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Original Article

Enhancing iron oxidation efficiency by a native strain of *Acidithiobacillus ferrooxidans* via response surface methodology, and characterization of proteins involved in metal resistance by proteomic approach

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Abstract

The effects of different factors on growth and bio-oxidation efficiency of a native strain of *Acidithiobacillus ferrooxidans* have been evaluated by the utilization of response surface methodology, RSM. Medium pH and iron concentration were found to be the most significant factors while temperature and ammonia concentrations had the least weight within the ranges investigated. Optimum operational conditions for maximizing Fe^{2+} oxidation were found to be 31 ^{o}C, 7 g/Liron concentration, 4.5 g/Ltotal ammonium salt concentration and medium pH 1.85. Maximum recovery of 98% of Zinc is the main outcome of results as observed at 7 g/Lof Fe^{2+}, under optimized experimental conditions. The response of a bacterial strain to metals toxicity also studied. The isolate showed good resistance to most of the toxic metals. The proteomics approach was used to identify the differentially expressed proteins under heavy metal stress. Four of the differentially expressed proteins were identified as major outer membrane protein of *A. ferrooxidans*, ribulose large bisphosphate carboxylase subunit, and holo synthase.

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Introduction

Mining operations using bacteria have become popular as the conventional methods utilize cost effective and energy intensive processes. Previously this technique was used for extraction of copper at industrial scale but today it is applied for extraction of other sulphide minerals. In recent years, bioleaching has been widely accepted at industrial scale due to its advantages of low cost and environment friendly procedures, social benefits to the communities within which bioleaching companies operate, without strict requirement of raw material composition and is suitable for the treatment of complex and low grade ores [1-3].

Although a variety of iron and sulfur oxidizing microorganisms are likely to be involved in the oxidation of sulphide minerals, mesophilic bacteria viz. *A. ferrooxidans*, *Leptospirillum ferrooxidans* and *A. thiooxidans* are commonly used microorganisms for the bioleaching of sulfide minerals [4].

These microorganisms can only be effective if they are maintained at their optimal ambient conditions required for growth and activity. The bacterial efficiency of metal dissolution is growth dependent that is affected by the physico-chemical conditions within the bioleaching environment [5]. Metal bioleaching in acidic environment is influenced by a series of factors, like temperature, pH, redox potential, nutrient availability, iron (III) and (II) concentrations, carbon dioxide and oxygen contents. In addition, some of them are microbiological parameters such as microbial diversity, population density, microbial activities, metal tolerance etc. Among these factors, temperature, pH and Fe (II) concentration are the most important factors. Many strains of *A. ferrooxidans* are reported to be active in the pH rang of 1.5-5.

Therefore, it is clear that strict control of factors is necessary for the success of bioleaching process. In statistical-based approaches, response surface methodology (RSM) has been extensively used in fermentation media optimization [6-8]. RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions [9]. It is a statistically designed experimental protocol in which several factors are simultaneously varied [6]. In fact, the relationship between the response and the independent variables is usually unknown in a process; therefore the first step in RSM is to approximate the function (response) through analyzing factors (independent variables).

Efforts have been made to understand the mechanisms by which cells react when exposed to exogenous metals. Proteomics is an important tool for elucidating the role of various proteins in stress tolerance and adaptation. Proteomics provides direct information of the dynamic protein expression in tissues or whole cells, thus making global analysis possible. Owing to the significant accomplishments of genomics and bioinformatics,

systematic analysis of all expressed cellular components has become a reality in the post-genomic era, and attempts to grasp a comprehensive picture of biology have become possible [10]. Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) in combination with mass spectrometry is currently the most widely used technology for comparative bacterial proteomics analysis [11].

In this study RSM is used to determine the optimum pH, iron concentration, temperature, and nitrogen concentration, on the growth and activity of indigenous bacterium that has been isolated from Koshk Zn/Pb sulfide mine, central of Iran. In addition the ability of this bacterium for extracting zinc and lead has also been studied. We have also investigated the response of the strain to external exposure of the exogenous metals Pb^{2+} and Zn^{2+} and characterized the differential expression profile of protein expression by 2D PAGE and mass spectrometry.

Material and Methods:

Ore Samples

Ore samples were collected from Koshk zinc and lead sulfide mine located in Yazd province in central of Iran. This mine is a low-grade sulfide mine with more than 120 million tons of concentrate (one of the largest zinc sulfide mines in the world). The chemical and mineralogical analysis of the ore was carried out by XRD and XRF as shown in Table 1.

Isolation and Identification of Bacteria

A total of five acidophilic bacterial strains and one fungus were isolated from the samples collected from the mine by using three different media viz. TK medium [12], Leathen [13] and 9-K [14]. For isolating the microorganisms, 1 gram of mine sample was added to 100 ml of broth media and after 72 hour of incubation at 35°C, isolates were purified by transferring to Modified 9-K solid 2:2 medium. Modified 9-K solid 2:2 medium was prepared as described below, $Na_2S_2O_3.5H_2O$ (2g) was added to 10 ml of H_2O (Solution A); $FeSO_4.7H_2O$ (2g) was added to 10 ml of distilled water adjusted to pH 1.8 with 1 N H_2SO_4 (solution B) $(NH_4)_2SO_4$ (3g), KCl (0.1 g), $MgSO_4.7H_2O$ (0.5g), and K_2HPO_4 (0.5g) were dissolved in 500 ml of distilled water and pH was adjusted to 3.4 with 1 N H_2SO_4 (solution C) Agarose (Agarose for routine work from Sigma) (0.8 g) was added to 480 ml of distilled water (solution D). Solution A and B were both filter sterilized but solution C and D were autoclaved and then all solutions were mixed, the final pH was 3.

Table 1. Composition of the mine sample as analyzed by (A) XRD and (B) XRF

A

Component	%	Component	%
Pyrite (FeS_2)	35	Sphalerite (ZnS)	4
Calcite ($CaCO_3$)	24	Quarts (SiO_2)	2
Dolomite{$CaMg(CO_3)_2$}	21	Others	1
Gypsum ($CaSO_4.2H_2O$)	13	Total	100

B

Component	%	Component	%	Component	%
Fe	24.24	MgO	4.52	CuO	0.008
S	14.8	MnO	0.512	SiO_2	2.83
Zn	3.1	Na2O	0.53	TiO_2	0.169
Pb	1.27	BaO	0.065	Y_2O_2	0.003
Cl	0.11	K_2O	0.15	Al_2O_3	0.59
Co_3O_4	0.015	P_2O_6	0.12	Nb_2O_3	<0.001
CeO_2	<0.001	MoO_3	<0.001	V_2O_5	<0.001
Rb_2O	<0.001	NiO_3	<0.001	ThO_2	<0.001
ZnO_2	<0.001	WO_3	<0.001	U_3O_8	<0.001

DNA isolation for 16S rRNA analysis

The isolated bacterial strain was identified and characterized with conventional and molecular technique.

DNA of the isolate was extracted for 16S rRNA analysis. The late- exponential phase culture were harvested and washed with same basal media without iron, cells were suspended in 576µL of 10 mM Tris-EDTA- 150 mM NaCl (pH 8.0) and followed by lyses of cells by sodium dodecyl sulfate and deproteination by chloroform-isoamylalcohol (24:1 v/v) and phenol- chloroform- isoamylalcohol (25:24:1 v/v), followed by precipitation by ethanol [15].

16S rRNA sequencing

For identification of the bacterium, 16S ribotyping was carried out. The sequence analysis was done at Ribosomal Database Project (RDP) I & II and the Basic Local Alignment Search Tool (BLAST-n) analysis at National Center for Biotechnology Information (NCBI) server. The alignment of the sequence was done using CLUSTALW program at European Bioinformatics site. These sequence data have been submitted to the GenBank data bases under accession no. FJ485936.

Optimization of culture conditions by RSM

To achieve the best results for growth and activity of bacterium, the composition of the media and growth conditions were optimized by using four different culture conditions with the help of RSM. To develop the model, four input parameters were considered as temperature, pH, Fe^{2+} concentration, $(NH_4)_2SO_4$ concentration. The specified codes for each parameter and the range of the parameters used for modeling the iron oxidation are given in Table 2.As a result 21 experiments (Table 3) were performed. The culture samples were collected at time intervals until 96 hours of incubation. Ferric ion concentration was used as an indicator of bacterial activity that was measured spectrophotometrically using 5-sulfosalicylic acid as complexing agent for colormetric analysis [16]. The initial pH was maintained constant throughout the incubation by adding excess slurry of acid because as bioleaching progresses there are a gradual decline in pH. In addition, sterile controls were also run by adding 5 mL of 0.5 % (v/v) formalin in ethanol to the medium and replacing the bacterial inoculum by an equal volume of medium. This is

Table 2. Rage of the parameters used for modeling the iron oxidation and the specified codes for each parameter.

Variables	Coded values and the corresponding values of parameters				
	$-\alpha$	-1	0	+1	$+\alpha$
A:Temperature	21.00	26.00	31.00	36.00	41.00
B: pH	1.15	1.50	1.85	2.20	2.55
C: Fe^{2+}	1.00	4.00	7.00	10.00	13.00
D: $(NH4)_2SO_4$	1.50	3.00	4.50	6.00	7.5

followed by analyzing the experimental results using the analysis of variance (ANOVA) technique to find out which factors are having the most effective interactions and also which ones are most effectives for reduction of the surface tension.

Zinc and Lead extraction

To standardize the bioleaching process for zinc and lead, 140 mL of 9-k medium having different concentration of Fe^{2+} (3, 5, 7, and 9 g/L) was added in 500 ml Erlenmeyer flasks and pH was adjusted to 1.8. Each flask contained 10 g of ore sample and after autoclaving the contents, it was inoculated with 6% (v/v) of the bacterial culture.

The cultures were maintained on the orbital shaker at 35 ^{o}C. Each flask was sampled every 24 hours for analysis of total Zn and Pb by atomic absorption spectrometry (AAS). Sterile controls were also run by adding 5 mL of 0.5 % (v/v) formalin in ethanol to the medium and replacing the bacterial inoculum by an equal volume of medium.

Determination of minimum inhibitory concentration (MIC)

The MICs of 9 toxic metals, namely, Zn, Pb, Cu, Co, Ni, As, Hg, Mn, and Cr were determined by the micro-dilution method. The metal salts used for the determination of MIC were lead nitrate, cobalt sulphate, zinc sulphate, copper sulphate, sodium arsenite, nickel sulphate, manganese sulphate, mercury chloride, and chromium oxide. The MICs were determined in 50 ml conical flasks containing 10 ml of 9-k medium with 4 g/L Fe^{2+} at pH 1.5. The concentration range chosen for the various metals were as follow; Zn (mM): 200, 400, 600, 700 and 800, Pb (mM): 1, 2.5, 5, 7.5, 10, and 12, Cu (mM): 10, 25, 50, 75, and 100, Ni (mM): 50, 100, 150, and 200, Mn (mM): 200, 400, 600, 700 and 800, As (mM): 10, 20, 40, 60 and 80, Co (mM): 20, 40, 60, 80, 100, 120 and 140, Cr (mM): 1, 2.5, 5, 7.5, 10, and 12.5, Hg (mM): 0.001, 0.002, 0.003, 0.004, 0.005, and 0.006 mM. Ten microliters inoculum of the strain (5×10^6) was added to different concentrations of each metal and was incubated at 35 ^{0}C and 180 rpm. Iron oxidation was determined as an indicator for the growth and activity of the bacteria. An uninoculated control containing 9-K medium and sterile acidified water was used to measure the amount of auto-oxidation. Lead forms poorly soluble complexes with sulfate in the 9-K medium; hence, in order to obtain the actual bioavailable concentration of the metal in the medium, the amount of soluble lead was measured by atomic absorption spectrometry (AAS) and the insoluble part was removed by filtration.

Heavy metal tolerance studies

The growth profile of the organism was studied in the 9-K medium in the presence of 2 concentrations of each metal mentioned above. The following metal concentrations were used (mM): Zn, 400 and 650; Pb, 5 and 10; Cu, 25 and 50; Ni, 100 and 150; Mn, 500 and 700; as, 15 and 25; Co, 40 and 80; Cr, 5 and 10; and Hg, 0.005 mM. Iron oxidation was determined periodically as an indicator of bacterial growth.

Protein extraction

Bacterial cells were harvested at the late log phase by centrifugation at 10,000 rpm for 10 min. The pelleted cells were washed thrice with the same basic culture medium without ferrous ions. The cells were suspended in 200 µl lysis buffer and sonicated at 46 Hz for 2 min with an interval of 30 sec in ice. The cell lysate was centrifuged at 15,000 rpm for 30 minutes. The supernatant containing intracellular soluble proteins was collected and stored at –20^{o}C in sealed microcentrifuge tubes for further studies.

2D PAGE

2D PAGE was carried out to study the differential expression of proteins. Isoelectric focusing (IEF) was performed using a 7 cm tube gel of pH range 3–10. A total of 600 µg protein containing the control, zinc-treated, and lead-treated proteins of isolate and a standard pI marker (Sigma) was loaded on the first-dimensional gel and isoelectric focusing was conducted at 200 V for 30 min, 500 V for 30 min, and then 800 V for 7 h. After IEF, proteins were separated in the second dimension by using 12% SDS polyacrylamide gel and 80 V was maintained throughout while running the second dimension.Gels were stained using Coomassie Brilliant Blue (CBB R250). Images were acquired using Fluor-S-Multiimager (Bio-Rad) using a visible light source and the differentially expressed proteins were detected manually and treated as separate spots.

In-gel tryptic digestion

2D PAGE spots were excised and each spot was washed with 400 µl of 50% acetonitrile solution (thrice for 15 min) to remove the CBB stain. The spots were dehydrated by using 100% acetonitrile solution for 10–15 min until the spots turned opaque white and were then dried using speed vacuum. Spots were then reduced and alkylated by soakingin 100 µl of dithiothreitol (10 mM) solution and then in 100 µl of iodoacetamide (25 mM) solution.The spots were then washed with 25 mM ammonium bicarbonate solution and digested with sequencing grade bovine trypsin (Sigma) solution. Molar ratio of protein to trypsin was maintained approximately between 1:10 and 1:20. After overnight trypsin digestion, the peptides were extracted from the gel using 50% acetonitrile and 1% trifluoroacetic acid (TFA), dried, and then stored at –20^{o}C till further analysis. (r)

Mass spectrometry

Peptides were reconstituted in 5 µl of 50% CAN containing 0.1% TFA, and then spotted and air dried on 96 X 2 Teflon coated plate. CHCA (α-cyano hydroxyl cinnamic acid) was added as a matrix onto the pre-spotted peptide spots. The PMF was obtained using a Voyager-DE-STR MALDI-TOF mass spectrometer (Applied Biosystems). MALDI mass spectra were recorded in the mass range of 800–4000 Da in the reflector mode using delayed ion extraction and each measurement was performed using the following parameters: accelerating voltage, 20kV; grid voltage, 66.5%; delay time, 190 ns; and low mass ion gate, 500 Da. Peptide mass calibration was performed with external mass standard (Calmix 1 and 2; Applied Biosystems). All spectra were externally calibrated and processed using the Data Explorer software (Applied Biosystem). The peptide mass list was created and processed for advanced baseline correction, noise removal, and deisotoping.

Table 3. Experimental central composite design (CCD) runs in Design Expert 7.1 and corresponding results (the response). The results of iron oxidation are for 96 hours incubation; (F: Factore)

Run	F1: Temperature	F2: pH	F3: Fe^{2+} concentration	F4: $(NH_4)_2SO_4$	Response Iron Oxidation %
1	26	2.2	10	3	62
2	36	1.5	4	3	98.5
3	31	2.55	7	4.5	62
4	36	1.5	10	6	66
5	31	1.85	7	7.5	84
6	31	1.85	13	4.5	50
7	26	1.5	10	3	67
8	36	1.5	10	3	73
9	31	1.85	7	4.5	88
10	31	1.85	1	4.5	68
11	36	2.2	4	6	67
12	26	1.5	4	6	70
13	26	1.5	10	6	59
14	36	2.2	4	3	77
15	26	1.5	4	3	81
16	36	2.2	10	6	65
17	31	1.15	7	4.5	59
18	31	1.85	7	4.5	95
19	31	1.85	7	1.5	93
20	36	1.5	4	6	80
21	26	2.2	4	3	74
22	31	1.85	7	4.5	93
23	21	1.85	7	4.5	67
24	31	1.85	7	4.5	95
25	31	1.85	7	4.5	94
26	26	2.2	10	6	65
27	31	1.85	7	4.5	92
28	41	1.85	7	4.5	72
29	36	2.2	10	3	67
30	26	2.2	4	6	66

Protein identification

Two search engines, MASCOT and ProFound, were used for database interrogation. A protein was considered as identified when the first ID was found as the first hit in both ProFound and MASCOT searches.

The probability-based score of either 50 in MASCOT or 1.5 in ProFound was considered acceptable. Protein ID's were also examined with the following search parameters: mass error less than 100 ppm, with a maximum of 2 missed cleavages, "carbamidomethyl" and "methionine oxidation" as the chemical modifications, and "Proteobacteria" as the taxonomy.

Results and Discussions

Isolation and Identification of bacteria

In the present study we have isolated six obligate chemolithotrophic bacteria and fungi that have shown the bioleaching properties. One of the isolates, initially named MY_1can oxidize ferrous to ferric (65-70%), which finally was selected for further studies. The 16S ribotyping and BLAST-n analysis of the 762 base pairs was done at the National Center for Biotechnology Information (NCBI) server which confirmed the identity of organism i.e. it was a strain of *A. ferrooxidans* and we have named it as *A. ferrooxidans* D.F.1. *A. ferrooxidans* belongs to the group of chemolithotrophic organisms, which are rod-shaped, non-spore forming, gram-negative, motile, and single pole flagellated bacterium.

Optimization of iron oxidation

Medium composition such as carbon sources, nitrogen sources, salinity and other growth factors are strongly influenced cell growth and the accumulation of metabolic products, thus the optimization of these parameters can enhance the bacterial efficiency. RSM is a combination of mathematical and statistical techniques that is useful for analyzing the effects of several independent variables on the system response without the need of a predetermined relationship between the objective function and the variables [17, 18].With the help of the RSM, we can perform the statistical models and to evaluate the effect of parameters of a particular process as well as to optimize the conditions for desirable responses.

The factors affecting the bioleaching process have been extensively studied in recent years, but few of these studies used proper statistical tools for experimental design (r). The classical method of medium optimization consists in changing one variable at time and keeping the others at fixed level. However in this study we have optimized the growth conditions of the D.F.1 strain by the help of RSM for designing the experiments with aim to achieve highest rate of iron oxidation. Due to the complex nature of biological processes, it is very difficult to predict distinctively the effects of all parameters, which may have multiple interactions. Therefore, RSM was applied to build up an empirical model for modeling iron oxidation in

terms of the operational parameters of medium temperature, pH, initial iron concentration and ammonia concentration. Design-Expert 7.1 suggested a quadratic equation for enhance in iron oxidation, IO as

$$IO = 92.83 + 2.48(A) - 1.90(B) - 5.23(C) - 3.31(D) - 1.97(A)(B) - 0.84(A)(C) - 0.84(A)(D) + 2.47(B)(C) + 1.72(B)(D) + 2.09(C)(D) - 5.55(A)^2 - 7.80(B)^2 - 8.17(C)^2 - 0.80(D)^2$$

Where IO is iron oxidation (%) (Ferric iron production), A, B, C and D are coded values pertaining to the temperature, pH, initial iron concentration and ammonia concentration, respectively. The predicted versus experimental plot for IO showed that actual values were distributed near to the straight line, which indicated that actual values were very close to the predicted ones (r^2 = 0.96). Thus, it was a suitable model to predict the iron oxidation efficiency using aforementioned experimental conditions. ANOVA results of the quadratic model in Table 4revealed that the model equation derived by RSM by Design- Expert 7.1 could adequately be used to describe the iron oxidation under a wide range of operating conditions. For the model, the quadratic R^2 was 0.9613.

Acidity of the environment controls the bacterial activity within a system. The H^+ ion is in fact vital for acidophilic microorganisms since bacteria utilize it as a proton source for the reduction of O_2 [19]. Also optimum activity of each type of bacteria takes place in a relatively well-defined range of temperature at which these microorganisms grow with maximum efficiency. This indicates the temperature dependence of bioleaching processes [5]. Increasing temperature results not only in the usual augmentation in the chemical reaction rate but also, within limits, in faster microbial metabolism.

Table 4. The analysis of variance (ANOVA) for the response surface quadratic model.

Source	SS	DF	MS	F-value	Prob>F
Model	4942.14	14	353.37	26.62	<0.0001
B: pH	147.51	1	147.51	11.11	0.0045
C: Fe^{2+}	86.26	1	86.26	6.50	0.0222
D: $(NH4)_2SO_4$	656.26	1	656.26	49.44	<0.0001
A:Temperature	263.34	1	263.34	19.84	0.0005

SS: Sum of squares; DF: Degree of freedom; MS: Mean Square

Figure 1(A) presents temperature and pH effects on iron oxidation at fixed iron and ammonia concentration of 7 g/Land 4.5 g/L, respectively. As can be seen from Figure 1(A) the pH had a definite influence on the oxidation of iron by the strain D.F.1.Increase in the acidity beyond pH 1.8 has an adverse effect on the growth and efficiency of the bacterium, because extreme acidity is toxic to these organisms [20]. It is also observed that an increase in the pH from 1.2 to 2.1 decreases the efficiency of bacteria for ferrous to ferric conversion which may be due to precipitation induced by ferric complex on bacterial surface, which hinders the diffusion of protons [21]. Therefore this indicates that the upper and lower pH limit in bioleaching environment should be controlled and maintained to preclude the formation of ferric precipitation and prevent the decline in the conversion efficiency. The optimum pH determined in our experiments (1.5-1.8) are in comparable range with previous studies [22-24] those have reported the optimum pH between 2 – 2.5 for the growth of *A. ferrooxidans*. This is one of the advantages of this isolate that grows in extremely low pH which ultimately increase the bioleaching efficiency by decreasing the chance of jarosite precipitation.

The iron oxidation efficiency and growth of the bacterium tended to increase with increasing the temperature. Optimal temperature for growth and activity of the bacterium was between 30 to 35^{o}C and further experiments were performed at 31^{o}C. These results are comparable with the previous reports [22, 24, 25] those have found the optimum temperature of 30-35^{o}C for the oxidation of iron by *A. ferrooxidans.*Howeverat lower and higher temperatures than 31 °C almost there were equal declines in iron oxidation. Therefore, we can conclude that for a particular bacterial strain there is a specific optimum growth temperature and another specific temperature at which the oxidation efficiency is maximum. Low pH values (1.6 – 1.8) at high temperatures (32 - 37 °C) may have a positive effect on iron oxidation whereas at high pH values and low temperatures iron oxidation was significantly decreased. A culture medium is mixtures of chemical compounds, which provide all the elements required for cell growth and biosynthesis. In the culture medium of *Acidithiobacillus* (9-K) after iron, $(NH_4)_2SO_4$ is the main component of medium and is essential for growth. So we have studied the effect of different $(NH_4)_2SO_4$ concentrations on growth and activity of the bacterium.

Figure 1(B) shows the effects of ammonium sulphat concentrations and temperature on iron oxidation at fixed iron concentration of7 g/Land pH 1.85. Increasing the ammonium concentration had no significant effect on iron oxidation. The strain showed maximum iron oxidation with 4 g/L ammonium sulphat supplementation.

These results are as expected since Gomez et al. (1999) [23] have investigated the influence of five different media with varying the concentration of salts and they have observed significant increase in bacterial growth and activity with medium containing higher concentration of nitrogen source. The possible reason for lower oxidation activity of bacterium at higher concentration of $(NH_4)_2SO_4$ compare to 4 g/L, could be due to the possible precipitation of phosphate, potassium and ammonium as jarosite complex due to excess concentration of this salt in the medium. This problem is prominently encountered while working with 9-k liquid medium [5]. Lower ammonium concentration at high temperatures (32 – 37^{o}C) can enhance the efficiency of the iron oxidation whereas high ammonium at very low or very high temperatures may have negative effect on iron oxidation.

The joint effects of ammonium sulphat concentration and pH are shown in Figure 1(C) with iron concentration fixed at 7 g/L and temperature 31 °C. Increment in iron oxidation gradually increased with decreasing pH and ammonium concentration reaching a final plateau value near 1.7 for pH and 3 g/L for ammonium concentration. However at high ammonium concentrations and pH range 2-2.3 iron oxidation was lowest.

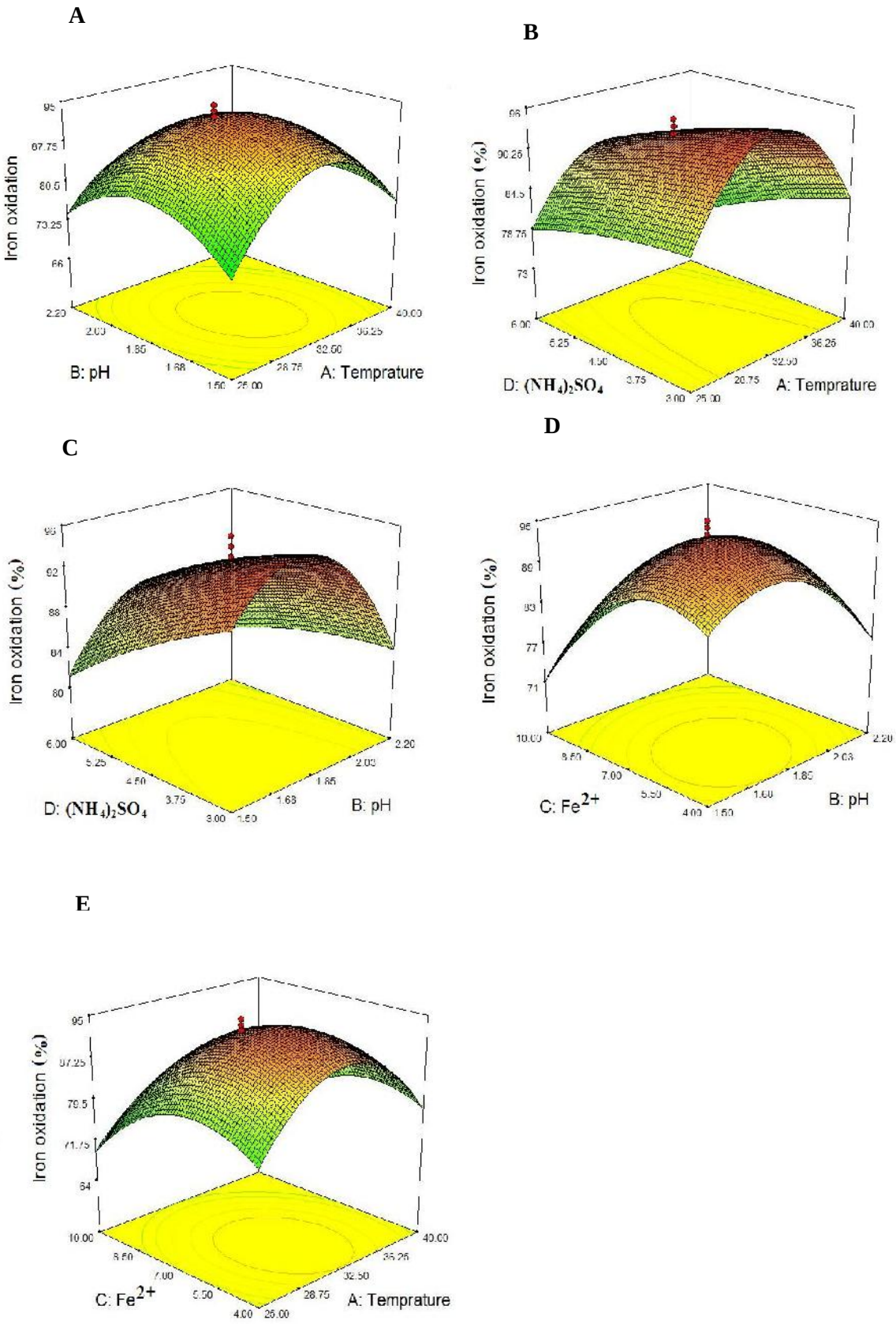

Figure 1. Three dimensional plots for maximum iron oxidation. RSM plots were generated using the data presented in Table 3. Inputs were the 30 experimental runs carried out under the conditions established by the CCD design. (A) Increment in iron oxidation as a function of pH and temperature (B) Increment in iron oxidation as a function of iron oxidation and ammonium sulphat concentration (C) Increment in iron oxidation as a function of pH and ammonium sulphat concentration (D) Increment in iron oxidation as a function of pH and iron concentration (E) Increment in iron oxidation as a function of temperature and iron concentration.

Figure 1(D) shows iron concentration and medium pH effects on iron oxidation at fixed temperature of 31 °C and ammonium concentration of 4.5 g/L. The iron concentration was found to be one of the critical parameter in the efficiency of bacterial strain on iron oxidation, in the absence or low concentration of the iron activity and growth was very low. The iron oxidation by D.F.1 strain was consistently increased by increasing the iron concentration, 7 g/L initial iron shown to be the best concentration for the maximum oxidation activity. The production was declined at higher concentrations of iron. The response surface of increase in iron oxidation gradually increased with increasing iron concentration from 2 g/L up to 7 g/L at fix pH value of 1.8. However at higher pH and high iron concentration activity of bacteria and iron oxidation was lowest.

It has been observed that at higher concentration of Fe^{2+}, there is a prolong lag phase (24-28 h) but at a lower concentration of Fe^{2+} the lag phase was reduced to 10-15 hours which shows the duration of the lag phase is dependent on the initial concentration of ferrous iron. Our results are in agreement with the studies reported by Mousavi *et al*. (2006) [22]. Unlike the lag phase, the specific growth rate of the bacterium was significantly high at higher concentrations of iron, compared with the lower concentrations. Bacterial efficiency was assessed by measuring the concentration of Fe^{3+} in solution after every 2 hours. Initial Fe^{2+} concentration of 7 g/L led to the maximum bio-oxidation rate (0.265 g/L) with shorter lag phase and better specific growth rate in comparison with 6.0256 g/L. It may be due to the precipitation of jarosite, which in turn may be due to the increases in the concentration of iron (Mousavi et al. 2006) [22]. The bio-oxidation rate of 0.265 g/L at initial Fe^{2+} concentration of 6 g/L is comparable with previous reports of Mousavi et al. (2006) [22]; Nemati & Harrison (2000) [27], which have reported 0.2 g/L and 0.09 g/L respectively at similar concentration of initial ferrous iron.

Figure 1(E) shows iron concentration and temperature effects on iron oxidation at fixed pH of 1.85 and ammonium concentration of 4.5 g/L. In the figure, increase in iron oxidation increases almost linearly with temperature in the range of 30 to 34°C, while increasing the iron concentration. Like joint effect of iron and pH here also at high concentration of iron and high pH lowest activity of bacterial strain took place, but at temperature range of 32–36°C and iron concentration of 8 g/L maximum iron oxidation was recorded.

Extraction of zinc and lead

Since this bacterium was isolated from zinc and lead mines, we have studied the ability of isolates for recovery of zinc and lead at optimum ambient conditions of bacterial activity which have been found initially with varying the initial ferrous iron concentration. Figure 2 illustrates the efficiency of extraction of zinc from the ore sample by this bacterium at different concentrations of iron viz. 3, 5, 7 and 9 g/L of Fe^{2+} concentrations. Results indicate that maximum zinc extraction takes place at 7.0 g/Lof Fe^{2+} (over 98%). These results are comparable with the results obtained by Shi et al. (2005) and Deveci *et al*. (2004) [28, 29]. They have reported over 90% extraction of zinc by *A. ferrooxidans* under similar conditions. We also tried other concentrations of iron like 3, 5 and 9 g/L of Fe^{2+}, but the extraction of zinc was less.

Similar results were also observed for recovery of lead but there is a same rate of lead extraction at all concentrations of iron except at 9 g/L of Fe^{2+}. However, for lead, the extraction efficiency is 20 – 25 % (Fig. 2). Less extraction of lead as compare with zinc could be related to the toxicity effect of lead on bacterial growth while leaching process progresses and its concentration exceeds more than the bacterial tolerance limit. In our experiments we did not observe selective extraction of metals which has been reported by Deveci *et al*. (2004) [28] where, *A. ferrooxidans* strain showed the selective recovery of zinc without the extraction of lead.

Determination of MIC

The effects of 9 different heavy metals that are mostly available in mine environments were studied on the growth of this isolate; the results are given in table 5. The organisms exhibited remarkable resistance to zinc, manganese, nickel, and cobalt. In the case of metals like Zn and Mn, our isolate was highly resistant, the concentrations of 700 mM of Zn and 750 mM of Mn were among the highest concentrations to which the bacterial resistance has been reported earlier [30,31].

Growth profile in response to metals

The growth profile of the organism was studied in 9-K medium in present of 2 different concentrations of 9 toxic metals. The organisms' growth responses were different in

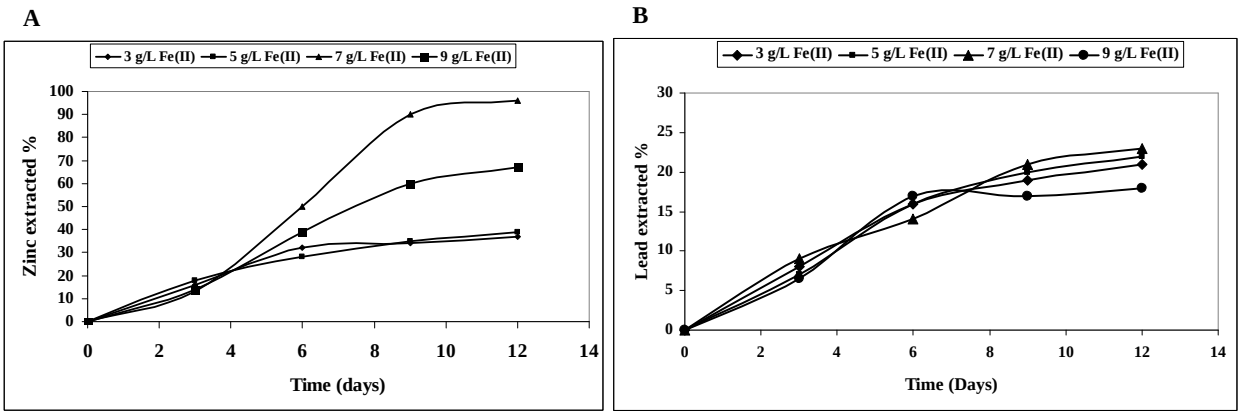

Figure 2. The effect of Fe^{2+} concentrations on the efficiency of zinc extraction (A) and lead extraction (B)

Table 5. The MIC of nine different toxic metals during the growth of bacterial strain.

Metals	Zn	Mn	Ni	Co	Cu	As	Cr	Pb	Hg
MIC (mM)	700	750	200	100	75	40	12	12	0.006

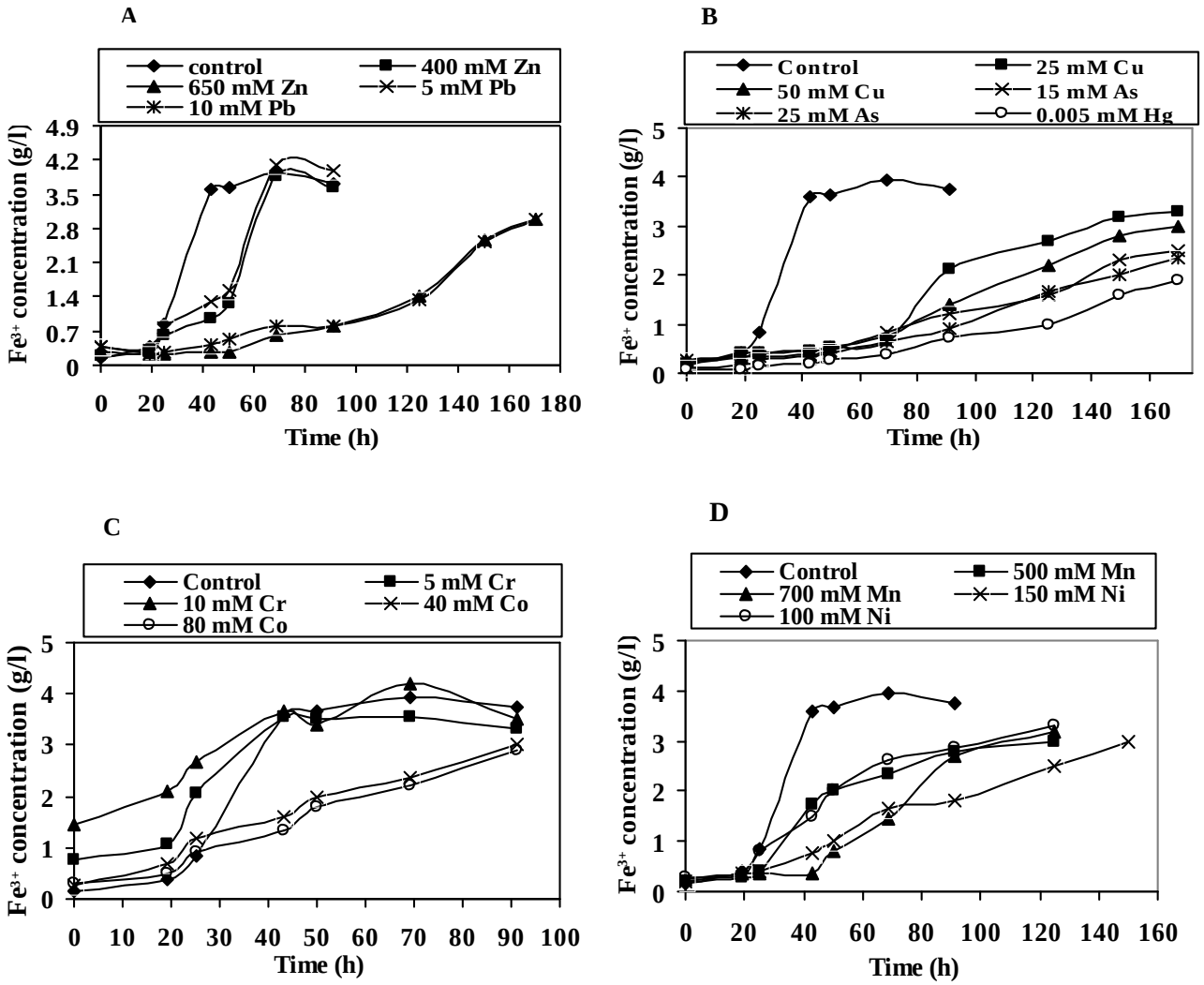

Figure3. Growth profile of an isolated strain in presence of different concentrations of 9 toxic metals. (A) Zinc and lead, (B) Arsenate, Mercury, and Copper (C) Chromium and Cobalt, (D); Manganese and Nickel

all metal salts when compared with control as shown in Figure 3. The lag phase of bacterial growth increased significantly in the presence of metals. Under the control condition, it took 45 h for the complete oxidation of ferrous iron whereas in presence of 650 mM Zn and 10 MmPb oxidation of ferrous iron was completed in 160 h. Similar results were also obtained for manganese, nickel, and copper where more than 140 h were required for the complete oxidation of ferrous iron. These results were expectable [31, 32].

Characterization of proteins using Peptide Mass Fingerprinting

2D PAGE and peptide mass fingerprinting techniques were previously used for the proteomic analysis of *A. ferrooxidans.* Proteins induced under heat shock [33], pH stress [34], phosphate limitation [35], or the presences of heavy metals such as copper have been previously reported [30].

The differential expression of protein of a strain in the presence of Zn^{2+} and Pb^{2+} were as shown in Figure 4. The proteins in spot nos. 1, 2, 3 and 4 are over-expressed in lead- and zinc-

Treated cells when compared with control, while those in spot nos. 7, 9, and 12 are over-expressed only in zinc-treated cells. The proteins in spot nos. 5, 10, 11, and 13 are down-regulated in metal-treated cells when compared with the control, while the protein in spot no. 8 is down-regulated only in lead-treated cells.

All the differentially expressed proteins were subjected to Peptide Mass Fingerprinting analysis; spot nos. 1, 2, and 11 gave a significant ProFound score (Table 6). These proteins were identified as the major outer membrane

protein of *A. ferrooxidans* (OMP40), the large subunit of ribulose bisphosphate carboxylase (RuBisCo) of *A. ferrooxidans*, and Holo-(acyl carrier protein) synthase (AcpS).

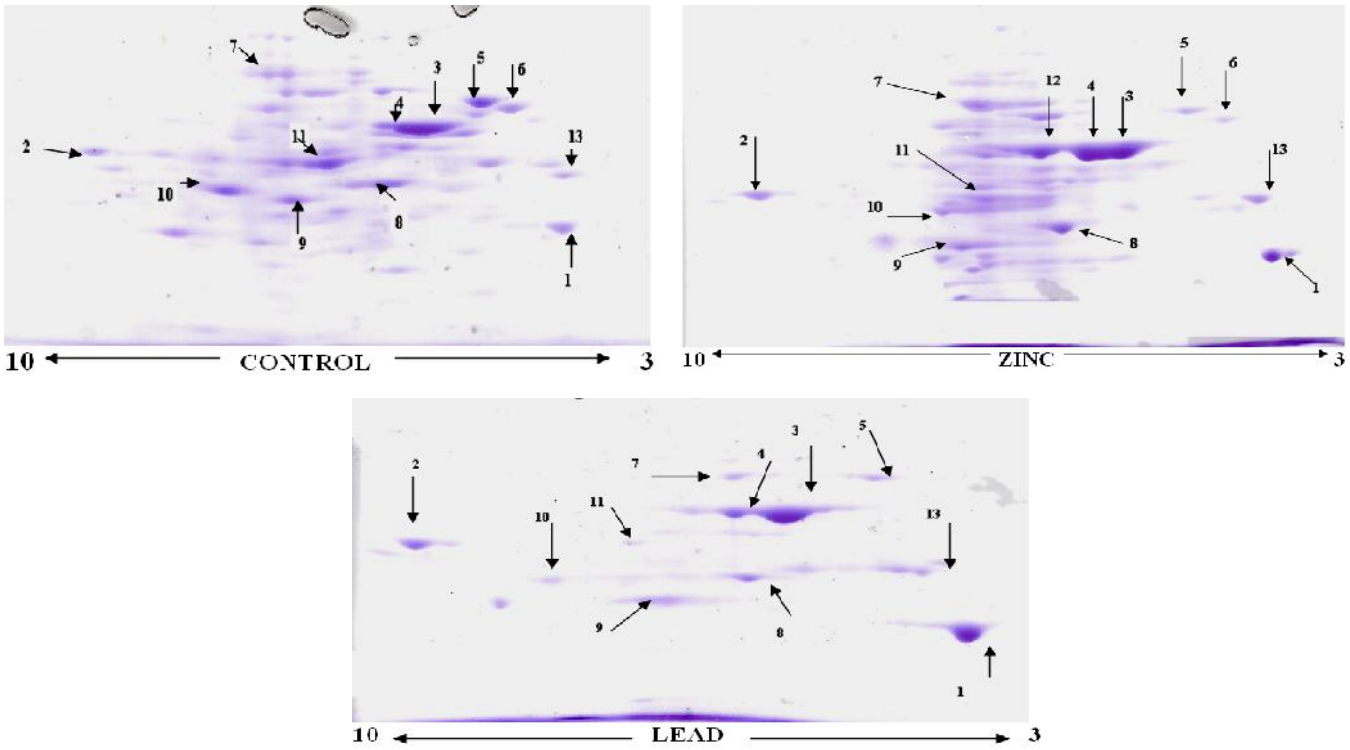

Figure 4. 2D PAGE diagram showing the differential expression of intracellular proteins in bacteria grown in the presence of metals. Proteins in spot nos. 1, 2, 3, and 4 are over-expressed in lead- and zinc-treated samples. Proteins in spot nos. 7, 9, and 12 are over-expressed only in zinc-treated cells. Proteins in spot nos. 5, 6, 10, 11, and 13 are down-regulated in metal-treated cells when compared with control. The protein in spot no. 8 is only down regulated in lead-treated cells.

Table 6. MALDI-TOF analysis of 2D spots using ProFound and MASCOT search engines

Spot No	**1**	**11**	**2**
Protein	Major Outer Membrane Protein of *A. ferrooxidans*	Riboluse bisphosphate carboxylase	Holo-synthase (*Syntrophicus aciditrophicus*)
NCBI Accession No	Gi|4138616|emb|CAA10107.1|	gi|4836660|gb|AAD30508.1|	Q2LYJ7_SYNAS
MW (kDa) Exp/pred	35 / 42.23	60 / 53	55 / 20
PI exp/pred	4.5 / 4.9	6 / 5.8	9.5 / 9.7
No. of peaks matched	10 / 28	18 / 69	4 / 15
ProFound / MASCOT score	2.25/ -	2.25/ 115	1.64/ -
Level of expression	Over expressed in lead and zinc	Down regulated in lead and zinc	Over expressed in lead and zinc
Function	Porin	Carbon fixation	transferring non-standard substituted phosphate groups

The molecular characterization of OMP40 protein suggests that OMP40 is a porin [36]. This protein has been reported to respond to external pH, phosphate starvation [37], and heavy metals [30]. Since an increased concentration of metal ions (Zn and Pb) has an inhibitory effect on the growth of bacterial cells, the cell must develop a mechanism that allows it to control the free passage of metal ions from the outside. The results obtained in this study implied that OMP40 is involved in the adaptation of cell to the increased concentration of toxic metals.

RuBisCo is an enzyme that is used in the Calvin cycle to catalyze the first major step of carbon fixation. RuBisCo is very important in terms of the biological impact and it is very vital and important in chemolitotrophic bacteria for carbon fixation. The reason of down regulation of this enzyme in metal-treated cells is the decrease in the growth and activity of the bacteria due to the toxicity effect of heavy metals and ultimately the activity and level of this enzyme are also down-regulated in metal-exposed cells.

The Holo-(acyl carrier protein) synthase (AcpS) belongs to the transferase family, specifically those transferring non-standard substituted phosphate groups. It is the central coenzyme of fatty acid biosynthesis and also has previously been identified and shown to be essential for *E. coli* growth. Over-expression of this protein in the presence of metals suggests that the organism tried to

adapt to the high concentration of metals by over-expressing this protein, which can helps in enhancing the biosynthesis of fatty acids for adaptation to the toxic metals. The later three proteins which are differentially expressed have not been reported earlier to be expressed under heavy metal stress.

Conclusions

It is important to know that the ubiquitous bioleaching bacterial population since such bacterial flora can be isolated with little difficulty where oxidizable ore bodies are exposed to the surface. However, newly isolated bacterial populations cannot be expected to oxidize ores at maximum rates until determined under laboratory conditions. Hence it would be beneficial to isolate the bacterial strain with the ability of rapid growth and oxidation as well as tolerance to the adverse conditions of mine habitat viz. low pH and high concentration of heavy metals and then making efforts to improve its abilities to enhance the efficiency of the oxidation process and metals extraction. In the present investigation, we have successfully isolated the new strain of *A. ferrooxidans* which itself had a good ability for bioleaching applications. By optimizing the growth conditions we could increase its efficiency more than 35%. One of the advantages of this bacterium is the ability to grow at very low pH with the optimum of 1.5-1.8, which in turn increases the bioleaching efficiency by decreasing the accumulation of jarosite precipitates. Another advantage of this bacterium is its bioleaching capability that could extract more than 98% of zinc from the mine sample. To the best of our knowledge, this is the first study involving the understanding of the mechanism of resistance to lead in *A. ferrooxidans* at the protein level by the proteomics approach. Thus, the functional analysis of the identified proteins, though very few, explained the tolerance of this bacterium to heavy metals at a certain concentration.

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