Indian Journal of Experimental Biology
Vol. 58, March 2020, pp. 219-227

Gene expression response of mutagenic breeding of *Acidithiobacillus* sp. FJ2 to different concentrations of uranium low grade ore

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Received 11 June 2018; *revised 03 February 2019*

Acidithiobacillus ferrooxidans (*At. f*) is a bacterium involved in the bioleaching process. In the present study, we investigated the effects of *Acidithiobacillus* sp. FJ2 induced with diethyl sulfate (DES) as mutagen on the bioleaching of low grade uranium ore. The comparison was carried out within 5, 10, 15, 25 and 50% uranium ore pulp densities in the bioleaching system. The impact of the induction of *Acidithiobacillus* sp. FJ2 was determined by Eh and pH values, concentrations of Fe^{2+} and uranium extraction rates at 24 h intervals. The results showed that DES with 0.8% may lead to an obvious change on bacteria leading to improvement of bioleaching capability in 5, 10, 15% pulp densities. However, the bioleaching activity of the original bacteria was more efficient than DES-induced bacteria at 25 and 50% pulp densities. The gene expression results in 0.8% DES treated bacteria indicated that the bacteria attempt to adjust in the bioleaching systems (with different pulp densities) through decreased *cyc2* and increased *rus*, *cyc1* and *coxB* levels. These results suggest that uranium may induce oxidative stress in the wild and treated strains in the high pulp density, while the bacteria tried to survive and gain more energy from the iron oxidation. However, when the amount of uranium increased, the mutants couldn't cope up woth the enhanced stress in 25 and 50% pulp densities. It may be due to inhibitory effect of uranium toxicity on adaptive processes which may change the trends.

Keywords: Bioleaching, Diethyl sulfate (DES), Metal sulphide

Microbial leaching technology is considered as more effective method to treat low grade tailings compared to the conventional methods, as it involves simple equipment, low investment, fast processing, low cost, easy management, wide range of applications and environment friendly. Bioleaching method has been widely used in the processing of low grade tailings[1,2].

Acidithiobacillus ferrooxidans (*At. ferrooxidans* or *At.f*) is a Gram-negative, acidophilic, diazotrophic bacterium that obtains its energy from the oxidation of ferrous iron and sulphur[3,4]. The oxidation mechanism of Fe(II) has been well studied, and the components involved with this system have been identified[5,6]. The *A. ferrooxidans* respiratory chain follows the pathway:

Fe(II) → Cyc2 → rusticyanin → cytochrome → c_4 cytochrome oxidase aa_3 → O_2, encoded by the *rus* operon genes[7,8]. The *cyc2* gene encodes an outer membrane *c*-type cytochrome[7] and the *cyc1* gene encodes cytochrome c_4 (or cytochrome c_{552}) localized in the periplasm[9]. The *coxBACD* genes encode the subunits of a cytochrome oxidase (aa_3-type) localized in the inner membrane and the *rus* gene encodes the rusticyanin, a periplasmatic blue copper protein[8].

At present, one of the most severe limitations in increasing the efficiency of bioleaching is metal toxicity. Since, high efficiency strains are extremely scarce and the oxidation ability of bacteria or the ability to resist harsh environments is inadequate, mutation breeding has become the most popular method for enhancing bioleaching activity of strains[10]. Although some researches on breeding of leaching bacteria have been reported[11-14], there are still several challenges for producing optimal strains for industrial application[10,15].

The main bacteria breeding methods include domestication, mutagenesis and genetic engineering[16]. Mutation breeding has become the most effective method for enhancing bioleaching activity of strains. In this study, diethyl sulfate (DES) as a chemical factor for *At. F* mutation was used due to the lower cost and its stability[17]. There have been few reports on uranium bioleaching by DES-induced *At. f*. Hence,

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the concentration of DES mutation was optimized and the bioleaching characteristics by DES-induced *At. F* was investigated in this work for the first time, expecting to improve the uranium bioleaching process. To gather more information on relation between *rus* operon genes expression, mutation and also pulp density, we compared their expression profiles using real-time polymerase chain reaction method, in wild type *Acidithiobacillus* sp. FJ2 and its mutant grown in the presence of different uranium pulp densities.

Materials and Methods

Materials

Culture medium of Leathern 9K comprised $FeSO_4.7H_2O$ (20 g L^{-1}), K_2HPO_4 (0.5 g L^{-1}), $(NH_4)_2SO_4$ (3 g L^{-1}), $MgSO_4.7H_2O$ (0.5 g L^{-1}), KCl (0.1 g L^{-1}) and Ca $(NO_3)_2.4H_2O$ (0.01 g L^{-1}). Our previous results indicated that optimum cell growth and iron oxidation by *Acidithiobacillus* sp. FJ2 was achieved at 20 $gL^{-1}FeSO_4.7H_2O$ concentration[18]. The pH of culture medium was adjusted to 2.0 by 10N H_2SO_4 solution. Uranium ore with particle size 106 μm, which contained 465 ppm U, 49.67% Fe_2O_3, 24.83% SiO_2,19.03% MgO and 0.75% S, was from Saghand uranium deposit in Yazd, Iran[19].

Bacteria and Growth conditions

In this study, the isolated bacteria (*Acidithiobacillus* sp. FJ2) were from sulfur springs in Ramsar province, Iran[20]. To enrich *At. f*, 10% of inoculums were added to 250 mL of 9K medium and then cultured in a shaker with a speed of 180 r/min at 30°C. Lastly, the *Acidithiobacillus* sp. FJ2 was prepared for experiments after centrifuging at 4500 rpm for 20 min, while the concentration of the bacteria was adjusted to 1×10^8 L^{-1} at logarithmic phase.

DES mutation

In order to induce mutation by DES, 10 mL of pre-cultured suspension of *Acidithiobacillus* sp. FJ2 was transferred to the plates. Then, the suspended cells were treated by 0.8, 1 and 1.2% of DES concentrations. The plates were shaken for 15 min and the reaction was terminated with 25% sodium thiosulfate. After DES mutation, the aliquots were kept away from the light in fridge at 4°C for 12 h to enhance the effect of induction. The bacterial mutants selected based on Fe^{2+} oxidation in uranium ore bioleaching experiments[21,22]. To determine the genetic stability of the induced ones, the obtained bacteria was serially passaged in Leathern 9K medium and the oxidation activity was measured in each generation (data not shown). Based on these screening, the Fe^{2+} oxidation activity of the induced and wild type strain was different in Leathern 9K in every generation that confirms the stability of mutation[17].

Uranium bioleaching

The wild and induced *Acidithiobacillus* sp. FJ2 were inoculated (10%) in 1000 mL 9K culture medium which was supplemented with 0.5 gL^{-1} peptone, 1.0 gL^{-1}tryptic soy broth (TSB) and 0.01% yeast extract[23,24]. The pulp densities of uranium ore were 5, 10, 15, 25, 50% (w/v), and the initial pH of the culture was adjusted to 2 with H_2SO_4 (10N). Flasks were incubated at 30°Cand 150 rpm in a rotary shaker. During bioleaching process, the pH was monitored at 8 h intervals and the Eh (redox potential) of the supernatant also was measured with an Eh meter (Metrohm, model 827). Samples were taken at regular intervals (8 h) to determine uranium extraction rates and concentrations of ferrous iron (data not shown). The uranium extraction was analyzed by ICP method (Perkin Elmer). In addition, concentrations of ferrous were analyzed by Golmohammadi's technique as colorimetric measurement[25]. Also, negative controls for bioleaching experiment (abiotic control) were carried out by addition of 100 mL methanol-formaldehyde solution (10:1) to the medium instead of inoculums.

Extraction of total RNA& RT-PCR

To determine the effect of uranium concentrations on the expression of the selected genes, the cells were adapted to grow in the presence of the different concentrations of uranium ore. When, uranium extraction reached the highest amount, total RNA was extracted from each culture condition. In addition, the control culture was also designed in order to understand the effect of mutation (under the same conditions of growth).

To minimize RNA degradation and cell damage during storage, the bacteria harvested by centrifugation were immediately processed for RNA extraction and cDNA synthesis following real-time PCR transcriptional expression determinations. Total RNA was prepared from wild and induced *At. F* cultures with the Gene JET RNA purification kit (Thermo Scientific). The quality of total RNA was measured at OD_{260} and OD_{280} with Nano Drop 2000 spectrophotometer (Thermo Scientific). To ensure that the sample of extracted RNA was not

contaminated with DNA, DNase treatment was carried out using DNase I (Thermo Scientific, RNase-free). Further, cDNA was synthesized using random hexamer primers and the RevertAid first strand cDNA synthesis kit (Thermo Scientific). It is noticeable that, total RNA was quantified by NanoDrop 2000 spectrophotometer (Thermo Scientific). The same amount of RNA (ng) was used in each reaction for cDNA synthesis.

Design and specificity of PCR primers

Primers for real-time PCR were designed with the Gene Runner software Version 3.05. Blast N searches were used to check primer specificity. Primers used in this study are listed in Table 1. The specific fragments were amplified and then checked by 1% agarose gel electrophoresis to ensure the specificity of primers and the size of PCR products.

Real-time PCR

The expression of selected genes was carried out with step one Real-Time PCR System (Applied Bio systems).The reaction mixture contained 5 µL of SYBR® Green Real-time PCR Master Mix (Takara Premix Ex Taq kit) which contains Taq DNA polymerase, dNTP, $MgCl_2$, and SYBR Green I dye, 0.2 µLof a 10 mM solution of sense/anti-sense primer, 0.5 µL of template cDNA, 0.2 µL of ROX and H_2O added to a total of 10 µL. The negative controls (without cDNA template) were also designed. Thermal cycling conditions were carried out by an initial denaturation stage at 95°C for 30 s, followed by 40 cycles at 95°C for 15 s, 60°C for 20 s, and 72°C for 20 s. Fluorescence measurements were recorded at 95, 60, 95 °C for 15, 60, 15 s, respectively.

To determine the specificity of the PCR amplification, the melting curve for T_m, its symmetry, and the lack of non-specific peaks were checked. All tests were conducted in triplicate. The expression ratio was recorded as the fold difference in quantity of real-time PCR product from samples with mutation *vs*. control sample (without mutation). Each mRNA expression value was normalized against a housekeeping gene expression (16S rRNA).

Statistical analysis

The results were subjected to one-way ANOVA (SPSS software) followed by Tukey's HSD using version 22 of software and presented as means ± Standard Error of Mean (SEM) of three samples (triplicate). Significant levels were defined as P <0.05.

Results

Bioleaching experiments by original and DES-induced *Acidithiobacillus* sp. FJ2

In this study on the effect of different concentrations of DES (0.8, 1 and 1.2%) on *Acidithiobacillus* sp. FJ2, the results of wild and induced bacterial activity in bioleaching systems with 5, 10, 15,25 and 50% uranium pulp densities are shown in Fig. 1. The Eh values increased in all of the bacteria (P <0.05), triggered by the oxidation of ferrous iron and sulfur. As culture time progressed, Fe^{2+} was oxidized to Fe^{3+} and decreasing the concentration of Fe^{2+} in the medium reflecting the change of Eh in the medium. When Fe^{2+} was oxidized completely, the Eh reached its highest value (P <0.05). In addition, the results showed that during the incubation periods of the bacteria, increase in uranium extraction was observed in mediums. The pH trend in the bioleaching systems was characterized by an increase up to first days (1-3 days) and then by a decrease in following days (P <0.05).

The bioleaching results from 5, 10, 15% pulp densities (Fig. 1 A-C) indicate that the optimum concentrations of DES for mutation of *Acidithiobacillus* sp. FJ2 was 0.8% (P <0.05). After two days of incubation, the highest value Eh and uranium extraction of induced *Acidithiobacillus* sp. FJ2 was 585.33 mV and 96.3% when the concentration of DES was 0.8% in 5% pulp density (Fig. 1A).These results also indicate that after 1 day incubation, the ferrous iron concentration in bioleaching system with 0.8% DES induced bacteria reached the lower amount, which is 4 times lesser than the wild strain (Fig. 1 A). In addition, the pH value in Fig. 1 for abiotic conditions showed increase while, it had a reverse trend in inoculated systems.

Table 1 — Primers sequences used in the study

Gene	Sequence		Amplicon length
	Forward primer (5'- 3')	Reverse primer (5'-3')	(bp)
cyc2	CCGCCAGAGTAGGTCAAATGC	AACTCTAATGCGGGTGCTTCTC	122
cyc1	TTCTGGGCGTTGAAGTAATCCG	TGAAAGCGTATAAGGACCACTCC	121
rus	GGCATAACCGCATAAGGAGGT	GAACCCGACCTTGGAGATTCC	120
coxB	GCTCCCTATCTGGTCAAACAACT	CGTGATCCCAAATGAAGTGCG	114
16S	CCTACGGGAGGCAGCAG	CGGTGCTTCTTCTTGGATTCACG	165

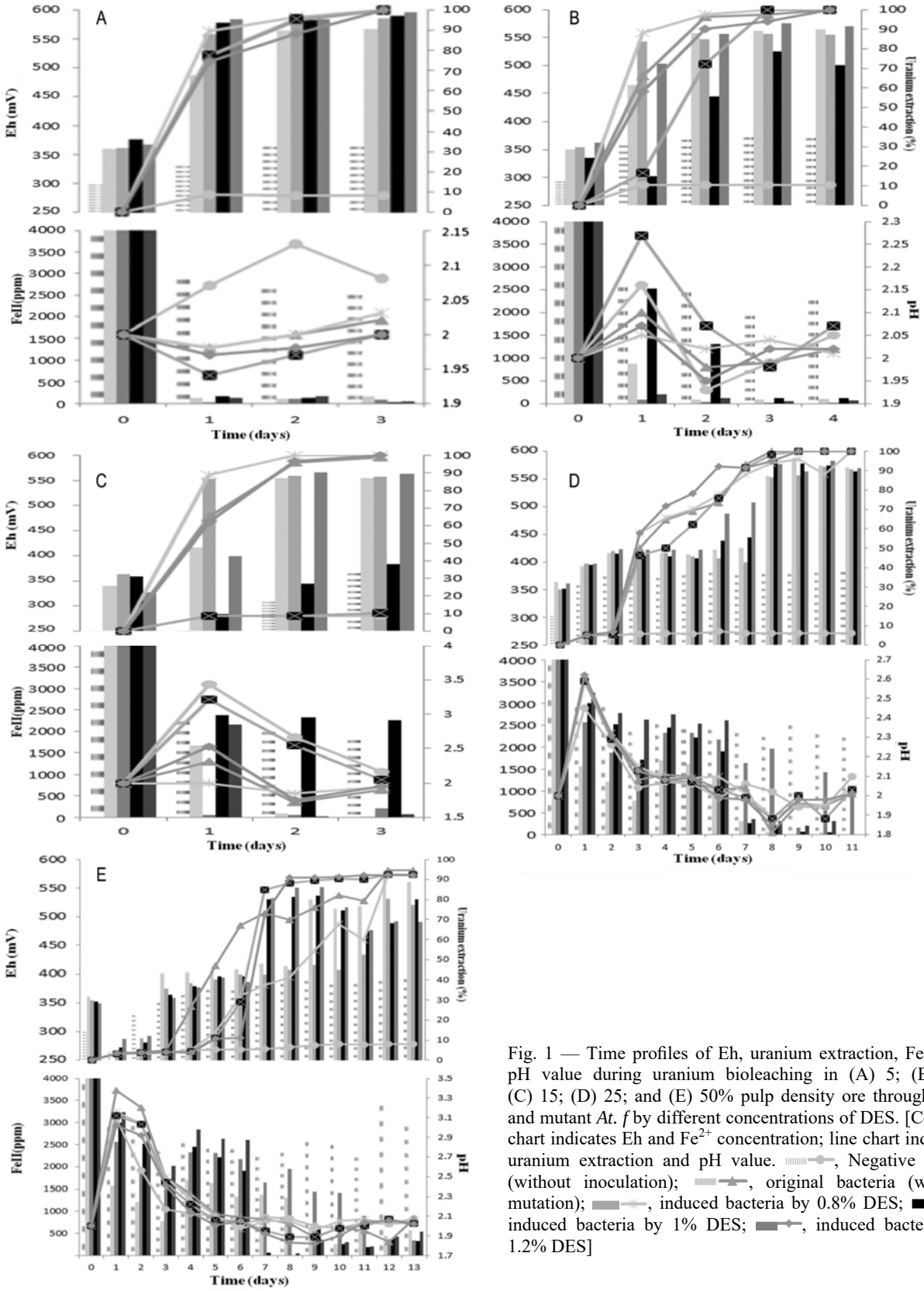

Fig. 1 — Time profiles of Eh, uranium extraction, Fe^{2+} and pH value during uranium bioleaching in (A) 5; (B) 10; (C) 15; (D) 25; and (E) 50% pulp density ore through wild and mutant *At. f* by different concentrations of DES. [Column chart indicates Eh and Fe^{2+} concentration; line chart indicates uranium extraction and pH value. ——●——, Negative contol (without inoculation); ——▲——, original bacteria (without mutation); ——✳——, induced bacteria by 0.8% DES; ——■——, induced bacteria by 1% DES; ——◆——, induced bacteria by 1.2% DES]

Effects of 10% ore pulp density on the bioleaching system with wild and DES-induced *Acidithiobacillus* sp. FJ2 are shown in Fig. 1B. The yield of uranium extraction showed that the induced bacteria with 0.8% DES has better activity in 10% pulp density. From Fig. 1B, it can be seen that uranium extraction reached 88.04% after 1 day in 0.8% DES, while in wild, 1 and 1.2% DES induced bacteria, uranium extraction reached 65.86, 16.84 and 58.04%, respectively at this time. The Eh and ferrous concentration also proved that 0.8% DES has better effect on bacteria in bioleaching system (P <0.05). In every experiment, a continuous pH increase and decrease was also observed.

The results of oxidative activity by wild and induced *Acidithiobacillus* sp. FJ2 in 15% uranium ore pulp density at 3 days is shown in Fig. 1C. It is shown that induced bacteria by 0.8% DES extracted all the uranium ions within 2 days, while the wild, 1 and 1.2% DES induced bacteria needed 4, 8 and 3 days, respectively (Fig. 1C). After 1 day incubation, Eh value of 0.8% DES was the highest and also nearly all the ferrous iron was oxidized in 15% pulp density (P <0.05).As it is clear from Fig. 1C, the amount of pH in original and induced bacteria, increased during the first 24 h of incubation. Afterward, the amount of pH decreased within 3 days. It is noticeable that induced bacteria with 1% DES lags behind the other bacteria (P <0.05) (data not shown) which had no significant variation in bioleaching results compared to the abiotic test (P <0.05).

The variations of the pH, Eh, uranium and Fe^{2+} concentrations with reaction time in the leaching slurry of 25% ore pulp density were determined (Fig. 1D). As depicted in Fig. 1D, uranium extraction reached 100% after 8 days in wild bacteria, while in induced bacteria with 0.8, 1 and 1.2% DES, uranium dissolution reached 93.96, 98.27 and 94.82%, respectively at this time. Besides that, uranium extraction of induced bacteria with 0.8, 1and 1.2% DES, reached to the highest amount after 11, 9 and 9 days, respectively (Fig. 1D). It shows that in this pulp density, induced bacteria with 0.8% DES lags behind the other bacteria, while this bacteria had better activity in lower pulp densities (5, 10 and 15%).

From Fig.1D, it can be seen that the introduction wild and DES-induced *Acidithiobacillus* sp. FJ2 into the reaction mixture caused the Eh variation. Also, Eh value of wild bacteria was the highest (587 mV) when the uranium extraction reached to the highest amount after 8 days (P <0.05).

Fig. 1D additionally shows that, when original and DES-induced *Acidithiobacillus* sp. FJ2 were inoculated, most of the ferrous ions were oxidized to the ferric state within 8 days, whereas oxidation of ferrous ions lags behind in the present of the induced bacteria with 0.8% DES (P <0.05). In addition, little of ferrous ions oxidized in the absence of the biological agent in negative control experiment. In every experiment, a continuous pH increase and decrease was observed, which was triggered by the oxidation of iron ions by microbiologically mediated mechanism (P <0.05).

The efficiency of uranium bioleaching enhanced with the increased pulp density up to 15%. Further increase in pulp density shows reveres trend, as the bacteria require more time to extract all uranium ions. The pH values, uranium extraction, Fe^{2+} concentrations and the Eh of the culture solution with 50% pulp density ore were shown in Fig. 1E. The results showed that uranium extraction in 50% ore pulp density did not reach 100% even after 13 days of incubation. In addition, uranium dissolution reached 94.62% after 13 days in original bacteria, while in induced bacteria with 0.8, 1 and 1.2% DES induced bacteria, uranium extraction reached 92.47, 92 and 92.06%, respectively at this time. It is noticeable that the uranium extraction is close to a stable state by at least 24 h, between 94 and 94.64% within 12 to 13 days in the culture system of original bacteria. The results demonstrate that the oxidation activity of wild bacteria is better than those the induced bacteria at end day of bioleaching.

The results in Fig. 1E showed that the pH increased at first and subsequently decreased. Also, as culture time progressed, Fe^{2+} was oxidized to Fe^{3+} and the concentration of Fe^{2+} in the medium decreased which reflects the change of Eh (P <0.05).

Relative genes expression results of *Acidithiobacillus* sp. FJ2 and its induced one in contact with different pulp densities of ore

The real-time PCR assay was used to determine the relation between gene expression and oxidation activity of *Acidithiobacillus* sp. FJ2 among its induced one in the bioleaching environments with different uranium pulp densities. As reported in Table 2, wild and induced cells responded with large changes in the expression level of selected genes (≥2-fold) during bioleaching process. According the bioleaching results, induced bacteria with 0.8% DES

Table 2 — List of Fold change values of *Acidithiobacillus* sp. FJ2 and its mutants (P <0.05) in different uranium pulp densities

Gene symbol	Bacteria sample	Fold change in different pulp densities (%)				
		5	10	15	20	25
cyc2	Control	1±0.234	1±0.041	1±0.051	1±0.263	1±0.347
	DES 0.8%	0.947±0.513	0.437±0.20	0.318±0.138	13367.5±0.069	2.93±0.07
	DES 1%	0.315±0.25	0.765±0.136	6.291±0.013	438.92±0.254	0±0.293
	DES 1.2%	5.566±0.206	0.32±0.045	3.499±0.311	5362.04±0.032	0.011±0.169
cyc1	Control	1±0.93	1±0.188	1±0.043	1±0.001	1±0.254
	DES 0.8%	43.65±1.09	3.735±0.271	1.078±0.082	40434.68±0.762	0.005±0.154
	DES 1%	11.232±0.114	1.983±0.096	0.005±0.017	5349.662±0.841	0±0.243
	DES 1.2%	67.07±0.58	2.409±0.31	0.035±0.046	236504.81±0.246	0.001±0.18
rus	Control	1±0.069	1±0.048	1±0.007	1±0.234	1±0.176
	DES 0.8%	0.768±0.049	0.71±0.055	0.332±0.008	90133. 922±0.238	9.424±0.04
	DES 1%	0.016±0.086	1.341±0.021	0.034±0.287	6166.866±0.285	0.727±0.131
	DES 1.2%	0.309±0.198	0.715±0.076	0.119±0.301	33766.242±0.136	0.007±0.066
coxB	Control	1±0.047	1±0.103	1±0.023	1±0.465	1±0.009
	DES 0.8%	4.635±0.093	0.226±0.017	0.76±0.163	11.127±0.560	62.998±0.108
	DES 1%	1.275±0.76	0.7±0.072	0.191±0.057	0.524±0.062	1.487±0.313
	DES 1.2%	3.601±0.243	0.104±0.076	0.36±0.014	2.03±0.311	0.041±0.84

has better oxidation activity (P <0.05) rather than other bacteria in 5, 10 and 15% pulp densities (Fig. 1 A-C), while, the original bacteria has more efficient uranium extraction in 25, 50% pulp densities (Fig. 1 D & E). The results of *cyc2* gene expression in induced bacteria with 0.8% DES showed a small decrease in the presence of 5, 10 and 15% uranium pulp densities (Table 2), as the expression of this gene shows increases in high pulp densities (25,50%). The expression of *cyc1* gene (0.8% DES) enhanced up to 25% pulp density compare with the original bacteria (control test) but it has significant decreases in 50% pulp density (Table 2). Furthermore, the results showed that the expression of *rus* gene in induced bacteria with 0.8% DES decreases with increasing pulp density up to 15% along with further increase in pulp density which shows different trend (P <0.05). Also, the gene expression profile of induced bacteria with 0.8% DES showed that the expression of *coxB* gene at 10 and 15% pulp densities was lower than the other pulp densities(P <0.05).

Discussion

Little is known about the expression and regulation of the *rus* operon genes when *At.f*is kept in contact with metal sulfides. Also, no study reported the effects of DES induced *At.f*in the case of uranium extraction. So, in this study, the bioleaching experiments were carried out following consideration the expression of selected genes in original and induced *At.f*in different uranium ore pulp densities.

The variations of the Eh, pH, Fe^{2+} and uranium concentrations with reaction time in the leaching slurry were shown in bioleaching results (Fig. 1). The results demonstrated that as culture time progressed, Eh value increased and the concentration of Fe^{2+} in the medium decreased. Also, the results showed that the pH increased slightly at first day and subsequently decreased. The pH value is an important factor in the culture of bacteria and also, a change of pH will affect bacteria growth and oxidation of Fe^{2+}[26]. In the first cultivation stage, the main reaction was oxidation of Fe^{2+} in the medium; this reaction can be accelerated by the involvement of bacteria, which leads to the pH increasing rapidly[26]. As culture time progressed, Fe^{2+} was oxidized to Fe^{3+} and the concentration of Fe^{3+} in the medium increased. Fe^{3+} was involved in the hydrolysis reaction and led to the formation of H^{+}. Hence, the pH decreased slightly in the late cultivation stage[10]. Besides that, the changes in the concentration of Fe^{2+} and Fe^{3+} in the medium can reflect the change of Eh. Oxidation of Fe^{2+} provides the required energy for growth of bacteria and increases the concentration of Fe^{3+} and Eh in solution gradually during the bioleaching process[10]. In uranium leaching, the bacteria do not directly attack the uranium mineral. However, the bacteria generate Fe(III) from pyrite and soluble Fe(II) in the medium. Fe (III) (as a oxidant) readily attacks minerals incorporating U(IV), thus converting it to U(VI) which is soluble in dilute sulfuric acid[27-29].

Mutations are heritable changes in genotype that can occur spontaneously or be induced by chemical or physical treatments. It was documented that DES acting primarily as base-pair substitution mutagens which it is known as strong specificity for

G → C to A → T transitions. In addition, Low levels of frame shift mutations of the deletion type are also likely[30]. In this study, induced bacteria were selected based on the Fe^{2+} oxidation activity compared to reference strains are called wild type. Results in our tests (data not shown) showed that Fe^{2+} oxidation activity of induced ones are different in culture medium of Leathern 9K as selective media. This screening was done every generation before inoculation of bacteria into uranium bioleaching and the results confirmed that the characteristics of the induced bacteria were heritable and stable in generations. Besides that *Ru-an et al.*[17] demonstrated that the enhancement of oxidative activity of bacterium by DES mutation is beneficial to the leaching of soluble phosphorus (P) from rock phosphate (RP). The results of this study also showed that treated cultures with 0.8% DES, achieve higher levels of uranium leaching yields in low pulp densities (5, 10 and 15%) than cultures without mutation and other induced ones (Fig. 1 A-C). However, the bacteria without mutation showed more better activity in high pulp densities (25,50%) than induced bacteria (Fig. 1 D & E). The negative effect of metal toxicity may cause this completely opposite results in 15 and 25% pulp density. These results suggest that uranium induces oxidative stress in wild and induced strain in high pulp density (25,50%), and they tried to survive in the stress and gain more energy from the iron oxidation but with increasing amount of uranium, the induced one cannot combat the enhanced stress of uranium in 25 and 50% pulp density. Also, the trend of gene expression changes also showed that induced strains tried to survive in 25% pulp density with significant increasing in the expression levels of *cyc2*, *cyc1*, *rus* but negative effect of uranium decreases the gene expression in 50% pulp density.

The gene expression results showed that the decreased expression of *cyc2* gene in induced bacteria with 0.8% DES at low pulp densities (Table 2), could be a mechanism enabling *Acidithiobacillus* sp. FJ2 to change in the permeability of outer membrane resulted into the decreasing the entrance of toxic ions to the cell. Also, some studies showed that the expression of outer membrane proteins such as Omp40 in the presence of uranium and copper were downregulated[31,32]. Since *Cyc2* is an outer membrane protein, it can be inferred that the expression of *Cyc2* is decreased in order to adjust to the toxic medium. The decreased expression of *rus* gene in 0.8% DES induced bacteria with the increase pulp density up to 15% (Table 2), may be the results of metal toxicity. Results of another study clearly proved that the expression of proteins, which are important to the *A. ferrooxidans* electron pathway from ferrous iron to oxygen, is repressed when the organism is grown in sewage sludge[33]. In addition, the results in different pulp densities indicated that the expression of *cyc1* gene in induced bacteria with 0.8% DES have increases up to 25% pulp density compare with the original bacteria while, it showed a significant decrease in 50% pulp density (Table 2). A research indicated that the transcript levels of the genes which encoded by the *rus* operon quickly increased during the active phase of ferrous iron oxidation[34]. Besides, cytochrome c_{552} proteins up regulated on ferrous iron during growth on metal sulfides containing iron, such as pyrite and chalcopyrite[35]. Thus, the increase in the expression of this gene might be related to the amount of Fe(II) available in the medium containing Saghand ore (the ore has iron in its composition).

Operons are often controlled by more than one mechanism. Physiological adaptations are often associated with changes in metabolic activities. The flow of metabolites through particular biochemical pathways can be controlled both by regulating the synthesis of specific enzymes and by altering the activities of existing enzymes[36]. Carlos *et al.*[16] explained the expression difference of the gene *rus* and the gene *cyc1* by information content presented at the Translation Initiation Site (TIS) of both genes.

On the other hand, transcription of the *rus* operon genes remains obscure and complex, and it is suggested that this operon has at least three promoters, two before *cyc2* and one (P_{rus}) between *coxD* and *rus*[37]. Since, there are two promoters (PI, PII) upstream from cyc2, cyc1, coxB regulated at the transcriptional level, depending on the energy source[37]. Yarzábal *et al.*[37] showed that PII and P_{rus} are regulated mainly by the presence of Fe(II), and PI is regulated mainly by S^0. As Saghand uranium ore contains 49.67 Wt% Fe_2O_3 and 0.75 Wt% S in its composition, these transient expressions may be related to the presence of both iron and sulfur in the bioleaching system, the reverse trend of the *rus* gene expression in different pulp density is in agreement with the previous study. Martínez *et al.*[38] reported that Rus in addition to its energetic role, has important role in copper resistance because excess copper in the periplasm can bind and also overexpressed sulfur in

the presence of copper. Our results also showed the unusually high transcription levels of selected genes in at 25% pulp density.

The results in 10 and 15% pulp densities showed decrease in the expression of *coxB* gene in induced bacteria with 0.8% DES and increases in other pulp densities (Table 2). These gene expression profiles suggest that *Acidithiobacillus* sp. FJ2 quickly read justs its transcript levels to a new steady state at different pulp densities, thereby allowing the bacterium to survive in the stress. Also, the effect of metal toxicity for each gene is different in bioleaching system with different pulp densities. Our previous gene expression analysis indicated that *Acidithiobacillus* sp. FJ2 try to survive in the stress with increasing the expression levels of *cyc2*, *cyc1*, *rus* and *coxB*, while the metal toxicity has a negative effect on the gene expression in different pulp densities[39].

Conclusion

Bioleaching results showed that the induced bacteria with 0.8% DES could increase the extraction of uranium and could shorten the leaching time at 5, 10 and 15% pulp densities. However, this improvement does still not meet the demands of industry, because the wild bacteria has better oxidation activity in high pulp density (25, 50%). Also, the results showed that metal pulp density has inhibitory effect on the oxidation activity of the bacteria which can affect Eh, pH, Fe oxidation and uranium extraction yields. According to the gene expression results in 0.8% DES induced bacteria, the bacteria try to adjust to the mediums with different pulp density with decreases in the *cyc2* and increases in *rus*, *cyc1* and *coxB* levels. However, the metal toxicity has inhibitory effect on adaptive processes and can change the trends.

Conflict of interest

The authors declare no conflict of interests.

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