

## MOLECULAR INVESTIGATION OF BIOLOGICAL NITROGEN FIXATION: A REVIEW

### Microbiology

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### ABSTRACT

The better understanding of the diazotrophic communities is an important topic to explore the possible applications of biological nitrogen fixation. Present work provides an outline on how to utilize the molecular tools to study the changes in the diazotrophs community structure in terrestrial ecosystem. Molecular methods help us to understand the ecology, significantly elevating our knowledge of this process in soil. The mRNA study for the investigation of *nifH* transcripts in soil will add an influential tool to analyze the activity of the diazotrophs in more detail. The knowledge gained will make it possible to focus on enhancing biological nitrogen fixation in the field, either through amendments, optimized soil management, or by inoculation with specifically engineered or selected microorganisms. However, such technology will also leads to descriptive control and monitoring of the effectiveness of such future applications.

### KEYWORDS:

diazotrophs, community structure, mRNA, *nifH*, biological nitrogen fixation

### Introduction

Nitrogen is a limiting factor for plant growth on earth.<sup>1</sup> The atmosphere of earth is made up of 78% of nitrogen in non-reactive form. This form cannot be utilized directly by plants or animals. Bacteria are capable of fixing atmospheric nitrogen into ammonium ( $\text{NH}_4^+$ ) and nitrate ( $\text{NO}_3^-$ ) which is consumed by plants.<sup>2</sup>

As we know that plants are not able to utilize the atmospheric nitrogen, essential to convert the nitrogen into useable form. The processes involved in the nitrogen cycle are entirely implemented by microorganisms therefore plants are dependent on the microbes. Nitrogen cycle involves different steps for transformation, during which nitrogen undergoes changes in oxidation states from +5 ( $\text{NO}_3^-$ ) to -3 ( $\text{NH}_3$ ). These steps include the biological nitrogen fixation (BNF), mineralization, nitrification and denitrification.

It is the transformation of atmospheric nitrogen ( $\text{N}_2$ ) into the inorganic nitrogenous form ammonium ( $\text{NH}_4^+$ ). Fixation of nitrogen is not completely a biological process. It is also conducted by lightning which oxidize nitrogen to nitrate and this nitrate washes out with precipitation from the atmosphere and gets deposited in ecosystems. However, fixation of nitrogen by abiotic process contributes little to global N input (20 Tg N in one year) in comparison of BNF (175 Tg N in one year). This makes BNF the primary source of nitrogen. N requirement of all living beings including plants, animals and microorganisms depend on the fixed N for their. N-fixation is an important process of the N-cycle because N is an essential component of the all living being (Fig. 1).<sup>3</sup> In biological nitrogen-fixation (BNF) process atmospheric nitrogen ( $\text{N}_2$ ) is reduced into ammonium ( $\text{NH}_4^+$ ). These compounds are the preliminary molecules for the biosynthesis of amino-acids and other N containing biomolecules. Only prokaryotes (*member of Archaea and Bacteria*) are capable of fixing nitrogen. On land ecosystem, a number of processes continuously remove N from the biosphere and soil. Denitrification process (convert  $\text{NO}_2^-$  into  $\text{N}_2\text{O}$ , NO and  $\text{N}_2$ ) return nitrogen into the atmosphere. Nitrogen reaches to the hydrosphere in soluble or particulate form by leaching, erosion or sedimentation. BNF is an important natural source of N on land ecosystems (Fig. 1).

Diazotrophs are very diverse and have the genetic capability for biological nitrogen fixation. There are three types of life strategies of diazotrophs: symbiotic, associative and free living. All processes of nitrogen fixation require differ source of energy for different fixing

ability.<sup>4</sup> The symbiotic process of nitrogen fixation is in which both partners are in mutual beneficial relationship. Earlier reports have observed that this process mainly involves rhizobium bacteria and leguminous plant. Other symbiotic association for nitrogen fixation is present between *Frankia* and non-leguminous plant like *Casuarina*. Under nitrogen limitation conditions, symbiosis between rhizobia and leguminous plants leads to development of new organs of plants like the nitrogen fixing nodules that usually developed on roots but due to limiting nitrogen in the environment these nodules originate from stems in few plants. Bacteroids, differentiated forms of rhizobia fix molecular nitrogen inside the nodule which is assimilated by the plants. Symbiotic mutualism is not important due to its great ecological and agricultural value but due to its influence on plant organogenesis, plant-microbe interactions and signaling.<sup>5</sup>

Associative nitrogen fixation is a process in which bacteria live in close to the plants roots or leaves.<sup>6</sup> Bacterial species like *Acetobacter* or *Azospirillum* require plant exudates and secretions as an energy source for fixing nitrogen, however, 90% of the nitrogen fixed is only available when the bacterium dies.

Free living form of nitrogen fixation is done by bacterial species like *Azotobacter* or *Klebsiella*.<sup>7</sup> They have capability of photosynthesis to produce own energy and fix nitrogen but fix only small amounts of nitrogen. Globally, it has been estimated that asymbiotic nitrogen fixation contributes 30% part of the BNF.

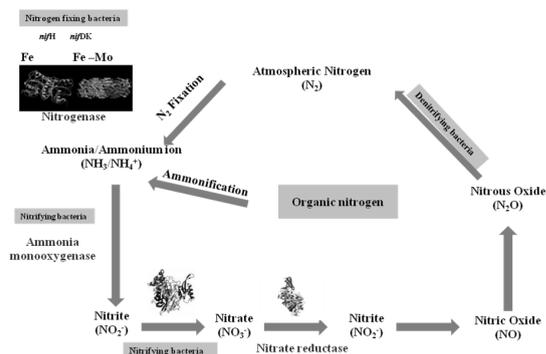


Fig 1: Nitrogen cycle

### Asymbiotic nitrogen fixation: future prospective for agriculture

For agricultural purpose, the prospective of asymbiotic nitrogen fixation is a matter of discussion. Earlier reports shown that crop production increased by inoculation of diazotrophs while negative results were also reported.<sup>8,9</sup>

Diazotrophic bacteria are also known as plant growth promoting rhizobacteria (PGPR), due to competitive benefit in carbon affluent and nitrogen poor environments.<sup>10</sup> These PGPR were reported to produce hormones such as auxin, gibberellin and cytokinin which stimulate growth of plants.<sup>11</sup> According to earlier reports seed germination and plant growth is enhanced due pretreatment of seeds with *Azotobacter* suspension.<sup>12</sup> Few diazotrophs have shown the activity of phosphate solubilization.<sup>12</sup> Numerous studies showed that inoculation of free living diazotrophs like *Azotobacter*, *Pseudomonas* and *Azospirillum* enhanced the rice productivity by 20–55% and diazotrophic bacterial strain Burkholderia improved the rice plant biomass upto 69%.<sup>13,14</sup>

Some studies showed that the activity of free living indigenous diazotrophs can differ greatly, depending on factors like soil pH, clay content and availability of the moisture.<sup>15</sup> Further research is essential to choose suitable diazotrophs for specific soil and to better understand the mechanisms which inhibit the potential of free living diazotrophs. The application of new methods especially molecular tools help to understand these processes.

### Diversity and evolution of diazotrophs

#### Earlier reports on nitrogen-fixation and diazotrophs

As discussed previously, symbiotic mechanism is the most significant source of biological nitrogen in different terrestrial ecosystems.<sup>2</sup> So BNF is most comprehensively studied in the legume-*Rhizobia* symbiotic system, first studied by Beijerinck, 1901, although the soil fertility-improving effect of legumes was reported earlier to this.<sup>7</sup> Asymbiotic free-living soil microorganisms were also reported first time with symbiotic systems, in the late 19<sup>th</sup> century. The capability of fixing atmospheric N<sub>2</sub> was first described by Berthelot, 1888, who found a net gain of nitrogen in non-sterile soil as compared to sterile soil. *Clostridium pasteurianum* was the first heterotrophic isolated diazotroph.<sup>16</sup> During those early days, discoveries of new diazotrophs continued and revealed diverse group of prokaryotes capable of performing fixation of nitrogen. The molecular tools shown the capacity of performing BNF through the discovery of the genes involved in it.<sup>17</sup>

#### Physiological and phylogenetic diversity of diazotrophs

Diazotrophs are phylogenetically diverse group of micro-organisms. Ability to fix nitrogen is detected in various groups of phototrophic bacteria like aerobic phototrophic (*Cyanobacteria*)<sup>18</sup> anaerobic purple-sulfur phototrophs (*Chromatium*), and green-sulfur phototrophs (*Chlorobium*)<sup>19</sup>. BNF has been observed in organisms growing chemolithotrophically (*Alcaligenes*, *Thiobacillus*)<sup>20</sup> or *Azospirillum lipoferum* and heterotrophic bacteria like *Clostridium* (anaerobes), like *Herbaspirillum* (microaerophiles), and *Azotobacter* (aerobes).<sup>20</sup> Although large number of diazotrophic genera reported, many isolate which have never been checked for their nitrogen fixing ability. Biological nitrogen fixation might have been even more widespread than the current status.<sup>21</sup> Though less number of diazotrophs species play role in symbiosis, still free-living diazotrophs have received less attention of the researchers. While for free-living *Cyanobacteria* in oceans and lakes detail studies have been reported,<sup>22</sup> yet free living diazotrophs of soil are poorly described. Free-living diazotrophs found in soil include aerobes like *Azotobacter*, *Beijerinckia* and *Derrxia* but the majorities are microaerophilic like *Azospirillum*, *Herbaspirillum* or facultative and obligate anaerobes such as *Klebsiella*, *Clostridium* and *Erwinia*.<sup>23</sup>

### Molecular approach for the analysis of diazotrophic diversity and ecology

#### The Application of molecular tools in soil microbiology

Two decades ago, ecology of soil microbes was typically restricted to describing bacterial activities in soil, utilizing biochemical measurements or enzyme assays or to the enrichment and isolation of microbes by culturing them on suitable media. These culture dependent methods have certain biases for describing soil microbial community. It is understood that the plate count or MPN counts are greatly lesser than the numbers studied by microscopic counts.<sup>24</sup> Moreover, the pioneer study verified that the several times higher soil

microbial diversity earlier estimated,<sup>25</sup> and it is supposed that the cultured organisms may be less than 1%.<sup>26</sup> The classic cultured dependent approaches raised numerous important questions of exact performance of isolated organism as in natural environment, cultured strains represents the soil activities properly, organisms play key functions cannot be cultured in laboratory, combined function of soil microbial communities and physiological features of organisms represents the whole community.

Due to limitations of the cultivation-dependent methods, the answers to these questions were mysterious; due to this microbial diversity dynamics considered as a black box.<sup>27</sup> Within the last two decades, the molecular tools and their application in microbial ecology has permitted researchers to answers many of these questions with new challenges. The rRNA methods allowed the great understanding of the microbial diversity in natural environment.<sup>29</sup> After this another milestone in the field of molecular methods is polymerase chain reaction (PCR), make the understanding and investigation of small quantity genetic material very easy.<sup>30</sup> Extraction of bacterial DNA from soil samples formed the chance to develop an arsenal of molecular tools for soil microbial analysis. Since these establish works, a constantly rising number of studies have required understanding of the soil microbial diversity using molecular tools, most importantly using the rRNA genes as genetic markers.<sup>29</sup> This work significantly expands our information related to microbial populations, their dynamics and response to environmental variations.

### Molecular investigation of diazotrophs: Marker genes

To describe the diversity of the diazotrophs using cultivation method have limitation as previously explained, mainly when focus was on the physiological diversity.<sup>31</sup> Because diazotrophs are phylogenetically diverse group of microorganisms, they cannot be chosen as an only cluster by rRNA gene-targeting probes or primers. Thus, specific molecular approaches were developed that aim the functional genes involved in BNF.<sup>32</sup> For this, it is necessary to know the conserved nature of the genes involved in it, besides phylogenetic information. *nifH*, *nifD* and *nifK* have been homologous genes in all diazotrophs. The gene *nifDK* (nitrogenase) and *nifH* (nitrogenase reductase) contain much conserved regions, showing the strict structural need of the nitrogenase complex to do proper functioning catalytically. However, less conserved regions can be used as molecular markers for fixing nitrogen in the environmental samples as they let to do phylogenetic classification of sequences.<sup>33</sup>

### Environmental studies using *nifH* as a marker gene

As *nifH*, *nifK* and *nifD* genes have all been studied phylogenetically, only *nifH* gene gives better phylogenetic information. Therefore, *nifH* is the gene which is commonly utilized for BNF studies as compared to other *nif* genes. The phylogenetic analyses of *nifH* has been done to understand the dynamics of uncultivated nitrogen fixing bacteria in various ecosystems like pasture and agricultural soils, wetland ecosystem,<sup>28</sup> and rhizospheric soil,<sup>34</sup> aquatic environments including marine,<sup>35</sup> freshwater<sup>36</sup> and estuaries<sup>37</sup> have been explored much. All these reports have deeply improved our knowledge of the functional capability and diversity of free-living diazotrophs for global N-cycle.<sup>38</sup>

### Biological nitrogen-fixation and *nifH*: A model system

BNF and *nifH* is an excellent model system to understand the community structure, gene expression and activity. The main benefits of this system are: large database and sufficient phylogenetic information for *nifH* gene, high physiologically differences to gather a significant understanding of sequence data, definite and easily quantifiable microbial activities and *nifH* which is directly linked to it, ubiquitous nature of diazotrophs throughout terrestrial ecosystems, tight transcriptional regulation of BNF, and easily availability of strains. So, BNF represents ideal model to develop mRNA methodology in microbial ecology, which will increase the knowledge of the connection between phylogeny and activity.

### Current challenges

#### Improving detection methods for *nifH*

Most of the earlier reports on the diversity of diazotrophic diversity in natural ecosystems were performed by utilizing PCR based methods with universal primers which amplified the *nifH* from whole microbial communities.<sup>39</sup> Few reports showed that these methods generally have difficulty to amplify the immense range of sequences.<sup>40</sup> As the application of highly degenerate universal primers are beneficial for screening function. So, quantitative analysis of *nifH* gene abundances

obtained with these methods must be explained with more care. The development of species or group-specific primer may help us to explore the specific group of the microbes which might be hidden by presence of other genotypes. In addition to this, a high detailed understanding of the diazotrophic communities would be achieved with universal approaches. Moreover, the PCR methods with less degenerate primers would lower the chance of PCR biases, leading the outcome more acquiescent and more reliable.

### Development of methods to identify active microorganisms in complex environments

Present Challenge of this study is to understand the relation between microbial community structures obtained with molecular methods to the activities of these communities.<sup>41</sup> The identification of an uncultured organism using SSU rRNA sequence analyses gives very less knowledge about the physiology of the organism. This problem is noticeable when microbial communities were studied using genetic fingerprinting methods. To know the mechanistic knowledge of microbial populations as dynamic ecological entities, new tools must be developed which make the understanding of the structural and functional relationship.

There are many approaches have been proposed:

- Information of microorganism's function under environmental stress conditions using molecular tools
- *In situ* measurement of metabolic potential utilizing autoradiography, stable- or radio-isotope probing of nucleic acids and other molecular markers, e.g. phospho-lipid fatty acids.
- Quantification of the expression of functional genes using mRNA to understand physiology and its relation between gene and protein synthesis.

All these methods have advantages and disadvantages, but the study of mRNA looks especially attractive due to its direct application into environmental samples. Additionally, the analyses of gene regulation under environmental conditions are an attractive research objective; since the natural environment (soils) conditions greatly vary from the laboratory optimum growth conditions.

### Conclusion

The aim of the current work is to summarize molecular investigation of BNF to understand changes in the diazotrophic populations with time in agricultural system. This improves the nitrogen status of soil using mRNA approach to understand the functional efficiency, *nifH* gene expression in soil samples and impact of inoculated microbial strains on the community structure native population of soil diazotrophs. These methods should come up with a sound basis for quantitative work in the soil environment to get new information on the dynamics and functioning of diazotrophs.

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