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A metagenomic lens into endosphere microbial communities, promises, and discoveries

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Abstract

The word endosphere represents the internal tissues of plants harboring diverse microbes capable of producing active biological products for various biotechnological and agricultural applications. The discreet standalone genes and interdependent association of microbial endophytes with plants can be an underlining factor in predicting their ecological functions. Yet-to-be-cultured endophytic microbes have geared the invention of metagenomics in various environmental studies to determine their structural diversity and functional genes with novel attributes. This review presents an overview of the general concept of metagenomics in microbial endophytic studies. First, the endosphere microbial communities were introduced, followed by metagenomic insights in endosphere biology, a promising technology. Also, the major application of metagenomics and a short brief on DNA stable isotope probing in determining functions and metabolic pathways of microbial metagenome were highlighted. Therefore, the use of metagenomics promises to provide answers to yet-to-be-cultured microbes by unraveling their diversity, functional attributes, and metabolic pathways with prospects in integrated and sustainable agriculture.

Significance and impact of study

Metagenomics approach in the understanding of the diverse endophytic microbiome, their functions, and the prospect of developing eco-friendly agriculture sustainably.

Keywords: bioinformatics tools, DNA extraction, metagenome, next-generation sequencing (NGS), root endophytes

Introduction

The current knowledge about endosphere biology has been predicted to discover an unseen microbial world colonizing the internal tissues of plants with the potential of promoting plant growth for improved crop production (Hallmann et al. 1997, Compant et al. 2010). Endosphere represents the interior parts of plant tissues that harbor innumerable microorganisms (Adeleke and Babalola 2022). The microbes capable of inhabiting the internal tissue of the plant and completing their life cycle without eliciting any disease symptoms are called microbial endophytes (Compant et al. 2020). The fact remains that endosphere microorganisms majorly recruited during plant development originated from the rhizosphere. Most of them infiltrate plant roots from the root's external environment and colonize the internal tissues. Still, it is also suggested that a certain number of microorganisms are transmitted vertically via seed (Dubey et al. 2020). The three microbial domains, microeukaryotes (i.e. fungi), bacteria, or archaea associated with plants are found in different environments such as tropical, temperate, and cold regions (Saxena et al. 2016). To a large extent, the abundance and functions of these microbes have significantly made their study exciting and promising as applied in agriculture, medicine, pharmaceutical, and industries. Both biotic and abiotic factors

usually influence the growth and survival of plants. As such, this review elucidates the metagenomic studies of endosphere microbes for novel inventories, their exploration, and possible applications in environmental research.

Studies have established that out of the total endophytic microbes in nature, only 1% have been cultured and biotechnologically explored (Huang 2018, Abedinzadeh et al. 2019, Zhang et al. 2019). Microbial endophytes can be found below ground and above ground. The microbial endophytes' interaction with plants can be mutual, neutral, pathogenic, or beneficial (Adegbeye et al. 2020, Tedersoo et al. 2020). However, few studies have indicated certain changes in environmental conditions, hosts' physiology, and other biological factors could mediate a "switch" across mutualistic and pathogenic lifestyles (Schulz et al. 1999, Schulz and Boyle 2005, Bamsile et al. 2021). The beneficial microbes referred to as plant growth-promoting endophytes modulate plant physiology and survival, thus contributing to the host plant's general functioning by stimulating plant growth effectors and other metabolites in the control of phytopathogens (Santoyo et al. 2016, Adeleke et al. 2022).

The roots endosphere community is sometimes referred to as a subset of the rhizosphere community since its origin is directly linked to the rhizosphere. The rhizosphere represents a

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dynamic soil region below ground that closely adheres to the plant roots (Nwachukwu and Babalola 2021). Rhizosphere biology is influenced by root exudation from plant roots, and associated microorganisms called root microbiomes. The competition for food and nutrients by plant tissue colonizers with other microbes is usually less in the endosphere than in the rhizosphere, perhaps due to their direct colonization of the internal tissues of plants (Brader *et al.* 2017). The host-plant response to environmental factors as well as plant tissue location can determine the extent of microbial endophytes' diversity and functions. However, studies have established various endophyte communities from the stems, leaves, and roots of most economically viable plants (Mashiane *et al.* 2017, Adeleke *et al.* 2021, Krishnamoorthy *et al.* 2021).

The plant microbes of the same species can be differentiated from each other based on their strain. Currently, metagenomics as employed by environmental microbiologists to study endosphere microbiomes has facilitated scientific discoveries in (i) redefining and elucidating the kind of associations existing between plants and the associated microbes, and (ii) figuring out the exact functions, metabolic pathway/activity, and phylogenetic relatedness of the possible identifiable novel genes expressed by plant endophytic microbiomes (Prasannakumar *et al.* 2020). Hence, the rapid insight into metagenomic analysis has made the interpretation of complete metagenome sequenced data reads ranging from hundreds to millions of most endophytic bacteria and fungi easier than the genomic reference library (Almeida and De Martinis 2019).

The metagenomics approach employed in analyzing microbial endophyte communities has revolutionized microbiological techniques without undergoing conventional laboratory routines of culturing (Selvin *et al.* 2019). Over the last 20 years, metagenomic studies have progressed significantly in many fields of research, such as agricultural, clinical, and environmental. The term metagenomics was used in 1997 but only to clone fragments of DNA extracted from DNA purified from soil to discover new functions, a kind of complementation of functions (Handelsman *et al.* 1998). Nevertheless, the metagenomics approach employed by the authors only revealed the stability of the DNA fragments after lysis, but without an account of the lysis procedure, which might likely be biased. The downstream application of next-generation sequencing (NGS) in identifying microbiomes revealing their metabolic functions has led to the identification of new microbes, with a detailed understanding of their dynamic physiological and biochemical attributes. The metagenomics approach has assured the study of whole genomic components of both cultured and yet-to-be-cultured microorganisms from environmental samples. Conversely, the major challenge encountered by scientists is the high level of professional skills required in the computational analysis and interpretation of metagenomic sequenced data reads (Vincent *et al.* 2017). Hence, researches on metagenomics and its application aim to unravel the diversity, functions, and metabolic pathways of plant microbes with prospective integration for sustainable agriculture. Therefore, this review aimed to elucidate the metagenomics approach to the endophytic microbes, promises, and discoveries with diverse ecological services.

Why metagenomics studies?

Metagenomics facilitate the direct identification of plant microbiomes and the detection of possible genetic materials,

which underline their environmental functions. In 1995, an account of the first successful screening of a functional gene library has been demonstrated by Healy *et al.* (1995), while performing an investigation on the cloned DNA fragments from a continuous fermentation system, and screening for activities before sequencing. Handelsman elucidated the metagenomics concept 3 years later to evaluate the genetic composition of known and unknown, identifiable and nonidentifiable, and culturable and nonculturable environmental microbes (Handelsman *et al.* 1998). According to the encyclopedia of metagenomics, metagenomics is defined as a modern method employed in analyzing environmental microbes using online computational software in accessing their complete genomic composition and functions without previously being cultured in the laboratory (Bouhajja *et al.* 2016).

The genomic DNA can be extracted from environmental samples (soil, water, plants, etc.) and microorganisms. The application of metagenomics tools has been employed in the retrieval of DNA-sequenced metagenome data from plant samples using different bioinformatics tools in determining microbial genetic relatedness. The microbial DNA can be sequenced using various Illumina platforms (MiSeq, HiSeq, or TruSeq). The most commonly used in the metagenomic study of plant endophytes is HiSeq for diversity amplicon study, i.e. metagenome 2×150 bp 10 million with host depletion. The sequencing of DNA has resulted in generating large metagenome data using bioinformatics tools to annotate and predict the function and diversity of microbes possessing novel genes (Langille *et al.* 2013).

Generally, two approaches can be used to analyze microbes from plant samples, which include (i) shotgun metagenome and (ii) metabarcoding. The advantages of shotgun metagenomics sequencing over targeted-amplicon sequencing are that (a) it reveals the entire genomic contents in samples without targeting a specific group of microorganisms, and (b) it is less biased to the taxonomical classification of multiple microbial genomes and easy identification of novel genetic traits in microbes (Mangrola *et al.* 2015). Currently, metagenome sequencing is becoming affordable, and scientists have employed a combination of metabarcoding and metagenome in various experiments. However, some of the disadvantages of metagenomics use may include, the overrepresentation of abundant species in the metagenome dataset, unnoticeable rare species from the metagenome sequences, and the high cost of sequence analysis. The challenge encountered using shotgun metagenomics is that large host DNA from biological samples, i.e. plants are often sequenced alongside the small fraction of the microbial DNA present in the samples, making it difficult to distinguish the microbial DNA sequence from the abundance host sequence.

Several thousands of bacterial genomes were binned in recent years without culturing the organisms. For example, 154 723 microbial genomes of the global human microbiome were recently reconstructed from 9 428 metagenomes. The metagenomic analysis of the large sequenced reads determines the real structure and functions of the microbial domains present in the environmental samples. Their comparative studies have placed the microbes into different taxa correspondingly to gene libraries with a significant level of difference (Mandal *et al.* 2015). Apart from metagenomics studies, other omics technologies such as metabolomics and proteomics have been used in determining microbial diversity (Li *et al.* 2020). The success recorded using NGS to access metagenome sequences has been remarkable in medicine,

Table 1. Metagenome pipelines.

Database/Naming	Target data	Supporting function	URL/Web online
IMG/M	All	Pipeline	http://img.jgi.doe.gov
EBI Metagenomics	All	Pipeline	https://ebi.ac.uk/metagenomics
MG-RAST	All	Pipeline	http://metagenomics.anl.gov
MetaMicrobesOnline	All	Pipeline	http://meta.microbesonline.org
METAGENassist	All	Tools	http://metagenassist.ca
MetaABC	All	Pipeline	http://metaabc.iis.sinica.edu.tw
METAREP	All	Pipeline	http://jcvl.org/metarep
CoMet	All	Pipeline	http://comet.gobics.de
MyTaxa	All	Pipeline	http://enve.omics.ce.gatech.edu
METAVIR	Virus/Microbes	Tools	http://metavir-meb.univ-bpclermont.fr
VIROME	Virus/Microbes	Tools	http://virome.dbi.udel.edu
CAMERA	Marine/Microbes	Tools	http://camera.calit2.net

agriculture, pharmaceutical, food, and environmental studies using various metagenome pipelines (Table 1). Currently, metagenomics is still working on endophytic microbiota and its promise and bioprospecting are highlighted below.

- (i) Metagenomic studies reveal complete taxonomic classification and functional and metabolic pathways of the active endophytic microorganisms (Forbes et al. 2017). It is used to determine total microbiomes from yet-to-be-cultured environmental microbes, and this has geared the interest of many scientists toward metagenomics studies despite the skillfulness and professionalism involved in the computational analysis and interpretation of the metagenome sequenced data.
- (ii) Generating a lengthening sequence reads from metagenomics studies influences the metagenomics sequence analysis. Currently, the use of real-time Nanopore sequencing analysis in clinical metagenomic studies has emerged and been applied in the detection of viral pathogens (Greninger et al. 2015, Hamza and Bibby 2019).

Conversely, scant information is available on the use of Nanopore sequencing in environmental studies, perhaps due to the problem of accurate identifications and the complexity of environmental samples.

- (i) Metagenomics studies help unravel the microbial community structure, functional genes, and metabolic pathways for the recovery of secondary metabolites through functional-based analyses; whereas, sequence-based analysis reveals the genetic functions linked to certain secondary metabolites. Still, difficulties might be experienced in recovering the target genes. For example, Faoro *et al.* (2011) reported using targeted gene function to recover novel enzyme lipases from flat sediments, the Atlantic rainforest, and esterase from South China Sea sediments.
- (ii) The metagenomics approach in the discovery of novel genes and enzymes for industrial purposes is advancing, though, to an extent, its application is limited as it only focuses on the analysis of the genomic sequences of relative microbial biomass. In an engineered environment or environment with low microbial diversity, metagenomics help to achieve and complete genomic coverage in metagenomic libraries with available sequence while in nutrient-rich environments such as soil, containing about 10^5 cells per sample material; capturing all the taxa might be a challenge, as studies

have shown that only 400 genomes have been captured from 40 000 fosmid library with an average size of 4000 base pairs (Lynch and Neufeld 2015). The aforementioned limitations associated with microbial diversity can be addressed by selectively determining the type of samples to be collected before metagenomics analysis for the targeted microbes and their metabolites with defined activities profiling.

- (iii) Screening of the metagenome library might help scientists explore genes of interest based on their most metabolic function assignments are putative. Screening of targeted genes from metagenome-sequenced data can as well be obtained from the metagenome library (Zainun and Simarani 2018). Creating a metagenomics library for a particular dataset requires screening for the genes of interest, and this can be achieved in two ways. (i) DNA sequence on the clone fragments or (ii) functional analysis of the clones. Metagenomics libraries of various samples, including soil, freshwater and seawater, sewage sludge, marine drainage, alkaline water, and non-alkaline water collected from different sites globally, have been documented (de Almeida et al. 2019). Metagenomic libraries have also been constructed from diverse microbial communities associated with eukaryotic organisms such as earthworms, marine sponges, termites, shrimps, ruminants, and human microbiota.
- (iv) The studies of metagenomics have enabled scientists to characterize microbial biodiversity as a premise for constructing metagenomic libraries to better understand various metabolites such as enzymes, antibiotics, etc., from screened microbes. The ideology behind sample collection from different locations limits the research scope for metagenomics and the desire to develop their current metagenomics libraries.

In addition, high throughput sequencing primarily reveals relative abundance and microbial diversity without preselection, which negates the benefit of direct and unbiased sampling techniques. The large datasets generated from this method pose challenges in data analysis. The computational analysis of different sequenced data based on a sequencing platform using online software packages for each initial investigation to identify possible microbial genes and functions has made information available on the novel microbial species (Nilsson et al. 2019). Hence, increased metagenomics projects across many fields could make sequenced reads information available and accessible in databases by setting up

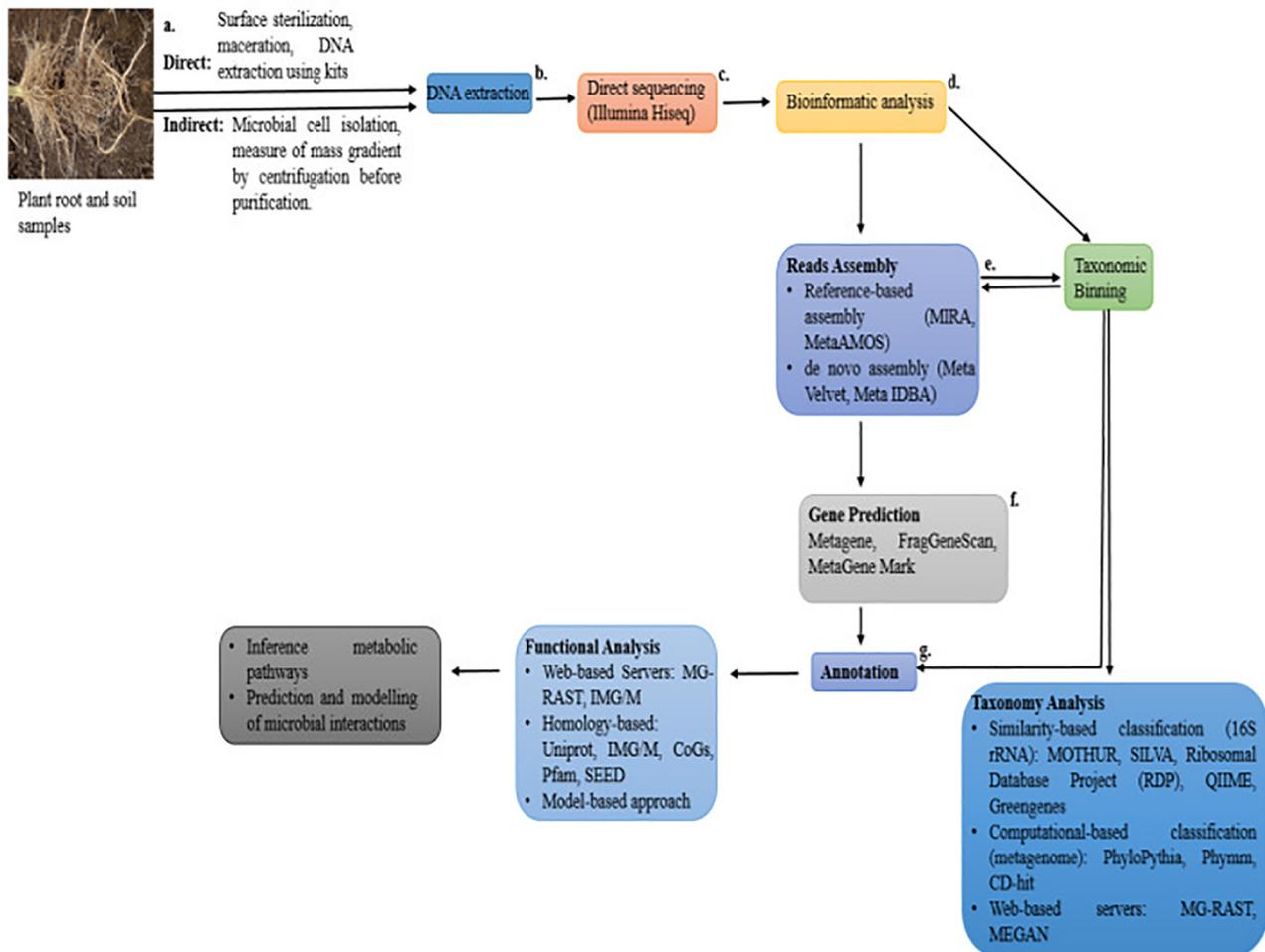


Figure 1. Diagram of metagenomic analysis.

free and quickly operational pipelines for the metagenomics sequence data. Hence, setting genome annotation for particular endophytic microbes may allow automated annotation of the sequence fragments from plant metagenomics projects, their further phylogenetic grouping, and reconstruction of the metabolic pathway.

Endosphere metagenomic promises and discoveries

The application of metagenomics in microbial endosphere studies is promising, and this has made it possible the discoverability of their keen bioactive functional novel genetic traits as an innovation to showcase the metagenomics approach in revealing the potential of yet-to-be-cultured unidentified microbes present in different plant organs and other environmental samples (Fig. 1). Below are some of the relevant applications of metagenomics in the study of endophytic microbes and their potential discoveries as applied in agriculture, medicine, and ameliorating environmental degradations

Metagenomics and discovery of enzymes

Metagenomics studies are becoming the most potent tool for studying yet-to-be-cultured endophytic microbes and unidentified novel enzymes with industrial and biotechnological

applications (Bilal et al. 2018). Commercializing industrial enzymes as an economic asset is currently dominating the world market. Reports have shown that the enzyme lipase market value is envisaged to reach USD 590 million by 2023 (de Almeida et al. 2019). Metagenomics is now employed to discover microbial genes coding for novel enzymes with specific functions. The high demand for enzymes for industrial purposes is enormous, and research is ongoing using a metagenomics approach to identify novel enzymes with unique traits from plants and microorganisms. Plant endophytic microbes stand as a potential source of industrial enzymes.

The use of metagenomic techniques for the identification of uncultured endophytic microbes could facilitate the evaluation of novel enzymes and their possible biotechnological and agricultural applications (Fig. 2).

Many microbial enzymes express high catalytic actions under various optimized conditions, which can be measured via appropriate screening. The screened of an extracellular enzyme such as lipase from some plants has been referenced from dataset metagenomics libraries (de Almeida et al. 2019). The use of metagenomic techniques to assess genes coding for novel enzymes from endophytic microbes are less studied. With current ongoing research findings, the application of modern molecular methods in microbial endophyte studies is promising for new enzyme commercialization.

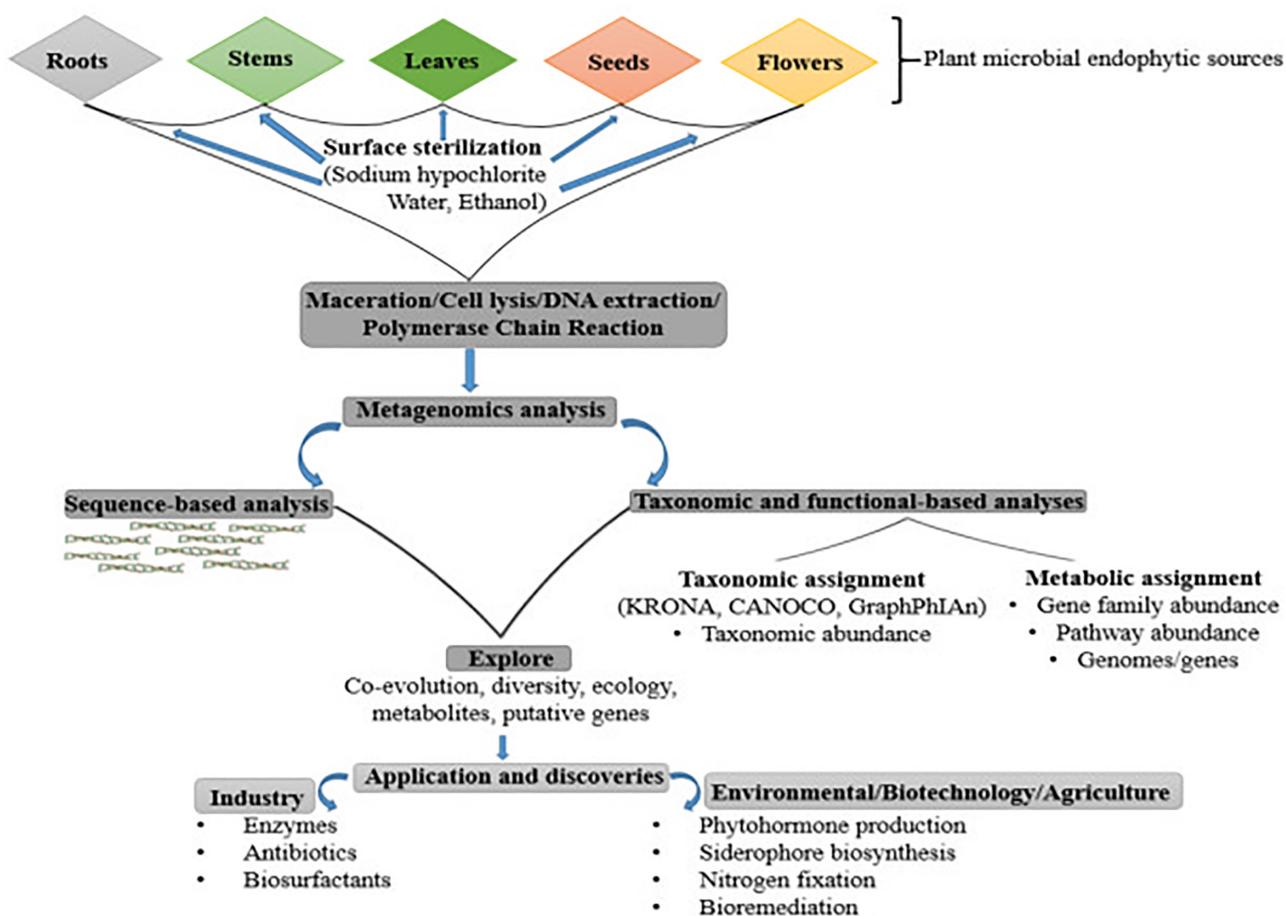


Figure 2. Agricultural, biotechnological, and industrial attributes of endophytic microbes revealed by metagenomics.

Harnessing microbial endophytes through metagenomics study for genes coding enzymes would help in overcoming problems of enzyme shortage and supply to industries (Ngara and Zhang 2018). For decades, enzymes have been explored from plant microbes for industrial and commercial purposes. Every year, new metagenomics enzyme libraries are developed. The scope of developing the metagenomics library has gone beyond collecting new plant samples to screening and developing metagenomics libraries for desirable activities. High-throughput sequencing of the plant endophytes metagenome libraries has advanced in discovering new genes that encode for specific enzymes exhibiting high catalytic activity under high environmental conditions as applied in various industrial processes.

The conventional cultivation and metagenomics methods employed in the study of diverse endophytic microbial communities associated with the Brazilian mangroves for the possible discovery of cellulase as applied in the biodegradation of cellulose biomass as well as their taxonomic and functional attributes have been reported (Thompson et al. 2013). A study by Montella *et al.* (2017) unraveled the use of the metagenomic approach in the analysis of complex microbial community structure and taxonomic diversity from lignocellulosic energy food crops, *Populus nigra*, *Eucalyptus camaldealensis*, and *Arundo donax*. Authors further reported cellulase coding genes from the plant microbes, thus suggesting their future in the hydrolysis of cellulose and lignocellulose materials from the environment. The screening of fungal and

bacterial endophytes isolated from medicinal plants by conventional plate assay for extracellular enzymes such as xylanase, amylase, lipase, cellulase, mannanase, pectinase, and protease on solid media has been reported (Sunitha et al. 2013, Popovic et al. 2015). Similarly, the enzymatic activities of cultural endophytic bacteria isolated from *Plectranthus tenuiflorus* have as well been documented (El-Deeb et al. 2013). Furthermore, studies have also revealed the varied enzymatic activities of endophytic fungi isolated from the stems, leaves, and roots of epiphytic orchids, namely, *Asocentrum curvifolium*, *Dendrobium anosmum*, *Dendrobium phylum*, *Doritis pulcherrima*, and *Aerides falcata* (Sopalun and Iamtham 2020).

Metagenomics and discovery of antibiotics

The metagenomics approach in medicine, environmental studies, and clinical microbiology has enabled scientists to identify diverse microorganisms that possess resistant genes that render the use of certain antibiotics ineffective in the treatment of human diseases caused by pathogens (Breitwieser and Salzberg 2020). The microorganisms isolated from plant samples could be a potential source of antibiotic and antimicrobial agents. The conventional method of extracting natural products from endophytic microorganisms associated with medicinal plants may hinder the full exploration of antibiotics of interest because of the low input of media composition and uncontrolled optimized process parameters. Hence, the need

for an alternative method to discover new drugs becomes imperative using metagenomics in the endosphere microbial community studies. Findings on the extraction of natural products such as didemnin B (Aplidine™) and thiocoraline from marine microorganisms via screening, genomics, metagenomics, and synthetic biology, and their trial in clinical and preclinical studies in the treatment of cancer have been reported (Xiong et al. 2013). The antibiotic potential of uncultured endophytic microbes can be revealed via biochemical and biosynthetic pathways (Oberhofer et al. 2022). Metagenomics screening of clinical samples has assured efficiency and accuracy in clinical diagnosis.

The limitations associated with exploring the natural product have stemmed from the search for an alternative to biological products upshot, which can be achieved by genetic engineering that alters biosynthetic pathways to synthesize new bioactive products. Natural products can selectively be derived from microbes with notable roles in clinical practice. It has been reported that 70 out of the ~90 antibiotics marketed in the years 1982–2002 were obtained from natural products. Notably, the classification of some synthetic antibiotics can be based on their chemical structure that resembles the natural products, i.e. quinolones or fluoroquinolones resembling quinine. Furthermore, the scaffolding approach has been employed in the modification of the physical and chemical structure of synthetic molecules for possible pharmacological use (Müller et al. 2016).

Despite several research findings into the exploration of microbial endophyte resources, the enormous diversity of endophytic microbes is still underexplored, thus creating an avenue for studying novel strains. Culture-dependent methods have been employed in the characterization of potentially active metabolites from endophytes, yet, some are nonculturable. This has prompted researchers to search for alternative molecular techniques in the analysis of microbial endophyte metagenomes for the detection of novel compounds. The metagenomics approach has been applied in determining the whole microbial genome contents, thus describing their whole functions and biosynthetic pathways. The structural and taxonomy differences, and genome sequencing data obtained from bacteria, *Streptomyces* spp. RTd22 isolated from the roots of sunflowers with the potential of synthesizing new bioactive metabolites has been reported (Silva-Lacerda et al. 2016, Hug et al. 2018).

Plant endophytic microbes could form a reservoir of potential genes that are laborious to harness by employing cultivation methods. The discovery of total microbial genes using a metagenomics approach has strategically allowed genetic access to the functional and taxonomic diversity of uncultivated endophytic microbes at the community level. Metagenomics techniques have been successfully employed in the accumulation of novel data in the discovery of bioactive compounds. Müller et al. (2016) reported the use of metagenomics in the discovery of cyclosporine and ecomycins from the endophytic fungus *Tolyposcladium inflatum*, and plant endophytes *Pseudomonas viridiflava* with broad antifungal activities. Similarly, the bioactive compounds produced by microbial endophytes exhibit antimicrobial properties and antiviral activity such as xiamycin from *Streptomyces* GT2002/1503. The novel antibiotics camptothecin produced by the endophytic fungus *Taxomyces andreanae* isolated from the bark of Pacific yew has been reported (Gupta et al. 2020). The genes encoding the natural compounds, ecomycins, munumbicins, xiamycins,

kakudumycins, and pseudomycins in the genome of bacterial endophytes, *Streptomyces* species enabled them to exhibit antibiotic activities. This has validated the prospect of bacterial endophytes as a source of novel metabolites (Christina et al. 2013, Kealey et al. 2017, Singh et al. 2017). Although, Fortman and Mukhopadhyay (2016) argued that, despite the use of metagenomics in the discovery of novel genes for antibiotics synthesis from the endophytic microbes, the research approach is still in infancy, hence, *in silico* and biosynthetic pathways in natural products discovery will be a mature process with conceptualizing prospects.

Metagenomics and degradation of pollutants

Most wastes generated from various environments contain nonbiodegradable pollutants and other xenobiotic compounds. The domestic and industrial wastes released into the environments have endangered terrestrial and aquatic habitats either by spillage, incomplete combustion of fossil fuels, or surface runoff. In addition, the yearly turn-out of petroleum hydrocarbons from industries to the environment contaminates and affects ecosystems. To mitigate against these effects, exploring the potential plant-microbe and their resources in the biotransformation of environmental pollutants may greatly facilitate the breaking down of the aromatic structure in benzene, toluene, and xylene and mineralizing their carbon skeleton (Glick and Stearns 2011).

The bioremediation process involves the use of viable microorganisms or their metabolites to degrade hazardous or toxic substances such as aromatic hydrocarbons, pesticides, and polychlorinated biphenyls (PCBs) from the environment (Ufarté et al. 2015). Some wastes can resist the degradation of recalcitrant materials for many years in the soil. The toxicity of heavy metals that persist in the environment has influenced plant growth and soil properties, mainly in the soil contaminated with nonbiodegradable pollutants (Bouhajja et al. 2016, Ayangbenro et al. 2019). The physicochemical analysis of polluted soil determines microbial actions in biostimulation, bioaugmentation, or natural attenuation as precursors to recover biological resources and applications to develop eco-friendly agriculture (Adetutu et al. 2015). The acclimatization of the microbial community on waste-polluted soil depends on their high tolerance to the utilization of available nutrients as sources of carbon, nitrogen, or energy, which facilitate their degradable ability of pollutants into simpler intermediates until complete mineralization to release carbon (IV) oxide, methane, ammonia, and corresponding final products. Some plant microbes co-metabolize in the degradation of some environmental pollutants. For example, the co-metabolic activities of plant microbes in the degradation of hydrocarbons such as polycyclic aromatic hydrocarbons of chlorinated biphenyls, chlorinated monoaromatics, and chlorinated aliphatics with more than five aromatic rings have been reported (Nzila 2013, Bouhajja et al. 2016).

The microbial degradation pathways of environmental pollutants such as PCBs and ring-structure (aromatic) hydrocarbons have been studied using an identified strain in the treatment of pollutants. However, a vast number of uncultured plant microbes exist and are yet to be explored. Employing a metagenomics approach would provide a detailed plant microbial profile with biodegradation potential (Gillan et al. 2015). Thus, metagenomic studies focusing on the sequencing of DNA from microbial endophytes in generating huge data

Table 2. Metagenomics approach with targeted genes and metabolic pathways.

Metagenomics study	Target gene/Pathway	Reference
Taxonomic profile and functional analysis of endophytic microbes in maize	16S rRNA genes and various metabolic genes	(Fadiji et al. 2020)
Assessment of endophytic bacterial diversity in rose by high-throughput sequencing analysis	16S rRNA genes	(Xia et al. 2020)
Structure and function of the bacterial root microbiota in wild and domesticated barley	16S rRNA gene amplicon and various metabolic genes	(Bulgarelli et al. 2015)
Dynamics of endophytic and epiphytic bacterial communities of Indian cultivated and wild rice (<i>Oryza</i> spp.) genotypes	16S rRNA gene amplicon and various metabolic gene	(Banik et al. 2017)
Bacterial endophytes of sweet potato tuberous roots affected by the plant genotype and growth stage	16S rRNA genes and proteins encoding genes.	(Marques et al. 2015)
Metagenomic analyses of bacterial endophytes associated with the phyllosphere of a Bt maize cultivar and its isogenic parental line from South Africa	16S rRNA genes and protein-encoding genes.	(Mashiane et al. 2017)
Metagenomic insights into communities, functions of endophytes, and their associates with infection by the root-knot nematode, <i>Meloidogyne incognita</i> , in tomato roots	16S rRNA genes	(Tian et al. 2015)
Identification of nitrogen-fixing <i>Bradyrhizobium</i> associated with roots of field-grown sorghum by metagenome and proteome analyses	16S rRNA genes and nitrogen cycling pathways	(Hara et al. 2019)

are on the increase, and a lot of projects have been carried out, with some currently in progress, at Joint Genome Institute (<http://img/jgi.doe.gov/>) on the understanding of metabolic interactions between microbes in the environment and various ways they can be explored in the biotransformation of environmental pollutants (Table 2).

Metagenomics and biosurfactants

Surfactants exist either as biological or chemical agents that modify the adsorption between liquid and non-liquid compounds across the surfaces, thus lowering the surface tension. Surface-active agents are classified into high molecular and low molecular molecules. The former bind to the solid surface while the latter can lower the surface tension. Currently, interest is being geared toward biosurfactants than chemical surfactants, due to their nondegradation potential and related environmental issues (Shalini et al. 2017).

Plant microbes can produce biosurfactants. However, the application of biosurfactants produced by endophytic bacteria *Pseudomonas fluorescens* RE1 and *P. fluorescens* RE17 in the bioremediation of complex nonbiodegradable xenobiotic and heavy metals such as cadmium, nickel, zinc, chromium, and lead have been reported (Karnwal 2018). Marchut-Mikolajczyk *et al.* (2018) reported the biosurfactant production and hydrocarbon degradation activity of endophytic bacteria isolated from *Chelidonium majus* L. Also, the biosurfactant production potential of *Piper hispidum* endophytic fungi has been reported (Silva et al. 2019).

Some examples of biosurfactants produced by endophytic microbes include lipopolysaccharides, polysaccharides, and lipoproteins. The characterization of glycolipid-type biosurfactant from endophytic *Acinetobacter* spp. ACM25 has been documented (Shalini et al. 2017).

In recent times, the process of biosurfactant discovery has been improved by making use of modern molecular techniques. Metagenomic techniques have been employed in the discovery of novel biosurfactants useful in ecosystems (Jackson et al. 2015). The recent information on biosurfactants as stemmed by bioinformaticians and computational biologists has acceded to developing a computational approach

tailored to biosurfactants discovery and accumulation of data/knowledge in the central online resources. Specifically, the database online resource “BioSurf” is now employed to provide collective information about biosurfactants, proteins, organisms, metabolic pathways, and related bioinformatics and algorithms tools in biosurfactant discovery (Mosa et al. 2016). The biosurfactants from plant microbial endophytes and their applications have been discussed in full in many reports (Shalini et al. 2017, Wu et al. 2018, Silva et al. 2019). Furthermore, the metagenomics approach for constructing DNA libraries from oil-contaminated samples, followed by the screening of biosurfactants-producing clones should be developed.

16S rRNA, gene expression, and stable isotope probing

The knowledge about microbial diversity possessing distinct novel traits in determining their functional characteristics has been demonstrated experimentally and analyzed computationally. The gene expression studies of microbial genome composition rely on sequence-based techniques in characterizing whole genomic functions. Despite this, the targeted analysis of microbial gene content is still complicated.

The success recorded in the past few decades using a culture-independent method in understanding the abundance, diversity, functions, and distribution of endosphere microbial communities makes it a critical factor in studying the whole microbial genomic sequence (Chen and Murrell 2010). The cultivation of microorganisms using the conventional agar method is known. The invention of culture-independent methods has changed the old concept of isolating and analyzing microorganisms from plant samples. The conventional design of metagenomics studies, a culture-independent way to extract and analyze the total genomic component from plants, has made it possible to understand endophytic microbes and their proteins encoding genes. Despite the former assertions, the understanding of the metabolic functions of endophytic microbes is still understudied. However, the current use of isotope probing has made it possible to determine microbial

metabolic pathways and functions for easier elucidation of the ecophysiological stability of endophytic communities associated with the host plants (Gutierrez and Kleindienst 2020).

Stable isotope probing

Stable isotope probing (SIP) has generated a lot of scientific interest and is a promising method for studying carbon flow and plant–microbe interactions. Recently, DNA-SIP has been gaining interest in filtering selected DNA from microorganisms (Coyotzi et al. 2016). DNA-SIP has also been employed in molecular biology by stable isotope incorporation with microbial biomass to enhance the quick recovery of already labeled stable isotope atoms (^{13}C , ^{15}N , ^{18}O , and $^{34/36}\text{S}$) or nucleic acids from active microbial DNA during microcosm or *in situ* activation of such substrates in identifying the functions of environmental microbes despite their initial abundance and culturability (Lünsmann et al. 2016).

Literarily, DNA-SIP has been developed to probe the functions and metabolic pathways of microorganisms from the plant source. Developing SIP has advanced the linkage of microbial endophyte functions via retrieving a stable isotope atom from a specific substrate into microbial cells with detailed phylogenetic and functional information such as DNA or RNA and lipid (Gutierrez and Kleindienst 2020). The DNA-SIP technique is now being used together with metagenomics, thus making the concept a reality in the identification of microbial genes and their systemic metabolic pathway. Hence, the combination of DNA-SIP with metagenomics made it easy to detect the functional gene diversity and abundance of microbial species from plants compared with metagenomic libraries and the discovery of novel secondary metabolites (enzymes) and other biologically active compounds. Metagenomics also allows access to microbial genomes with unique metabolic activities of interest. Under certain experimental conditions, the use of metagenomics in analyzing DNA gotten from DNA-SIP incubations has been used in the recovery of novel metabolites applicable in biodegradation, bio-transformation, and biosynthesis (Nkongolo and Narendrula-Kotha 2020).

The application of the DNA-SIP approach in the identification of grass rhizosphere microbial endophytes population through exudates secretion and influence on the diversification of active bacterial communities colonizing the root-adhering soil and inhabiting the intracellular spaces in roots tissues has been reported (Guyonnet et al. 2018). Similarly, the report had shown the use of DNA-SIP in the identification of active fungal endophytes colonizing the roots of the rice plant (Zhang et al. 2020). The spatial differences in a microbial population inhabiting the ryegrass rhizosphere and exudates utilization potential using PLFA-SIP (Phospholipid Fatty Acid) are documented (Butler et al. 2003, Li et al. 2016).

Conclusion

Researchers are gaining interest in studying plant–microbe interactions, mostly endophytes, which are presumed to achieve success in due time. Culture-dependent methods of microbial isolation are surrounded by limitations, considering that only 1% of the total microbes in the environment can be identified. As such, it is presumed that these unknown, uncultured, and unidentified microbes from the environment might possess novel genetic attributes that can be explored for

agricultural and biotechnological purposes. The need for the identification of yet-to-be-cultured endophytes using omics technology and metagenomics becomes imperative. Metagenomics can be functional or sequence-based, and this gives more understanding of the structural, functional, and metabolic pathways of the long metagenome sequenced data read. The computational analysis of the sequences has enabled researchers to analyze and explain the phylogenetic relatedness and taxonomic functions of endophytic microbes.

Many research findings have been carried out on the plant–microbe interactions both in the internal and external plant environment. Yet, many uncertainties still surround microbial endophyte studies, such as the nonspecificity of microbes interacting with plants. Hence, new findings regarding the role of endophytes and an understanding of their interactions with plants continue to emerge. In addition, a combination of omics and metagenomics has provided answers to some questions relating to the relative abundance, diversity, and taxonomy of environmental microbes.

NGS methods have revolutionized metagenomics in analyzing relative abundance, diversity, and functions of microbiota from the environment. It is established that culture-dependent methods only study minor microbial cell mass (1%) from the total microbial community in a sample. Hence, NGS or metagenomic sequencing needs to be adopted to analyze the whole microbial genome. Metagenomics studies can be performed by targeting specific genes of known functions. The advancement in NGS using shotgun metagenomics high-throughput sequencing has revealed the genetic composition of endophytic communities about root metagenomics libraries for comparison with the hundreds to millions of sequence data reads from uncultured microbes. Furthermore, the metabolic functions of endophytes' metagenome data can be determined using DNA-SIP. DNA-SIP has made a great advancement in molecular biology, linking the functional potential of microbial endophyte communities by retrieving a stable isotope atom from a specific substrate into components of microbial cells with corresponding phylogenetic information. Hence, the combination of DNA-SIP with metagenomics has made the detection of diverse microbial species from metagenomic libraries easier with the identifiable novel genes and their systemic metabolic pathways.

Conflict of interests

None declared.

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Authors contributions

B.S.A.: conceptualization, writing—original draft, and revisions; D.M.: writing—review and editing; and O.O.B.: conceptualization, writing—review and editing, supervision, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Data availability

There are no data associated with this article.

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