PROTEOMICS OF PHANEROCHAETE CHRYSOSPORIUM UNDER HEAVY METAL STRESS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

VOLKAN YILDIRIM

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

BIOLOGY

OCTOBER 2014

Approval of the thesis:

PROTEOMICS OF PHANEROCHAETE CHRYSOSPORIUM UNDER HEAVY METAL STRESS

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ABSTRACT

PROTEOMICS OF PHANEROCHAETE CHRYSOSPORIUM UNDER HEAVY METAL STRESS

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October 2014, 156 pages

In this study, time-dependent heavy metal response of the white rot fungus *P. chrysosporium* was analyzed by using 2D-PAGE-MS for soluble cytosolic fraction and GeLC-MS approach for membrane enriched fraction.

After the 2D-PAGE-MS analysis, a total of 123 protein spots were detected as differentially expressed for Cu-exposed samples and 89 of them were identifed. Further analysis revealed that the 89 protein spots are the products of the 58 distinct ORFs. Overall, strongly up-regulated proteins included (i) Ras and NAC signalling, (ii) defense against oxidative damage and redox metabolism, (iii) translation and transcription and (iv) yet unknown function represented by putative proteins.

For the cadmium-exposed cells, a total of 130 spots were detected as differentially expressed after Cd exposure for 1h, 2h, 4h and 8h in *P. chrysosporium* proteome. MALDI-TOF analysis of these 130 protein spots identified 89 of them. Further analysis revealed that these 89 protein spots are the products of 58 distinct ORFs. Similar to Cu exposed samples, strongly up-regulated protein groups included (i) Ras and NAC signalling, (ii) defense against oxidative damage (iii) heat shock proteins (HSPs) and chaperons, (iv) transcription and carbohydrate metabolism and (v) poorly characterized hypothetical proteins.

Membrane proteins were successfully obtained by disrupting the cells with the help of lysing enzymes and enriched by employing PEG/Dextran aqueous two phase separation method. For quantification of the membrane enriched proteins, ^{15}N based SILAC method was utilized. GeLC-MS analysis of the Cu-exposed samples resulted in identification and quantification of more than 700 proteins for the Cu exposure of 2h, 4h and 8h. Subcellular location prediction analysis revealed that 20% of the identified proteins were plasma membrane proteins. Among the identified proteins, 83 were found to be as upregulated and 55 were found to be down-regulated. In addition to these, 32 proteins were only detected in Cu exposed samples and 18 of the proteins could not be detected after Cu treatment.

Key words: *Phanerochaete chrysosporium*, heavy metal stress, 2-DE, MALDI-TOF-MS, SILAC, geLC-MS/MS,

ÖZ

AĞIR METAL STRESİ ALTINDA PHANEROCHAETE CHRYSOSPORIUM'UN PROTEOMİĞİ

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Ekim 2013, 156 sayfa

Bu çalışmada, sitozolik proteome fraksiyonu için 2D-PAGE-MS ve membran proteinleriyle zenginleştirilmiş proteom fraksiyonu için GeLC-MS yöntemi kullanılaraki *P. chrysosporium*'un ağır metal yanıtı analiz edilmiştir.

2D-PAGE-MS analizinden sonra, Cu ile muamele edilmiş örneklerde toplam 123 proteinin farklı olarak ifade edildiği tespit edilmiş ve bunların 89 tanesi tanımlanabilmiştir. Tanımlanan bu 89 spotun 58 farklı genin ürünü olduğu belirlenmiştir. Genel olarak bakıldığında miktarınca önemli artış görülen protein gruplarının: (I) Ras ve NAC sinyal proteinlerini, (ii) redoks metabolizması ve oksidatif hasara karşı savunma mekanizması proteinlerini, (iii) translasyon ve transkripsiyon proteinlerini, ve (iv) fonksiyonu henüz belirlenememiş proteinleriiçerdiği belirlenmiştir.

Cd ile muamele edilmiş örneklerin 2D-PAGE-MS analizi ise toplam adet protein spotunun farklı olarak ifade edildğini göstermiştir. Bu 130 protein spotundan 89 tanesi tanımlanabilmiştir. Tanımlanan 89 spotun toplam 58 farklı gen ürününü temsil ettiği tespit edilmiştir. Cu ile muamele edilmiş örneklerden alınan sonuçlara benzer biçimde, Cd'ye maruz bırakılmış örneklerden tanımlanan ve miktarlarında artış görülen proteinlerin şu fonksiyonel gruplara ait oldukları görülmüştür: (i) Ras ve NAC sinyal proteinleri, (ii) anti-oksidan proteinleri, (iii) şaperon sıcaklık

şoku proteinleri, (iv) transkripsiyon ve karbohidrat metabolizması proteinleri ve (v) fonksiyonu henüz belirlenememiş olan proteinler.

Hücreleirn liziz enzimleri yardımıyla parçalanması ve iki fazlı PEG/Dextran ayrıştırma yönteminin kullanılması sayesinde membran proteinleri başarılı bir şekilde zenginleştirilmiştir. Membran proteinlerinin kantitasyonu için ise ^{15}N temelli SILAC yöntemi kullanılmıştır. 2, 4, ve 8 saat boyunca Cu'ya maruz bırakılmış örneklerin GeLC-MS yöntemiyle analizi sonrası 700'den fazla protein tanımlanmıştır. Hücre içi lokasyon analizi sonuçları, tanımlanabilen proteinlerin yaklaşık %20'sinin plasma membran proteini olduğunu göstermiştir.

Tanımlanmış proteinlerden 83 adedinin miktarının Cu etkisiyle arttığı, 55 tanesinin ise miktarının azaldığı görülmüştür. Buna ek olarak 32 adet protein sadece Cu'ya maruz bırakılmış örneklerde tespit edilirken, 18 adet protein ise sadece kontrol örneklerinde görülmüş ancak Cu'ya maruz kalmış örneklerde tespit edilememiştir.

Miktarında artış görülen proteinlerin dahil olduğu ana fonksiyonel gruplar: (i) enerji üretimi ve çevrimi proteinleri, (ii) hücre içi trafiği, sekresyon ve vesiküler taşıma proteinleri, (iii) posttranslasyonel modifikasyon proteinleri ve şaperonlar (iv) translasyon ve ribozomal biyogenez proteinleri ve (v) hipotetik proteinler olarak belirlenmiştir.

Sadece Cu etkisiyle ortaya çıkan, ancak Cu'ya maruz kalmamış kontrol örneklerinde belirlenemeyen protein grupları ise (i) hücre içi trafiği, sekresyon ve vesiküler taşıma proteinleri, (ii) sinyal transdüksiyon mekanizması proteinleri ve (iii) postranslasyonel modifikasyon proteinleri ve şaperonlar olarak belirlenmiştir.

Anahtar sözcükler: *Phanerochaete chrysosporium*, ağır metal stresi, 2-DE, MALDI-TOF-MS, SILAC, GeLC-MS/MS.

To my family
and
the phoenix on my shoulder

ACKNOWLEDGEMENTS

The writing of this dissertation has been one of the most significant academic challenges I have ever had to face. Without the support and help of the following people, this study would not have been completed. It is to them that I owe my deepest gratitude.

I would like to express my deepest gratitude and sincerest appreciation to Prof. Dr. Gülay Özcengiz who undertook to act as my supervisor despite her many other academic and professional commitments. Her supervision, invaluable help, continuous advice, patience and knowledge to the highest standards inspired and motivated me during this long process.

My special thanks go to Assoc. Prof. Dr. Servet Özcan for his encouragement, kindness, guidance, advices and for sharing all his work experiences with me.

I would like to express my great indebtedness to the people in the University of Greifswald Department of Microbiology, especially Andreas Otto, Martin Moche, Florian Bonn, Dr. Dirk Albrecht and Prof. Dr. Michael Hecker for their hospitality, kindness, endless help and making available all the equipment for MS analysis done in this work. Very special thanks go to Dr. Dörte Becher and Knut Büttner for their kindness, unique hospitality and invaluable help both at work and daily life during my stay in Germany.

I would like to acknowledge my former labmate and life time friend Dr. Burcu Tefon for her friendship, support and help during this long process. I thankfully acknowledge my former and current labmates Dr. Sezer Okay, Dr. Aslıhan Kurt, Çiğdem Yılmaz, Elif Tekin, Mustafa Demir, Mustafa Çiçek, Alper Mutlu, Eser Ünsaldı, İbrahim Sertdemir, Ayça Çırçır, İsmail Cem Yılmaz for their friendship and cooperation.

I fully appreciate the financial support which was granted in part by TUBİTAK-TBAG (project no.108T937) and Middle East Technical University Research Fund METU-09-11-DPT.2002K120510.

I express my warm thanks to my friends Muammer Sonsuz and Seçkin Doğaner for their sincere friendship and endless support. A very special thank goes to my beloved Derya Gürcan for her love, understanding, patience and help during this long process.

The last but not least, I would like to express my heartful gratitude to my mother Hatice Yıldırım, my father Ürfettin Yıldırım and my brother Atakan Yıldırım for supporting me and being there for me whenever I need throughout the my whole life.

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LIST OF ABBREVIATIONS

1DE	One Dimensional Electrophoresis
2D-PAGE-MS	Two Dimensional Polyacrylamide Gel Electrophoresis Mass Spectrometry
2DE	Two Dimensional Electrophoresis
CID	Collision Induced Dissociation
ESI	Electrospray Ionization
GeLC	Gel Electrophoresis Liquid Chromatography
IEF	Isoelectric Focusin
ITRAQ	Isobaric Tag for Relative and Absolute Quantitation
kDa	kilo Dalton
LC	Liquid Chromatography
LTQ	Linear Trap Quadrupole
MALDI-TOF	Matrix-assisted Laser Desorption Ionization Time of Flight
MS	Mass Spectrometry
MudPIT	Multidimensional Protein Identification Technique
ORF	Open Reading Frame
P. chrysosporium	*Phanerochaete chrysosporium*
pI	Isoelectric Point
PMF	Peptide Mass Fingerprint
PTM	Posttranslational Modification
ROS	Reactive Oxygen Species
RP	Reverse Phase
SCX	Strong Cation Exchange
SDS	Sodium Dodecyl Sulfate
SILAC	Stable Isotope Labeling with Amino acids in Culture

CHAPTER 1

INTRODUCTION

1.1. *Phanerochaete chrysosporium*

Since its discovery in 1974 (Burdsall and Eslyn, 1974), the white rot basidiomycete *Phanerochaete chrysosporium* has been subjected to many scientific work in order to understand its nature and investigate possible usage of the organism for industrial benefits. As of June 2014, a pubmed search for the "*Phanerochaete chrysosporium"* query results in 1399 publications. There are two main reasons which make this organism very interesting for the scientific world: Firstly, as a white rot fungus, the organism has a unique oxidative system and it could mineralize lignin completely and leaves the cellulose and hemicellulose as white crystals, which is the base for the name "white rot fungi". Because of this unique oxidative enzyme system, *P. chrysosporium* is very important for the carbon recycling in nature (Kirk and Farrell, 1987; Martinez, 2002). This system has been challenged for degradation of various xenobiotic compounds and dyes within many studies. Therefore, *P. chrysosporium* is one of the best-studied white-rot fungi shown to be very promising for treatment of phenolic effluents from pulp and paper, for coal conversion, and in the textile and olive oil industries (Pérez et al., 2002; Wesenberg et al., 2003). Secondly, the live and dead biomasses of *P. chrysosporium* were shown to have high metal bioaccumulating potential for treating industrial effluents and contaminated resources (Gopal et al., 2002; Pakshirajan and Swaminathan, 2009; Say et al., 2001; Yetis et al., 1998).

Taxonomy of the model wood decay fungus *Phanerochaete chrysosporium* was described by Burdsall and Eslyn (1974). The relevant classification of the organism is as follows:

Kingdom:	Fungi
Division:	Basidiomycota
Class:	Hymenomycetes
Subclass:	Homobasidiomycetes
Order:	Aphyllophorales
Family:	Corticiaceae
Genus:	Phanerochaete

The *P. chrysosporium* genome is organized in 10 chromosomes which is approximately 30 Mb in size. Sequencing of the organism's genome was accomplished by DOE Joint Genome Institute (Martinez et al., 2004) which constitutes the first completed basidiomycete genome project. Sequencing of the organism's genome resulted in a database including 10047 ORF, which was released as database v2.1.

1.2. Interactions of heavy metals with living organisms.

By definition, if an element has the specific gravity above five, it is called as heavy metal. However, the term “heavy metal” mostly refers to toxic metals, e.g., cadmium (Cd), copper (Cu), chromium (Cr), lead (Pb), zinc (Zn) as well as hazardous metalloids e.g., arsenic (As), boron (B), which causes negative effects on an organism's growth and development (Hossain et al., 2012).

1.2.1. Cadmium toxicity

One of the major environmental hazards is the contamination of soil and water by heavy metals. The highly toxic heavy metal cadmium (Cd) is one of these pollutants. Anthropogenic sources of the Cd are mining and industrial activities, land applications of sewage sludge, use of phosphorus fertilizers and atmospheric deposition (Toppi and Gabbrielli, 1999). Except that once it has been reported as an enzyme cofactor (Lane et al., 2005), Cd is a non-essential metal. For the last two decades Cd has been listed as number 7 in the Substance Priority List of Agency for Toxic Substances and Disease Registry (ATSDR). Based on data obtained from human occupational exposure, International Agency for Research

on Cancer (IARC) as a part of the World Health Organization, declared the Cd and Cd-containing compounds as group-1 human carcinogens. After the toxic effect of cadmium is recognized, necessary precautions are taken and the emission of the Cd in the air has been reducing due to improved technology for the production, use, and disposal of Cd and Cd-containing products (Moulis and Thévenod, 2010). However the world consumption of Cd by industries increases every year. In addition to that, as a chemical, Cd cannot be degraded and its concentration in the environment increases steadily.

Cd is a class B metal, it has a high affinity with sulfur ligands. It is supposed that its toxic effect can be explained by its strong interaction with sulfhydryl groups in proteins. Cd may also interfere with cellular homeostasis of several essential metals including Zn, Ca, Fe, Mg and Cu. Nonetheless, it has been reported that Cd exposure causes the production of reactive oxygen species (ROS) within the cell (Hassoun and Stohs, 1996). In addition to that, Cd also diminishes the main antioxidant compounds in the cells by inactivating enzymes and other antioxidant molecules (Filipic et al., 2006), which leads to lipid peroxidation (Sarkar et al., 1995).

1.2.2. Copper toxicity

Environmental Pollution Agency (EPA) sets the maximum contaminant level for Cu in drinking water as 1.3 mg Cu/l (Fitzgerald, 1998). In soil, Cu is found of an average concentration of 50 ppm (Barceloux, 1999). Atmospheric concentration of the copper is 5 to 20 ng Cu/m^3 (Barceloux, 1999). The main sources of environmental copper pollution are mining, smelting and refining of copper, industries producing copper based products such as wire, pipes and sheet metal, and fossil fuel combustion.

As being one of the essential elements, copper can catalyze redox reactions and it is a cofactor of many enzymes such as cytochrome C oxidase, Cu, Zn-superoxide dismutase (SOD), and several ATPases (Festa and Thiele, 2011). However,

exposure to excess Cu can be toxic or even lethal for biological systems, depending on the duration and/or amount of the exposure. Although several different mechanisms are suggested, generally the toxicity of the copper is directly linked to its ROS generation ability. Both cupric and cuprous Cu ions can participate in oxidation and reduction reactions. In the presence of superoxide (*O_2-) or any reducing agent like GSH, Cu^{2+} can be reduced to Cu^{+}. Then the reduced Cu^{+} can catalyze the formation of hydroxyl radicals (OH^{-}) from hydrogen peroxide (H_2O_2) via the Haber-Weiss reaction as it is depicted below (Bremner, 1998; Kadiiska et al., 1993).

$$^{\bullet}O_2^{-} + Cu^{2+} \rightarrow O_2 + Cu^{+}$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + OH^{-} + OH^{\bullet}$$

The hydroxyl radical is known to be the most powerful oxidizing radical and it can practically react with any biological molecule (Buettner, 1993).

1.3. Proteome and proteomics

The term proteome was first mentioned by Wilkins et al., (1996) and basically it can be defined as the proteins expressed by an organism in a certain time and space. While “time” refers to a specific developmental stage in the organism's life cycle, “space” could be a specific organ, organelle, tissue or the body fluid. Although an organism has one definitive genome, proteome of the organism is a dynamic entity which changes according to different environmental conditions. Proteomics is being used to define all the approaches together that investigate the proteomes in any state of the organisms. By the help of proteomics, one could get invaluable information about changes in protein synthesis and degradation rates, posttranslational modifications and protein-protein interactions (Han et al., 2008). Although the genomic technologies have been useful for revealing the abiotic stress response mechanisms, changes at transcriptomic level do not always reflect

the proteomic level (Gygi et al., 1999a).

A typical proteomic workflow includes the following steps: i) protein isolation from the sample organism/tissue, ii) fractionation and/or separation of the proteins, iii) tryptic digestion of the proteins to peptides, iv) measurement of the peptides by the help of a mass spectrometer and v) identification of the proteins by searching the databases. These steps are performed variously according to aim of the study and the chosen method for proteome analysis.

1.3.1. Classification of proteomic workflow and definitions

Proteomic studies can be classified as "*Gel-based*" and "*Non-gel-based*" approaches. While gel-based approaches employ the 1D or 2D gels for separation of the proteins, the non-gel-based approaches refer to liquid phase separation of the proteins/peptides. In most of the cases 1D-gel separated proteins are further separated by an HPLC system after tryptic digest. This setup is named as "*GeLC*" approach.

Proteomic approaches can also be classified as "*bottom-up*" or "*top-down*" methods. In top-down proteomics, intact protein entities are sprayed into the mass spectrometer by the help of ESI (Electro Spray ionization). The intact proteins are then fragmented within the mass spectrometer and the fragments are measured. On the other hand, in the bottom-up approach, proteins are separated by a separation method like gel electrophoresis and submitted to tryptic digestion (in-gel digestion). Resulting peptides are measured by a suitable MS (Mass Spectrometer) system (e.g. MALDI-ToF, LC-QToF). Alternatively, the crude protein extract can be digested without any protein separation step and then resulting peptides are separated by an HPLC system. The HPLC system is connected to an ESI-MS system and peptides are measured. This type protein identification is called as shotgun proteomics.

1.3.2. Sample preparation

1.3.2.1. Protein extraction for proteomics

Protein extraction and isolation is the first and the most crucial step in a proteomic study. Although the main aspects of the protein extraction process are common, there is not a simple de facto extraction method for every sample. Methods can differ according to aim of the study and the type of the organism. Protein extraction starts with the disruption of the cells. For most of the bacteria, sonication in a lysis buffer and french press are choice of the cell disruption (Eymann et al., 2007; Suvarna et al., 2000)). Plants and fungi have strong cell wall of cellulose and chitin respectively, therefore grinding under liquid nitrogen is being utilized mostly for these organisms (Yıldırım et al., 2011)). After discarding the debris the remaining supernatant is rich in cytosolic proteins. The proteins in the supernatant can be used directly or used after a concentration step such as TCA-acetone precipitation or phenol extraction (Özcan et al., 2007; Wolff et al., 2006). However if the target is the membrane-associated proteins some extra steps are needed to enrich the membrane proteins in the supernatant such as aqueous two phase extraction (Nühse et al., 2007), methanol/chloroform extraction (Rolland et al., 2006). Subfractination of the cells is another approach in which the cells are broken and target organelles such as mitochondria, chloroplast, nucleus etc. are extracted or enriched by the help of different protocols (Boisvert et al., 2010; Huber et al., 2003).

1.3.3. Protein separation for proteomics

To analyze, quantify and identify, proteins must be first separated based on intrinsic properties of proteins, e.g. molecular weight, isoelectric point, charge and polarity. The aim of the study mostly determines the choice of protein separation. 2DE based approaches are are still advantageous since they can be used to analyze phosphoproteomes. Moreover, while some proteins can't be detected by LC-MS based tecniques, they can be detected by 2DE-based approaches. However 2DE

approach is quite poor in analyzing membrane proteins since it is incompatible with hydrophobic proteins. The choice of separation also has an impact on the choice of MS system. Today's preotomic technologies are mainly based on two approaches: 2D gel separation followed by a MALDI-MS analysis and liquid chromatography based separation followed by an ESI-MS analysis based approach. Besides these methods, use of GeLC approach has also been emerging within the last decade.

Membrane proteins are special interests of many studies owing to being at the interfacing position between the environment and the cell. Main functions of the membrane proteins are to regulate the imports and exports of the information, ions, and metabolites between the cell and the surrounding and all these functions are accomplished by membrane proteins. However analyzing membrane proteins are one of the major challenges of proteomic studies. Majority of the problems arise from difficulty of the extraction of membrane proteins. Accordingly low recovery rates, protein hydrophobicity and contamination from other organelles are the major problems of the membrane protein extraction. Traditionally plasma membrane (PM) is enriched by lysing the cell and afterwards employing a series of gradient centrifugation steps. Simply, cells are collected and disrupted via different methods (e.g. sonication, glass bead homogenization, dounce homogenization) then to improve purity and solubility of plasma membrane proteins different methods are utilized such as high pH carbonate precipitation, high salt precipitation and addition of detergents like Triton-X to the extraction buffers. Afterwards partially pure PM proteins are centrifuged in a density gradient which is established by using sucrose. Finally the fractions including the PM proteins are collected, pooled and pelleted (Helbig et al., 2010; Orsburn, 2011). Alternatively membrane proteins can be enriched by using two-phase aqueous solution systems. In this system, a solution having both hydrophobic and hydrophilic phases is prepared. When the crude membrane extraction is mixed with this solution, while hydrophobic proteins (membrane proteins) tend to stay at the hydrophobic phase, hydrophilic ones tend to stay at hydrophilic phase. Most

commonly used two-phase system is PEG/Dextran mixture. In addition to that, use of certain detergents (e.g. Triton X-100, Tween 80, Nonident P40) in the two-phase systems increases the membrane protein solubility.

1.3.3.1. Gel-based protein separations

The very early steps of the proteomics start with the introduction of 2D gel electrophoresis technique (2DE). 2DE is a method in which the proteins are separated in sequential two steps: IEF and SDS-PAGE (O'Farrell, 1975). In IEF (IsoElectric Focusing) as a first dimension, proteins are separated electrophoretically according to their isoelectric points (*pI*) in a denaturing environment which keeps charges on the proteins untouched. The second dimension of the 2DE technique is a standard Laemli SDS-PAGE (Laemli, 1970). After the IEF run gel is put on SDS-PAGE horizontally. When SDS-PAGE starts, proteins migrate from IEF gel to SDS gel where they are further separated according to their molecular weights. The IEF technique, in the form it was discovered by Farrell et al. (1975), had a poor reproducibility, in spite of the fact that it provides a superior protein resolution. However, immobilizing the carrier ampholites by crosslinking them to the acrylamide gel surpassed the reproducibility problems to a large extent (Görg et al., 1988) .

1.3.3.2. Liquid chromatography based (LC) based protein separation

Liquid chromatography is a powerful method to separate complex protein/peptide mixtures. By using the method, proteins can be separated according to their intrinsic properties such as affinity (affinity chromatography), ionic charge (ion-exchange chromatography), hydrophobicity (reverse phase chromatography), and size (size exclusion chromatography). LC separation is especially important for proteomic research in separation of the hydrophobic proteins since the IEF method has some compatibility issues with the hydrophobic proteins, such as precipitation and low solubilization (Speers and Wu, 2007). As an advanced LC technique, the high pressure liquid chromatography is directly coupled with ESI-

MS systems. Today the nano-HPLC is a standard method for several proteomic studies (Breci et al., 2005; Hwang et al., 2009; Percy et al., 2013; Yates et al., 2009). Reverse phase (RP) columns are the most used LC columns in proteomic studies. Lack of salt in the buffer systems and relatively higher separation power makes the RP columns most preferable. An RP column composed of C8 or C18 alkyl groups which are attached to 3-5 μm diameter silica beads. Because of these nonpolar alkyl groups, the more nonpolar the protein causes the more delayed exit from the column. If the diameter of the silica beads are decreased (less than 2 μm), a better resolution can be obtained. However this kind of setup needs higher pressures and the system is called as Ultra High Pressure Liquid Chromatography (UPLC) (MacNair et al., 1997). Because of its high resolution power, studies employing this method have been emerged recently (Hahne et al., 2010; Percy et al., 2013; Want et al., 2010).

1.3.3.3. Multidimensional LC separation

Although the UPLC separation is a powerful method, its separation is not sufficient for highly complex samples, which can include a thousands of proteins or peptides. Two or more separation techniques are combined to obtain a better separation results for this highly complex samples. This approach is named as Multidimensional protein identification technique (MudPIT) (Washburn et al., 2001; Wolters et al., 2001). In MudPIT each individual separation method uses different molecular property of the protein to separate them. In one of the first attempts of this approach, Opiteck et al. (1997) successfully identified *Escherichia coli* proteins by coupling a strong cation exchange column (SCX) as the first dimension and an RP column as the second dimension. This conventional setup was improved by Motoyama et al. (2006), when they obtained higher sensitivity and doubled the protein coverage by using the UPLC chromatography in the multidimensional separation approach.

1.3.3.4. GeLC method

GeLC is a technique in which 1D gel electrophoresis is coupled with a liquid chromatographic separation. In GeLC, the separation by 1-D GE is employed as a first dimension and as a second dimension a reversed-phase chromatographic separation is used. In this method, proteins are separated by 1-D GE, visualized in the gel and then each lane in the gel is cut into 10-12 equal pieces. Following the gel cut, an in-gel digestion procedure is applied to each of the gel pieces. Afterwards peptides are extracted and measured by reversed-phase nanoLC-MS and MS/MS. In many reports GeLC was proved to be superior to mudPIT and other techniques in terms of number of identified proteins (Breci et al., 2005; de Godoy et al., 2006). It is speculated that by using GeLC up-to 5-fold higher number of proteins could be identified (de Godoy et al., 2006). Since the majority of the protein content in a crude protein extract is represented by a small group of housekeeping proteins, identifying lower abundance proteins is a difficulty. This problem is overcome by pre-fractionating the sample on the protein level and effectively realized with 1D gel electrophoresis (Nesatyy and Suter, 2007).

1.4. Mass spectrometry analysis

The first step for the invention of the mass spectrometry was the discovery of an electron in a cathode tube by JJ. Thomson in 1913 (Thomson, 1913). A mass spectrometer is a device in which the mass/charge ratio (m/z) of a molecule can be measured so that the total mass of the molecule could be interpreted. Typically a mass spectrometer composed of three main parts: an ionization source, a mass analyzer and a detector (Fig. 1.1). The molecules to be analyzed in an MS should be in gas-phase first. This gas-phase transition is achieved by the ionization source. Once the molecules switched to the gas-phase and ionized they run through the analyzer in which by the help of electric field, magnetic field and radio frequencies they are separated and filtered. Those molecules are detected at the detector and each one is assigned with an m/z value.

The MS analysis was not suitable for the biological molecules until the discovery of the soft ionization techniques like MALDI (Matrix Assisted Laser Desorption Ionization; Tanaka et al., 1988) and ESI (Electro Spray Ionization; Yamashita and Fenn, 1984). While MALDI is suitable for solid samples, ESI is suitable for the samples dissolved in different solvents. MALDI or ESI sources could be coupled with different types of analyzers like ToF (Time of Flight), LTQ (Linear Trap Quadrupole), Orbitrap and Quadrupole to achieve various types of MS analysis having different sensitivity and precision.

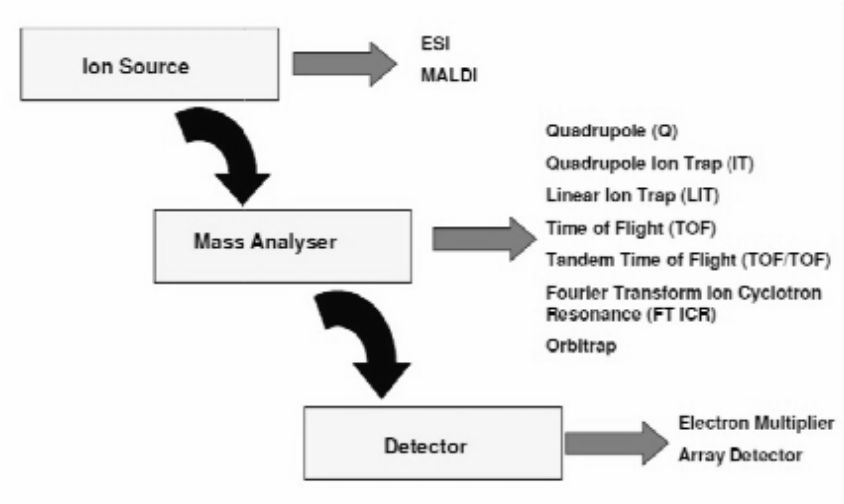

Figure 1.1. Simple representation of a mass spectrometer **(Graham et al., 2007).**

1.4.1. Matrix assisted laser desorption ionization (MALDI)

Matrix Assisted Laser Desorption Ionization technique requires the sample is co-crystalized with a matrix. There are three most commonly used matrices today:

i) 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid),

ii) α-cyano-4- hydroxycinnamic acid (alpha-cyano or alpha-matrix) and

iii) 2,5- dihydroxybenzoic acid (DHB).

Practically one of the above matrix compound is dissolved in 1:1 acetonitrile:water mixture and then it is mixed with the sample and spotted on a

MALDI sample target as droplets, either manually or by using an automated spotter. The co-crystallized sample/matrix mixture is beamed with a UV laser, and sample molecules got volatile and protonated because of the matrix. Theoretically, it is thought that when the laser hits, firstly the matrix is desorbed and ionized and then transfers a proton to the analytes (e.g., protein molecules), thus charging the analyte (Knochenmuss, 2006). MALDI system have a mass range of 1-300kDa, which is quite high. Thus it has been proved to be method of choice for the identification of biomolecules in complex samples, including peptides, proteins, oligosaccharides and oligonucleotides. (Marvin et al., 2003).

1.4.2. Electro spray ionization (ESI)

Electro spray ionization technique was first introduced by Dole et al. (1968). However it was not used until 1984 for biomolecules (Yamashita and Fenn, 1984). In an ESI devices, the liquid sample coming from an LC system is sprayed through a 1µm diameter steel or silica needle, by the help of a nebulizer gas and it is turned into a droplet nebula. Each droplet in the nebula includes both the solvent and the molecules to be analyzed. After the spraying, the solvent in the droplets starts to evaporate and at a certain point droplets explode because of the "Coulomb" effect (Figure 1.2) (Iribarne and Thomson, 1976). When the ions switch to the gas-phase they move towards the detector because of the applied voltage. Finally all the m/z values each molecule's is detected, recorded and the chart showing all the m/z values with its respective intensity is called as mass spectrum.

The mass spectrum of each protein species is unique and because of this uniqueness it is called as peptide mass fingerprint (PMF) (Pappin et al., 1993). PMFs are searched in a database by the help of some softwares like MASCOT® or SEQUEST® to find the identity of a given protein. However PMF data doesn't include amino acid sequences but only the masses of the peptides. The success rate of the PMF matches to the database can be improved by adding the amino acid sequence data which is obtained by CID mechanism by which the gas-phase

molecule ions are further fragmented by colliding neutral molecules such as helium, argon, nitrogen. By the help of CID from the fragments of the peptides, amino acid sequences could be predicted. Together the PMF data and the sequence information results in higher protein identification rates. To acquire a CID data an MS system has to have two mass analyzer divided by a collision induction cell and this type of MS systems are called as MS/MS or tandem MS (Wells and McLuckey, 2005).

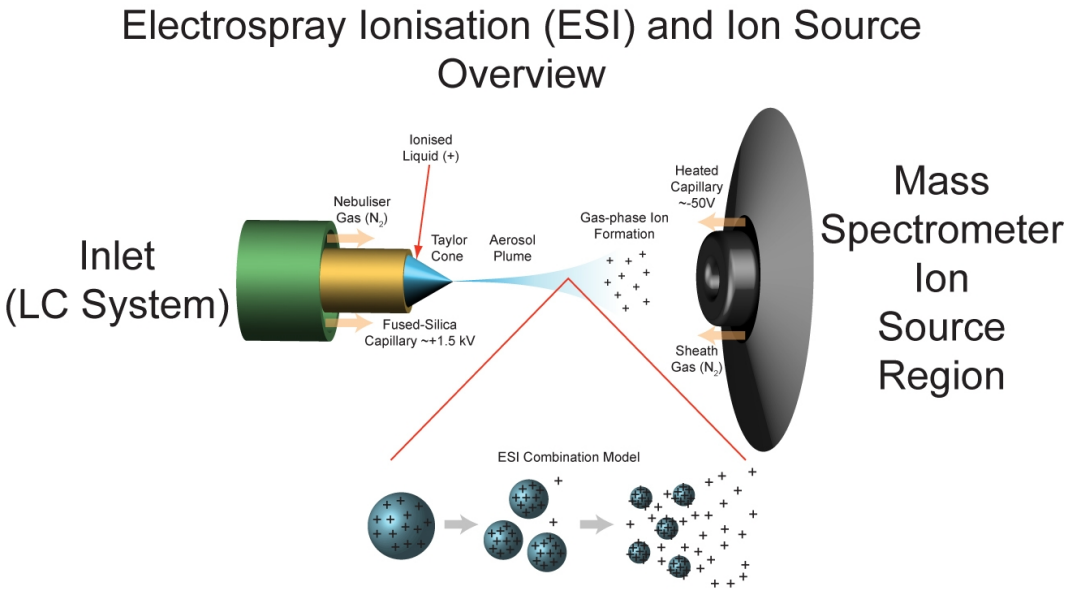

Figure 1.2. Scheme of ESI ion source and its working principle. (Courtesy of Lamondlab.com)

1.4.3. Mass analyzers

Quadrupole, Time of Flight (ToF), Lineer ion trap(LTQ), Quadrupole ion trap (QIT), Fourier Transform-Ion Cyclotron Resonance (FT-ICR), and the Orbital ion trap (Orbitrap) are the most known mass analysers used in the proteomic studies. Comparison of the different analyzers are tabulated at Table 1.1.

1.4.3.1. Quadrupole mass analyzer

A quadrupole analyzer is composed of four axial rods positioned parallel to the flight path of the ions. Of these four rods the neighboring rods have the opposite

signs and the diagonal opposites have the same sign electric poles. If a direct current (DC) and a radio frequency (RF) is applied to the rods, the ions passing through the quadrupole could be filtered according to the magnitude of the electric field created within the analyzer. By adjusting the RF, ions having various m/z values could be focused on the detector and a mass spectrum could be established (Paul and Steinwedel, 1960).

1.4.3.2. 3D ion trap

A paul trap or a 3D ion trap is made-up with two hyperbolic electrode having the hyperboles turned to each other and another ring electrode placed between them. Because of the applied DC and RF fields, when the ions get into the 3D trap, they start to oscillate and can't go out. By adjusting the DC and RF fields a spectrum of ions could go out the trap and establish the mass spectra (Paul and Steinwedel, 1960).

1.4.3.3. Lineer ion trap (LIT)

The structure of the LIT is almost identical to the quadrupole mass analyzer. However the tips of the axial rods are separated and have an opposite sign of the middle parts, so that an ion can be trapped within the created electrical field. Therefore, by adjusting magnitude of the electric field, ions can be filtered and a mass spectra could be obtained (Schwartz et al., 2002).

1.4.3.4. Orbital ion trap(Orbitrap)

The orbitrap technology is rather new in comparison to above mentioned mass analyzers. The orbitrap was first developed by Makarov (2000) and it was started to be used for proteomic purposes by Hu et al. (2005). Basically an orbitrap is composed of two cylindric shaped electrodes, one is hollow and big, and the other one passes through the big one at the same axial center. When an ion enters the electrical field created within this chamber at a perpendicular angle to the axial center it starts to a circular movement on the orbit of the inner electrode. The sum

of the forces from electrical field and centrifugal movement keeps the ions on the orbit, so are trapped (Figure 1.3)

1.4.3.5. Fourier Transform-Ion Cyclotron Resonans (FT-ICR)

In an FT-ICR mass analyzer both electrical field and magnetic field is employed to analyze molecules. While the ions are in the magnetic field, because of the applied electrical field, the ions started to oscillate in the chamber. This oscillation is called as "Ion Cyclotron Resonans" (ICR). This ICR could be measured and converted to m/z values with the help of "Fourier Transform", which in turn gives the mass spectra (Marshall et al., 1998).

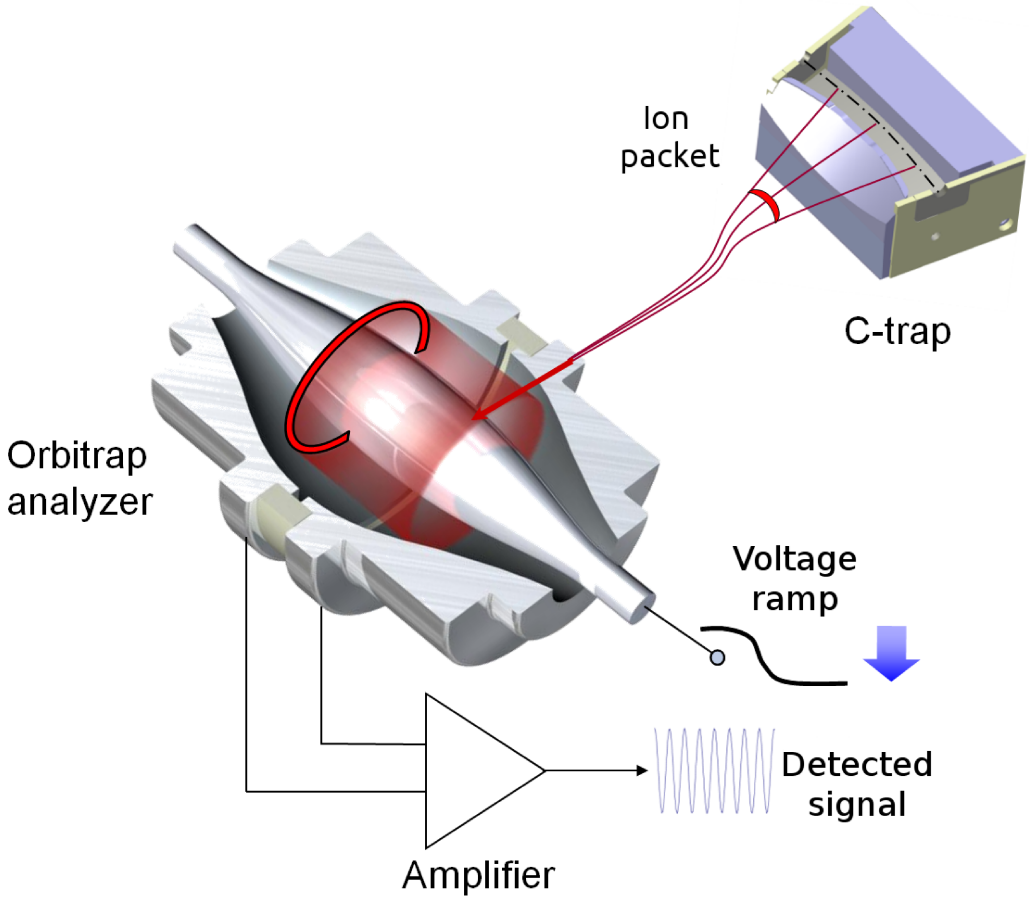

Figure 1.3. Representative scheme of an orbitrap analyzer (Courtesy of Thermo Fisher Scientific).

1.4.4. Hybrid analyzers and MS/MS analysis

Each of the mass analyzers used in an MS system has its own advantages and drawbacks. By using these analyzers in tandem, these drawbacks could be overcome and the MS system becomes more powerful with combined advantages. Moreover, by using two analyzers together it is possible to get an MS/MS spectra which leads to better protein identification rates (Glish and Burinsky, 2008). In such a system two of the analyzers are serially linked and mostly collision induction cell is placed between them (Figure 1.4). Some of the commonly used hybrid analyzers are ToF/ToF, Quadrupole-ToF, triple quoadrupole, Q-trap and Linear ion trap-orbitrap.

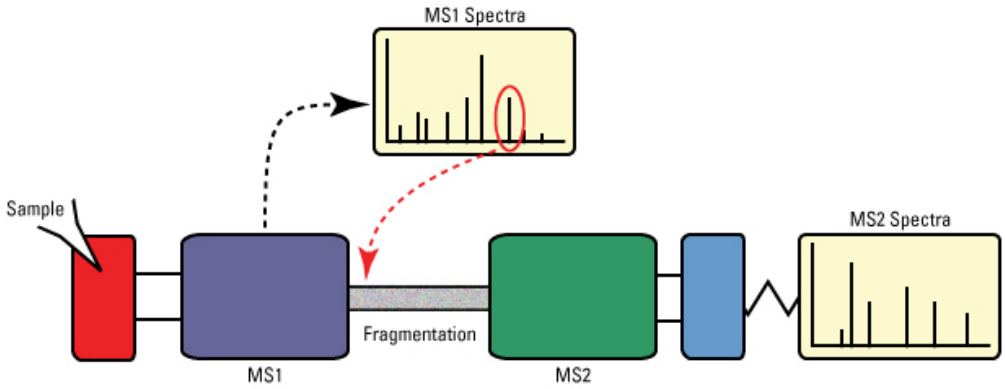

Figure 1.4. Diagram of tandem mass spectrometry (MS/MS) (Courtesy of Thermo Scientific http://www.piercenet.com/method/overview-mass-spectrometry).

1.5. Quantification methods in proteomics

After the genomic revolution, researchers could explain the biological processes and systems by comparing the gene expression patterns obtained from cells or tissues of different states. For such a comparison, a precise measurement of the gene expression is essential. The method of choice for gene expression measurement differs according to the essence of the study. For instance, in genomic studies the most known gene expression profiling method is the microarray technology (Kawasaki et al., 2001; Yasokawa et al., 2008). However in proteomics, measurement of the abundance of individual protein species can be

achieved via measuring the spot intensity on the 2DE gels, counting the peptides in MS results or comparing the intensities of isotope labeled peptides on the MS spectra.

1.5.1. Quantification by measuring spot intensities on 2DE

Magnitude of the spot intensities on a 2DE gel gives sufficient information about the expression level of the individual protein species. After the gel run, proteins on a 2DE gel is fixed and stained with different protein specific stains such as Coomassie blue, silver stain or the sensitive florescence stains emerged within the last decade. The choice of the stain depends on the required sensitivity of the study, MS compatibility and economical reasons. For example, the silver stain is a quite sensitive stain but it has a poor compatibility with MS and its spot density depends on the time spent for development. Coomasie blue staining has a better reproducibility and MS compatibility but has a poor sensitivity in comparison to silver staining. On the other side, the commercially available florescent dyes are (e.g. SYPRO® Ruby, Flamingo™, Lava purple, Krypton) MS compatible, highly sensitive and reproducible, but they are not as cost efficient as coomassie or silver staining.

After the gel is stained, its image is digitalized by using a scanner or a digital camera and the obtained image is analyzed by specific image analysis software. Decodon's Delta2D, Bio-Rad's PDQuest and GE Healthcare's ImageMaster can be listed as most popular 2DE image analysis softwares. These softwares can measure each spot's intensity and can make comparisons between the 2DE gels. By this way protein expression profiles can be obtained for different conditions.

Table 1.1. Comparison of performance characteristics of commonly used mass spectrometers for proteomics (Han et al., 2008)

Instrument	Mass resolution	Mass accuracy	Sensitivity	m/z range	Scan rate	Dynamic range	MS/MS capability	Ion Source	Main applications
QIT	1000[a]	100–1000 ppm	picomole	50–2000; 200–4000	moderate	1 E3	MS^n [d]	ESI	protein identification of low complex samples; PTM identification
LTQ	2000[a]	100–500 ppm	femtomole	50–2000; 200–4000	fast	1 E4	MS^n [d]	ESI	high throughput large scale protein identification from complex peptide mixtures by on-line LC-MS^n; PTM identification
Q-q-Q	1,000	100–1000 ppm	attomole to femtomole	10–4000	moderate	6 E6	MS/MS	ESI	quantification in selected reaction monitoring (SRM) mode; PTM detection in precursor ion and neutral loss scanning modes
Q-q-LIT	2000[a]	100–500 ppm	femtomole	5–2800	fast	4 E6	MS^n [d]	ESI	quantification in selected reaction monitoring (SRM) mode; PTM detection in precursor ion and neutral loss scanning modes
TOF	10,000–20,000	10–20 ppm[b]; <5 ppm[c]	femtomole	no upper limit	fast	1 E4	n/a[e]	MALDI	protein identification from in-gel digestion of gel separated protein band by peptide mass fingerprinting
TOF-TOF	10,000–20,000	10–20 ppm[b]; <5 ppm[c]	femtomole	no upper limit	fast	1 E4	MS/MS	MALDI	protein identification from in-gel digestion of gel separated protein band by peptide mass fingerprinting or sequence tagging via CID MS/MS.
Q-q-TOF	10,000–20,000	10–20 ppm[b]; <5 ppm[c]	femtomole	no upper limit	moderate to fast	1 E4	MS/MS	MALDI; ESI	protein identification from complex peptide mixtures; intact protein analysis; PTM identification
FTICR	50,000–750,000	<2 ppm	femtomole	50–2000; 200–4000	slow	1 E3	MS^n [d]	ESI; MALDI	top-down proteomics; high mass accuracy PTM characterization
LTQ-Orbitrap	30,000–100,000	<5 ppm	femtomole	50–2000; 200–4000	moderate to fast	4 E3	MS^n [d]	ESI; MALDI	top-down proteomics; high mass accuracy PTM charaterization; protein identification from complex peptide mixtures; quantification

[a] mass resolution achieved at normal scan rate; highesr resolution achievable at slower scan rate
[b] with external calibration
[c] with internal calibration
[d] n>2, up to 13
[e] fragmentation acheivable by post-source-decay

1.5.2. Mass spectra based quantification

An MS based quantification could be achieved either by label free peptides or isotopically labeled peptides. In the label free method, the number of identical identified peptides (e.g. how many times the same peptide is identified) are counted and by using a formula, an RSC (Relative spectral count) value is calculated to obtain protein expression profiles (Beissbarth et al., 2004).

The principle of the isotope labeling approach is the two different isotopes of a molecule/peptide having the same LC retention times, however having a different m/z values in the MS spectra. Therefore if the same proteins/peptides coming from two different samples (each of them labeled with two distinct isotopes) are analyzed simultaneously via MS, they could be differentiated and their intensities on the MS spectra can be used as a representative figure of protein expression. Some of the isotopic labeling methods are discussed below.

1.5.2.1. SILAC (Stable Isotope Labeling with Amino acids in Culture)

This method is also named as metabolic labeling since the culture media includes one of the heavy ^{15}N, ^{13}C, ^{18}O isotopes as amino acids or mineral salts. Therefore the isotopes are incorporated into the proteins metabolically. This method was first used to quantify proteomes of model organisms such as *Saccharomyces cerevisiae* and *Deinococcus radiodurans* (Conrads et al., 2001; Oda et al., 1999; Washburn et al., 2003). In the cultivation step, organism is cultured in two separate media simultaneously, one containing the light isotope and the other containing the heavy isotope for each experimental condition. After the cultivation and protein isolation the protein extract from both media is mixed with 1:1 ratio and the analysis procedure goes on as usual. The resulting mass spectra is analyzed by a software which can differentiate between “heavy and light” spectra and calculates the relative intensities. Census (Park and Yates, 2010) is such software which can analyze the SILAC data. The main drawback of the approach is to feed the large animals (e.g. mammals) with the heavy isotope only food,

which increases the cost of the approach and makes it difficult to apply.

1.5.2.2. ITRAQ (Isobaric Tag for Relative and Absolute Quantitation)

Isobaric tagging is a peptide labelling method in which proteins are labeled with specific tags that are chemically identical but distinguishable by MS/MS due to their distinct fragmentation pattern. The most commonly used tags are N-hydroxysuccinimidyl esters which are linked to primary amino groups of the peptide chains. For instance N-methylpiperazine based 4-plex iTRAQ reagents generate ions of 114, 115, 116 and 117 m/z ratios. By comparing the relative intensities of these peaks on the mass spectra gives the information about relative abundancies of the respective sample (Ross et al., 2004). By comparing relative abundancy of two samples or as Quaglia et al. (2008) reported, by using synthetic peptides as standard, an absolute quantification can be achieved. Since the labelling is done at the protein level, it can be employed in any kind of study regardless of the type of the organism. However cost of iTRAQ reagents is still considerably high and only the peptides fragmented in CID are considered in quantification. Therefore the unselected peptides for MS/MS are missed which results in low proteome coverage.

1.5.2.3. ICAT(Isotope Coded Affinity Tags)

ICAT is known to be the first robust and universal approach in which the proteins/peptides are differentially labeled for quantitative LC-MS. The system is based on modification of the thiol groups on the cysteins by using isotopic iodoacetamide tags. In the first report mentioning ICAT usage, researchers successfully identified and quantified proteins of yeast growing either on galactose or ethanol in a single analysis (Gygi et al., 1999b). As in the iTRAQ technique, since there is no need to add isotopic tags to the culture media, the technique is quite suitable for a wide range of applications. However, this labeling with tags can also negatively affect the sake of the experiments and can cause a lower coverage of the proteome.

1.6. Physiological proteome studies for abiotic stresses

Abiotic stresses like cold, heat, drought, oxidative stress, some toxic chemicals and heavy metals cause some alterations in the organism's metabolism. Currently, proteomics is the method of choice to investigate physiological responses of the organisms to these abiotic stresses at the protein level. The model plant *Arabidopsis thaliana* was investigated for proteome changes in response to cold stress by utilizing the 2DE based DIGE method (Amme et al., 2006). Researchers successfully identified that 22 proteins were differentially expressed at both 6 °C and 10 °C degrees. 18 of the identified proteins found to be upregulated. An RNA binding protein CP29, three dehydrin proteins and a low temperature induced protein were among the upregulated proteins at 6 °C.

Oxidative stress response were also studied by exposing the human opportunistic pathogen *Candida albicans* to hydrogen peroxide and thiol oxidizing agent diamide (Kusch et al., 2007). The authors revealed that 21 proteins were upregulated in response to the both stressors. Antioxidant enzymes like catalase or thioredoxin reductase and a set of oxidoreductases were especially the proteins of interest among the identified proteins.

Salt basically causes an osmotic stress on the organism when it is in high concentrations. Direct targets of the high concentration of salt in the environment are the microorganisms and the plants. Therefore proteomic salt stress response studies focus on microorganisms and plants. For instance a 2DE based physiological proteomics study was performed on *Bacillus subtilis* in response to salt stress (Höper et al., 2006). Authors have revealed that synthesis of the vegetative proteins is decreased just after the salt stress application. However the synthesis rate increased again once the organism adapted to salt stress. In addition, these authors showed that intracellular levels of the majority of the enzymes functioning in the glucose metabolism remained constant during the salt stress. In that study, it is proved that salt stress also triggers some general stress response mechanisms such as σ^B stress regulon. In another study, three weeks old rice

seedlings were exposed to salt stress for 24h, 48h and 72 hours (Yan et al., 2005). According to this study, 1100 protein spots were detected on the 2DE gels and the authors could identify ten proteins as upregulated in response to salt. Among these proteins, four of them were previously shown to be salt responsive and six of them were found to be novel salt responsive proteins. The authors claimed that these findings can provide a base for future investigations of the functional genetics studies.

A comprehensive 2D gel based proteomics study was accomplished to investigate salt, cadmium and peroxide stress responses in *Candida albicans*(Yin et al., 2009). A total of 47 spots were found to be upregulated in response to salt. Functional classes carbohydrate metabolism, protein folding and degradation, amino acid synthesis, were major groups represented in these 47 spots. Especially HSP proteins and carbohydrate metabolism proteins were prominent. Peroxide stress caused upregulation of 39 proteins spots. Only three of them were identified as known redox protection proteins. Additionally, nine putative chaperone, six translation related protein and three proteolysis proteins were also among the identified proteins in response to H_2O_2. Cadmium exposure resulted in 119 upregulated protein spots on the 2D gels. The major functional category among the cadmium-induced proteins were carbohydrate metabolism especially the enzymes of glycolysis. Two of the pentose phosphate pathway protein were also upregulated, which may resulted in overproduction of NADPH to reduce the key antioxidants. In addition to that 12 of these protein spots were also found to be upregulated in peroxide stress, which suggests that there is an overlapping response mechanism.

1.6.1. Proteomics of heavy metal stress responses

As a natural habitant in the soil, bacteria and fungi are the first components of the biota affected by heavy metals (Nesatyy and Suter, 2007). To understand the impact of the heavy metals on the cellular physiology, proteomics has been of a great interest for the last decade. For instance, proteome of the metal resistant

bacteria *Ralstonia metallidurans* was studied in its response to zinc (Noël-Georis et al., 2004). As their preliminary findings the authors found three upregulated spots and two downregulated spots on the 2DE gels. Upregulated spots were identified as a product of a single ORF named as CzcB which belongs to Copper-zinc-cobalt response operon. Additionally the two downregulated protein spots were also found to be expressed from the same ORF, with no function could be assigned.

Thomson et al. (2010) revealed a core molecular response to acute chromate exposure in *Pseudomonas putida.* According to the authors the response of the organism to chromate depends on the composition of the medium. When the cells were grown on LB, secondary metabolite biosynthesis and catabolism, inorganic ion transport and amino acid metabolism proteins were found to be upregulated**.** By contrast, cells grown on defined minimal medium responded the chromate exposure by upregulating the proteins involving in the cell envelope biogenesis, inorganic ion transport, and motility.

Vido et al. (2001) exposed the yeast *Saccharomyces cerevisiae* to Cd and reported the proteome changes. The authors identified 54 induced and 43 repressed proteins. They also observed an increase in the glutathione synthesis and antioxidant proteins. In addition to that, proteins depending on Yap1p transactivator were also found to be upregulated including thioredoxin and thioredoxin reductase. In this report, it was indicated that the two cellular thiol redox systems, glutathione and thioredoxin are essential for Cd defense mechanism.

To reveal heavy metal-plant interactions, the model organism *Arabidopsis thaliana* has been subjected to many investigations. Cadmium response of *A. thaliana* was reported by Roth et al. (2006). A 2DE approach was utilized in the study and the authors could identify 25 spots representing 17 genes differentially expressed. Some of the significant proteins among them were ATP sulphurylase, glycine hydroxymethyltransferase, trehalose-6-phosphate phosphatase and

glutathione-S-transferases, which are mostly stress response related proteins.

The yeast *Schizosaccharomyces pombe* was also subjected to a heavy metal response study (Bae and Chen, 2004). The organism's proteome after Cd exposure was analyzed by employing the SILAC method. The authors could identify 1133 proteins, of which 106 upregulated and 55 downregulated in response to Cd. The major class among the upregulated proteins was constituted by the proteins of protein biosynthesis. The other significant groups of upregulated proteins included oxygen and radical detoxification, heat shock response, and other stress responsive proteins. They also found that different isozymes of glutathione-S-transferase are upregulated. Authors also suggested that *S. pombe* produces a large amount of inorganic sulfide to immobilize cellular Cd^{2+} as a form of CdS nanocrystallites.

The ascomycete plant pathogen *Botrytis cinerea* was challenged with copper, zinc, nickel or cadmium heavy metals and the secretome of the organism was analysed by 2DE approach (Cherrad et al., 2012). The authors detected 116 differentially expressed spots in response to at least one of the heavy metals. Fifty five of these proteins were identified and majority of them were the members of oxidoreductases, proteases, allergen-like proteins and the proteins with unknown funtions. Additionally, 31 of these proteins found to be coded with genes having a pH responsive PacC/Rim101 promotor region, which implies a link between pH regulation and metal response.

Yeast (*S. cerevisiae*) mitochondrial proteins were investigated under copper stress by the help of 2DE approach (Banci et al., 2011). A total of 90 protein spots were detected and analyzed in this study. Investigation of proteomic data revealed that mitochondrial proteins were diminished and oxidative stress response proteins were activated. The functional categories of these identified proteins included the main eukaryotic biological processes like protein synthesis, translocation and folding, cellular respiration, amino acids/pyruvate/ethanol metabolism, metal trafficking and homeostasis and oxidative stress response.

A yeast pathogen *Trichosporon asahii* was exposed to sodium arsenite ($NaAsO_2$) and cadmium ($CdCl_2$) (Ilyas et al., 2014). 2D gel analysis of the arsenite exposed samples revealed upregulation of 3-isopropylmalate dehydrogenase, phospholipase B, alanine-glyoxylate aminotransferase, ATP synthase alpha chain, 20S proteasome beta-type subunit Pre3p proteins and 4 additional hypothetical proteins. Authors also found extensive proteomic alterations in response to arsenate, especially the proteins of amino acid metabolism and, protein folding and degradation were found to be affected.

The pigmented yeast *Rhodotorula mucilaginosa* were exposed to Cu stress for 48 h and proteome of the organism was investigated by the help of 2D gel electrophoresis (Irazusta et al., 2012). A total of 16 spots were detected as upregulated in response to Cu stress. 10 of these 16 proteins were identified as heat shock proteins (HSP) andSOD, beta glucosidase and a methyl transferase were among the other upregulated proteins. The authors concluded that *R. mucilaginosa* uses HSP proteins as the main resistance mechanism against Cu stress. In addition, an antioxidant protein (SOD) and a methyl transferase are also the part of the response mechanism to counteract Cu originated ROS.

In a study that our group has conducted, a 2D map of the *P. chrysosporium* was constructed for the first time and some preliminary findings about the Cu and Cd response was reported (Özcan et al., 2007). In the 2D map a total of 517 spots representing the 314 distinct ORFs, could be identified. Of the 314 ORFs, 118 yielded multiple spots on the master gel. According to KOGG classifications, the functional class of PTMs, protein turnover and chaperones was represented with the highest number (63) of the identified ORFs. The reference map was efficiently used in the identification of the proteins differentially expressed under cadmium and copper stress. Three new ribosomal proteins as well as zinc-containing alcohol dehydrogenase, glucose-6-phosphate isomerase, flavonol/cinnamoyl-CoA reductase, H1-transporting two-sector ATPase, ribosomal protein S7, ribosomal protein S21e, elongation factor EF-1 alpha subunit were demonstrated as the most strongly induced.

Pb response of the *P. chrysosporium* was also investigated by our group (Yıldırım et al., 2011). The organism was subjected to different doses (25, 50 and 100 μM) of Pb (II) and proteome changes were characterized by 2DE in combination with MALDI-TOF-MS. A total of 14 up-regulated and 21 down-regulated proteins were identified upon the analysis. The induction of an isoform of glyceraldehyde 3-phosphate dehydrogenase, alcohol dehydrogenase class V, mRNA splicing factor, ATP-dependent RNA helicase, thioredoxin reductase and actin required a Pb (II) dose of at least 50 μM. Besides, proteome dynamics of mid-exponential phase cells of *P. chrysosporium* subjected to 50 μM lead was also investigated at exposure times of 1, 2, 4 and 8h, respectively. This approach resulted in identification of 23 proteins in increased and 67 proteins in decreased amounts. Overall, the newly induced/strongly up-regulated proteins involved in (i) amelioration of lipid peroxidation products, (ii) defense against oxidative damage and redox metabolism, (iii) transcription, recombination and DNA repair and (iv) a yet unknown function represented by a putative protein. This study implicated the particular role of the elements of DNA repair, post-transcriptional regulation and heterotrimeric G protein signaling in response to Pb (II) stress, as shown for the first time for a basidiomycete.

1.7. Aim of the study

For a comprehensive understanding of the molecular dynamics of the stress response and stress adaptation, proteomics is an ideal tool because the proteins are the actual players in cells. Considering the fact that post-transcriptional and post-translational controls give the final active level of proteins in the cellular machinery, a “protein first” strategy is quite useful to characterize any stimulon. While gel-based proteomics have the power to address post-translational modifications, protein stability and turnover, protein activity, protein localization and protein-protein interaction networks, certain proteins such as the hydrophobic membrane proteins, very acidic or basic proteins as well as very large or small proteins are poorly represented by current 2-DE technology, so prefractionation is quite useful before MS analyses. This is why a combination of LC and MS has

proven to be more effective in proteomics investigations, including stress proteomics. The aim of the present study is to reveal molecular response mechanisms of *P. chrysosporium* to two different heavy metals, copper and cadmium, in a dynamic (time-dependent) manner by analyzing both cytosolic and membrane proteome of the organism. For this aim, (i) proteome response to copper and cadmium was analyzed as a function of time with 2DE approach followed by MALDI MS MS and newly induced, upregulated and downregulated proteins were identified and relatively quantified, (ii) membrane proteome enrichment was next performed by using three different methods and their efficacy to yield more membrane proteins were compared. Using the most effective protocol (cell wall lysis followed by aqueous two phase separation) for membrane protein enrichment, a SILAC analysis of stress response was undertaken in a dynamic fashion by exposing the cultures to copper, and GeLC-MS technique was used this time for the identification and relative quantification of differentially expressed proteins. In this way, many differentially expressed proteins that could not be determined with 2 DE approach because of their size, charge or location restrictions were successfully identified.

CHAPTER 2

MATERIALS AND METHODS

2.1. Microorganism and its maintenance

The white-rot fungus, *Phanerochaete chrysosporium* (ATCC 24725) which was kindly provided by Prof. Filiz B. Dilek (Env. Eng. Dept., METU) was used in this study. The fungus was inoculated to Sabaroud Dextrose agar slants and incubated for 4 days at 35 °C for spore formation. The organism was transferred monthly and stored at 4 °C for further use.

2.2. Growth conditions, biomass preparation and exposure to heavy metal stress

Biomass of *P. chrysosporium* was prepared as reported by Yetiş et al. (1998). *P. chrysosporium* spores were separated from Sabaroud Dextrose agar slant surfaces by scrapping. The spores was gently suspended to homogeneity by using a teflon homogenizer. A spore suspension was prepared to contain $2.5x10^6$ spores/mL at an absorbance of 0.5 at 650 nm using a Shimadzu UV-1208 spectrophotometer. The spore suspension was then transferred into 500 mL Erlenmayer flasks each containing 250 mL of the growth medium. The medium used to grow *P. chrysosporium* in this study is described by Prouty, (1990). It is composed, in g/L of glucose, 10; KH_2PO_4, 2; $MgSO_4$, 0.5; $CaCl_2$, 0.1; NH_4Cl, 0.12 and thiamine, 0.001 and adjusted to a pH of 4.5. The cultures were incubated for 40h at 200 rpm in a rotary shaker at 35 °C. When the growth was reached to 40th h, the cultures were still in exponential growth (Özcan et al., 2007) and the bead-like mycelia of the fungi had a diameter of 2.5 to 4 mm. After 40h of growth, $Cd(NO_3)_2$ or

$Cu(NO_3)_2$ solutions were added to the medium to get a final concentration of 100 μM. Heavy metal treated cells were harvested at time points of 1h, 2h, 4h and 8h exposure with their corresponding non-exposed controls. Cells were harvested by filtration, washed with dH_2O and frozen in liquid nitrogen. Frozen cells were stored at -80 ˚C until they are processed for protein extraction.

2.3. Protein extraction for 2D-PAGE

For the extraction of proteins to be used in 2D-PAGE analysis, a modified version of TCA-acetone extraction method of Damerwal et al. (1986) was performed. Briefly, after breaking of harvested mycelium in liquid nitrogen, 5 mL of 10 % TCA in acetone containing 0.07 % β-mercaptoethanol was added and vortexed, then incubated at –20 ˚C for 45 min and centrifuged at 15.000 xg for 15 min. The supernatant was decanted and the pellet was rinsed with 5 mL of cold acetone containing 0.07 % β-mercaptoethanol two times by incubating at -20 ˚C for 1h (mixed every 15 min intervals by vortexing) and centrifuging as above. After discarding the supernatant, the remaining pellet was vacuum-dried and stored as a powder at -20 ˚C. Finally 10 mg of the powder was solubilized in 500 μl rehydration buffer containing 8 M urea, 2 M thiourea, 0.3% DTT, 1% (w/v) CHAPS, 0.3% (w/v) DTT, 0.5% (v/v) ampholyte pH 3-10 and incubated for 30 min at room temperature. Final resuspension was centrifuged at 15000 g for 15 min and the supernatant was collected. The proteins obtained by using this procedure represented total cellular proteins since the debris contained whole soluble protein complements of the cells.

2.4. Protein amount estimation

To determine total protein concentrations, the modified Bradford assay described by Ramagli and Rodrigez (1985) was used. 5X Bradford reagent (containing 500 mg Comassie Brillant Blue G-250, 250 mL of 96% ethanol and 500 mL of 85 % ortho-phosphoric acid; completed to a 1 L with dH_2O) was diluted 1:3 with dH_2O and filtered at least two times using Whatman No. 1 filter paper. For determination of the total protein concentration, 10 mg of a mycelium extract

powder was dissolved in 500 µL of rehydration buffer [containing 8 M urea, 2 M thiourea, 0.3 % DTT, 1% (w/v) CHAPS, 0,3 % (w/v) DTT, 0,5% (v/v) ampholyte pH 3-10]. The suspension was mixed and incubated at room temperature for 1h and then centrifuged at 12 000 xg for 5 min. To 20 µL of aliquots of the supernatant, 80 µL of 0.1 N HCl were added to protonate samples and mixed thoroughly. To this mixture, 3.5 mL of 1:3 diluted 5X Bradford reagent was added, incubated at room temperature for 10 min and absorbance was measured at 595 nm. Bovine Serum Albumin (BSA) fraction number V was used as a standard for the construction of calibration curves.

2.5. 2D-PAGE

2.5.1. Isoelectric focusing (IEF)

Isoelectric focusing was performed in 18 cm IPG-strips (pH range 3-10 non-linear, Amersham Biosciences, Uppsala, Sweden). IPG strips were passively rehydrated by applying 360 µl of rehydration buffer including 300 µg protein for 16 h. The isoelectric focusing was performed with the MultiphorII unit (Amersham Biosciences) employing the following voltage profile; linear increase from 0 to 500 V for 2500 Vh, 500 V for 2500 Vh, linear increase from 500 to 3500 V for 10.000 Vh and a final phase of 3500 V for 35 000 Vh.

2.5.2. SDS-PAGE

After IEF, focused strips were equilibrated according to Görg et al. (1988). Equilibration included the reduction step; shaking of the IPG strips for 15 min in equilibration buffer A (6 M urea, 30% (w/v) glycerol and 2% (w/v) SDS in 0.05 M Tris-HCl buffer, pH 8.8.) containing 100mg/10ml DTT and an alkylation step; shaking of the IPG strips for 15 minutes in equilibration buffer B containing 250mg/10ml Iodoacetamide. Equilibrated IPG strips was placed on top of the 1 mm thick, continuous polyacrylamide gels of 12.5% T and 2.6% C according to Leammli (1970). SDS-PAGE was performed by using the Millipore

electrophoresis system (Millipore, MA. USA) by applying approximately 2 W per gel. After electrophoresis, protein spots were stained with colloidal Coomassie blue (CCB) by using the procedure of Neuhof et al. (1988) (Appendix A). CCB-stained gels were stored in a little amount of water in dark cold room for image analysis.

2.6. Evaluation of 2-DE data

Scanning of preserved gels was done by using a X-Finity scanner (Quato Graphics Braunschweig, Germany). Spot pattern analyses and labeling of the spots to be cut were accomplished by using the 2D image analysis software Delta2D version 3.4 (Decodon, Germany).

2.7. Sample preparation and analysis by mass spectroscopy

The protein spots were excised from the stained 2D gels using a spot cutter (Ettan Dalt Spot cutter). Cut spots were transferred into 96 well microtiter plates. The tryptic digest with subsequent spotting on a matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF MS target) was carried out automatically with the Ettan Spot Handling Workstation (Amersham Biosciences, Uppsala, Sweden) using the following protocol: The gel pieces were washed twice with 100µl of a solution of 50% ACN and 50 % 50 mM NH_4HCO_3 for 30 min and once with 100µl 75 % ACN for 10 min. After drying at 37 ºC for 17 min 10µl trypsin solution containing 20 ng/µl trypsin (Promega, Madison, WI, USA) was added and incubated at 37 ºC for 120 min. For extraction, the gel pieces were covered with 60 µl 0.1 % trifluoroacetic acid (TFA) in 50 % ACN and incubated for 30min at 40 °C. The peptide containing supernatant was transferred into a new microtiter plate and the extraction was repeated with 40 µl of the same solution. The supernatants were dried at 40 °C for 220 min completely. The dry residue was dissolved in 3 µl of 0.5 % TFA in 50 % ACN and 0.4 µl of this solution were directly spotted on the MALDI target. Then 0.4 µl of a saturated α-cyano-4-hydroxy cinnamic acid solution in 70 % ACN

were added and mixed with the sample by aspirating the mixture five times. The samples were allowed to dry on the target for 10 to 15 min before measurement in MALDI-TOF. The MALDI-TOF measurement was carried out on the Proteome-Analyzer 4800 (Applied Biosystems, Foster City, CA, USA). This instrument is designed for high throughput measurement, being automatically able to measure the samples, calibrating the spectra and analyzing the data using the 4700 ExplorerTM Software. The spectra were recorded in a mass range from 900 to 3700 Da. If the autolytical fragment of trypsin with the mono-isotopic (M+H) +m/z at 2211.104 reached a signal to noise ratio (S/N) of at least 10, an internal calibration was automatically performed as one-point-calibration using this peak. After calibration the peak lists were created by using the "peak to mascot" script of the 4700 ExplorerTM Software. The three highest peaks were chosen automatically for recording an MS/MS spectrum. The peak lists of the MS/MS experiments were included to the mascot search to improve the reliability of the search result.

2.8. MALDI-TOF protein identification

P. chrysosporium whole genomic DNA sequence data which are available in the organism's genome project (Joint Genome Institutes) web address (http://genome.jgi-psf.org/Phchr1/Phchr1.home.html database version 2.1) have already been translated to amino acid sequences in FASTA format and published at the same web address. In order to construct a database for PMF search for *P. chrysosporium* proteins, the whole database was downloaded and processed for theoretical trypsin digestion of amino acid sequences by the use of MASCOT program to construct a theoretical peptide mass fingerprint (PMF) database. The peak lists of each protein spot (peptide mass fingerprint and MS/MS data) obtained from MALDI TOF MS measurement were analyzed with the aid of "Peptide Mass Fingerprint" and "MS/MS Ion Search" engines of MASCOT software (Matrix Science Inc., Boston, MA, USA) against the above-mentioned *P. chrysosporium* PMF database. Of the results given by the MASCOT software, those having a probability score value higher than 53 (which was calculated

statistically by the software as base score for a meaningful identification according to genome size of *P. chrysosporium*) were considered for protein identification. To find out putative functions, protein accession numbers of the identified spots which were included in the MASCOT output were searched in the website of the Joint Genome Institutes for *P. chrysosporium*. The JGI database uses InterPro approach which includes an integrated documentation resource for protein families, domains and sites, for putative function assignment for each protein in their databases. For the proteins which were certainly assigned to the particular genes, i.e. the identified ones, the functional classification was made by consulting to the functional categories list contained the JGI website.

2.9. Membrane protein enrichment

2.9.1. Membrane shaving

Shaving was first developed and applied by Speers et al., (2007). The method simply includes extracting the intact cell membranes and shaving proteins off from the membrane surface by the help of proteinase-K. Afterwards the shaved membrane is destroyed by the help of a strong detergent like RapiGest®. Since the membrane is lysed, transmembrane helices (TMH) embedded in the membrane are released to the solution. In the final step the detergent is hydrolyzed by the help of a strong acid like HCl and obtained peptide solution is analyzed by an LC-MS system.

2.9.2. Membrane protein extraction by ß-glucorinidase

Removing the cell wall enzymatically is one of the methods employed in order to obtain a crude cell membrane. Because of enzymes' high specificity and capabilities, this method is frequently chosen over other methods. The examples are lysozyme for lysing the *E. coli* cell wall, zymolase for yeast cell wall and cellulase for plant cell wall. Hernández-Macedo et al., (2002) used a similar approach to identify iron related proteins of *P. chrysosporium* by applying the

method of Bowman et al., (1981). According to the protocol the mycelia was grounded to fine powder in liquid nitrogen mycelia was suspended in solution A (0.59 M sucrose, 5mM EDTA, 50mM NaH_2PO_4, 70 µL β-mercaptoethanol, 133 mg/mL *Helix pomatia* β-glucoronidase type H-1 enzyme and incubated for 1h at 30˚C. The suspension was homogenized in a glass tissue grind tube and centrifuged at 4000 g for 10 min at 4˚C. The pellet was suspended in solution B (0.33 M sucrose, 1 mM EGTA, 0.3% bovine serum albumin, pH 7.1), homogenized again, and centrifuged at 1000 g for 10 min. The supernatant was centrifuged for 30 min at 11700g and then the obtained supernatant was recentrifuged for 40 min at 40000 g. Finally, the pellet was and suspended in 6 mM Tris-HCl, pH 6.8, 2% SDS, and 5% β-mercaptoethanol. 50 µl of this crude membrane protein extract was run on 1D-PAGE and the gel were processed for GeLC-MS approach.

2.9.3. Cell wall lysis followed by aqueous two phase separation

The procedure for membrane associated protein enrichment was adapted from Shary et al. (2008) and Hernández-Macedo et al. (2002). Basically, the collected biomass was ground with liquid nitrogen and suspended in homogenization buffer containing 1.5 mM MOPS pH 7.8, 1 mM EDTA, 1 mM DTT, 0.25 M sucrose, 1 mM PMSF and 1mg/mL lysing enzymes from *Trichoderma harzianum* (Sigma L1412). After incubating the suspension for 30 min at RT to digest chitin cell wall, it was homogenized in a teflon homogenizer. Homogenized solution was centrifuged for 10 min at 10000 g and supernatant was collected. Another round of 10 min at 10000 g centrifugation was accomplished and the pellet was discarded. The protein amount determination of the supernatant was performed by the standard Bradford technique (Bradford, 1976)**.** This mixture was centrifuged for 10 min. at 10000g two times. Resulting supernatant were then centrifuged for 1h at 100000g and the pellet was suspended in 500 µl of 5mM phosphate buffer (pH 7.8) including 1mM DTT. The suspended pellet was further separated by using aqueous two phase separation system to enrich membrane proteins. The two

phase separation system consisted of 6.4% w/v PEG-3350, 6.4% w/v dextran T-500, 0.25 M sucrose, 4.7 mM phosphate buffer pH 6.8. Five mL of this solution was gently mixed and stored at +4 °C until it is used. 500 µl of suspended pellet was mixed with 5 mL of two phase PEG/dextran solution. Final mixture was centrifuged for 5 min at 1000 g. The upper PEG phase containing hydrophobic proteins were collected and further centrifuged for 1h at 100000g. The final pellet was resuspended in 5 mM phosphate buffer and used as membrane enriched protein fraction.

2.10. SILAC experiments

For SILAC experiments, a set of minimal medium flasks was prepared as described in Section 2.2 and labeled as light medium. Additionally, another set of minimal medium was prepared in which light NH_4Cl was replaced by its ^{15}N isotope-containing form and labeled as heavy medium. Both of the sets were inoculated and cultivated simultaneously. At the 40th h of incubation, $Cu(NO_3)_2$ treatments were started. Finally, the cells were harvested at time points of 2h, 4h and 8h following heavy metal exposures. The supernatants obtained from the heavy medium cultures (^{15}N containing), for each time point, both control and heavy metal treated ones, were mixed in a proportion that each provides an equal amount of protein. This mixture was designated as the “standard”. The samples obtained from light medium-grown cells were mixed with the standard in a ratio of 1:1 in terms of protein amount. Finally these mixtures were centrifuged and membrane proteins were enriched by the aqueous two phase separation as described in Section 2.9.3.

2.11. GeLC-MS analysis

Membrane protein enriched samples containing 30 µg of proteins were mixed with Laemli loading buffer containing 2X more SDS and loaded on the SDS-PAGE and run at 120V. After the running gels were fixed in a fixer solution containing %40 EtOH and 10% acetic acid for at least 1 hour. Gels were stained

with colloidal coomassie staining overnight. After staining gels were washed with dH_2O and stored at +4°C.

2.12. In-gel digestion for 1D-PAGE

In-gel digestion was performed according to Dreisbach et al. (2008). Each lane of the samples were cut from the gel and divided into 10 equidistant pieces. Each of these lanes were further sliced into 1-2 mm^2 pieces and washed 2-3 times with washing solution including 30% acetonitrile and 1.6% ammonium bicarbonate. The final cut gel pieces were dried by the help of a speedvac and covered with a trypsin solution (10 ng/mL in water). After rehydration of the gel pieces excess of trypsin solution is discarded and the gel pieces were incubated over night at 37˚C. At the end of incubation time, the gel pieces were covered with MS grade milliQ water and incubated in an ultrasonic water bath for 5-10 minutes, which helps the peptides crawl out from the gel to milliQ water. The elutes were transferred to empty low protein binding Eppendorf tubes and concentrated to 10 µl by a speedvac drier.

2.13. Zip-tip cleaning

To clean-up the impurities from the extracted peptides, Zip-tip® Millipore™ cleaning tips were used according to the following procedure. For this purpose, the tips were first wet with 70% ACN (acetonitrile) and then equilibrated with 3% ACN including 0.1% acetic acid. 10 µl of the sample were pipetted ten times to bind peptides to ZipTip column material. At washing step, 10 µl of 0.1% acetic acid was pipetted and discarded 2 times to get rid off the impurities. Finally peptides were eluted by pipetting 10 µl of 60% ACN including 0.1% acetic acid three times. Final eluates were vacuum dried to get rid of ACN and resuspended in 5 µl of dH_2O.

2.14. Nano-LC MS/MS analysis

The nano-LC-MS/MS analysis of peptides derived from tryptic in-gel digestion was performed on a linear trap quadrupole (LTQ) Oribtrap (Thermo Fisher Scientific, Waltham, MA) equipped with a nano- ACQUITY UPLC (Waters, Milford, MA). Peptides were loaded onto a trapping column (nanoAcquity Symmetry UPLC column, C18, 5 μm, 180 μm by 20mm; Waters) at a flow rate of 10 μl/min and washed for 3 min with 99% buffer A. Peptides were then eluted and separated via an analytical column (nanoAcquity BEH130 UPLC column, C18, 1.7 μm, 100 μm by 100 mm; Waters) with a decreasing buffer gradient (from 99% buffer A to 60% buffer B (0.1% acetic acid, 90% acetonitrile, distilled water [dH_2O]) in a time frame of 80 min. The mass spectrometric analysis started with a full survey scan in the Orbitrap (m/z 300 to 2,000, resolution of 60,000) followed by collision-induced dissociation and acquisition of MS/MS spectra of the four most abundant precursor ions in the LTQ. Precursors were dynamically excluded for 30 s, and unassigned charge states as well as singly charged ions were rejected.

2.15. Bioinformatic analyses

Sequest peak list files (*.dta) were generated from the raw instrument data (*.raw) using RawXtract with default settings implemented in BioworksBrowser 3.3.1 SP1. The database search was performed with SEQUEST (version 27, rev. 12), and the result files (*.out) were combined and filtered with DTASelect 1.9 and Contrast (Tabb et al., 2002). The searched database contained the target sequences, which include the complete proteome set of *P. chrysosporium* and a set of common laboratory contaminants as well as a decoy database. The precursor tolerance was set to 10 ppm, and tolerance for fragment ions was set to 1 atomic mass unit (amu). In these searches, only fully tryptic peptides, two missed cleavage sites, and methionine oxidation as differential modification were allowed. Only b- and y-ion series were included in the database search. All searches were performed for either light or heavy (with ^{15}N as fixed modification) peptides using the same set of parameters. Peptide filter criteria were adjusted by

keeping the empirically determined false-positive rate (FPR) for each set of combined ^{14}N and ^{15}N search results on a protein level below 3% and on a peptide level below 1%. The following filter criteria were sufficiently stringent: minimum XCorr scores were set to 2.2, 2.8 and 3.5 for +2, +3 and +4 charged ions, respectively, and a ΔCN value of 0.1 was demanded.

2.15.1.1. Quantification, normalization, and filtering of proteomic data.

The quantification of metabolically labeled peptides was performed using the quantification program Census (Park and Yates, 2010). MS1 files were generated from the raw instrument data with the RawExtractor program (version 1.9.3) and SEQUEST search results of heavy and light peptides were combined with Contrast and filtered with DTA-Select 1.9, applying the above-mentioned filter criteria. Both MS1 files and the combined search results were passed to Census, and quantification was performed using the default setting for high-resolution mass spectrometry data. Quantified peptides were extracted from Census, using a default filter and a stringent determinant score of 0.8. Singleton peptides (i.e., peptides with one isotopomer below the detection threshold) as well as proteins with only one quantified unique peptide were discarded. Protein ratios were calculated from peptide ratios using the weighted average approach implemented in Census.

Weighted average protein ratios were transformed to Z-scores and mean centered. The final data set retained only those proteins that were quantified with at least two ^{14}N- or ^{15}N-labeled peptides per sample and that had at least two expression values from both of the biological replicates.

WolfPsort Protein subcellular localization predictions were obtained from PSORT web server (http://wolfpsort.seq.cbrc.jp/) by submitting amino acid sequences of the identified proteins to this server. The existence of signal peptide sequences was checked in the public web server of signal peptide prediction program SignalP version 3.0 (http://www.cbs.dtu.dk/services/SignalP/). Number of

transmembrane helices are predicted by TMHMM algorithm (Krogh et al., 2001).

$^{15}N/^{14}N$ based quantification is accomplished by analyzing the resulting MS data via a series of softwares and scripts. Bioworks Sequest and Sorcerer were used to identify proteins from the *P. chrysosporium* protein database. DTASelect algorithm (Tabb et al., 2002) and Census (Park et al., 2008) software were used to analyze quantification data.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Growth curve results

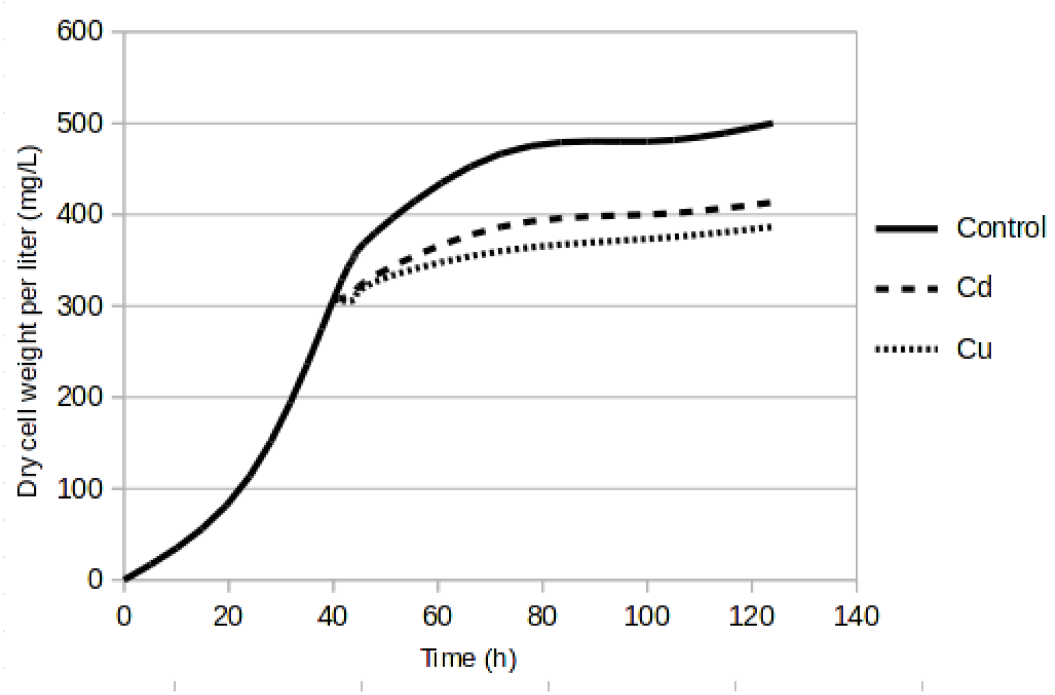

Figure 3.1. Growth curve of *P. chrysosporium* under normal conditions and Cd and Cu stress.

Growth curves of the *P. chrysosporium* for normal conditions and heavy metal stress were established (Figure 3.1). It was revealed that Cd and Cu causes an arrest on the growth rate immediately after the exposure. This delay were observed especially after 2h and 4h exposure. However after 8h exposure this effect seemed to be faded and growth rate of the organism was similar to the that of control sample for the rest of the incubation. This results suggests that after 8h of exposure the organism starts to adapt the heavy metal stress returns to its normal life cycle.

3.2. 2DE analysis results of copper and cadmium response in *P. chrysosporium*

To understand physiological response of P. chrysosporium to copper exposure, the organism was exposed to 100 μM copper stress for 8h. Copper treatment was started at 40 h of cultivation and a total of four sampling was accomplished at the time intervals of 1h, 2h, 4h and 8h exposure.

3.2.1. 2DE analysis of copper-induced proteins

Proteome profile of each sample was analyzed by using 2D-PAGE approach. Differentially expressed proteins were identified on the 2D gels by comparing them with their respective control samples, which were harvested exactly at the same time point from the cultures without heavy metal treatment.

A total of 123 spots were detected as differentially expressed after Cu exposure for 1h, 2h, 4h and 8h on *P. chrysosporium* proteome. By employing the MALDI-TOF analysis of these 123 protein spots 89 could be identified. Further analysis revealed that the 89 protein spots are the product of the 58 distinct ORFs. The Id numbers of the identified protein spots are depicted on each gel through Figure 3.2 to 3.5. A detailed comparison of relative abundance of each spot and their functions are tabulated at Table 3.1.

3.2.1.1. Intracellular trafficking, secretion, and vesicular transport

A Ran GTPase protein (123314) was found to be upregulated in response to Cu 1, 2, and 8h treatments. In our previous studies, a Ran GTPase with the same Id was found to be responsive against both Cd and Pb exposures (Özcan et al., 2007; Yıldırım et al., 2011), providing a strong evidence for the existence of Ras signalling pathway in *P. chrysosporium.* This pathway interacts with actin-related proteins and triggers accelerated programmed cell death in *C. albicans* under harsh environmental conditions (Phillips et al., 2006).

3.2.1.2. Posttranslational modification, protein turnover, chaperones

14-3-3 family multifunctional chaperones play a regulative role by interacting with various proteins. This interaction is achieved by the means of phosphorylation. In other words, 14-3-3 proteins are a part of intracellular signal transduction cascade. One of the target proteins of the 14-3-3 are the H^+ATPases. In a study, cotton plants transfected with 14-3-3 gamma gene was found to be resistant against drought stress(Yan et al., 2004). Similarly, 14-3-3 transfection increased the activity of plasma membrane H^+ATPases of sugar beet plants under the cold and osmotic stress (Chelysheva et al., 1999). In our study 14-3-3 proteins (139500) were found to be upregulated in response to copper stress throughout the copper exposure. An H^+ATPase also found to be upregulated after 8h Cu exposure. These results are consistent with the present stress response literature.

The function of the 20S proteasomes were well documented previously and it is known that 20S proteasome complex degrades oxidized proteins in particular (Davies, 2001). In some cases, this degradation process requires the ubiquitinylation, and it is called as ubiquitin–proteasome pathway (Shang and Taylor, 2011). As a canonical oxidative stress response protein, it was expected to see elevated levels of the 20S proteasome complex proteins after 1h Cu treatment. As our 2D analysis results indicates two of the 20S proteaseome complex subunits were found to be upregulated.

AAA+-type ATPase (127435) was significantly upregulated for both Cu and Cd exposures. A similar upregulation of AAA+-type ATPase in response to Cu was also shown in our previous work (Özcan et al., 2007), and elevated levels of the protein in response to Cd was shown in a study conducted with *S. pombe* (Bae and Chen, 2004).

The most significant feature of Hsp90 (128865) protein, which distinguishes it from other HSP proteins, is that most of its substrates are signal transduction proteins like kinases (Young, 2001). Although their main roles involves correction of the misfolded proteins, they also play important roles intracellular signal

transduction, cell cycle control, and protein trafficking. Nollen and Morimoto, (2002) has revealed that osmolite addition to the environment triggers the interaction of Hsp90 and Hsp70 proteins with tyrosine, serin/threonin kinases and cell cycle regulators. HSP90 is one of the house keeping proteins, however its expression increases dramatically when cells encounter with a stressor (Krishna et al., 1997). The HSP90 was upregulated in response to both of the metals. Thus, we can assume that the main targets of the Cu damage are intracellular signal transduction proteins, which in turn induce the HSP90 to repair the damaged ones.

3.2.1.3. Transcription proteins

The plant-specific NAC transcription factors family consists of three subfamilies: NAM(No Apical Meristem), ATAF (Arabidopsis Transcription Activation Factor) and CUC (Cup-Shaped Cotyledon) and they play key roles in both biotic and abiotic stresses. Their roles in stress response mechanisms were extensively reviewed (Nakashima et al., 2012; Nuruzzaman et al., 2013; Puranik et al., 2012). Basically a NAC-TF regulates the transcription of stress responsive genes having a NAC recognition sequence (NAC-RS), called as NAC genes. Abiotic stresses induces NAC and related transcription factors and afterwards NAC binds to NAC-RS to start the transcription of stress responsive genes. Increased levels of NAC-Tf are proven to be beneficial against abiotic stresses. For instance, it was shown that plants overexpressing a NAC transcription factor showed increased resistance against drought and salt stress (Hu et al., 2006; Le et al., 2011). A NAC domain containing protein found to be upregulated in response to all of the Cu treatment in the present study, suggesting that a similar pathway also exists in *P. chrysosporium* as a stress response mechanism.

The transcription factor BTF3 was first extracted from HeLa cells(Moncollin et al., 1986). It is known that BTF3 attatches to RNA polymerase II to make an active transcription complex. In this study,BTF3 was found to be upregulated after 1h Cu treatment. It can be proposed that BTF3 is required for the transcription of the heavy metal responsive proteins.

3.2.1.4. Translation and ribosomal structure

Mitochondrial elongation factor Tu (EF-Tu) is a multifunctional protein. To be counted among its functions are the transport of aminoacyl-tRNA complexes to the ribosome during protein biosynthesis, chaperone activity preventing aggregation of proteins because of environmental stresses, degradation of the N-terminally blocked proteins by the proteasome and participation to the immune responses (Fu et al., 2012; Suzuki et al., 2007). It was also shown that EF-Tu is essential for transcription of heat responsive genes (Fu et al., 2012). In the present study, we recorded increased levels of over 2 fold of this protein with a predicted mitochondrial location at different time points of Cu exposure.

3.2.1.5. Amino acid metabolism

Aspartate-aminotransferase (AAT) is an enzyme catalyzing the α-amino group transfer between aspartate and glutamate, therefore it is an important enzyme in amino acid metabolism. Easlon et al., (2008) conducted a study with three different yeast strains over-expressing AAT1, MDH1 (malate dehydrogenase) and Gut2 (glycerol-3-phosphate dehydrogenase), respectively. According to the study, overexpression of these enzymes resulted in an expanded life span in yeast. Aat1 and Mdh1, convert mitochondrial malate into aspartate, with the reverse reactions occurring on the cytoplasmic side of the mitochondrial membrane. When the mitochondrial NAD/NADH ratio increases, it leads to the production of aspartate from malate through the malate dehydrogenase (Mdh1) and the aspartate aminotransferase (AAT1). It can be assumed that, in the present study, copper-originated oxidative stress might have caused an imbalance to NAD/NADH ratio(Kumari et al., 2008). Thus, an early upregulation of three different isomers of an AAT enzyme upon Cu exposure could be a way to restore this balance.

Three spots identified as thiamine pyrophosphate-requiring enzyme from the database were found to be significantly upregulated (up to 10 fold) in response to Cu-treatment. Blast search for this protein revealed that, it has high similarity with

pyruvate decarboxylase. Pyruvate decarboxylase removes a carboxyl group from pyruvate and produce acetaldehyde and CO_2 as a part of the fermentation process. It was shown that the enzyme is upregulated under anoxic conditions (Kürsteiner et al., 2003). Still the link between its upregulation and heavy metal stress response is missing and open to further investigation.

Cobalamin-independent methionine synthase II is an enzyme catalyzing the final step in methionine synthesis by converting homocystein to methionine. This enzyme has been shown to be inactivated by oxidative stress in both eukaryotes (Muratore et al., 2013) and prokaryotes (Hondorp and Matthews, 2004). When the methionine synthase activity decreases due to oxidative stress, accumulating homocysteine is redirected to cysteine production via the intermediate cystathionine. This redirection of the pathway provides a huge source of cysteine for glutathione (GSH) synthesis, which in turn gives the organism an important arsenal against oxidative agents (Muratore et al., 2013). In the present study two isoforms of this enzyme were found to be significantly upregulated in Cu-exposed samples. Especially the level of one isoform was 3-4 fold higher and this increment reached to ca. 8 fold at 8^{th} h of exposure, ,suggesting that the organism might have been trying to replenish the damaged enzyme.

3.2.1.6. Carbohydrate metabolism

6-phosphogluconate dehydrogenase (6PGD) is one of the best known stress response proteins which appeared to be upregulated in various studies (Dorts et al., 2011; Li et al., 2009; Teixeira et al., 2006; Yasokawa et al., 2008). It is an important enzyme in the pentose phosphate pathway. This pathway results in two NADPH molecule in every cycle. NADPH is an important intracellular antioxidant, thus it is reasonable to suggest that oxidative stress causes a metabolic shift within the cell from glycolysis towards the pentose phosphate pathway. Upregulation of 6PGD after the Cu induction proves that *P. chrysosporium* uses the same mechanism to counteract against the oxidative stress created by Cu.

Like 6PDG, transketolase is an important element of pentose phosphate pathway which leads to an increase in the cytosolic NADPH production. This enzyme transfers a 2-carbon fragment from D-xylulose-5-P to the aldose erythrose-4-P thus producing a fructose-6-P and a glyceraldehyde-3-P molecule. Increase in the transketolase levels in the face of the oxidative stress was confirmed by different studies. For instance, Rapala-Kozik et al. (2008) reported elevated levels of this enzyme in corn when the plant was exposed to salt and oxidative stress. Because of the ROS generation ability of copper, greatly upregulated levels of the three isoforms (5 to 10 fold during exposure for one isoform) of transketolase in this study accord well with earlier findings.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyses the reaction in which glyceraldehyde-3-phosphate is converted to 1,3-bisphosphoglycerate while converting NAD^+ into NADH. This is a crucial step in glycolytic energy metabolism. However, GAPDH is a multifunctional enzyme and its functions include DNA repair, tRNA export, membrane fusion and transport, cytoskeletal dynamics, and cell death (Tristan et al., 2011). It is known that the active-site of GAPDH can be S-thiolated by hydrogen peroxide (Shenton and Grant, 2003), which makes the enzyme one of the major targets of oxidative stress. We recently demonstrated that five isoforms of this enzyme are involved in lead-response in *P. chrysosporium* (Yıldırım et al., 2011). However, in the present study GAPDH gave a single spot which was 3 fold upregulated throughout Cu exposure. Since the oxidative stress deactivates the active site of this enzyme, it is possible that the organism compensates its lack by upregulating its expression.

3.2.1.7. Energy production and conversion

With two isomers, one is 2-fold induced rather late (8^{th} h), but the other being upto 4-5 fold elevated levels throughout the Cu exposure, citrate synthase was another Cu-responsive protein. As its name implies, citrate synthase catalyzes production of six-carbon citrate from two carbon acetyl coenzyme A and four-carbon oxaloacetate in the Krebs cycle. Studies conducted with citrate synthase showed

that it is also an important element of abiotic stress tolerance. For instance, yeast mutants having the citrate synthase gene (*cit1*) deletion shows hypersensitivity to heat and aging induced apoptosis (Lee et al., 2007). In contrast, over expression of the enzyme in *Arabidopsis thaliana* boosted its growth on a phosphorus limited soil (Koyama et al., 2000). Some studies reported that Cu inhibits phosphate uptake and reduces phosphorus concentration in some plants (Lin and Wu, 1994; Mateos-Naranjo et al., 2008; Xing et al., 2010). Therefore we can assume that upregulation of the citrate synthase probably helps the organism's tolerance to phosphorus shortage due to Cu exposure.

UDP-glucose pyrophosphorylase (UGPase) catalyzes a reversible production of UDPG and pyrophosphate (PPi) from Glucose-1-P and UTP. UGPase was shown to as a novel salt stress-responsive protein in rice (Yan et al., 2005). We also reported an upregulation of the UGPase in response to Pb in our previous work (Yıldırım et al., 2011). It was shown that UGPase is strongly upregulated when there is phosphorous deprivation (Ciereszko et al., 2001). As discussed above it is known that Cu exposure causes phosphorus limitation in some plants, therefore it can be proposed as UGPase upregulation is required to supply phosphorus demand of the organism under Cu stress. Nonetheless, this finding shows that UGPase is clearly a member of the environmental stress response proteins.

Fumarase is another Krebs cycle protein that is affected by heavy metal exposure. It was shown that heavy metals can bind to active site of the fumarase and inactivate the enzyme (Xu and Imlay, 2012). In another study, one of three *E. coli* fumarases (Fumarase C) was found to be upregulated under Cd stress (Helbig et al., 2008). The present study also indicates upregulated fumarase (135713) under Cu stress, possibly implying an effort to replenish the inactivated fumarases.

F0F1-type ATPases catalyze ATP synthesis when the H^+ gradient is favorable. If the gradient is abolished, the K_{eq} favors ATP hydrolysis instead. In a previous work conducted by our group, F0F1-type ATPase was found to be upregulated in response to Pb stress in *P. chrysosporium* (Yıldırım et al., 2011). Although there is

limited knowledge about F0F1-type ATP synthase upregulation under abiotic stress responses, there is some evidence that organisms can go to apoptosis because of abiotic stresses and since the apoptosis is a highly regulated process involving a number of ATP-dependent steps, it requires more ATP (Zamaraeva et al., 2005).

3.2.1.8. Inorganic ion metabolism

Superoxide dismutases (SOD) are one of the best known enzymes for their antioxidant properties. SODs catalyze the production of H_2O_2 and O_2 by dismutating the superoxides. SODs found to be upregulated in response to stress responses in different studies. For instance, nickel induction resulted in upregulation of SOD enzyme in *Burkholderia vietnamiensis* (van Nostrand et al., 2008). Manganese superoxide dismutases (MnSOD) are a family of SODs which require manganese as a cofactor and are localized in mitochondria. Besides the antioxidant function of the enzyme, it was shown that MnSOD can bind heavy metals other than manganese (Beyer and Fridovich, 1991; Whittaker and Whittaker, 1997). Since the minimal culture media used in the present study does not contain any externally added manganese, two upregulated MnSOD isoforms suggest that the enzyme may be used by the cells as a heavy metal chelator.

3.2.1.9. Secondary metabolites biosynthesis

Uricases (urate oxidases) catalyze the oxidation of uric acid to allantoin and play an important role in purine metabolism (Wu et al., 1994). The synthesis of two uricase isoforms were found to be stimulated at almost all time points during Cu exposure. Although the correlation between uricase and stress response is not clear, it can be speculated that Cu stress increases cellular demand for purine catabolism.

3.2.1.10. Hypothetical proteins

The present 2DE-based analysis resulted in identification of two isoforms of a

hypothetical protein (Id number 4311) significantly increased at 4th and 8th h of Cu exposure. As for every hypothetical protein, demonstration of its cellular function and its relation with stress response awaits further experiments for a much better understanding of the organism's stress physiology.

As a poorly characterized protein, an mRNA splicing factor (Id 134635) was found to be upregulated at a moderate level only at an early period of 1st h of Cu exposure. The same protein was reported to be elevated at much higher levels in response to Pb stress (Yıldırım et al., 2011), suggesting that a given protein may not be equally important in response to stress induced by different heavy metals.

3.2.1.11. Downregulated proteins in response to Cu

A total of 16 proteins were found to be downregulated in response to copper. Posttranslational modifications, protein turnover and chaperones were the major group negatively affected from Cu, as the 4 of the dowregulated proteins belong to this group. The spot corresponding to small heat-shock protein Hsp26/Hsp42 (Id 137747) was found to be downregulated at the time points of 2h, 4h and 8h. Interestingly, another spot of this protein was found to be upregulated at the same time points. It was shown that Hsp26 is stored as oligomers in the cell and disaggregated in response to heat-shock (Haslbeck et al., 1999). With the present study, we have the clues pointing to a similar mechanism in *P. chrysosporium* in response to Cu. Thus, we can propose that an oligomeric protein undergoes conversion to a monomeric form with accompanying up/down-regulation of its expression in response to heavy metal stress.

Downregulation of tubulin genes in response to low temperature were reported by Chu et al., (1993). According to the authors, tubulin expression is mainly controlled at a posttranscriptional level and low temperature causes shorter half life of tubulin mRNAs. The downregulation of the beta tubulin in the present study could therefore be the resulted from shorter half life of the tubulin mRNA when the cells were exposed to Cu..

Some other significantly downregulated proteins were Nucleolar GTPase, Ubiquitin-conjugating enzyme E2, FKBP-type peptidyl-prolyl cis-trans isomerase, dipeptidyl aminopeptidase, molecular chaperones GRP170/SIL1, ribosomal protein RPL1/RPL2/RL4L4, 40S ribosomal protein S3A, enolase and three predicted hypothetical proteins.

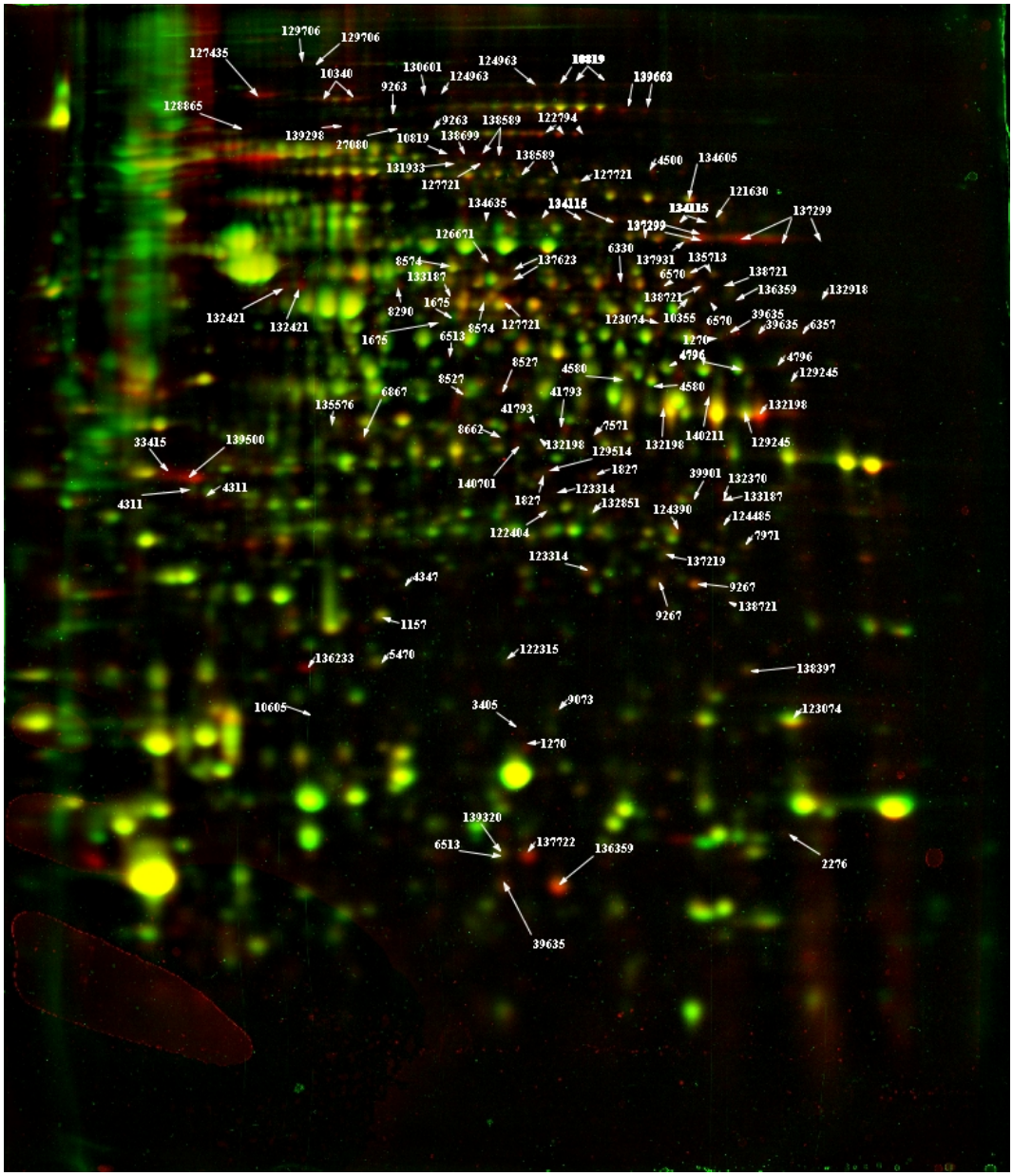

Figure 3.2. Comparison of control sample 2DE with that of 1h 100 µM Cu exposed sample.

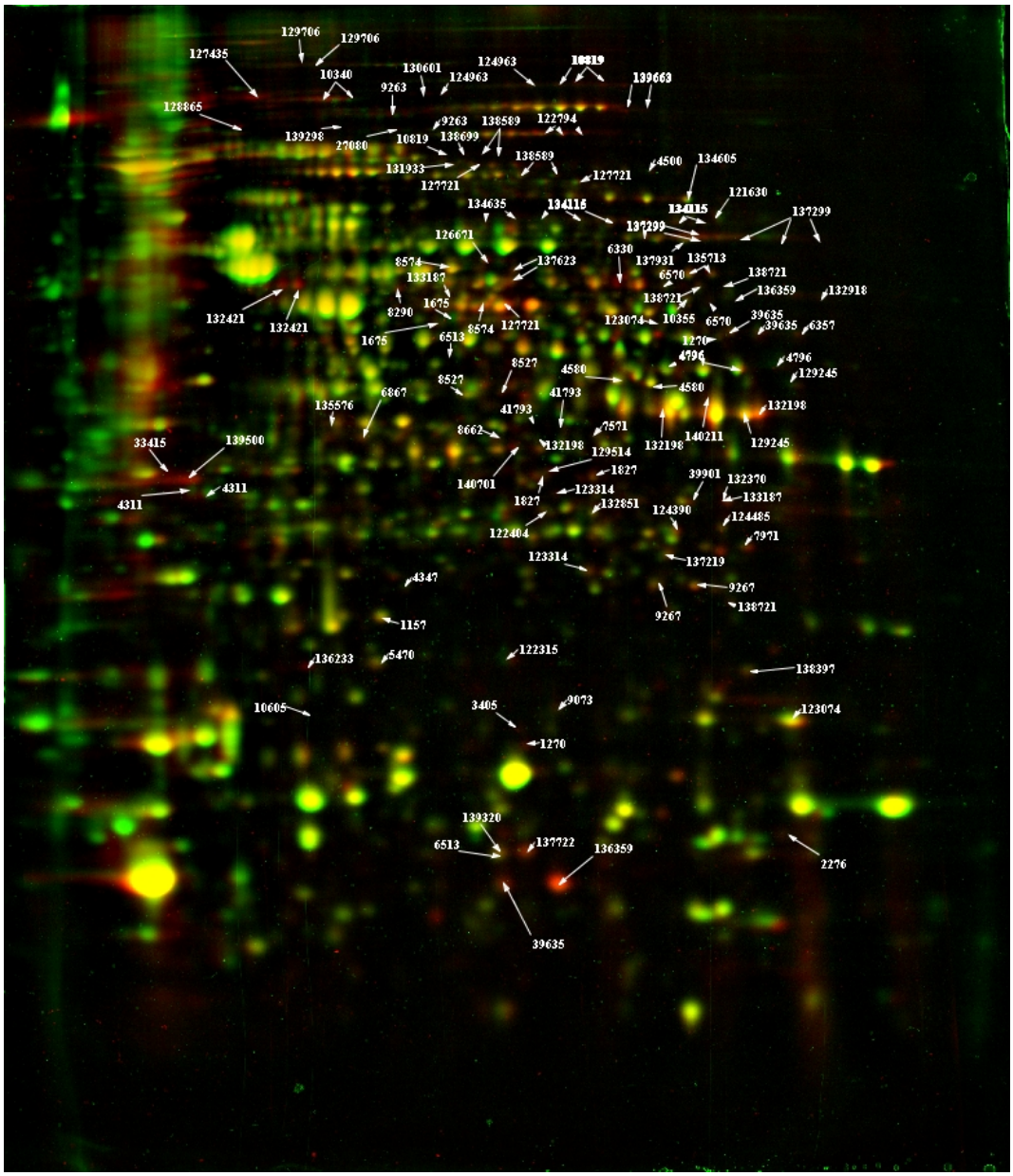

Figure 3.3. Comparison of control sample 2DE with that of 2h 100 µM Cu exposed sample.

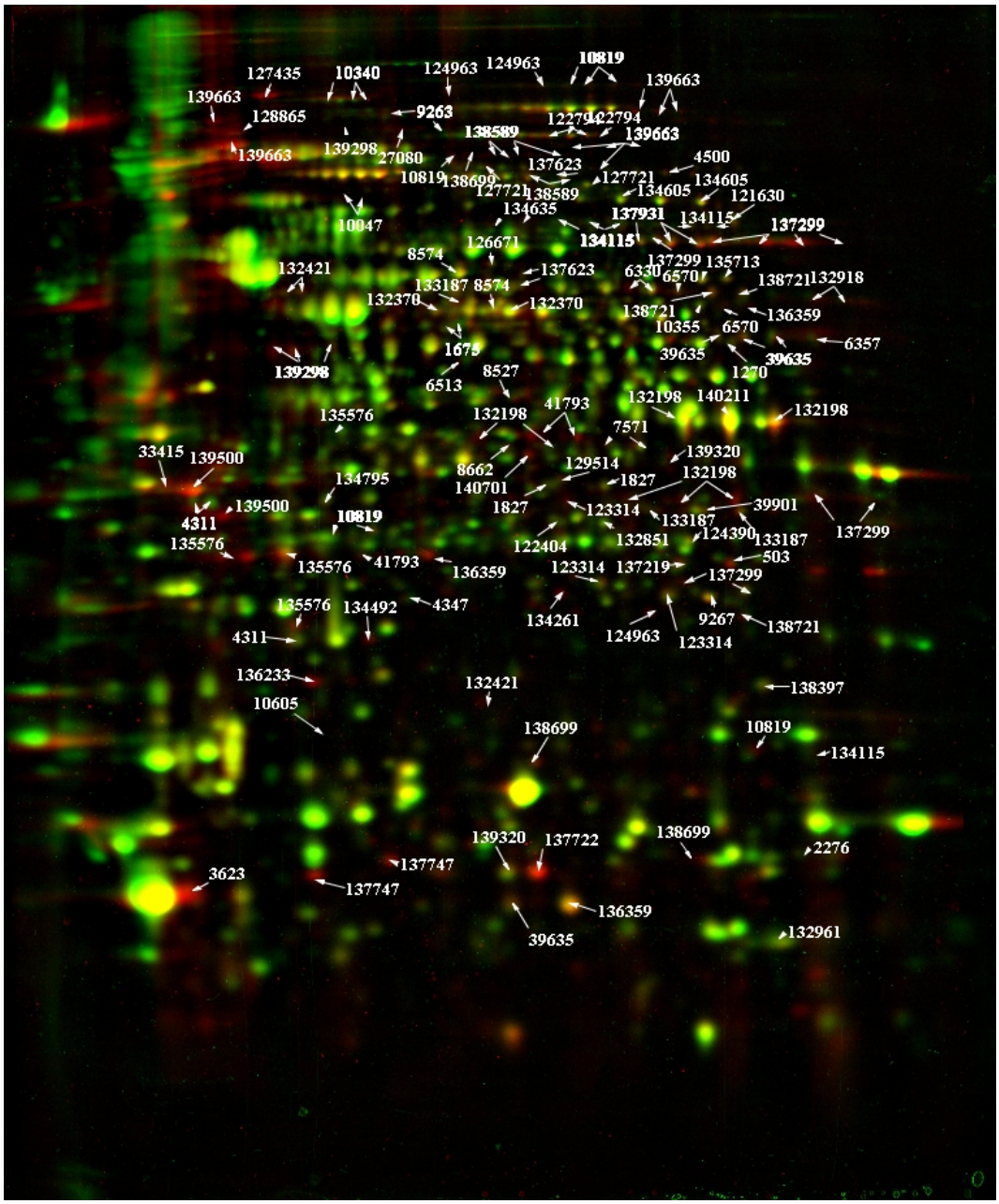

Figure 3.4. Comparison of control sample 2DE with that of 4h 100 µM Cu exposed sample.

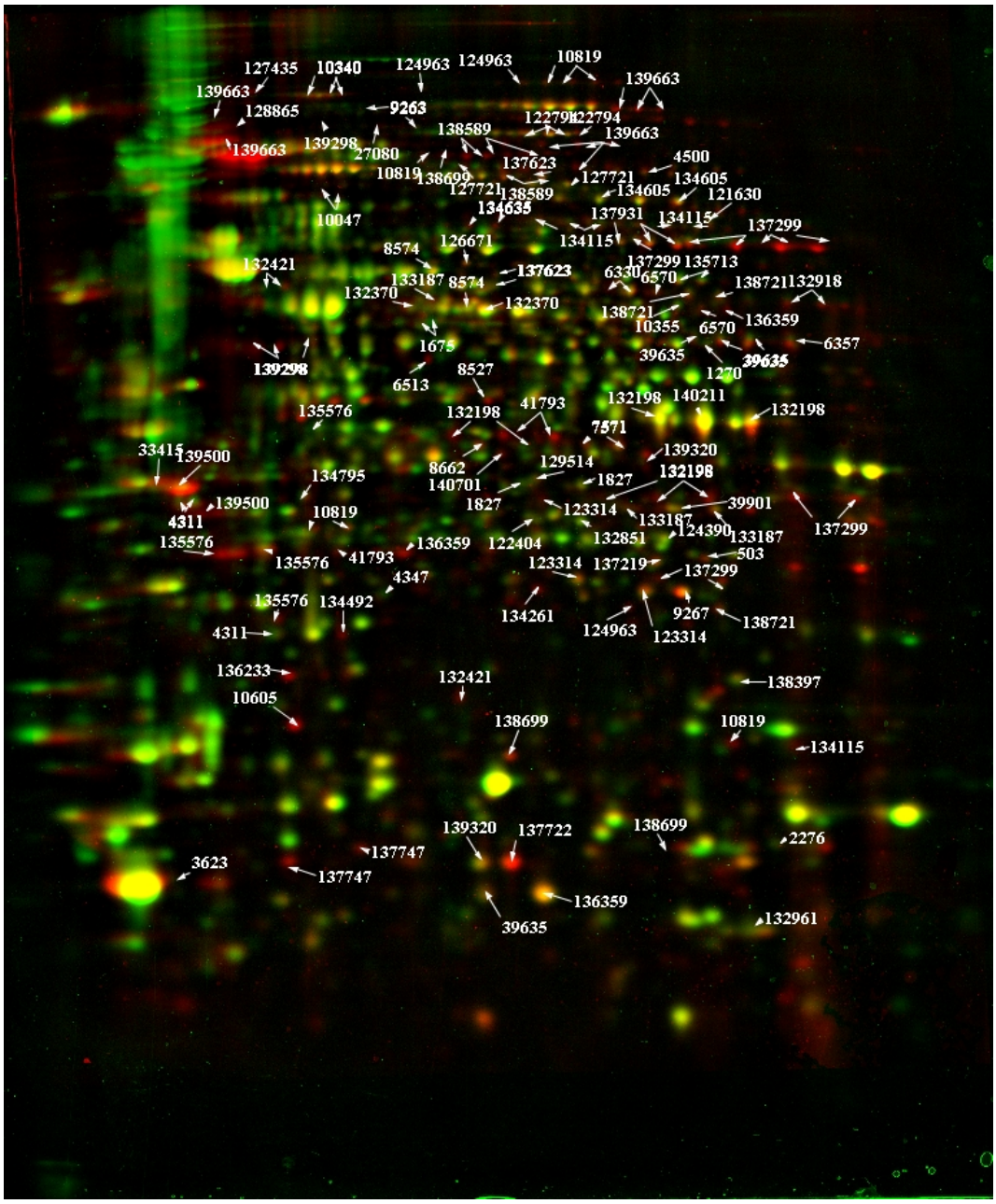

Figure 3.5. Comparison of control sample 2DE with that of 8h 100 µM Cu exposed sample.

Table 3.1. List of upregulated proteins detected on 2D gels in response to 100 µM Cu.

KOG Group	KOG Class	Protein Function	Protein ID	Cellular location	Cu 1h	Cu 2h	Cu 4h	Cu 8h
CELLULAR PROCESSES AND SIGNALING	Cell wall, membrane, envelope biogenesis	UDP-glucose 4-epimerase/UDP-sulfoquinovose synthase	39901	mito	1,53	1,33	1,88	**2,19**
	Cytoskeleton	WD40 repeat stress protein/actin interacting protein	4500	extr	1,49	1,15	1,52	**2,36**
		Actin-related protein Arp2/3 complex, subunit Arp3	126671	cysk	**3,59**	**2,53**	**2,08**	**2,42**
		Actin-binding protein Coronin, contains WD40 repeats	127721	mito	**3,00**	1,74	**2,42**	**2,12**
		Actin-binding protein Coronin, contains WD40 repeats	127721	mito	**2,15**	**2,41**	**2,39**	**2,67**
		Actin and related proteins	139298	cysk	**4,99**	**2,49**	1,48	1,60
	Intracellular trafficking, secretion, and vesicular transport	GTPase Ran/TC4/GSP1 (nuclear protein transport pathway), small G protein family	123314	cyto	**2,36**	1,88	**4,60**	**5,40**
		GTPase Ran/TC4/GSP1 (nuclear protein transport pathway), small G protein family	123314	cyto	1,59	1,28	1,41	**2,00**
	Posttranslational modification, protein turnover, chaperones	20S proteasome, regulatory subunit alpha type PSMA6/SCL1	1827	cyto_mito	**3,20**	**3,62**	**3,06**	**3,48**
		Thioredoxin-like protein	4347	nucl	1,01	1,09	1,24	**2,57**

Table 3.1. List of upregulated proteins detected on 2D gels in response to 100 µM Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	Cellular location	Cu 1h	Cu 2h	Cu 4h	Cu 8h
CELLULAR PROCESSES AND SIGNALING	Posttranslational modification, protein turnover, chaperones	Thioredoxin reductase	8527	mito	1,79	1,96	1,73	**2,31**
		Mitochondrial processing peptidase, beta subunit, and related enzymes (insulinase superfamily)	8574	mito	1,48	**2,34**	**2,18**	**2,28**
		Molecular chaperones HSP105/HSP110/SSE1, HSP70 superfamily	10340	cyto	1,62	**3,04**	**2,84**	**2,28**
		Molecular chaperones HSP105/HSP110/SSE1, HSP70 superfamily	10340	cyto	**5,25**	1,47	**3,19**	**3,89**
		Chaperone HSP104 and related ATP-dependent Clp proteases	124963	cyto	1,26	1,85	**3,74**	1,45
		Chaperone HSP104 and related ATP-dependent Clp proteases	124963	cyto	**2,30**	1,45	**2,04**	*0,42*
		AAA+-type ATPase	127435	nucl	**3,01**	**4,35**	**3,61**	**3,65**
		Molecular chaperone (HSP90 family)	128865	cyto_nucl	**2,28**	**2,03**	**6,62**	**8,12**
		20S proteasome, subunit alpha PSMA7/PRE6	133187	cyto	**2,16**	**2,03**	**3,05**	**2,86**
		20S proteasome, subunit alpha PSMA7/PRE6	133187	cyto	**2,00**	**2,82**	**3,24**	**4,51**
		Multifunctional chaperone (14-3-3 family)	139500	nucl	**4,91**	**3,35**	**7,01**	**7,97**

Table 3.1. List of upregulated proteins detected on 2D gels in response to 100 µM Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	Cellular location	Cu 1h	Cu 2h	Cu 4h	Cu 8h
INFORMATION STORAGE AND PROCESSING	Transcription	Hsp27-ERE-TATA-binding protein/Scaffold attachment factor (SAF-B)	8662	nucl	1,15	0,99	0,94	**2,04**
		Transcription factor containing NAC and TS-N domains	33415	cyto	**10,02**	**9,42**	**13,26**	**15,63**
		RNA polymerase II general transcription factor BTF3 and related proteins	136233	cyto	**2,84**	1,99	**3,48**	**3,77**
	Translation, ribosomal structure and biogenesis	Mitochondrial translation elongation factor Tu	6570	mito	1,49	1,30	1,56	**2,74**
		Mitochondrial translation elongation factor Tu	6570	mito	**2,12**	1,27	**2,10**	**2,15**
		Translation initiation factor 5A (eIF-5A)	10605	cyto	0,66	0,92	1,08	**5,89**
		Elongation factor 2	10819	cyto	**2,99**	1,58	**3,01**	**4,60**
		Elongation factor 2	10819	cyto	**2,56**	1,28	1,93	1,50
		Elongation factor 2	10819	cyto	1,67	**2,23**	1,58	1,61
		Elongation factor 2	10819	cyto	**2,73**	**2,43**	**2,07**	**2,12**
		Translation initiation factor 4F, helicase subunit (eIF-4A) and related helicases	132421	nucl	**5,05**	**7,69**	**5,18**	0,99
		Translation initiation factor 4F, helicase subunit (eIF-4A) and related helicases	132421	nucl	**2,04**	**2,57**	1,45	1,13
		40S ribosomal protein S19	138699	cyto	**3,58**	1,36	1,99	**2,27**

Table 3.1. List of upregulated proteins detected on 2D gels in response to 100 µM Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	Cellular location	Cu 1h	Cu 2h	Cu 4h	Cu 8h
METABOLISM	Amino acid transport and metabolism	Lysine-ketoglutarate reductase/saccharopine dehydrogenase	9263	cyto	0,88	**2,32**	**2,28**	0,46
		Lysine-ketoglutarate reductase/saccharopine dehydrogenase	9263	cyto	0,77	1,08	1,69	**2,28**
		Alpha-isopropylmalate synthase/homocitrate synthase	27080	cyto	1,50	1,49	**3,76**	**2,24**
		Aspartate aminotransferase/Glutamic oxaloacetic transaminase AAT1/GOT2	39635	cyto	**2,19**	1,85	**2,35**	**2,43**
		Aspartate aminotransferase/Glutamic oxaloacetic transaminase AAT1/GOT2	39635	cyto	**2,30**	1,67	1,76	**3,17**
		Aspartate aminotransferase/Glutamic oxaloacetic transaminase AAT1/GOT2	39635	cyto	**2,34**	**2,69**	**2,51**	**3,27**
		Aminomethyl transferase	137722	cyto	**4,13**	**2,88**	**6,65**	**7,63**
		Glycine/serine hydroxymethyltransferase	137931	cyto	1,81	1,90	**2,26**	**2,61**
		Thiamine pyrophosphate-requiring enzyme	138589	cyto	1,16	0,96	1,39	**2,93**
		Thiamine pyrophosphate-requiring enzyme	138589	cyto	**4,36**	1,60	1,77	**6,00**
		Thiamine pyrophosphate-requiring enzyme	138589	cyto	**6,01**	1,88	**3,98**	**10,75**

Table 3.1. List of upregulated proteins detected on 2D gels in response to 100 µM Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	Cellular location	Cu 1h	Cu 2h	Cu 4h	Cu 8h
METABOLISM	Amino acid transport and metabolism	Glutamate/leucine/phenylalanine/valine dehydrogenases	138721	cyto	**2,59**	1,62	**2,49**	**2,67**
		Methionine synthase II (cobalamin-independent)	139663	cyto	1,70	1,37	**2,06**	**4,05**
		Methionine synthase II (cobalamin-independent)	139663	cyto	**3,37**	**3,92**	**3,19**	**7,94**
		Isocitrate dehydrogenase, alpha subunit	139320	mito	1,29	1,52	1,95	**2,59**
	Carbohydrate transport and metabolism	6-phosphogluconate dehydrogenase	6357	cyto	**2,15**	1,08	**2,98**	**2,59**
		Transketolase	122794	cyto	**5,25**	**4,73**	**5,49**	**10,01**
		Transketolase	122794	cyto	**3,92**	**2,40**	**3,13**	**4,08**
		Transketolase	122794	cyto	**3,62**	**2,92**	**3,13**	**3,76**
		Glyceraldehyde 3-phosphate dehydrogenase	132198	cyto	**3,20**	**2,64**	**3,23**	**3,35**
		UDP-glucose pyrophosphorylase	134115	cyto_nucl	1,80	1,66	1,42	**2,17**
		UDP-glucose pyrophosphorylase	134115	cyto_nucl	1,80	0,91	0,67	**2,01**
		UDP-glucose pyrophosphorylase	134115	cyto_nucl	**2,11**	1,77	1,16	1,62
		UDP-glucose pyrophosphorylase	134115	cyto_nucl	**2,29**	1,71	1,23	**2,20**
METABOLISM	Carbohydrate transport and metabolism	Phosphoglucomutase	137623	cyto	**2,50**	**2,48**	**2,05**	1,70
		Phosphoglucomutase	137623	cyto	**2.02**	1,89	1,46	1,68
		S-adenosylmethionine synthetase	132370	cyto	1,93	1,03	**4,06**	**3,50**

Table 3.1. List of upregulated proteins detected on 2D gels in response to 100 µM Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	Cellular location	Cu 1h	Cu 2h	Cu 4h	Cu 8h
METABOLISM	Energy production and conversion	Vacuolar H+-ATPase V1 sector, subunit G	2276	nucl	1,24	1,67	1,58	**2,10**
		NADH:flavin oxidoreductase/12-oxophytodienoate reductase	6330	cyto	**2,79**	**4,92**	**3,18**	**3,33**
		Cytochrome c oxidase, subunit VIa/COX13	6513	mito	**2,32**	0,94	1,99	**5,96**
		Sulfide:quinone oxidoreductase/flavo-binding protein	132918	mito	1,34	1,26	1,69	**2,32**
		Nucleosome remodeling factor, subunit NURF38	135576	nucl	1,79	**2,68**	0,90	1,28
		Fumarase	135713	mito	1,72	**2,24**	1,69	1,66
		Fumarase	135713	mito	1,98	**2,12**	1,82	1,91
		Citrate synthase	136359	mito	1,32	0,79	0,70	**2,29**
		Citrate synthase	136359	mito	**3,60**	**4,18**	**3,86**	**5,15**
		Glyoxylate/hydroxypyruvate reductase (D-isomer-specific 2-hydroxy acid dehydrogenase superfamily)	140211	cyto	1,53	**2,01**	1,86	1,41
	Inorganic ion transport and metabolism	Manganese superoxide dismutase	9267	mito	1,93	1,53	**2,26**	**2,96**
		Manganese superoxide dismutase	9267	mito	**2,19**	1,82	**2,29**	**4,25**

Table 3.1. List of upregulated proteins detected on 2D gels in response to 100 µM Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	Cellular location	Cu 1h	Cu 2h	Cu 4h	Cu 8h
METABOLISM	Energy production and conversion	F0F1-type ATP synthase, alpha subunit	137299	mito	**2,05**	1,52	1,66	1,89
		F0F1-type ATP synthase, alpha subunit	137299	mito	**3,62**	**2,60**	**3,12**	**2,55**
		F0F1-type ATP synthase, alpha subunit	137299	mito	**2,15**	0,99	2,48	1,20
		F0F1-type ATP synthase, alpha subunit	137299	mito	**3,68**	1,43	**2,40**	**2,01**
	Lipid transport and metabolism	Mevalonate pyrophosphate decarboxylase	10355	mito	1,47	1,43	1,39	**2,10**
		Methylthioadenosine phosphorylase MTAP	129514	cyto	**2,49**	**2,09**	**2,61**	**3,41**
	Secondary metabolites biosynthesis, transport and catabolism	Sorbitol dehydrogenase	1675	cyto	1,39	**2,34**	1,77	**3,38**
		Uricase (urate oxidase)	41793	cyto	**3,18**	**2,24**	**5,23**	**5,89**
		Uricase (urate oxidase)	41793	cyto	**2,12**	0,60	**2,21**	**3,88**
POORLY CHARACTERIZ	General function prediction only	Aldo/keto reductase family proteins	7571	cyto	**2,10**	1,48	1,32	2,22
		mRNA splicing factor	134635	cyto	**2,42**	1,83	1,56	1,79
Unknown	Unknown	Hypothetical protein	4311	cyto	**2,41**	**2,98**	**6,07**	**9,07**
Unknown	Unknown	Hypothetical protein	4311	cyto	1,18	0,82	1,91	**2,14**
Unknown	Unknown	Hypothetical protein	138397	extr	1,66	1,50	1,89	**2,08**
Unknown	Unknown	Hypothetical protein	140701	nucl	1,60	**2,05**	**2,79**	**2,14**

***Cyto**; cytoplasmic, **Extr**; extracellular, **Mito**; mitochondrial, **Nucl**; Nuclear, **Cysk**; cytoskeleton, **Cyto_nucl**; cytoplasmic_nuclear

TMH: Number of predicted transmembrane helices

Table 3.2. List of downregulated proteins detected on 2D gels in response to 100 μM Cu.

KOG Group	KOG Class	Protein Function	Protein Id	Cellular location*	Cu 1h	Cu 2h	Cu 4h	Cu 8h
CELLULAR PROCESSES AND SIGNALING	Cytoskeleton	Beta tubulin	125674	nucl	0.40	0.47	0.39	0.29
	Nuclear structure	Nucleolar GTPase/ATPase p130	465	nucl	0.26	0.40	0.16	0.12
	Posttranslational modification, protein turnover, chaperones	Molecular chaperone (small heat-shock protein Hsp26/Hsp42)	137747	cyto_nucl	0.55	0.39	0.09	0.03
		Ubiquitin-conjugating enzyme E2	2075	cyto	0.25	0.46	0.13	0.07
		FKBP-type peptidyl-prolyl cis-trans isomerase	44306	cyto	0.28	0.32	0.08	0.03
		Dipeptidyl aminopeptidase	5061	extr	0.20	0.12	0.17	0.10
		Molecular chaperones GRP170/SIL1, HSP70 superfamily	5586	cyto_mito	0.38	0.34	0.47	0.26
INFORMATION STORAGE AND PROCESSING	RNA processing and modification	Ribosomal protein RPL1/RPL2/RL4L4	128104	extr	0.61	0.41	0.29	0.65
	Translation, ribosomal structure and biogenesis	40S ribosomal protein S3A	139199	nucl	0.36	0.30	0.24	0.07
METABOLISM	Carbohydrate transport and metabolism	Enolase	135734	cyto	0.56	0.61	0.41	0.17
		Beta-galactosidase	9466	extr	0.35	0.43	0.75	0.68

Table 3.2. List of downregulated proteins detected on 2D gels in response to 100 µM Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein Id	Cellular location	Cu 1h	Cu 2h	Cu 4h	Cu 8h
METABOLISM	Energy production and conversion	UDP-glucuronosyl and UDP-glucosyl transferase	3221	mito	0.47	0.59	0.30	0.15
		UDP-glucuronosyl and UDP-glucosyl transferase	3221	mito	0.72	0.51	0.77	0.09
POORLY CHARACTERIZED	General function prediction only	Multiple inositol polyphosphate phosphatase	121720	cyto	0.31	0.39	0.21	0.10
		Predicted hydrolase related to dienelactone hydrolase	6857	cyto	0.51	0.29	0.04	0.04
Unknown	Unknown	Hypothetical protein	1249	mito	0.59	0.42	0.47	0.76

***Cyto**; cytoplasmic, **Extr**; extracellular, **Mito**; mitochondrial, **Nucl**; Nuclear, **Cysk**; cytoskeleton, **Cyto_nucl**; cytoplasmic_nuclear

TMH: Number of predicted transmembrane helices

3.2.2. 2DE analysis of cadmium-induced proteins

Mid-log cultures (40h) of *P. chrysosporium* were exposed to 100 μM cadmium for 8h. At the time intervals of 1h, 2h, 4h and 8h, the samples were collected together with their respective controls. Each of the samples was analyzed by using 2D-PAGE approach. After the detection of the differentially expressed proteins, they were submitted to MALDI-ToF analysis for identification.

A total of 130 spots were detected as differentially expressed after Cd exposure for 1h, 2h, 4h and 8h in *P. chrysosporium* proteome, respectively. MALDI-TOF analysis of these 130 protein spots identified 89 of them. Further analysis revealed that the 89 protein spots are the products of 58 distinct ORFs. Protein Id numbers of the identified spots are depicted on each gel through Figure 3.6 to 3.9. A detailed comparison of the semiquantitative abundance of each spot and their functions are tabulated in Table 3.3.

3.2.2.1. Elements of cytoskeleton

A total of 4 proteins which are the elements of cytoskeleton were identified as upregulated after Cd exposure: Two actin related proteins (1 26671, 139298) and two WD40 repeat containing actin binding proteins (127721, 4500). Protein having the Id number 127721 was found exist in three spots in the cells. Proteins containing WD40 repeats function in a wide range of cellular processes, such as vesicular trafficking, transcription regulation and cell cycle control (Srinivasan et al., 2007). It is thought that WD40 sites work as a docking site for other proteins, thus coordinating the assembly of multiprotein complexes. It was also shown that a WD40 protein (RACK1) regulates stress response by interacting diverse stress related proteins in A*rabidopsis* (Kundu et al., 2013). Many extracellular stimuli cause rapid changes in cytoskeleton, and these changes may be accepted as signals by other pathways within the cell which regulate cellular responses. Zhao et al. (2013) showed that Arp2/3 complex protein regulates salt stress response of the A*rabidopsis*. Since WD40 proteins and actins were found to be upregulated in

different time points in this study, it can be concluded that these proteins together play a pivotal role in the stress response throughout Cd exposure.

3.2.2.2. Intracellular trafficking, secretion, and vesicular transport

Ras GTPase superfamily consists of several signaling GTPases such as Ran, Ras, Rab, Arf, Rad etc (Wennerberg et al., 2005). Ras related nuclear protein is designated as Ran GTPase and it was found to be constantly upregulated during the Cd exposure (protein id: 123314). There are reports indicating that Ran GTPases are linked to stress response cascades of cold stress (Xu and Cai, 2014), oxidative stress (Datta et al., 2014) and salt stress (Wang et al., 2013). Cd exposure led us to see the function of GTPase Ran protein (123314) as another time (Section 3.1.1.1.) as a heavy metal responsive protein, acting in both Cu and Cd stresses. As to Cd exposure, it was found to be upregulated constantly, but with the peaks in between 2 to 4h of Cd exposure. Moreover, in our previous studies, Ras GTPase was found to be responsive against Cd and Pb exposures (Özcan et al., 2007; Yıldırım et al., 2011) providing a solid evidence for the existence of Ras signaling pathway in *P. chrysosporium*. This pathway interacts with actin-related proteins and triggers accelerating programmed cell death in *C. albicans* under harsh environmental conditions, as explained earlier in this text (Phillips et al., 2006)

Intracellular trafficking protein, mitochondrial inner translocase TIM23 subunit (137219) was found to be upregulated after 4h Cd exposure. In a recent study, Rainbolt et al. (2013) showed that degradation of the TIM17A, a core subunit of TIM23 complex of mammalian cells, is required to resist the arsenite stress. TIM23 complex transports the proteins in to the mitochondria. However under the mitochondrial stress, this continuous transportation has to be ceased to avoid misfolded protein aggregation and to ease the burden on the chaperones. Thus the TIM17A is degraded rapidly to stop the transportation under the stress conditions. Upregulation of this protein not immediately, but after 4h of cadmium exposure suggests that more mitochondrial proteins are required to be transported into the

mitochondria at this time. In addition, when the overall data are examined, a slight increase in the number of upregulated mitochondrial proteins could be observed after 4h Cd exposure.

A mitochondrial peptidase which actually belongs to the functional class of postranslational modification and protein turnover chaperons, is also found to be upregulated. Mitochondrial peptidases are essential for the maturation process of the proteins which are translocated into the mitochondria via TIM23 complex (Lionaki and Tavernarakis, 2013). Detection of the upregulation of the protein only in 2h exposed samples indicates that there occurs an increasing demand for the maturation of the proteins in mitochondria at this time upon Cd exposure.

3.2.2.3. Posttranslational modification, protein turnover, chaperones

It is known that Cd causes ROS production and oxidative stress (Szuster-Ciesielska et al., 2000; Wang et al., 2004). 20S proteasome complex degrades especially oxidized proteins (Davies, 2001). Three of the subunits of the 20S proteasome complex were found to be upregulated in response to Cd and two subunits were found to be upregulated also in response to Cu, implying Cu or ROS induced regulation of 20S proteasome complex subunits.

Chaperones are one of the main elements of the stress response mechanisms. In this study, several chaperones were found to be upregulated in response to heavy metal stress. A HSP70 family chaperon (10340) was upregulated and identified as multiple spots on the 2DE gels of E Cd treated samples. Hsp70 proteins recognize N-terminal sequences of the mitochondria targeted proteins and deliver them to outer membrane translocases (TOM) of mitochondria (Abe et al., 2001). There are many reports mentioning about HSP70 upregulation in fungi like *S. cerevisiae*, *Rhizopus nigricans* and the aquatic hyphomycete *Heliscus lugdunensis* in response to environmental stresses (Boorstein et al., 1994; Cernila et al., 2000; Miersch and Grancharov, 2008). Two spots identified as HSP70 were being upregulated in 1h to 4h Cd exposed samples. These results further prove that Cd

causes increasing intracellular protein trafficking towards mitochondria.

The other chaperones, HSP90 (128865) and HSP104 (124963) were also upregulated. HSP90 is known to work together with HSP70 to deliver mitochondrial proteins (Lionaki and Tavernarakis, 2013). HSP104 is a well known general stress response chaperone (Sanchez and Taulien, 1992). It is mostly important for deggragating misfolded proteins as caused by heavy metals (Tamás et al., 2014). In a study conducted with *S. cerevisiae* deletion mutants of HSP104, it was found that the deletion of ATP-binding sites made the yeast cells cadmium sensitive (Lee and Ueom, 2001). HSP104 was found to be upregulated for 1h, 2h and 4h Cd exposures,reaffirming that this chaperonin is a very important element of the response metabolism. Another continuously upregulated chaperon was 14-3-3 family chaperone (139500) which together with H^+ATPases (127435) plays a key role in regulating the stress response against Cu, as explained in a previous section. The finding that 14-3-3 protein is upregulated in between 1 to 4h cadmium exposure shows the regulation by this protein is valid for both Cu and Cd response.

Two antioxidant proteins namely, alkyl hydroperoxide reductase (6867) and thioredoxin reductase (8527) were also upregulated. Upregulation of thioredoxin reductase was also recorded in our previous work investigating the response to Pb stress (Yıldırım et al., 2011). In *E. coli,* alkyl hydroperoxide reductase (Ahp) was defined as the primary scavenger for endogenous hydrogen peroxide (Seaver and Imlay, 2001). In another study of interest, the investigators speculated that an alkyl hydroperoxide reductase protein may possess stress-induced chaperone property in response to oxidative stress in *Helicobacter pylori* (Chuang et al., 2006). Both of these studies, along with that of us indicate that Ahp is an element of the response mechanism against ROS generating toxicities Since Cd is known to induce ROS production, upregulation of these ROS scavengers should be a part of antioxidation mechanisms of *P. chrysosporium*.

3.2.2.4. Elements of transcription

A highly upregulated transcription factor containing NAC and T-SN domains (33415) were detected in all of the Cd-exposed samples. As explained earlier, this protein was also found to be upregulated in all of the Cu-exposed samples. It appears that this transcription factors one of the main characteristics of the stress response in *P. chrysosporium*. Upregulation of the transcription factor BTF3 as mentioned and discussed for Cu-exposed samples shows that the organism is in a process to produce more transcripts requiring BTF3 activation under the conditions of heavy metal stress..

3.2.2.5. Translation and ribosomal structure

The upregulated translational factors in response to Cd were identified as mitochondrial translation elongation factor Tu, translation initiation factor 5A (eIF-5A), elongation factor 2, and translation initiation factor 4FA (eIF-4FA). Mitochondrial elongation factor Tu (EF-Tu) is a multifunctional protein which was found to be associated with Cu response and already discussed . Although it is suggested that eIF5A plays a role in mRNA turnover, its precise function is only partially understood. EIF5A is apparently the only hypusine-containing protein (post-translationally synthesized polyamine-derived amino acid) (Park, 2006)). The expression levels of OseeIF5A-1 and OseIF5A-2 were investigated in response to copper in rice suspension cells (Chou et al., 2004). The authors reported that mRNA levels of OseIF5A-1 and OseIF5A-2increased within first three hours of the copper exposure and continued to accumulate throughout a period of 24 h. eIF4A is one of the subunit of the initiation complex eIF4F. Under abiotic stress conditions, the subunits of this complex are affected via various mechanisms and formation of this complex is prevented (Muñoz and Castellano, 2012). Shenton et al., (2006) has suggested that these damaged proteins have to be replenished by upregulation to provide a signal for recovery.

3.2.2.6. Amino acid metabolism

Proteins belonging to amino acid metabolism were also found to be upregulated after Cd exposure. Aspartate aminotransferase, thiamine pyrophosphate and methionine synthase II (cobalamin-independent) are among such proteins that were also found to be upregulated in the Cu-exposed samples. As it was discussed in the relevant sections, Cu exposure results in aspartate aminotransferase upregulation which is in turn a result of NAD/NADH ratio change while cobalamin-independent methionine synthase upregulation appeared to be most likely due to the organism's effort to replenish the ROS-damaged enzyme. On the other hand, a possible direct link between the upregulation of thiamine pyrophosphate and heavy metal response looks like a puzzle with missing pieces at the current stage.

3.2.2.7. Carbohydrate metabolism

Upregulated proteins of this class included 6-phosphogluconate dehydrogenase, transketolase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), UDP-glucose pyrophosphorylase, pyruvate kinase, and phosphoglucomutase. Upregulation of glycolysis in response to oxidative stress already stands as a proposed mechanism for human cells (Kondoh et al., 2007; Wu and Wei, 2012). The enzymes 6-phosphogluconate dehydrogenase and transketolase are the members of the pentose phosphate pathway. Cd causes a similar response with Cu, forcing the organism to use this pathway. Redirection of metabolic flux from glycolysis to pentose phosphate pathway in response to oxidative stress was extensively reviewed by Grant (2008). It was suggested that this metabolic redirection supplies more NADPH, the reducing power required for the main cellular redox systems coping with the relevant stress.. These mechanisms are believed to be highly conserved in organisms ranging from yeast to human. The upregulated enzymes of these two pathways in response to Cd and Cu indicates that the same mechanism is utilized by the white rot fungus too, in order to fight with heavy metal-induced toxicity.

3.2.2.8. Hypothetical proteins

Cadmium treatment resulted in upregulation of 4 distinct proteins which are not identified in the current *P. chrysosporium* database. Id numbers of these proteins are as follows: 1270, 4311, 138397 and 140701. These proteins deserve further investigations with respect to their functions and relations with the heavy metal stress response.

3.2.2.9. Downregulated proteins in response to Cd

A total of 17 proteins were found to be downregulated in response to Cd stress. "Posttranslational modifications, protein turnover and chaperones" constituted the major group negatively affected by Cd, as the 5 of the downregulated proteins belonged to this group. As it was detected in the case of Cu exposure, the small heat-shock protein Hsp26/Hsp42 (137747) was found to be downregulated at one spot and upregulated at another spot at the time points of 1h 2h, 4h and 8h. As speculated earlier, this could be due to activation of the protein in response to stress by switching its form from an oligomer to monomer (Haslbeck et al., 1999).

The other significantly downregulated proteins were nucleolar GTPase, ubiquitin-conjugating enzyme E2, FKBP-type peptidyl-prolyl cis-trans isomerase, dipeptidyl aminopeptidase, molecular chaperones GRP170/SIL1, ribosomal protein RPL1/RPL2/RL4L4, 40S ribosomal protein S3A, enolase and three predicted hypothetical proteins, as in the case of Cu exposure.

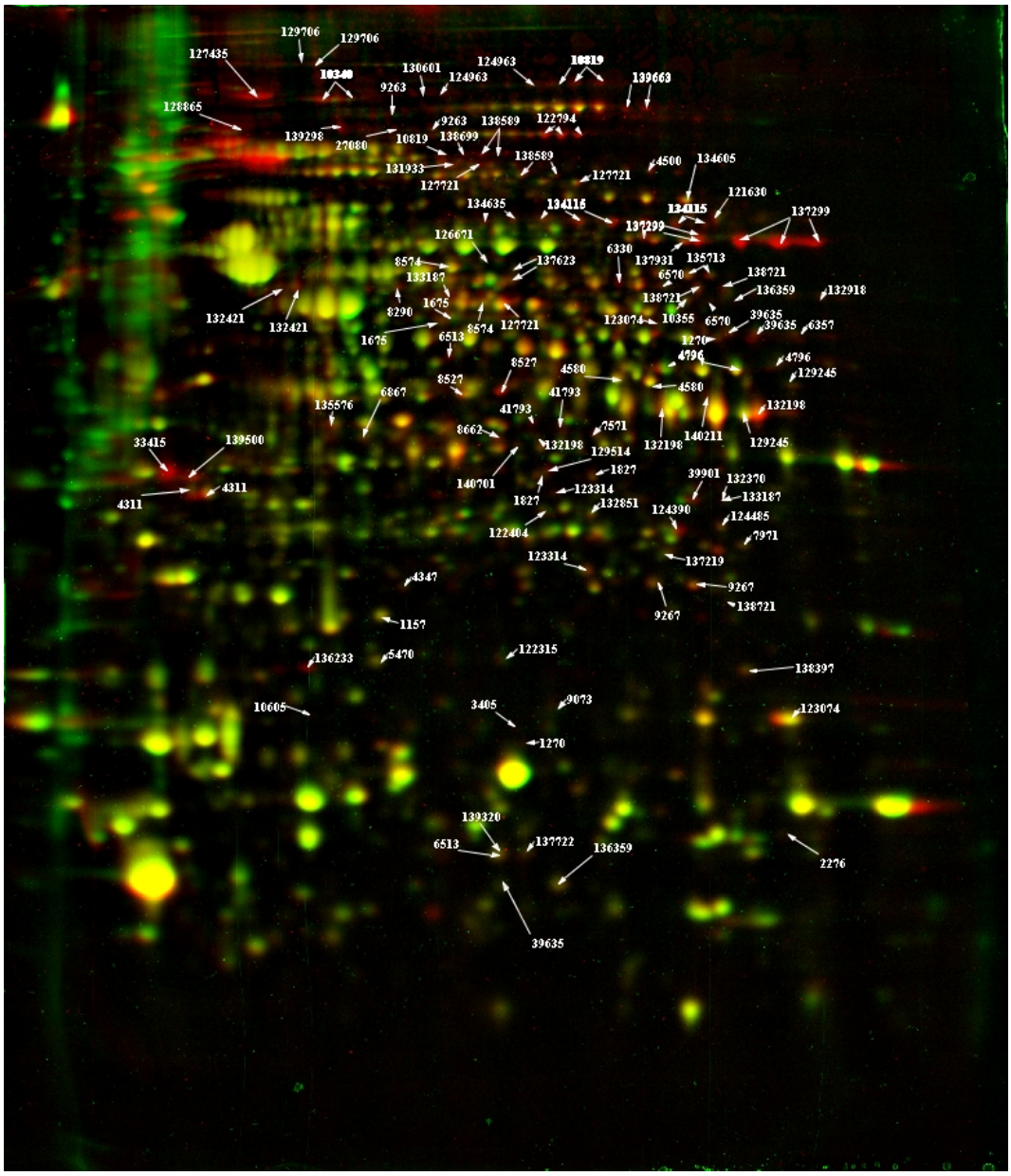

Figure 3.6.Comparison of control sample 2DE with that of 1h 100 µm Cd exposed sample.

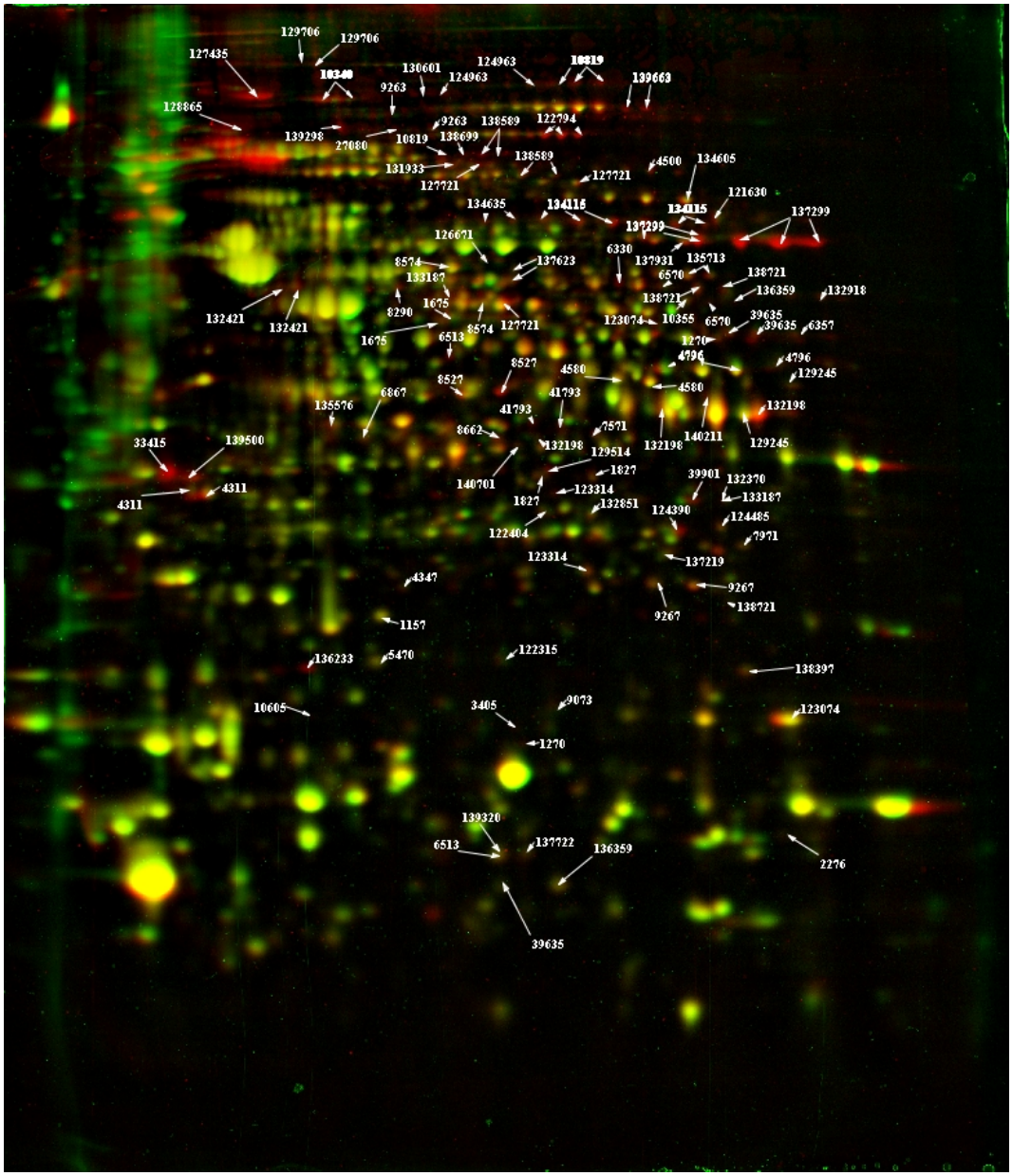

Figure 3.7. Comparison of control sample 2DE with that of 2h 100 µm Cd exposed sample.

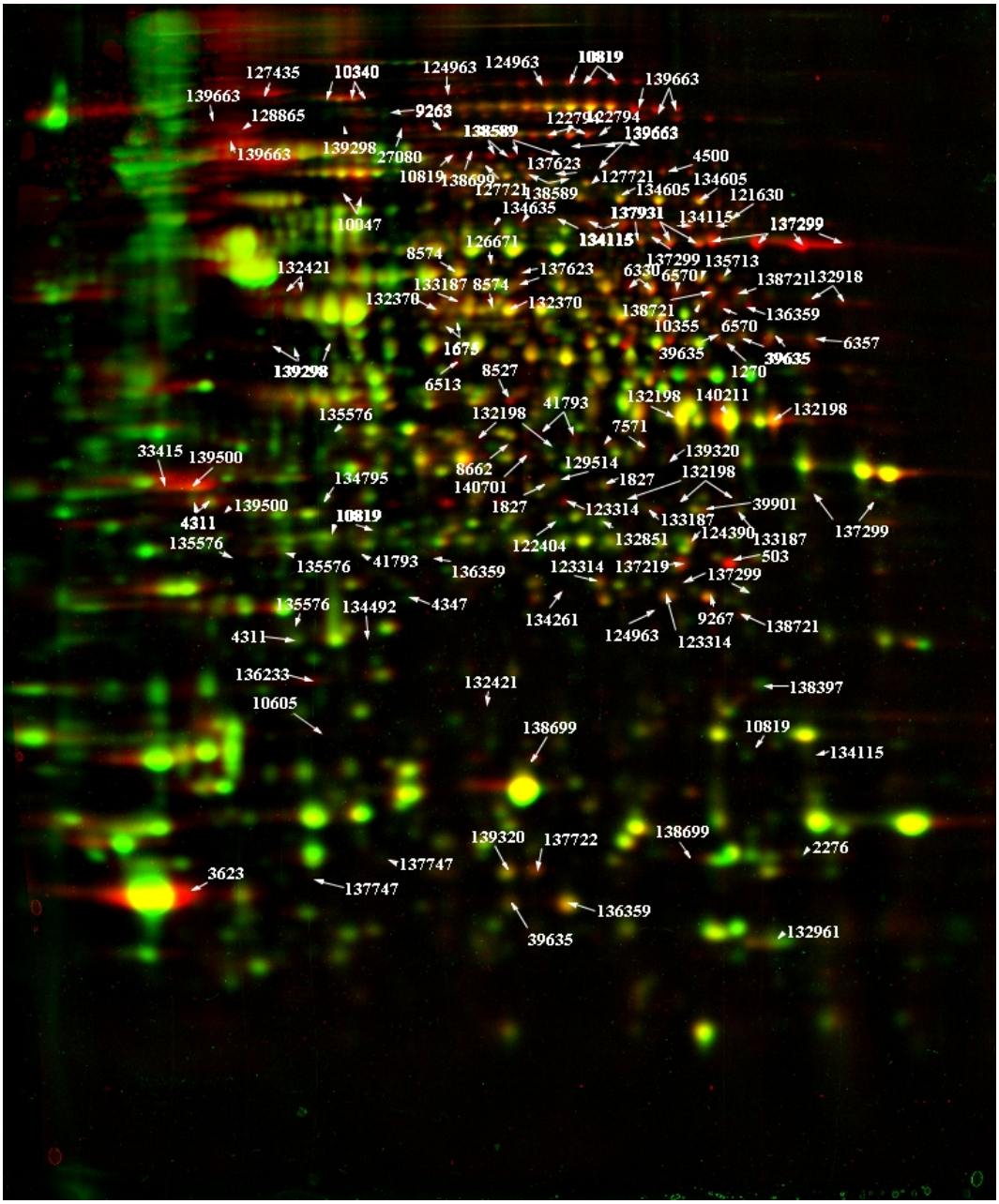

Figure 3.8. Comparison of control sample 2DE with that of 4h 100 µm Cd exposed sample.

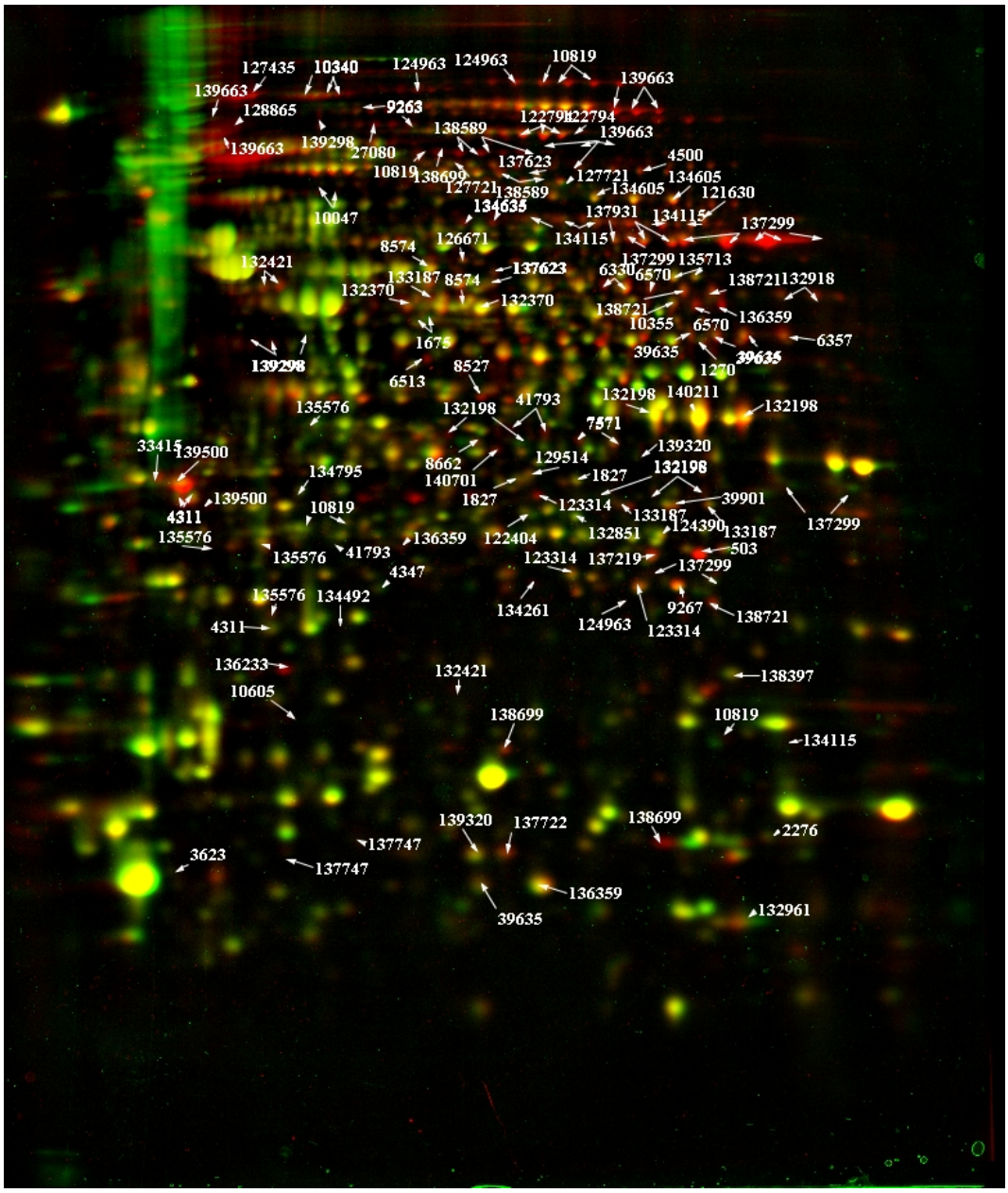

Figure 3.9. Comparison of the control sample 2DE with that of 8h 100 µm Cd exposed sample.

Table 3.3. List of upregulated proteins on 2D gels in response to 100 µm Cd

KOG Group	KOG Class	Protein Function	Protein ID	Cellular location	Cd 1h	Cd 2h	Cd 4h	Cd 8h
CELLULAR PROCESSES AND SIGNALING	Cytoskeleton	WD40 repeat stress protein/actin interacting protein	4500	extr	**2,39**	**2,33**	**2,27**	0,83
		Actin-related protein Arp2/3 complex, subunit Arp3	126671	cysk	1,50	**2,55**	1,68	1,10
		Actin-binding protein Coronin, contains WD40 repeats	127721	mito	**3,82**	**4,28**	**2,70**	1,30
		Actin-binding protein Coronin, contains WD40 repeats	127721	mito	1,76	**2.01**	**1,97**	1,50
		Actin-binding protein Coronin, contains WD40 repeats	127721	mito	**2,09**	**2,41**	**2,44**	**2,09**
		Actin and related proteins	139298	cysk	**8,35**	1,73	**2,15**	1,00
	Intracellular trafficking, secretion, and vesicular transport	GTPase Ran/TC4/GSP1 (nuclear protein transport pathway), small G protein superfamily	123314	cyto	**2,33**	**3,79**	**4,20**	**2,00**
		Mitochondrial import inner membrane translocase, subunit TIM23	137219	cyto	1,16	1,77	**2,23**	1,12
	Posttranslational modification, protein turnover, chaperones	20S proteasome, regulatory subunit alpha type PSMA6/SCL1	1827	cyto_mito	**2,63**	**2,90**	**3,29**	**3,15**
		20S proteasome, regulatory subunit beta type PSMB1/PRE7	130601	cyto	1,97	**2,96**	**4,19**	1,33
		20S proteasome, regulatory subunit alpha type PSMA7/PRE6	133187	cyto	**2,15**	**2,12**	**2,69**	1,56

Table 3.3. List of upregulated proteins on 2D gels in response to 100 µm Cd (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	Cellular location	Cd 1h	Cd 2h	Cd 4h	Cd 8h
CELLULAR PROCESSES AND SIGNALING	Posttranslational modification, protein turnover, chaperones	Mitochondrial processing peptidase, beta subunit, and related enzymes	8574	mito	1,20	**2,25**	1,62	1,75
		Molecular chaperones HSP105/HSP110/SSE1, HSP70 superfamily	10340	cyto	**3,60**	**2,61**	**2,10**	1,17
		Molecular chaperones HSP105/HSP110/SSE1, HSP70 superfamily	10340	cyto	**3,42**	**4,90**	**6,58**	1,43
		Chaperone HSP104 and related ATP-dependent Clp proteases	124963	cyto	**3,61**	**5,04**	**5,98**	1,30
		Chaperone HSP104 and related ATP-dependent Clp proteases	124963	cyto	**3,03**	**2,86**	**4,81**	1,44
		AAA+-type ATPase	127435	nucl	**8,22**	**6,22**	**6,54**	0,42
		Molecular chaperone (HSP90 family)	128865	cyto_nucl	**10,97**	**10,84**	**11,81**	0,48
		Alkyl hydroperoxide reductase, thiol specific antioxidant and related enzymes	6867	mito	1,33	1,92	**2,08**	1,91
		Thioredoxin reductase	8527	mito	**2,33**	**2,36**	**2,69**	1,41
		Multifunctional chaperone (14-3-3 family)	139500	nucl	**3,89**	**6,05**	**8,03**	**2,20**
INFORMATION STORAGE AND PROCESSING	Transcription	Transcription factor containing NAC and TS-N domains	33415	cyto	**18,92**	**9,80**	**18,06**	**8,85**
		RNA polymerase II general transcription factor BTF3 and related proteins	136233	cyto	**2,33**	**2,90**	**2,42**	0,79

Table 3.3. List of upregulated proteins on 2D gels in response to 100 μm Cd (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	Cellular location	Cd 1h	Cd 2h	Cd 4h	Cd 8h
INFORMATION STORAGE AND PROCESSING	Translation, ribosomal structure and biogenesis	Mitochondrial translation elongation factor Tu	6570	mito	1,68	**2,81**	**3,77**	1,53
		Mitochondrial translation elongation factor Tu	6570	mito	1,77	**3,06**	**2,79**	0,76
		Translation initiation factor 5A (eIF-5A)	10605	cyto	0,67	**4,98**	1,34	1,27
		Elongation factor 2	10819	cyto	**3,57**	**5,44**	**7,55**	1,63
		Elongation factor 2	10819	cyto	**3,96**	**4,90**	**5,57**	**2,66**
		Elongation factor 2	10819	cyto	**3,83**	**3,22**	**3,30**	1,29
		Elongation factor 2	10819	cyto	**4,01**	**5,06**	**6,07**	**2,04**
		Translation initiation factor 4F, helicase subunit (eIF-4A) and related helicases	132421	nucl	**4,70**	**5,39**	**6,12**	**2,32**
		Translation initiation factor 4F, helicase subunit (eIF-4A) and related helicases	132421	nucl	1,97	**2,18**	1,56	0,63
		40S ribosomal protein S19	138699	cyto	**2,47**	**4,64**	**3,93**	0,62
METABOLISM	Amino acid transport and metabolism	Lysine-ketoglutarate reductase/saccharopine dehydrogenase	9263	cyto	**2,96**	**2,25**	1,39	0,97
		Lysine-ketoglutarate reductase/saccharopine dehydrogenase	9263	cyto	**2,67**	**3,57**	**4,64**	0,98
		Alpha-isopropylmalate synthase	27080	cyto	**3,78**	**4,24**	**4,21**	0,41

Table 3.3. List of upregulated proteins on 2D gels in response to 100 µm Cd (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	Cellular location	Cd 1h	Cd 2h	Cd 4h	Cd 8h
METABOLISM	Amino acid transport and metabolism	Aspartate aminotransferase/Glutamic oxaloacetic transaminase AAT1/GOT2	39635	cyto	**2,09**	**2,15**	**2,32**	1,51
		Aspartate aminotransferase/Glutamic oxaloacetic transaminase AAT1/GOT2	39635	cyto	**2,57**	**2,78**	**3,16**	1,42
		Aspartate aminotransferase/Glutamic oxaloacetic transaminase AAT1/GOT2	39635	cyto	1,03	1,07	1,78	**2,13**
		Delta-1-pyrroline-5-carboxylate dehydrogenase	121630	cyto	1,88	**2,13**	1,80	1,18
		Aminomethyl transferase	137722	cyto	1,47	1,80	**2,35**	1,50
		Glycine/serine hydroxymethyltransferase	137931	cyto	**2,10**	1,80	**2,25**	0,98
		Thiamine pyrophosphate-requiring enzyme	138589	cyto	1,67	**2,54**	1,90	1,66
		Thiamine pyrophosphate-requiring enzyme	138589	cyto	**4,29**	**8,88**	**5,34**	1,15
		Thiamine pyrophosphate-requiring enzyme	138589	cyto	**9,22**	**16,74**	**11,20**	0,77
		Glutamate/leucine/phenylalanine/valine dehydrogenases	138721	cyto	1,60	**2,29**	**2,40**	1,12
		Glutamate/leucine/phenylalanine/valine dehydrogenases	138721	cyto	**2,89**	**3,33**	**4,16**	1,80
		Methionine synthase II (cobalamin-independent)	139663	cyto	**4,60**	**5,37**	**5,25**	1,17
		Methionine synthase II (cobalamin-independent)	139663	cyto	**4,35**	**5,03**	**7,44**	1,83

Table 3.3. List of upregulated proteins on 2D gels in response to 100 μm Cd (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	Cellular location	Cd 1h	Cd 2h	Cd 4h	Cd 8h
METABOLISM	Energy production and conversion	Citrate synthase	136359	mito	1,11	**2,36**	**3,02**	0,89
		Citrate synthase	136359	mito	1,28	**2,18**	**2,98**	**3,18**
		F0F1-type ATP synthase, alpha subunit	137299	mito	**2,69**	**2,31**	**2,59**	0,87
		F0F1-type ATP synthase, alpha subunit	137299	mito	**4,59**	**4,47**	**5,17**	1,64
		F0F1-type ATP synthase, alpha subunit	137299	mito	**4,21**	**3,79**	**3,16**	0,65
		F0F1-type ATP synthase, alpha subunit	137299	mito	**4,03**	**3,48**	**3,58**	1,12
		F0F1-type ATP synthase, alpha subunit	137299	mito	**5,04**	**7,54**	**7,54**	1,22
		Glyoxylate/hydroxypyruvate reductase (D-isomer-specific 2-hydroxy acid dehydrogenase superfamily)	140211	cyto	**2,00**	**2,18**	**3,59**	1,19
	Inorganic ion transport and metabolism	Manganese superoxide dismutase	9267	mito	1,79	**2,01**	**2,31**	1,56
		Manganese superoxide dismutase	9267	mito	1,87	**2,62**	**2,79**	1,98
		Manganese superoxide dismutase	131933	mito	**2,55**	1,69	1,94	1,07
	Lipid transport and metabolism	Mevalonate pyrophosphate decarboxylase	10355	mito	1,65	1,33	**2,42**	1,69
		Isoamyl acetate-hydrolyzing esterase	122404	nucl	1,30	1,16	0,81	**2,03**
METABOLISM	Secondary metabolites biosynthesis, transport and catabolism	Sorbitol dehydrogenase	1675	cyto	1,76	1,59	**2,82**	**2,07**

Table 3.3. List of upregulated proteins on 2D gels in response to 100 µm Cd (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	Cellular location	Cd 1h	Cd 2h	Cd 4h	Cd 8h
METABOLISM	Secondary metabolites biosynthesis, transport and catabolism	Uricase (urate oxidase)	41793	cyto	1,06	1,74	**2,35**	0,81
		Uricase (urate oxidase)	41793	cyto	1,59	**2,28**	1,48	0,67
POORLY CHARACTERIZED	General function prediction only	Aldo/keto reductase family proteins	7571	cyto	**2,12**	**2,86**	1,87	0,88
Unknown	Unknown	Hypothetical protein	1270	mito	**2,55**	**2,14**	**2,43**	1,64
		Hypothetical protein	4311	cyto	**3,79**	**2,65**	**2,79**	**2,30**
		Hypothetical protein	4311	cyto	**2,30**	**2,38**	**2,30**	1,06
		Hypothetical protein	138397	extr	1,73	1,07	1,45	**2,02**
		Hypothetical protein	140701	nucl	1,33	1,42	**2,10**	0,44

*Cyto; cytoplasmic, Extr; extracellular, Mito; mitochondrial, Nucl; Nuclear, Cysk; cytoskeleton, Cyto_nucl; cytoplasmic_nuclear

TMH: Number of predicted transmembrane helices

Table 3.4. List of downregulated proteins on 2D gels in response to 100 µm Cd.

KOG Group	KOG Class	Protein Function	Protein ID	Cellular location	Cd 1h	Cd 2h	Cd 4h	Cd 8h
CELLULAR PROCESSES AND SIGNALING	Cytoskeleton	Beta tubulin	125674	nucl	**0.48**	**0.46**	0.82	0.51
	Nuclear structure	Nucleolar GTPase/ATPase p130	465	nucl	**0.50**	**0.17**	**0.11**	**0.50**
	Posttranslational modification, protein turnover, chaperones	Molecular chaperone (small heat-shock protein Hsp26/Hsp42)	137747	Cyto_ nucl	**0.45**	**0.25**	**0.19**	**0.06**
		Ubiquitin-conjugating enzyme E2	2075	cyto	0.67	**0.18**	**0.08**	**0.16**
		FKBP-type peptidyl-prolyl cis-trans isomerase	44306	cyto	**0.41**	**0.33**	**0.44**	**0.40**
		Dipeptidyl aminopeptidase	5061	extr	**0.26**	**0.21**	**0.27**	**0.45**
		Molecular chaperones GRP170/SIL1, HSP70 superfamily	5586	cyto_mito	**0.38**	**0.31**	**0.10**	**0.45**
INFORMATION STORAGE AND PROCESSING	RNA processing and modification	Ribosomal protein RPL1/RPL2/RL4L4	128104	extr	**0.34**	**0.42**	**0.39**	0.85
	Translation, ribosomal structure and biogenesis	40S ribosomal protein S3A	139199	nucl	**0.47**	**0.27**	0.52	**0.41**
METABOLISM	Carbohydrate transport and metabolism	Enolase	135734	cyto	**0.42**	**0.44**	**0.02**	0.64
		Beta-galactosidase	9466	extr	**0.46**	**0.23**	**0.34**	0.57
	Energy production and conversion	UDP-glucuronosyl and UDP-glucosyl transferase	3221	mito	**0.36**	**0.34**	**0.35**	0.58
		UDP-glucuronosyl and UDP-glucosyl transferase	3221	mito	**0.37**	**0.35**	**0.31**	0.75

Table 3.4. List of downregulated proteins on 2D gels in response to 100 μm Cd (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	Cellular location	Cd 1h	Cd 2h	Cd 4h	Cd 8h
POORLY CHARACTERIZED	General function prediction only	Multiple inositol polyphosphate phosphatase	121720	cyto	**0.40**	**0.32**	**0.34**	0.58
		Predicted hydrolase related to dienelactone hydrolase	6857	cyto	**0.42**	**0.30**	**0.10**	**0.21**
Unknown	Unknown	Hypothetical protein	1249	mito	**0.46**	**0.40**	0.74	0.81
		Hypothetical protein	35714	cyto	**0.43**	**0.06**	**0.38**	2.47

***Cyto**; cytoplasmic, **Extr**; extracellular, **Mito**; mitochondrial, **Nucl**; Nuclear, **Cysk**; cytoskeleton, **Cyto_nucl**; cytoplasmic_nuclear
TMH: Number of predicted transmembrane helices

3.3. Membrane enriched proteome analysis

3.3.1. Shaving method

In the present study, shaving method was utilized as the first time to analyze the membrane proteome of *P. chrysosporium*. The crude membrane extract was obtained by the modified method described in the Materials and Methods Section 2.9.1. A 100 µl of this extract was used in shaving procedure and analyzed by an LC-Orbitrap (Thermo Scientific™ LTQ Orbitrap™) system. As a result, a total of 90 proteins were identified with 2 or more distinct peptides, i.e. all identifications were valid. 17 of these were found to have one or more TMH (Trans membrane helices) and only 8 of the 17 proteins were predicted as plasma membrane proteins (Table 3.5).

3.3.2. Enzymatic cell wall distruption

By using this protocol a total of 284 proteins could be identified. The number of the predicted plasma membrane proteins among the identified ones was 29. In addition to this, a total of 60 predicted proteins were having more than one TMH (Table 3.6).

Table 3.5. Membrane proteins identified by shaving method.

No	Protein ID	Cellular location	TMH	Protein function (KOG)
1	137220	plas	12	Predicted transporter (major facilitator superfamily)
2	39702	plas	11	Ca^{2+}/H^{+} antiporter VCX1 and related proteins
3	28102	plas	10	Permease of the major facilitator superfamily
4	10757	plas	9	H^{+}/oligopeptide symporter
5	134084	plas	8	Putative protein
6	126811	plas	7	Plasma membrane H^{+}-transporting ATPase
7	363	plas	7	Putative protein
8	134565	mito	3	Mitochondrial ADP/ATP carrier proteins
9	4924	plas	2	COPII vesicle protein
10	137219	cyto	2	Mitochondrial import inner membrane translocase, subunit TIM23
11	4549	E.R.	2	Oligosaccharyltransferase, delta subunit (ribophorin II)
12	1722	cyto	1	Putative protein
13	138125	extr	1	GO Desc: hydrolase activity, hydrolyzing O-glycosyl compounds
14	137302	extr	1	Cytochrome C1
15	122715	cyto	1	M13 family peptidase
16	26890	extr	1	Multicopper oxidases
17	123329	nucl	1	SNARE protein Syntaxin 1 and related proteins

***Cyto**; cytoplasmic, **Extr**; extracellular, **Mito**; mitochondrial, **Nucl**; Nuclear, **Cysk**; cytoskeleton, **Cyto_nucl**; cytoplasmic_nuclear **TMH**: Number of predicted transmembrane helices

Table 3.6. Membrane proteins identified by the β-glucoronidase type H-1 enzyme treatment method

No	Protein ID	TMH	Cellular location	Function
1	3390	13	plas	1,3-beta-glucan synthase/callose synthase catalytic subunit
2	6110	12	plas	Multidrug resistance-associated protein/mitoxantrone resistance protein, ABC superfamily
3	3668	12	plas	Multidrug resistance-associated protein/mitoxantrone resistance protein, ABC superfamily
4	2466	11	plas	Dolichyl-phosphate-mannose:protein O-mannosyl transferase
5	1367	10	plas	Permease of the major facilitator superfamily
6	102	10	plas	electron transport
7	198	9	plas	Ca^{2+} transporting ATPase
8	8051	8	plas	Predicted Dolichyl-phosphate-mannose-protein mannosyltransferase
9	424	8	plas	N-methyltransferase
10	7495	7	plas	Mannosyltransferase
11	1061	7	plas	Chitin synthase/hyaluronan synthase (glycosyltransferases)
12	363	7	plas	ion channel activity
13	7544	6	plas	Predicted membrane protein
14	904	6	plas	Transporter, ABC superfamily (Breast cancer resistance protein)
15	7054	4	plas	Putative protein
16	5306	4	plas	Putative protein
17	3692	4	plas	Predicted membrane protein
18	6711	3	plas	Putative protein
19	3404	3	plas	HVA22/DP1 gene product-related proteins
20	332	3	plas	Oligosaccharyltransferase, gamma subunit
21	4924	2	plas	COPII vesicle protein
22	2476	2	plas	Putative protein
23	7753	0	plas	Cullins
24	7498	0	plas	Thioredoxin

Table 3.6 Membrane proteins identified with the β-glucoronidase type H-1 enzyme treatment method (Cont'd)

No	Protein ID	TMH	Cellular location	Function
25	5833	0	plas	Uncharacterized conserved protein
26	5579	0	plas	Uncharacterized conserved protein
27	4453	0	plas	Putative protein
28	3605	0	plas	Putative protein
29	1726	0	plas	Uncharacterized conserved protein
30	5828	4	mito	Inner membrane protein translocase involved in respiratory chain assembly
31	4885	3	mito	Sterol reductase/lamin B receptor
32	2707	3	mito	Farnesyl cysteine-carboxyl methyltransferase
33	1226	3	mito	Predicted membrane protein
34	3244	2	mito	Uncharacterized protein, induced by hypoxia
35	2142	2	mito	Integral membrane protease of the rhomboid family involved in different forms of regulated intramembrane proteolysis
36	166	2	mito	Putative protein
37	6157	1	mito	NADH-dehydrogenase (ubiquinone)
38	4947	1	mito	Prohibitin-like protein
39	4591	1	mito	Predicted membrane protein
40	3771	1	mito	Ubiquinol cytochrome c reductase, subunit QCR8
41	3112	1	mito	Putative protein
42	1249	1	mito	Putative protein
43	801	1	mito	Ca2+-binding transmembrane protein LETM1/MRS7
44	304	1	mito	Putative protein
45	157	1	mito	Signal recognition particle receptor, beta subunit (small G protein superfamily)
46	8097	3	extr	E3 ubiquitin ligase
47	6851	3	extr	Putative protein
48	730	3	extr	Alkaline phosphatase
49	6433	2	extr	Predicted divalent cation transporter
50	4675	2	extr	emp24/gp25L/p24 family of membrane trafficking proteins
51	4079	2	extr	Putative Protein

Table 3.6. Membrane proteins identified with the β-glucoronidase type H-1 enzyme treatment method (Cont'd)

No	Protein ID	TMH	Cellular location	Function
52	7174	1	extr	Putative protein
53	6768	1	extr	Predicted membrane protein
54	6450	1	extr	Putative protein
55	6205	1	extr	Uncharacterized conserved protein
56	6163	1	extr	Putative protein
57	5463	1	extr	bZIP transcription factor MafK
58	4553	1	extr	MAPK-activating protein DENN
59	2111	1	extr	Nucleolar GTPase/ATPase p130
60	894	1	extr	Hydrolase activity
61	4549	2	E.R.	Oligosaccharyltransferase, delta subunit (ribophorin II)
62	7259	3	cyto	Integral to membrane
63	6833	2	cyto	Putative protein
64	6171	1	cyto	Pentafunctional AROM protein
65	1722	1	cyto	Putative protein
66	1491	1	cyto	Cytochrome b5

3.3.3. Membrane enrichment with aqueous two phase separation followed by GeLC analysis results

By using the method Shary et al., (2008) followed by GeLC-MS analysis, more than 700 proteins could be identified. Bioinformatic analysis revealed that almost 20% of these proteins are predicted to have at least one TMH. Interestingly, some of the plasma membrane proteins were predicted as having no TMH, thus 21 % of the total identified proteins were actually the membrane proteins. To note, the samples used in GeLC analysis were two simultaneously cultivated biological replicates which were pooled from 3 repeated cultivations. Only the proteins that were identified and quantified in both of the replicas were counted as valid identification/quantification.

3.4. GeLC-MS results of Cu-responsive proteins

In Cu exposure experiments, 700-800 proteins could be identified and quantified in each of the time points. The success rate of the quantification was 70-80 %. Theoretically *P. chrysosporium* genome has a total of 10047 ORF (Martinez et al., 2004) and WolfPsort (wolfpsort.org, Horton 2007) analysis of the whole protein database of the organism revealed that 1110 proteins were predicted as plasma membrane proteins, constituting 11% of the total predicted proteome. In a study conducted by Shary et al. (2008), LC-MS analysis of the *P. chrysosporium* revealed 10% of the total identified ones as the membrane proteins. In the present study, the membrane enrichment resulted in a much better resolution in that we ended up with 20% membrane protein identification.

After 2h Cu exposure, a total of 767 proteins were identified and quantified. 29 of the 767 proteins were found to be upregulated (Table 3.7) while 24 proteins were down-regulated (Table 3.8). In addition to this, 14 proteins were detected only in Cu-exposed cells, but not in untreated control cells. On the other hand, 8 of the proteins that were detectable in untreated control were absent from Cu-exposed samples.

GeLC analysis of 4h exposure resulted in identification and quantification of a total of 789 proteins. Among those quantified, 35 were upregulated and 45 were downregulated. Moreover 8 proteins were only detected after 4h Cu exposure and 3 could not be detected anymore after 4h Cu exposure.

From the samples of 8h Cu-exposed *P. chrysosporium* cultures, a total of 705 proteins could be identified and quantified. Quantification analysis revealed that 8h Cu exposure caused upregulation of 27 proteins and downregulation of 30 proteins. Moreover 15 proteins were only detected after 8h Cu exposure and 8 could not be detected after 8h exposure.

3.4.1. Upregulated proteins in response to copper identified by GeLC-MS

3.4.1.1. Inorganic ion transport and metabolism

One of the basic resistance mechanisms for toxic metals is metal efflux by the help of the pumps of the membrane. ATPases and MDR (Multi Drug Resistance) proteins are best representative of such efflux pumps. There are several studies that confirm the role of these proteins in the heavy metal resistance in bacteria (Mergeay et al., 2003), yeast (Fernandes and Sá-Correia, 2001) and plant (Bernard et al., 2004). In the present study, increased level of MDR protein (136846) after 4h Cu exposure showing that this efflux system also exists in *P. chrysosporium* and significant upregulation of ATPase (34990, Z-score:13.6), shows that the organism heavily uses this efflux system.

3.4.1.2. Proteins of energy production and conversion

The vacuolar ATPases (V-ATPase) are responsible for the compartmentalization of the toxic molecules/metals by pumping them from cytosol to vacuoles so that they are prevent the harm to other organelles such as mitochondria, chloroplast and golgi (Hall, 2002). Additionally, the intracellular ion homeostasis is disturbed because of heavy metal stress and V-ATPases are in charge to restore the balance to intracellular ion balance. Our study confirms the upregulation of four V-ATPase subunits at 8h Cu treatment (2276, 124930, 134368, 135018). This finding suggests that *P. chrysosporium* uses compartmentalization as a part of its resistance mechanism against heavy metal toxicity.

Voltage-dependent anion channels (VDAC) supply the transportation of molecules like ATP, ADP and malate between cytoplasm and mitochondria. Another function of the VDACs is the transportation of the Ca^{2+} ions (Huang et al., 2013). Since the Ca^{2+} ions are the cofactor of the isocitrate dehydrogenase enzyme, the activity of the VDAC is crucial for the energy metabolism and homeostasis. In the present study, it was found out that 3 distinct isocitrate dehydrogenase enzyme subunits (1651, 9983 and 139320) were upregulated upon 2h Cu exposure. A VDAC

protein (138775, porin) was also induced after 2h Cu treatment (Table 3.9). In the light of these findings, we can hypothesize that the organism requires more energy to resist the heavy metal stress and these upregulated proteins reflects the elevated levels of energy metabolism to cope with high energy demand of the cells.

3.4.1.3. Carbohydrate metabolism

Bacteria, plants, insects, and fungi can produce the disaccharide trehalose. In fungi, trehalose functions both as a reserve carbohydrate and as an element of stress response mechanism (Al-Bader et al., 2010). Trehalose molecules prevent aggregation of denatured proteins and scavenge free radicals (Singer and Lindquist, 1998). When the organism is exposed to a stressor, increase in the trehalose concentration is always associated with a drastic activation of trehalase and a rapid increase of the cAMP level. In addition, evidence for the activation of trehalase *in vitro* by AMP-dependent protein phosphorylation was obtained for these fungi. Upregulation after 4h of Cu treatment of a neutral trehalase (140627) in the present study is in a complete agreement with the above mentioned response mechanism.

C1-tetrahydrofolate synthase is a crucial enzyme for one-carbon metabolism. Although the knowledge about its stress response relevance is limited, it was recently shown to be upregulated in response to zinc in liver cells (Bondzio et al., 2013). Our study suggests a link between one-carbon metabolism and stress response by revealing upregulation of C1-tetrahydrofolate synthase after 8h Cu exposure. Nevertheless, such a relevance is needed to be clarified with further investigations.

3.4.1.4. Lipid metabolism

One of the mechanisms that organisms utilize to prevent oxidative stress is methylation of the hydroxyl groups of the biomolecules. In case of heavy metal exposure, heavy metals can not react with hydroxyl groups and production of ROS is prevented by the help of these methylations (Zhu et al., 1994). Kunstman

et al. (2009) have shown that overproduction of SAM-dependent methyltransferase prevents the oxidative damage and delays the cellular aging in fungus *Podospora anserina*. The authors also showed that the deletion of the gene expressing the SAM-dependent methyltransferase causes a strain to become more prone to oxidative stress in comparison to wild type. S-adenosylmethionine (SAM)-dependent methyltransferase is one of such methylating enzymes. We found a SAM-dependent methyltransferase (126764) as upregulated in response to 4h Cu exposure, providing evidence that the organism uses this enzyme to counteract with Cu-hydroxyl groups interaction.

3.4.1.5. Secondary metabolites transport

A multi drug resistance-associated protein (136846) was found to be upregulated upon 4h Cu exposure. Together with the upregulated multidrug resistance proteins and ATPases this finding confirms that organism employs the efflux pump system to resist Cu exposure.

3.4.1.6. Intracellular trafficking, secretion and vesicular transport

p24 family proteins are known to be essential components of the coated vesicles which facilitate the transportation of cargo molecules from the ER to the Golgi complex (Hwang et al., 2008). During ER stress, these proteins are especially needed to deliver proteins out of ER, so to avoid protein aggregation within ER (Hwang et al., 2008; Satpute-Krishnan et al., 2014). A p24 protein family member membrane trafficking protein (123547) found to be upregulated, suggesting a probable ER stress induction because of Cu stress.

In order to sequester heavy metals, most of the organisms produce small cysteine-rich peptides having 2-11 repeats of a dimeric amino acid. namely phytochelatins (PCs). PCs chelate heavy metal ions, thus mediate heavy metal tolerance in plants, fission yeast, and *Caenorhabditis elegans*. Synthesis of the PCs are achieved by a specific dipeptidyl transferase from glutathione (Grill et al., 1989). Although *S. cerevisiae* can synthesize PCs upon exposure to heavy metal ions, the *S.*

cerevisiae does not have a PC synthase homologue. However, one study has revealed that vacuolar serine carboxypeptidases can be responsible for PC synthesis in yeast (Wünschmann et al., 2007). GeLC-MS technology is not capable of identifying PCs yet. For this reason, in the present study, any identification and quantification for PC proteins were not expected. Still, a serine carboxypeptidase was found upregulated upon 8h Cu exposure. In addition, the subunits of vacuolar H+-ATPase V1 sector were found to be significantly upregulated. These V-type ATPases are known as extremely essential for the organisms' fight against heavy metal stress since they are responsible for transportation of the conjugated phytochelatin-heavy metal compound into the vacuole (Schneider et al., 2009). These findings provide indirect evidence for the use by *P. chrysosporium* phytochelatin-like peptides to inactivate heavy metals, the latter, as conjugated molecules are then being transported into the vacuoles, the organelles of limited metabolic activity.

3.4.1.7. Energy production and conversion

It was previously shown that cytochrome C is released from the mitochondria as a stress response upon oxidative stress (Sun et al., 2000) and ER stress (Ito et al., 2001). The level of a putative cytochrome C assembly protein was upregulated after 4h Cu exposure in the present work. Increased levels of this protein is most probably associated with the cytochrome C release. In a study conducted by (Ishii et al., 1998), deletion of succinate dehydrogenase and cytochrome B of the electron transport chain gave rise to an oxidative stress,. Here in this study, upregulation of the latter proteins after 4h Cu exposure can be interpreted as a relevant defense of the cells

The mitochondrial carrier protein family was generally shown to be overrepresented in the stress-responsive genes suggesting that stress induces altered needs for metabolite transport across the mitochondrial inner membrane (Van Aken et al., 2009). Mitochondrial oxaloacetate carriers exchange malate and oxaloacetate between mitochondria and cytosol. This exchange pumps reducing

equivalents from a relatively reduced compartment to a relatively oxidized compartment. Because of an increased NADH/NAD-ratio, mitochondria is more reduced, therefore the oxaloacetate transporter tends to export reducing equivalents (Krömer and Heldt, 1991; Wigge and Krömer, 2006). An upregulation of the mitochondrial oxaloacetate carrier protein (135598) after 4h of Cu exposure is most probably due to an abolished oxidation/reduction balance between the mitochondria and cytosol. Besides these, it was also shown that malate has a protective effect by avoiding the metals binding to fumarase's active site (Xu and Imlay, 2012).

Another protein found to be upregulated was fumarate reductase flavoprotein subunit. In *E. coli,* fumarate reductase is an enzyme which converts fumarate to succinate as an important step in the anaerobic metabolism. There are several literature reports about the relation of this enzyme with anoxic/oxic switch in facultatively anaerobes like *E. coli* and with hypoxia especially in helminthic parasites, human intracellular pathogens and malignant cells. Moreover, fumarate reductase is also known to cause a small size oxidative stress in the studied cell types since it produces ROS (Meehan and Malamy, 2012; Storz and Imlay, 1999). Another redox enzyme is cytochrome b5 which that reduces the other eukaryotic P450 enzymes. Recently, it was proven that cytochrome b5 reductase transfers electrons to P450 CYP63A2 of *P. chrysosporium* (Syed et al., 2011). A secretome analysis of *P. chrysosporium* also confirmed that cytochrome b5 is upregulated in the presence of lignin and cellulose (Manavalan et al., 2011). It was reported that this protein was downregulated in response to salt stress in rice root tissues (Cheng et al., 2009). However, it was shown that the protein was upregulated when the stress conditions are removed, as a sign of organism switching to the normal metabolism after stress response (Dankel et al., 2010). In the present study, both fumarate reductase and a cytochrome b5 (1491) reductase were found to be upregulated at 8^{th} h of Cu stress, which might be interpreted as the signals for restoration of normal metabolism and recovery after a prolonged exposure in *P. chrysosporium*

3.4.1.8. Translation and ribosomal structure

The canonical translation machinery of the eukaryotes starts with the initiation factor eIF4E. eIF4E interacts with eIF4G and with eIF4A and they all together form a complex known as eIF4F. Afterwards this complex recruits the preinitiation complex 43S, which includes 40S ribosomal subunit, the ternary complex eIF2/GTP/tRNA and initiation factors eIF3, eIF1 and eIF1A. When this complex is bound to the initiation codon, the 60S ribosomal subunit is loaded and elongation starts. However, under abiotic stress conditions this process is interrupted by different mechanisms by affecting the activity of the initiation factors eIF2α, eIF4E, eIF4E, and eIF4A (Muñoz and Castellano, 2012). In the present study, several translation machinery proteins were upregulated, initiation factor eIF4, 40S ribosomal protein subunits, S2, S26, S24, S7, S19 being among the upregulated ones. As an oxidative stress initiator, Cu causes damage on most of the biomolecules and ribosomal proteins are no exception. Shenton et al., (2006) has studied the elements of oxidative stress response on yeast and reported that 2mM of H2O2 caused upregulation of several ribosomal proteins. Because of this findings, authors proposed that the organism needed to replenish damaged ribosomal proteins by upregulating ribosomal proteins production. Since our study yielded similar findings, we can propose that high levels of ribosomal protein production is crucial to replace the damaged ones after an oxidative stress exposure. Another three ribosomal proteins found to be upregulated were 60S ribosomal proteins L7, L7A (RPL7, RPL7A) and 40S ribosomal protein S12 (RpS12). In a study conducted by breast cancer cell line, Zhu et al. (2001) reported that RPL7A is upregulated in response to alcohol. On the other hand, RpS12 was reported as downregulated under different stress conditions (Becker 2001, Qasim et al., 2011). Many reports indicate that ribosomal proteins are generally upregulated under stress conditions (Kawasaki et al., 2001). Taken together, we can conclude that the elements of protein synthesis machinery is upregulated either to meet increasing protein production demand or to replenish damaged ribosomal proteins.

Hydrolases are another class of proteins that are known to be important regulators of biotic and abiotic stresses in plants and fungi (Lee et al., 2014; Zhang et al., 2009; Liu et al., 2008). In the present study, an exposure to Cu for 4h Cu caused upregulation of three putative proteins having hydrolase activity (122884, 122483, 133689).

Renewal of the damaged biomolecules present within various compartments of the cell is a must for stress adaptation process. To replenish these, newly synthesized ones are to be transported by an intracellular vesicle trafficking system (Mazel et al., 2004). One of the members of this system is adaptin protein. Adaptins select cargo molecules and include them into coated vesicles in the late secretory and endocytotic pathways (Murphy et al., 2005). Another member of this pathway is the synaptic vesicle protein (containing EH domain). Proteins having the EH domain are mostly associated with the synaptic vesicle trafficking. These two proteins (128445 and 654) were found to be upregulated at the 8h Cu-treated samples. Having elevated levels of these two proteins after 8h Cu treatment, it might be suggested that the organism produce them at higher rates to ensure their replenishment.

3.4.1.9. Posttranslational modification, protein turnover, chaperones

Several molecular chaperones were found to be upregulated throughout the 8h Cu treatment: Hsp26/Hsp42 and mortalin, the latter being a Hsp70 family chaperon. Especially the mortalin chaperones are highly interesting since they act as a swiss army knife in various cellular processes such as cell proliferation, stress response, maintenance of the mitochondria, unfolding of proteins outside the mitochondria, unidirectional translocation across mitochondrial membranes by using membrane potential, and by acting as an ATP-driven motor completion of biomolecule import (Wadhwa et al., 2002). Upregulation of such a multifunctional protein deserves more detailed investigation. However, from the other upregulated proteins we can assume that the organism is in the process of recovery. Thus we can speculate that, mortalin is required to control the folding of newly synthesized

proteins of mitochondria and helps to import the biomolecules into the mitochondria as well as a part of this process. Our results indicated that five different molecular chaperones are upregulated in response to 2h Cu exposure. WolfPsort results revealed that three of these five chaperones are located at cytoplasm, one is located at mitochondria and the other located at nucleus. Increasing levels of different chaperones at different intracellular locations shows that Cu causes a globular protein damage and organism responses by a much wider range of chaperones.

Overproduction of aspartyl proteases are mostly correlated with programmed cell death. In particular, prolonged ER stress causes cell death which is mediated by proteases (Egger et al., 2007). However there are some other reports revealing that aspartyl proteases functions as an anti-cell-death component in plants (Ge et al., 2005). Similarly an aspartyl protease (135608) were found to be upregulated for 4h and -induced samples.

A copper amine oxidase (CuAO, 36058) was another protein detected to be stimulated after 8h of Cu exposure. CuAOs catalyze oxidation of various biogenic amines. They function in the process of exogenous and endogenous amines degradation (including neurotransmitters and mono-, di-, and polyamines) and the cross-linking of collagen and elastin by the oxidation of lysine peptides (McGrath et al., 2011). CuAO activity results in H_2O_2 production. Although the organism produces additional H_2O_2 internally, deamination of the biological molecules should be more beneficial for the organism after 8h Cu exposure though this view remains to be supported or disproved by some necessary experiments.

A transferrin receptor-like protein (129890) was among those upregulated lately, after 8h of Cu exposure. Transferrin receptor-like proteins are actually endopeptidases functioning as protein degradation enzymes (Stimpson et al., 2006). In a study conducted by *Schizosaccharomyces pombe* Tre proteins were shown to be important to regulate the heavy metal transporter proteins (Stimpson et al., 2006). The authors suggested that transferrin receptor like proteins (Tre1,

Tre2, Tre3) serve as adaptors linking unwanted metal transporters to the quality control mechanism, thus guaranteeing their removal. Thus, the present study points to the existence of a similar mechanism in white rot fungi.

There is another UPR (Unfolded Protein Response) system which is triggered by disturbed protein homeostasis at the mitochondria, so called mitochondrial UPR (UPRmt). UPRmt is activated in response to any kind of stress which causes protein folding problems within the mitochondria and UPRmt induces expression of the nuclear DNA-coded mitochondrial chaperones (Haynes and Ron, 2010). A mitochondrial chaperon (HSP60, protein Id number 134348) was upregulated after 2h Cu exposure.

3.4.1.10. Transcription

Several studies have shown the homeodomain proteins play a crucial role in mating and sexual differentiation in fungi (Astell et al., 1981; Casselton and Olesnicky, 1998; Hull et al., 2005). Another interesting study with obese people showed that homeobox proteins are upregulated after fat removal surgery (Dankel et al., 2010). More importantly, the authors indicated that there was a significant downregulation of stress responsive proteins following the surgery. Quite reminiscent of this kind of pattern, a homeobox transcription factor (2305) increased its level by 3.56 fold most probably indicating preparation of the organism for sexual differentiation after eliminating the effects of Cu stress.

3.4.1.11. Poorly characterized and hypothetical proteins

Ketol-acid reductoisomerase is a bi-functional enzyme which functions during the biosynthesis of the amino acids valine, leucine and isoleucine (Durner et al., 1993). After its catalysis, the enzyme produces an NADPH molecule. NADPH molecules, as stated whenever appropriate in the present dissertation, are used to prevent the oxidative stress within the cell (Zhang et al., 2011). Accordingly, the upregulation of a protein having ketol-acid reductoisomerase activity (132767) in response to 8h Cu treatment might indicate that the organism has not yet given up

its needs for antioxidants to fight with the ROS generated during the treatment.

The hypothetical proteins upregulated upon 8h Cu exposure included those with Id numbers: 7825, 5249, and 4671. Also, the level of one protein increased upon a shorter Cu exposure of 2h.

3.4.2. Downregulated proteins in response to copper identified by GeLC-MS

A total of 84 proteins were detected as downregulated upon Cu stress response. Two KOG class, Posttranslational modification, protein turnover, chaperones and hypothetical proteins were found to be mostly affected from Cu exposure.

Subtilisin-related proteases are called as the plant alternative for the animal caspases (Vartapetian et al., 2011). Caspases play a crucial role in apoptotic pathways by cleaving a variety of key cellular proteins. In a report by Down-regulation of a caspase protein in breast cancer were associated with chemoresistance (Devarajan et al., 2002). In this study a subtilisin related protein (138363) was found to be downregulated in both 2h and 4h Cu exposures. Moreover this protein was not detected in 8h Cu exposed samples. Therefore, we can speculate that *P. chrysosporium* also utilizes a similar mechanism to escape from programmed cell death by downregulating the caspase like protein.

A 26S proteasome subunit, ATPase RPT5 (29020), was found to be downregulated after 2h Cu exposure. RPT5 helps the 26S proteasome complex to attach chaperone Nas2/p27 (Lee et al., 2011). It was reported that the structure of the 26S proteasome complex is disturbed under oxidative stress, therefore can't degrade the ubiquitinylated proteins (Wang 2010).

DnaJ superfamily molecular chaperones (Hsp40) are essential for the interaction of the denatured protein and Hsp70 proteins and at the face of environmental stress it is known to be upregulated (Lin et al., 2013). However downregulation of this protein after 8h of Cu exposure is puzzling since it is required to fight against effects of environmental stresses.

Several hypothetical proteins were also found to be down-regulated. Some of these hypothetical proteins were predicted as having metal binding domains like, metalloendopeptidase, iron binding and iron permease.

Table 3.7. List of upregulated proteins identified by GeLC-MS analysis in response to 100 μM Cu.

KOG Group	KOG Class	Protein Function	Protein ID	TMH	Cellular location	2h	4h	8h
CELLULAR PROCESSES AND SIGNALING	Cell wall/membrane/envelope biogenesis	Chitinase	2495	0	mito	**2.71**	-0.46	0.14
		Glucosamine 6-phosphate synthetases, contain amidotransferase and phosphosugar isomerase domains	136680	0	cysk	**4.43**	0.97	-0.55
	Intracellular trafficking, secretion, and vesicular transport	Succinate dehydrogenase membrane anchor subunit and related proteins	2221	0	mito	1.08	**2.23**	0.10
		emp24/gp25L/p24 family of membrane trafficking proteins	123547	1	cyto	-0.58	**2.44**	-0.75
	Posttranslational modification, protein turnover, chaperones	Molecular chaperone (small heat-shock protein Hsp26/Hsp42)	843	0	nucl	**6.47**	**2.85**	**6.50**
		Chaperonin complex component, TCP-1 beta subunit (CCT2)	1846	0	cyto	**9.62**	NQ	NQ
		Molecular chaperones mortalin/PBP74/GRP75, HSP70 superfamily	7166	0	mito	1.91	-0.14	**2.42**
		Transferrin receptor and related proteins containing the protease-associated (PA) domain	129890	0	cyto	NQ	NQ	**2.80**
		Mitochondrial chaperonin, Cpn60/Hsp60p	134348	0	mito	**2.00**	0.43	1.52
		Aspartyl protease	135608	0	extr	NQ	**2.38**	**2.61**
		Molecular chaperone (small heat-shock protein Hsp26/Hsp42)	137747	0	Cyto_nucl	**2.51**	NQ	-0.11
		Serine carboxypeptidases (lysosomal cathepsin A)	140512	0	extr	-1.48	-1.04	**2.01**

Table 3.7. List of upregulated proteins identified by GeLC-MS analysis in response to 100 µM Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	TMH	Cellular location	2h	4h	8h
CELLULAR PROCESSES AND SIGNALING	Signal transduction mechanisms	Proteins containing Ca2+-binding EGF-like domains	682	0	extr	0.24	0.41	**2.07**
		Synaptic vesicle protein EHS-1 and related EH domain proteins	977	0	nucl	-1.23	**-2.57**	**2.25**
		G protein beta subunit-like protein	10373	0	cyto_nucl	**2.52**	1.13	NQ
INFORMATION STORAGE AND PROCESSING	RNA processing and modification	Fibrillarin and related nucleolar RNA-binding proteins	132476	0	cyto	-0.02	**2.15**	NQ
	Transcription	Homeobox transcription factor prospero	2305	0	nucl	0.86	-1.97	**3.56**
		Transcription factor, Myb superfamily	6340	0	nucl	1.80	**2.03**	NQ
		RNA polymerase II, large subunit	29074	0	extr	1.48	**2.10**	1.73
	Translation, ribosomal structure and biogenesis	60s ribosomal protein L18	1312	0	mito	**2.03**	0.76	0.98
		Mitochondrial/chloroplast ribosomal protein L12	1364	0	mito	**2.07**	-0.08	0.79
		60S ribosomal protein L7A	6222	0	mito	1.89	0.37	**2.11**
		40S ribosomal protein S2/30S ribosomal protein S5	10998	0	mito	0.58	**2.13**	0.71
		60S ribosomal protein L10A	11013	0	mito_nucl	0.60	**2.17**	0.76
		40s ribosomal protein S26	120998	0	mito	0.70	**3.02**	NQ
		Ribosomal protein S7	123753	0	cyto	1.25	**2.07**	0.46
		ATP-dependent RNA helicase	127255	0	nucl	**2.07**	0.99	-0.76
		Protein containing adaptin N-terminal region	128445	0	cyto	NQ	NQ	**2.34**

Table 3.7. List of upregulated proteins identified by GeLC-MS analysis in response to100 μM Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	TMH	Cellular location	2h	4h	8h
INFORMATION STORAGE AND PROCESSING	Translation, ribosomal structure and biogenesis	40S ribosomal protein S7	129681	0	nucl	0.17	**2.06**	0.28
		Translation initiation factor 4F, helicase subunit (eIF-4A) and related helicases	132421	0	nucl	1.44	**2.29**	0.57
		60S ribosomal protein L7	133217	0	cyto	0.93	0.91	**2.29**
		40S ribosomal protein S24	134927	0	mito	0.68	**2.87**	NQ
		40S ribosomal protein S4	137380	0	mito	NQ	**2.12**	-0.06
		40S ribosomal protein S19	138699	0	cyto	NQ	**2.03**	0.23
		Aspartyl-tRNA synthetase	6758	0	nucl	**2.46**	NQ	NQ
		40S ribosomal protein S12	125300	0	cyto	**2.02**	0.84	0.79
		Translation elongation factor EF-3b	10554	0	cyto	**3.27**	NQ	NQ
	Chromatin structure and dynamics	SWI-SNF chromatin-remodeling complex protein	213	0	nucl	**2.76**	NQ	NQ
METABOLISM	Amino acid transport and metabolism	Isocitrate dehydrogenase, alpha subunit	9983	0	mito	**3.06**	NQ	NQ
		H+/oligopeptide symporter	10757	9	plas	0.01	**2.43**	-0.95
		Aspartate aminotransferase/Glutamic oxaloacetic transaminase AAT1/GOT2	39635	0	cyto	1.58	**2.07**	1.87
		Isocitrate dehydrogenase, alpha subunit	139320	0	mito	**2.58**	-1.69	0.07
	Carbohydrate transport and metabolism	Glycosyl transferase, family 8 - glycogenin	2562	1	mito	**-3.15**	**2.77**	0.60
		Transaldolase	134261	0	cyto	**2.29**	**2.21**	0.53

Table 3.7. List of upregulated proteins identified by GeLC-MS analysis in response to 100 µM Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	TMH	Cellular location	2h	4h	8h
METABOLISM	Carbohydrate transport and metabolism	Neutral trehalase	140627	0	extr	1.87	**4.10**	1.68
		C1-tetrahydrofolate synthase	123437	0	cyto	0.94	NQ	**2.73**
		S-adenosylmethionine synthetase	132370	0	cyto	1.22	**2.06**	-0.74
	Energy production and conversion	Cytochrome b5	1491	1	cyto	1.75	0.14	**2.11**
		Vacuolar H+-ATPase V1 sector, subunit G	2276	0	nucl	0.28	-1.44	**2.09**
		Mitochondrial solute carrier protein	10978	0	mito	1.46	**2.24**	NQ
		Putative cytochrome C oxidase assembly protein	41906	1	cyto	NQ	**2.31**	**-2.02**
		Fumarate reductase, flavoprotein subunit	123932	0	cyto	0.89	-0.79	**2.00**
		Vacuolar H+-ATPase V1 sector, subunit F	124930	0	cyto	NQ	-1.55	**2.15**
		Mitochondrial tricarboxylate/dicarboxylate carrier proteins	128101	0	mito	**2.71**	0.19	NQ
		Aldehyde dehydrogenase	133924	0	cyto	**2.27**	NQ	-1.05
		Vacuolar H+-ATPase V1 sector, subunit E	134368	0	nucl	NQ	-1.26	**2.02**
		Vacuolar H+-ATPase V1 sector, subunit C	135018	0	nucl	-0.11	-1.44	**2.24**
		Mitochondrial oxaloacetate carrier protein	135598	2	plas	1.04	**2.36**	0.06
		MAM33, mitochondrial matrix glycoprotein	136828	0	mito	**2.22**	NQ	NQ
	Inorganic ion transport and metabolism	Cation transport ATPase	34990	8	plas	**13.62**	NQ	NQ

Table 3.7. List of upregulated proteins identified by GeLC-MS analysis in response to 100 μM Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	TMH	Cellular location	2h	4h	8h
METABOLISM	Lipid transport and metabolism	Acyl-CoA synthetase	5315	0	plas	**2.93**	1.20	NQ
		Acetyl-CoA acetyltransferase	10015	0	cyto	**2.42**	-0.53	0.73
		SAM-dependent methyltransferases	126764	0	cyto	0.52	**3.03**	-1.44
		Cytochrome P450 CYP4/CYP19/CYP26 subfamilies	137072	2	plas	**2.01**	**5.06**	-0.71
METABOLISM	Secondary metabolites biosynthesis, transport and catabolism	Copper amine oxidase	36058	0	cyto	-1.17	-0.02	**2.83**
		Multidrug resistance-associated protein/mitoxantrone resistance protein, ABC superfamily	136846	14	plas	0.07	**2.49**	1.02
POORLY CHARACTERIZE	Function unknown	Uncharacterized membrane protein	10140	1	mito	**3.93**	0.31	-0.98
		Uncharacterized membrane protein	3798	4	plas	**3.54**	NQ	NQ
Unknown	Unknown	Hypothetical protein	166	2	mito	0.73	**2.00**	-1.00
		ion channel activity	363	7	plas	**2.53**	1.65	**2.50**
		hydrolase activity	894	1	extr	-0.13	-0.68	**3.28**
		Hypothetical protein	4459	0	mito	0.82	**3.53**	0.91
		Hypothetical protein	4671	0	cysk	**2.19**	NQ	**3.45**
		Hypothetical protein	5249	1	cyto	0.06	0.67	**3.14**
		Hypothetical protein	7825	0	mito	NQ	0.86	**2.31**

Table 3.7. List of upregulated proteins identified by GeLC-MS analysis in response to 100 µM Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	TMH	Cellular location	2h	4h	8h
Unknown	Unknown	Hypothetical protein	8405	1	cyto	1.33	**2.88**	-0.07
		hydrolase activity, hydrolyzing O-glycosyl compounds	122483	0	extr	NQ	**2.20**	-0.21
		glucan 1,3-beta-glucosidase activity	122884	1	plas	-0.10	**2.34**	0.26
		carbohydrate metabolism	124827	0	cyto_nucl	1.44	1.19	**2.07**
		ketol-acid reductoisomerase activity	132767	0	mito	**2.01**	**-2.32**	**2.11**
		hydrolase activity	133689	1	extr	NQ	**2.02**	1.93
		Hypothetical protein	134241	1	extr	1.58	1.84	**2.06**
		IMP dehydrogenase activity	134829	1	cyto	1.61	**2.95**	-0.88

***Cyto**; cytoplasmic, **Extr**; extracellular, **Mito**; mitochondrial, **Nucl**; Nuclear, **Cysk**; cytoskeleton, **Cyto_nucl**; cytoplasmic_nuclear, **plas**; plasma membrane, **extr**; extracellular,

TMH: Number of predicted transmembrane helices

NQ: Not quantifiable

Table 3.8. List of downregulated proteins identified by GeLC-MS analysis in response to 100 μM Cu.

KOG Group	KOG Class	Protein Function	Protein ID	TMH	Cellular location	2h	4h	8h
CELLULAR PROCESSES AND SIGNALING	Cell wall membrane envelope biogenesis	Fasciclin and related adhesion glycoproteins	6614	1	extr	-0.42	**-2.21**	-0.70
		Chitin synthase/hyaluronan synthase (glycosyltransferases)	7247	6	plas	-0.48	**-6.03**	-1.37
	Cytoskeleton	Alpha tubulin	10047	0	cysk	-1.50	**-2.02**	**-2.25**
	Defense mechanisms	Ras GTPase activating protein RasGAP/neurofibromin	9174	0	mito	NQ	**-2.01**	-0.04
	Intracellular trafficking, secretion, and vesicular transport	Preprotein translocase subunit Sec66	43328	1	cyto	-0.30	0.38	**-2.18**
		Membrane protein involved in ER to Golgi transport	122171	4	plas	NQ	NQ	**-2.28**
	Posttranslational modification, protein turnover, chaperones	Predicted E3 ubiquitin ligase	1465	0	mito	NQ	**-3.63**	-0.35
		Dipeptidyl aminopeptidase	5061	0	extr	NQ	**-3.01**	0.40
		AAA+-type ATPase containing the peptidase M41 domain	6527	0	mito	NQ	1.50	**-3.57**
		Alkyl hydroperoxide reductase, thiol specific antioxidant and related enzymes	10009	0	cyto	**-2.02**	ND	ND
		26S proteasome regulatory complex, ATPase RPT5	29020	0	cyto	**-3.49**	**-4.67**	0.28
		Aspartyl protease	29558	0	extr	-0.73	**-2.97**	-0.63
		Molecular co-chaperone STI1	32973	0	nucl	-0.36	0.16	-2.19
		Hydrolytic enzymes of the alpha/beta hydrolase	37542	0	cyto	**-2.23**	NQ	0.94

Table 3.8. List of downregulated proteins identified by GeLC-MS analysis in response to 100 μM Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	TMH	Cellular location	2h	4h	8h
CELLULAR PROCESSES AND SIGNALING	Posttranslational modification, protein turnover, chaperones	Aspartyl protease	129956	0	cyto	NQ	**-2.77**	NQ
		Molecular chaperone (DnaJ superfamily)	137352	0	nucl	0.25	1.76	**-2.77**
		Subtilisin-related protease/Vacuolar protease B	138363	0	extr	**-2.52**	**-3.23**	NQ
		Multifunctional chaperone (14-3-3 family)	139500	0	nucl	0.43	-1.61	-2.11
	Signal transduction mechanisms	Synaptic vesicle protein EHS-1 and related EH domain proteins	654	0	nucl	-1.81	**-2.23**	0.63
		Synaptic vesicle protein EHS-1 and related EH domain proteins	977	0	nucl	-1.23	**-2.57**	**2.25**
		RasGAP SH3 binding protein rasputin, contains NTF2 and RRM domains	4531	0	mito	ND	ND	**-2.04**
		Sexual differentiation process protein ISP4	10276	15	plas	**-7.19**	**-3.36**	1.56
		Armadillo/beta-Catenin/plakoglobin	139181	0	mito	**-2.01**	-0.37	NQ
INFORMATION STORAGE AND PROCESSING	RNA processing and modification	ATP-dependent RNA helicase	126823	0	nucl	ND	-2.03	ND
	Translation, ribosomal structure and biogenesis	Ribosomal protein S4	8697	0	nucl	-0.14	1.18	**-2.08**
		40S ribosomal protein S6	42805	0	cyto_nucl	**-2.34**	**-4.09**	0.78
		60S ribosomal protein L14/L17/L23	124435	0	mito	**-2.17**	NQ	ND
METABOLISM	Amino acid transport and metabolism	D-3-phosphoglycerate dehydrogenase, D-isomer-specific 2-hydroxy acid dehydrogenase superfamily	40191	0	cyto	**-2.10**	-1.16	-1.93

Table 3.8. List of downregulated proteins identified by GeLC-MS analysis in response to 100 μM Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	TMH	Cellular location	2h	4h	8h
METABOLISM	Amino acid transport and metabolism	Cystathionine beta-lyases/cystathionine gamma-synthases	130629	0	cyto	0.97	**-2.24**	1.02
	Carbohydrate transport and metabolism	Glycosyl transferase, family 8 - glycogenin	2562	1	mito	**-3.15**	**2.77**	0.60
		Beta-galactosidase	9466	0	extr	NQ	**-4.50**	-1.71
		Beta-N-acetylhexosaminidase	37522	0	cyto	**-2.31**	-0.03	0.31
		Chitinase	39872	0	extr	-1.45	**-2.90**	-0.90
		3-phosphoglycerate kinase	122435	0	cyto	NQ	**-2.68**	-0.28
		Maltase glucoamylase and related hydrolases, glycosyl hydrolase family 31	135833	0	extr	NQ	**-2.34**	NQ
		Dihydroxyacetone kinase/glycerone kinase	137211	0	cyto	ND	ND	-2.04
	Cell cycle control, cell division, chromosome partitioning	Septin family protein (P-loop GTPase)	134975	0	nucl	-0.27	**-2.22**	1.70
	Energy production and conversion	Mitochondrial F1F0-ATP synthase, subunit delta/ATP16	134058	0	mito	**-2.30**	0.34	-0.16
		UDP-glucuronosyl and UDP-glucosyl transferase	3221	0	mito	NQ	-1.82	**-2.66**
		Succinate dehydrogenase, Fe-S protein subunit	7113	0	mito	0.91	**-2.17**	**-5.42**
		Putative cytochrome C oxidase assembly protein	41906	1	cyto	NQ	**2.31**	**-2.02**

Table 3.8. List of downregulated proteins identified by GeLC-MS analysis in response to 100 μM Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	TMH	Cellular location	2h	4h	8h
METABOLISM	Energy production and conversion	F0F1-type ATP synthase, beta subunit	123444	0	mito	0.90	NQ	**-2.06**
		Vacuolar H+-ATPase V1 sector, subunit A	123500	0	cyto	0.11	NQ	**-2.05**
		Dihydrolipoamide dehydrogenase	131879	0	cyto	-0.06	**-2.16**	0.58
		Mitochondrial F1F0-ATP synthase, subunit delta/ATP16	134058	0	mito	**-2.30**	0.34	-0.16
		Vacuolar H+-ATPase V1 sector, subunit E	134368	0	nucl	NQ	-1.26	**-2.20**
		Mitochondrial tricarboxylate/dicarboxylate carrier proteins	134570	0	mito	NQ	0.66	**-2.10**
		Betaine aldehyde dehydrogenase	135533	0	extr	ND	-1.07	**-2.48**
		Aldehyde dehydrogenase	137014	0	cyto	ND	NQ	**-2.05**
	Inorganic ion transport and metabolism	Manganese superoxide dismutase	9267	0	mito	0.25	**-2.13**	0.38
		Heavy metal exporter HMT1, ABC superfamily	35720	5	plas	-0.06	1.15	**-2.57**
	Lipid transport and metabolism	Phosphatidylinositol transfer protein SEC14 and related proteins	2149	0	mito	-1.32	**-3.37**	0.23
		Lysosomal & prostatic acid phosphatases	8617	1	extr	-2.31	NQ	NQ
		3-oxoacyl CoA thiolase	10096	0	mito	1.67	**-2.65**	NQ
		Cytochrome P450 CYP4/CYP19 subfamilies	129940	1	plas	**-3.89**	**-3.58**	**-6.41**

Table 3.8. List of downregulated proteins identified by GeLC-MS analysis in response to 100 µM Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	TMH	Cellular location	2h	4h	8h
METABOLISM	Secondary metabolites biosynthesis, transport and catabolism	Cytochrome P450 CYP3/CYP5/CYP6/CYP9 subfamilies	4557	1	plas	**-4.63**	NQ	**-4.24**
		Multicopper oxidases	26890	1	extr	**-3.13**	**-2.22**	**-8.65**
		Pleiotropic drug resistance proteins (PDR1-15), ABC superfamily	131145	12	plas	ND	ND	**-2.41**
		Cytochrome P450 CYP11/CYP12/CYP24/CYP27 subfamilies	138737	1	plas	-2.06	0.28	**-4.72**
		Cytochrome P450	140188	1	cysk	-0.81	1.23	**-2.51**
POORLY CHARACTERIZED	Function unknown	Uncharacterized conserved protein	8889	0	extr	-1.11	**-2.38**	-1.97
	Function unknown	Uncharacterized conserved protein	131243	11	plas	**-3.34**	**-3.16**	NQ
	General function prediction only	Peroxidase/oxygenase	2255	0	plas	NQ	NQ	-1.21
		Predicted Dolichyl-phosphate-mannose-protein mannosyltransferase	8051	8	plas	NQ	**-2.70**	0.84
		Glucose dehydrogenase/choline dehydrogenase/ mandelonitrile lyase (GMC oxidoreductase family)	38091	0	mito	-1.82	**-3.58**	NQ
		Multiple inositol polyphosphate phosphatase	121720	0	cyto	**-3.20**	**-4.05**	-0.74
		Glucose dehydrogenase/choline dehydrogenase/ mandelonitrile lyase (GMC oxidoreductase family)	127396	0	extr	0.77	**-2.29**	-0.23
		Predicted integral membrane protein	132827	8	plas	-1.05	**-2.33**	NQ

Table 3.8. List of downregulated proteins identified by GeLC-MS analysis in response to 100 µM Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein ID	TMH	Cellular location	2h	4h	8h
POORLY CHARACTERIZED	General function prediction only	Reductases with broad range of substrate specificities	135868	0	cyto	0.22	**-2.65**	NQ
		Predicted DNA repair exonuclease SIA1	137752	1	extr	-0.95	-0.71	**-3.03**
Unknown	Unknown	Hypothetical protein	1179	4	plas	**-2.73**	NQ	**-2.56**
		Hypothetical protein	4580	0	cyto	-0.89	-0.96	**-2.91**
		Hypothetical protein	124204	0	mito	NQ	ND	**-2.43**
		metalloendopeptidase activity	37396	0	cyto	**-2.12**	**-2.51**	1.25
		Hypothetical protein	3383	0	extr	NQ	**-4.53**	ND
		Hypothetical protein	5300	0	extr	-1.22	**-4.35**	-0.38
		Hypothetical protein	5735	0	mito	NQ	**-2.93**	ND
		Hypothetical protein	35714	0	cyto	NQ	**-2.73**	1.67
		iron permease activity	121155	7	plas	**-3.58**	**-2.41**	-0.38
		Hypothetical protein	121728	0	extr	NQ	**-3.64**	NQ
		ion channel activity	130224	7	plas	-0.70	**-3.13**	NQ
		ketol-acid reductoisomerase activity	132767	0	mito	2.01	**-2.32**	2.11
		iron ion binding	138825	0	cyto	-0.15	**-2.73**	NQ
		catalytic activity	3312	1	extr	**-3.98**	NQ	-0.12

***Cyto**; cytoplasmic, **Extr**; extracellular, **Mito**; mitochondrial, **Nucl**; Nuclear, **Cysk**; cytoskeleton, **Cyto_nucl**; cytoplasmic_nuclear, **plas**; plasma membrane, **extr**; extracellular,

TMH: Number of predicted transmembrane helices

3.4.3. Proteins detected only after Cu exposure

A total of 34 proteins were detected after Cu induction, while they were not detected under their respective control conditions. 16 of them were detected after 2h exposure, 8 of them after 4h exposure and 15 of them after 8h exposure.

3.4.3.1. Intracellular trafficking, secretion, and vesicular transport

Endoplasmic reticulum (ER) is an organel which processes the proteins and directs the intracellular protein traffic according to motifs on the proteins. In addition to that ER has the capability of folding processing and quality control of newly synthesised proteins. Under normal conditions there is a dynamic balance between protein synthesis and ER's protein process capacity. However if this balance is disturbed and the ER can't process and fold the proteins then the unfolded proteins accumulated in the ER lumen. This leads to condition known as ER stress and ER stress leads to unfolded protein response (UPR) (Rutkowski and Kaufman, 2004; Schröder and Kaufman, 2005). UPR involves the reducing the protein synthesis rate, degredation of the accumulated proteins in the ER's lumen and increasing the ER's protein process capacity. Recently it was revealed that the heavy metal exposure can causes ER stress and it activates unfolded protein response mechanism (Shinkai et al., 2010, Hiramatsu et al., 2007). Calnexin/calretulin is a 90 kDa, internal ER transmembrane chaperone. It regulates the folding and oligomerization of the newly synthesized proteins (Hebert and Molinari, 2007). In the present study calnexin/calcireticulin and other ER related proteins such as clathrin and COPI were detected only after 2h Cu exposure. This findings may indicate Cu exposure causes and ER stress on *P. chrysosporium* and a UPR system is triggered in response.

In different studies it was proved that mitochondrial phosphate carrier (MPT) proteins have different functions in different organisms (Hamel et al., 2004; Liu et al., 2011; Mayr et al., 2011). Generally an MPT protein responsible for intake of the cytosolic inorganic phosphate to the mitochondria. It is suggested that there is

a link between stress response and overexpression of MPT proteins (Zhu et al., 2012). An MPT overexpressor *A. thaliana* was found to be sensitive against salt stress. Zhu et al. suggested that intracellular ATP concentration has an impact on the stress response. In this study an MPT (2160) was detected after 2h Cu exposure. Although it is contradictory with the findings of Zhu et al. we can speculate that the increased levels of the MPT is required in the cell to supply the increased levels of phosphate needs which is the result of active transport of the heavy metals outside of the cell in an expense of ATP.

3.4.3.2. Posttranslational modification, protein turnover, chaperones

Chaperonin proteins are known as general stress proteins and they stabilizes the proteins under stress conditions. Chaperonin containing TCP-1 (CCT) is consist of 8 or 9 different subunits and it is essential for growth, and especially for the assembly of tubulin and/or actin (Stoldt et al., 1996). CCT were reported as a stress response protein in the cases of cold shock and arsenate treatment in yeast (Somer et al., 2002; Yokota et al., 2000). Although the CCT is constitutively expressed under normal conditions, subunits of this complex (2978, 133339, 134073, 131251, 133830, 134073 and 126013) could only be detected after 2h and 4h Cu exposure by GeLC approach in this study. In addition to that, there is also a great possibility that upregulation of actin and related proteins are relevant with the induction of CCT subunits.

3.4.3.3. Signal transduction mechanisms

Serine/threonine protein phosphatases are known to be important in the signal transduction of stress response in plants and in yeast (País et al., 2009; Santhanam et al., 2004). It was also shown that it was upregulated during the ER stress in human (Christen et al., 2007). Detection of a PP2A catalytic subunit (45101) protein is no surprise, since this study also revealed that Cu exposure causes ER stress in *P. chrysosporium*.

Aconitase/3-isopropylmalate dehydratase balances the amount of citrate and

isocitrate in the cytoplasm, which in turn creates a balance between the amount of NADPH generated from isocitrate by isocitrate dehydrogenase. After Cu exposure an aconitase protein was detected. This finding also confirms the mechanism in which the organism tries to produce more NADPH to counteract the ROS.

Glycogen Synthase Kinase-3 (GSK-3) is a serine/threonine protein kinase that adds phosphate molecules to serine and threonine amino acid residues. GSK3 has a key role in the regulation of many cellular functions, including signalling by insulin , growth factors and nutrients, control of cell division, apoptosis and microtubule function. In addition to that it was also shown that cells have evolved some mechanisms to switch off GSK3 activity in response to different signals (Cohen and Frame, 2001). Hirata et al., (2003) showed that GSK-3 is also required for the transcription of stress responsive genes in yeast. In the present study, detection of a GSK-3 protein (132466) after Cu exposure indicates that *P. chrysosporium* also uses a similar signal transduction mechanism to transcribe stress responsive genes.

3.4.3.4. Translation and ribosomal structure

Sensory transduction histidine kinases were shown to be as a salt stress sensors in cyanobacterium *Synechocystis sp.* (Marin et al., 2003). Similarly a sensory transduction histidine kinase were found to be induced after 4h of Cu exposure which suggest that *P. chrysosporium* also requires a histidine kinase to sense the abiotic stress

Additionally five hypothetical proteins were newly detected after Cu exposure. Further studies are required to reveal the functions and relations of these proteins with the Cu stress response mechanism.

Table 3.9. List of newly induced proteins identified by GeLC-MS analysis in response to 100 μM Cu.

KOG Group	KOG Class	Protein Function	Protein Id	TMH	Cellular location	2h	4h	8h
CELLULAR PROCESSES AND SIGNALING	Cytoskeleton	Actin-related protein Arp2/3 complex, subunit ARPC3	1540	0	cyto		*	
	Intracellular trafficking, secretion, and vesicular transport	Vesicle coat complex COPI, beta subunit	36104	0	cyto			*
		Vesicle coat protein clathrin, heavy chain	125193	0	cyto_nucl	*		
	Posttranslational modification, protein turnover, chaperones	26S proteasome regulatory complex, ATPase RPT3	361	0	nucl	*		
		Chaperonin complex component, TCP-1 epsilon subunit (CCT5)	2978	0	cyto		*	
		Chaperonin complex component, TCP-1 eta subunit (CCT7)	126013	0	cyto	*		
		26S proteasome regulatory complex, subunit RPN7/PSMD6	131251	0	cyto	*	*	
		Chaperonin complex component, TCP-1 delta subunit (CCT4)	133339	0	mito	*		
		Molecular chaperone (small heat-shock protein Hsp26/Hsp42)	133830	0	cyto_nucl		*	
		Chaperonin complex component, TCP-1 zeta subunit (CCT6)	134073	0	cyto	*		
		Glutaredoxin and related proteins	135692	0	mito	*		
		Calnexin	7453		extr	*		

Table 3.9. List of newly induced proteins identified by GeLC-MS analysis in response to Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein Id	TMH	Cellular location	2h	4h	8h
CELLULAR PROCESSES AND SIGNALING	Signal transduction mechanisms	OTU-like cysteine protease	111	1	nucl			*
		Serine/threonine protein phosphatase 2A, catalytic subunit	45101	0	cyto	*		
		Sensory transduction histidine kinase	140795	0	cysk		*	
INFORMATION STORAGE AND PROCESSING	Chromatin structure and dynamics	SWI-SNF chromatin-remodeling complex protein	213	0	nucl			*
	RNA processing and modification	ATP-dependent RNA helicase	136188	0	nucl	*		
	Translation, ribosomal structure and biogenesis	60S ribosomal protein L11	2657	0	mito			*
		60s ribosomal protein L2/L8	9996	0	mito			*
		60S ribosomal protein L21	130471	0	mito			*
		40S ribosomal protein S11	134847	0	mito	*		
METABOLISM	Lipid transport and metabolism	Putative phosphoinositide phosphatase	124764	2	plas			*
	Inorganic ion transport and metabolism	Na+/K+ ATPase, alpha subunit	123319	10	plas			*
		Protein involved in inorganic phosphate transport	136625	2	cyto			*
		Porin/voltage-dependent anion-selective channel protein	138775	0	Cyto	*		

Table 3.9. List of newly induced proteins identified by GeLC-MS analysis in response to Cu. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein Id	TMH	Cellular location	2h	4h	8h
METABOLISM	Carbohydrate transport and metabolism	UDP-glucose:glycoprotein glucosyltransferase	24966	0	extr			*
		Glycogen synthase kinase-3	132466	0	cyto	*		*
	Amino acid transport and metabolism	3-isopropylmalate dehydratase (aconitase superfamily)	10011	0	cysk	*		
	Energy production and conversion	Mitochondrial phosphate carrier protein	2160	0	mito	*		
	Cell cycle control, cell division, chromosome partitioning	Cullins	124505	0	nucl			*
POORLY CHARACTERIZED	General function prediction only	Ras-related small GTPase, Rho type	5909	0	mito			*
	Function unknown	FOG: Low-complexity	5811	0	Nucl		*	
		protein involved in ubiquitination and degradation at the ER surface	6163	1	extr		*	
Unknown	Unknown	Hypothetical protein	6906	1	mito	*		
Unknown	Unknown	Unknown	2552	0	cyto			*

***Cyto**; cytoplasmic, **Extr**; extracellular, **Mito**; mitochondrial, **Nucl**; Nuclear, **Cysk**; cytoskeleton, **Cyto_nucl**; cytoplasmic_nuclear, **plas**; plasma membrane, **extr**; extracellular,

TMH: Number of predicted transmembrane helices

Table 3.10. List of proteins that could not be detected after Cu treatment.

KOG Group	KOG Class	Protein Function	Protein Id	TMH	Cellular location	2h	4h	8h
CELLULAR PROCESSES AND SIGNALING	Intracellular trafficking, secretion, and vesicular transport	GDP-fucose transporter	3721	7	plas	*		
	Posttranslational modification, protein turnover, chaperones	Predicted E3 ubiquitin ligase	1465	0	mito	*		
		Thioredoxin	7498	0	plas	*		
		Subtilisin-related protease/Vacuolar protease B	138363	0	extr			*
	Signal transduction mechanisms	Ca2+/calmodulin-dependent protein kinase, EF-Hand protein superfamily	135087	0	cyto			*
METABOLISM	Energy production and conversion	Mitochondrial solute carrier protein	10978	0	mito			*
		Pyruvate dehydrogenase E1, alpha subunit	563	0	mito			*
		Zinc-binding oxidoreductase	133678	0	cyto			*
		Dihydrolipoamide acetyltransferase	871	0	cyto	*		
		NADH:ubiquinone oxidoreductase, NDUFS2/49 kDa subunit	126052	0	mito	*		
	Inorganic ion transport and metabolism	Cation transport ATPase	137802	8	plas	*		
POORLY CHARACTERIZED	General function prediction only	Ras-related small GTPase, Rho type	5909	0	mito	*	*	
		N-methyltransferase	424	8	plas		*	
		Predicted endoplasmic reticulum membrane protein Lec35/MPDU1	128941	5	plas			*

Table 3.10. List of proteins that could not be detected after Cu treatment. (Cont'd)

KOG Group	KOG Class	Protein Function	Protein Id	TMH	Cellular location	2h	4h	8h
Unknown	Unknown	Hypothetical protein	140681	0	cyto		*	
Unknown	Unknown	Hypothetical protein	6434	3	plas			*
Unknown	Unknown	Hypothetical protein	121062	1	extr			*
	Function unknown	Uncharacterized conserved protein	125135	0	mito	*		

***Cyto**; cytoplasmic, **Extr;** extracellular, **Mito;** mitochondrial, **Nucl;** Nuclear, **Cysk;** cytoskeleton, **Cyto_nucl;** cytoplasmic_nuclear, **plas;** plasma membrane, extr; extracellular,

TMH: Number of predicted transmembrane helices.

CHAPTER 4

4. CONCLUSION

- In this study, physiological proteome of *P. chrysosporium* were investigated under copper and cadmium stress. Two main approach were employed to investigate the physiological proteome of the organism: 2D-PAGE-MS and GeLC-MS based SILAC.

- 2D-PAGE-MS analysis of the Cu response revealed a total of 123 differentially expressed protein spots. 89 of these spots could be identified and further analysis revealed that the 89 protein spots are the products of the 58 distinct ORFs. Major functional classes of the identified proteins were (i) posttranslational modification, chaperones, (ii) energy production and conversion and (iii) amino acid metabolism.

- Analysis of the Cd response by the help of 2D-PAGE-MS revealed a total of 130 differentially expressed protein spots. 89 of these spots could be identifed and further analysis revealed that the 89 protein spots are the products of the 58 distinct ORFs. Major functional classes of the identified proteins were (i) posttranslational modification, protein turnover, chaperones, (ii) energy production and conversion and (iii) amino acid metabolism.

- To enrich the membrane proteins in the protein extracts three different approaches were tested and cell wall disruption with lysing enzymes followed by PEG/dextran aqueous two phase separation was found to be superior over membrane shaving approach and cell wall disruption by beta glucoronidase. By using this method, membrane protein fraction

percentage was elevated up to 20% within the identified proteins.

- To quantify and compare membrane enriched proteomes, SILAC technique was successfully employed and more than 700 proteins were identified and quantified by the help GeLC method.

- By the help of GeLC-MS based SILAC method, from the Cu treated samples 83 proteins were found to be upregulated and 55 were found to be down-regulated in the membrane enriched protein fraction. Additionally, 32 proteins were only detected in Cu exposed samples and 18 of the proteins could not be detected after Cu treatment.

- In this study, GeLC-MS method was successfully applied and resulted in identification and quantification of not only the membrane proteins but also the cytosolic proteins that could not be detected by the 2D-PAGE-MS method.

- Molecular chaperones, Hsp60, Hsp70, Hsp40, Hsp26/Hsp42, mortalin and chaperonin complex subunit TCP-1 were among the most drastically upregulated proteins, indicating that organism uses Hsp proteins heavily to counter the heavy metal effect.

- Upregulation of several ribosomal proteins including 60S RpL18, mitochondrial/chloroplast RpL12, 60S RpL7A, 40S RpS2/30S RpS5, 60S RpL10A, 40s RpS26 and RpS7 were also monitored throughout the 8h heavy metal exposure. The highly upregulated levels of ribosomal proteins seems to be a key element in responding to heavy metal induced damage.

- A Ran GTPase upregulation implies that heavy metal stress response was also accomplished by the Ras GTPase signalling cascades.

- In the 8h samples, the finding that the upregulated proteins belonging to usual metabolism like a homeobox protein implied that the negative effects of the heavy metal starts fading at this late hour and organism

returns to its normal life cycle.

- Proteins of energy metabolism and conversion were also found to be upregulated which may be explained by two main reasons: (i) to supply the energy demand of the organism which increases during stress and (ii) to produce more NADPH to increase the organism's antioxidant capacity.

- By identifying the main heavy metal stress responsive proteins and pathways, this study establishes a firm groundwork for future studies. Although several of the proteins identified in this work were demonstrated in many other studies investigating physiological stress responses, there were many other newly identified proteins. The involvement of the latter, points to the need for more detailed investigations in order to reveal their function and/or their relation to stress response.

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APPENDIX A

STOCK SOLUTIONS

A) Colloidal Coommassie Staining

1-Coomassie Brilliant Blue (CBB) Stock

Coomassie Brilliant Blue G-250	5 g
dH_2O	100 mL

2-Fixation Buffer

40 % Ethanol	125 mL
10 % Acetic Acid	25 mL
50 % dH_2O	100 mL

The gel is shaked in this solution for 1 to 2 hours

3-Washing

Gels are washed with dH_2O two times for 10 min.

4-CCB dye solution

Ammonium sulfate	100 g
85% phosphoric acid	12 mL
CBB stock solution	20 mL
Distilled water add to	1000 mL

5-CCB staining

CCB dye solution	200 mL
Methanol	50 mL

Gels are put in to 200 mL CCB dye solution. 50 mL methanol is added afterwards. Gels are kept in this solution for 24 hours.

6-Washing

The gel is washed with dH_2O until protein spots are clearly visible.

B) Membrane enrichment buffers

1- Homogenization buffer

Sucrose	0.25M
Trichoderma harzianum lysing enzymes	1 mg/ml
MOPS	50 mM
EDTA	1 mM
DTT	1 mM
Roche c0mplete protease inhibitor cocktail	1 tablet /100 ml
PMSF	1 mM
pH	7.5

2-PEG/Dextran

PEG	6.4 %
Dextran	6.4 %
Sucrose	330 mM
KCl	5 mM
EDTA	1 mM
DTT	1 mM
K_2HPO_4/KH_2PO_4 (pH: 7.8)	5 mM

APPENDIX B

CHEMICALS AND THEIR SUPPLIES

Chemicals	Supplier
Acetic acid	Merck
Acetone	Merck
Acrylamide	Sigma
Ammonium sulfate	Merck
Ampholines pH (3-10)	Fluka
Bis-acrylamide	Sigma
Bovine Serum Albumin (BSA)	Sigma
$CaCl_2$ H_2O	Merck
CH3CN	Applichem
CHAPS	Merck
Comassie Brillant Blue G 250	Sigma
DTT	Fluka
Ethanol	Merck
Glucose	Merck
Glycerol	Merck
Glycine	Merck
H_3PO_4	Merck
HCI Merck	Merck
IPG strips	BioRad
KH_2PO_4	Merck
Lysing enzymes	Sigma
Methanol	Merck
$MgSO_4$ $7H_2O$	Merck
Molecular Weight Standard (14,400-116,000)	Fermentas
NaOH	Merck
NH_4Cl	Merck
$^{15}NH_4Cl$	CIL
NH_4HCO_3	Applichem
SDS	Sigma
TEMED	Sigma

TFA	Applichem
Thiourea	Fluka
Trichloroacetic acid (TCA)	Merck
Tris- HCI	Sigma
Urea	Fluka

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WORK EXPERIENCE

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LIST OF PUBLICATIONS

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1. Özcan, S., Yıldırım, V., Kaya, L., Albrecht, D., Becher, D., Hecker, M., and Ozcengiz, G. (2007). Phanerochaete chrysosporium soluble proteome as a prelude for the analysis of heavy metal stress response. Proteomics 7, 1249–1260.

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