PROSPECTS FOR DIATOMS IDENTIFICATION USING METAGENOMICS: A REVIEW

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Abstract. The marine environment is the largest ecosystem, richest in biodiversity and biological activity. Diatoms are almost omnipresent and common in the plankton and benthos in both freshwater and marine environments. They have a major influence on their environment whereas representing a significant part in primary production and carbon fixation in marine ecosystems. Microscopic examination and cell culture technique have been applied to detect, isolate and study the diatom species. On the other hand, molecular methods have contributed to overcome the drawbacks of the classical methods and also to accomplish the research objectives more efficiently. Metagenomics is an advanced molecular method utilizing direct collected microbial samples and has been used in various fields of microbial studies. It provides obvious details on the taxonomy, biodiversity, ecology and further information around the potential functions. Consequently, it can explore the biochemical components that have significant importance in various biotechnology, ecology, biomedicine and industry applications. This review focused on the effectiveness of the metagenomics method for exploring microbial communities and recruiting this to discover the diatom community.
Keywords: *microbial communities, genomic, sea water, molecular technique, gene markers*

Introduction

Diatoms are silicified, ubiquitous and highly abundant microalgae that are rich in minerals, metabolites and various biochemical compounds. These compounds have significant roles in various fields such as industry, pharmaceutics, biotechnology, and biodegradation (Vanelslander et al., 2009). Some species are used in the biomonitoring of ecological assessment as a response to the environmental fluctuations and changes in water habitats (Poikane et al., 2016). Diatoms have been described and characterized traditionally based on morphological characteristics (Falasco et al., 2009), by microscopic examination which does not require cultivation in a laboratory. Furthermore, valuable biochemical components of diatoms have been detected through growing, isolating and studying characteristics of the species after being cultivated under laboratory conditions (Araújo and Garcia, 2005). Despite the long history of the study of diatom diversity and the numerous researches about its importance, knowledge on the diversity of diatoms and their potential applications still limited. Consequently, the ecological conditions and various interactions related to them continue to be misleading.

In the 1980s, molecular techniques were applied to diatom studies for the first time (Medlin et al., 1988). Molecular phylogenetic researches have been widely performed to overcome morphological limitations to identify and classify diatoms (Evans et al., 2007; Jahn et al., 2007; Mann et al., 2010; Moniz and Kaczmarska, 2010). Powered by advances in the technology of next-generation sequencing, metagenomics has the possibility to explore the taxonomic and functional diversity of the microbes sampled from a certain

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natural environment. Moreover, metagenomic analysis aims to determine the genome sequences of uncultivable and rare microbes, for describing microbial ecosystems and for discovering the novel genes and gene products (Wrighton et al., 2012; Yatsunenko et al., 2012; Albertsen et al., 2013).

This review shows the traditional methods used in diatom community studies and obstacles that hindered the advancement of diatoms' researches and applications. In addition, it illustrates the metagenomic approach as an alternative method to diatoms discovery and discusses the potential capabilities and applications.

General information of diatoms

Diatoms (Bacillariophyta) represent one of the most common and high diversity group of microalgae and have an important role in the food web and ecosystems (Mann, 1999). They are photosynthetic, microscopic, unicellular and eukaryotic microalgae (McLaughlin, 2012). There are more than 200,000 species of diatoms reported worldwide (Mann and Droop, 1996), but only about 12.000 species have been defined (Guiry, 2012).

Diatoms are almost omnipresent, they exist wherever water is present, in water bodies and terrestrial ecosystems as well as in aerosols (Jahn et al., 2007). They occur in terrestrial environments such as mosses, wet rocks and soils (Falasco et al., 2014; Tofilovska et al., 2014). Most of diatoms are common in the plankton and benthos in all aquatic habitats including freshwater and marine (Smol and Stoermer, 2010), except the warmest and most hypersaline environments (Round et al., 1990). Benthic diatoms are found in large quantities on the surface of intertidal sediments that are covered with water at high tide and exposed to the atmosphere within low tide status (Admiraal, 1984; Round et al., 1990; Haubois et al., 2005). Their dominant and diversity in the tidal flats contribute to the maintenance of the functional ecosystems such as primary production, algal biomass, nutrient cycling and sediment stabilization (Admiraal, 1984; Sullivan and Moncreiff, 1988; Underwood and Kromkamp, 1999).

The wide distribution of diatoms makes them fitting tools for a variety of applications both, as living and fossils organisms (Atazadeh and Sharifi, 2010). Marine diatoms contribute to around 40% of the total primary production in marine ecosystems and 20% of global carbon fixation. They feed the food chains of aquacultures with sufficient levels of amino acids and vitamins (Brown, 1991). Moreover, diatoms contain a high abundance of diverse biochemical compounds that make them important sources for applications in various fields (Caldwell, 2009; Falkowski et al., 1998). Some strains have been selected as the best candidates for biofuel production (D'Ippolito et al., 2015). Biochemical compounds are also used in other applications such as in therapeutic (e.g. anticancer and anti-tuberculosis) (Lauritano et al., 2018; Hussein and Abdullah, 2020), for foods, food supplements and nutrition industries (Chauton et al., 2015). In addition, they can be applied for biomineralization, biomaterials synthesis, degradation of wastes, and nanotechnology (Dolatabadi and de la Guardia, 2011; Jamali et al., 2012). Besides, diatoms play role in forensic science as one of independent techniques used to diagnose a cause of death by drowning (Levkov et al., 2017). Some species, especially benthic diatoms, are highly sensitive to changes in the condition of the environment. Therefore, they are used as bio-indicators for the ecological assessment of water habitats around the globe (Stevenson and Smol, 2015; Poikane et al., 2016).

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Traditional methods applied for studying diatoms

Many of the studies depended on the traditional methods to determine taxonomic information, cellular composition and the functional roles of diatoms. Despite the long history of the study of diatom diversity and the numerous studies about its importance, the information on the diversity of benthic diatoms still limited.

Microscopy examination

Morphological taxonomic identification is the traditional methodology for determining the diatoms used to date. This method is performed by microscopic inspection of the outline and details of the silicified frustule or valve (Shape, Raphe, Puncta and Cingula), and the examination slid is prepared according to the condition of the study sample (McLaughlin, 2012). The morphological method is distinguished by its ability to examine the sample directly without the need for culture, and to identify a large majority of the taxa and lower taxonomic levels, until genus and species, shown in (*Fig. 1A*) (Falasco et al., 2009; Rivera et al., 2018).

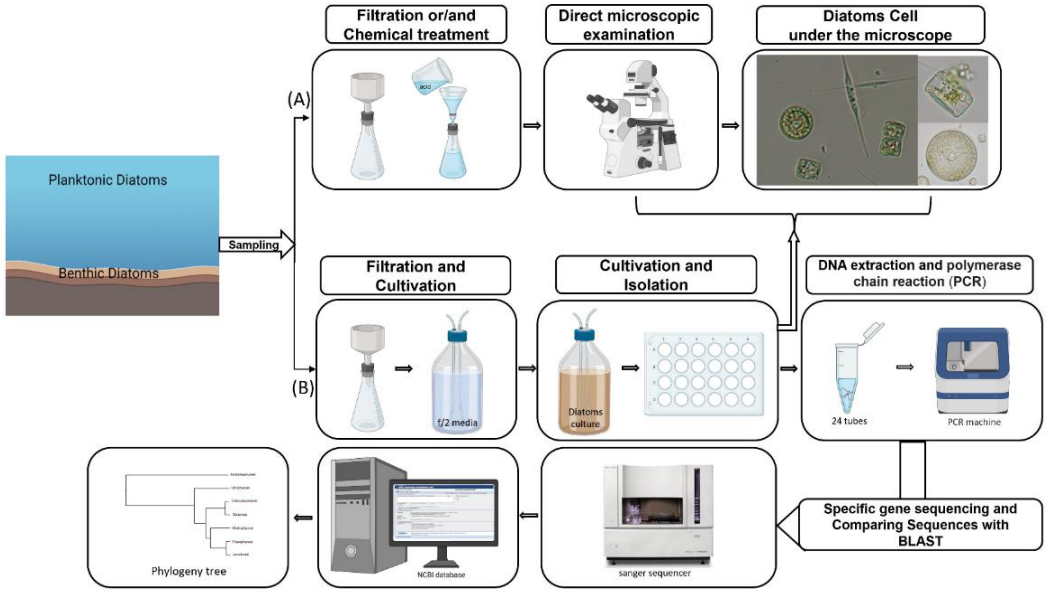

Figure 1. *Illustration of the steps of the traditional method for studying diatom species; (A) Microscopic examination, (B) Cultivation and Sanger sequencing*

However, the morphological method is an impediment for continuous studies for several reasons: 1) depends on the simple forms and small size of benthic diatoms. Also, challenges arise when differentiating between morphologically near species, 2) it needs an extensive experience, 3) requires a great deal of time and effort (Sullivan and Currin, 2002; Brotas and Plante-Cuny, 2003; Underwood and Barnett, 2006; Kahlert et al., 2012). In addition, before applying morphological analysis, some samples need to be treated with acidic treatment, because these samples contain a lot of sediment particles and organic material. As a result, this processing removes all organic material and makes it impossible to know whether the frustules of diatoms are dead or alive (Baldi et al., 2011).

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Cell culture technique

Diatom cells can be used for numerous applications of biotechnology such as using silicon originated from frustules in aquaculture, using of intracellular and extracellular metabolites extracted from the cells in cosmetic, industrial and pharmaceutical applications (Lebeau and Robert, 2002). Given the importance of diatoms, species cultivation is the reliable method for growing, isolating and studying the characteristics of the species and its valuable components. This method is based on preparing an artificial environment in the laboratory, with suitable ingredients for culturing diatoms (*Fig. 1B*). In addition, the procedure for the culture involves other aspects such as maintenance of the culture in the laboratory scale, as well as control the quantity and quality of products throughout careful control of environmental factors and conditions, such as temperature, aeration, availability of nutrient, pH and light intensity, and their constant supply to the aqua farmers in different phases of growth (Lebeau and Robert, 2003; Perumal et al., 2015). Cell culture technique may be able to grow individual species that have not been well defined, to isolate those important for biotechnology or to increase cells number for species to be studied (Nanjappa et al., 2013).

Although culturing diatoms has many advantages, there are many drawbacks that make it a tedious and costly process, such as 1) imbalance of any component of the media leads to the limitation of diatom growth, 2) it requires certain light intensity and photoperiod, 3) it causes overproduction of the beneficial products with a decrease in biomass production as a consequence of metabolic stress conditions, 4) some species may not grow in the presence of others or they may appear transparent (Sullivan and Currin, 2002; Lebeau and Robert, 2003; Kahlert et al., 2012; D'Ippolito et al., 2015). In addition, microalgae are generally challenging in axenic culture without any contamination (Ashokkumar et al., 2015).

Microscopy examination and cell culture technique are the conventional approaches that have been used extensively in the taxonomy and biotechnology. Nevertheless, there were many studies needed more examinations, such as chemical, physical and biochemical tests and statistical analysis, to complete the results of the study. Despite that, these methods are still used up to date.

Molecular approaches

To identify and characterize the taxonomy of cultured and uncultured diatoms, there is a need for molecular methods that are more effective, more accurate, rapid and highly specific for the detection of diatom species, as an alternative method to microscopic examination (Siaut et al., 2007; Nguyen et al., 2011). Such morphologically similar species are not necessarily similar in their genetic content, therefore these methods provide precise identification at the species level. Accordingly, protein expression is different within species. Sanger sequencing is one of these approaches based on PCR sequencing of a specific gene, where it can accurately read an average length of 800 base pairs. These methods rely on the extraction, storage, amplification and sequencing of DNA from environmental samples (*Fig. 1B*) (Lear et al., 2018). Therefore, using the Sanger sequencing method is suitable for individual gene sequencing from a pure culture containing a single strain. In addition, this method can be run for 96 individual specimens at one time (Kim et al., 2014). Sanger sequencing has been used to study the diversity of microalgal communities but in low-diversity environments (Aliaga Goltsman et al., 2009; Bates et al., 2012; Park et al., 2015). Although this technique is in use, yet it does not fit

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large-scale experiments and the complex microbial communities study. As such, sequencing technologies were developed to coincide with the modern studies that have focused on microbiota and microbiome (Turnbaugh et al., 2007; Thursby and Juge, 2017; Song et al., 2018). Furthermore, metagenomics is a powerful molecular approach used for the comprehensive analysis of microbial community which replaces a series of traditional studies.

Metagenomics

Metagenomic analysis is an advanced molecular method that offers comprehensive portraits of microbial communities isolated directly from their environment (Dutilh, 2014; Kumar Awasthi et al., 2020). It provides more significant data and a deep insight into the taxonomic composition and diversity, functional genes, novel genes exploration, the structure and organization of genomes, metabolic products and biocatalysts, which were difficult to find through classical laboratory methods as the conditions were optimized for those finding which missed a lot of important aspects to human needs and knowledge (Tringe et al., 2005; Felczykowska et al., 2015; Roumpeka et al., 2017). Metagenomics is primarily a microbial community science, with a primary emphasis on describing and predicting the interactions between different populations of microbes and the interaction between species or among species (Marx, 2013).

Nevertheless, metagenomics is a culture-independent analysis of the genomic content of entire microbiome into their structure and function in a given environment (Handelsman et al., 1998). It depends on two procedures which are next-generation sequencing (NGS) and bioinformatics (*Fig. 2*). NGS provides millions to billions of nucleotide short reads in massively parallel analysis and high-throughput with low cost (Metzker, 2010; Mardis, 2011). These sequencing outputs are digital and hence enable direct quantitative comparisons through bioinformatics tools (Kulski, 2015; Jünemann et al., 2017). Therefore, this combination produces huge data obtained from environmental DNA and processes it to provide an obvious perception of their biosynthesis.

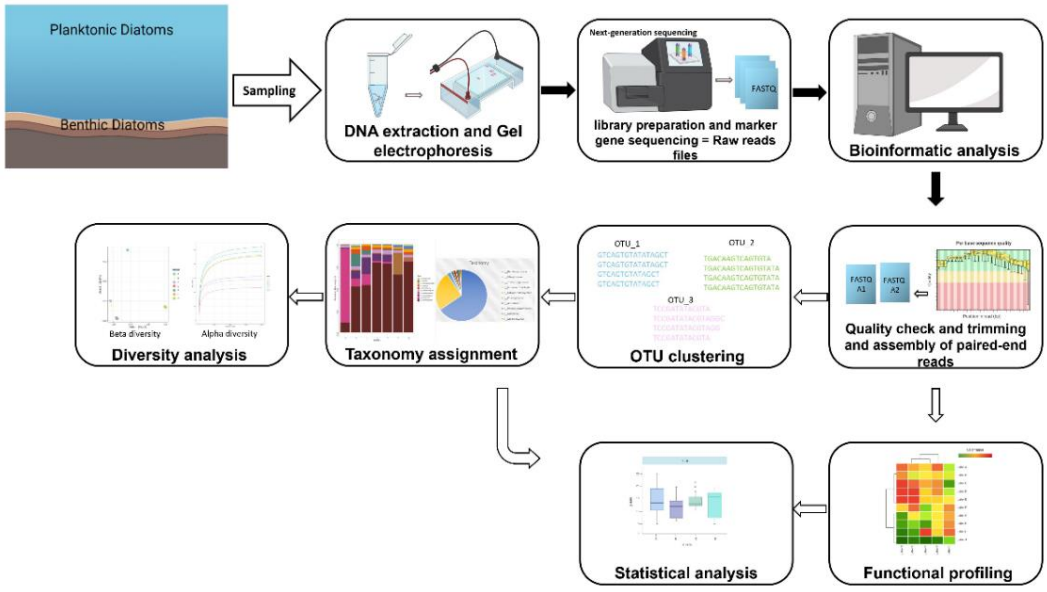

Figure 2. *Schematic illustrates the amplicon metagenomics workflow for studying diatom community*

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Marine microbial metagenomics

Communities of microorganisms- archaea, bacteria, viruses, phages, fungi, microalgae and other eukaryotic microorganisms- are an integral part of, and play important roles in, various ecosystems. The marine environment is the largest ecosystem, richest with biodiversity and biological activity (Zhao, 2011; Felczykowska et al., 2012). The microbial community was estimated in the oceans which host about 3.6 $x10^{29}$ microbial cells. While soil represents the most diverse environment contained 2.6 x 10^{29} cells of the bacterial community (Torsvik and Øvreås, 2002; Sogin et al., 2006). As for the human level, the intestine is the most densely part of the microbial community in the human body, where bacterial counts reaching $10^{12\text{-}14}$ cells (Prescot et al., 1999; Tremaroli and Bäckhed, 2012; De Mandal et al., 2014). Although of the numerous studies on microbes and their ecological importance, the marine microbial community is still not completely exploited yet. The main reason for not characterizing these microbes is the difficulties in detecting under microscopy and in reproducing or culturing microbes by classical techniques under laboratory conditions (Epstein, 2013).

Nowadays, metagenomics can be considered an advance technology to investigate genetic information of marine microbiome (Barone et al., 2014). The taxonomy of metagenomics aims to detect the exact species of microbes and to classify the data of various microbial groups through the analysis of specific genes that reside in a given environment (Kumar Awasthi et al., 2020). Metagenomics can recognize the microbial genes that carry out specific functions and can detect the ecological factors that shape the microbial diversity of community structures (Delmont et al., 2011). Moreover, it contributes to found microbial fingerprints of diverse environments (Behzad et al., 2016). Using metagenomics term was began when Handelsman and colleagues extracted the collection of microbial DNA from soil samples (Handelsman et al., 1998). For 20 years, metagenomics has been used to explore microbial communities in soil, marine water, activated sludge, animal waste and, human and animal guts (Poroyko et al., 2010; Beale et al., 2018; Cabral et al., 2018; Cai et al., 2018; Lekunberri et al., 2018; Yang et al., 2018). For example, Hess et al. (2011) applied high-throughput sequencing to analyze the samples from the rumen of fistulated cows samples in an extended metagenomic study. They discovered more than 2.5 million novel genes and the nearly entire genomes of 15 microorganisms that had never cultured in the laboratory. They also recorded exceeding 27,000 putative carbohydrate-active enzymes with cellulolytic function (Hess et al., 2011). Besides, metagenomic studies provide significant visualization into previously unidentified terrestrial and marine microorganisms (Daniel, 2005; Simon and Daniel, 2011).

High-throughput sequencing

Metagenomics depend on NGS which works on the principle of high-throughput sequencing (HTS) that was successfully implemented in the microbial community environment, where it becomes essential in studies on genomics, epigenomics and transcriptomics (Sogin et al., 2006). There are two major methods based on high-throughput sequencing for studying the microbiome: whole-genome-shotgun (WGS) and marker-gene (amplicon) metagenomics. WGS is sequencing complete genomes of all microorganisms in a certain environment at a single time. In contrast, the sequencing-based on marker gene targets a specific region of gene for microbial community existent in an ecological sample (*Fig. 2*) (Pérez- et al., 2020). Both approaches are suitable option

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applicable in different conditions according to the target study. Nevertheless, marker gene analysis is useful for long- term projects or studies due to the fact that it consumes mostly short time, because of their low cost, and the simplicity and easiness of analyzing results for large numbers of samples, compared to WGS (Knight et al., 2018).

Generally, high- throughput sequencing involves PCR amplification of a marker gene using primers designed according to the marker. All genetic markers are conserved genes including one or more hypervariable regions, which can be used to discriminate different or closely related lineages. The ideal marker (1) composed of a short sequence which amplified and sequenced easily during one read following a standardised laboratory technique, (2) the global primers is flanked by the conserved region, and (3) it has the ability which resolving the different organism's species (Stoeckle, 2003; Hebert et al., 2003; Moritz and Cicero, 2004). Various gene regions have been used as markers for different microorganisms. The mitochondrial cytochrome oxidase I gene (cox1 or COI) has been used for the analysis of eukaryotes communities (Seifert et al., 2007; Stern et al., 2010). The widest marker that contributes to studying microbial diversity and community structures is nuclear-encoded small subunit ribosomal RNA genes (SSU-rRNA). This marker includes the 16S rRNA gene (16S rDNA) and the 18S rRNA gene (18S rDNA) that has been used extensively to examine prokaryotic and eukaryotic diversity, respectively (Janda and Abbott, 2007; Zhan et al., 2013; Hugerth et al., 2014; Saghaï et al., 2015; Groendahl et al., 2017; Yergeau et al., 2017; Winand et al., 2019). Furthermore, another nuclear marker is the internal transcribed spacer region (ITS), which is a common one in the fungal community study (Tedersoo et al., 2010; Schoch et al., 2012; Kemler et al., 2013; Nilsson et al., 2013), and already been used as a marker in some protists (Stern et al., 2010; Molins et al., 2018). To study photosynthetic microorganisms, microalgae, the chloroplast gene encoding, ribulose-1,5-bisphosphate carboxylase large-subunit (rbcL) is utilized as a molecular marker (Patel et al., 2018; Pujari et al., 2019).

Marker genes of diatoms

For diatom communities, the 18S rRNA gene, COI, ITS and rbcL already have shown potential for use as marker (Moniz and Kaczmarska, 2009). Nevertheless, two markers of them are more useful to analysis diatom communities, 18S rRNA gene and rbcL gene, because they are less heterogeneous between individuals when distinguishing between species (Mann et al., 2010; Zimmermann et al., 2011). The highly conserved region, 18S rRNA gene, uses for deep phylogenetic analyses and biodiversity screening. It contains nine highly variable regions; V1 to V9, but V4 and V9 regions are the best candidates (Stoeck et al., 2010; Pawlowski et al., 2012; Zimmermann et al., 2015). Both of them are the most variable regions of the 18S rRNA gene, however, the V4 region is strongly approximated to the variability of the entire 18S gene (Dunthorn et al., 2012). The V4 represents the largest and most complex of the highly variable regions within the 18S locus, while its length ranges 390–410 bp long fragment of the 1800 bp long 18S rRNA gene (Nelles et al., 1984; Nickrent and Sargent, 1991). The efficiency of using the V4 region in environmental studies is due to its ease of amplification with the universal primers and its ability identification to the species level. The 18S rRNA gene is distinguished by its high representation in databases and more extensive compared with other genetic markers (Zimmermann et al., 2011).

On the other side, rbcL marker also demonstrated the ability to distinguish among diatom taxa and study of the phylogenetic (Trobajo et al., 2010; MacGillivary and

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Kaczmarska, 2011; Rimet et al., 2018; An et al., 2018). Use the rbcL gene as a genetic marker is recommend due to its ease of amplification and alignment, and low susceptibility to contamination by heterotrophic pollutants (Evans et al., 2007; MacGillivary and Kaczmarska, 2011). Furthermore, it affords better resolution recognition of diatom species than 18S rRNA, in addition to it lacks long branch artefacts among its phylogeny and not influenced by sequencing errors (Beszteri et al., 2001; Lenaïg Kermarrec et al., 2013; An et al., 2017). Despite of it is appropriate as a marker for the study of taxonomy and phylogenetic analyses of benthic diatoms, it is more conserved than the 18S rRNA gene and failed to cluster the entire diatoms assemblage (Guo et al., 2015; An et al., 2017).

Metagenomics- next-generation sequencing applied in diatoms study

Next-generation sequencing (NGS) is the most effective technique to analyze diatom community structures and functions, where render it possible in a short time, less effort and minimal cost (Visco et al., 2015). It enables the rapid and accurate classification of benthic diatom and contributes extremely to study the biodiversity. It can also reveal hidden and small cells that cannot appear under the microscope (Zhan et al., 2013). NGS includes various platformers and three of them were commoly applied in diatom studies, Roche 454 System, Ion torrent and Illumina sequencer.

The 454 pyrosequencing was applied to study microbial communities in various environments for different purposes (Tedersoo et al., 2010; Cheval et al., 2011; Mayo et al., 2014; Groendahl et al., 2017). It was a convenient tool for the taxonomic characterization of diatom communities and within the context of biomonitoring (Zimmermann et al., 2011; Kermarrec et al., 2013; Lenaïg Kermarrec et al., 2014; Nanjappa et al., 2014; Piredda et al., 2018). Ion Torrent Personal Genome Machine (PGM) has been used for structure and function analyses of fungal, bacterial and archaeal communities (Whiteley et al., 2012; Kemler et al., 2013; Groendahl et al., 2017). Most diatoms studies that applied HTS by Ion Torrent machine, targeted rbcL gene, compared the ability of the molecular approach to assess the composition of diatom community, quality indices (the Specific Pollution sensitivity Index (SPI)) score and ecological status, to the ones generated by the morphology-based method (Rivera et al., 2017; Rivera et al., 2018; Bailet et al., 2019). The third platform which used for studying diatom was Illumina platform, it which has been used combined with the rbcL in most studies to explore the structure and diversity of diatom community with estimating the environmental condition and the effect of spatial and temporal changes on diversity and distribution of communities (Rimet et al., 2019; Tapolczai et al., 2019). Recently, most studies are targeted to describe benthic diatoms communities by using Illumina sequencer with rbcL or 18S rRNA gene (An et al., 2018, 2020; Mora et al., 2019; Bailet et al., 2020; Huang et al., 2020; Stoof-Leichsenring et al., 2020).

Metagenomics applications

Identification of microbial communities is followed by detecting their functional genes and metabolic activities, therefore, leading to employment of microbes to useful various applications. Metagenomics is an effective tool to achieve that and reveal the secrets of countless microbial communities. The metagenomic approach has the potential to improve knowledge in various fields, such as biomedicine, Agriculture, pharmaceutical, industrial and ecological applications (*Fig. 3*).

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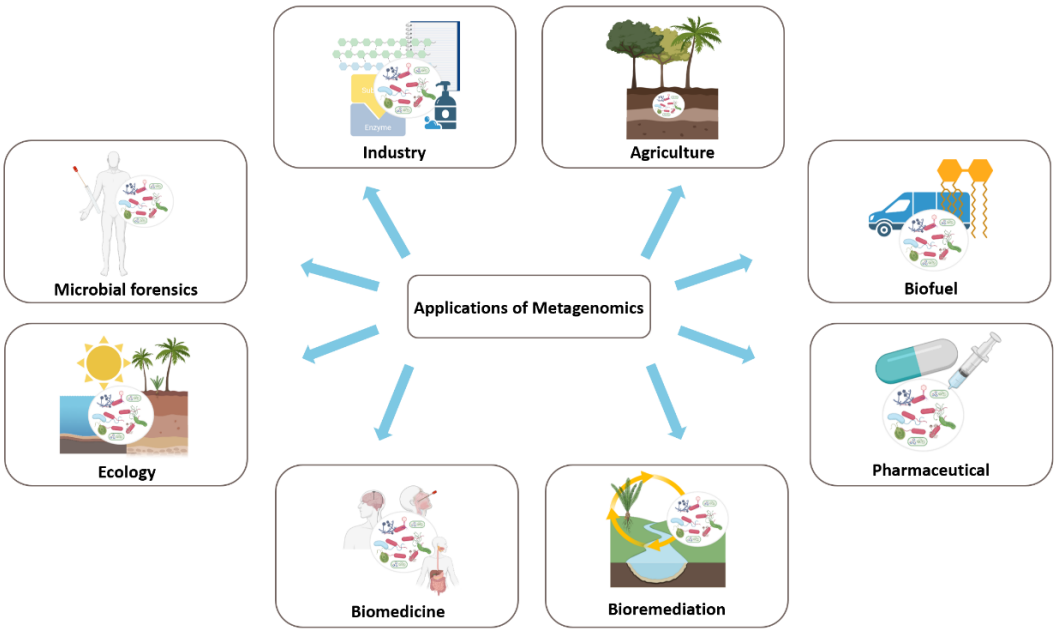

Figure 3. *The various fields of metagenomic applications*

In biomedical sciences, metagenomics has been used to develop strategies of novel diagnostic and treatment after describing the human microbiome role in healthy and patient individuals and also in populations. Zhao et al. (2021) presented a description of the relationship between the gut microbiome of patients with lung cancer and the clinical outcomes of chemotherapy based on the metagenomic analysis. Although most of the natural and bioactive compounds of marine microbes remain unknown to date, metagenomics was able to identify microbial communities, that have diverse biosynthetic capacities, and then the uncovering some distinct biocatalysts that were used in biotechnological applications (Turnbaugh and Gordon, 2008; Jeon et al., 2009; Coughlan et al., 2015). It was also utilized for explorating enzymes from nature to be taken advantage of in industrial applications, such as cellulase, lipase and protease (Lopez-Lopez et al., 2014; Schröder et al., 2014; Pessoa et al., 2017). Furthermore, metagenomics has been used in marine biomonitoring field through study the microbial community during an intense dinoflagellate bloom (Nowinski et al., 2019). Using the metagenomic method in agriculture applications helped in monitoring the ecological status and the early detection of problems affecting the microbial population in agricultural environments (Goss-Souza et al., 2019). In addition to these applications, there is a wide spectrum of research areas that have utilized metagenomics.

Accordingly, it is expected that diatom applications based on metagenomics will discover completely unknown components, functions and characteristics for various diatom species, as well as detailed information on previously studied functions with other methods.

Conclusion

Marine ecosystems tend to be rich in bioactive compounds produced by various marine microorganisms. Diatoms are one of the most common and more active microorganisms

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found in the seawater. They have, however, received insufficient attention due to used classical methods limitations. The advancement of molecular methods and their effectiveness for detecting microbial communities contributed to improve the output of microbial studies. Since the majority of marine microbes are not cultivable, the metagenomic method holds great promise for discovering their communities and novel applicable compounds of significant biological activities. It is expected that metagenomics will be used increasingly in the future to detect previously unknown diatom communities and to broaden the scope of diatom applications. Nevertheless, even with substantial advances in the development of molecular tools used for the assessments and monitoring of microbial diversity, the morphological approach remains critical in the eco-genomic era in species identification and detection. Finally, further research in metagenomics field is expected to provide new fascinating discoveries and exciting findings.

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