

Isolation of Novel Acid Soil-tolerant Isolates of *Rhizobium* from "Pigeon Pea" and Proteomic Characterization by Utilizing MALDI-TOF/TOF and "Peptide Mass Fingerprinting" Approach to Identify Genes Associated with Acid-soil Tolerance

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Abstract Biological processes account for approximately 60% of the biosphere's fixed nitrogen. As concerns mount to the growing input of reactive nitrogen into environment, as part of a 'nitrogen cascade', an increased need to understanding 'Biological Nitrogen Fixation (BNF)' has become of paramount importance. *Rhizobium* spp. survival in soil is influenced by a combination of many variable parameters, with soil-acidity being prominent ones. Using *16SrRNA Ribotyping analysis* we have identified novel strains of *Rhizobium* (GenBank Accession Numbers KF309195, KF309203 and KF309204) from pigeon pea, which are tolerant to acidic soil pH regimes. 'Two-Dimensional Gel Electrophoresis (2-DE)', followed by MALDI-TOF-TOF (*Peptide-Mass Fingerprinting*) was performed to characterize several important 'Unique' protein differences amongst acid tolerant / acid in-tolerant *Rhizobium* isolates. Analysis of 14 (Fourteen) 'Unique' protein spots identified the genes implicated in the acid-soil tolerance. These genes were found to encode a wide range of functions, which are implicated in modulation of the chemo-taxis system, required for virulence, enzymes that catalyze redox transformations, biosynthesis / intermediary metabolism / detoxification, export / import of a wide variety of substrate, receptors for osmotic solutes produced, movement of diverse solutes, catalysis of the transfer of a methyl group, transport large folded proteins / Na⁺ dependent unidirectional secondary transporters and cellular metabolism respectively. One of the important protein was identified as 'Chain A, Structure Of Periplasmic Binding

Protein' (Accession Number: gi88192851, Molecular weight: 33,300Da and PI: 7.80). The periplasmic binding proteins serve as chemo-receptors, recognition constituents of transport systems, and initiators of signal transduction pathways. The existence of such unique proteins in the acid-tolerant isolates of *Rhizobium* is believed to explain the molecular basis of the factors responsible for imparting selective acid tolerance and better understanding of the molecular basis of leguminous plant nodules interaction, structure and function.

Keywords Biological Nitrogen Fixation, *Rhizobium*, Two-Dimensional Gel Electrophoresis, Mass Spectrophotometry

1. Introduction

Rhizobia are soil inhabiting, gram-negative bacteria, which are able to establish a nitrogen-fixing symbiotic relationship with legume plants [1]. Once the symbiosis is established, the rhizobia convert atmospheric N₂ into ammonia to their legume host plant. These bacteria may be considered as Plant Growth Promoting Bacteria (PGPB), since they directly affect plant growth development. Generally, rhizobia are aerobic or facultative anaerobic, rod shaped, and do not produce endospores. Rhizobia cells are mobile with one polar or sub-polar flagellum with two

to six peripheral flagella [2]. They show a typical translucent, viscid, slimy growth on Yeast Mannitol Agar media with individual colonies having domed shape, elevated feature with entire margins [3]. Rhizobia produce nodulation (*nod*) factors after a specific interaction with their host plant, and thus it has been assumed that rhizobia have co-evolved with their host plants [4]. The strong correlation between host plant nodulation genotypes denotes the importance of the host on nodulation genotypes [5,6,7,8]. Rhizobia have been utilized in agriculture to increase the yield of leguminous plants [9], through their use as inoculants to seed or, less often, soil. Symbiotic nitrogen fixing bacteria are represented by phylogenetically disparate class of alpha- and beta-proteobacteria, but the majority of the symbiotic species are represented in the alpha- proteobacteria order Rhizobiales, which, amongst many others, contain the agriculturally important nitrogen fixing genera of *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium* (Ensifer) and *Azorhizobium* [10].

Nitrogen is one of the most important elements in biological system, comprising the main building blocks of nucleic acid, enzymes and proteins among its multiple functions. In nature it exists primarily in the gaseous form and constitutes approximately 78% of the atmosphere. Despite its abundance, nitrogen (N) is one of the most growth-limiting nutrients in terrestrial and aquatic ecosystems [11], because its gaseous form is inert and unusable by most living organisms except for nitrogen fixing microorganisms. Biological Nitrogen Fixation (BNF) represents the major source of N input in agricultural soils including those in arid regions. The major N₂-fixing systems are the symbiotic systems, which can play a significant role in improving the fertility and productivity of low-N soils. The behavior of some N₂-fixing systems under severe environmental conditions such as salt stress, drought stress, acidity, alkalinity, nutrient deficiency, fertilizers, heavy metals and pesticides is matter of concern in enhancing the agricultural productivity. These major stress factors suppress the growth and symbiotic characteristics of most rhizobia; however, several strains, distributed among various species of rhizobia, are tolerant to stress effects. The climate changes and anthropogenic activities, such as urban development, road construction, industrial processes, mining and inadequate agricultural practices, are resulting in the eutrophication and pollution of soils and fresh water resource, soil degradation, loss of soil fertility, and desertification [12,13,14]. So, augments in crops production will need to be achieved, despite a significant deterioration of much prime agricultural lands, and will require the utilization of large areas now considered marginal. Moreover, the demand for nitrogen increased greatly in the last 5 years, especially in the emerging countries, such as India and China in Asia, and Brazil and Argentina in Latin America [15]. However, the use of nitrogen fertilizers accelerates the depletion of large

amounts of fossil fuels non-renewable energy resources, and it contributes substantially to environmental pollution, through atmospheric emission and leaching of nitrogenous compounds to ground or surface water [16,17,18,].

Rhizobia bacteria are able to establish symbiosis with legumes. They are considered to be the most important nitrogen-fixing agents in agriculture. This particular symbiotic relationship plays an important role in agriculture in grain and forage legumes, such as bean, pea and chickpea, increasing crop productivity without the requirement of chemical nitrogen fertilizers, and thus contributing to the sustainable agriculture as-well-as for the pollution reduction [19,20]. Furthermore, this association is also important as legume crops can improve the soil nitrogen availability to other crops, for instance cereals, as-well-as in land remediation [21]. The specific symbiosis association between rhizobia and leguminous plants results in the formation of specialized structures, called root nodules, where bacteria can convert di-nitrogen into ammonia, and supply it to the host plant in exchange for carbohydrates [22]. This prokaryotic-eukaryotic intimacy is based on a complex molecular cross-talk between both partners, which is initiated by the secretion of flavonoids and other compound to rhizosphere by legume plants, inducing the rhizobia lipo-chito-oligosaccharides, termed as *Nod* factors [23]. A complete and efficient nitrogen fixation in legume rhizobia symbiosis requires the co-ordinated interaction of several major classes of genes present in rhizobia, namely the co-ordinated interaction of several major classes of genes present in *rhizobia*, the *nif* genes and *fix* genes [24] for atmospheric nitrogen fixation, and the *nod*, *nol* and *noe* genes for nodulation [25] respectively.

Pigeon pea [*Cajanus cajan* (L.) Millsp.] is an important grain legume crop of rain- field agriculture in the tropics and sub-tropics. Compared with other grain legumes, pigeon pea ranks only sixth in area and production, but it is used in more diverse ways than others [26,27,28,29]. The extracts or components of pigeon pea are commonly used all over the world for the treatment of diabetes, dysentery, hepatitis and measles, as febrifuge to stabilize the menstrual period [30,31,32]. Now-a-days, pigeon pea leaves are used for the treatment of wounds, aphtha, bedsores and malaria, as-well-as diet- induced hypercholesterolemia [33,34,35,36]. Pigeon pea is hardy, widely adaptable, and more tolerant to drought and high temperature than most other crops. It is widely grown in about 14 countries in over 4 million ha. The major producer of pigeon pea in the world includes: India, followed by Uganda, Tanzania, Kenya, Dominican Republic and Mozambique in Africa; the Dominican Republic, Puerto Rico and the West Indies in the Caribbean region and Latin America; Burma and Thailand; Indonesia and Philippines in Asia and Australia [37].

Soil acidity is the term used to express the quantity of hydrogen (H⁺) and aluminum (Al³⁺) cations (Positively

charged ions) in soils, which also serves as an indicator of soil acidity. Agricultural practices and climate changes increase the amount of land affected by acidity, and thus limit legume crop productivity. Worldwide, more than 1.5 Gha of acid soils limit agriculture production [38], and as much as 25% of the earth's crop-lands are impacted by problems associated with soil acidity [39]. Legumes and their rhizobia exhibit varied response to soil acidity. Most leguminous plants require a neutral or slightly acidic soil for growth, especially when depending on symbiotic nitrogen fixation [40,41,42]. However, differences in acid soil tolerance by legumes have been reported. Some species, like Lucerne (*Medicago sativa*), are extremely sensitive to acidity [40], while others, such as *Lotus tenuis* tolerate relatively low soil pH [43]. The major factors contributing towards soil acidity include, amount of rainfall received, weathering of minerals, acid rain, uses of fertilizers, mine spoil, leaching of nitrogen and plant root activity respectively. Soil acidity is very prominent in the state of Jharkhand. Acidic soils in Jharkhand constitutes about 10 lakh hectares of the total geographical area. Based on the soil acidity, the soils in the state of Jharkhand are classified as, extremely acidic, very strongly acidic, strongly acidic and moderately acidic respectively. Aluminum is the third abundant element after oxygen and silicon present in the earth's crust [44,45]. It belongs to the non-essential category of metals, thus does not exert any known function in plant metabolism [46]. However, the metal is considered to be a major growth-limiting factor particularly in acid soils (pH<5.0), which are estimated to be approximately 30-40% of arable lands in the world [47]. Depending on pH, aluminium exists in a number of different forms in the soil [48]. Under acidic conditions, aluminium is solubilized in to $[Al(H_2O)]^{3+}$, generally referred as Al^{3+} , which is highly toxic to many plant species [49]. Despite the fact that many plants find it difficult to withstand against the aluminium toxicity, its most common forms i.e. oxides and aluminosilicates are harmless to plants [44,46]. Besides contributing to soil acidity, aluminium affects the plants by causing alterations in vast variety of functions [50-63]. On the other hand, although resistance to temperature stress may constitute a prerequisite for symbiotic nitrogen fixation in tropical areas, heat may be also affecting other properties. In several organisms, including bacteria, plants and animals, the acquired thermo-tolerance appeared to be dependent on the protein synthesis. All organisms so far examined respond to a sudden increase in growth temperature by inducing synthesis of small number of heat-shock molecular chaperons Hsp's [64]. Some of them highly conserved throughout evolution, which may be indicative of an important cellular function of these proteins [65]. All organisms respond to a sudden increase in growth temperature by inducing the synthesis of a number of Heat Shock Proteins (HSPs). HSPs consist of chaperons (such as GroEL, DnaK, DnaJ), small Heat Shock Proteins (sHsps),

and proteases. Soil acidity causes major problem in agriculture production worldwide by lowering the crop productivity [42,66]. Low pH values can affect all the steps in the *Rhizobium*-legume symbiosis, ranging from the survival of the bacteria in the soil, root hair infection, nodule development and nitrogen fixation [67]. This reduction in the nodulation rate is especially evident when legumes grow in pH values under 5.0, due to the inability of the rhizobial inoculums to survive in these severely adverse conditions [68]. Rhizobia have evolved several mechanisms for maintaining intracellular pH. The prominent strategies employed by rhizobia bacteria include, decrease membrane permeability, internal buffering, amelioration of external pH, proton extrusion / uptake, and prevention of metal ions respectively [69]. A number of genes, such as *actA*, *actP*, *exoR*, *lpiA*, *actR*, *actS*, and *phrR*, are essential for growth at low pH [70,71]. Acidity also becomes a challenge in nodule development affecting both the plant and bacteria, as-well-as their interaction. Several genes have been characterized as essential for acid-resistance [72,73,74]. It has been reported that glutathione participates in acid resistance in *R. tropici*, and is essential for it to be able to grow at low pH. In acidic stress rhizobia produces Acid Shock Proteins (ASP's). These proteins are known to contribute to acid tolerance by conferring acid protection on the bacteria, but do not alter the internal pH of the cell. Several genes, such as *actA*, *actP*, *exoR*, *lpiA*, *actR*, *actS*, and *phrR* are shown to be essential for rhizobia growth at low pH [75-77,70,71,73].

Proteomics is an ideal tool for the dissection of plant-microbe interactions. It provides a broad overview of the proteins produced by both partners during their constant signal exchange. It also allows the detection of signal transduction pathways by following phosphorylation changes of proteins [78] that are important for protein function [79,80]. The recent discovery of several plant receptor kinases responsible for early detection and signal transduction of Nod factor perception [81,82], and auto-regulation of nodule numbers [83,84], suggests, that many early plant-microbe signaling events are regulated by phosphorylation events and key receptor kinases. MALDI-TOF-MS (Matrix-Assisted Laser Desorption Ionization Time Of Flight Mass Spectrometry) has become a popular and versatile method to analyze a range of macro-molecules from biological origin from cell to tissues. The techniques stands out as the preferred method of analysis due to its high accuracy and sensitivity, combined with its wide mass range (1-300kDa). The "Proteome" represents the array of proteins that are expressed in a biological compartment (cell, tissue or organ, as the case may be), at a particular time, under a particular set of conditions. In the present study, characterization and identification of "Candidate Proteins" from acid soil-tolerant isolates of *Rhizobium* from pigeon pea collected from the acidic soils of the State Of Jharkhand was carried out by utilizing MALDI- TOF-TOF approach

("Peptide Mass Fingerprinting"). Subsequently, genes which might be implicated to play crucial roles in imparting acid soil-tolerance to *Rhizobium* isolates of pigeon pea were identified from the isolates collected from acid soils of the State Of Jharkhand.

2. Materials and Methods

Sample Collection and 16SrRNA Analysis

Sample collection was performed for pigeon pea crop for *Rhizobium* isolates from the acidic soils of the State Of Jharkhand. *Rhizobium* isolates were purified from the root nodules of pigeon pea crop, and were characterized by various methods. 16SrRNA "Ribotyping Analysis" of various isolates from pigeon pea collected from acidic soil regimes of the State Of Jharkhand was performed. "Nucleotide Accessions" have been released by NCBI (NIH, USA) for acid-tolerant *Rhizobium* isolates from pigeon pea, with Accession Numbers, KF309195, KF309203 and KF309204 collected from soil pH 5.5, 4.5 and 4.5 respectively. The details of various *Rhizobium* isolates are available on <http://www.ncbi.nlm.nih.gov>.

Protein Isolation from *Rhizobium* Isolates

The bacterial cultures were inoculated in 3ml of YEMA media, and multiplied O/N at 200 rpm, 28°C to generate the starter culture. The O. D. was measured at 620_{nm} using UV spectrophotometer the following day, to ensure optimal growth of the cultures. Large-scale culture was set-up by employing the starter culture. Subsequently, the bacterial pellet was isolated by centrifugation of culture at 6,000rpm, 4°C, 10'. The supernatant was discarded, and the bacterial pellet was stored at -86°C till further processing. The lysis of bacterial cells was performed by employing freeze-thaw method using liquid N₂, and as per the recommendations of "Bacterial Isolation Q Proteome Kit (Qiagen, Germany)" provided by the manufacturer.

Two-Dimensional Gel Electrophoretic Analysis of Samples

The proteins present in the supernatant were precipitated O/N at -20°C in SS-34 tubes by employing precipitation buffer (10% TCA, 20 mM DTT prepared in acetone). Subsequently, the proteins were pelleted by centrifugation at 11,000g, 4°C, 10'. The protein pellets were washed with acetone + DTT solution and kept at -20°C for 5 minutes. The pellet was subsequently dried under vacuum and dissolved in re-hydration buffer (8M Urea, 2M Thiourea, 0.1mM DTT, 2% CHAPS, few crystals of bromophenol blue dye and 2% IPG Buffer pH 3-10). Protein quantification of samples was performed by employing 2-D Quant Kit (GE Healthcare, Sewden Uppasla), as per the manufacturer's recommendations. A total of 150 µg protein was loaded for each of the samples by the

re-hydration method (O/N) to the Immobiline IPG Strips (GE Healthcare, Sweden) with pH range 3-10L, 7cm. Subsequently, 1st Dimensional Electrophoresis was performed by employing IPG Phor 3 instrument (GE Healthcare, Sweden), as per the optimized protocol (6,000 Volt-Hours, 50µA / Strip @ 20°C). Upon completion of the IPG strip's run, the IPG strips were equilibrated in "Equilibration Buffer" (50mM Tris-HCl, pH: 6.8, 6M Urea, 30% Glycerol, 2% SDS, few crystals of bromophenol blue) consisting of DTT (64mM), followed by Iodoacetamide (135mM), before performing 2nd Dimensional Gel Electrophoresis by employing 12.5% Linear SDS-PAGE gels (1.0mm, 30mA Constant / Gel, 10°C).

Staining of the Gels

The gels were stained by employing Silver Nitrate (Silver Staining) by employing PlusOne Silver Staining Kit, Protein (GE Healthcare, Sewden Uppasla), as per the manufacturer's recommendations, and by employing "Automated Gel Staining Instrument (GE Healthcare, Sweden)". The protein spots obtained were analysed and subsequently marked for MALDI-TOF-TOF analysis.

MALDI-TOF-TOF Analysis of Protein Spots

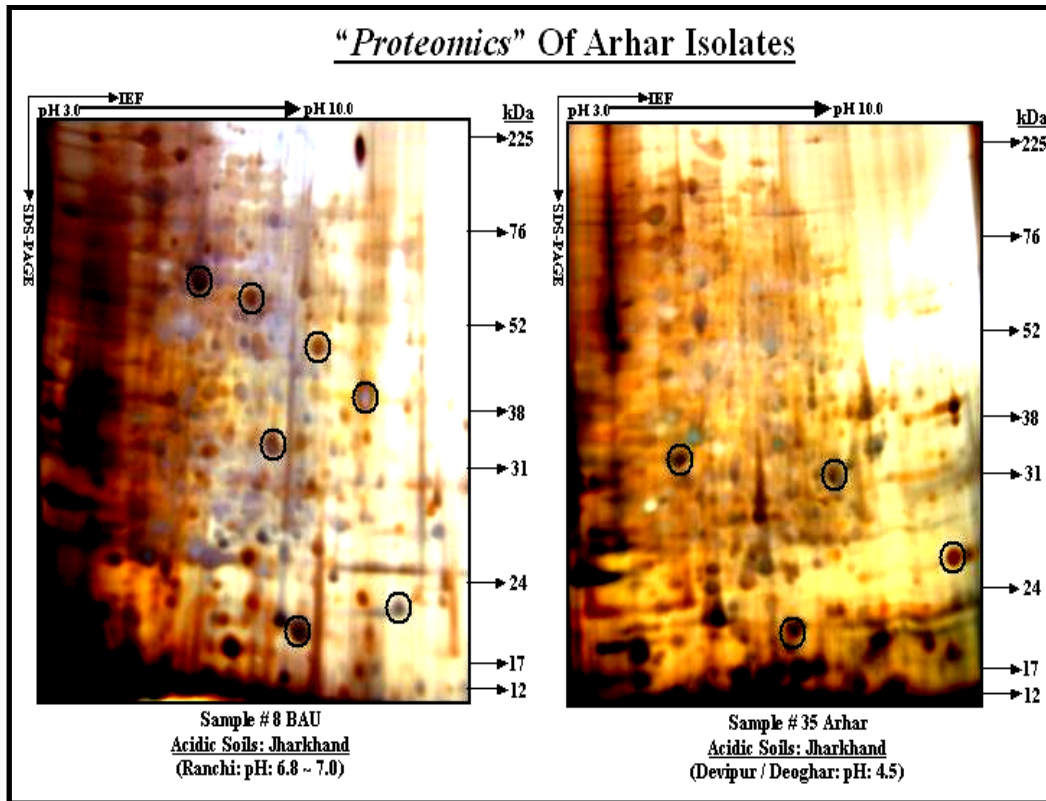
The selected protein spots were carefully excised from the silver stained gels by employing a sterile scalpel blade. The spots were then shipped under sterile conditions for the MALDI-TOF-TOF analysis. The analysis of MS (Mass Spectrophotometry) data was performed by employing the Mascot search engine.

3. Results and Discussion

The soil environment is constantly changing which can relatively be stressful for both macro- and micro-organisms. Changes such as, fluctuation in pH, temperature, salinity and nutrient availability greatly influence the growth, survival and metabolic activity of soil micro-organisms and plants, and thus interfere with their ability to enter into symbiotic interactions. In case of *Rhizobium*, it greatly affects the Biological Nitrogen Fixation (BNF) process. Soil acidity is the most prominent problem for agriculture development. Therefore, the knowledge of the factors and genes which play crucial roles towards the adaptation of *Rhizobium* spp. towards abiotic stresses, in this case, soil acidity, is of paramount importance. We have identified 14 genes from acid soil-tolerant *Rhizobium* isolates of pigeon pea, which play crucial role in acid soil-tolerance mechanism of *Rhizobium*. It is hypothesized, that these genes play crucial roles towards imparting tolerance to the *Rhizobium* isolates for adaptation to the acidic soil regimes. The two-dimensional gel analysis of *Rhizobium* isolates native to the neutral soil pH (6.8-7.0) versus isolates native to acidic soil pH (4.5 and 5.0 respectively) revealed lots of

“Unique Protein Changes”, which are not found to be present in the neutral samples (Figure 1.0). The major protein changes between the isolates were documented and subsequently selected for the MALDI-TOF-TOF analysis (Figure 2.0). The comparison of *Rhizobium* isolates of pigeon pea collected from acidic soil regimes with isolates collected from neutral pH soil regimes identified many

genes which perform varied functions in order to help bacterium survive in the acidic soil regimes (Table 1.0). In the subsequent paragraphs below, we discuss in detail the probable roles the identified genes might play towards imparting acidic soil tolerance to the *Rhizobium* isolates analyzed in our study (Figure 1.0 and 2.0, Table 1.0):



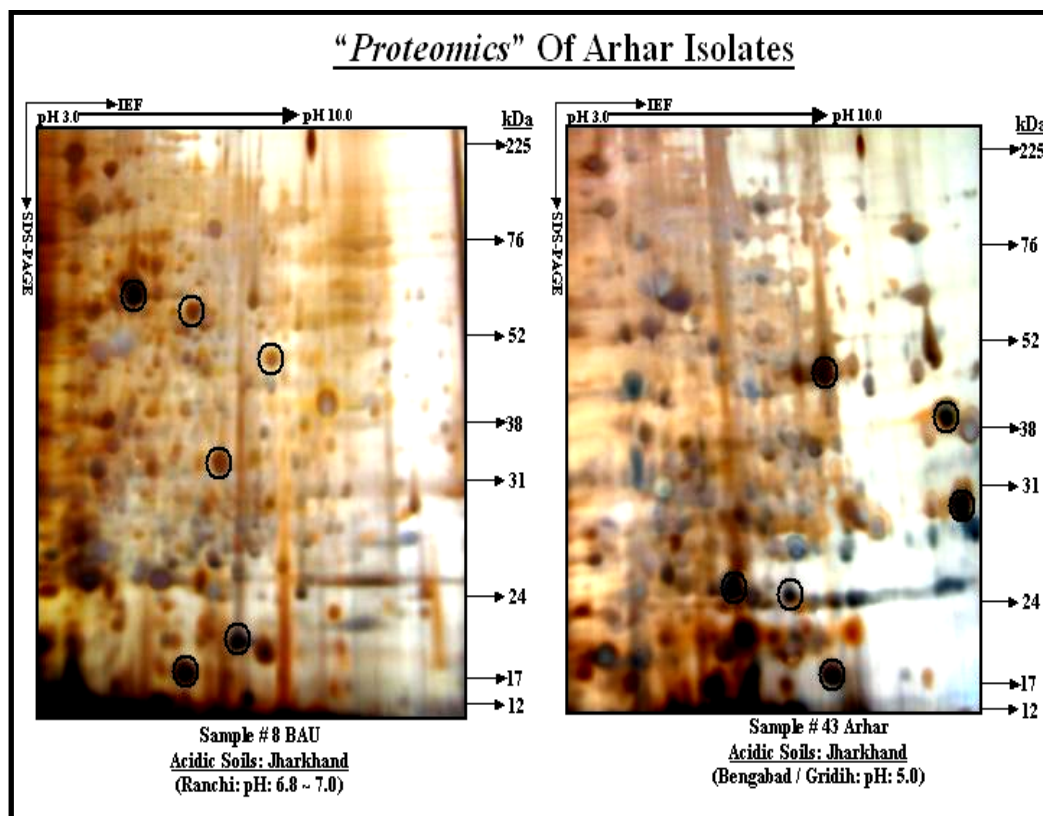
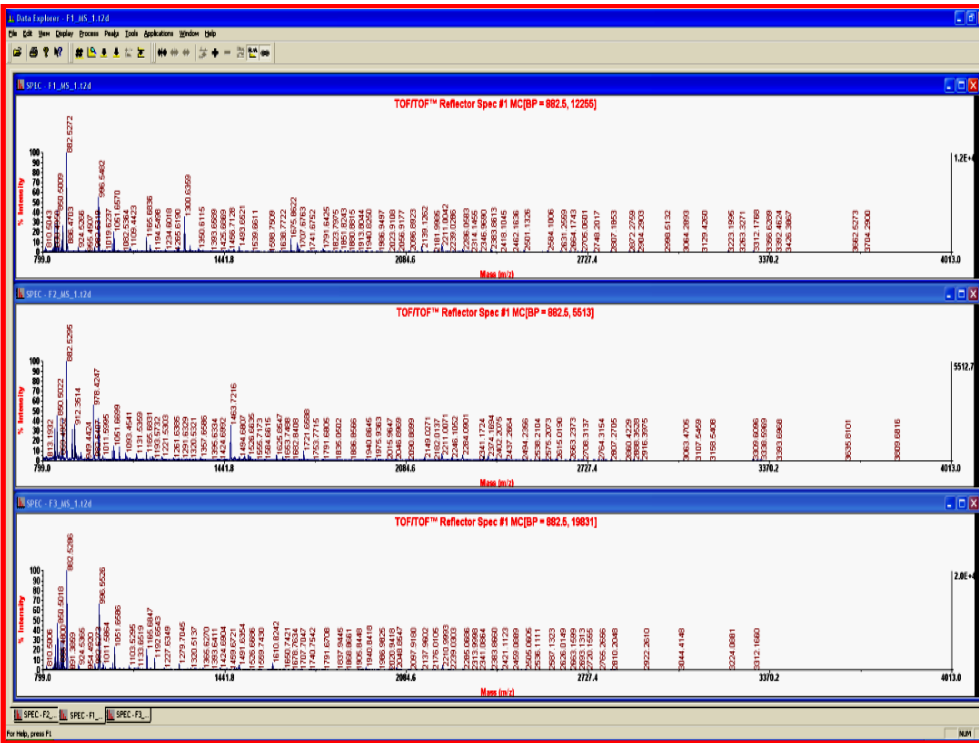
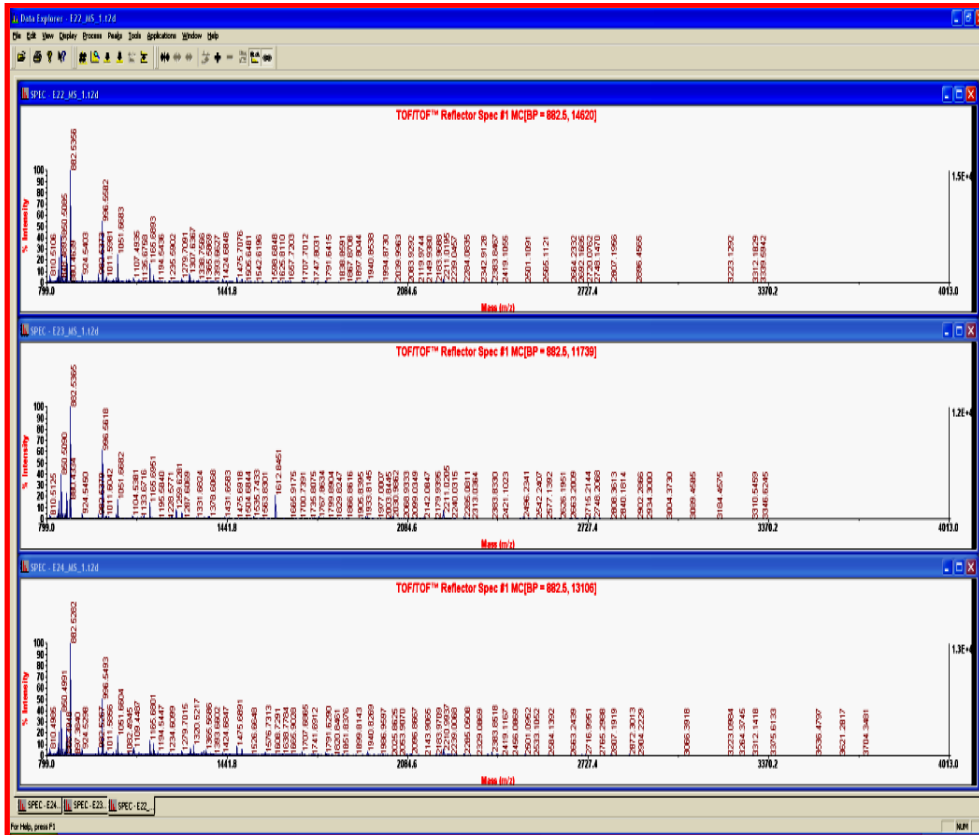
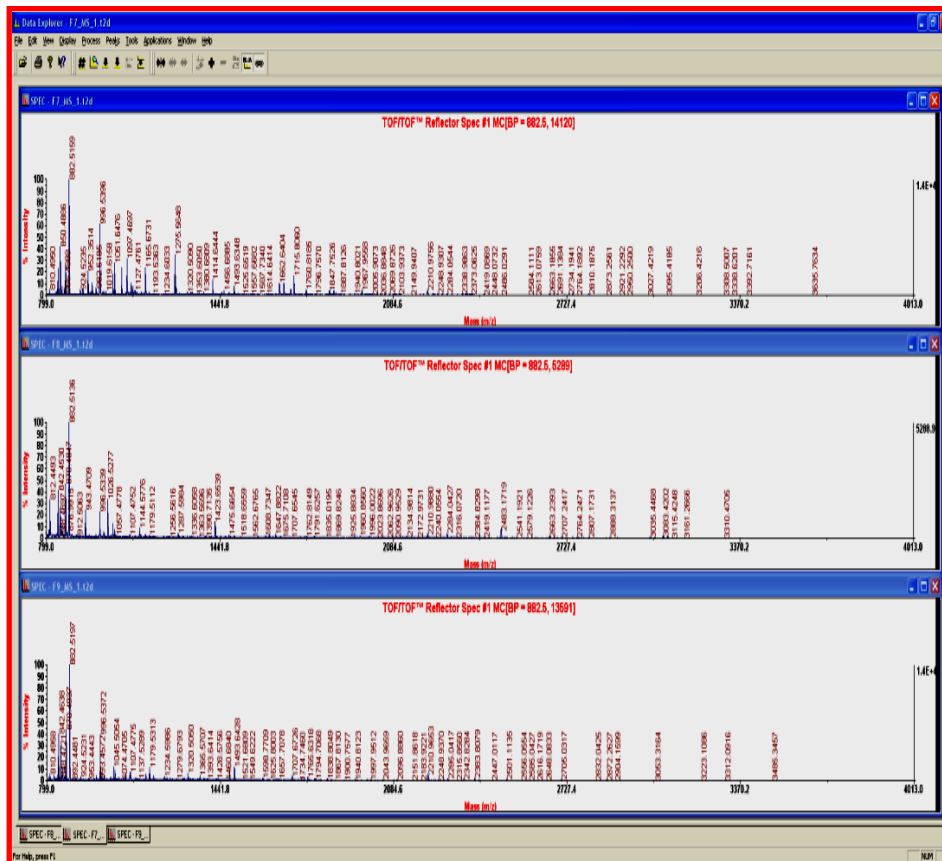
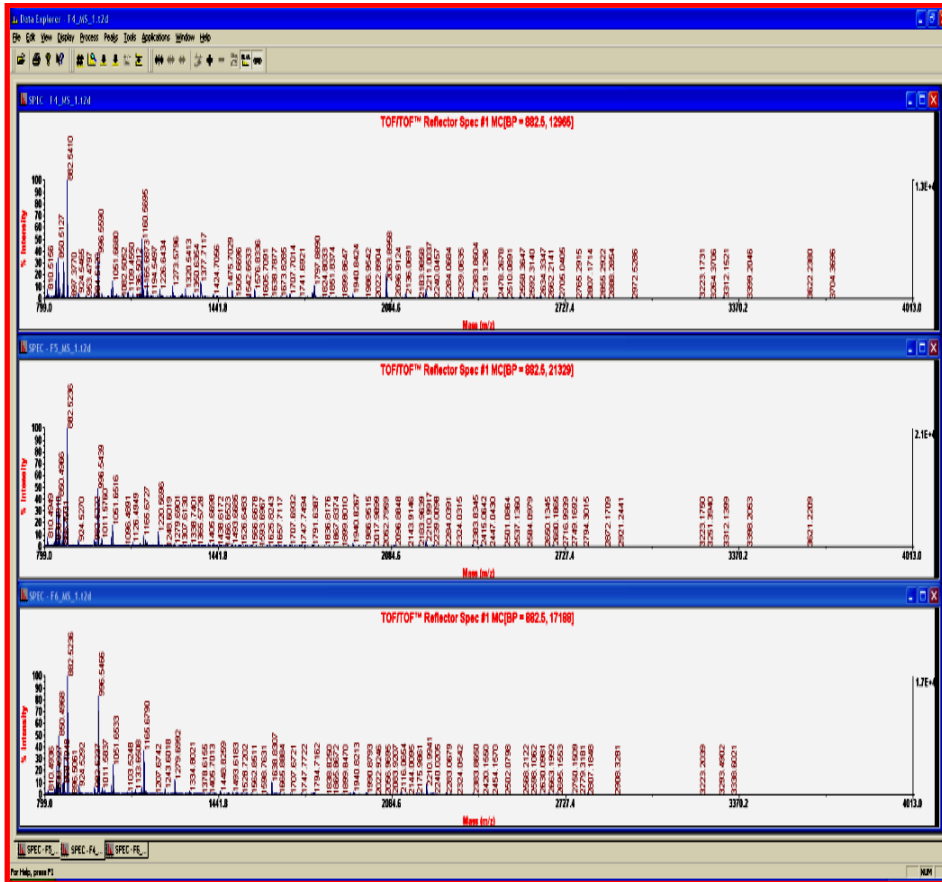


Figure 1. Two-Dimensional Gel Electrophoretic analysis of samples: Silver stain gel profiles of *Rhizobium* isolates collected from acidic soil regimes of the State Of Jharkhand. 150 µg total protein was employed for running the Immobiline IPG Strips (GE Healthcare, Sweden) with pH range 3-10L, 7cm. 2nd Dimensional Gel Electrophoresis was performed by employing 12.5% Linear SDS-PAGE gels (1.0mm, 30 mA Constant / Gel, 10°C). Left-most panel represents Sample #8BAU (pH: 6.8-7.0), while right-most panel represents Sample #35 Arhar (pH; 4.5) and Sample # 43 (pH: 5.0) respectively. The circled protein spots depict, “Unique Spots”, not present in either of the corresponding samples analyzed.



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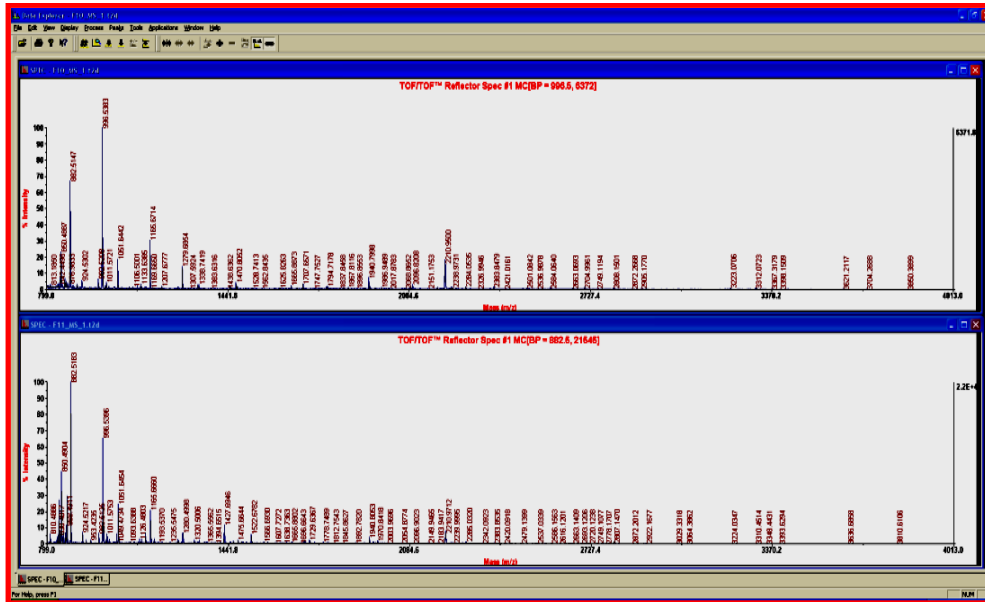


Figure 2. MALDI-TOF-TOF spectrums of the protein spots analyzed

Table 1. MALDI-TOF-TOF data analysis of the protein spots and their attributed functions

S. No.	Spot ID	Protein Identification (MALDI-TOF-TOF)	Attributed Functions	Accession No.	MW (Da)	Score	(CI %)	Engine
1.	123/E22	Chemotaxis Protein CheR [<i>Myxococcus xanthus</i> DK]	Involved in modulation of the chemo-taxis system	gi 108758057	47574.70	87.00	96.984	Mascot
2.	124/E23	TTT family tricarboxylate transporter, receptor protein, [<i>Achromobacter piechaudii</i> ATCC 43553]	Constituents of primary and secondary active transport systems	gi 293603568	34718.00	203.0	100.00	Mascot
		Extra-cytoplasmic solute receptor family protein 18 [<i>Achromobacter xylosoxidans</i> A8]	Involved in solute transport, involved for the recruitment of the solute and its presentation to the membrane complex	gi 311104125	34716.00	185.0	100.00	Mascot
		Exported protein [<i>Achromobacter xylosoxidans</i> C54]	Required for virulence	gi 422322729	34444.90	177.0	100.00	Mascot
3.	125/E24	Aldo / keto reductase [<i>Rhizobium etli</i> Brasil 5]	Super-family of enzymes that catalyze redox transformations, involved in biosynthesis, intermediary metabolism, and detoxification	gi 218510830	38205.90	87.00	97.249	Mascot
		Valyl-tRNA synthetase [<i>Microscilla marina</i> ATCC 23134]	Cellular metabolism	gi 124009039	104529.30	85.00	95.739	Mascot
		Deoxyribodipyrimidine photolyase [<i>Mariniradius saccharolyticus</i> AK6]	Cellular metabolism	gi 440749714	50970.10	85.00	95.328	Mascot
4.	126/F1[1] 31564	Molybdate ABC super-family ATP binding cassette transporter, binding protein [<i>Achromobacter piechaudii</i> ATCC 43553]	Uses hydrolysis of ATP to energize diverse biological systems, involved in the export or import of a wide variety of substrates ranging from small ions to macromolecules, major function is to provide essential nutrients to bacteria	gi 293606431	26681.10	238.0	100.00	Mascot
5.	127/F2[1] 31564	Branched-chain amino acid ABC super-family ATP binding cassette transporter, amino acid-binding protein [<i>Achromobacter piechaudii</i> ATCC 43553]	Provide movement of diverse solutes, facilitates net uptake of solutes into bacterial cells	gi 293602433	48964.90	198.0	100.00	Mascot
		Exported protein [<i>Achromobacter xylosoxidans</i> C54]	Required for virulence	gi 422323639	48992.90	154.0	100.00	Mascot

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6.	128/F3[1] 31564	Extra-cytoplasmic solute receptor family protein 4 [<i>Achromobacter piechaudii</i> HLE]	Involved in citrate uptake, functions as receptors for osmotic solutes produced and accumulated by bacterial cells under stress (salt)	gi 421483536	33818.80	231.0	100.00	Mascot
		TTT family tricarboxylate transporter, receptor protein [<i>Achromobacter piechaudii</i> ATCC 43553]	Constituents of primary and secondary active transport systems	gi 293602883	33701.80	222.0	100.00	Mascot
7.	129/F4[1] 31564	ABC super-family ATP binding cassette transporter, binding protein [<i>Achromobacter piechaudii</i> ATCC 43553]	Provide movement of diverse solutes, facilitates net uptake of solutes into bacterial cells	gi 293603959	40476.20	341.0	100.00	Mascot
		Receptor family ligand binding region family protein 10 [<i>Achromobacter piechaudii</i> HLE]	Cellular metabolism	gi 421482768	40344.20	298.0	100.00	Mascot
8.	130/F5[1] 31564	Chemotaxis protein CheR [<i>Myxococcus xanthus</i> DK 1622]	Catalysis of the transfer of a methyl group from S-adenosyl-L-methionine to a substrate	gi 108758057	47574.70	89.00	98.264	Mascot
9.	131/F6[1] 31564	Tat pathway signal sequence domain-containing protein 9 [<i>Achromobacter piechaudii</i> HLE]	Operates in plant thylakoid membranes and the plasma membranes of most free-living bacteria. In bacteria, it is responsible for the export of a number of proteins to the periplasm, outer membrane or growth medium, can transport large folded proteins (even oligomeric proteins) across the tightly sealed plasma membrane	gi 421483968	34071.90	159.0	100.00	Mascot
		TTT family tricarboxylate transporter, receptor protein [<i>Achromobacter piechaudii</i> ATCC 43553]	Constituents of primary and secondary active transport systems	gi 293604353	33999.80	145.0	100.00	Mascot
		Secreted protein [<i>Achromobacter xylosoxidans</i> C54]	Gram-negative bacteria secrete a wide range of proteins, functions include biogenesis of organelles (pili and flagella), nutrient acquisition, virulence, and efflux of drugs and other toxins. Six distinct secretion systems have been shown to mediate protein export through the inner and outer membranes of Gram-negative bacteria.	gi 422322292	34052.90	103.0	99.926	Mascot
10.	132/F7[1] 31564	TRAP-T family tripartite ATP-independent periplasmic transporter, binding protein [<i>Achromobacter piechaudii</i> ATCC 43553]	Ubiquitous in prokaryotes, but absent from eukaryotes, are high-affinity, Na(+)-dependent unidirectional secondary transporters	gi 293606006	39984.20	209.0	100.00	Mascot
11.	133/F8[1] 31564	3-Hydroxyisobutyrate dehydrogenase MmsB [<i>Janthinobacterium sp.</i> HH01]	Cellular metabolism	gi 445497174	31050.80	81.00	89.298	Mascot
12.	134/F9[1] 31564	Pyruvate Kinase [<i>Xylella fastidiosa</i> Temecula1]	Cellular metabolism	gi 28199715	52452.20	90.00	98.683	Mascot
13.	135/F10 [1] 31564	Aspartyl-tRNA synthetase [<i>Rhodopirellula baltica</i> SH28]	Cellular metabolism	gi 421610931	63689.40	14.00	66.000	Mascot
14.	136/F11 [1] 31564	UvrB/UvrC protein [<i>Thermaerobacter marianensis</i> DSM 12885]	Celullar metabolism	gi 317123128	20738.60	86.00	96.616	Mascot

Chemotaxis protein cheR (*Myxococcus Xanthus* DK): Chemotaxis describes the cellular processes that control the movement of organisms towards favorable

environments. These proteins are involved in modulation of the chemotaxis system. CheR methyl-transferases catalyze methylation of the cytosolic signaling domain of

chemo-receptors, and are among the core proteins of chemo-sensory cascades [85]. TTT family tri-carboxylate, transporter, receptor protein (*Achromobacter piechandii* ATCC 43553): These proteins are constituents of primary and secondary active transport system [86]. Active transport system helps in accumulation of high concentration molecules that the cell needs, such as ions, glucose and amino acids. Primary active transport system utilizes energy in the form of ATP to transport molecule across membrane against their concentration gradient. Secondary active transport, is transport of molecules across the cell membrane utilizing energy in the other form than ATP. This energy comes from the electro-chemical gradient created by pumping ions out of the cell. Extra-cytoplasmic solute receptor family protein 18 [*Achromobacter xylosooxidans* A8]: Extra-cytoplasmic solute receptors are constituents of primary and secondary active transport system. This protein is involved in solute transport, involved for the recruitment of the solute and its presentation to the membrane complex [87]. Exported protein: [*Achromobacter xylosooxidans* C54]: Export of proteins is necessary for a variety of essential bacterial functions including expression of virulence factors on the cell surface, release of effector proteins to the extra-cellular milieu nutrient acquisition, and organelle biogenesis. To export proteins, the gram negative bacterium faces the triple barrier of transporting the polypeptide first across the inner membrane, then through the periplasmic space, and finally across the outer membrane. The task of passing through the periplasm is not trivial since proteins may fold and form disulfide bonds in this space before reaching and crossing the outer membrane. Aldo / keto reductase [*Rhizobium etli* Brasil5]: The Aldo-Keto Reductase (AKR) super-family comprises of several enzymes that catalyze redox transformations involved in biosynthesis, intermediary metabolism and detoxification. Substrates of the family include, glucose, steroids, glycosylation end products, lipid peroxidation products, and environmental pollutants [88]. Valyl-tRNA synthetase [*Microscilla marina* ATCC 23134]: Valyl-tRNA synthetase, a large monomeric enzyme in a free state, forms a class- Ia sub-family, which characteristically have an α - helix bundle domain near the C-terminus to recognize the tRNA anti-codon. It is involved in cellular metabolism, and catalyzes the attachment of val to tRNA. Deyoxyribopyrimidine photolyase [*Mariniradius saccharolyticus* AK6]: It is involved in cellular metabolism. Cyclobutane pyrimidine dimers are major DNA photo-products induced by the UV component of solar radiation. Photo-reactivating enzyme (DNA photolyase) repairs DNA by utilizing the energy of visible light to break the cyclobutane ring of the dimer [89]. Molybdate ABC super-family ATP binding cassette transporter binding protein [*Achromobacter piechandii* ATCC 43553]: ABC transporters belong to the ATP-Binding Cassette (ABC) super-family, which uses the hydrolysis of ATP to

energise diverse biological systems. ABC transporters minimally consist of two conserved regions: a highly conserved ATP Binding Cassette (ABC) and a less conserved Trans-Membrane Domain (TMD). These can be found on the same protein or on two different ones. Most ABC transporters function as a dimer, and therefore, are constituted of four domains, two ABC modules, and the two TMDs. ABC transporters are involved in the export or import of a wide variety of substrates ranging from small ions to macro-molecules. The major function of ABC import system is to provide essential nutrients to bacteria. They are found only in prokaryotes, and their four constitutive domains are usually encoded by independent polypeptides (two ABC proteins and two TMD proteins). Prokaryotic importers require additional extra-cytoplasmic binding proteins (one or more per systems) for function. In contrast, export systems are involved in the extrusion of noxious substances, the export of extra-cellular toxins, and the targeting of membrane components. They are found in all living organisms, and in general, the TMD is fused to the ABC module in a variety of combinations [90]. Branched-chain amino acid ABC super-family ATP binding cassette transporter, amino acid-binding protein [*Achromobacter piechandii* ATCC 43553]: They provide movement of diverse solutes, and facilitate net uptake of solutes into bacterial cells. Exported protein: Required for virulence. Extra- cytoplasmic solute receptor family protein 4 [*Achromobacter piechandii* HLE]: Extra-cytoplasmic solute receptors are the constituents of primary and secondary active transport systems. They are involved in citrate uptake, function as receptor for osmotic solutes produced, and are accumulated by bacterial cells under stress. TTT family tri-carboxylate, transporter, receptor protein (*Achromobacter piechandii* ATCC 43553): These proteins are constituents of primary and secondary active transport systems [86]. Active transport system helps in accumulation of high concentration molecules that the cell needs, such as ions, glucose and amino acids. ABC super- family ATP binding cassette transporter, binding protein [*Achromobacter piechandii* ATCC 43553]: ABC transporters utilize the energy of ATP binding and hydrolysis to transport various substrate across cellular membranes. Bacterial ABC transporters are essential in cell viability, virulence, and pathogenicity. They perform important role in the import of essential nutrients, and the export of toxic molecules [91]. They provide movement of diverse solutes, and also, facilitate net uptake of solutes in-to bacterial cells. Receptor family ligand binding region family protein 10 (*Achromobacter piechandii*): It is involved in cellular metabolism. Chemotaxis protein cher [*Myxococcus Xanthus* DK1622]: Catalyzes the transfer of a methyl group from S-adenosyl 1- L-methionine to a substrate. Tat pathway signal sequence domain-containing protein 9 [*Achromobacter piechandii*]: In chloroplast, the Tat component are found in thylakoid membranes, and direct the import of proteins from the stroma. In bacteria,

they are found in plasma membrane. The Twin-Arginine Translocation (TAT) pathway is responsible for the export of folded proteins across the cytoplasmic membrane of bacteria. Secreted protein [*Achromobacter xylosoxidans* C54]: Gram- negative bacteria secrete a wide range of proteins. Their functions include biogenesis of organelles (pili and flagella), nutrient acquisition, virulence, efflux of drugs and other toxins. Six distinct secretion systems have been shown to mediate protein export through the inner and outer membranes of gram-negative bacteria. TRAP-T family tripartite ATP independent periplasmic transporter, binding protein [*Achromobacter piechaudii* ATCC 43553]: Tripartite ATP-independent periplasmic transporters represent a novel type of secondary active transporter, that functions in conjunction with an extra-cytoplasmic solute-binding receptor [92], and are ubiquitous in prokaryotes, but notably absent from eukaryotes. They are high-affinity, Na⁺ dependent uni-directional secondary transporters [93]. 3-Hydroxyisobutyrate dehydrogenase: It is involved in cellular metabolism. Pyruvate kinase [*Xylella fastidiosa* Temecula]: It is involved in cellular metabolism. Aspartyl-tRNA synthetase [Rhodopirellula baltica SH28]: It is involved in cellular metabolism. UvrB / UvrC protein [*Thermocrobacter marianensis*]: It is involved in cellular metabolism.

MALDI-TOF-MS is important tool for protein identification, because of its high throughput, sensitivity, and high mass accuracy. This highly effective approach of protein identification is based on the accurate mass measurement of a group of peptides derived from a protein by sequence-specific proteolysis. After proteolysis with a specific protease e.g. trypsin, proteins of different amino acid sequence produce a series of peptides of different amino acid sequence, which can be detected by MALDI-TOF-MS. The spectrum of identified peptide masses is unique for a specific protein, and is known as a "Mass Fingerprint" against databases of known protein sequence (e.g. SwissProt-TrEMBL). This process subsequently enables the identification of most proteins. The development of large biological databases based on sequence identification has been the major driver for the application of MALDI- TOF-MS and proteomics in general.

The analysis of the "Candidate Proteins" by "Mass Spectrophotometry (MS)" followed by "Peptide Mass Fingerprinting" analysis revealed corresponding genes to be involved in various pathways of the cellular metabolism. Our analysis has elucidated identities of the corresponding genes with respect to the

"Signature Proteins" implicated in the adaptation of the isolates to various acidic soil pH regimes. The "Proteome Maps" generated in our analysis pin-point to the genes which may be contributing towards imparting selective acid soil-tolerance amongst various *Rhizobium* isolates analyzed. The expression of selective genes, as depicted by our analysis suggest to the changes, which occur at the

cellular level towards imparting tolerance to the acidic soil pH regimes to various isolates. Through our analysis, we have been able to identify the candidate genes which are implicated / involved towards facilitating the adaptation of *Rhizobium* isolates towards the harsh environment, in this case, acidic-soil. Knowing the candidate genes involved in the adaptation processes, opens up avenues towards better understanding of the complex phenomenon of abiotic stress adaptation mechanisms in micro-organisms of agricultural importance.

Acknowledgements

Dr. Himanshu Dubey (Principal-Investigator, AINP-SBB, BAU, Ranchi Centre) great-fully acknowledges the funding support made available from the project entitled "All India Network Project On Soil Biodiversity-Biofertilizers (AINP-SBB)" by ICAR (New Delhi) (India) under which the research work was carried out.

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