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ORIGINAL RESEARCH ARTICLE

Variability in Protein Expression in Marine-Derived *Purpureocillium lilacinum* Subjected to Salt and Chromium Stresses

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Abstract Abiotic factors can cause substantial limitation of growth of microbes. A combination of salinity stress along with chromium (Cr^{+6}), one of the carcinogen, can pose an immediate threat to any living system. To understand how salinity (0, 35 and 100 PSU) and Cr(VI) stress (0, 100 and 500 ppm), affects cells at the molecular level, the cellular response of *Purpureocillium lilacinum* to the individual as well the combination of both the stresses were studied by peptide mass fingerprinting technique. The study reports 1412 proteins, of which 105 proteins were found to be present across all conditions. The most prevalent functional class expressed was genetic information processing. Proteins involved in free radical scavenging were up-regulated in response to the oxidative stress generated due to both the applied stresses while expression of metal chelators, transporters systems, indicated towards multiple stress tolerance mechanisms to combat synergistic effects of salt and Cr stress.

Keywords Filamentous fungi · Glutathione · Heat shock proteins · LCMS QToF · Proteomics · Protein expression · Salinity stress · Stress tolerance mechanisms

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Introduction

Purpureocillium lilacinum, previously known as *Paecilomyces lilacinus* is one of the most widely reported endophytic filamentous fungi found in various terrestrial as well as estuarine habitats [1]. This hyphomycete is profusely studied for its use as a biocontrol agent against plant-parasitic nematodes as well as for production of various biologically active secondary metabolites leading to many medical discoveries in human research [2]. Although a wealth of literature is available on the medical importance of this fungus, its ability to tolerate heavy metals has never been addressed before, which can give a new insight for its growth in the presence of heavy metals as endophyte as well independently its applications in industrial waste remediation [3]. *Purpureocillium* sp. has been reported to protect mangrove plant *Kandelia candel* under copper stress [4]. Heavy metals especially chromium, pose a significant threat to the environment as well as human health. High solubility and redox-active nature of Cr(VI), allows it to damage cellular organelles oxidatively and is therefore termed carcinogenic. Due to functional and structural similarities between various genes in mammals and lower eukaryotes, cellular response of such toxic metals in lower organisms will provide a better understanding of mechanisms in human cells [5]. Despite the estuarine occurrence of *P. lilacinum*, mechanisms of tolerating salinity stress by this isolate have also never been formulated. Active efflux of cations and accumulation of osmolytes have been widely documented as mechanisms for salt tolerance in many organisms [6–8] while resistance nodulation cell division (RND) driven export, cation diffusion facilitators (CDF) and *P*-type ATPases have been reported to mediate efflux of divalent metal ions out of the cell [9]. But a combination of both stresses, salt, and metal,

would give a better insight into the mechanisms adapted by lower eukaryotes to evade deleterious effects of stress. Therefore by using modern proteomics tools, protein profiling will provide accurate and comprehensive information of the expressed proteins in an organism under stress at transcriptional as well as translational levels [5]. This study aimed to analyze the proteins expressed by marine-derived *P. lilacinum*, subjected to varying salinity and Cr(VI) stresses, individually and in combination.

Materials and Methods

Strain Isolation

Purpureocillium lilacinum, #NIOSN-SK56-S76 culture was isolated from off-shore sediment collected from Arabian Sea (15°30′N, 73°40′) on Zobell Marine agar (ZMA) and identified using molecular technique of ITS sequencing (ITS sequence submitted to NCBI with accession no KY788339 and pure culture submitted to MTCC, Chandigarh, India) and maintained on Czapek Dox agar (CDA). This isolate has been previously studied for its tolerance to chromium in broth as well as on agar [3].

Growth Under Saline Condition and Determination of MIC for Chromium

The culture was grown in 100 mL Erlenmeyer flasks with 20 mL of Czapek Dox broth prepared in distilled water (CDB-dw) and incubated at 28 °C till the onset of sporulation. On sufficient amount of growth, the mycelia were crushed using sterile glass beads, and aliquots of one mL were aseptically inoculated in 20 mL of CDB containing varying salinities (0, 35, 50, 100 and 250 PSU). The flasks were incubated at 80 rpm for a week at 28 °C. The contents were then filtered through 0.45 µm pore sized filter paper, and the residue was lyophilized to determine dry weights of the biomass. To determine the minimum inhibitory concentration (MIC) of chromium, the isolate was grown as mentioned above. The mycelia was crushed and inoculated in CDB (dw) containing varying concentrations of Cr(VI) (0, 250, 500, 750, 1000, 1250 ppm) prepared from 100 mM stock of $K_2Cr_2O_7$, where the pH was adjusted to 5, and incubated at 80 rpm at 28 °C for a week. The contents were filtered and lyophilized to determine biomass weight. Aliquots of crushed mycelia were also added to pre-weighed tubes to determine initial biomass added.

Protein Extraction and Sample Processing

Purpureocillium lilacinum was grown till the onset of sporulation in 20 mL of CDB-dw and crushed using sterile beads to add aliquots of one mL into experimental flasks. The crushed mycelia were inoculated into CDB prepared with varying salinities (0, 35 and 100 PSU), each comprising of varying Cr(VI) concentrations (0, 100 and 500 ppm) giving rise to nine different conditions, and incubated for a week at 80 rpm at 28 °C. After incubation, contents of the flasks were filtered and lyophilized before weighing and extraction of proteins. Aliquots of crushed mycelia were also added to pre-weighed tubes to determine initial biomass added.

Approximately 30 mg of lyophilized biomass was taken from all nine conditions for protein extraction using a previously standardized 3-buffer extraction method (unpublished work). In short, the sample was suspended in 500 µL of buffer I [Tris HCl, CHAPS, $MgCl_2$, and dithiothreitol (DTT)] and homogenized (FastPrep, MP Biomedicals, USA) using zirconia beads, twice at 6.5 m s^{-1} for 60 s. On centrifugation at 14000 rpm, the supernatant was collected in a clean tube and stored in ice. The homogenized biomass was further suspended in 500 µL of buffer II (urea, CHAPS, and DTT) and homogenized similarly. The resultant supernatant on centrifugation was collected in the same tube used previously to obtain the supernatant from buffer I. The sample tube was homogenized for the last time using 500 µL of buffer III (urea, thiourea, CHAPS, and DTT) and the supernatant was collected in the tube with supernatants from buffer I and II. At all the homogenization steps, five µL of protease inhibitor cocktail was added to the sample to prevent degradation of proteins, also taking care that the entire procedure was carried out in ice. Pooled supernatants were thoroughly mixed and quantified using Folin Lowry method [10]. On quantification, 250 ng of protein was used to carry out SDS-PAGE on a 12% resolving gel with 6% stacking gel. The samples were electrophoresed at 90 V for two h. The gel was stained using silver staining technique [11] to check the quality of proteins before processing them further for MS analysis.

Tryptic Digestion and LC–MS Separation

After checking the protein profile on the SDS gel, the protein sample was further processed for in-solution digestion (modified from [12]). For this, 100 µL of protein extract was precipitated using methanol (HPLC grade). The air-dried precipitates were then suspended in urea solution to dissolve the proteins. These proteins were reduced using DTT and alkylated using iodoacetamide (IAA) before digesting with trypsin. The digested samples (100 µL) were transferred into MS vials and analyzed using in-house LCMS QToF facility (6538 UHD Accurate Mass QTOF LC/MS, Agilent Technologies, USA). Four replicate injections of 8 µL each of the digested protein samples were carried out through auto-sampler. Protein chip used was 150 II 300A

C18 150 mm column. The LC separations were carried out using aqueous phase (deionized water, 0.22 μm filtered and autoclaved) and an organic phase (HPLC grade acetonitrile) across a gradient (3–97%) using formic acid (0.1%) as an adduct in both the phases over 100 min. A range of 50–2000 m/z in positive mode was maintained for MS/MS of separated ions, at 2 ppm accuracy. The spectral data after MS/MS was acquired with the help of Mass Hunter software ver 5.0 (Agilent Technologies, USA) and was searched against the organism-specific database (NCBI txid 33203) in NCBI using Spectrum Mill MS Proteomics Workbench ver. B.04.01.141 (Agilent Technologies, USA) at an accuracy of 50–100 ppm. The MS searches were then auto-validated at a false discovery rate (FDR) of 1.2%, followed by generation of protein summary files (Supplementary Data) and MPP files. The files generated in Spectrum Mill were processed in Mass Profiler Professional ver. 13 (MPP13, Agilent Technologies, USA) for further analysis. Since this is a high throughput technique, samples were run as technical replicates as opposed to biological replicates and were grouped on the basis of salinities and Cr(VI) concentrations.

Results and Discussion

Fungi surviving in environments with low amounts of biologically available water were often considered as contaminants, but are now recognized as integral part of saline microbial communities and often categorized as highly halotolerant rather than halophilic [13]. Halotolerant fungi have been found to grow and adjust under a wide range of salinities, and they can also survive without salt. This was observed in the current study with marine-derived *Purpureocillium lilacinum*, which grew at 0 PSU as well sodium saturated 100 PSU salinity. The isolate belonging to Hypocreales produced purple spores of circular to an oval shape (Supplementary Fig. 1) and was found to be halotolerant, growing in both absence and presence of salt in medium with appreciable biomass production at varying salinity conditions. Maximum biomass was produced under salt concentrations of 100 PSU but failed to grow at 250 PSU (Table 1). The culture was able to tolerate three times more the salt concentration than from which it was isolated. Apart from being halotolerant, the isolate was also able to withstand Cr(VI) stress and showed MIC value of 750 ppm for Cr(VI) (Table 1).

Protein Profiling of *Purpureocillium lilacinum* Subjected to Salinity and Cr(VI) Stress

The LC–MS/MS analysis used to elucidate the cellular response of *P. lilacinum,* to salinity and Cr(VI) stress, led to the identification of 1412 expressed proteins in total, across all nine conditions (0, 35 and 100 PSU at of 0, 100 and 500 ppm Cr(VI) concetrations each). All the detected proteins could be divided into 17 categories (Fig. 1) on the basis of their functional processes based on KEGG pathways and metabolic processes. Majority of the proteins belonged to basic cellular functional processes such as carbohydrate metabolism, amino acid metabolism, cellular processes, environmental information processing, genetic information processing, etc. It is important to note that a sizeable amount (15%) of proteins belonged to hypothetical proteins whose functions are unknown and these could also include proteins responsible for combating both the stresses. Amongst all the proteins procured, 105 proteins were commonly expressed in all the conditions whereas a number of unique proteins were also found in each condition (Table 2). Most of the commonly expressed proteins belonged to transcription/translation proteins (ribosomal proteins, elongation factors, Ran GTPase); carbohydrate metabolism (phosphoglycerate kinase, pyruvate decarboxylases, isocitrate dehydrogenase, transaldolases); Glutathione metabolism (ascorbate peroxidase, glutathione reductase, peroxiredoxins); amino acid metabolism (glutamine synthetase, aspartate aminotransferase, adenosylhomocysteinase); proteins involved in oxidative phosphorylation (ATP synthases, cytochrome c oxidases) and also commonly occurring stress response proteins (heat shock proteins, peroxidases). To understand whether salinity alone caused any variability in protein profiles, proteins produced by *P. lilacinum* across varying salinities (0, 35 and 100 PSU) were compared against each other (Fig. 2). It was observed the number of proteins detected did not vary much across varying salinities. The maximum

Table 1 Biomass produced by *P. lilacinum* at varying salinities and concentrations of Cr(VI)

Salinity (PSU)	Biomass (g)	Cr(VI) concentration (ppm)	Biomass (g)
0	0.175 ± 0.006	0	0.158 ± 0.009
35	0.229 ± 0.009	250	0.026 ± 0.001
50	0.225 ± 0.015	500	0.016 ± 0.001
100	0.236 ± 0.045	750	0.001 ± 0.001
250	0.001 ± 0.0	1000	0.0 ± 0.0
		1250	0.0 ± 0.0

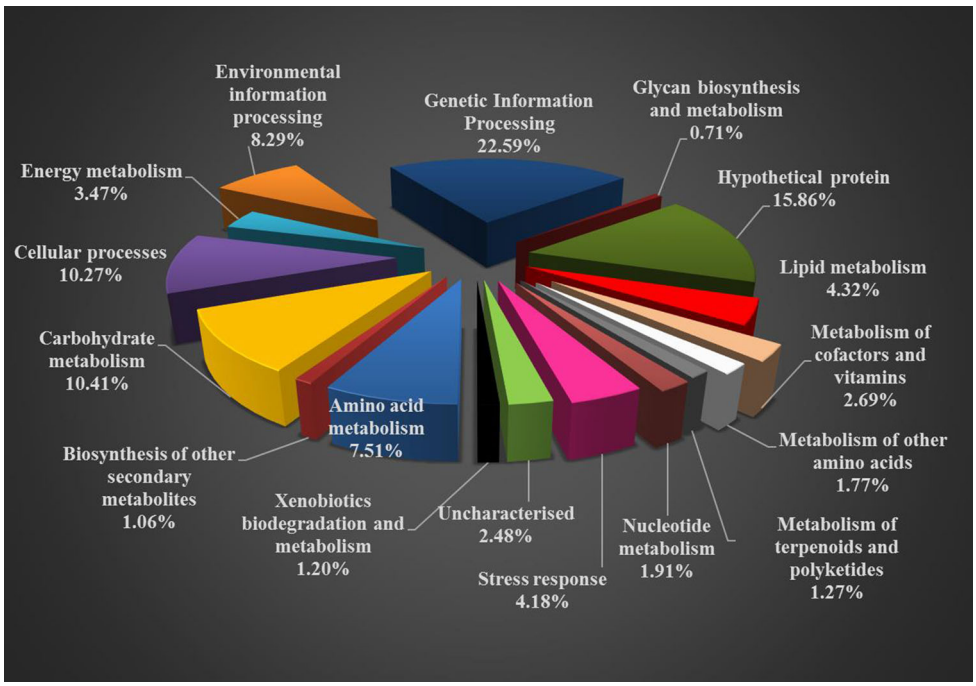

Fig. 1 Graphical representation of functional distribution of all proteins acquired under all conditions for *P. lilacinum*

Table 2 Variability in expressed protein numbers across different conditions

Salinity (PSU)	Cr(VI) concentration (ppm)	Number of proteins[##]	
		A	B
0	0	453	68
	100	449	48
	500	454	51
35	0	426	63
	100	380	32
	500	544	80
100	0	426	56
	100	483	49
	500	41	6

A Number of proteins expressed in one condition

B Number of unique proteins in one condition

[##]Total number of proteins identified across all conditions was 1412

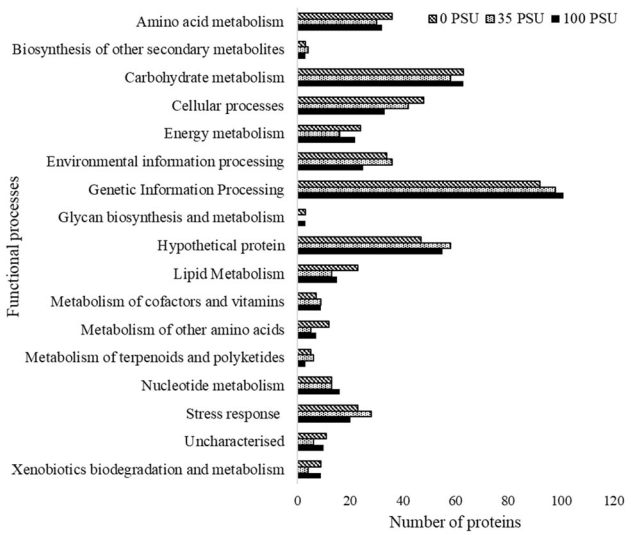

Fig. 2 Graphical representation comparing functional distribution of proteins acquired under varying salinity (0, 35 and 100 PSU) for *P. lilacinum*

number of proteins belonged to the category of genetic information processing which involves processes such as DNA replication, repair, transcription, translation along with folding and sorting of proteins. Pathways involved in Glycolysis, TCA cycle, Pentose shunt, pyruvate, and sugar metabolism were equally expressed under 0 and 100 PSU salinities. It was also observed that proteins involved in membrane transport and signal transduction belonging to environmental information processing were least expressed in 100 PSU salinity which also coincided with the less number of stress proteins expressed in this condition. Similarly, proteins involved in cellular processes such as cell cycle and apoptosis were also found to be decreased at 100 PSU. The primary response of fungi growing in the presence of salts is an accumulation of intracellular solutes to maintain osmotic balance and generate positive turgor pressure [14]. The most reported osmoprotective compounds accumulated by fungi as a response to salinity stress are been polyols (glycerol), sugars (trehalose, mannitol, erythritol, dulcitol, ribitol, inositol), amino acids (glutamine, proline, glycine), amino acid derivatives (peptides, N-acetylated amino acid) and other organic

solutes (betaine, ectoines) [7, 15–17]. This could be correlated with expression of glucose-regulated protein precursor, glycerol kinase, phosphatidylinositol phospholipase C which is involved in inositol metabolic process and sugar 1, 4- lactone oxidase proteins by *P. lilacinum*, in the presence of 35 and 100 PSU salinity which possibly play a role in osmoregulation. In addition to this, MAPK (Mitogen-Activated Protein Kinase) signaling protein, C2H2 zinc finger protein were expressed under 35 PSU along with other signal transduction proteins such as CMGC/SRPK protein kinase and rho guanyl nucleotide exchange factor. Proteins involved in transport such as Mg^{+2} transporter protein and vacuolar import and degradation protein 27, were also expressed at both these salinities (35 and 100 PSU). The osmotic adjustment has been reported by fungal cells by the exclusion of Na^{+2} ions out of the cells [16]. Nikolauo [18] reported activation of HOG (High Osmolarity Glycerol) and MAPK pathways play an important role in response to elevated osmotic stress apart from the accumulation of osmolytes in *S. cerevisiae*. Another study reported the expression of ABC transporters in cotton roots in response to salinity stress [19]. ABC transporters have multiple functions, of which transport of a variety of substrates as well as xenobiotic compounds across membranes is one [20]. Salinity stress which leads to the production of ROS also activates production of antioxidant enzymes and free radical scavengers. Enzymes such as superoxide dismutase, catalase, peroxidase are involved in detoxification of O_2^- radicals and H_2O_2, while oxidized antioxidants such as glutathione (GSH), glutathione reductase (GR), ascorbate help in removal of H_2O_2 from cellular compartments [7, 17] were also expressed in the current study. Heat shock proteins such as glomalin have been previously reported to be expressed under NaCl stress [21]. It was observed that a number of autophagy and cell senescence proteins along with stress/DNA repair proteins, were expressed by *P. lilacinum* under salt stress.

Proteomic analysis has helped in obtaining functional information regarding various microbial communities and their responses to heavy metals [22], and the same has been achieved here. Amongst all nine conditions, the least number of proteins were found under 500 ppm concentration of Cr(VI) at 100 PSU salt concentration. Although the isolate has been found to grow exceptionally well at 100 PSU salinity, the decrease in proteins expressed could be attributed to stress caused by Cr(VI) at 500 ppm. Interestingly, at the same concentration of Cr(VI) under 35 PSU salinity, the protein numbers were not affected in comparison to presence/absence of salinity or Cr(VI) stress condition. This proves that higher salt concentrations along with metal stress have a synergistic effect as toxic inhibitors for growth. Since metals cannot be synthesized nor degraded, their homeostasis can be achieved via transport in/out of the cell, chelation and sequestration [23, 24]. One of the most widely reported responses towards heavy metal stresses is the production of molecular chaperones and heat shock proteins/stress proteins. The primary function of chaperones is the prevention of inappropriate protein aggregation and degradation of misfolded or damaged proteins [20, 25]. They also deliver metal ions to organelles and metal requiring proteins [23]. Heat shock proteins such as HSP70 and HSP90 group are an evolutionarily conserved set of proteins that assist in many processes such as protein folding, transport, maturation and degradation [25, 26], hence have been considered as useful universal biomarkers for stress. The current study detected the expression of metal chelating proteins such as pyoverdine/tyrosine biosynthesis protein along with F1F0-ATP synthase regulatory factor Stf2 involved in the transport of H + ions and GDP/GTP exchange factor Sec2p involved in vesicular transport, at 500 ppm Cr(VI) under 35 PSU salinity. Metal chelators have been previously reported in literature where picolinic acid, a transition metal ion chelator [27], hemopexin, free radical scavenging glycoprotein [20], glomalin, an insoluble glycoprotein that binds to toxic heavy metals [26], have been expressed in the presence of metals. Other metal chelators such as metallothioneins and phytochelatins decrease metal toxicity by forming stable metal–organic complexes via Cys-thiol groups [28, 29]. The production of metal chelators by *P. lilacinum* under the saline condition, coupled with production of various transporter proteins, directs towards the mechanism of chelation of Cr(VI) and then transporting it to vacuoles. The final step of heavy metal detoxification is sequestering of the metal ions or metal complexes into cell vacuoles which are usually mediated by *P*-type ATPases, ABC transporters and tonoplast-bound cation/proton exchangers [30, 31].

Each condition under this study also showed unique proteins. Some unique proteins produced under Cr(VI) stress (0, 100, 500 ppm) in 0 PSU salinity are listed in Table 3. On comparison within conditions, i.e., between Cr(VI) concentrations in 0 PSU salinity, proteins such as Hsp90 co-chaperone, aminopeptidase Y which is involved in glutathione metabolism, and proteins belonging to PX domain, tubulin-specific chaperone c, protein EFR3, which are involved in transport, were expressed. At 100 ppm Cr(VI) in 35 PSU salinity, DNA repair proteins such as DNA mismatch repair proteins were expressed along with proteins involved in amino acid metabolism such as chorismate synthase protein, phosphoserine aminotransferase, peptidase A4 protein) while at 500 ppm Cr(VI), metal chelator protein (pyoverdine/dityrosine biosynthesis protein) was expressed along with proteins involved in rectification of misfolded proteins (FR47, LSM, v-snare) and vesicular transport were expressed in response to metal

Table 3 Unique proteins expressed under varying Cr(VI) concentration at 0 PSU salinity

0 PSU_0 ppm Cr(VI)		0 PSU_100 ppm Cr(VI)		0 PSU_500 ppm Cr(VI)	
Protein name	Function	Protein name	Function	Protein name	Function
Membrane-associating domain	ABC transporters	Calcium-proton exchanger	Calcium transport	WD40 repeat-domain	Cellular senescence
Protein TEM1	Cell growth and death	Cyclophilin type peptidyl-prolyl cis–trans isomerase/CLD	Folding, sorting and degradation	Tctex-1	Cytoskeleton proteins
TRAPP complex component Bet3	Folding, sorting and degradation	Ubiquitin C-terminal hydrolase	Folding, sorting and degradation	Amidohydrolase	D-Glutamine and D-glutamate metabolism
Ubiquitin-protein ligase (Hul4)	Folding, sorting and degradation	Protein EFR3	Membrane trafficking	PHD and RING finger domain	Folding, sorting and degradation
Bicyclomycin resistance protein	MFS transporter	Gibberellin 20-oxidase	Metabolism of terpenoids and polyketides	Aminopeptidase Y	Glutathione metabolism
fmi2 protein	Oxidative phosphorylation	Coatomer subunit delta	Oxidative phosphorylation	Phosphoserine aminotransferase	Glycine, serine and threonine metabolism
Endodeoxyribonuclease-like protein	Replication and repair	Cytochrome c oxidase polypeptide VIb	Oxidative phosphorylation	Imidazole glycerol phosphate synthase hisHF	Histidine metabolism
Ribonuclease/ribotoxin	Replication and repair	FMI1 protein	Oxidative phosphorylation	Golgin subfamily A member 7/ERF4	Membrane trafficking
C3HC zinc finger protein	Signal transduction	C2 domain protein	Signal transduction	Tropomyosin	Membrane trafficking
CNH domain	Signal transduction	HET domain	Signal transduction	Isopenicillin N synthetase	Penicillin and cephalosporin biosynthesis
Ferritin ribonucleotide reductase	Signal transduction	GTPase activating protein	Signal transduction	Metallo-beta-lactamase superfamily protein	Penicillin and cephalosporin biosynthesis
Protein mesA	Signal transduction	SAM binding domain	Signal transduction	pps1 dual specificity phosphatase	Signal transduction
Rheb small monomeric GTPase	Signal transduction	Cytoplasm to vacuole targeting Vps64	Signal transduction	SAM-dependent methyltransferase	Signal transduction
Protein sey1	Stress response	GTP-binding protein GUF1	Signal transduction	GRAM domain	Signal transduction
AP-1 complex subunit beta-1	Transport and catabolism	HRQ family protein 2	Signal transduction	Dim2-associated protein 1, variant 1	Signal transduction
Protein-vacuolar targeting protein Atg18	Transport and catabolism	Protein GCN20	Signal transduction	IQ motif, EF-hand binding site	Transport and catabolism
MFS toxin efflux pump	Transporters	Hsp90 co-chaperone Cdc37	Stress response	PX domain	Transport and catabolism
Pinin/SDK/MemA protein	Uncharacterised	Disintegrin-like metalloprotease	uncharacterised	Tubulin-specific chaperone c	Transport and catabolism
Pol protein	Uncharacterized	Regulator of chromosome condensation (RCC1) repeat	Uncharacterized	PP-loop family protein	Uncharacterized
TrkA-N domain-containing dehydrogenase	Uncharacterized	Spc97/Spc98 family protein	Uncharacterized	Stomatin family protein	Uncharacterized

(Table 4). Further at 100 PSU salinity without Cr(VI) showed expression of autophagy proteins such as VPS15 protein kinase, Ariadne ring protein. Protein 5-oxoprolinase involved in glutathione metabolism was also expressed (Table 5). Universal stress proteins, DNA mismatch repair proteins, heat shock proteins, cellular

Table 4 Unique proteins expressed under varying Cr(VI) concentration at 35 PSU salinity

35 PSU_0 ppm Cr(VI)		35 PSU_100 ppm Cr(VI)		35 PSU_500 ppm Cr(VI)	
Protein name	Function	Protein name	Function	Protein name	Function
UV-damage endonuclease	Cell growth and death	AP-2 complex subunit mu-1	Transport and catabolism	Vacuolar protein sorting protein DigA	Autophagy
FKBP-type peptidyl-prolyl isomerase	Chaperones and folding catalysts	P-loop containing nucleoside triphosphate hydrolase	Nucleotide excision repair	Reg SU protein	Cell growth and death
ino80 chromatin remodeling complex protein	DNA repair	D-lactate dehydrogenase	Pyruvate metabolism	Pyoverdine/dityrosine biosynthesis protein	Chelators for metals/transport
Subtilase family domain	Folding, sorting and degradation	Two-component osmosensing histidine kinase (Bos1)	Signal transduction	DNA replication licensing factor mcm3	DNA replication
UV-damaged DNA binding protein	Nucleotide excision repair	ATP-dependent RNA helicase DBP5	Spliceosome	COPII vesicles protein Yip3	Endocytosis
Alternative NADH-dehydrogenase	Oxidative phosphorylation	Glycosyltransferase family 32	Starch and sucrose metabolism	FR47-like protein domain	Folding, sorting and degradation
Ferric reductase	Oxidoreductase	DNA mismatch repair protein Msh1	Stress/DNA repair	LSM domain	Folding, sorting and degradation
DNA polymerase delta catalytic subunit	Replication and repair	DNA mismatch repair protein Msh4	Stress/DNA repair	v-snare	Folding, sorting and degradation
CMGC/SRPK protein kinase	Signal transduction	Rhodanese-like protein	Sulfur metabolism	Replication factor A 1, rfa1	Nucleotide excision repair
Rho guanyl nucleotide exchange factor	Signal transduction	Diphosphomevalonate decarboxylase	Terpenoid backbone biosynthesis	Prolidase pepP	Proline metabolism
wsc domain	Signal transduction	Pentatricopeptide repeat protein	Transfer RNA biogenesis	DNA polymerase subunit delta-2	Replication and repair
Heat shock protein 78	The stress response	Sporulation protein RMD8	Two-component system	Calcium channel subunit Cch1	Signal transduction
DNA excision repair protein (rad2)	Stress/DNA repair	RINT-1 family protein	Ubiquitin-mediated proteolysis	Disheveled, Egl-10, and Pleckstrin domain	Signal transduction
TMEM1 family protein	Transport	Restless-like transposase	Uncharacterized	Leo1-like protein	Signal transduction
RhoGAP domain-containing protein	Transport and catabolism	SNARE-dependent exocytosis protein (Sro7)	Uncharacterized	F1F0-ATP synthase regulatory factor Stf2	Transport of h + ions
SH3 domain	Transport and catabolism	Transmembrane and coiled-coil domain	Uncharacterised	SSU processome component Utp10	Uncharacterised
Pogo transposase/Cenp-B/PDC2, DNA-binding HTH domain	Uncharacterised	CobW domain	Vitamin B12 biosynthesis	GDP/GTP exchange factor Sec2p	Vesicular transport

Table 5 Unique proteins expressed under varying Cr(VI) concentration at 100 PSU salinity

100 PSU_0 ppm Cr(VI)		100 PSU_100 ppm Cr(VI)		100 PSU_500 ppm Cr(VI)	
Protein name	Function	Protein name	Function	Protein name	Function
VPS15 protein kinase	Autophagy	Maltose permease	ABC transporters	Aldehyde dehydrogenase	Ascorbate and aldarate metabolism
Ran exchange factor Prp20/ Pim1	Cell cyle	Siderochrome-iron transporter	ABC transporters	Ubiquitin-protein ligase E3 component	Folding, sorting and degradation
Major intrinsic protein domain	Cell growth and death	Serine/threonine protein kinase	Cell cycle	Heat shock protein DnaJ	Stress response
Spindle pole body component	Cell growth and death	Sit4-associated protein	Cell cycle	Eukaryotic translation initiation factor 3 subunit EifCa	Translation
Glutaredoxin domain	Chaperones and folding catalysts	WD domain-containing protein	Cellular senescence	Tropinone reductase	Tropane, piperidine and pyridine alkaloid biosynthesis
RTC4-like domain	Folding, sorting and degradation	WD repeat protein	Cellular senescence	Taurine catabolism dioxygenase TauD, TfdA family protein	Metabolism of other amino acids
5-oxoprolinase	Glutathione metabolism	PI31 proteasome regulator	Folding, sorting and degradation		
Ariadne ring protein	Meiosis/Autophagy	Sec23/Sec24 family protein	Folding, sorting and degradation		
ATP-dependent permease MDL1	Oxidative phosphorylation	Ubiquitin-activating enzyme E1 1	Folding, sorting and degradation		
coatomer beta subunit	Oxidative phosphorylation	Calcium permeable channel	Ion channels		
DNA topoisomerase 2	Replication and repair	DNA-binding WRKY domain	Signal transduction		
ATP-dependent RNA helicase	Spliceosome	MADS-box MEF2 type transcription factor	Signal transduction		
DNA ligase (polydeoxyribonucleotide synthase)	Stress/DNA repair	Epsilon subunit of F1F0-ATP synthase N-terminal	Oxidative phosphorylation		
DNA repair and recombination protein RAD54	Stress/DNA repair	1-phosphatidylinositol-3-phosphate 5-kinase (Fab1)	Signal transduction		
Sulfite oxidase	Sulfur metabolism	Universal stress protein	Stress response		
Guanine nucleotide exchange factor	Transport and catabolism	DNA mismatch repair protein MutL	Stress/DNA repair		
Pfs, NACHT and WD domain	Uncharacterized	Sugar porter (SP) family MFS transporter	Transport		
WLM domain	Uncharacterised	Vacuolar calcium ion transporter/H(+) exchanger	Transport		

senescence proteins such as WD repeat protein and ABC transporter proteins such as siderochrome- iron transporter, were found to be expressed under the presence of Cr(VI) at 100 PSU salinity.

Protein Regulation Under Cr(VI) Stress at Varying Salinities

Analysis of the detected proteins using MPP13, pointed to the regulation pattern of proteins that differed across different salinities with respect to Cr(VI) concentrations. At different salinities of 0, 35 and 100 PSU, 30, 8 and 110 proteins were found to be up-regulated at 100 ppm Cr(VI) concentration in comparison to absence of Cr(VI) respectively, while the same condition showed down-regulation of 9, 33 and one proteins respectively. Similarly, 22, 71 and one proteins were found to be up-regulated at 500 ppm Cr(VI) concentration while 11, 1 and six proteins were down-regulated at 0, 35 and 100 PSU respectively. Amongst the commonly up-regulated proteins, proteins involved in free radical scavenging such as peroxidases, peroxiredoxins showed significant fold change values (Table 6). ROS and its reaction products have been reported to be counteracted by both enzymatic (superoxide dismutase, catalase, guaiacol peroxidases) and non-enzymatic (ascorbic acid, GSH, tocopherols, phenolics) antioxidants [32, 33] and have been mentioned previously. Enzymes which are involved in the direct removal of ROS such as glutathione peroxidase, glutathione reductase as well as glutathione dehydrogenase have been expressed at 100 and 500 ppm Cr(VI) under varying salinities. Peroxiredoxin (Prx) which is an important antioxidant and plays multiple roles such as detoxification of hydroperoxides, cell signaling, enzyme activation and redox sensing [31], was found to be up-regulated in the presence of 100 and 500 ppm of Cr(VI) under 0 and 35 PSU salinity. A similar trend was seen with respect to S-(hydroxymethyl) glutathione dehydrogenase, isocitrate dehydrogenase, RNP domain protein, ATP synthase subunit 4. Different types of stresses hamper the normal metabolic functioning of the organism. Oxidative stress can often affect carbohydrate metabolism, amino acid metabolism and also affect pathways in oxidative phosphorylation. Fructose-bisphosphate aldolase involved in carbohydrate metabolism was one such protein that was down-regulated at both Cr(VI) concentrations across all salinities (0, 35 and 100 PSU). Pathways involving glycolysis, TCA cycle, pentose phosphate shunt appeared to have been affected due to synergistic effect of salt and Cr(VI) stress, as can be seen from the down-regulation of respective enzymes (ATP citrate synthase, glucose-6-phosphate isomerase, phosphoglycerate kinase) (Table 6), proving that carbohydrate metabolism gets severely affected due to Cr(VI) stress. Redox-sensitive enzymes such phosphoglycerate kinase, malate dehydrogenase, translation elongation factor 2, were identified in *S. cerevisiae* [34]. Another study reported replacement of three key glycolytic enzymes by sulphur-depleted isozymes in *S. cerevisiae*. This replacement allowed channeling of sulphur-containing amino acids towards the synthesis of thiol metabolite glutathione, which is excellent metal detoxifier [35].

Dramatic physiological changes in the patterns of protein synthesis and gene expression in a living system, is often a result of a direct response to the diverse stresses, thereby helping the organisms to survive in an altered environment [27, 36]. Stresses such high salt concentrations and presence of heavy metals are often detrimental to organisms. Elevated sodium concentrations result in water-deficient cells causing inhibition of metabolic and cellular activities [17]. Alterations in lipid patterns and production of reactive oxygen species (ROS), triggering oxidative stress, are also a result of increased salinity stress [36, 37]. Redox-active metals such as chromium, pose a threat as it is very toxic and intrinsically persistent. Heavy metals have strong binding affinities to sulfhydryl groups of proteins such as thiol or histidyl moieties, resulting in displacement of essential metal ions from biomolecules, conformational modifications or inactivation of enzymes, disruption of cellular integrity along with production of free radicals and ROS [5, 31, 38]. Proteins absorb 70% of these radicals and its derivatives that cause reversible/irreversible changes to amino acid side chains leading to protein aggregation, inactivation or degradation [34]. ROS also participate in deleterious reactions such as peroxidation of lipids, causing membrane disruption and also affect DNA, RNA, ribosome synthesis and enzyme systems in organisms [7, 34, 39]. Therefore to overcome such life-threatening toxic effects, microbes need to devise robust cellular responses.

Purpureocillium lilacinum is a potential biocontrol fungus which has found its way into agricultural and medical applications, was studied for its halotolerance and Cr(VI) tolerant abilities in the current study. Elevated salt and metal concentrations were found to have synergistic toxic effects and altered the protein profiles at these conditions. As opposed to one stress one mechanism, cultures have to embrace multiple mechanisms to survive the combination of stresses. Multiple stress responsive mechanisms put forth by this organism were intracellular chelation, enzymatic scavenging of free radicals, elevated expression of transporter proteins as well as possible vacuolar accumulation of metal complexes. Although this study provides an insight into the protein expression under stressful conditions, a lot more research and experimental evidence are required to prove the existence of these mechanisms.

Table 6 Protein regulation and their fold change values across varying salinities with respective to Cr(VI) stress

Commonly up-regulated proteins						Commonly down-regulated proteins							
Protein names	NCBI ID	0 PSU		35PSU		Protein names	NCBI ID	0 PSU		35PSU		100 PSU	
		A	B	A	B			A	B	A	B	A	B
40S ribosomal protein S15	1032884361	1.08	0.79	1.81	3.19	26S protease regulatory subunit 6A-B	1032874680	− 1.19	− 1.01	− 0.99	− 1.01	*	*
40S ribosomal protein S5	1032873501	3.45	4.32	1.52	1.62	Alcohol dehydrogenase I	1032873610	− 1.49	− 0.83	− 1.64	− 1.48	*	*
ATP synthase subunit 4	1032877544	1.99	2.13	2.21	3.55	ATP-citrate synthase subunit 1	1032873526	− 2.45	− 0.12	− 2.13	− 1.12	*	*
Citrate synthase	1032873133	0.83	1.53	0.73	2.14	CFEM domain	1032868822	− 1.59	− 2.59	− 0.98	− 1.25	*	*
Cystathionine beta-lyase	1032879849	5.96	6.21	*	*	Cytochrome c oxidase polypeptide IV	1032872224	*	*	*	*	− 2.47	− 3.32
Heat shock protein 70	1032873271	1.17	1.14	0.12	1.42	Fructose-bisphosphate aldolase	1032884790	− 0.77	− 1.38	− 1.06	− 1.92	− 1.28	− 5.93
Isocitrate dehydrogenase subunit 2	1032880026	2.32	2.48	1.28	3.30	Glucose-6-phosphate isomerase	1032880807	− 1.68	− 1.48	− 1.73	− 0.08	*	*
NADP-dependent glycerol dehydrogenase	1032872240	4.05	4.73	2.96	3.61	Glutamine synthetase	1032868609	− 1.81	− 3.51	− 1.61	− 3.89	*	*
Peroxidase/catalase 2	1032881229	2.83	0.76	1.80	1.89	Glutathione reductase	1032873462	− 0.53	− 1.00	− 1.53	− 0.49	*	*
Peroxiredoxin type-2	1032879586	4.04	4.48	4.65	4.70	Nucleoside diphosphate kinase	1032879126	*	*	− 1.16	− 0.41	− 1.08	− 6.40
RNP domain	1032879985	0.85	2.05	0.42	1.60	Phosphoglycerate kinase	1032862591	− 1.06	− 0.42	− 1.38	− 1.90	*	*
Superoxide dismutase	1032879402	*	*	2.06	2.47	Pyruvate kinase	1032870961	− 2.71	− 1.41	− 2.35	− 2.33	*	*
S-(hydroxymethyl) glutathione dehydrogenase	1032876161	0.93	1.85	0.47	3.81	Superoxide dismutase	1032879402	*	*	*	*	− 3.20	− 5.83
Vacuolar protease A precursor	1032864819	1.77	1.14	0.12	2.17	Transketolase 1	1032874216	− 2.44	− 1.75	− 2.11	− 0.76	*	*
Woronin body major protein	1032879473	1.46	1.29	0.72	1.44	Xylulose-5-phosphate phosphoketolase	1032872482	− 3.15	− 2.20	− 4.32	− 2.71	*	*

A Fold change values of proteins expressed at 100 ppm as compared to 0 ppm Cr(VI)

B Fold change values of proteins expressed at 500 ppm as compared to 0 ppm Cr(VI)

*Protein not detected

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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