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Heavy Metal Transport Genes and Bioinformatics Analysis of At. Ferrooxidans

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This paper analyzes the resistance mechanism of At. Ferrooxidans to heavy metal ions, and compares the tolerance of different strains to Cu2+ and the expression of At. Ferrooxidans strains under different concentrations of Mn2+, Zn2+ and Cd2+. The results show that: the three selected At. Ferrooxidans strains, DY1, DY2 and DY3, differ in terms of Cu2+ toleration and S and Fe2+ oxidizing abilities. The maximum tolerable Cu2+ concentrations of DY1, DY2 and DY3 are 0.41mol/L, 0.20mol/L and 0.04mol/L, respectively. DY1 has the strongest oxidizing ability to S element, but the weakest oxidizing ability to Fe2+ ions among the three strains; DY3 has the strongest oxidizing ability to Fe2+ ions. There are differences in the adaptability of different strains to the leaching system. The copper resistance gene of the strains are stimulated to a certain degree by the Cu2+ in the culture solutions. the Cu2+ resistance of the strains is proportional to the resistance gene of the strain itself. The stronger the resistance of the strain to Cu2+, the more unlikely for the relevant resistance gene to be expressed in large quantities at low Cu2+ concentration in the culture solution. The maximum tolerated concentrations of Mn2+, Zn2+ and Cd2+ are 0.38 mol/L, 0.18 mol/L and 0.08 mol/L for the selected At. Ferrooxidans strains. The three heavy metal elements on the strains are ranked as Mn2+ <Zn2+ <Cd2+ by toxic effect in descending order. The relative expression of the four genes of the strains is proportional to the concentration of heavy metal elements. The proteins encoded in the four genes are involved in the transport of heavy metals.

1. Introduction

Acidithiobacillus Ferrooxidans (At. Ferrooxidans) is a leaching bacterium proven to be an efficient reducing agent for metals like gold, copper, manganese and zinc (Sasaki et al., 2009). It can also oxidize Fe2+, S2+ and other cations and absorbs energy (Paulino et al., 2002; Luo et al., 2008). Living in high concentrations of heavy metals, At. Ferrooxidans boasts a strong tolerance to heavy metals. Recent years has seen more and more attention been paid to the strong heavy metal tolerance of At. Ferrooxidans, and the gradual application of the bacterium in bio-metallurgy and heavy metal pollution control (Rawlings, 2005; Dopson et al., 2014). The DNA sequence of the standard strain was detected in 2007, but the principle of heavy metal transport genes in the strain has not been scientifically explained. Therefore, it is of great theoretical and practical significance to study the relative expression of metal transport genes in At. Ferrooxidans strains, and to analyze the mechanism of At. Ferrooxidans to withstand high concentrations of heavy metal ions (Qin et al., 2011; Pittman, 2005; Silver and Phung, 2005).
This paper analyzes the resistance mechanism of At. Ferrooxidans to heavy metal ions, and compares the tolerance of different strains to Cu2+ and the expression of At. Ferrooxidans strains under different concentrations of Mn2+, Zn2+ and Cd2+, laying the theoretical basis for relevant research.

2. Test materials and methods

2.1 Test materials

The At. Ferrooxidans strains were taken from the acidic abandoned pits in several large mines. The strains were cultured in the following conditions: temperature 26°C; shaking speed 165r/min; concentration 0.48×107cell/ml. The 9k medium was divided into 4 groups. The solutions in Group 1 was added 0.1-0.4mol/L

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Mn2+; the solutions in Group 2 was supplemented with 0.04—0.12 mol/L Zn2+; the solutions in Group 3 was treated with 0.04—0.06 mol/L Cd2+; the solutions in Group 4 was provided with 0.02—0.1 mol/L Cu2+. The original strains were put into the medium for short culture, and centrifuged to extract ENA. The test adopted Tiangen DNA extraction kit, Tiangen DNA recovery kit, TRIzol RNA extraction reagent and Qiagen RNA purification reagent. Fe2+ was extracted from the strains by potassium dichromate.

2.2 Measuring the tolerance of at. Ferrooxidans to metal elements

Different concentrations of Mn2+, Zn2+, Cd2+ and Cu2+ were used to create stress environments. At. Ferrooxidans strains were inoculated into the culture solutions. In the control group, the culture solution is replaced with clean water. The pH value of the culture medium was tested every 12 hours. The extracted RNA was purified by Qiagen, some of the RNA was subjected to reverse transcription, and the cDNA obtained by transcription was measured by NanoDrop. After measurement, the solutions were diluted to 180 mg/L and stored.

2.3 RT-PCR and bioinformatics analysis

The general PCR was amplified and purified based on cDNA, and the purified PCR was diluted in 12 folds. The general PCR was produced in the following conditions: pre-denaturation (4min) and denaturation (30s) at 95°C; extension (40s) at 70°C (40 cycles); annealing at 50°C-90°C (the temperature increased by 1°C in each cycle). Five parallel tests were performed for each heavy metal test, and 16SrRNA was selected as the reference gene.

The bioinformatics method was employed for target gene analysis. Specifically, the search for target similarity was based on BLAST; the ORF Finder was used to search the related gene reading frame; the protein isoelectric points, relative molecular weight and protein cell structure were calculated and evaluated by ExPASy and PSORTb; Tmhmm and Ncbi were the transmembrane structure and conserved region of protein.

3. Test results and analysis

3.1 Leaching of copper pyrites using At. Ferrooxidans

Three types of At. Ferrooxidans strains were selected from different sources and denoted as DY1, DY2 and DY3, respectively. The strains were separately placed in Cu2+ solutions for Cu2+ resistance test. It was found that the highest Cu2+ resistances of the three strains were 0.41mol/L, 0.20mol/L and 0.04mol/L, respectively, signifying significant difference in Cu2+ resistance of the three At. Ferrooxidans strains. In descending order, the ranking of Cu2+ resistance is DY1> DY2> DY3.

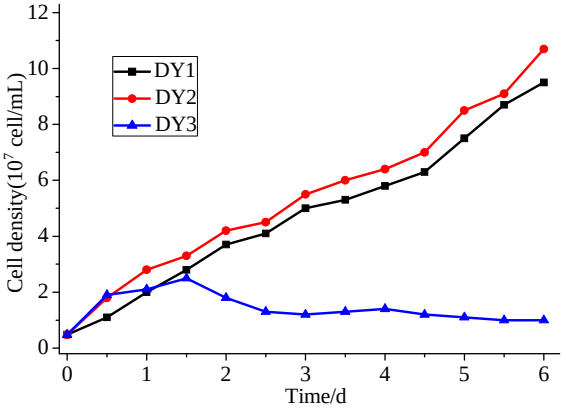


Figure 1: Growth curves of 3 types of strains

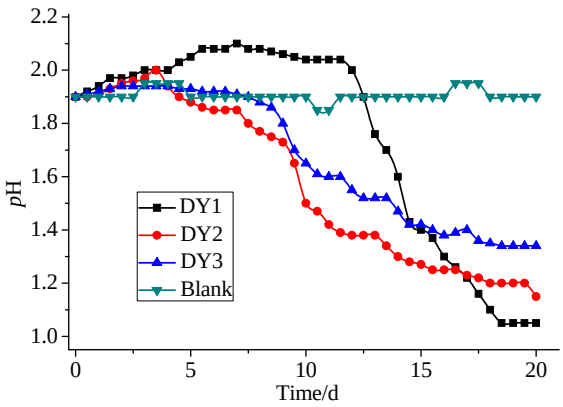


Figure 2: pH variation curves of 3 types of strains

Figure 1 illustrates the growth curves of the three strains when the culture mediums were added with the S element. Figure 2 shows the pH variation curves of the culture solutions of the three strains, where “Blank” stands for the control group that replaced the culture solution with clean water. It can be seen from Figure 1 that DY1 and DY2 increased linearly, and the cell densities of the culture solutions after 6 days were 1.08×108cell/ml and 9.7×108cell/ml, respectively. DY3, however, increased before the ultimate decrease; After 6 days, the cell density was merely 0.9×107cell/ml, up by 0.4×107cell/ml. This means the growth of DY3 strain was inhibited. As shown in Figure 2, DY1 suffered the most significant pH variation as its pH increased before eventually decreasing to 1.07; the pH value plunged deeply on the 12th day. In contrast, the pH values

of DY2 and DY3 were in a gradual decline. The final pH values of DY2 and DY3 were 1.18 and 1.39, respectively. Therefore, the three strains should be ranked as DY1> DY2 >DY3 by the sulfur oxidizing ability.

When the substance of energy source of the culture solutions was changed to Fe2+, the cell density growth curves and Fe2+ oxidizing abilities of the three strains were as shown in Figure 3. According to the illustration, the results were different from those in the S element culture solutions. After 42h, the three strains were ranked as DY2> DY3 >DY1 by the cell density in Fe2+ culture solution. The cell density in DY3 grew by a wider margin than in DY1 and DY2. In the latter two strains, the growth of cell density was slower than that of S element culture solutions. After 40h, the Fe2+ oxidation rates of DY2 and DY3 both reached 100%, but that of DY1 was only 81%. Comparing Figures 1-3, it is clear that DY1 has the strongest oxidizing ability to S element, but the weakest oxidizing ability to Fe2+ ions among the three strains; on the contrary, DY3 has the strongest oxidizing ability to Fe2+ ions. From the above analysis, it is concluded that the appropriate At. Ferrooxidans strains should be selected for different heavy metal pollution sources to maximize the survival rate of the strains and the leaching effect of metal minerals.

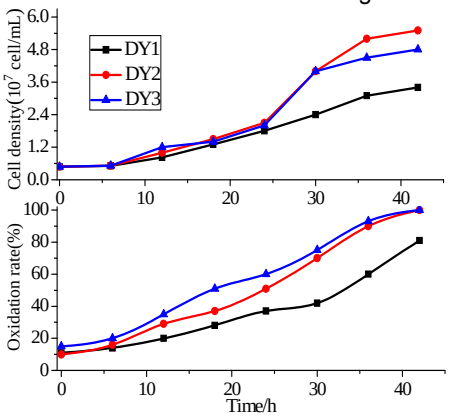


Figure 3: Cell densities and oxidation rates with the resource of Fe^{2+}

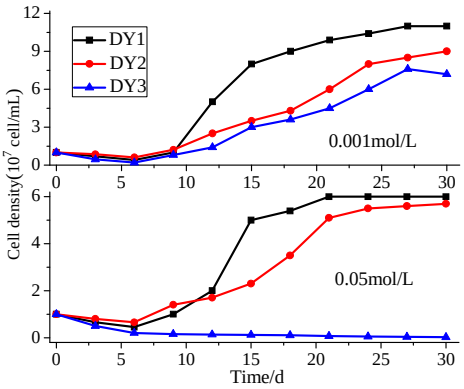


Figure 4: Cell density with different initial Cu^{2+} energy concentrations

Figure 4 shows the strain growth when the solutions were treated with different concentrations of Cu^{2+} ions. The concentrations of Cu^{2+} ions in the solutions were set to 0.001mol/L and 0.05mol/L, respectively. The initial cell density was set as 1.0×10^7cell/mL. After 30 days of soaking, the results show the decline of cell density across the three strains in the first 6 days. In particular, the cell density decreased by 60% at the solution concentration of 60%. The phenomenon reflects that the three strains died in large numbers as they were not adapted to the new culture environment. Since the 6^{th}day, the three strains have gradually adapted to the Cu^{2+} environment, resulting in a gradual increase in the cell density. However, the growth differs from strain to strain, which again reveals the different adaptability of different strains to the leaching system.

It can also be inferred that the strains of DY1, DY2 and DY3 maintained different growth rates when the Cu2+concentration was 0.001mol/L; nevertheless, when the Cu2+concentration was 0.05mol/L, the growth of the three strains was significantly inhibited, leading to continuous decline in cell density. The cell density of DY3 even suffered negative growth. After 30 days, the cell densities of DY1 and DY2 were 6.1×107cell/ml and 5.8×107cell/ml, respectively. This also demonstrates the advantage of DY1 in actual leaching, that is, the DY1 strain can extract Cu2+ at high concentration of the ion by extending the reaction time, thus saving the cost of frequent replacement of extracted ores and improving the economic efficiency.

Figure 5 displays the leaching of copper ore concentrates after the three types of At. Ferrooxidans strains had been immersed for 30 days in culture solutions of different Cu2+ concentrations (0.001mol/L and 0.05mol/L). According to the figure, the leaching amount of copper was very limited in the first 6 days and the cell densities were very low because none of the three strains had adapted to the heavy metal environment; After 6 days, the strains started to grow rapidly, accompanied by the gradual increase in copper leaching amount and leaching efficiency. At the Cu2+ concentration of 0.001mol/L, the inhibition of the growth and bioactivity of the strains was small. After 30 days, the leaching amounts of the three strains were DY1-2.958g, DY2-2.633g and DY3-2.392g. When the concentration of Cu2+ reached 0.05mol/L, the bioactivities of the three strains were obviously inhibited, and the leaching efficiencies were suppressed. After 30 days, the leaching amounts of the three strains was DY1-1.825g, DY2-1.603g and DY3-0.326g. Hence, high Cu2+ concentration has a negative effect on the growth and decomposition of the strains.

Table 1 presents the up-regulated folds of copper resistance gene in the three strains DY1-DY3 under different Cu2+ concentrations. According to previous studies, when the concentration of Cu2+ in the culture

solution is lower than the maximum tolerable Cu2+ concentration of At. Ferrooxidans strain, the gene Afe0022 in the strain will be up-regulated. It can be seen from the table that, under the maximum tolerable Cu2+ concentration, the gene were up-regulated by 18.26, 54.56 and 185.58 folds in DY1-DY3, respectively.The copper resistance gene of the strains were stimulated to a certain degree by the Cu^{2+} in the culture solutions. Featuring stronger Cu^{2+} resistance than the other two strains, DY1 has the highest gene up regulated fold, followed by DY2 and DY3 in descending order. This shows that the Cu^{2+} resistance of the strains is proportional to the resistance gene of the strain itself. The stronger the resistance of the strain to Cu^{2+}, the more unlikely for the relevant resistance gene to be expressed in large quantities at low Cu^{2+} concentration in the culture solution.

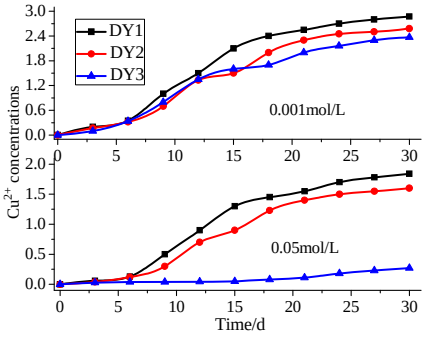


Figure 5: Cu^{2+} leaching curves with different initial Cu^{2+} concentrations

Table 1: Differentially expression of 3 types of strains under different Cu2+ concentrations

Strain	Cu^{2+} concentration (mol/L)	Up-regulated fold
DY3	0.02	1.35
	0.04	18.26
DY2	0.02	1.40
	0.04	11.42
	0.20	54.56
DY1	0.04	1.21
	0.20	72.18
	0.41	185.58

3.2 Analysis of the resistance of At. Ferrooxidans strains to Mn2+, Zn2+ and Cd2+

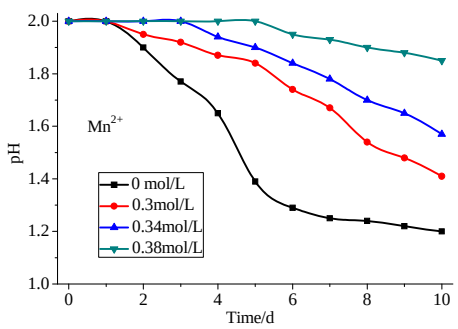
Figure 6 shows the pH variations in each culture solution after 10 days of culture of *At. Ferrooxidans* strains at different concentrations of Mn^{2+}, Zn^{2+} and Cd^{2+}. The substance of energy source in the solutions is S element. 0mol/L stands for the control group in which the culture solution did not contain any heavy metal ion. As shown in the figure, the pH of the control group fell from 2 to 1.20 after 10 days of culture. In comparison, the final pH values of the culture solutions were increased in different degrees after 10 days of culture in solutions added with Mn^{2+}, Zn^{2+} and Cd^{2+}. In other words, the strain growth and decomposition were suppressed. The less the variation in pH, the more inhibited the activity of the strains. As can be seen from Figure 6(a), the final pH values of the culture solutions stood at 1.41, 1.56 and 1.90 when the Mn^{2+} concentrations were 0.3mol/L, 0.34mol/L and 0.38mol/L, respectively. As the strain activity was almost completely inhibited at the Mn^{2+} concentration of 0.38mol/L, the maximum tolerable Mn^{2+} concentration of the strains is 0.38mol/L; similarly, the maximum tolerable Zn^{2+} concentration of the strains is 0.18mol/L for the final pH values of the culture solutions were 1.32, 1.57 and 1.93 when the Zn^{2+} concentrations were 0.06mol/L, 0.12mol/L and 0.18mol/L; the maximum tolerable Cd^{2+} concentration (0.08mol/L) is obtained in similar manner.

The difference in the maximum tolerable concentration of Mn^{2+}, Zn^{2+} and Cd^{2+} is an evidence to the varied degrees of toxic effect of the three heavy metal ions on the bacterium. As two of the essential elements for biological growth, suitable amounts of Mn^{2+} and Zn^{2+} are conducive to strain growth while excessive amounts of the two ions will hinder the growth. Cd^{2+}, however, is not a biologically essential element. Any trace of Cd^{2+} will harm the bacterium in the surrounding environment.

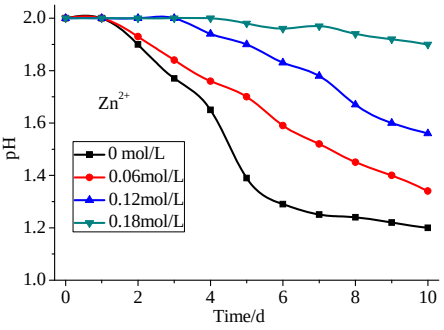
Figure 7 describes the relative expression levels of the genes in the strains stimulated by Mn^{2+} and Zn^{2+}. The abscissa 1-4 in the figure represent genes (Afe-671, Afe-674, Afe-1143 and Afe-1144) in the strains, respectively. As shown in Figure 7(a), the expression levels of Afe-671, Afe-1143 and Afe-1144 were increased by 2 times, 3.5 times and 2.7 times, respectively, when Mn^{2+} was 0.1 mol/L in the culture solutions.

When the concentration of Mn^{2+} was increased to 0.4mol/L, the expression levels of the four genes were increased by 428.2 times, 688.1 times, 289 times and 620.6 times, respectively. According to Figure 7(b), the expression levels of Afe-671, Afe-674, Afe-1143 and Afe-1144 in the culture solutions were increased by 1.35 times, 1.47 times, 5.23 times and 6.2 times, respectively, when Zn^{2+} concentration was 0.04mol/L; when Zn^{2+} concentration was raised to 0.16mol/L, the expression levels of the four genes were increased by 229 times, 86.4 times, 216.6 times and 153.7 times, respectively. To sum up, Figure 7 demonstrates that the expression levels of the genes in the strains experienced different degrees of upregulation with the increase in the concentrations of heavy metal ions in the culture solutions. Comparatively speaking, the four genes in the At. Ferrooxidans strain were more sensitive to Mn^{2+} and Zn^{2+}.

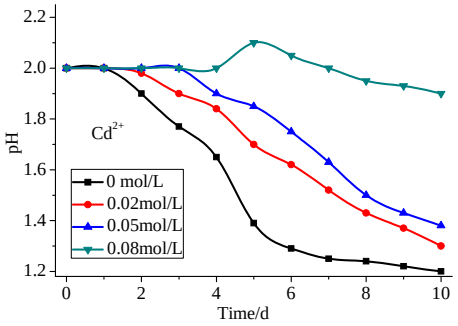
The genes in the above four genes were then analyzed by bioinformatics. The reading frame of Afe-671 is 3114bp, which encodes the protein COG3696. Known as the silver efflux pump, it is mainly responsible for the transport of inorganic ions. The open reading frame lengths of Afe-671, Afe-1143 and Afe-1144 are 363bp, 1320bp and 3111bp, respectively. In the test, the proteins encoded by the four genes participated in the transport of heavy metals. The proteins encoded in Afe-671 and Afe-1144 are multiple transmembrane proteins, while the protein of Afe-1143 is a metal ion transport protein.



(a) Mn2+

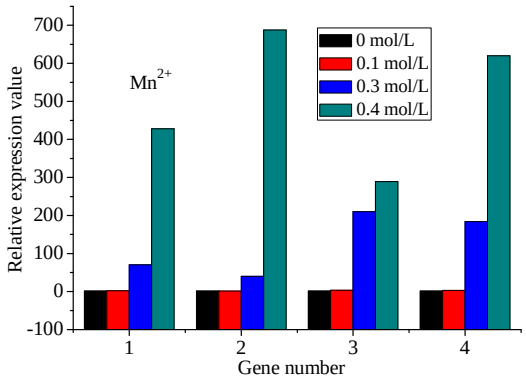


(b) Zn^{2+}

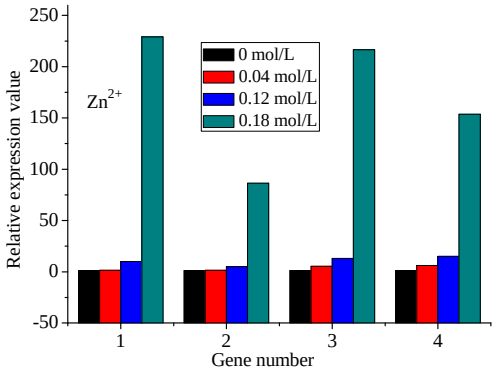


(c) Cd2+

Figure 6：pH variation curves at different concentrations of metal ions



(a) Mn2+



(b) Zn2+

Figure 7：Differentially expressed of metal genes under different metal ions concentrations

4. Conclusion

This paper analyzes the resistance mechanism of At. Ferrooxidans to heavy metal ions, and compares the tolerance of different strains to Cu2+ and the expression of At. Ferrooxidans strains under different concentrations of Mn2+, Zn2+ and Cd2+. The conclusions are as follows:

(1) The three selected At. Ferrooxidans strains, DY1, DY2 and DY3, differ in terms of Cu2+ toleration and S and Fe2+ oxidizing abilities. The maximum tolerable Cu2+ concentrations of DY1, DY2 and DY3 are 0.41mol/L, 0.20mol/L and 0.04mol/L, respectively. DY1 has the strongest oxidizing ability to S element, but the weakest oxidizing ability to Fe2+ ions among the three strains; DY3 has the strongest oxidizing ability to Fe2+ ions. There are differences in the adaptability of different strains to the leaching system. The copper resistance gene of the strains are stimulated to a certain degree by the Cu2+ in the culture solutions. The Cu2+ resistance of the strains is proportional to the resistance gene of the strain itself. The stronger the resistance of the strain to Cu2+, the more unlikely for the relevant resistance gene to be expressed in large quantities at low Cu2+ concentration in the culture solution.

(2) The maximum tolerated concentrations of Mn2+, Zn2+ and Cd2+ are 0.38 mol/L, 0.18 mol/L and 0.08 mol/L for the selected At. Ferrooxidans strains. The three heavy metal elements on the strains are ranked as Mn2+<Zn2+<Cd2+ by toxic effect in descending order. The relative expression of the four genes of the strains is proportional to the concentration of heavy metal elements. The proteins encoded in the four genes are involved in the transport of heavy metals.

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