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Comparison of Uranium Extraction by Bio-product of 3 Bacteria Strains Isolated from Shihongtan Uranium Deposite, Xinjiang, China

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It is well known that bacteria produce short-chain organic acids and element-specific ligands (siderophores) that are able to change pH and enhance chelation, which results in increased mobilization of uranium. This research work represents the extraction of Uranium by Siderophores (bio-products) producing microbial species Myroides Odoratimimus, Pseudomonas Aeruginosa and Pseudomonas Fluorescens were incubated in chemically defined medium supplemented with uranium ore. Batch leaching tests were performed in neutral conditions for 7 days. With Myroides Odoratimimus, uranium was leached out in test about 20 mg/L, with Pseudomonas Aeruginosa, it was about 12 mg/L and with Pseudomonas Fluorescens it was about 10 mg/L. Uranium extraction with Myroides odoratimimus was higher as compared to others was due to the production of greater siderophores as compared to other two strains. This comparative study showed that the strain produced greater siderophores production leached out more uranium as compared to other strains. For future suggestion, better siderophores producing bacterial strains may be used at industrial scale to extract heavy metals like uranium.

Keywords: Siderophores (Bio-Products), Uranium Extraction at neutral conditions, Myroides odoratimimus, Pseudomonas Aeruginosa and Pseudomonas Fluorescens

Introduction

Bio-Leaching is an environment friendly technique to extract metals from their ores as compared to other unconventional mining techniques. Uranium Bioleaching is certainly one of the most promising and regarding the most revolutionary solution to the serious environmental problems, and metal grade decreasing in metal minerals after long time exploitation, compared to pyrometallurgy or chemical metallurgy. The bioleaching process, which employs microorganisms such as bacteria and fungi that produced siderophores as the leaching catalysts, is known to be economical and environmentally acceptable[1]. Microorganisms synthesize secondary metabolites called ‘‘-siderophores-’’, under iron-deficient conditions, which strongly scavenge extracellular Fe(III), then solubilize it, and transport it inside the cells. These siderophores which further help in the leaching of uranium from their ore[2-4]. Different types of siderophores form complexes with radioactive elements such as uranium and thorium which reveals that these kinds of siderophores can be used to mobilize or leach out uranium from their ore[5]. In the earlier study work, *P. aeruginosa* produced element-specific ligands (siderophore) that was able to change pH and enhanced chelation of Th^{4+} and UO_2^{2+}. The produced siderophore at pH 5.3 had the ability to bioleach and was complexed with 68.00% of uranium and 65.00% of thorium[6]. In another earlier study, to better understand the effects of biologically mediated leaching of metals from mine waste, *P. fluorescens* was cultivated in the presence of processed ore from the former uranium mine in Ranstad, southern Sweden. Data from this study supported siderophores production by bacteria that allowed mobilization of essential nutrients from the processed ore. However, the availability of potentially toxic metals like Ni and U may also be enhanced. Microbial-promoted mobilization could contribute to leaching of toxic metals in current and historic mining areas[7, 8]. In another earlier study, The siderophore producing bacterium Pseudomonas fluorescens was grown in batch cultures for 5 to 8 days with naturally weathered (unprocessed) uranium ore (0.0029%Uby weight), kolm (0.52%Uby weight) and acid-leached ore (0.0099%Uby weight) in chemically defined media (unbuffered and buffered). *P. fluorescens* was grown with ore and unbuffered medium changed the pH from 4.7 to 9.3 and leached out 0.016 to 0.9% (normalized to surface area) of the total amount of U from the different ores. Incubation of the acid-leached ore with bacteria in buffered medium leached out 0.04% of the

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total U. Uranium was leached out selectively at all conditions. Thus, the production of microbial chelators could contribute to the elevated metal concentrations in the drainage water from the closed Ranstad mine[9].

In the present work, a comprehensive study on comparison was studied between three siderophore-producing bacteria. 1) *M. Odoratimimus* is a common bacterium found in soil and water. It is an aerobic gram-negative bacterium.[10] 2) *P. Aeruginosa* is also a common gram-negative, rod-shaped bacterium and 3) *P. Fluorescens* is also a common gram-negative, rod-shaped bacterium. These bacteria were selected for present study by investigating and studying the earlier studies on siderophore producing bacteria. They were investigated that which bacterial strain can produce maximum siderophores. A universal assay was used described by Schwyn and Neilands (1987)[11] to detect and calculate the production of siderophores. The bacterial strains were also compared which one can leach out maximum uranium from the 721 uranium mineral ore.

Materials and Methods

Uranium Ore

A hard-rock type of Uranium ore from 721 uranium mine which is one of the largest uranium mine in Jiangxi province of China was used in this research. The uranium ore was weathered and crushed to size of 74µm. The uranium ore was sterilized before used in leaching experiments. The chemical composition of uranium ore is presented in Table 1.

Table 1; Chemical Composition of uranium ore

Elements														
%														
SiO_2	Al_2O_3	Fe_2O_3	MgO	CaO	Na_2O	K_2O	MnO	TiO_2	P_2O_5	LOI	FeO	SO_3	Or C	IOr C
64.30	14.30	3.63	0.853	3.88	2.77	3.68	0.192	0.398	0.769	4.58	1.88	0.44	0.13	0.21
Ppm														
Li	Be	Sc	V	Cr	Co	Ni	Cu	Zn	Ga	Rb	Sr	Y	Zr	Li
104	4.64	9.73	41.4	16.5	7.67	12.8	12.8	83.9	19.1	273	394	104	184	104
Mo	Ba	La	Ce	Pr	Nd	Cs	Dy	Yb	Th	Ta	W	U	U(IV)	U(VI)
155	398	47.7	91.5	10.8	41.1	40.0	12.8	21.3	453	2.44	10.6	2496	1965	531

Media Composition

The experiments were conducted in unbuffered chemically defined medium consisting of 6.0 g/L Na-Lactate, 0.2 g/L KH_2PO_4, 0.3 g/L NH_4Cl, 0.1 g/L $MgCl_2.6H_2O$, 0.15 g/L $CaCl_2.6H_2O$, 0.5 g/L KNO_3 and 0.2 g/L $MgSO_4.7H_2O$. The medium was adjusted to pH of 6.9 (adjusted with 0.3M NaOH). All chemicals were purchased from different organizations i.e. AR, Chembasebio in China and were of analytical grade.

Bacterial Strains

Three bacterial strains were used in leaching experiments, as follows; a) A strain named M.O.1 *M. Odoratimimus* was used in leaching experiment, which was isolated from 738 uranium mine in northwest of China, then purified and identified by 16sRNA gene sequencing, which showed 99% identity with 16sDNA of *Myroides odoratimimus (T45)* Accession No. KF758445.1.

b) A strain named G16X-D (dated 24-01-2017) *P. Aeruginosa* was taken from Culture Collection of state key nuclear laboratory of East China university of Technology, Nanchang, China and was used in leaching experiment.

c) A strain named CGMCC 1.1802 (dated 16-05-2013) *P. Fluorescens* was taken from China General Microbiological Culture Collection Centre, Beijing, China and was used for leaching experiment.

All bacterial strains were inoculated in liquid nutrient broth medium for 24 hours prior used in leaching experiments.

Siderophore Production and Detection Assay

All bacterial strains were observed to detect the presence and production of siderophores. (Figure 2) For this purpose, a universal assay was used described by Schwyn and Neilands (1987) was used to detect the presence and production of siderophores in the filtered microbial supernatants. The assay is based on the decrease in absorbance at 630 nm of a chrome azurol S (CAS) dye–Fe(III) complex, from which the Fe(III) is removed by a strong chelator, such as a siderophore, changing the color of the solution from blue to orange red. Chemicals used were purchased from company named Solarbio from China.[12] For the detection of siderophore on blue agar plates, CAS assay dye solution was mixed with liquid broth medium and filtered cultures supernatant were inoculated in 3mm diameter of holes which were made by hole puncture on blue agar plates to see the production of siderophores as shown in (Figure 1a). For the production of siderophore assay, 0.5-ml aliquots of CAS assay solution were added to 0.5 ml of filtered culture supernatants, blanks standards. Thereafter, 10 µl of shuttle solution (0.2 M 5-sulfosalicylic acid) was added. The absorbances were read at 630 nm using a UV-Vis Spectrophotometer (Model # Rayleigh UV-1601).

Siderophore production was calculated using the following equation:

$$\text{Siderophore\%} = (A_{ref} - A_{sample})/A_{ref} \times 100 \quad (1)$$

where Siderophore % is the siderophore production in percent, A_{ref} is the absorbance measured when no siderophores are present, A_{sample} is the absorbance measured in a sample with siderophores present. To get the siderophores production percentage, all bacterial strains were inoculated in nutrient broth medium and checked the absorbances of samples to calculate the siderophore production percentage.

Other experimental material

All glassware used was soaked for >2 h in 10% (v/v) HNO_3 and rinsed five to six times with deionized H_2O. The material used in the leaching experiments, including flasks, forceps, medium, and mineral powder, was autoclaved and sterilized prior to the experiments.

Experimental Methods

Three sets of aerobic batch experiments were completed at 30±1 C°, namely:

Set#1-3: 250-ml Erlenmeyer glass flasks were loaded with 10 g of ore in 200 ml of the chemically defined medium described previously and inoculated with 5 ml of *M. odoratimimus* (Set 1), *P. Aeruginosa* (Set 2) and *P. Fluorescens* (Set 3). Samples of 5 ml were removed every day for 5 days and on day 7, upon termination of the experiment, and submitted for uranium analysis and pH determination. 5 ml sample were taken every day because 3 ml sample was used to detect the presence of produced siderophores and the rest of sample used for further analysis.

Besides experimental setups, controls were ore without bacteria. All experiments were done in triplicate. The flasks were swirled carefully in shaking incubator at 120 rpm throughout upon termination of experiment. The supernatant was taken as samples, after centrifuge the samples at 10000 rpm for 3 minutes. pH was detected by lab scale pH meter (model Starter 3100, OHAUS). Uranium concentration analysis was determined on ICP-OES (model 5100, Agilent Technologies, USA).

Results and Discussion

Bacteria Isolation

The bacterial strain M.O.1 *M. Odoratimimus* was isolated from 738 uranium mine which was inoculated in liquid broth medium and solid broth medium and then it's pure strain was isolated and identified as shown in (Figure 2).

Siderophore Production

All bacterial strains were observed to detect the presence and production of siderophores (Figure 1a and 1b). The siderophores produced by bacterial strain M.O.1 M. Odoratimimus showed maximum quantity of siderophore as compared to other two bacterial strains. The siderophore production percentage was much higher about 90 % in bacterial strain M.O.1 M. Odoratimimus as compared to other two strains about 50 % in strain G16X-D and about 40 % in strain CGMCC 1.1802 as shown in (Figure 3a, 3b, 3c). This result showed that this bacterial strain M.O.1 M. Odoratimimus can produced more siderophore as compared to other two strains.

Figure 2: Different stages of bacterial strain M.O.1 *Myroides odoratimimus* a) in liquid broth medium b) in Solid Medium c) SEM picture of bacterial strain

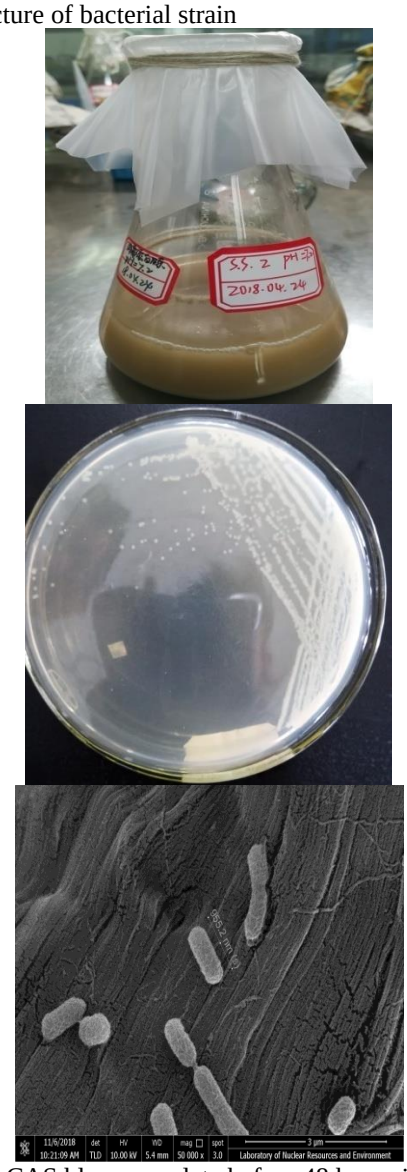


Figure 1a: CAS blue agar plate before 48 hours inculation.

Figure 1b: Siderophore produced and detected on CAS blue agar plates (orange circles) after 48 hours of incubation; by

bacterial strain (a) *M. Odoratimimus* (b) *P. Aeruginosa* and (c) *P. Fluorescens*

Figure 3a: Siderophore production verses time is expressed in percentage.

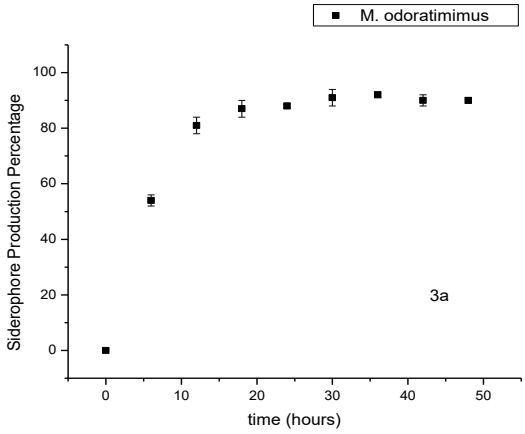


Figure 3b: Siderophore production verses time is expressed in percentage

Cultures with all the bacterial strains *M. Odoratimimus, P. Aeruginosa and P. Fluorescens* showed approximately doubled the amount of cells with 10 g of ore as compared to without ore, this may be due to these species extract more nutrients from ore. This strain also showed greater concentration of Uranium as compared to other as shown in (Figure 4)

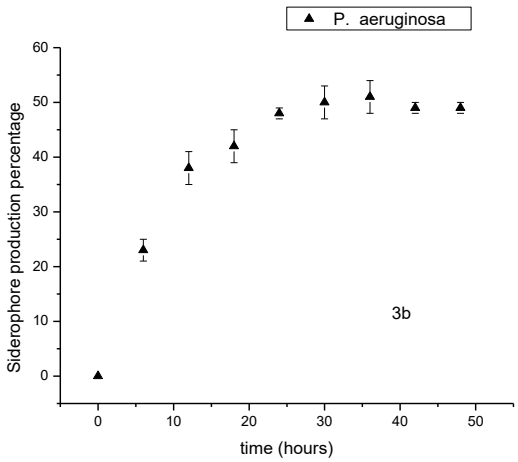
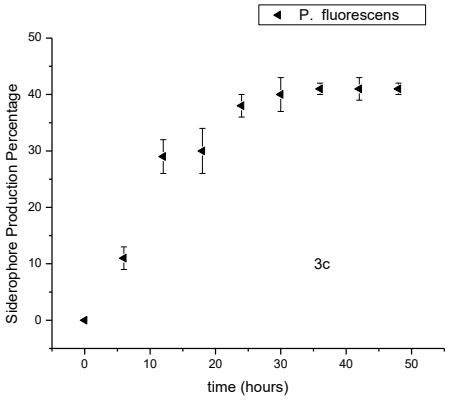


Figure 3c: Siderophore production verses time is expressed in percentage



Cell Growth

M. odoratimimus is the only one of the three species tested that appears to thrive in the presence of either ore and consequently showed more extensive growth than seen without these ores. After 7 days, the cell numbers reached 0.35±2 x 10^9 cells ml^{-1} and 1.45±3 x 10^9 cells ml^{-1} for *M. odoratimimus* without and with ore, respectively (Table 2). The opposite trend was observed for *P. Aeruginosa*. The cell numbers in cultures with *P. Aeruginosa* were lower by a factor of 100 when grown with either ore as compared with cultures grown without ore. In actual numbers, this corresponds to 0.85±3 x 10^7 cells ml^{-1} and 0.25±3 x 10^7 cells ml^{-1} without and with leached ore, respectively. Cell numbers were also lower for samples incubated with *P. Fluorescens* as compared with the other two species, amounting to 0.15±3 x 10^6 cells ml^{-1} and 0.25±3 x 10^6 cells ml^{-1} after 7 days when grown without and with ore, respectively (Table 2).

Table 2:
Direct counting by microscope of bacteria grown with ore and without ore respectively, in cells ml^{-1}

Type of Ore	Days	Medium only	Medium + m.o[a] (x 10^9)	Medium + m.o[a] + ore (x 10^9)	Medium + p.a[b] (x 10^7)	Medium + p.a[b] + ore (x 10^7)	Medium + p.f[c] (x 10^6)	Medium + p.f[c] + ore (x 10^6)

	2	n.d	0.02±1	0.22±2	<0.12±1	0.10±2	<0.002±1	<0.01±1
721	3	n.d	0.03±1	0.43±1	0.01±3	0.13±1	0.003±3	<0.03±1
Uranium	4	n.d	0.05±2	0.65±2	0.15±2	0.15±2	0.05±2	0.06±2
Ore	5	n.d	0.21±1	1.31±1	0.31±2	0.21±1	0.11±4	0.13±1
	7	n.d	0.35±2	1.45±3	0.85±3	0.25±3	0.15±3	0.25±3

95% confidence intervals were used for the number of cells and error calculations.

a) m.o. = *M. Odoratimimus*, b) p.a = *P. Aeruginosa*, p.f. = *P. Fluorescens*, n.d. = no cells detected,

Siderophore Analysis

After 2 days of incubation, all the cultures used, reached stationary phase and turned yellow as a result of the production of fluorescent Pyoverdine siderophores (Figure 1b).

The universal assay by Schwyn and Neilands (1987) shows that siderophores were already being excreted during the adaptation phase (lag phase) in all cultures but greater excreted with culture *M. Odoratimimus* as compared to others (Figure 3a). In cultures, the siderophore production seemed to be connected to the amount of cells in the culture, as the start of the log phase and detection of siderophores occurred simultaneously. In cultures supplemented with ore the siderophore production seems to be lower as compared with cultures without ore. This also suggests that *M. Odoratimimus* managed to solubilize more Fe(III) as compared to other two strains. Due to more solubilization of Fe(III), this metabolizes and consequently produces more siderophores as compared to other two strains. However, by the end of the experiment, analyses using the universal assay indicated that all the bacterial ligands produced in the presence of ore during the experiment had formed organo-metallic complexes, as no free siderophores were detected.

Uranium Concentration

Uranium concentrations in the experiments with ore incubated with bacterial cultures were initially low on day 1. However, after 7 days, the uranium concentration in the experiments with *M. Odoratimimus* increased to 19.785 (±0.2) mg/L for the ore, (Figure 4). Whereas, on day 7, uranium concentration in cultures with *P. Aeruginosa* and *P. Fluorescens* were lower (12.12 ±0.1 mg/L and 9.89 ±0.14 mg/L) respectively as compared to concentration with *M. Odoratimimus*. The percentage of uranium concentration increase was quite higher near about 81% on day 7 in experiment with *M. Odoratimimus* as compared to uranium concentration in experiment with ore only (Figure 6). The difference in uranium concentration between three species was most likely due to iron release coupled with the production of siderophores, as iron is more or less insoluble in the pH range of the experiment. Uranium followed the same pattern. The iron concentration decreased with time in ore with cultures as shown in (Figure 5) while uranium concentration increased with time. The explanation to that

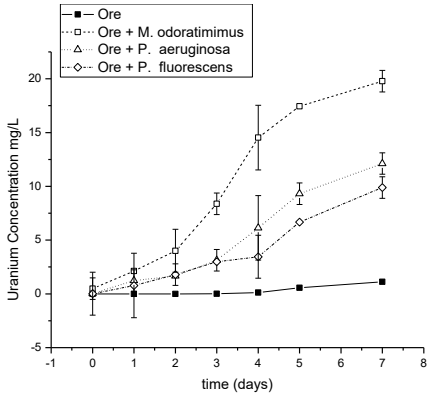


Figure 4: Uranium concentration analyzed by ICP-OES in experiments, error bar shows standard deviatic

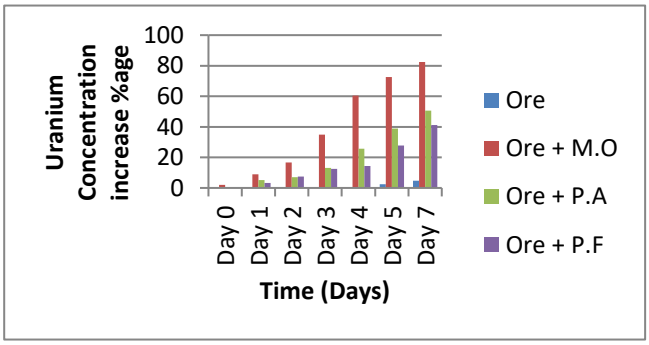


Fig 6: Uranium concentration percentage increase with time, ore without bacteria and with bacterial strains. *M.O = M. Odoratimimus*, *P.A = P. Aeruginosa*, *P.F = P. Fluorescens*,

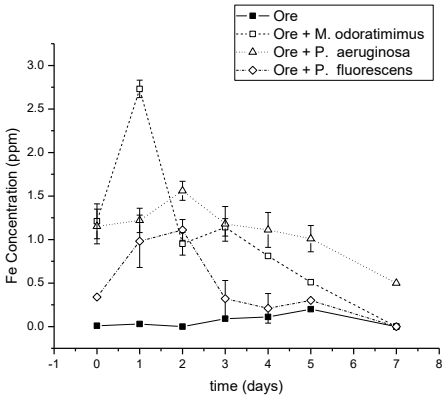


Figure 5: Iron concentration analyzed by ICP-OES in experiments. Error bars showed standard deviation.

the iron concentrations decrease whereas U concentration increases with time is most likely due to the elevation in pH (Table 2) as UO_2^{2+} readily forms water soluble complexes

with carbonate whereas iron forms hydroxides and precipitate at near

Table 2: pH data of samples of uranium ore only and with ore and incubated with *M. odoratimimus, P. aeruginosa* and *P.fluorescens* , respectively

Days	Ore	Ore + *M.O*	Ore + *P.A*	Ore + *P.F*
0	6.90	6.90	6.90	6.90
1	6.92	7.09	7.72	7.22
2	6.95	7.30	7.78	7.77
3	7.01	8.25	8.40	8.35
4	6.98	8.47	8.55	8.42
5	6.98	8.65	8.63	8.44
7	7.02	8.89	8.68	8.50

M.O = M. Odoratimimus, P.A = P. Aeruginosa, P.F = P. Fluorescens,

neutral pH and above. Bouby et al. (1998) [5] have reported that Pyoverdine ligands complex uranyl ions with higher affinity in degraded form than when the siderophore molecule is intact. Iron and other metals will most likely precipitate when these complexes are degraded and thus become immobilized, whereas uranium will still be complexed and mobile.

Remarkable, the uranium concentration is higher in experiment with *M. Odoratimimus* and ore as compared to other two bacterial strains.

Conclusions

In present study, all the bacterial cultures used are managed to produced siderophores but *Myroides odoratimimus* species produced greater siderophores as compared to other two species, and also extract higher uranium concentrations from ore as compared to other experiments. This study using laboratory incubations showed that mobilization of uranium from ore can occur aerobically at neutral to alkaline. Theoretically, if the appropriate conditions are met, such as uranium mobilization rate and exponential growth is maintained, then this microorganism could continue to extract uranium. This study was conducted under close to optimal laboratory conditions, the next step, at least for *M. Odoratimimus*, would be to investigate how higher uranium and possibly other metals are released from their ore in in-situ.

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