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REVIEW

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Potential of proteomics to probe microbes

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Abstract

An organism exposed to a plethora of environmental perturbations undergoes proteomic changes which enable the characterization of total proteins in it. Much of the proteomic information is obtained from genomic data. Additional information on the proteome such as posttranslational modifications, protein–protein interactions, protein localization, metabolic pathways, and so on are deduced using proteomic tools which genomics and transcriptomics fail to offer. The proteomic analysis allows identification of precise changes in proteins, which in turn solve the complexity of microbial population providing insights into the microbial metabolism, cellular pathways, and behavior of microorganisms in new environments. Furthermore, they provide clues for the exploitation of their special features for biotechnological applications. Numerous techniques for the analysis of microbial proteome such as electrophoretic, chromatographic, mass spectrometric-based methods as well as quantitative proteomics are available which facilitate protein separation, expression, identification, and quantification of proteins. An understanding of the potential of each of the proteomic tools has created a significant impact on diverse microbiological aspects and the same has been discussed in this review.

KEYWORDS

microbial proteomics, proteomic applications, protein separation, label-based methods

1 | INTRODUCTION

Proteomics is described as the investigation of the complete protein complement expressed in a cell at a given time under specific environmental conditions [1]. The whole-genome sequencing of several microorganisms provides information on the entire set of proteins that can be expressed in a cell. Investigation of proteomic changes to understand microbial adaptation strategies using genomic tools renders inadequate information on metabolic capacity and physiological responses to environmental perturbations whereas the proteomic approach provides detailed information on the protein concentration and their functional activity [2,3]. Proteomics can be grouped into three major fields namely comparative, structural, and functional proteomics. Comparative proteomics aids in the analysis of differentially expressed and repressed proteins under normal and altered conditions providing insights into the response of cells to environmental perturbations, while structural proteomics deals with structural characterization of proteins in addition to posttranslational modifications. Functional proteomics constitutes an analysis of the biological function of unknown

proteins, cellular mechanisms as well as the signaling pathways [4–6]. The characterization of proteins by comparative proteomics has undergone a major transition by application of Edman sequencing and mass spectrometry tools. Two-dimensional (2D) gel electrophoresis is one of the earliest techniques applied in proteomics for the separation and analysis of all the proteins expressed by a microbe for adaptation to extreme environmental conditions. Studying the impact of environmental changes upon protein expression profiles of microbial communities would provide information on the functional significance of the altered protein in the specific environment. Mass spectrometry is a powerful tool for the qualitative identification of extracellular, membrane, periplasmic, and cytosolic microbial proteins [7]. There is also a demand for quantitative protein analysis in microbiology. Quantitative proteomics involving both label-based and label-free approaches is an efficient tool to study the protein–protein interactions, metabolic pathways and alterations in protein abundance. The merits and demerits, and application of comparative and quantitative proteomics tools applied in microbiology are discussed in the present review.

2 | TECHNIQUES TO STUDY COMPARATIVE PROTEOMICS

Comparative proteomics involves protein separation and identification as shown schematically in Figure 1.

2.1 | Electrophoresis-based protein separation

2.1.1 | Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and native polyacrylamide gel electrophoresis

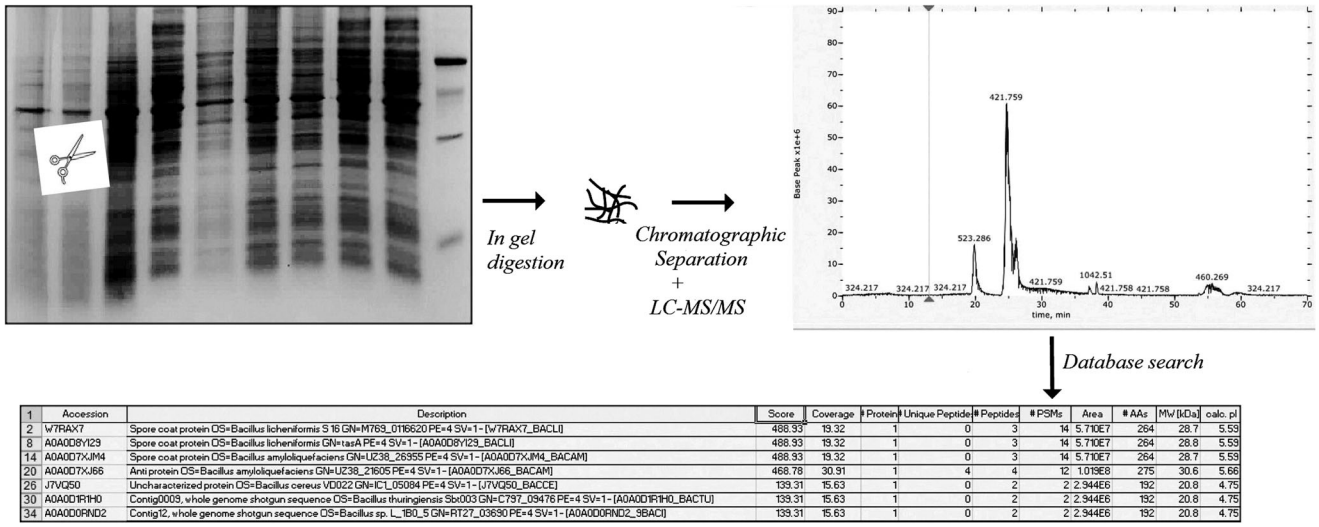
Sodium dodecyl sulfate (SDS) is an anionic detergent which denatures the proteins and binds to the uncoiled molecules resulting in rod-shaped polypeptides with equal charge densities. The denatured proteins are separated as a function of their molecular weights in an electric field. Disulfide bonds, if any, in the proteins are reduced by the addition of reductants (e.g., DTT or β-mercaptoethanol) [8]. The functions of the proteins cannot be studied by the SDS-polyacrylamide gel electrophoresis (PAGE) method as the proteins undergo denaturation before electrophoresis. To address this issue, native PAGE was introduced in which the proteins escape the denaturation step. Though the native PAGE retains the functional activity of proteins, a high resolution of separation is not obtained as in SDS-PAGE [9]. To overcome the drawbacks of both methods, native SDS-PAGE was introduced to achieve separation with excellent resolution without the loss of the nativity of proteins [10].

2.1.2 | 2D gel electrophoresis

2D gel electrophoresis enables efficient separation of a complex mixture of proteins and the identification of proteome variations in an altered cell. Proteins are separated in the first dimension on the basis of isoelectric point by isoelectric focusing and further separated on the basis of relative molecular mass by SDS-PAGE in the second dimension. In the first dimension, the pH gradient is created using immobilized pH gradients (IPG strips) or the carrier ampholytes. The IPG strips are desirable due to the reproducibility of results. They are available in different sizes and pH ranges. The proteins are then stained with Coomassie blue, silver nitrate, SYPRO Ruby CyDye or deep purple dye to detect the proteins [11]. For quantitative analysis of the protein spots, the gel images are analyzed using software such as PDQuest (Bio-Rad), Decyder 2D (GE Healthcare), Melanie (GeneBio), Delta2D (Decodon), and Progenesis SameSpots (Nonlinear Dynamics). They perform spot detection, image alignment, background adjustment, spot matching, calculation of spot volume, and statistical comparison of differentially expressed proteins. As 2D gel electrophoresis is capable of separating thousands of proteins in a single gel, it enables the simultaneous study of several differentially expressed proteins. The proteins separated by 2D PAGE are almost pure, and hence can be excised from the gels and subjected to Edman degradation for protein sequencing. Despite the wide application, 2D gel electrophoresis suffers from several drawbacks such as cumbersome time-consuming procedures and low reproducibility [12]. The transfer of samples from the first dimension to the second might also lead to reduced reliability. Another major drawback is that only a few samples can be analyzed per gel. 2D gel electrophoresis also fails to catalog low abundant and hydrophobic proteins as well as proteins with extreme molecular size (5 and >150 kDa) and pH range (<3.5 and >10) [13]. These disadvantages have been overcome by the advent of differential gel electrophoresis (DIGE).

2.1.3 | Differential gel electrophoresis

DIGE involves the use of a single gel to detect the differences between two different protein samples which are labeled with different fluorophores namely Cy2, Cy3, and Cy5 before isoelectric focusing [14]. These fluorophoretic



	Accession	Description	Score	Coverage	# Protein	# Unique Peptide	# Peptides	# PSMs	Area	# AAs	MW [kDa]	calc. pI
2	W7RAX7	Spore coat protein OS=Bacillus licheniformis S 16 GN=M769_0116620 PE=4 SV=1 - [W7RAX7_BACLI]	488.93	19.32	1	0	3	14	5.710E7	264	28.7	5.59
8	A0A0D8Y129	Spore coat protein OS=Bacillus licheniformis GN=tasA PE=4 SV=1 - [A0A0D8Y129_BACLI]	488.93	19.32	1	0	3	14	5.710E7	264	28.8	5.59
14	A0A0D7XJM4	Spore coat protein OS=Bacillus amyloliquefaciens GN=U238_26955 PE=4 SV=1 - [A0A0D7XJM4_BACAM]	488.93	19.32	1	0	3	14	5.710E7	264	28.7	5.59
20	A0A0D7XJ66	Anti protein OS=Bacillus amyloliquefaciens GN=U238_21605 PE=4 SV=1 - [A0A0D7XJ66_BACAM]	468.78	30.91	1	4	4	12	1.019E8	275	30.6	5.66
26	J7VQ50	Uncharacterized protein OS=Bacillus cereus VD022 GN=IC1_05084 PE=4 SV=1 - [J7VQ50_BACCE]	139.31	15.63	1	0	2	2	2.944E6	192	20.8	4.75
30	A0A0D1R1H0	Contig0009, whole genome shotgun sequence OS=Bacillus thuringiensis Sbt003 GN=C797_09476 PE=4 SV=1 - [A0A0D1R1H0_BACTU]	139.31	15.63	1	0	2	2	2.944E6	192	20.8	4.75
34	A0A0D0RND2	Contig12, whole genome shotgun sequence OS=Bacillus sp. L_1B0_5 GN=RT27_03690 PE=4 SV=1 - [A0A0D0RND2_9BACI]	139.31	15.63	1	0	2	2	2.944E6	192	20.8	4.75

FIGURE 1 Schematic representation of steps involved in protein identification. The protein of interest was excised from the sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subjected to in-gel digestion. The digested peptides were separated and identified by liquid chromatography/mass spectrometry followed by database search. The in silico theoretical spectra from a protein sequence database was compared with the experimental spectra to find the best match

dyes carry the *N*-hydroxysuccinimidyl ester reactive group that binds to the amino group of lysine residues in proteins. The single positive charge of the dye replaces the positive charge of the lysine at neutral acidic pH, maintaining the same isoelectric point of the protein. The labeled proteins are pooled and separated on a 2D gel. The protein spots are detected upon excitation of the fluorophores and two separate images are acquired. DIGE allows simultaneous separation of two protein samples minimizing the use of gels.

2.1.4 | Gel-based proteomics tools in comparing the protein expression pattern of microbes

Initially, gel-based proteomics was applied in taxonomical studies. The ribosomal proteins of *Propionibacterium acnes* and *Propionibacterium granulosum* display differences in their mobility on 2D PAGE [15] and this is helpful in analyzing different taxonomic groups. The gel-based protein separation tools have proven to be efficient in comparing the alteration in protein patterns in response to external stimuli. Certain antibiotic-resistant bacteria like methicillin-resistant *Staphylococcus aureus* have emerged as a major threat to human health. To combat the bacterial infection caused by the resistant organism, a phytotherapeutic agent, *Quercus infectoria,* was used to test its antibacterial effect towards the resistant organism. Analysis of the differentially expressed proteins in methicillin-resistant *S. aureus* by SDS and 2D PAGE revealed the upregulation of proteins such as 3-hydroxyacyl-CoA dehydrogenase, NAD binding domain protein, formate C-acetyltransferase, 3-hydroxyacyl-(acyl-carrier-protein) dehydratase FabZ, NAD-dependent epimerase/dehydratase family protein, and phosphopantothenoyl cysteine decarboxylase when treated with *Q. infectoria* indicating that the bacterial proteome participated in energy metabolism and protein stress [16]. The study provided an insight into the resistant mechanisms of the bacteria through the identification of proteins. The differential protein expression of a metal-resistant *Alcaligenes faecalis* VITSIM2 under chromium stress was investigated by SDS-PAGE and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [17]. Two proteins namely flagellin and 50S ribosomal L36 protein were differentially expressed which had a probable role in helping the organism to move away from the toxicant and aiding in survival. Analysis of the proteome expressed under low-temperature conditions (4°C and 8°C) by antarctic bacterium *Pseudoalteromonas haloplanktis* by 2D-DIGE revealed that apart from proteins involved in translation, antioxidant activity, protein unfolding, and membrane integrity, the one responsible for trigger factor was the major protein expressed under cold stress. Trigger factor assists in the rapid and efficient folding of the newly synthesized peptides, and also functions when the activity of other chaperones like DnaK and GroEL is repressed [18]. 2D gel electrophoresis profiling is used to identify proteins involved

in basic cellular metabolism as well as in stress conditions. Gel-based proteomics has been used to explore the metaproteome of the ruminal digesta microbial community. Proteins from the digesta of dairy cows and beef cattle were precipitated by trichloroacetic acid, and the protein extracts were analyzed on 1D and 2D PAGE. Identification of the proteins expressed by *Methanobrevibacter ruminantium* using LC-MS/MS revealed the presence of 5,10-methylenetetrahydromethanopterin reductase and methyl-coenzyme M reductase which are the key enzymes involved in methane metabolism [19]. These gel-based tools play a vital role in gaining insight into ruminal methanogenesis and developing methods to reduce methane emissions from ruminants. Though several techniques are available to determine the protein content and protein pattern alterations, only a few techniques like the blue native PAGE are available to study the protein interactions. Analysis of protein–protein interactions provides insights into bacterial physiology and pathogenesis. In *Methanococcus thermoautotrophicus*, methane is released as the end product of metabolism. The protein complexes involved in methanogenesis were analyzed by native PAGE and SDS-PAGE. The protein complexes involved in anabolic, catabolic, and general cellular events were identified [20]. 2D gel electrophoresis was applied to study the protein profile of *Lactobacillus plantarum* under bile stress [21]. Two glutathione reductases involved in protection against oxidative damage caused by bile salts, a cyclopropane-fatty-acyl-phospholipid synthase responsible for the maintenance of cell envelope integrity, a bile salt hydrolase, an ABC transporter, and a FoF1-ATP synthase were overexpressed in bile-related stress. The capacity of gel-based proteomics to study the protein expression on a large scale is evident from these investigations.

2.2 | Chromatography-based separation

Proteins have different characteristic features such as size, charge, and binding affinity, and hence chromatographic techniques find application in the separation of proteins. In ion-exchange chromatography, the matrix consists of charge opposite to that of the protein to be separated. Proteins are separated either by altering the pH or ionic strength of the buffer solution [22]. Gel permeation chromatography separates proteins based on their molecular size. Larger molecules pass rapidly between the spaces of the pores whereas smaller molecules pass slowly diffusing into the pores. In affinity chromatography, a particular ligand is immobilized or attached to a solid support. When the sample is passed through the column, the molecules which possess a binding affinity to the ligand binds to it and the remaining components are washed away. The bound molecule is eluted from the original sample using a suitable eluant [23].

2.2.1 | Applications of chromatography in the purification of microbial enzymes and proteins

Chromatography enables separation, identification and purification of components of a mixture for qualitative and quantitative analysis. In cheese, bacteria such as *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *lactis, Streptococcus salivarius* subsp. *thermophilus*, and *Propionibacterium freudenreichii* release several proteins during the ripening process. To reduce the complexity of proteins, 2D PAGE is carried out. Size exclusion chromatography is used to prefractionate the bacterial and milk proteins, and the fractions are identified by mass spectrometry. Using this approach, proteins involved in proteolysis, glycolysis, stress response, DNA and RNA repair, and oxido-reduction were identified. Different peptidases were found to be released into cheese providing information about the casein degradation mechanisms [24]. The copper-binding proteins (heat shock proteins, rubisco, α and β-tubulins, ATP synthase beta subunit) of four species of algae namely, *Dunaliella tertiolecta*, *Tetraselmis* sp., *Phaedactylum tricornutum*, and *Ceratoneis closterium*, were purified and identified by Cu-immobilized affinity chromatography and liquid chromatography–electrospray ionization–tandem mass spectrometry (LC ESI-MS/MS) [25], respectively. The copper-binding proteins play a role in metal detoxification [26]. Bacteriocins of *Lactobacillus viridescence* which display antimicrobial activity against a wide range of food-borne pathogens were purified by gel filtration chromatography [27]. This study with the main objective of the purification and characterization of bacteriocins revealed that bacteriocins could serve as potential food preservatives. Another study focused on the purification of alkaline phosphatase, an enzyme used in the degradation of organophosphorus pesticides. Alkaline phosphatase from lactic acid bacteria was purified by ion-exchange chromatography. The findings of the study reveal that alkaline phosphatase could be used in the clean-up of pesticides in raw materials used in the food industry [28].

2.3 | Protein identification

Once the proteins are separated, they are subjected to the process of identification. Earlier identification of

proteins was based on Edman degradation [29], which is tedious and expensive. The time-consuming technique is replaced by mass spectrometry-based identification methods. Before identification, the target protein electrophoresed on the gel is proteolytically digested to yield a mixture of peptides in a process called in-gel digestion. The procedure includes excision of gel band, stain removal, reduction, alkylation, proteolytic cleavage, and peptide extraction. The digested peptides are chromatographed to obtain a homogenous mixture of peptides. This approach is termed as the bottom-up approach, whereas in a top-down approach, the intact proteins are subjected to mass spectrometric analysis.

2.3.1 | MS-based identification

An MS generates ions from a sample and separates according to the mass to charge ratio (m/z). The steps involved in MS include sample ionization, separation of ions according to mass to charge ratio and ion detection (Figure 2).

Ionization of sample

The biological sample to be analyzed is ionized by the use of soft ionization tools, namely ESI and matrix-assisted laser desorption/ionization (MALDI), converting the peptides into ions by the addition or loss of protons. In ESI, the sample flows from a high voltage microcapillary tube and passes on to a gaseous ion phase before entering into the MS [30,31]. As the solvent evaporates, the size of the droplets decreases, leading to the formation of desolvated ions [30]. In MALDI, the sample is incorporated into matrix molecules and then subjected to irradiation by a laser resulting in the formation of molecular ions [32].

Separation and detection of ions

Several mass analyzers, namely quadrupole, quadrupole ion trap, time of flight (TOF) tandem mass spectrometer, and Fourier-transform ion cyclotron resonance can be used. In a quadrupole mass analyzer, a quadrupolar electric field is created by supplying a positive and negative direct current and radiofrequency voltage to two pairs of rods. Only ions with selected mass to charge ratio traverse the quadrupole while the remaining ions fail to reach the detector. In the ion trap mass analyzer, a rotating 3D electrostatic field effectively captures the ion of interest. The ions of successive mass/charge ratio caught at the trajectories become successively unstable and exit the trapping field on the basis of their mass/charge ratio. A TOF MS estimates the time taken by the ions to travel from the ion source to the detector based on the mass of the ions. Tandem mass spectrometers function by merging two mass analyzers to operate as a single instrument.

Collision-induced dissociation

Collision-induced dissociation is a method of fragmentation where the precursor ions are accelerated to higher kinetic energy collisions with inert gas atoms like helium or nitrogen. The kinetic energy is converted into internal vibration energy, resulting in peptide cleavage at the amide bond generating b and y fragment ions [33]. The ions pass through the ion detector, which is usually

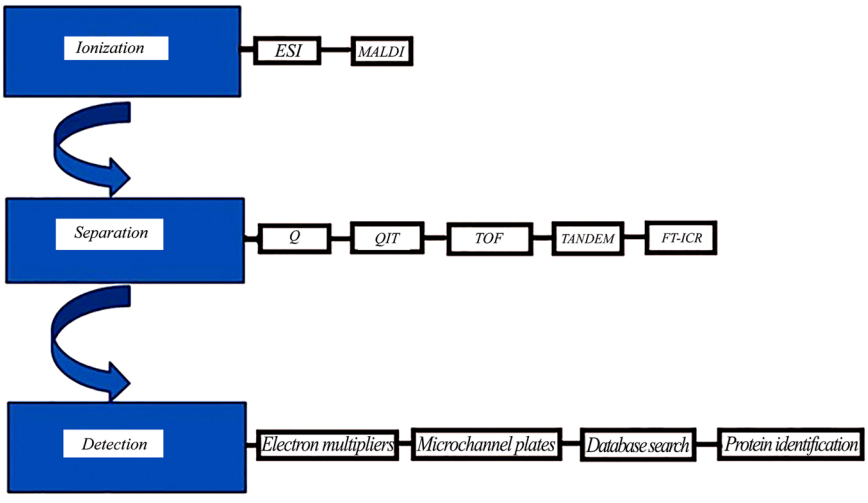


FIGURE 2 Steps involved in a mass spectrometry analysis. The samples ionized by electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) are separated by mass analyzers such as quadrupole (Q), quadrupole ion trap (QIT), time of flight (TOF) tandem mass spectrometer and Fourier-transform ion cyclotron resonance (FT-ICR). The ions pass through the detector, that is, electron multipliers or microchannel plates followed by database search and protein identification

electron multiplier or microchannel plate. The resulting MS/MS spectrum is generated as a plot of the intensities of all the fragment ions against the m/z values. There are different types of computational approaches that assign peptide sequence to the MS/MS spectra. In one method, the peptide sequences are extracted directly from the spectra and in the other, the experimental and theoretical spectra are correlated for each peptide present in a protein database [34]. In most proteomic studies, the database search approach appears to be the primary method of protein identification except in cases where the proteome of the organism remains unsequenced.

Application of Edman sequencing and MALDI-TOF in microbial identification

Biochemical tests and morphological features are the primary sources of identification of microbes based on the phenotype. As the phenotype alters in response to environmental perturbations, these methods of identification become least preferred whereas the genotype is independent of such conditions. Though 16S rRNA and 18S rRNA gene sequencing has been employed in the routine identification of microbes, MALDI-TOF-MS, which is an indirect genome analysis has emerged as a powerful tool in the recent years to identify the organism at the genus and species level [35]. Several studies have reported that MALDI-TOF-MS has performed better than the conventional techniques of microbial identification (16S and 18S rRNA gene sequencing) in terms of speed, cost, and reliability [36–38]. Rapid identification of the bacteria is required for the early diagnosis of bacterial infections in clinical samples [39,40], contamination in fermented food [41], drinking water, and environmental samples [42], and bacteria and fungi responsible for antibiotic resistance [43,44] and identification of yeast species [45]. The technique has also certain lacunae as it is dependent on the availability of spectral database containing genomic information of a specific genus and local databases that contain information on the altered phenotype and genotype due to geographical variations [46].

MALDI-TOF in microbial protein characterization

Protein identification helps to unveil the metabolic pathways and the mechanisms underlying the cellular process. Protein identification necessitates the use of Edman sequencing and mass spectrometry. *P. acnes* are associated with acne vulgaris, and to understand the role of the organism in the pathogenesis of acne, a proteomic analysis of the sebaceous follicular casts is carried out. Proteins involved in wound healing, inflammation, and tissue formation were identified by mass spectrometry. The most abundant *P. acnes* proteins were identified to be CAMP factors, and surface-exposed dermatan sulfate adhesions [47]. The variation in protein expression of different clonal types of *S. aureus* at various stages of biofilm formation was determined by coupling 2D gel electrophoresis and LC-MS/MS [48]. The differentially expressed proteins of *Dunaliella salina,* a halotolerant organism, were identified by MALDI-TOF/TOF as molecular chaperones and proteins involved in respiration, photosynthesis, and amino acid synthesis [49]. The findings of the study provide insights into the functions of various proteins involved in the salt-stress of the marine algae. MALDI-TOF-MS and LC-MS/MS were used to identify the differentially expressed protein in *Pseudomonas aeruginosa* exposed to chromium. Overexpressed proteins included stress proteins, proteins involved in protein biosynthesis, energy production, and free radicals detoxification by the glutathione system and outer membrane proteins, MucD, while downregulated proteins were isocitrate dehydrogenase and 30S ribosomal protein S1 [7]. In all these studies, protein identification and characterization contributed to the understanding of molecular and metabolic processes underlying the response to stress conditions. Although MS-based identification of proteins is an efficient tool, it suffers from certain drawbacks arising from the fact that the peptides with varying sequences possess different ionization capabilities and intensities in an MS not matching with their abundance. Moreover, MSs allow a low percentage of sampling of total peptides present in the sample. These difficulties led to the emergence of label-free and label-based quantitation to aid mass spectrometry in nonbiased identification of several differentially expressed proteins.

3 | QUANTITATIVE PROTEOMICS

3.1 | Label-based techniques

3.1.1 | Stable isotope labeling by amino acids in cell culture

This method employs the use of heavy or light isotopes of an essential stable (nonradioactive) amino acid, which allows the proteomes to be differentiated by MS. Usually the medium is amended with labeled lysine and arginine, producing a high coverage of isotopically labeled tryptic peptides; but arginine when present in excess in the media may get converted to proline leading to reduced reliability [50]. The technique allows multiple samples to be investigated simultaneously. After labeling, the samples are pooled, as they can be differentiated on the basis of peptide masses. The proteins are extracted, digested, and the peptides are analyzed by MS. The ratio between the heavy and light peptides indicates the abundance of the peptides in the two conditions. The advantage of this method is that as the heavy and light samples are pooled,

the downstream processing is carried out for both the samples at the same time and the errors in sample analysis are reduced. The limitation of this approach is that the technique might not be amenable to cell lines, which are difficult to grow or which are sensitive to alterations in the culture media [51].

3.1.2 | Isotope-coded affinity tag

Isotope-coded affinity tag (ICAT) is the first introduced chemical labeling technique which acts on the sulfhydryl groups of cysteine residues [52]. The technique employs ICAT reagent which comprises a thiol-reactive group (iodoacetamide), an isotopic linker region, and a cleavable biotin moiety. The samples are labeled with light and heavy ICAT reagents followed by pooling, digestion, and chromatography [53]. Although the sample complexity is reduced and the low abundant proteins and peptides are identified, the disadvantage of this method is that it does not allow quantitation of peptides lacking in cysteine residues.

3.1.3 | Isobaric tags

Isobaric mass tags possess identical mass but the position of the isotopes is different. The tag comprises three functional groups, namely an amine-reactive group (*N*-hydroxysuccinimide) coupled to a bipartite mass tag of 305 Da and an isotopic reporter group (*N*-methylpiperazine; 113–121 Da) linked by an isotopic balancer group (carbonyl; 184–192 Da) for the normalization of the total mass of the tags. This variability of the mass tag allows multiplexing of up to eight different samples at present. The proteins from different samples are subjected to protease digestion before labeling [54]. The most popular techniques of isobaric labeling include iTRAQ labels that react with the primary amine group of tryptic peptides, that is, *N*-termini and ε-amino groups of lysine side chains. Modification of primary amino groups is not susceptible to labeling by isobaric tags [55]. To avoid bias and reduce the steps involved in sample preparation like labeling, label-free approaches were developed.

3.2 | Label-free approach

3.2.1 | Spectral peak intensity

This technique detects the intensity of the ions to quantify the proteins present in the sample. The peak intensities of MS alerts are combined across retention times with an outlined mass window termed as the area under the curve. In this method, proteins are isolated from the sample and subjected to liquid chromatographic separation. The peptides eluted have a specific m/z ratio and specific retention time [56]. The area under the curve, which is directly proportional to the quantity of peptide, is calculated based on the m/z ratio, retention time (elution profile), and signal intensities, by the use of automated software. Although the technique is more reliable, it is also technically difficult as the m/z, peak area, and retention time have to be aligned properly [57].

3.2.2 | Spectral counting

This quantitative approach is based on the principle that higher the number of MS/MS spectra of peptides, the higher the quantity of protein from which they were derived. In this approach, the samples are digested to generate tryptic peptides. These peptides are subjected to liquid chromatography followed by tandem mass spectrometry. The peptides are eluted and different retention time and chromatographic peaks data are obtained, which differentiate the spectral count of each peptide. Methods available to calculate the peptide count are (a) protein abundance index [58], (b) exponentially modified protein abundance index [59], and (c) absolute protein expression [60]. The spectral count is an easy approach to implement, but the low number of spectral counts for low abundant proteins makes the quantitation less reliable resulting in statistical issues [61].

3.2.3 | Applications of quantitative proteomics in microbiology

Protein levels have a direct impact on protein–protein interactions, the cellular process, and molecular phenotype [62]. Quantification of protein abundance is essential to gain a thorough insight into the functions and biological activities occurring inside a cell. The protein expression of *Chlamydomonas reinhardtii* to salt-stress was studied by stable isotope labeling by amino acids in cell culture (SILAC) [63]. The results indicated that the metabolic response was associated with amino acid metabolism. The study provides insights into the stress adaptation of higher photosynthetic organisms. SILAC labeling enabled the study of proteome dynamics in the organism. The proteomic analysis of *Methanococcoides burtonii* studied by ICAT at low and optimum temperature conditions revealed repression of proteins related to cellular component biosynthesis and expression of enzymes involved in methanogenesis [64]. The study was the first report on the exploitation of ICAT technology to

TABLE 1 Selected publications of different proteomic tools employed in microbiological studies

Organism	Proteomic tool	Purpose of the study and reference	The outcome of the study
Methanococcoides burtonii	ICAT, LC-MS/MS	Cold adaptation in the organism [64]	Cold adaptation in the organism involved specific changes in membrane lipid unsaturation
Methanococcus maripaludis Porphyromonas gingivalis	Spectral count	Quantify and compare the protein abundance in a wild type and mutant lacking key enzyme in energy metabolism [68]	Metabolic labeling with stable isotopes appears to allow generation of a larger number of biologically meaningful and statistically significant expression ratios
Pseudomonas fluorescens	2DE, MALDI-TOF	Differential protein expression in the presence of lead, copper, and cobalt [69]	Proteins spo VG and enolase were upregulated whereas xylosyltransferase, ORF 18 phage phi KZ, OMP H1 and translational elongation factor EF-Tu were synthesized on exposure and were responsible for the survival of the organism
Fusarium graminearium	iTRAQ	Analyze the proteome under mycotoxin inducing conditions [70]	The study provided a basis for the probable mechanisms, markers of host invasion and novel antifungal targets
Lactococcus lactis	2DE, MALDI-TOF/TOF	Study the differentially expressed proteins in response to osmotic stress [71]	The role of glutathione in protecting *L. lactis* against multiple environmental stresses; by improving the robustness and stability of dairy starter cultures
Acinetobacter baumannii	2DE, MALDI-TOF/TOF MS	Proteins expressed under iron-rich and chelated conditions [72]	Iron-repressed protein spots represent outer membrane siderophore receptors, some of which could be involved in the utilization of siderophores produced by other bacteria. The proteins induced by iron were OmpA, Bfr and AcnA, AcnB, GlyA, SdhA, and SodB
Synechocystis salina Chlorella vulgaris	SDS-PAGE, Native PAGE, LC-MS/MS	Effect of UV-B and temperature stress on secreted proteins of Antarctic and mesophilic strains [73]	Antarctic *S. salina* secreted cysteine, serine, and metalloproteases, while mesophilic strain secreted mainly cysteine proteases.
			This suggested different mechanisms for withstanding UV-B and temperature stress in these strains
Pseudomonas putida Pseudomonas monteilli	2DE, LC-MS/MS	Identify the differentially expressed proteins upon cadmium exposure [74]	The czc gene present on the plasmid DNA involves metal binding and an efflux mechanism of resistance which was responsible for cadmium resistance of the organism
Synechocystis sp.	2DE, MALDI-TOF	Protein pattern alterations under cobalt, cadmium, and nickel stress conditions [75]	Some proteins were commonly regulated in response to the different metal ions, including ribulose-1,5-bisphosphate carboxylase and the periplasmic iron-binding protein FutA2, while proteins like chaperones, were specifically induced by each metal
Termitomyces heimii	2D-DIGE, MALDI-TOF/TOF	Protein profile at each developmental stage of the organism [76]	There was a correlation between the changes in protein expression that occur during different developmental stages.

TABLE 1 (Continued)

Organism	Proteomic tool	Purpose of the study and reference	The outcome of the study
			Enzymes related to cell wall synthesis were most highly expressed during fruiting body formation compared with the mycelium and primordial stages and enzymes involved in cell wall component degradation were upregulated in the earlier stages of mushroom development
Pseudomonas aeruginosa	SILAC	Study the antibiotic resistance in the organism [77]	A clear distinction between resistant and susceptible isolates was possible for all antibiotics (meropenem, tobramycin, and ciprofloxacin) with different modes of action. The use of SILAC technology in the study contributes to accelerated and reliable susceptibility testing
Candidatus thiodictyon syntrophicum	2D-DIGE, MALDI-TOF	Monitor the global proteomic changes in the presence and absence of light [78]	The abundant presence of enzymes involved in the autotrophic dicarboxylate/4-hydroxybutyrate cycle in the dark suggested a possible reason for the high capacity shown by PSB, to fix CO_2 in the absence of light
Streptococcus pneumonia	2DE, MALDI-TOF/TOF	Identify the proteins involved in copper resistance [79]	The differentially expressed proteins were mainly involved in the biosynthesis of the cell wall, protein, purine, and pyrimidine,
L. lactis subsp	2DE, MALDI-TOF/TOF	Study the proteome profiling in the presence of cadmium [80]	The study suggested that *L. lactis* subsp. lactis resist cadmium stress through antioxidant approach and enhanced energy metabolism
Vibrio parahaemolyticus	iTRAQ-based LC-MS/MS	Examine the protein profile of the organism at different growth conditions [81]	The findings of the study revealed that the prevalence of *V. parahaemolyticus* in a variety of common seafood consumed domestically in China and provides insights into the dissemination of antibiotic-resistant strains
Lactobacillus plantarum	iTRAQ	Investigate the mechanisms of the cadmium-induced stress response by comparing the protein profiles of resistant *L. plantarum* strain CCFM8610 and the sensitive CCFM191 strain in the presence and absence of cadmium [82]	Both strains displayed physiological alterations in energy metabolism, purine and pyrimidine metabolism, global stress responses, lipid, and amino acid metabolism, metal-binding properties, cell wall biosynthesis, and transporters in response to Cd exposure
Anabaena sp	MALDI-TOF-MS/MS	Carry out the physiological and proteomic analysis of halotolerant *Anabaena* sp [83]	Fifty salt responsive proteins were expressed upon salt treatment. The proteins identified include proteins involved in photosynthesis, protein folding, energy metabolism, and cell organization. The salt tolerance varied depending upon the salinity levels
Anabaena azolla	2DE, MALDI-TOF-MS/MS	The proteome profile of the cyanobiont was investigated upon exposure to salinity [84]	Proteins involved in protein synthesis and cell signaling were found to aid in the adaptation to salinity

(Continues)

TABLE 1 (Continued)

Organism	Proteomic tool	Purpose of the study and reference	The outcome of the study
A. azolla	2DE	The symbiotic nature of *A. Azolla* was studied by different protein extraction methods and 2DE [85].	The proteins of the whole plant, roots, and chloroplast obtained by different extraction methods were compared by 2DE. The phenol extraction method was found to be a suitable method

Abbreviations: DIGE, differential gel electrophoresis; ICAT, isotope-coded affinity tag; LC, liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SILAC, stable isotope labeling by amino acids in cell culture; TOF, time of flight.

study the cold adaptation in archaea and other members of the methanogenic archaea. The protein profile of *Thermobifida fusca* was studied in the presence and absence of cellulose by iTRAQ. The technique relatively quantified many hydrolyzing enzymes such as cellulases, hemicellulases, glycoside hydrolases, proteases and peroxidases, and protein translocating transporter proteins that play a role in energy metabolism and cell wall synthesis [65]. The study highlights the importance of using iTRAQ based on quantitative proteomics for microbial secretome analysis. The differentially expressed proteins of arsenate resistant microalga *D. salina* were quantified by the area under the curve [66]. Proteins involved in energy metabolism, protein synthesis and folding, reactive oxygen species scavenging, phosphate transport, membrane trafficking, and amino acid synthesis were observed to be the differentially expressed proteins. The relative abundance of the proteins was expressed in *Thalassiosira pseudonana* under phosphorous replete, and deficient conditions were calculated by spectral counting [67], and alteration in the abundance of proteins and transcripts were coordinated to various biochemical pathways including glycolysis and translation. The authors have reported that this was the first work to demonstrate that diatoms have a cell-surface-associated alkaline phosphatase, that may be involved in tightly coupling hydrolysis and uptake. The various applications of proteomics in microbiology have been presented in Table 1.

4 | CONCLUSION AND FUTURE PERSPECTIVES

The potential of different proteomic tools has been discussed in detail in this review. Due to the availability of genome sequences, microbes serve as excellent models for proteomic studies. Proteomics has the potential to contribute to environmental microbiology by enabling researchers to study the protein profiling of microbes, understand the stress response and adaptive strategies of extremophiles, and uncover the pathways behind the cellular processes. The complexity of environmental samples has been posing challenges, and proteomics in combination with multiple omic technologies would enable one in tackling them. In the field of medical microbiology, proteomics aids in the analysis of drug resistance of microbial pathogens, understanding the epidemiology and taxonomy of human microbial pathogens as well as the pathogenesis of microbes. The performance and reproducibility of already existing proteomic tools have to be enhanced for their routine use in clinical laboratories. Proteomics helps to identify the biomarkers of food contaminants, food-borne pathogens and to investigate their impact on food safety and human health, thus expanding research in food microbiology. Evaluating the risks associated with genetically modified foods has been challenging due to the seldom use of proteomics as a result of lack of interdisciplinary approach in food safety research on one hand and lack of good proteomic experts on the other.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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