenomics has expanded and reshaped our understanding of certain microbial properties in a diverse environment in terms of their structure, diversity, richness, and dynamics of microbial communities. The current application of metagenomics has bred several laudable findings and discoveries especially in unravelling the majority uncultured microbial world, and the apt shift to the use of NGS technology has generated an increasing amount of data that is indirectly proportional to its biological significance. Consequently, the development of novel high-throughput bioinformatics tools and platforms highly specific and efficient for sequences analysis is paramount to care for the massive data generated in this research circle. This is expected to further unravel more beneficial uncultured communities and novel genes with biodegradation and biocatalytic potentials for industrial exploitation and bioremediation applications.

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