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ORIGINAL PAPER

Differential expression of peroxidase and ABC transporter as the key regulatory components for degradation of azo dyes by *Penicillium oxalicum* SAR-3

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Abstract Fungal species are potential dye decomposers since these secrete spectra of extracellular enzymes involved in catabolism. However, cellular mechanisms underlying azo dye catalysis and detoxification are incompletely understood and obscure. A potential strain designated as *Penicillium oxalicum* SAR-3 demonstrated broad-spectrum catabolic ability of different azo dyes. A forward suppression subtractive hybridization (SSH) cDNA library of *P. oxalicum* SAR-3 constructed in presence and absence of azo dye Acid Red 183 resulted in identification of 183 unique expressed sequence tags (ESTs) which were functionally classified into 12 functional categories. A number of novel genes that affect specifically organic azo dye degradation were discovered. Although the ABC transporters and peroxidases emerged as prominent hot spot for azo dye detoxification, we also identified a number of proteins that are more proximally related to stress-responsive gene expression. Majority of the ESTs (29.5%) were grouped as hypothetical/unknown indicating the presence of putatively novel genes. Analysis of few ESTs through quantitative real-time reverse transcription polymerase chain reaction revealed their possible role in AR183 degradation. The ESTs identified in the SSH library provide a novel insight on the transcripts that are expressed in *P. oxalicum* strain SAR-3 in response to AR183.

Keywords Quantitative real-time PCR · Suppression subtractive hybridization · Azo dye · *Penicillium oxalicum* · Bioremediation

Introduction

Earth inhabits a plethora of microbial strains which shall prominently be deployed for combating the hazardous effects posed by textile industrial effluents containing ecotoxic azo dyes. Azo dyes are diazotized amines attached to an amine or phenol with characteristic chromophoric azo group (–N=N–), accounting for 60–70 % of all the 7×10^5 tonnes of dyes produced annually (Ghodake et al. 2009). They are widely prevalent environmental contaminants that are recalcitrant to biodegradation processes and have detrimental biological effects and impose toxicity, mutagenecity, and carcinogenicity for a number of vertebrates and the human population (Elisangela et al. 2009). Their ubiquitous occurrence, recalcitrance, bioaccumulation potential, and carcinogenic nature pose an immense technical challenge to the detoxification of these compounds. A number of basdiomycetes mainly white rot fungi, viz. *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Bjerkandara adusta*, *Trametes versicolor*, etc., had been reported to be rapid dye decolorizers (Soo and Jin 1998; Heinfing et al. 1998; Binupriya et al. 2008; Singh and Pakshirajan 2010). However, some other genera such as *Penicillium sp*., *Aspergillus niger*, *Funalia trogii*, etc., were also observed to decolorize azo dyes (Fu and Viraraghavan 2002; Park et al. 2007; Gou et al. 2009). Fungal strains respond to toxic azo dyes in various ways either by biosorption and subsequent decolorization or biodegradation that leads to complete mineralization of the dyes into non-toxic metabolites. However, the elucidation of molecular responses underlying their versatile character to degrade a number of pollutants is still in its infancy. Hence, deciphering the complex network of molecular mechanisms

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underlying the fungal propensity to degrade toxic azo dyes is pertinent to develop specialized and eco-friendly bioremediation technology.

Penicillium oxalicum SAR-3 strain used in this study was isolated from dye-contaminated soil and was observed to have notable dye decolorization potential. It is a potential strain producing spectra of catabolic enzymes which can effectively be employed for biodegradation/detoxification of a broad range of environmental pollutants (Opasols and Adewoye 2010). However, molecular cascades underlying detoxification of azo dyes by this fungal strain are still unexplored.

The present study was aimed towards the analysis of dye decolorization potential of *P. oxalicum* SAR-3 and to decipher the impact of azo dyes on enzymatic induction. Furthermore, the degradation by UV-visible and Fourier transform infrared (FTIR) spectroscopy and toxicity assessment of the metabolites generated were also ascertained. The notable potential of this strain to degrade and detoxify AR183 led us to the analysis of the molecular cascade regulating the degradation. Suppression subtractive hybridization was employed for exploring the genes that were differentially expressed during degradation of ecotoxic azo dye Acid Red 183 by the fungus. The cDNA library was constructed at the stage whereby both the dye decolorization and enzymatic activity were at their maximum. Expressed sequenced tags (ESTs) obtained by SSH were analyzed and validated using real-time reverse transcription polymerase chain reaction (qRT-PCR). The work undertaken represents, to the best of our knowledge, the first study investigating the transcriptional profile of a fungal strain underlying azo dye degradation.

Materials and methods

Dyes and chemicals

The azo dye Acid Red 183 (AR 183) (Fig. 1a), having a chromophoric azo group, was purchased from MP Biomedicals (USA). Strain *Saccharomyces cerevisiae* BY4741 (MTCC 3156) was procured from Micobial Type Culture Collection, IMTECH, Chandigarh, India. Hydrogen peroxide was obtained from Sigma Chemical Company (St. Louis, MO, USA). $MnSO_4$ and other fine chemicals were purchased from HiMedia (Mumbai, India).

Culture media

Potato dextrose agar was used for maintenance of the fungal strain. Modified Kirk's basal salt medium [(grams per liter): ammonium tartarate, 0.22; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.05; $CaCl_2$, 0.01, Thiamine, 0.001; 1 mL L^{-1} of 10 % Tween-80 solution; 100 mM veratry1 alcohol; 4 % carbon source, and 10 mL L^{-1} trace elements solution were added. Trace elements solution had the following composition (grams per liter)—0.08 $CuSO_4$, 0.08; 0.05 H_2MoO_4, 0.05; $MnSO_4.4H_2O$, 0.07; $ZnSO_4{\cdot}7H_2O$, 0.043, and $Fe_2(SO4)_3$, 0.05] (Robinson et al. 2001).The pH of the medium was adjusted to 7.5, autoclaved at 121 °C for 15 min, and then inoculated.

Isolation of fungal strain and culture conditions

Soil samples collected from the vicinity of Amarkattha leather industry, Agra, Rakshi dyes, Farrukhabad, U.P., India, were used for isolation of microbial strains having azo dye decolorization ability. An isolate denoted as SAR-3 demonstrated maximum decolorization ability to azo dye AR183, and notable levels of MnP activity were identified to be a strain of *P. oxalicum* on the basis of 18S and internal transcribed spacer region sequencing (Saroj et al. 2014). To study the dye decolorization potential of *P. oxalicum* SAR-3 and the enzymatic profile of the strain, the actively growing culture was used as inoculum for 50 mL of modified Kirk's medium and after 48 h of initial growth at 30 °C under shaking conditions (200 rpm), the flasks were supplemented with 100 mg L^{-1} of dye azo dye AR183. Unsupplemented flasks were used as controls. Aliquots (3 mL) of culture supernatant were withdrawn at different time intervals from each flasks, centrifuged (5,000 rpm, 10 min) to separate the mycelial cell mass, and thereafter used for estimating enzyme activity and determining the decolorization level of dye. The decolorization of the dyes was assayed spectrophotometrically by measuring the decrease in absorbance at the λ_{max} of AR 183 dye, i.e., 494 nm (percentage decolorization=Initial absorbance−Absorbance observed/Initial absorbance×100) (Chander et al. 2004; Khelifi et al. 2009). All experiments were run in triplicates. Pure fungal cultures were stored on potato dextrose agar slants at 4 °C (Sukumar et al. 2009).

Estimation of MnP activity

Manganese peroxidase activity was determined as described by Paszczynski et al. (1988). Reaction mixture contained 100 mM sodium tartarate buffer (pH 5.0), 0.1 mM $MnSO_4$, 0.1 mM H_2O_2, and 50 μl of enzyme extract. The product Mn (III) forms transiently stable complex with tartaric acid, showing characteristic absorbance at 238 nm (ε=6500). Reaction was initiated by addition of H_2O_2, and increase in absorbance was monitored at 238 nm for 1 min. One unit of peroxidase oxidizes 1 μmol of Mn (II)min^{-1}.

Protein concentration was estimated using the Bradford method with crystalline bovine serum albumin as standard. Protein concentrations in the fractions were determined from absorbance values at 280 nm.

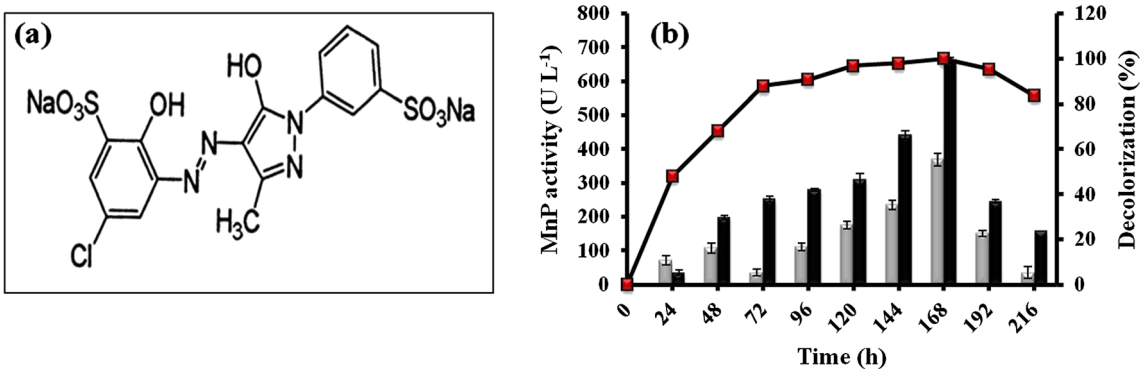

Fig. 1 **a** Chemical structure of azo dye AR183; **b** MnP activity profile in the absence of dye (*solid grey bar*) and in the presence of dye (*solid black bar*) and percentage of decolorization, (*dash-and-black square line*), at 100 mg L^{-1} of Acid Red 183 by the isolate *P. oxalicum* SAR-3

Analysis of degradation products through UV–VIS and FTIR

The metabolites produced during the biodegradation of Acid Red 183 after 168 h were extracted with equal volumes of ethyl acetate. The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator. The crystals obtained were dissolved in small volume of HPLC-grade methanol and were used for UV–VIS spectral analysis and FTIR. UV–VIS spectral analysis was carried out using CARY UV–VIS spectrophotometer (100 Bio), and changes in its absorption spectrum (400–800) were recorded. FTIR analysis was carried out using Perkin-Elmer 1600 series spectrometer at room temperature, and changes in percentage transmission at different wavelengths were observed.

Toxicity analysis of degradation metabolites of azo dye

Toxicity assays were performed based on the inhibitory effects of AR183 and its metabolites obtained following degradation, on the growth of the yeast *S. cerevisiae* BY4741 (Mendes et al. 2011). Initially, the yeast cells were grown up to a mid-exponential phase, and afterwards, inoculum was prepared from the above culture, which was centrifuged and suspended to OD_{640nm}=0.15 in a triple strength minimal growth medium that contained (per liter)—1.7 g YNB without amino acids (Sigma, USA), 20 g glucose (HiMedia, India), 2.65 g $(NH_4)_2SO_4$ (HiMedia, India), 20 mg methionine, 20 mg histidine, 60 mg leucine, and 20 mg uracil (Sigma, USA). All experiments were run in triplicates, for the toxicity analysis; 50 μL of the standardized yeast cell suspension was mixed with 100 μL of test or control solutions in 96-well polystyrene micro-plates (TARSONS, India) that were sealed and incubated at 30 °C for 16 h with constant agitation. Growth of the yeast cell population was assessed by measuring the optical density (OD_{750nm}, which did not interfere with any of the maximal absorption wave-length of the dye tested) attained after 16 h of incubation. The toxicity was measured on the basis of the percentage of inhibition of yeast growth defined as $1-OD^{x}_{750nm}/OD^{0}_{750nm}\times 100$ where OD^{x}_{750nm} and OD^{0}_{750nm} are the absorbance values attained by the yeast cell population in the presence and in the absence of each test solution, respectively.

Construction of subtracted cDNA library

Extraction of total RNA was carried out using frozen, powdered mycelia using RNA-XPressT M reagent (HiMedia, Laboratories, India) following manufacture's protocol, and poly A^+ RNA was purified by mRNA isolation kit (Roche Applied Science, Manheim, Germany). Two micrograms of poly A^+ RNA from each sample was used for the construction of forward [168 h AR183 (100 mgL^{-1}) treated *P. oxalicum* mycelia as tester and 168 h *P. oxalicum* mycelia without AR183 as driver] subtracted cDNA library by using CLONTECH PCR-Select cDNA subtraction kit (CLONTECH Laboratories, Palo Alto, CA) following manufacturer's protocol. Enriched cDNA fragments obtained after subtraction were directly cloned into $pGEM^R$-T Easy vector system-I (Promega Co. USA). The recombinant (white) colonies obtained were picked up and cultured overnight at 37 °C in 96-well plates with 150 μL LB medium supplemented with ampicillin.

EST sequencing and analysis

Recombinant plasmids isolated were sequenced with ABI sequencer, version no.3770 using M13 forward primer. Expressed sequence tags obtained were screened for vector and adaptor sequences by VECSCREEN program available at NCBI database (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). Homology search and annotation were performed by BLASTX and BLASTN algorithms (Altschul et al. 1997). ESTs that showed smaller *E*-value, i.e., $\leq 10^{-5}$ and were >100 nucleotides in length were considered significant. Functional classification of the ESTs generated above was carried out according to MIPS data base (http://mips.helmholtz-muenchen.de/proj/funcatDB/searchmainframe.html).

All these sequences were submitted to the EST database of NCBI GenBank.

Relative quantification of mRNAs expression by real-time quantitative PCR

Eleven ESTs were chosen on the basis of their possible role in dye decomposition for qRT-PCR analysis. Primers used for qRT-PCR analysis for target genes were designed with Primer3web version 4.0.0 available at http://primer3.wi.mit.edu/ and listed in Table 1. qRT-PCR analysis was carried out as described by Jayaraman et al. (2008). All experiments were performed in triplicate. The Ct values for all transcripts were normalized to the Ct value of an internal control gene and actin, and subsequent analysis of the relative mRNA expression level of the transcripts was done using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Results

AR183 decolorization and enzymatic analysis

The isolate SAR-3 was observed to have notably higher ability of decolorization (87.9–100 %) to a highly recalcitrant azo dye AR183 (100 mg L^{-1}) within 72 to 168 h (Fig. 1b). There was a significant level of increase in MnP activity from 367.87±18.18to 660.5±13 U L^{-1} in the presence of dye AR183 that substantiates the role of peroxidases in dye decolorization by SAR-3 (Fig. 1b). Results obtained indicated that manganese peroxidase has prominent role in azo dye decolorization.

Degradation product analysis through UV–visible spectroscopy

UV–Vis spectrum (400–700 nm) (Fig. 2) of supernatants withdrawn at different time intervals showed decreased absorbance for AR183, indicating the adsorption of dye to fungal mycelium. Apparently, dyes were adsorbed on fungal biomass and then were degraded by fungal extracellular enzymes, since it has been observed that with the increase in MnP levels the mycelia retained back its original color. Peak observed at 494 nm for AR183 was observed to decrease gradually after 120 h to complete decolorization of the medium following 168 h of growth (Fig. 2).

FTIR analysis of degradation products

Comparison of FTIR spectrum of control dye with extracted metabolites after complete decolorization clearly indicated the biodegradation of the AR183 dye by *P. oxalicum* SAR-3 (Fig. 3). Control AR183 spectrum (Fig. 3a) showed characteristic peaks at 3,440.46 cm^{-1}, 1,631.36 cm^{-1} corresponding to –OH stretching vibration, –N=N– stretching in azo group.

Table 1 Primers used for qRT-PCR analysis

S. no.	Primer ID	GenBank acc. no.	Forward sequence (5 -3), reverse sequence (5 -3)	Amplicon length (bp)
1.	PEROX	JK747284	GAAAAAGTCCGACCACTGGA ACCAAACTCCTCCTCCCACT	181
2.	TAP	JK747316	GACGATCGACCCATGAACTT GTTTTGAAGACGGGCATGTT	182
3.	PTGE	JK747359	CCAATCCCACTCGACACTCT CGCGAAGATTGAAAAGAAGG	179
4.	HPCYT	JK747350	GACCTGTCCAGCCCTGATAA TGCACCCTTGAACAATTTGA	181
5.	CYT	JK747318	GCTTCTTCGACGTCACCTTC CATGGTTCGGACGAGACTTT	188
6.	ABCT	JK747281	CAATCAACTTTCACCCACGA CAGACTGGAGGAAATGTGAA	169
7.	MTHFR	JK747323	TCATCTCACCAGCGTTCTTG ACACGTGGCTTGTCTGTGTC	158
8.	POX4	JK747324	CGGGATCATTTTGGATTGTC GGGTGGTCAGAGGAAAAACA	169
9.	POX 1	JK747277	TTGTTGGAGCCTGTCTGATG TACAATCCCCTCGTCTCCAG	190
10.	HSP	JK747415	ACAAAGAAAGCTCCGCTTCA TGAAGCTCTGGGGAGAAAGA	166
11.	NDFR	JK747265	GTCCCAGAAGGTCTTCCAC CTTCCAGATTGCCGAGACT	174

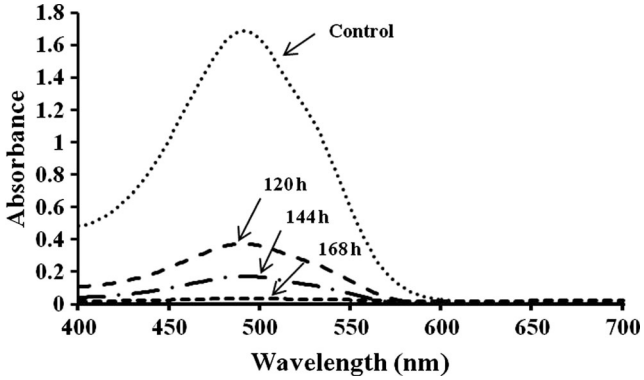

Fig. 2 UV–Vis spectra of dye AR183 at different time intervals during decolorization by *P. oxalicum* SAR-3

The peaks between 523.63 and 614.88 cm^{-1} could be assigned to substituted benzene, indicating the aromatic nature of dye. Peak between 1,095.88 –and 1,035.81 cm^{-1} corresponds to asymmetric stretching vibration of the $–SO_3Na$ group. Peak at 1,410.43 cm^{-1} represents bending frequencies of methyl group on aromatic ring. The FTIR spectrum of the extracted metabolites after degradation of AR183 denoted appearance of new peaks, indicating the production of intermediates in the degradation process (Fig. 3b). Absence of peaks at 523.63 and 614 cm^{-1} had suggested loss of aromaticity or benzene ring, and broadening of peak at 3,345.28 cm^{-1} shows –OH stretching. Appearance of peaks at 1,408.42 and 1,554.83 cm^{-1} were indicative of C–H deformation of methyl group. Appearance of new peaks at 1,742.27 cm^{-1} denotes C=O stretching vibration and at 2,925.26 cm^{-1}, C–H stretching of alkanes.

Toxicity analysis of AR183 over *S. cerevisiae* BY4741 growth

The toxicity of the azo dye AR183 and its residual metabolites following degradation of azo dye by *P. oxalicum* SAR-3 was enumerated based on the inhibitory effects on the growth of *S. cerevisiae* BY4741 (Fig. 4). AR183 when used at 200 to 1,000 mg L^{-1} had adversely affected the growth of the yeast cells, and inhibition of growth from 31.9±2.34 % to 96.9±3.2 %, respectively, was observed. Metabolites generated following incubation of dyes (200 to 400 mg L^{-1}) with *P. oxalicum* SAR-3 had affected into reduced inhibition to about 40–20 % of yeast growth, indicating, therefore, a notable decrease in the toxicity level of the dye following degradation of dye. Higher concentration of the dye 600 to 1,000 mg L^{-1} used was inhibitory (68.9 % to 86.9 %) probably as the dye remained largely undegraded at these concentrations.

Construction of SSH library

A total of ~500 white colonies representing recombinant clones were obtained from the SSH library. SSH is specifically employed to identify distinctively expressed genes in response to varying nutritional or environmental conditions. Incorporation of normalization step by SSH to equalizes the relative quantity of the cDNA and thus enriches the mRNA population containing sets of mRNA induced during the catabolism of azo dyes by the organism. It augments the probability of identifying the increased expression of low-abundance transcripts (Diatchenko et al. 1996; Gurskaya et al. 1996). Successful SSH was indicated by gel electrophoresis of PCR-amplified cDNAs that showed a reduction in the quantity and size diversity of SSH samples in comparison to unsubtracted controls (Fig. 5). After single-pass sequencing, 286 sequences of high quality were obtained from 6-day-old culture forward library. Out of 286 recombinant clones, 183 unique ESTs were obtained and were deposited in the GenBank database under accession numbers: JK747250–JK747432. BLAST analysis of the 183 unique ESTs revealed that the obtained SAR-3 sequences were mostly similar to *Penicillium sp.* and *Aspergillus sp.* as shown in Supplementary Table 1, which were subsequently classified into 12 functional categories according to their putative functions (Fig. 6). The largest category (29.5 %) contained EST sequences with hypothetical/unknown function or with no similarities to previously sequenced genes, which indicated possibly the putative novel genes that were induced and may be specific to SAR-3 (Fig. 6). A large number of hypothetical transcripts observed in the library denote the effectiveness of subtractive libraries in identifying new genes that are overlooked by large scale EST projects. Hence, these transcripts might be induced to counteract the harmful effects of azo dyes. The second largest category (17.48 %) comprised of metabolism-related transcripts (Supplementary Table 1). The library largely constituted transcripts related to receptor/signalling and steps associated to transcription/post-transcription and translation/post-translation modifications.

qRT-PCR analysis of subtracted transcripts

Eleven genes were selected on the basis of their putative function inferred from sequence comparison for quantitative real-time PCR analysis at different time intervals in response to azo dye AR183. Out of 11 transcripts chosen, eight showed the perceptible levels of differential expression. The responses of these 11 genes of *P. oxalicum* SAR-3 are shown in Figs. 7a–c and 8. Quantitative evaluation of the relative mRNA accumulation of eleven genes, namely putative peroxidase protein (PEROX), transcriptional accessory protein (TAP), post-transcriptional gene expression (PTGE), hypothetical protein cytochrome P450 (HPCYT), cytochrome p450, ABC transporter (ABCT), methylenetetrahydrofolate reductase (MTHFR), unknown protein (POX4), unknown protein (POX1), heat shock protein (HSP), and NADH-dependent FMN reductase (NDFR) was performed

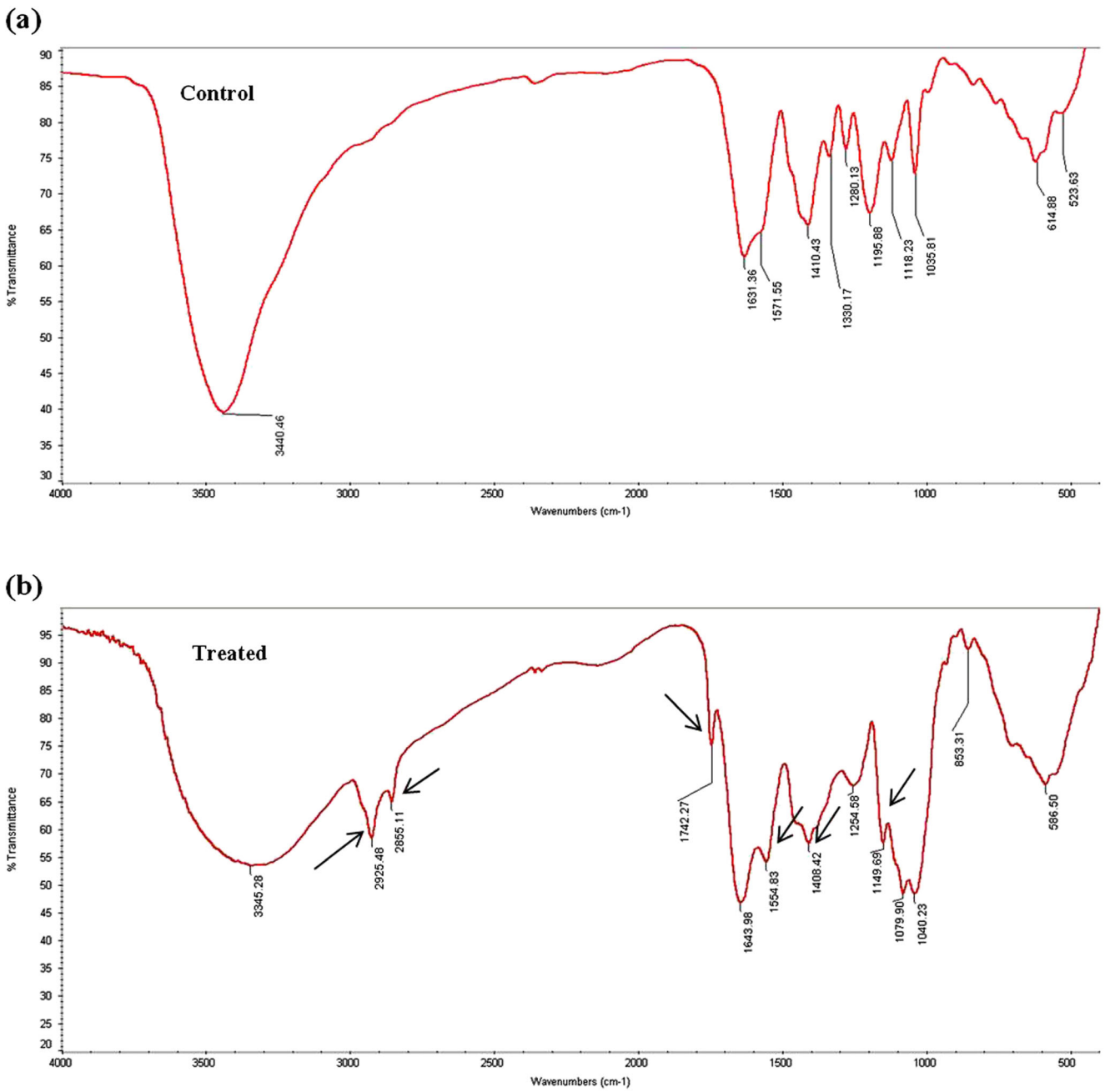

Fig. 3 FTIR spectra of products extracted with ethyl acetate formed after degradation of AR183 (**a**, control; **b**, treated) by *P. oxalicum* SAR-3

(Table 2). Gene expression studies were done with samples taken at different time intervals (0, 24, 72, 120, 168, and 216 h) under submerged fermentation condition in presence of 100 mg L^{-1} of AR183. The results obtained indicate towards the differential expression of transcripts, as observed and is somehow related to responses of various organisms to the toxic pollutants present in the environment as discussed below.

Manganese peroxidase has been found to be the key enzyme assisting in the degradation of azo dye AR183, the qRT-PCR analysis of PEROX (#JK747284) transcript had showed fourfold induction at 168 h that gradually dropped after 216 h (Fig. 7a). Upregulation of this transcript is concurrent with the maximum level decolorization of the dye that suggests its potential involvement in azo dye degradation. TAP (transcriptional accessory protein) and PTGE (post-transcriptional gene expression) are fungal-specific transcription factors. The qRT-PCR analysis of TAP (# JK747316) and PTGE (# JK747359) transcripts showed constant decline in mRNA accumulation in the presence of azo dye AR183 in *P. oxalicum* SAR-3 (Fig. 7a). HPCYT (#JK747350) transcript showed ≥2.5-fold induction at 168 h, whereas CYT (#JK747318) transcript

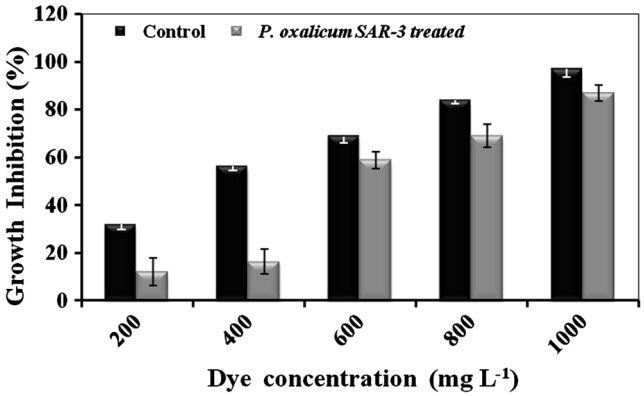

Fig. 4 Inhibitory effects of intact azo dye AR183 (*solid green bar*) and after treatment (*solid red bar*) with *P. oxalicum* SAR-3 over *S. cerevisiae* BY4741 growth

expression was observed to be downregulated (Fig. 7b). Prominent involvement of ABC transporters in azo dye degradation was observed in present study as the ABCT (#JK747281) transcript showed 30–40-fold induction at 120 and 168 h, respectively, strongly suggesting their role in AR183 degradation (Fig. 7b). qRT-PCR analysis of MTHFR (#JK747323) transcript showed 0.5-fold induction at 120–168 h (Fig.7c), indicating its possible role in maintaining plasma membrane redox system. Transcripts with unknown/unclassified functions abbreviated as Pox4 (#JK747324) and Pox 1 (#JK747277) were observed to be upregulated to one- and 0.5-fold as revealed by qRT-PCR analysis, respectively (Fig. 7c). The differential expression of these transcripts indicates the presence of putative novel genes that are vital for azo dye degradation by *P. oxalicum* SAR-3.

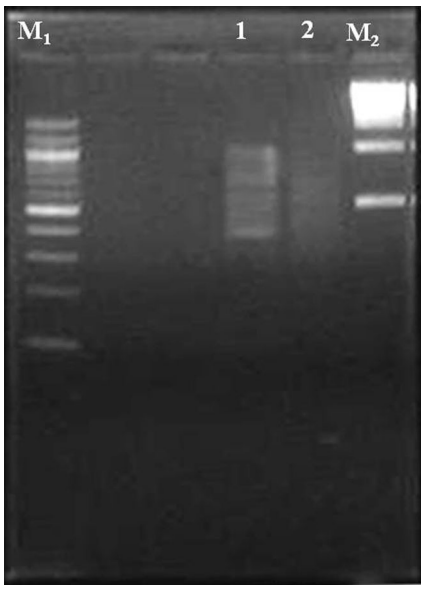

Fig. 5 PCR-amplified suppression subtractive hybridization (SSH) cDNA (*lane 2*) and unsubtracted cDNA control (*lane 1*) derived from *P. oxalicum* mRNA isolated in presence and absence of AR183 M_1—100 bp molecular weight marker; and *2*: secondary PCR product of control and subtracted cDNA; M_2 1 kb molecular weight marker

qRT-PCR analysis had revealed 1.7- to 4.3-fold induction of the heat shock protein transcript (#JK747415) at 120–168 h (Fig. 8). It has been reported that several members of chaperone gene families have increased levels of expression, and thus, the amount of chaperones is elevated under stressful conditions. NADH-dependent FMN reductases (NDFR) have role in azo dye degradation and have been reported in filamentous fungi, bacteria, and yeast (Solís et al. 2012). qRT-PCR analysis had showed 2.5- to 3-fold induction of NDFR transcript (#JK747265) at 120–168 h (Fig. 8).

Discussion

The work undertaken had enumerated the potential of strain *P. oxalicum* SAR-3 to degrade and detoxify the azo dye AR183 to an appreciable extent. Involvement of MnP as key regulatory enzyme had strongly suggested the role of extracellular peroxidases in the decolorization process. Earlier observations had also shown that the decolorization of azo dyes is reliant on the relative contributions of manganese peroxidases, lignin peroxidases, and laccases and may vary among different species (Pointing and Vrijmoed 2000; Novotny et al. 2004; Boer et al. 2004; Kalyani et al. 2008). Decolorization of azo dyes could be due to adsorption by microbial cells or to the catabolism of the dyes (Asad et al. 2007). FTIR analysis of the degradation products had also denoted the catabolism of the azo dyes.

Reduction in toxicity of metabolites generated after degradation is a prerequisite to the development of effective ecofriendly bioremediation technology. In the current investigation, significant reduction in toxicity of the degradation metabolites was observed, and these results are comparable with the earlier observations of Mendes et al. (2011) that reported more than 50 % reduction in azo dye toxicity following treatment with laccase enzyme. The results therefore obtained denote that *P. oxalicum* SAR-3 is a potential strain for remediation of toxic azo dyes to the non-toxic metabolites.

A number of differentially expressed transcripts had been observed in the cDNA library. The expression of functionally different transcripts that may be involved in the catabolism of AR183 has been discussed as below.

Regulatory aspects of metabolism related transcripts

Considerably increased numbers of metabolism-related transcripts like JK747272, JK747425, JK747329, JK747378, etc., in the library denote their role in catabolism to generate energy for various cellular processes. Extent of azo dye decoloration using microbial strains is influenced by the presence of carbon and nitrogen sources. Different microbial metabolic characteristics may lead into differences in the uptake of sources, thus affecting azo dye decoloration. Since dyes are deficient in

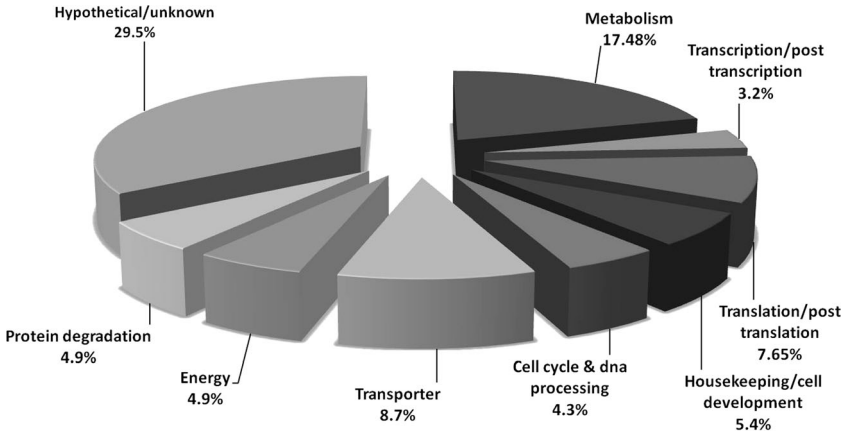

Fig. 6 Functional classification of 183 differentially expressed transcripts in the presence of dye AR183 in SAR-3 identified from the SSH library

carbon, biodegradation without an extra carbon source is cumbersome; also, the strain under investigation required significant amounts of glucose for decolorization of AR183. It had also been shown that the abundance of metabolism-related transcripts might be to generate precursor metabolites for biomass production and energy for various cellular processes (Zhang et al. 2007).

Involvement of proteases and other stress- and defense-related genes

Apart from transcripts involved in metabolism, few chaperones and proteases were also obtained, as these are important for the refolding or degrading of stress-damaged proteins. Heat shock proteins (JK747415, Hsp90 co-chaperone Cdc37; JK747416,

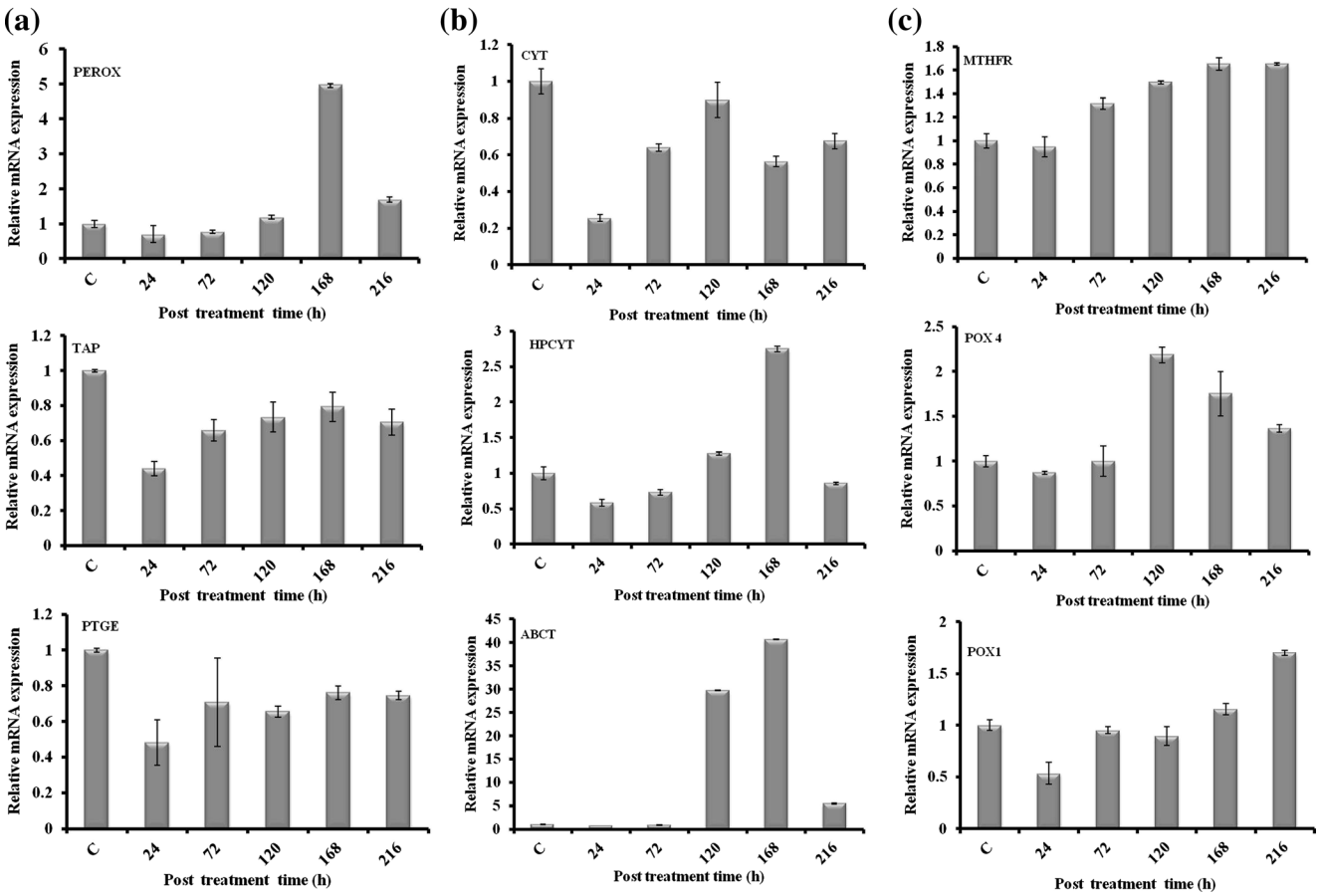

Fig. 7 **a** qRT-PCR analysis of peroxidase protein (PEROX), transcriptional accessory protein (TAP), and post-transcriptional gene expression (PTGE) in response to azo dye AR183 in *P. oxalicum* SAR-3. **b** qRT-PCR analysis of cytochrome P450, hypothetical protein cytochrome P450 (HPCYT), and ABC transporter (ABCT) in response to azo dye AR183 in *P. oxalicum* AR-3. **c** qRT-PCR analysis of methylenetetrahydrofolate reductase (MTHFR), unknown protein (POX4), and unknown protein (POX1) in response to azo dye AR183 in *P. oxalicum* SAR-3

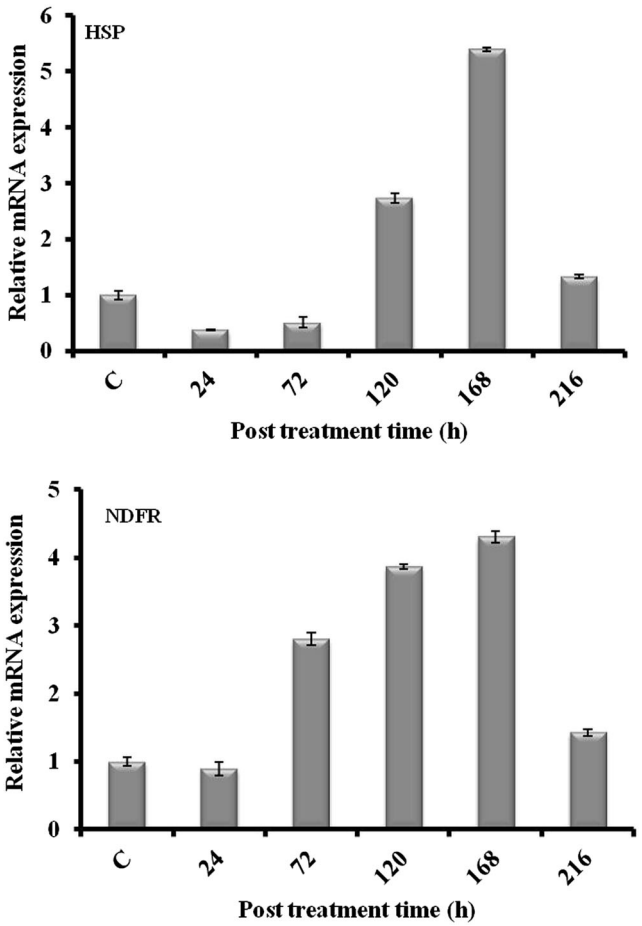

Fig. 8 qRT-PCR analysis of heat shock protein (HSP) and NADH-dependent FMN reductase (NDFR) transcripts in response to azo dye AR183 in *P. oxalicum* SAR-3

molecular chaperone) have previously been reported to induce peroxidases in *Neurospora crassa* (Machwe and Kapoor 1993). Peroxidases are the important players in azo dye degradation according to our preliminary studies. HSPs with a large molecular size (70 to 110 kDa) appear to be implicated in several functions critical for the maintenance of the integrity of structural proteins and cellular enzymes under unfavorable conditions (Kapoor et al. 1995).

Role of fungal enzymatic machinery

Many dyes are polar and/or large molecules which are unlikely to diffuse through the cellular membrane (Zolinger 1991). Plethora of enzymes participating in azo dye mineralization involved peroxidases, azoreductase, FMN-dependant reductases (Dos Santos et al. 2007; Bürger and Stolz 2010). Pajot et al. (2011) had also reported that the presence of dyes in the culture media induced the production of two enzymes, manganese peroxidase, and tyrosinase, which confirms their potential role during the decolorization process. Significant levels of MnP have been observed in the strain *P. oxalicum* SAR-3, and presence of transcript corresponding to peroxidase (PEROX) (JK747284) validates the involvement of MnP in azo dye AR 183 decolorization/degradation.

Cytosolic flavin-dependent reductases have been reported as azo dye decolorizers, which transfer electrons via soluble flavins to azo dyes. It had also been suggested by Russ et al. (2000) that, in actively growing cells with integral cell membranes, a number of enzyme systems and/or other redox mediators are accountable for the reduction of azo dyes. Therefore, NADH-dependent FMN reductase (NDFR) (JK747265), found in the SSH library of *P. oxalicum* SAR-3, may be involved in degradation of the dye. Involvement of cytochrome P450 in dye (malachite green) degradation has also been suggested. The cytochrome P450 system of *C. elegans* mediated the *N*-demethylation reaction as well as the reduction of malachite green to leucomalachite green (Cha et al. 2001). Cytochrome P450 transcripts (JK747318, Cytochrome P450; JK747350, AN8952.2, cytochrome P450) have also been observed in the SSH library that directs towards their possible role in AR 183 transformation.

Transporters regulating azo dye elimination

Multidrug resistance (MDR) transporters are reported to protect cells from injuries caused by environmental exposure due to toxic chemicals of varying structure and function (Alarco et al. 1997). We have observed MDR transcripts (JK747340, multi-drug resistance-associated protein; JK747281, ABC transporter permease) that are likely to be involved in counteracting toxicity posed by azo dyes by enabling the increased extracellular secretion of peroxidases.

Involvement of transcripts related to energy generation during azo dye treatment

Aerobic degradation of azo dyes involves electrons routed through electron transport chain to the final electron acceptor that results into reduction and decolorization of azo dye and reoxidation of the flavin nucleotide (Robinson et al. 2001). Several transcripts showing homology to electron transport chain enzymes (JK747354, ATP synthase subunit beta; JK747329, hypothetical cytochrome C oxidase subunit IV; JK747327, cytochrome b subunit of succinate dehydrogenase, and JK747417, cytochrome C oxidase subunit IV) had also been observed in the current investigation. This therefore suggested the involvement of ETC members in the reduction and degradation of AR183 by *P. oxalicum* SAR-3.

Posttranslational modifications (e.g., glycosylation, phosphorylation, and methylation) are essential elements of the cellular mechanisms, and these are involved in regulating the proteins often by modulating thermodynamic, kinetic, and structural features of proteins. Since manganese peroxidases

Table 2 List of some differentially expressed transcripts having possible role in the degradation of azo dye AR183 by *P. oxalicum* SAR-3

S. no.	GenBank ID	Similarity of transcripts	Species	*E*-value
1	JK747272	Zn-dependent alcohol dehydrogenases	*P. chrysogenum* Wisconsin 54-1256	6.00E-69
2	JK747425	TPA: succinyl-CoA synthetase beta subunit, putative	*A. nidulans FGSC A4*	3.00E-82
3	JK747329	Pyruvate dehydrogenase dihydrolipoyltransacetylase	*P. chrysogenum* Wisconsin 54-1256	4.00E-44
4	JK747378	Pyruvate decarboxylase	*P. chrysogenum* Wisconsin 54-1255	1.00E-37
5	JK747323	Methylenetetrahydrofolate reductase (MTHFR)	*P. chrysogenum* Wisconsin 54-1257	3.00E-50
6	JK747359	Post-transcriptional gene expression	*P. chrysogenum* Wisconsin 54-1255	3.00E-05
7	JK747384	C6 finger domain protein, putative	*A. clavatus NRRL 1*	9.00E-59
8	JK747316	Transcriptional accessory protein	*P. chrysogenum* Wisconsin 54-1255	7.00E-25
9	JK747415	Hsp90 co-chaperone Cdc37	*P. chrysogenum* Wisconsin 54-1255	1.00E-120
10	JK747416	Molecular chaperone	*Rhizoplaca chrysoleuca*	2.00E-38
11	JK747284	Putative peroxidase	*P. chrysogenum* Wisconsin 54-1259	5.00E-42
12	JK747265	NADPH-dependent FMN reductase	*Ajellomyces capsulatus G186AR*	4.00E-41
13	JK747318	Cytochrome P450	*Leptographium longiclavatum*	3.00E-103
14	JK747350	Hypothetical protein AN8952.2, cytochrome P450	*A. nidulans FGSC A4*	2.00E-13
15	JK747340	Multi-drug resistance-associated protein	*P. chrysogenum* Wisconsin 54-1255	9.00E-105
16	JK747281	ABC transporter permease	Uncultured bacterium	4.00E-08
17	JK747318	Cytochrome P450	*Leptographium longiclavatum*	3.00E-103
18	JK747354	ATP synthase subunit beta	*A. niger CBS 513.88*	7.00E-55
19	JK747327	Cytochrome b subunit of succinate dehydrogenase, putative	*Neosartorya fischeri* NRRL 181	3.00E-14
20	JK747329	Hypothetical cytochrome C oxidase subunit IV	*P. oxalicum 114-2*	4e-12
21	JK747417	Cytochrome C oxidase subunit IV	*P. chrysogenum* Wisconsin 54-1255	8.00E-10
22	JK747277	Pc21g01830	*P. chrysogenum* Wisconsin 54-1257	7.00E-09
23	JK747324	Hypothetical protein	*P. chrysogenum* Wisconsin 54-1256	8.00E-26
24	JK747283	Pc13g15670	*P. chrysogenum* Wisconsin 54-1258	2.00E-05

are glycosylated proteins, these require addition of $(GlcNAc)_2$ Man_3 at Asn 103 for its proper folding and functioning (Limongi et al. 1995). Some glycosyltranferases (group 1 glycosyl transferase, galactosyltransferase) are also observed, and these may have role in proper functioning of glycosylated proteins involved in azo dye degradation.

Possible reasons behind observed unannotated transcripts

Many of the isolated ESTs encoded proteins with a wide variety of functions while a large fraction corresponded to hypothetical or unknown proteins. A large number of ESTs were obtained in the present study are similar to the results obtained by Nevarez et al. (2008); they obtained 445 differentially expressed ESTs in response to thermal stress (i.e., 40 °C, 120 min). Castillo et al. (2006) had conducted studies to evaluate effects of different nutritional growth conditions and isolated 37 genes of *P. chrysogenum* implicated in growth with penicillin-repressing or non-repressing carbon sources. For example, transcripts JK747277 and JK747283 have no annotation in the database but were observed to be induced at 216 and 120 h in the presence of AR 183.

Quantitative real-time PCR analysis of transcripts

Upregulation of transcript (#JK747284) to a notable extent had suggested the role of peroxidases in the AR183 degradation. Manganese peroxidases are reported to be involved in the biodegradation of lignocellulose and lignin and participate in the bioconversion of other diverse recalcitrant compounds, like polycyclic aromatic hydrocarbons, chlorophenols, industrial effluents of textile and petrochemical industries, and bioremediation of contaminated soils (Saroj et al. 2013). MnP has been observed to be induced during decolorization of the mixture of azo dyes at a concentration range of 10–200 mg L^{-1} each, and also MnP from *P. chrysosporium* sp. HSD has been reported to rapidly decolorize a higher concentration (up to 600 mg L^{-1}) of azo dyes (Singh and Pakshirajan 2010).

ATP binding cassette (ABC) transporters form a particular family of membrane proteins, characterized by homologous ATP-binding and large, multi-pass transmembrane domains. The possible reason behind upregulation of the ABCT transcript between 120 and 168 h lies behind the extracellular nature of peroxidases and is in agreement with the highest MnP yield and attainment of maximum level of decolorization

(Fig. 1b). Regarding yeast and fission yeast, in which Cd is able to form complexes either with glutathione (GSH) or phytochelatins (PC) subsequently transported into vacuoles via ABC transporters (Bovet et al. 2005), it is also very likely that some fungal ABC transporters are able to transport toxic dyes into subcellular compartments or outside of the cell. Furthermore, downregulation of TAP (# JK747316) and PTGE (# JK747359) transcripts in response to dye suggested that they might be acting as inhibitors to peroxidase activity that is downregulated when the cell induces increased levels of enzyme to counteract toxicity due to azo dyes.

HPCYT (hypothetical protein cytochrome P450) and CYT (cytochrome P450) are well-reported enzymes to detoxify harmful dyes like malachite green and leuco malachite green, and other toxic chemicals (Culp et al. 1999; Cha et al. 2001).

Methylenetetrahydrofolate reductase (*MTHFR*) gene had been reported to be involved in the plasma membrane redox system that is required for pigment biosynthesis in filamentous fungi. Heat shock proteins have previously been reported to induce peroxidases in *N. crassa* (Machwe and Kapoor 1993). Induction of heat shock proteins and NDFR transcript levels coincides with that of peroxidase transcripts that suggest their cumulative involvement in AR183 degradation.

To the best of our knowledge, this is the first ever report enumerating analysis of differential expression of transcripts in *P. oxalicum* SAR-3 in response to an azo dye and includes genes with known as well as unknown functions. Presence of large number of transcripts denotes that there is no single mechanism channelling azo dye AR183 decolorization by *P. oxalicum* SAR-3. In *P. oxalicum* SAR-3, they may be "common ecological stress response" genes and have similar trend of expression in response to varying environmental changes as those identified in studies on yeasts and fungi (Gasch et al. 2000; Chen et al. 2003; Nevarez et al. 2008). Expression and induction of ATP binding cassettes transporters (ABCT) and peroxidases turn out to be the major factors required for significantly acceptable levels of dye decomposition. The ESTs having hypothetical or unknown functions with no notable similarity in the database may represent new potential stress markers to characterize the physiological state of *P. oxalicum* SAR-3 in the presence and absence of azo dye. It is clear that additional inputs are necessary to define the interplay of integral genes and to fully understand the regulation of these complex adaptive responses. However, the various ESTs identified in this work provide new insights into multispectral domain of *P. oxalicum* SAR-3 biology, revealing new angles for functional genome analysis. This work will greatly enable understanding the molecular basis of dye detoxification, particularly the novel genes that may be involved in the process and, eventually, could be accountable to the development of a robust, eco-friendly, and an economical process for the textile waste water treatment.

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