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Validation and quality assessment of NifH protein structure in nitrogen-fixing *Azospirillum brasilense* using Ramachandran plot analysis

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Abstract
The Ramachandran plot is among the most central concepts in structural biology for ultra-high resolution and validation of three-dimensional structures of protein structures. Three-dimensional models of macromolecules such as proteins have been successfully described by a network of nodes and edges in the last two decades. Amino acid residues as nodes and close contact between the residues as edges have been used to study protein folding and stability of the proteins. In this study, Ramachandran plot analysis of NifH protein in nitrogen-fixing bacterium Azospirillum brasilense was carried out using I-TASSER and Raptor X models. It was observed that out of 255 amino acid residues used in I-TASSER model analysis, 87.5% amino acid residues were located in most favoured (red) region, whereas only 9.8% residues were observed in additional allowed (yellow) region. Seven residues were present in outlier or generously allowed (yellow) region. In Ramachandran plot analysis using Raptor X model, 92.5% amino acid residues resided in most favoured region and only 6.7% residues were observed in additional allowed region. The presence of more than 90.0% amino acid residues in the favored regions of NifH protein using Raptor X model represents best quality metrics of experimental structure models before structure deposition.

Keywords: *Ramachandran plot, NifH protein, Nitrogen fixation, Amino acids, Structural stability*

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I. INTRODUCTION

The limited availability of plant utilizable nitrogen for crops is a threat to sustainability of our agricultural systems, economy and food supply [1]. Some microorganisms (including bacteria, cyanobacteria, *Frankia* and archaea) possess the unique ability to reduce atmospheric inert nitrogen (N_2) to ammonical form in the soil using the nitrogenase enzyme [2–4]. The use of such nitrogen-fixing bacteria as biofertilizers reduce the use of synthetic chemical fertilizers in farms, prevents public health hazards and act as an environmental friendly technology for achieving sustainable restoration of soil fertility [5, 6]. The nitrogenase enzyme complex consists of as many as 20 *nif* (nitrogen fixation) genes [7, 8]. One of the important structural gene, *nif*H gene, which synthesize dinitrogenase reductase (NifH protein), plays critical role in transfer of electrons during nitrogen fixation process in diversified nitrogen-fixing microbes [3, 9]. Better understanding about the functioning of NifH protein using computational and bioinformatics tools will supplement the existing biotechnological efforts to improve nitrogen fixation process for sustainable agriculture [10].

Recent understanding of structural biology and biological processes such as nitrogen fixation involves the validation and quality assessment of three-dimensional structures of proteins [11, 12]. In addition to conventional experimental methods such as X-ray crystallography, nuclear magnetic resonance (NMR) and cryoelectron microscopy (Cryo-EM), Ramachandran plot analysis is currently being applied for the validation and quality assessment of various protein structures [13, 14]. It also acts as a key in interpreting the quality of models from the Protein Data Bank (PDB) repository [15]. Ramachandran plots describe the two-dimensional distribution of the torsion angles [phi (φ) and psi (ψ)] of the amino acids residues contained in a peptide, which constitutes protein backbone [16]. Insight into the three-dimensional structures of peptides/proteins using Ramachandran plot and subsequent validation using software packages indicates the number of residues belonging to "outlier, allowed, and favored" regions [17].

Among various nitrogen-fixing bacteria, *Azospirillum* species colonize the inner tissues of plants inter-cellularly, without causing any apparent damage to the host [18]. The inoculation of nitrogen-fixing *Azospirillum* strains has been found to enhance plant growth, which has been attributed to several mechanisms,

including biological N_2 fixation [6, 19]. Therefore, understanding and optimization of these N_2-fixing plant-bacteria associations have promising prospective for sustainable agriculture. NifH protein is actively involved in transfer of electrons to substrate (N_2) reduction site during nitrogen fixation by nitrogenase enzyme under microaerobic and nitrogen-limited conditions in different soil agri-ecosystems [8]. However, limited work has been carried out on the phylogenetic analysis and use of *in silico* methods of NifH protein using computational and bioinformatics tools [20–22]. Considering the importance and application of nitrogenase enzyme in agricultural field, Ramachandaran plot analysis and *in silico* modeling of NifH protein based on homology modeling technique was undertaken in *Azospirillum brasilense* for understanding of the biological nitrogen fixation process at molecular level.

II. MATERIALS AND METHODS

For understanding the biological information from genomic and proteomic databases, various computational and bioinformatics tools and techniques are applied [23]. In the present study, the quality of NifH protein structure was predicted by plotting of Ramachandran plots. We have shown that the simple counting of residue fractions that belong to favoured and outlier regions of the Ramachandran plot may provide basic information to validate protein backbone conformation.

Retrieval of NifH protein sequences in different nitrogen-fixing bacteria

The protein data base (PDB) allows a wide spectrum of queries through data integration to obtain complete information regarding the features of macromolecular structures. Database similarity search tool, FASTA, was applied that work on heuristic method of database searching. BLAST was used for searching of GenBank and other sequence databases for sequence similarity and homology among different nitrogen-fixing bacteria [24, 25].

In the present studies, NifH protein sequences from ninety seven different nitrogen-fixing bacterial strains and nodule-forming *Rhizobium* strains were retrieved from Uniprot KB Database. Amino acid sequences of NifH protein were further filtered out by ignoring partial, putative, hypothetical and uncharacterized sequences. BLAST was used for searching of GenBank and other sequence databases for sequence similarity and homology among different nitrogen-fixing bacterial strains.

In silico modeling of NifH protein in *Azospirillum brasilense*

The amino acid sequences and structure of template were retrieved from PDB and UniProtKB database. Alignment of the template and target sequences was performed using CLUSTALW and pairwise sequence alignment script. *In silico* 3D models were built by creating backbone/threading based on template structure using I-TASSER and Raptor X.

I-TASSER server: I-TASSER based algorithms for prediction of protein structure and function are implemented through on-line platform I-TASSER server [26] (Fig. 1). Use of I-TASSER based algorithms permits academic users to automatically create high-quality model predictions of 3D structure and biological function of protein molecules from their amino acid sequences [27, 28). I-TASSER Suite is downloadable package of independent PC programs, developed by Yang Zhang Lab for protein structure prediction and refinement, and structure-based protein function annotations (28). Additionally, I-TASSER structural and functional template library is weekly updated and freely accessible to the I-TASSER users.

Raptor X: Raptor X was developed by Xu group. It is protein structure prediction server, which predicts 3D structures for protein sequences without close homologs in the Protein Data Bank (PDB). Once input sequence has been provided, Raptor X predicts its secondary and tertiary structures, contacts, solvent accessibility, disordered regions and binding sites [29].

3Drefine web server: 3Drefine is interactive web server for consistent and computationally efficient protein structure refinement with capability to perform online statistical and visual analysis. 3D refine web servers takes into consideration protein structure refinement through text or file input submission, e-mail notification, and is freely accessible without any registration. This server also provides comprehensive analysis of submissions through different energy and statistical feedback using various protein model analysis tools [30]. The 3D refine web server has been made freely accessible, broadly tested and used by numerous users.

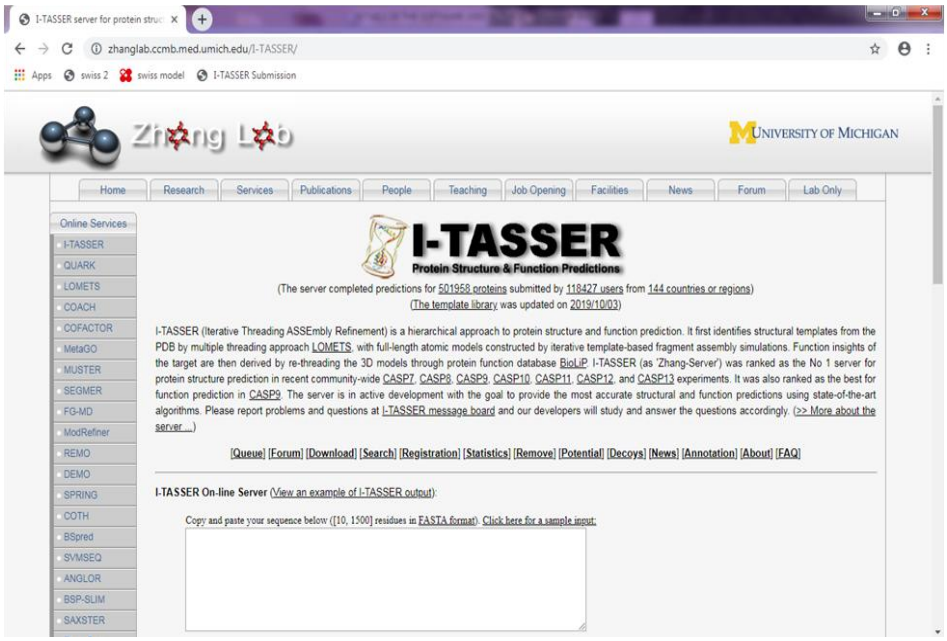

Figure 1: Interface of I-TASSER Server to identify structural templates and construction of atomic models from PDB utilizing multiple threading approach

III. RESULTS

Twenty distinct amino acids combine in particular arrangement for biosynthesis of various proteins through formation of peptide bonds in living cells. Amino acid sequence makes up the primary structure of the protein. Every amino acid has both a one-letter and three-letter abbreviation. However, chemical/ biological properties of the protein are reliant on the 3D or tertiary structure of the protein.

The amino acid sequences of NifH protein in 97 different nitrogen-fixing bacteria were obtained in FASTA format. The amino acid residues varied in different nitrogen-fixing bacteria. For example, 295 amino acids were found in NifH protein of *Azospirillum brasilense*, 291 amino acids in *Azotobacter chroococcum* strain, 294 amino acid residues in *Klebsiella pneumoniae*, where as 296 amino acid residues were observed in nodule-forming *Azorhizobium caulinodans* ORS 571 and *Sinorhizobium fredii* strain NGR 234. The amino acid sequences of the NifH protein in *Azospirillum brasilense* is provided below:

>***Azospirillum brasilense***

MSLRQIAFYGKGGIGKSTTSQNTLAALVELDQKILIVGCDPKADSTRLILHAKAQDTVLHLAAEAGSVEDLELED
VLKIGYKGIKCVESGGPEPGVGCAGRGVITSINFLEENGAYDDVDYVSYDVLGDVVCGGFAMPIRENKAQEIYIV
MSGEMMALYAANNIAKGILKYAHSGGVRLGGLICNERQTDKEIDLASALAARLGTQLIHFVPRDNIVQHAELRRM
TVIEYAPDSQQAQEYRQLANKVHANKGKGTIPTPITMEELEEMLMDFGIMKSEEQQLAELQAKEAAKA

The one-letter abbreviation of various amino acids found in NifH protein are as follows: Alanine (A), Arginine (R), Asparagine (N), Aspartic acid (D), Cysteine (C), Glutamine (Q), Glutamic acid (E), Glycine (G), Histidine (H), Isoleucine (I), Leucine (L), Lysine (K), Methionine (M), Phenylalanine (F), Proline (P), Serine (S), Threonine (T), Tryptophan (W), Tyrosine (Y) and Valine (V)

The Ramachandran plot of I-TASSER model showed that out of 255 amino acid residues used in analysis, 223 residues resided in most favoured (red) region, whereas only 25 residues were observed in additional allowed (yellow) region (Fig. 2). Rest seven residues were present in outlier or generously allowed (yellow) region. All of these amino acid residues were found very near to expected statistics. Number of glycine residues was depicted as 28 and only eight proline residues were shown in model. Analysis of Ramachandran plot indicated that 87.5% amino acid residues resided in favoured (red) region, where as only 9.8% residues were found in allowed (yellow) region.

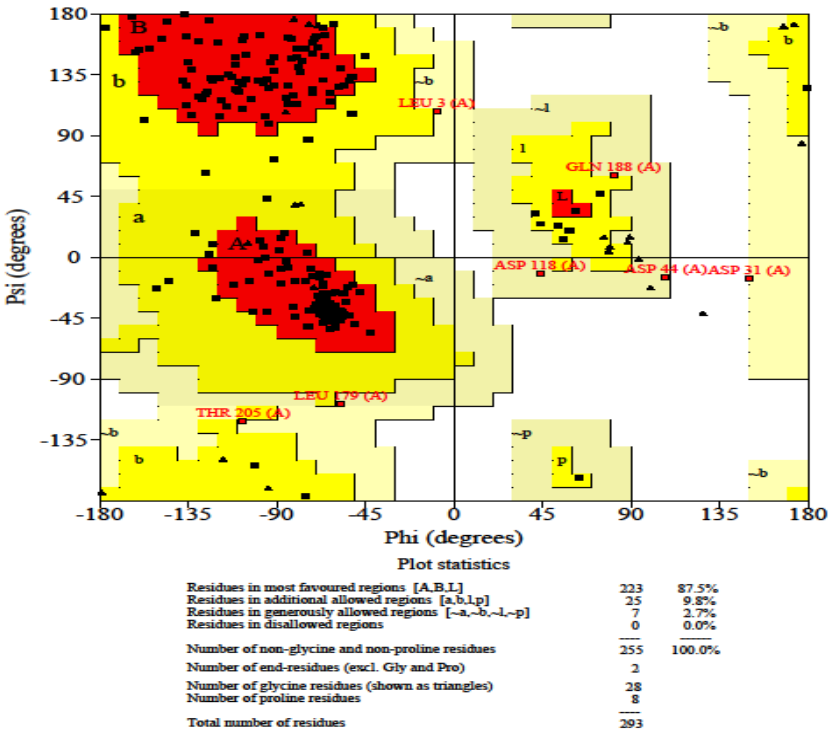

Figure 2: Ramachandran plot analysis of NifH protein in I-TASSER model

The Ramachandran plot analysis of Raptor X model showed that 236 residues resided in most favoured (red) region, whereas only 17 residues were observed in additional allowed (yellow) region (Fig. 3). Amino acid residues were not observed in outlier or generously allowed (yellow) region but two residues were observed in the disallowed regions. The number of glycine residues was depicted as 28 and only eight proline residues were shown in model. Analysis of Ramachandran plot indicated that 92.5% amino acid residues resided in favoured (red) region and only 6.7% residues were found in allowed (yellow) region.

IV. DISCUSSION

The Ramachandran plot was developed using theoretical methods [13], but its importance was realized with the beginning of mathematical calculations and *in silico* model building of the different protein structures [31]. The most important application of Ramachandran plot is the prediction of the quality of various protein structures determined using experimental methods such as X-ray crystallography [32], nuclear magnetic resonance (NMR) and cryoelectron microscopy (Cryo-EM) [14]. A good quality three-dimensional structure of macromolecule contains all the set of torsional angles in the allowed area where as, a bad quality (low resolution) protein structure is reflected as a number of torsional angles falling in the forbidden region. Besides experimental methods, protein structure obtained using homology modeling or ab-initio methods are also routinely checked by plotting Ramachandran plot. The interacting amino acids in proteins may act like other self-organized metabolic networks contributing towards structure validation, quality assessment and biological signaling pathways [33, 34].

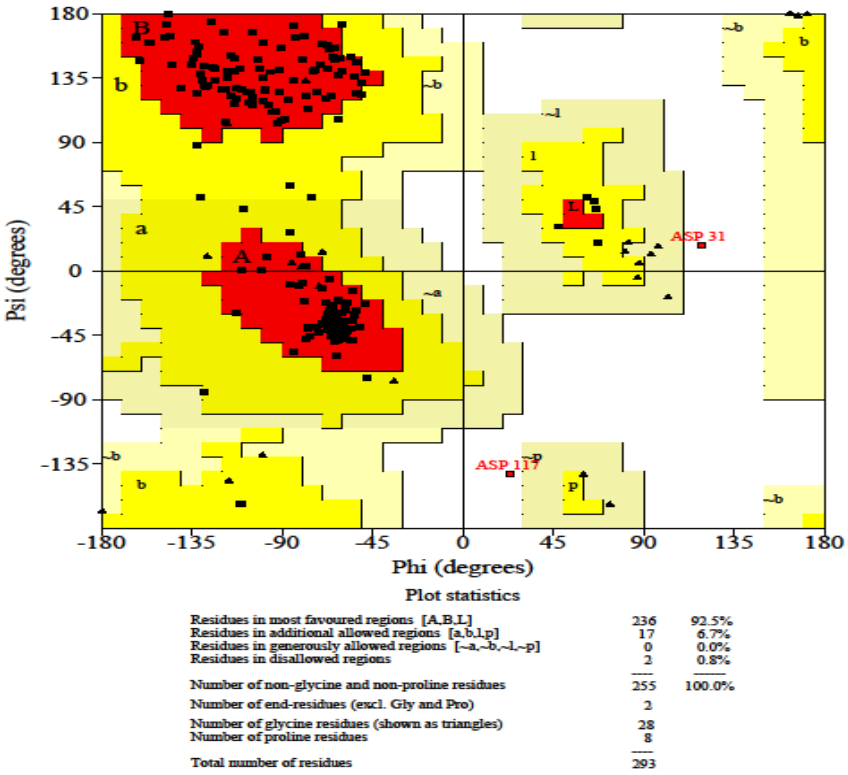

Figure 3: Ramachandran plot analysis of NifH protein using Raptor X model

In this study, we have demonstrated the utility of the Ramachandran plot by counting of amino acid residue fractions that belong to favored and outlier regions to validate protein backbone conformation. The Ramachandran plot analysis of NifH protein using I-TASSER model showed that out of 255 amino acid residues used in analysis, 223 residues (87.5%) were located in most favoured (red) region, whereas only 25 residues (9.8%) were observed in additional allowed (yellow) region (Fig. 2). Rest seven residues were present in outlier or generously allowed (yellow) region. Number of glycine residues was depicted as 28 and only eight proline residues were shown in model. In another study using Raptor X model, Ramachandran plot analysis showed that 236 residues (92.5%) resided in most favoured (red) region, whereas only 17 residues (6.7%) were observed in additional allowed (yellow) region (Fig. 3). Amino acid residues were not observed in outlier or generously allowed (yellow) region but two residues were observed in the disallowed regions.

Similar results regarding percentage distribution of amino acid residues in NifA protein of three rhizobial strains were reported during Ramachandran plot analysis [35]. It was observed that 93.80% amino acid residues of NifA protein resided in favoured (red) region, while 4.70% residues were found in allowed regions and rest 1.50% residues were available in outlier or generously allowed region. The distribution of more than 90% of residues in favoured region has been suggested as indicator of good quality model [36]. Analysis of Ramachandran plot during *in silico* structural analysis with 3D protein modeling of alkaline phosphatase enzyme in *Pseudomonas aeruginosa* indicated that 97.0% amino acid residues resided in favoured (red) region, while 4.70% residues were found in allowed (brown) regions and rest 2.60% residues were available in generously allowed regions (yellow) or outlier regions [37].

The traditional X-ray crystallography or nuclear magnetic resonance methods used for identification of 3D structure of proteins are tedious and costly [22]. Therefore, *in silico* analysis of genes and proteins has been receiving greater attention for designing effective chemicals and drugs and characterization of beneficial microbes for improving crop production [38–40]. Current advances at molecular and genomic level, development of new experimental methods, use of statistical and computational models, theoretical and *in silico* approaches along with multi-scale modeling and data integration along with validation and stability of structural proteins will provide innovative technologies for future synthetic biology and sustainable agricultural developments [20, 23, 41, 42]. Therefore, current scientific progress in understanding the structures of biological macromolecules, their validation, biological functioning of systems and their bioengineering holds enormous potential for further research in improving crop production, human health and ecofriendly environment.

V. CONCLUSION

For resolving the complexity of protein structures and to understand their natural conformations, novel informative presentations such as *in silico* 3D modeling along with mirrored and wrapped Ramachandran plots were introduced. Ramachandran plot provides a simple two-dimensional graphic representation of all possible protein structures in terms of torsion angles for models building. The ϕ, ψ-distributions for individual amino acid residues, which are influenced by the relative energetics of the 20 residue types and percentage distribution of amino acids that belong to favored and outlier regions of the Ramachandran plot contributes towards validatation of protein backbone conformation. In addition, the most important application of Ramachandran plot is the prediction of the quality of various protein structures determined using experimental methods including X-ray crystallography, NMR and Cryo-EM.

In the present manuscript, a valid approach has been offered for distinguishing native conformations of proteins based on percentage distribution of amino acids in the NifH protein of *Azospirillum brasilense* that belong to favoured and outlier regions of the Ramachandran plot. A significant improvement in the quality of macromolecular models is expected within a few years by application of improved validation tools to improve low-resolution refinement in atomic models. Further studies on structure validation, protein stability and their biological functioning will unveil novel pathway of protein folding along with characterization of active and catalytic sites in proteins. The modifications of active catalytic sites of NifH protein through mutations may improve the stability of protein leading to enhanced nitrogen fixation by diazotrophic bacteria [43, 44]. Use of such engineered nitrogen-fixing bacteria may increase the availability of fixed nitrogen in soil for use by crop plants leading to improvement in yield of different crops [45].

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