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Current Proteomics, 2018, *15*, 000-000

RESEARCH ARTICLE

Differential Protein Expression in *Shewanella seohaensis* Decolorizing Azo Dyes

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ARTICLE HISTORY

Received: March 06, 2018
Revised: June 21, 2018
Accepted: July 24, 2018

DOI:
10.2174/1570164615666180731110845

Abstract: ***Background***: Microbial remediation is an ecologically safe alternative to controlling environmental pollution caused by toxic aromatic compounds including azo dyes. Marine bacteria show excellent potential as agents of bioremediation. However, a lack of understanding of the entailing mechanisms of microbial degradation often restricts its wide-scale and effective application.

Objective: To understand the changes in a bacterial proteome profile during azo dye decolorization.

Method: In this study, we tested a Gram-negative bacterium, *Shewanella seohaensis* NIODMS14 isolated from an estuarine environment and grown in three different azo dyes (Reactive Black 5 (RB5), Reactive Green 19 (RG19) and Reactive Red 120 (RR120)). The unlabeled bacterial protein samples extracted during the process of dye decolorization were subject to mass spectrometry. Relative protein quantification was determined by comparing the resultant MS/MS spectra for each protein.

Results: Maximum dye decolorization of 98.31% for RB5, 91.49% for RG19 and 97.07% for RR120 at a concentration of 100 mg L^{-1} was observed. The liquid chromatography-mass spectrometry - Quadrupole Time of Flight (LCMS-QToF) analysis revealed that as many as 29 proteins were up-regulated by 7 hours of growth and 17 by 24 hours of growth. Notably, these were common across the decolorized solutions of all three azo dyes. In cultures challenged with the azo dyes, the major class of up-regulated proteins was cellular oxidoreductases and an alkyl hydroperoxide reductase (SwissProt ID: A9KY42).

Conclusion: The findings of this study on the bacterial proteome profiling during the azo dye decolorization process are used to highlight the up-regulation of important proteins that are involved in energy metabolism and oxido-reduction pathways. This has important implications in understanding the mechanism of azo dye decolorization by *Shewanella seohaensis*.

Keywords: Azo dye, decolorization, mass spectrometry, proteomics, *Shewanella seohaensis*, up-regulation.

1. INTRODUCTION

Currently, there are over 100,000 different synthetic dyes in use worldwide [1]. They find useful applications in textile, pharmaceutical, leather, paper, cosmetic and food industries [2, 3] and are classified by their chemical composition or by the types of fibers to which they can be applied [4]. Chemical classification system groups synthetic dyes based on the nature of their chromophores. As such, the various classes of industrially significant dyes include azo, anthraquinone, diazonium, sulfur, indigoid, triarylmethane, triphenylmethyl (trityl), nitro and phthalocyanine derivatives [5].

In India, the dye manufacturing industry contributes to almost 6.6% of the global dye production [6]. The textile industry accounts for two-thirds of the total dyestuff market. The process of dyeing of one kilogram of fiber with reactive dyes utilizes 70 to 150 L water, 0.6 to 0.8 kg NaCl and about 30 to 60 g dyestuff [7]. Depending on the class of dye used, the dyeing process discharges approximately 2% to 50% of dye into wastewater [8, 9]. Reactive dyes used for dyeing of cellulosic fibers, wool and silk have low rates of fiber fixa-

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tion [10]. Consequently, textile effluents contain large amounts of unbound dye which enter aquatic bodies directly and impart intense coloration. It is estimated that 280,000 tons of textile dyes are discharged in industrial effluents per year worldwide [11]. Disposal of large volumes of azo dyes in textile industrial effluent poses an environmental and organismic health hazard. If left untreated, the deeply colored water causes a reduction in the penetration of sunlight to subsurface layers of the receiving water bodies. This leads to decreasing photosynthetic activity and increasing biological oxygen demand [12]. Oliveira *et al*. [13] and Shah [14] reported that many mutagenic and carcinogenic synthetic dyes which are resistant to microbial degradation can become highly persistent in natural water bodies leading to ecosystem degradation and implicit hazards of various kinds.

Azo dyes typically contain one or more azo (-N=N-) groups and are the most extensively used textile dyes [15]. A complex chemical structure and synthetic nature make these dyes xenobiotic and recalcitrant to biodegradation [16]. The toxic nature of certain azo dye breakdown products has prompted the European Union to impose a ban on the use of certain azo dyes [17].

Many of the world's textile industry hubs are present in developing countries. However, due to a lack of stringent monitoring and inadequate environmental management controls, effluents are discharged without proper treatment directly into water bodies [18].

High economic costs coupled with low efficiency and limited application make conventional physicochemical treatments of dye-containing wastewater less appealing. Biological systems capable of degrading and decolorizing azo dyes are therefore of great significance in bioremediation efforts. Various mechanisms of dye decolorization by microorganisms have been proposed in recent times [19]. However, bacteria receive attention for their ability to decolorize a wide range of structurally different dyes. Many species of bacteria adopt a number of mechanisms to remove or decolorize azo compounds from the environment including biosorption, bioaccumulation, reduction, oxidation and sequential reduction-oxidation processes [20]. Among them, *Shewanella* species are reported to possess diverse respiratory metabolic pathways and are indeed known potential agents for bioremediation [21]. In this regard, understanding and evaluating the changes in the proteome of an organism during its growth would prove pivotal. For instance, Zhao and Poh [22] describe how different cellular protein components of bacteria are known to respond differently to various environmental stimuli.

The ease and reliability of reproducible proteome analyses are facilitating large-scale descriptions of proteins from various organisms. Essentially, the expression of proteins by the bacterium exposed to synthetic dyes under experimental alterations helps to elucidate the possible expression of pertinent proteins. As Linsen *et al*. [23] suggest, differential protein expression analysis enables the identification of proteins and the pattern of their expression under different conditions of growth. This is implicit of the fact that the proteins expressed differentially are useful to discern their functional involvement in a given process. In this regard, their identification would contribute to recognize the role of specific proteins in the pathway of dye decolorization. Such a comprehensive understanding would enable selective alteration of certain proteins for targeted development of strains with maximum efficiency of dye decolorization.

In this study, a gel-free proteomic approach was used to evaluate the changes in protein expression of *Shewanella seohaensis* NIODMS14 during the decolorization of three reactive dyes, Reactive Black 5 (RB5), Reactive Green 19 (RG19) and Reactive Red 120 (RR120). We used a Liquid Chromatography-Mass Spectrometry - Quadrupole Time of Flight (LCMS-QToF) system to identify the proteins expressed and to measure their abundance with respect to differential regulation. This was done to understand the contrast in the expression of proteins when *Shewanella seohaensis* NIODMS14 is grown in a medium with an azo dye versus growth in the dye-free medium. The results demonstrate differences in the proteome of *Shewanella seohaensis* NIODMS14 during the decolorization process of the three azo dyes.

2. MATERIALS AND METHODS

2.1 Microorganism and Culture Conditions

The proteomic profiles of a Gram-negative, salt tolerant bacterial isolate collected from an estuarine location in Goa, India were analyzed in this study. The strain NIODMS14 was identified as *Shewanella seohaensis* on the basis of 16S rRNA gene sequencing and the sequence was submitted to GenBank with the accession number KR922404. The pure culture of the isolate was deposited at the Microbial Type Culture Collection (MTCC) of the CSIR-Institute of Microbial Technology, Chandigarh, India, under the accession number MTCC 25079**.** The culture was purified on seawater nutrient agar (SWNA containing [l^{-1}]: peptic digest of animal tissue 5.0 g, sodium chloride 5.0 g, beef extract 1.5 g, yeast extract 1.5 g, agar 15 g, distilled water 500 mL, sea water 500 mL, pH 7.4 ± 0.2) and a loopful of seed culture from an isolated colony was pre-cultured in 10 mL nutrient broth (NB, containing [l^{-1}] 5.0 g peptone and 3.0 g yeast extract) at pH 7.0 for 12 hours at 30°C, 120 rpm on a shaker incubator. Fresh NB (100 mL) was further inoculated with 1% (v/v) of the pre-cultured broth and grown under the same conditions. This was then used as the inoculum for decolorization experiments. Stock solutions of the dyes (10 g L^{-1}) were prepared and sterilized by membrane filtration using a 0.2-µm pore size membrane filter (Axiva, Delhi). All chemicals used in this study were of analytical grade.

2.2. Decolorization Assay and Growth Profiles

Decolorization experiments using three commercial reactive azo dyes, viz. RB5, RG19 and RR120 (Sigma-Aldrich), were performed by growing the culture in 500 mL Erlenmeyer flasks containing 250 mL of sterile nutrient broth supplemented with dyes added at a concentration of 100 mg L^{-1}. Flasks were incubated at 30°C and samples were aliquoted every 3 hours until decolorization was complete. The samples were then centrifuged at 20,000 g for 10 minutes at 4°C. The cell-free supernatant color was read at the wavelength of maximum absorption of the dyes used, i.e. 597 nm

for RB5, 630 nm for RG19 and 522 nm for RR120, using a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan). Controls without dye and/or inoculum were maintained under identical conditions. The cell pellets were further used for protein extraction.

The decolorization efficiency was expressed using the following equation [24]:

$$\text{Decolorization (\%)} = (A_{initial} - A_{final})/ A_{initial} \times 100$$

where $A_{initial}$ is the initial absorbance and A_{final} is the absorbance after time t.

Bacterial growth was measured by determining the absorbance of culture samples at 600 nm [24].

2.3. Extraction of Proteins

The cell pellets, obtained at the time points 7 hours and 24 hours, were washed twice with 1X Phosphate Buffer (PB containing [l^{-1}]: disodium hydrogen phosphate 1.42 g, potassium dihydrogen phosphate 0.24 g, distilled water 1000 mL, pH 7.4 ± 0.2) and resuspended in 1 mL of protein extraction buffer along with zirconia beads and homogenized in a FastPrep24 homogenizer (MP Biomedicals, USA) at 6.5 m/s for 40 seconds. Whole cell proteins were extracted using a urea-thiourea buffer containing [ml^{-1}]: urea 0.42 g, thiourea 0.15 g, CHAPS 0.4 g, IPG buffer (Immobilized pH gradient) 80 microL, Dithiothreitol (DTT) 0.03 g and 1M tris solution 200 microL. Cell debris was removed by centrifugation at 20,000 g at 4°C for 20 minutes. The proteins in the supernatant were precipitated using methanol [25]. The vacuum dried samples were resuspended in 1 mL of TE buffer (pH 8.3) containing 10 µL of 1X protease inhibitor (Serva, Germany). The protein concentration was estimated using the Folin-Lowry method [26]. Based on this concentration, suitable aliquots were made and stored at -20°C until further processing.

2.4. Preparation of In-solution Digests

The method of in-solution trypsin digestion for the solubilization of proteins was followed (modified from Kinter *et al*. [27]). 6M urea was used as a chaotropic agent along with 200mM DTT and 200 mM iodoacetamide as reducing and alkylating agents, respectively. 100 µg of the extracted protein sample was digested with 1 mg mL^{-1} trypsin (20µg Proteomics grade Trypsin from porcine pancreas dissolved in 20µL of 1mM HCl, Sigma T6567, USA) at 37°C for 15 hours. After digestion, the samples were acidified to a pH of 2-3 using 100% formic acid (MS grade) before subjecting to mass spectrometry.

2.5. Mass Spectrometry and Protein Identification

Proteins were identified using the in-house LCMS-QToF facility (6538 UHD Accurate Mass QTOF LC/MS, Agilent Technologies, USA). Sub-volumes of 8 µL tryptic digests were injected into the auto-sampler. The LC separations were carried out on a Prot ID Chip 150 II 300A C18 150 mm column using water and 9:1 (v/v) acetonitrile: water. 0.1% Formic acid in water (1:999 v/v) was used as adduct in both the aqueous and organic phases (acetonitrile). The peptides were eluted using a standardized 71-minute protocol with a linear gradient of increasing acetonitrile concentration (2 to 90% for 50 min, 90% for 10 min and 90 to 2% for 3 min). The MS/MS spectra for the separated ions were acquired in the range of 50 to 2000 *m/z* in positive mode at 2 ppm accuracy. The spectral data was acquired using Mass Hunter Software ver. B.06.00 (Agilent Technologies, USA).

2.6. LC-MS/MS Data Analyses and Interpretation

Protein identification and quantification were performed using Spectrum Mill software ver. B.04.01.141 (Agilent Technologies, USA). The MS/MS spectra acquired were searched against the genus-specific UniProtKB/Swiss-Prot database for *Shewanella*. The following search criteria were used: minimum matched peak intensity of 50%, precursor mass tolerance of 50 ppm and product mass tolerance of 100 ppm. In addition, a maximum of 2 missed cleavages was allowed. Carbamidomethylation of cysteine residues was included as the fixed modification. The study was a high throughput discovery proteomics experiment and samples were run as four technical replicates.

2.7. Statistical Analyses

Mass Profiler Professional (MPP) software ver. 14.5 (Agilent Technologies, USA) was used to analyze the protein hits generated. The proteins were identified in each of the replicates and grouped for each sample (dye-free control, RB5, RG19 and RR120). Each identified protein entity in the sample was selected based on the frequency of occurrence in at least one condition. Auto-validation was carried out for the database matches using the default settings with a False Discovery Rate (FDR) of 1.2% (Supplemental file 1). Significant proteins had peptide scores in protein mode >1 with a scored peak intensity (SPI) of >10% for peptides. The differential significance between control samples (dye-free) and dye-amended samples was determined with a p-value cut-off of 0.2 and a fold-change cut-off of 2.0. The protein hits satisfying the cut-off criteria were functionally classified based on the Kyoto Encyclopedia of Genes and Genomes, ver. 37 (KEGG) pathways. Venn diagrams were created to identify proteins exclusively up-regulated during decolorization of different dyes and proteins common to all the dye conditions.

3. RESULTS

3.1. Cell Growth and Decolorization Assay

The growth assay of *Shewanella seohaensis* NIODMS14 and extent of decolorization of the three reactive dyes in dye-amended nutrient broth suggest rapid decolorization (Fig. **1**). The culture decolorized all three reactive dyes substantially. Dye decolorization was visible in the initial 3 hours of contact with the dye itself. In case of RB5, rapid decolorization (84.33%) was seen within the initial 3 hours and a maximum of 98.31% was achieved by 20 hours. Similarly, for RG19, decolorization of 78.5% was seen after 3 hours incubation of the culture with the dye whereas a maximum of 91.49% was achieved in 23 hours. Maximum decolorization of 97.07% of RR120 happened within 17 hours. It was observed that color reduction of the reactive dyes occurred concomitantly with

active cell growth and considerable decolorization occurred in the early stages of growth.

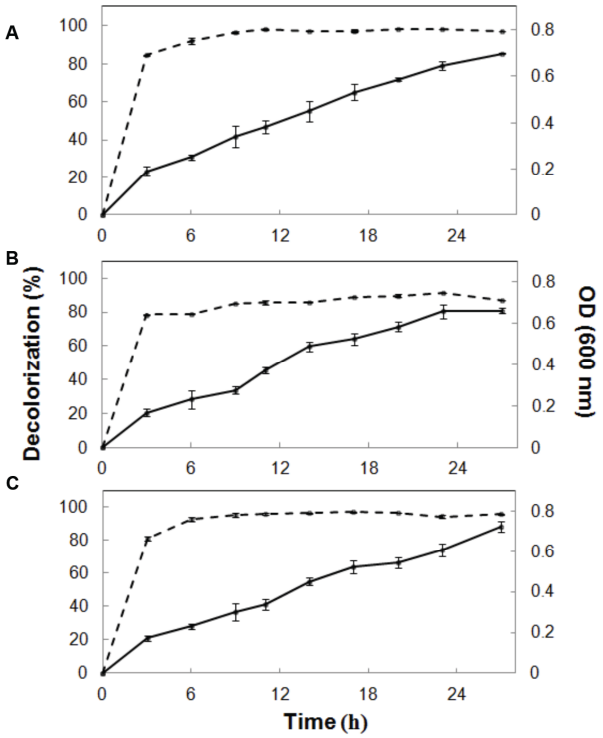

Fig. (1). Growth curve (solid line) and decolorization percent (dashed line) of *Shewanella seohaensis* NIODMS14 in nutrient broth amended with 100 ppm of (**A**) Reactive Black 5 (**B**) Reactive Green 19 (**C**) Reactive Red 120. Data are expressed as mean ± standard deviation (SD) of three replicate experiments.

3.2. Protein Identification

The numbers of expressed proteins in *Shewanella seohaensis* NIODMS14 in the medium with and without dyes (control) were compared to discern the changes in the protein profiles during dye decolorization. The accession numbers of the identified proteins were referenced using the UniProtKB database (www.uniprot.org).

Approximately, a total of 1182 functionally different protein hits were obtained from the data acquired using the MPP software ver. 14.5 (Agilent Technologies, USA). Of these, 507 entities were obtained from the culture grown in the absence of the dyes (control), whereas, 423, 417 and 677 proteins were detected in *S. seohaensis* NIODMS14 grown in medium with RB5, RG19 and RR120, respectively (Supplemental file 2).

3.3. Functional Characterization of Expressed Proteins

The identified proteins had a broad functional distribution and were classified as such. According to the physiological functions assigned using the Kyoto Encyclopedia of Genes and Genomes, ver. 37 (KEGG) metabolic pathways and Clusters of Orthologous Groups database (https://www.genome.jp/kegg/pathway.html), the proteins were classified into 15 functional categories (Fig. **2**). This allowed a comparison of differentially expressed proteins between control and dye treatment. It was observed that the number of proteins detected did not vary much with or without the presence of azo dyes. Major cellular processes were not affected during decolorization as the culture grew well and essential proteins were expressed in all three dye-challenged conditions. Proteins involved in the process of translation were maximal, contributing to 25% of the total classified proteins. The minimum numbers of proteins identified were those involved in the metabolism of other molecules including terpenoids, polyketides, flavonoids, etc., accounting for only 13% of the total classified proteins.

3.4. Comparison of Differentially Expressed Proteins

Significant differences in protein profiles were observed in *S. seohaensis* NIODMS14 grown in the presence of reactive azo dyes. At 7 hours of incubation, 176, 113 and

Table 1. Proteins up-regulated during decolorization of Reactive Black 5 azo dye.

Swiss-Prot ID	Protein Name	Gene Based on Protein ID	Protein Mass (Da)	Functional Category[a]	Fold Change[b]
A9L5Q8	Cytochrome c prime	*Sbal195_1300*	16506.6	Energy metabolism	16.00
P83223	Fumarate reductase flavoprotein	*SO_0970*	62903.3	Energy metabolism	1.29
A8GZW1	Oxidoreductase FAD/NAD(P)-binding domain protein	*Spea_0520*	26730	Energy metabolism	1.08
Q8EI38	NADH-quinone oxidoreductase, subunit K	*nuoK*	11211.3	Energy metabolism	16.00
A9KY42	Alkyl hydroperoxide reductase/ Thiol specific antioxidant	*Sbal195_1777*	22160.7	Peroxiredoxin activity	1.54
A4Y8D8	TRAP dicarboxylate transporter, DctP subunit	*Sputcn32_2500*	38006	Membrane transport	16.00
A9KTX7	Membrane protein involved in aromatic hydrocarbon degradation	*Sbal195_2866*	46196.6	Cellular process	3.59

[a]Functions are reported according to KEGG2 metabolic database (http://www.genome.jp/kegg/kegg2.html).

[b]Fold change compared to dye free control.

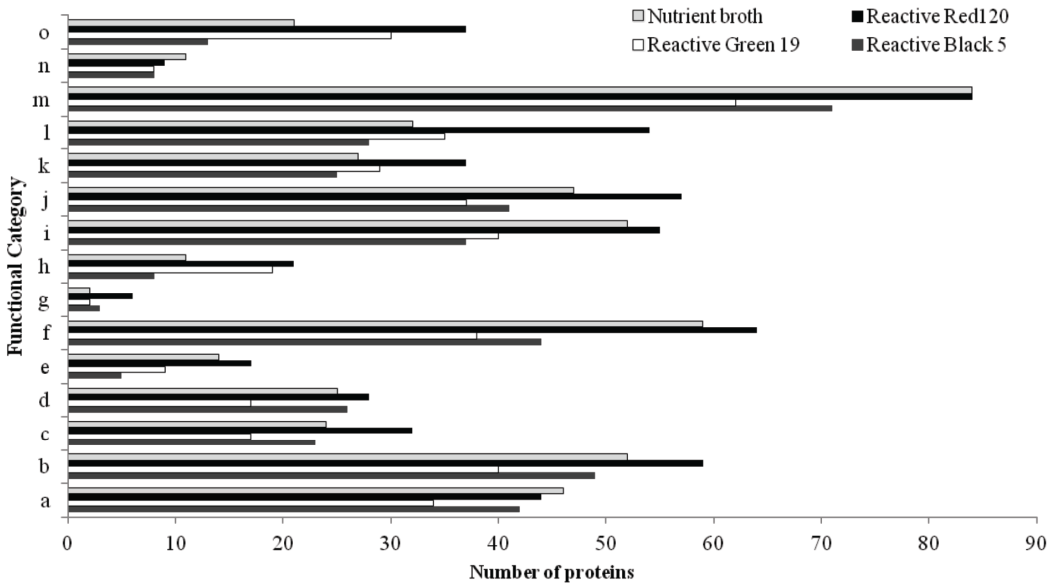

Fig. (2). Functional classification of identified proteins expressed in dye-free nutrient broth (light grey shaded bar) compared to those expressed during decolorization of the reactive dyes: RR120 (black shaded bar), RG19 (white shaded bar) and RB5 (dark grey shaded bar). Lowercase letters along the x-axis refer to the following categories of functional roles: (a) Carbohydrate metabolism; (b) Amino acid metabolism; (c) Lipid metabolism; (d) Nucleotide metabolism; (e) Cofactors and vitamins metabolism; (f) Energy metabolism; (g) Other metabolic processes; (h) Signal transduction; (i) Cellular process; (j) Transport; (k) Replication and repair; (l) Transcription; (m) Translation; (n) Protein folding, sorting and degradation; (o) Miscellaneous & Unknown function. (KEGG metabolic database, http://www.genome.jp/kegg/).

231 proteins were up-regulated during the decolorization of RB5, RG19 and RR120 respectively. Further, at 24 hours, as many as 138, 219 and 520 proteins were up-regulated in *S. seohaensis* NIODMS14 grown in RB5, RG19 and RR120 respectively as compared that grown in the dye-free control (Fig. **3**). As many as 29 up-regulated proteins were found to be common to all the three dyes at the early stages of the decolorization process (7 hours) while 17 commonly up-regulated proteins were identified in the samples after 24 hours of incubation (Fig. **3**). Of these, some prominent proteins expressed during the decolorization of Reactive Black 5 are listed in Table **1** along with their fold change and functional categorization. Similarly, up-regulated proteins identified during decolorization of Reactive Green 19 and Reactive Red 120 are summarized in Tables **2** and **3** respectively.

3.5. Changes in Abundance of Expressed Proteins Accompanying Dye Decolorization

Many proteins functioning in energy metabolism were predominantly up-regulated in the bacterium during dye decolorization, especially the cellular oxidoreductases. Proteins commonly expressed in all three dye decolorizing conditions were an oxidoreductase FAD/NAD (P)-binding domain protein (SwissProt ID: A8GZW1) and an alkyl hydroperoxide reductase (SwissProt ID: A9KY42). At 24 hours of exposure, the alkyl hydroperoxide reductase was up-regulated by 1.54, 1.25 and 7.68 folds under RB5, RG19 and RR120 dye decolorizing conditions respectively. This protein complex has oxido-reductase activity and can use either NADH or NADPH as an electron donor for direct reduction of redox dyes or of alkyl hydroperoxides. It functions in cellular response to oxidative stress and were notably absent in the control sample without dye.

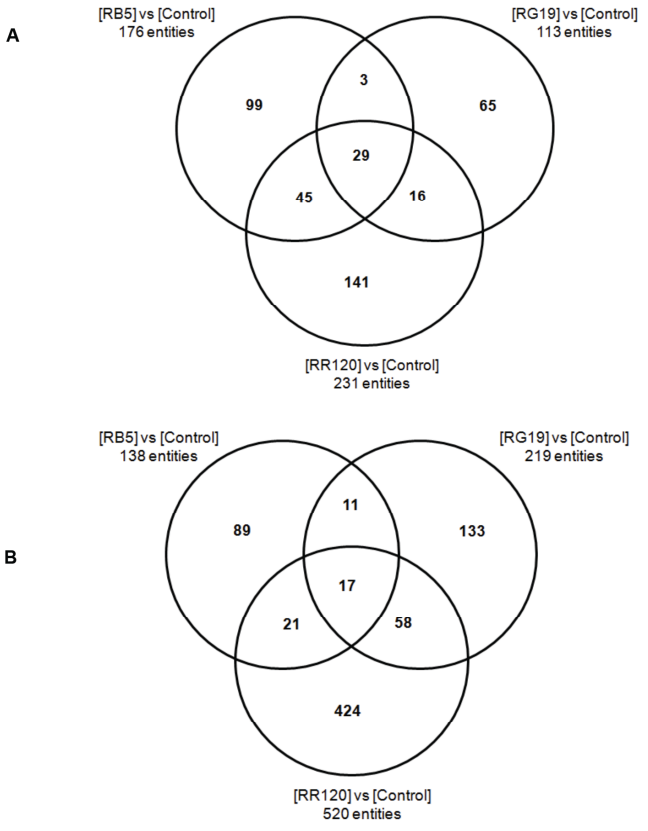

Fig. (3). Venn diagrams comparing up-regulated proteins entities in dye challenged *Shewanella seohaensis* NIODMS14 versus control (without dye). (**A**) Up-regulated entities at 7 hours; (**B**) Up-regulated entities at 24 hours of exposure to dyes.

Table 2. Proteins up-regulated during decolorization of Reactive Green 19 azo dye.

Swiss-Prot ID	Protein Name	Gene Based on Protein ID	Protein Mass (Da)	Functional Category[a]	Fold Change[b]
A9L5Q8	Cytochrome c prime	*Sbal195_1300*	16506.6	Energy metabolism	16.00
A8GZW1	Oxidoreductase FAD/NAD(P)-binding domain protein	*Spea_0520*	26730	Energy metabolism	2.94
A8FPZ7	FMN-dependent NADH-azoreductase	*azoR*	21205.9	Energy metabolism	16.00
P83223	Fumarate reductase flavoprotein subunit	*SO_0970*	62903.3	Energy metabolism	16.00
B0TT30	NADH:flavin oxidoreductase/NADH oxidase	*Shal_2030*	74360.4	Energy metabolism	1.49
A9KY42	Alkyl hydroperoxide reductase/ Thiol specific antioxidant	*Sbal195_1777*	22160.7	Peroxiredoxin activity	1.25
A8FYW2	Na(+)-translocating NADH-quinone reductase subunit A	*nqrA*	47902.9	Membrane transport	16.00
B1KNE0	TRAP dicarboxylate transporter, DctP subunit	*Swoo_1732*	39527.3	Membrane transport	16.00

[a]Functions are reported according to KEGG2 metabolic database (http://www.genome.jp/kegg/kegg2.html).

[b]Fold change compared to dye free control.

Table 3. Proteins up-regulated during decolorization of Reactive Red 120 azo dye.

Swiss-Prot ID	Protein Name	Gene Based on Protein ID	Protein Mass (Da)	Functional Category[a]	Fold Change[b]
A9L5Q8	Cytochrome c prime	*Sbal195_1300*	16506.6	Energy metabolism	1.18
A8GZW1	Oxidoreductase FAD/NAD(P)-binding domain protein	*Spea_0520*	26730	Energy metabolism	3.32
P83223	Fumarate reductase flavoprotein subunit	*SO_0970*	62903.3	Energy metabolism	1.66
BOTT30	NADH:flavin oxidoreductase/NADH oxidase	*Shal_2030*	74360.4	Energy metabolism	16.00
A9KY42	Alkyl hydroperoxide reductase/ Thiol specific antioxidant	*Sbal195_1777*	22160.7	Peroxiredoxin activity	7.68
A4Y3Y6	Na(+)-translocating NADH-quinone reductase subunit A	*nqrA*	47856.1	Membrane transport	1.36

[a]Functions are reported according to KEGG2 metabolic database (http://www.genome.jp/kegg/kegg2.html).

[b]Fold change compared to dye free control.

Proteins functioning in electron transport were observed to be up-regulated during dye decolorization. A fumarate reductase flavoprotein subunit (SwissProt ID: P83223) was up-regulated by 1.29, 16.00 and 1.66 in RB5, RG19 and RR120 decolorizing conditions respectively. Cytochrome c protein (SwissProt ID: A9L5Q8) which is also an electron transport protein, was identified in all three dye-challenged samples as well as in dye-free control with a fold change of 1.18 (in RR120 decolorizing condition) and 16.00 (in RB5 and RG19 decolorizing conditions). The up-regulation of these two proteins points to their role in dye reduction and decolorization by electron transfer. NADH: flavin oxidoreductase/NADH oxidase (SwissProt ID: B0TT30) was up-regulated by 1.49 fold in RG19 decolorizing condition and by 16.00 fold in RR120 decolorizing condition. Another protein with similar function, NADH-quinone oxidoreductase, subunit K found in the RB5 decolorized sample was up-regulated with a high fold change of 16.00. An FMN-dependent NADH-azoreductase was also obtained under RG19 decolorizing condition.

Two transport proteins were notably up-regulated wherein, Na (+)-translocating NADH-quinone reductase subunit A (SwissProt ID: E6XLW6) was detected during decolorization of RG19 (fold change of 16.00) and RR120 (fold change of 1.36) and TRAP dicarboxylate transporter (SwissProt ID: A4Y8D8; fold change of 16.00) was detected in *S. seohaensis* NIODMS14 decolorizing RB5 and RG19 and was not detected in the dye-free control. A membrane protein with 3.59 fold increase, reported to be involved in aromatic hydrocarbon degradation was identified in RB5 decolorizing condition at 7 hours. Gene Ontology (GO) annotation indicated that this 46.197 kDa polypeptide functions in membrane transport associated with the catabolism of aromatic hydrocarbons. The protein was identified based on amino acid similarity hit to the polypeptide present in *Shewanella baltica (strain OS195)*. Studies aimed at ex-situ expression of this and other up-regulated proteins would be highly valuable.

4. DISCUSSION

Shewanella strains have the ability to use a range of electron donors and acceptors, thereby reducing the toxic effects of many contaminants which have earned them the reputation of being excellent candidates for bioremediation [28]. Various cellular proteins which play a role in the reductive metabolism of *Shewanella* species include cellular oxidoreductases as well as *c*-type cytochromes and peroxiredoxins. Chen *et al*. [29] propose that some of these proteins have also been implicated in azo dye decolorization.

Various species of bacteria are known to accomplish decolorization and detoxification of azo dyes [30]. Pearce *et al*. [31] observed the lower efficiency of decolorization in case of complex structured and high molecular weight dyes such as azo dyes. However, in this study *Shewanella seohaensis* NIODMS14 exhibited rapid and efficient decolorization of three reactive diazo dyes at a concentration of 100 mg L^{-1}. In fact, complete decolorization of the dye RB5 was achieved within 24 hours. The strain also effectively decolorized more than 90% of RG19 and RR120 in a short time of 23 hours and 6 hours respectively. This is in contrast to previous reports by Wu *et al*. [32] where *Shewanella oneidensis* WL-7 was found to decolorize only 36% of 100 μM of RB5 in about 12 hours.

There are many studies aimed at identifying and characterizing the molecular components of the dye decolorization process [28, 33, 34, 35]. Kudlich *et al*. [36] first proposed the involvement of the bacterial electron transport chain in azo dye reduction. Dubbs and Mongkolsuk [37] suggested peroxiredoxins as a class of enzymes that play an antioxidant protective role in cells. The up-regulation of pertinent oxidoreductases (oxidoreductase FAD/NAD(P)-binding domain protein) and peroxiredoxins (alkyl hydroperoxide reductase) in dye-exposed *Shewanella seohaensis* NIODMS14 points to the involvement of these proteins in the dye decolorization process. Experimental validation of these proteins was not carried out as this was a discovery proteomic experiment.

As described by Hong and Gu [28], Hong *et al*. [33] and Wang *et al*. [35] fumarate reductase enhances decolorization by transferring electrons during bacterial azo reduction. The analysis of results showed a significantly up-regulated fumarate reductase flavoprotein subunit suggesting it plays a similar role in *Shewanella seohaensis* NIODMS14.

Transport proteins constitute an integral part of the response mechanism of bacteria to heavy metals and other environmental contaminants including azo dyes. The up-regulation of two transport proteins (Na (+) -translocating NADH-quinone reductase and TRAP dicarboxylate transporter) in *Shewanella seohaensis* NIODMS14 points to their possible involvement in transferring reducing electrons to the azo dye during the decolorization process (also described by Tang *et al*. [38]).

Brigé *et al*. [34] observed the involvement of cytochrome c proteins in the reduction of reactive dyes. These crucial components of the electron transport chain function in reducing dye molecules and thereby facilitating the decolorization process. In concurrence with these findings, multiple up-regulated cytochrome c proteins were observed in *Shewanella seohaensis* NIODMS14 associated with decolorization of the three reactive azo dyes.

The protein profiles generated from this study reveal the up-regulation of cellular oxidoreductases which may function in electron transfer during azo dye decolorization. However, it is necessary to further deduce the specific metabolic pathways in which these proteins function. This can be achieved by integrating computational and other 'omics' techniques.

CONCLUSION

Using a discovery proteomic approach, this study provides an insight into the induction, expression and catabolic pathway of certain proteins of *Shewanella seohaensis* NIODMS14 which rapidly decolorized three reactive azo dyes. Results obtained are used to directly correlate the function of various proteins to the process of azo dye decolorization based on observed differential regulation and/or expression and accompanying fold change values. During dye decolorization, the study identifies proteins in oxido-reduction pathways (for example, oxidoreductase FAD/NAD (P)-binding domain protein, NADH: flavin oxidoreductase/ NADH oxidase, alkyl hydroperoxide reductase and FMN-dependent NADH-azoreductase) to be up-regulated. Synergistic up-regulation of these proteins coupled with membrane transport proteins (Na (+)-translocating NADH-quinone reductase and TRAP dicarboxylate transporter) and electron transport (by fumarate reductase and cytochrome c proteins) serves to rapidly and efficiently decolorize azo dyes by *Shewanella seohaensis* NIODMS14.

Our results provide additional information to support that the electron transfer pathway contributes to azo dye recognition, metabolism and finally decolorization. This information has useful implications for targeted manipulation of specific dye decolorizing proteins in order to augment their activity and further design bioremediation strategies using *Shewanella seohaensis* in particular and other decolorizing bacterial strains in general.

LIST OF ABBREVIATIONS

DTT	=	Dithiothreitol
FDR	=	False Discovery Rate
GO	=	Gene Ontology
IPG	=	Immobilized pH gradient
KEGG	=	Kyoto Encyclopedia of Genes and Genomes
LCMS-QToF	=	Liquid Chromatography-Mass Spectrometry - Quadrupole Time of Flight
MPP	=	Mass Profiler Professional
MTCC	=	Microbial Type Culture Collection
NB	=	Nutrient broth
PB	=	Phosphate buffer
RB5	=	Reactive Black 5

RG19 = Reactive Green 19

RPM = revolutions per minute

RR120 = Reactive Red 120

SPI = scored peak intensity

SWNA = Sea water nutrient agar

TE = Tris-EDTA

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

This work was conceptualised by NR, SD and NdeS and exectued by NdeS, AP, MDeL. NdeS analyzed the data and wrote and revised this manuscript. SD, CM and NR supervised experimental design and helped revise this manuscript. BF and SD helped in carrying out proteomic experiments and acquisition of data. Ram Murti Meena helped in sequencing and identification of the isolate. All authors read and approved the final manuscript. The authors thank the Director, Council of Scientific and Industrial Research (CSIR) - National Institute of Oceanography for the facilities and encouragement. Financial support from CSIR funded project PSC0206 (MAR-BIOTECH) and BSC0111 (INDEPTH) is gratefully acknowledged. This is NIO contribution number 6275.

This study is funded by project Nos: PSC0206 and BSC0111. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article. Supplemental File 1 contains all the files of auto validation carried out on the MS results. Supplemental File 2 shows a complete summary of all protein entities.

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