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Review Article

Methods to Analyze Proteins from Soils

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Abstract: Recent advances in molecular techniques, especially system approaches at DNA, RNA and protein levels have opened an emerging field in microbial ecology. These approaches can be used to identify the specific microbial genes and their functions directly from environmental samples. Among the different 'omics' approaches, metaproteomics is used to study microbial ecology and it plays an important role in the determination of microbial functionality. It provides detail about the structure and function of microbial populations from soil samples. However, proteins isolation and purification is very challenging due to complexity of soil samples. Only a few methods give high quality of extracting proteins and other methods can only be used to separate proteins using SDS electrophoresis but they are unable to characterize and identify specific proteins present in a soil sample. This review has mainly focused on recent advances in metaproteomic strategies to understand the structure and function of soil microbial communities. Three methods for protein extraction from soil samples were explained here, e.g., (1) using the phenol extraction method, (2) cell lysis method using different concentrations of SDS and alkaline lysis method using NaOH. For purification and identification of proteins, HPLC, FPLC, 2D-LC, LC-MS, MALDI-TOF and shotgun proteomics analyses were explained.

Keywords: Metaproteomics, Halophiles, 2-D Electrophoresis, High performance liquid chromatography (HPLC) Mass spectrometry (MS).

1. INTRODUCTION

Soil represents a naturally occurring complex system in which different biological, chemical and physical components interact with one another. A specific level of each component is maintained by formation, transformation and decomposition of complex organic materials in soil into simpler available nutrients by soil microbial populations [1-3]. Some DNA and RNA based methods have been regularly used during 1990s to characterize and identify the function of different microbial proteins and enzymes from soil samples. For example, micro-autoradiography and *in situ* hybridization had been used as powerful tools to study function-based microbial diversity and to identify the specific protein-protein and protein-substrate interactions in individual bacterial cells from soil microbial communities [4, 5]. By using stable isotope probing (SIP) DNA or RNA molecules, bacterial species involved in bioremediation of toxic compounds can be identified [6, 7].

Recently, various meta-omics approaches such as metagenomics, metatranscriptomics and metaproteomics have been used to study microbial ecology and functional make up of natural environments [8, 9]. 'Metaproteomics' is defined as the characterization of whole microbial protein complement from an environmental sample at a specific time. Microbial communities from different environments such as soil, marine and fresh water and activated sludge have been studied by using metaproteomic approaches [10-13]. A number of experiments have been performed for proteomic analysis of individual cells or microbe, protein-protein interactions and identification of disease biomarkers, but metaproteomics technique can be used for the entire environmental sample at a time to study functional microbial community [14-16]. Metaproteomic based techniques can be used for identification of different microbial proteins and enzymes with potential biotechnological applications such as biodegradation of complex organic pollutants, biological nitrogen fixation and

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other environmental processes [17].

In proteome analysis from soil samples, to get high quality proteins is a very critical step because protein distribution varies with change in microbial populations, e.g. Gram-negative bacteria have variety of intracellular proteins while Gram-positive bacteria have a great variety of extracellular proteins [18, 19].Some other methodological challenges may be there to get high quality and maximum number of proteins from a complex soil samples. Physical characteristics of soil, such as salinity, pH, temperature and texture, microbial diversity and presence of high amount of extracellular enzymes may affect the protein extraction process [20] and ultimately hinder the expression of proteins profiles and characterization of microbial metabolic pathways in a specific soil sample [21].

A number of previous studies have discussed on importance of metaproteomics from different environments. Metaproteomics studies on the plant rhizosphere microbiome help to understand complex metabolic pathways, detection of multiple functions of microbial genes and proteins. This review has focused on the recent advances in soil metaproteomics analysis, such as identification of proteins through HPLC, 2D-LC, MS/MS and MALDI analysis.

2. SOIL SAMPLE PREPARATION

Soil is one of the most complex sample types regarding metaproteomics analysis. It contains organic matter, inorganic ions and complex microbial communities. Microbial diversity identified from the soil is more diverse and complicated as compared to diversity other samples such water, human, animal or plant tissue [9, 11]. It is very tricky to handle soil samples during metaproteomics analysis as they react very quickly to environmental changes. Proteins extraction from soils is especially difficult and critical because it is mostly clay soil with more salt concentrations and less permeable than the loam and sandy soils [22, 23].

3. PROTEIN EXTRACTION

Extraction of total proteins from an environmental sample especially from soil is very important for metaproteomics analysis [24, 25]. Lysis of Gram-positive bacteria such as *Bacilli* and *Cyanobacteria* is difficult as compared to Gram-negative bacteria. So, protein concentrations are low because of incomplete lysis of Gram-positive bacterial cells. Soil sample also contains humic substances which cause problems in the protein extraction process. These compounds mostly interfere with protein purification and estimation through colorimetric methods and SDS-PAGE analysis [26]. Some organic substances present in soil samples hindered the separation of individual peptides obtained by tryptic digestion [27]. Whiffen et al. [28] suggested that humic acid and polyphenolic compounds usually interfere with protein estimation from soil samples when the Bradford assay is used (Fig. 1). Three different methods have usually been used for protein extraction from soil samples: (1) by using ‘phenol method’ in which, lysis buffer with phenol used for cell lysis and extraction; (2) ‘cell lysis’ by using different concentrations of SDS; (3) protein extraction by cell lysis with alkaline solution e.g., NaOH. Recently, different kits have been used for extraction of total proteins from soil samples, e.g., Power Soil Protein kitand FastProtein™ with blue and red matrix. Soil protein extraction by using these kits is relatively easy and protein concentration is good as compared to previously described methods [29, 30].

4. PROTEIN PRECIPITATION

To concentrate and purify, protein samples are precipitated by using various inorganic or organic compounds. Salting out of some neutral salts such as ammonium acetate, and ammonium sulfate have been commonly used for protein extraction by protein-protein interactions [31, 32]. In this technique, to change the charge on the surface of proteins, salt concentration in solution is increased so that hydrophobic parts of proteins interacts and proteins can be precipitated from solutions easily.A number of previous studies reported that protein can be precipitated from soil samples by using ammonium acetate buffer[33]. Some organic solvents such as methanol, ethanol or acetone can be used for precipitation of proteins (Fig. 1). In this method, temperature is considered an important parameter to avoid denaturation of proteins. Some previous studies showed that protein precipitation

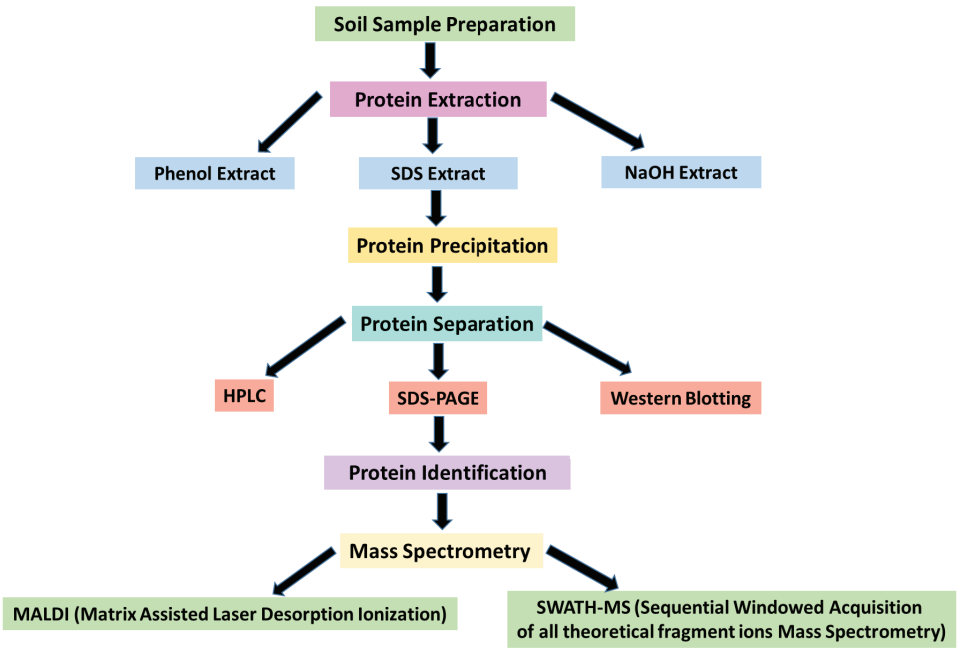

Fig. 1. Overview of soil metaproteomics strategies

has also been done by using polymers, such as polyethylene glycols and dextrans. This method has been used for proteins identification from biomaterials [34].

5. PROTEIN SEPARATION

Protein separation techniques play important roles in the development of metaproteomics of soil. For protein separation, a number of methods have been used, such as polyacrylamide gel electrophoresis (PAGE), western blotting and high performance liquid chromatography (HPLC).

5.1. SDS-PAGE

This technique is mostly used to separate and characterize different proteins from a mixture. Different proteins from a complex sample such as soil can be purified, analyzed and identified by using polyacrylamide gel electrophoresis (Fig. 1). Initially blue-native (BN-PAGE) has been used to separate a mixture of proteins [35, 36]. To address the resolution of complex mixtures of proteins, SDS-PAGE has been introduced. Various chemicals like detergents are used to denature the proteins which bind to individual proteins and help them to separate according to molecular mass [37, 38]. SDS-PAGE or one-dimensional electrophoresis has commonly been used to separate proteins on the basis of their molecular mass. Sodium dodecyl sulphate (SDS), a detergent is used in this method to denature the protein, so that they can be purified easily [28, 39]. It is a native technique which has been previously used to isolate and study enzymes and other proteins. In two-dimensional electrophoresis (2-D electrophoresis), proteins are separated in two directions: according to their isoelectric point in the 1stdimension and SDS-PAGE in the 2nd dimension to separate proteins on the basis of their molecular weights (Fig. 2). This technique has the main advantage of identification of proteins with some post-translation modifications [40, 41]. Now 2-D electrophoresis is widely used to study the expression profile of proteins both quantitatively and qualitatively (Fig. 1 and 2). The intensity of spots provides the information about the presence and absence of proteins expression. A number of softwares have been used to analyze complex images [42]. The main drawback of this technique is to study and characterize proteins with more hydrophobic parts, e.g., membrane proteins. Another problem associated with 2-D electrophoresis is the analysis of proteins with low abundance. This technique has been used to analyze and separate more complex and less purified proteins from a mixture. SDS-PAGE is used to measure the protein size and molecular weight of proteins, peptide mapping, estimate protein purity, comparison of the polypeptide composition of

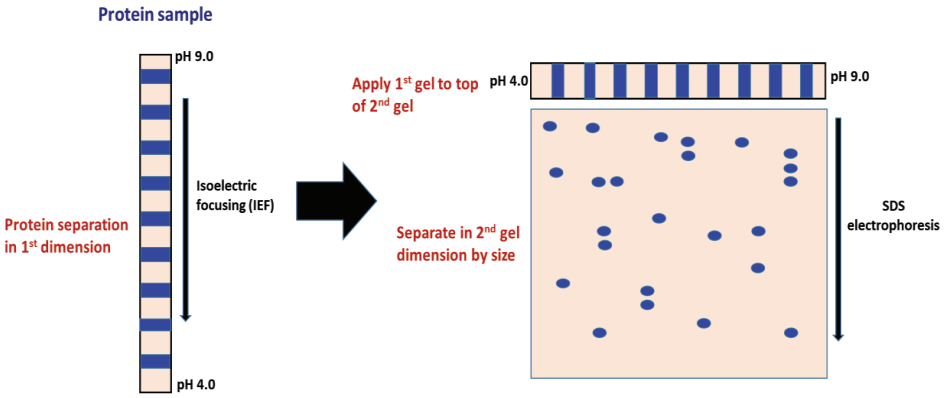

Fig. 2. Separation of different proteins from a sample by using 2-D SDS gel electrophoresis

different proteins and ubiquitination of proteins [43, 44].

5.2. High Performance Liquid Chromatography (HPLC)

It is highly dynamic technique in its nature especially depending on physiological conditions and abundance of proteins that are analyzed at the same time (Fig. 1). Same proteins show different expression in different cells and tissues [45, 46]. Reverse phase high performance liquid chromatography is the most commonly used technique which has been used for the separation, quantification and identification of peptides, proteins and other small organic molecules on the basis of their hydrophobicity. By using this approach, small molecules can also be detected with the application of high pressure on the rate of solvent flow in the separation process [47, 48]. In HPLC technique, different detector types are used to separate and identify all proteins but for identification of individual peptides, HPLC is not sufficient by only using UV spectrum [49]. Duration of separation of specific proteins is controlled by using a high-pressure pump and computerized system [50, 51]. HPLC is also used for identification of proteins with post-translational modifications. For this purpose, HPLC system has water as mobile phase for the accurate detection of peptides with post-translational modifications [52, 53].

5.3. Fast Protein Liquid Chromatography (FPLC)

It is a type of medium pressure liquid chromatography that can be used to purify proteins with high resolution and reproducibility. The distinctive feature of this technique is the stationary phase with small beads packed in plastic or glass columns which have high loading capacity [54]. The most common forms of FPLC are ion exchange, affinity and gel filtration chromatography. This technique can be used to purify different proteins and enzymes with applications in agriculture, industry, medicine and bioremediation of complex organic compounds [55].

5.4. Two Dimensional Liquid Chromatography (2D-LC)

The two dimensional liquid chromatography (2D-LC) is usually used to analyze two samples of separate liquid chromatographs for combined data analysis [56]. This chromatography technique can be used to analyze and separate complex mixtures with lots of proteins such as soils, liquid samples, e.g. blood, urine, waste and marine water [57]. The 2D-LC has important applications in proteomic and metabolomic studies of various environmental samples which are involved in the identification of targeted and non-targeted proteins [58].

5.5. Western Blotting

Western blotting is a technique used to study different proteins from a mixture or peptides from an individual protein. This technique can be applied for identification of proteins, protein-protein interactions, the kinase activity of proteins, cellular localization, monitoring of post-translational modifications, e.g., glycosylation, methylation and ubiquitinylation [59, 60]. Analysis of some proteins

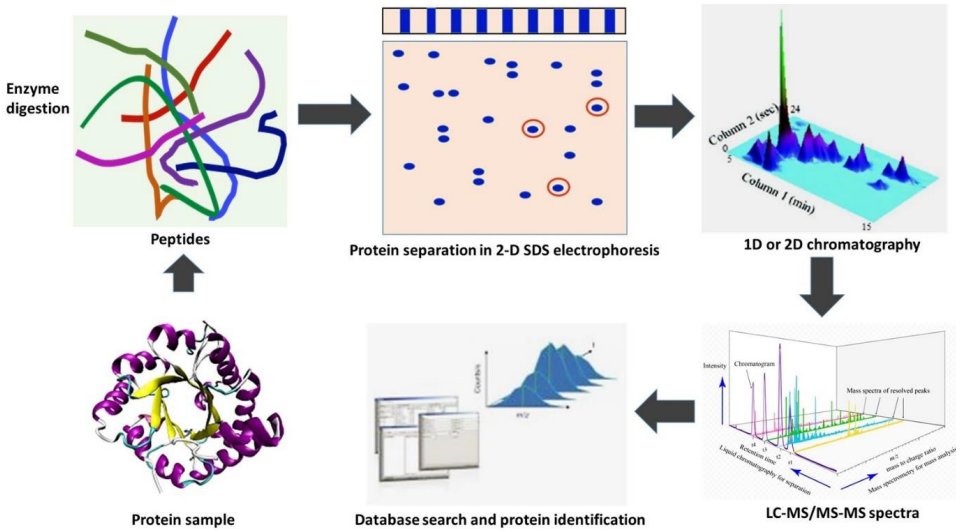

Fig. 3. Identification of proteins from a sample by using LC-MS/MS-MS data

through this approach may have some error due to variations at any step reducing the reproducibility and reliability [61]. Significant improvements have been made in this technique over the last decade, such as the modifications in methods used for sample preparation, the source and amount of primary antibodies used [62, 63]. In recent years, some new protocols have also been introduced, such as DqiWest automated microfluid western blotting, capillary and microchip electrophoresis and single cell resolution [64]. So, with the help of these innovative developments in the protocol and instrumentation, sensitivity and reproducibility of western blotting can be increased [64, 65]. The principle of the western blotting is mainly based on the nature of proteins (intracellular or extracellular), quantity of specific proteins, composition of gel matrix used for proteins separation and antigen-antibody binding during the identification of specific proteins (Fig. 1).

6. PROTEIN IDENTIFICATION

6.1. Mass Spectrometry

For the identification of proteins, experimental spectra are compared with theoretical spectra obtained from protein databases [66]. Mass Spectrometry (MS) ionizes the chemical compounds into charged molecules and measures its mass to charge ratio. Although the technique was discovered in 1900s, but the scope was limited until other potential tools emerged. Samples for MALDI-TOF are prepared by coating the sample with matrix. The matrix is an organic chemical compound with an ability to absorb energy. Crystallization of matrix consequently crystalizes the protein sample (Fig. 1). A laser beam ionizes the sample coated with matrix. Upon ionization, proteins from a specific sample get protonated and separated on the basis of charge and mass ratio upon acceleration on fixed potential. These proteins are identified and measured using different mass analyzers. Time of Flight (TOF) analyzer is preferred option for microbiological uses [45].

Tandem Mass Spectrometry is one of the most used techniques for proteomics after the digestion of proteins. The advancement in the field of matrix assisted laser desorption ionization (MALDI) has increased the scope of MS's application for protein identification [67]. In MALDI-TOF technique, mass to charge ratio is calculated by determining the time it requires to travel the tube [68]. Followed by MS analysis, theoretic peptides from protein databases are checked for proximal correlation with resulting spectra (Fig. 3). The technique's efficiency has provided the potential for the use of the developed approach for identification, quantification and detection in large-scale metaproteomics [69]. After the digestion, it matches the resulting spectra with theoretical spectra of the protein database [70]. The proteomic data obtained from mass spectrometry and protein sequences are added to the Proteome

Xchange Consortium [71]. Standard proteomic softwares are often incompatible for metaproteomics search because they do not provide sufficient data on un-sequenced species and complete taxonomy of microbial communities [66].

Sequential Windowed Acquisition of all theoretical fragment ions Mass Spectrometry (SWATH-MS) is a recent development in the field of MS. Advancements in this technique gives us more accurate and reproducible results of low abundance microbial proteins. SWATH-MS uses an approach that simultaneously scans all ionized fragments in a given sample. Spectral library is used to match and identify peptide sequences with already known peptides (Fig. 1 and 3). Moreover, the abundance of peptides is quantitatively measured by extracting the targeted signals [72]. Mass Spectrometry performance varies in analyzing different sample which is one of the factors that affects the reproducibility and accuracy of proteomics results. To resolve the issue, several techniques have been developed, i.e. tandem mass tags (TMT) and isobaric tags for relative ad absolute quantification (iTRAQ). TMT and iTRAQ multiplex several samples in one analysis, reducing the quantitation error [73, 74]. A further development in mass spectrometry could yield a better throughput. Unlike genomic studies, the metaproteomics technologies that are based on mass spectrometry have the potential to provide a deeper understanding of functional interactions between host and microbes [75, 76].

6.2. SIP-Protemics (Stable Isotope Probes Linking Proteins)

Metaproteomics provides the complete information about the different proteins and enzymes to be found in a specific environment and their possible origin. To determine the function of a given enzyme from a particular environmental sample, stable isotope probes can be used. In this technique, environmental samples such as soil are labeled with isotope ^{15}N or ^{13}C to detect the functional relationships among different microorganisms [6, 77]. Microorganisms in this environment are able to incorporate ^{15}N or ^{13}C into their molecules; DNA, RNA and proteins [78, 79]. By using stable isotope probes (DNA/RNA-SIP), microbial populations can be quantified and identified directly from environmental samples.

7. SHOTGUN PROTEOMICS ANALYSIS

For shotgun proteomics analysis, two methods, (1) data-independent acquisition and (2) data-dependent selection of proteins with specific function are commonly used. These approaches can be used for comparative analysis and functional analysis of different proteins. This technique can also be used for the whole proteome analysis of various environmental samples such as blood, water and soil [80, 81]. Washburn et al. [82] have used shotgun metaproteomics for the analysis of whole proteome of yeast (*Saccharomyces cerevisiae*). They identified more than 1400 known proteins and some unknown or rarely identified proteins such as protein kinases, DNA replication and transcription factor proteins.

8. STATISTICAL ANALYSES OF METAPROTEOMES

A number of statistical software's can be used to find the correlations among diversity analysis and different environmental factors. Multivariate analyses such as principal component analysis (PCA), correspondence analysis (CA), non-metric multidimensional scaling (NMDS) and analysis of similarity (ANOSIM) are the most common methods used for metaproteomic analyses [83, 84].

9. CONCLUSION

Metaproteomic studies of the rhizosphere soils have permitted the analysis of individual proteins that are involved in complex metabolic pathways. This approach provides a detailed study of the structure and functions of soil microbial communities together with metagenomics and transcriptomics. Thus, this review mainly focused on overview of the study of soil metaproteomics and improvements in methods for extraction, purification, and identification of soil proteins. A few protocols can be established and standardized for the extraction of soil proteins from different environments. For the separation and identification of peptides and proteins, especially proteins with small amount, specific strategies should be used. Due to lack of advanced software's and database gaps, metaproteomics technique needs some improvements to give an accurate and detailed picture of soil proteins. In future, advancement in metaproteomic techniques and

databases will be used for better understanding of functional microbial communities from different soils.

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