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Optimization of Bioleaching Conditions Using *Acidithiobacillus ferrooxidans* at Low Temperatures in a Uranium Mining Environment

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Abstract

Systematic studies were conducted at one of the uranium deposits in Kazakhstan. Native strains of *Acidithiobacillus ferrooxidans* bacteria were found in leaching solutions at the deposit. The modeling of iron species in the culturing medium was analyzed using Medusa software v.2.0.5. To intensify the process, the bacterial strains were propagated in laboratory conditions, and strains available in the laboratory were added. The ability of bacteria to oxidize divalent iron to trivalent iron at 8 °C in laboratory conditions was established, but the oxidation rate was low. It was found that the limiting stage of bioleaching use in deposit conditions is the temperature mode, the content of divalent iron, and oxygen. A biomass volume of 15 L was initially cultivated under laboratory conditions, and subsequently scaled up to 3 m^3 in production using three 1 m^3 pachucas with air aeration. In addition, pilot tests were carried out directly in production conditions and biomass in the volume of over 30 m^3 was produced. The kinetics of the oxidation process of divalent iron to trivalent iron in 1 g/h under production conditions was established. The features of the bioleaching process at the field are shown as follows: since production, the solution contains the main microelements for the nutrition and reproduction of bacteria, and recommendations for the use of bioleaching are proposed. Research has established that under conditions of a shortage of divalent iron in the solution, sulfuric acid is formed due to sulfur-containing substances. It was observed that for the effective conversion of divalent iron to trivalent iron, bacteria of the provided strain and air (oxygen) supply are sufficient. The corresponding recommendations were issued during the work.

Keywords: *Acidithiobacillus ferrooxidans*; bioleaching; biooxidation; uranium deposit; low-temperature bioleaching; field-scale bacterial leaching

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1. Introduction

In 2022 Kazakhstan produced the largest share of uranium from mines (43% of world supply), from the other side it is a significant effect on the environment [1].

In in situ leaching (ISL) of uranium, lixiviant solutions are used to dissolve uranium within the ore body. The review outlines several challenges, such as the high cost and complexity of site restoration, the formation of gypsum, elevated salinity levels, and the presence of certain heavy metals and radionuclides, following restoration efforts. A key

concern is whether natural attenuation alone can effectively restore groundwater quality and reestablish the geochemical balance in areas affected by acid in situ leaching [2,3].

Increasing the redox potential (Eh) of these lixiviants is one of the key strategies for intensifying the leaching process. For instance, pyrite leaching occurs much more rapidly at a solution redox potential (Eh) of 0.9 V compared to 0.7 V at pH 1 when using leaching agents such as HCl, H_2SO_4, and $HClO_4$. The strong influence of Eh indicates that the leaching process is electrochemically controlled and likely follows a similar mechanism at both 0.7 V and 0.9 V, though the reaction rates differ. The leaching rate follows the trend $HClO_4$ > HCl > H_2SO_4, at 0.7 V, while the order changes to HCl > $HClO_4$ > H_2SO_4 at 0.9 V [4,5]. These acids oxidize ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}), which in turn increases the redox potential of the solution [6,7].

However, these oxidizing agents are expensive and require special storage conditions. When introduced into the ore body, ferric iron oxidizes the relatively insoluble tetravalent uranium (U(IV)) into its soluble hexavalent form (U(VI)), thereby increasing the uranium concentration in the pregnant leach solution [8–10]. Given the recent price surge (nearly a twofold increase) for sulfuric acid, which is the primary chemical reagent in ISL, the application of costly chemical oxidants has become economically inefficient.

The central concept of nowadays is the development of a bioactivation technology for lixiviant solutions to intensify the in situ recovery of uranium [11]. The proposed bioactivation process involves the use of iron-oxidizing bacteria [12,13]. This technology can significantly accelerate the uranium leaching process, increase the concentration of uranium in the pregnant leach solution, and reduce costs associated with enhancing the redox potential of the solutions [14]. Recently a pilot-scale experiments demonstrated an increase in the redox potential of the activated solution from 360 mV to 420–450 mV, reflecting the effective performance of the biooxidation process. A strong correlation (0.91) was observed between the redox potential values and the concentration of ferric iron (Fe^{3+}), confirming that the redox potential is directly influenced by the Fe^{3+} content in the solution.

It is essential to investigate the mechanisms by which iron-oxidizing bacteria operate, including biomass growth and adaptation to technological solutions [15]. Among the key parameters in this process are the kinetics and mechanisms of lixiviant bioactivation, which are influenced by process conditions such as pH, Eh, biomass concentration, and activity. It is also necessary to identify rate-limiting steps and inhibitors of the bioactivation process [16].

The use of bacteria in uranium leaching has been investigated in numerous studies [17,18]. Laboratory and pilot-scale trials of the ISL process employing bacterial iron oxidation in circulating solutions were carried out at a uranium mine [19,20]. The effectiveness of using flow-through bioreactors to enhance the oxidation kinetics of ferrous iron in lixiviant solutions has been demonstrated [21]. Our research has achieved a tenfold increase in the rate of iron oxidation due to the innovative design of the bioreactor.

Previously, it was shown that bioactivation improves the redox potential of lixiviant solutions. It is crucial to establish the relationship between bioactivation conditions and uranium recovery in the pregnant solution [20]. Practically all lixiviants used by uranium mining companies contain ferrous iron, and it is well-known that converting Fe^{2+} to Fe^{3+} enhances solution redox potential. Importantly, it was established that biologically produced ferric iron is approximately 20% more active than chemically generated ferric iron due to the formation of complexes with amino groups. In this study, we discuss the bacterial leaching at various concentrations and ore types. The comparison of the current in situ leaching process at the deposit, the implementation of bioleaching technologies under industrial conditions has been evaluated.

2. Materials and Methods

2.1. Materials

KH_2PO_4, purity 99%; $MgSO_4 \times 7H_2O$, purity 99%; $(NH_4)_2SO_4$, purity 99%; KCl, purity 99%; $Ca(NO_3)_2$, purity 99%; $FeSO_4 \times 7H_2O$, purity 99%; and 10N H_2SO_4 were purchased from Sigma-Aldrich (Darmstadt, Germany).

Technological samples obtained from the pumping wells of the deposit, and throughout the entire cycle of the leaching process, including containers after columns with ion exchange resin, were analyzed.

2.2. Isolation of Iron-Oxidizing Bacteria

Isolation of chemolithotrophic iron-oxidizing bacteria from samples of mine water samples taken from the mine wells, their adaptation, activation for use in industrial conditions for intensive oxidation of divalent iron at low temperatures [22]. The task is to isolate pure cultures of chemolithotrophic iron-oxidizing bacteria capable of growth and oxidation of divalent iron at low temperatures; the biomass of *Acidithiobacillus ferrooxidans* culture with a cell titer of 1×10^8 cells/mL (NVCH) was obtained. Samples of mine waters were collected from the mine wells and isolates of iron-oxidizing bacteria *Acidithiobacillus ferrooxidans pcs. M.* were isolated. The nutrient medium 9K Silverman and Lundgren was used as an accumulative medium. The composition of the medium g/L: I solution was as follows: KH_2PO_4—3.0; $MgSO_4 \times 7H_2O$—0.5; $(NH_4)_2SO_4$—3.0; KCl—0.1; $Ca(NO_3)_2$—0.01; II solution: $FeSO_4 \times 7H_2O$—44.2; 10N H_2SO_4—1 mL.

The medium was sterilized at 110 °C for 30 min. The pH of the medium after sterilization was 2.5. The biomass was grown using the standard technique. The limiting dilution method was used to isolate pure cultures. The culture enrichment were diluted 10, 100, 1000 …. 1 million, and 10 million times and cultured on a shaker with a rotation speed of 200 rpm at a temperature of 8 °C. To assess the purity of the isolated isolates, the culture was seeded on 9K, MPA, and Czapek medium, cultivated on 9K medium at a temperature of 8 °C until a brown color appeared, at a temperature of 29 ± 1 °C on MPA for 3 days and on Czapek medium for 7 days. The obtained isolates were stored in a refrigerator at a temperature of 5 ± 1 °C.

2.3. Bioleaching Process

Pure cultures isolated from mine water samples from the mine borehole were used for the experiments. The pH and content of divalent and trivalent iron were determined in the working solution beforehand. Cultivation was carried out in test tubes with 3–4 mL of the solution and in Erlenmeyer flasks (250 mL) with 30 mL of the solution on a shaker with a rotation speed of 180,220 rpm at a temperature of 8 ± 1 and 18 ± 1 °C until the culture turned brown. Bacteria was not added to the control flasks.

2.4. Development of Biomass of the Acidithiobacillus ferrooxidans Culture

The culture of *Acidithiobacillus ferrooxidans* pcs. was cultured in 750 mL Erlenmeyer flasks containing 100 mL of 9K medium pH 2.2 on a shaker with a rotation speed of 200–220 rpm at a temperature of 29 ± 1 °C for 5 days [23]. The inoculum of 10% by volume was a three-day culture of *Acidithiobacillus ferrooxidans* strain M, grown on a shaker with a rotation speed of 200–220 rpm at a temperature of 29 ± 1 °C. The growth and development of isolates and the culture of *Acidithiobacillus ferrooxidans* strain M were assessed by the concentration of cells in the culture fluid, the most probable number of which was determined by the method of serial dilutions on 9K medium. The content of Fe^{2+} and Fe^{3+} was determined by well-known standard redox titration method by potassium dichromate [24,25].

2.5. Enrichment Cultures of Acidophilic Microorganisms from Process Solutions of a Uranium Mine

To obtain an enrichment culture of acidophilic thione microorganisms, Silverian and Lundgren 9K nutrient medium was used [26]. The prepared medium was poured into flasks and sterilized in an autoclave at a temperature of 121 ± 1 °C for 30 min (the pH of the medium after sterilization was in the region 3.0–3.3. After sterilization, 100 mL of the 9K nutrient medium and 10 (ml) of the sample were placed in 250 mL shaking flasks. Cultivation in a thermostatically controlled shaker-incubator with a rotation speed of 180 rpm at a temperature of +8 °C was carried out. The development of microorganisms was assessed by a decrease in the pH of the medium and microscopy data. Of the 8 samples seeded on the 9K nutrient medium, enrichment cultures were obtained in 6 mine water samples; the data are presented in Table 1.

Table 1. Characteristics of mine water samples collected from uranium mine boreholes.

Sample No.	Sample Name	Matrix (Sample Type)	Quantity	PH	Temperature (°C)	Moisture
531/10	UPPR-7-6-1 Pumped well	water	1	2.0 ± 0.5	6.0	Moist
532/10	Water after well development	Water	1	5.0 ± 0.25	10.0	Moist
533/10	Ore horizon water	Water	1	5.0 ± 0.25	10.0	Moist
534/10	Mechanical impurity removal cascade	Water	2	2.0 ± 0.5	15.0	Moist
535/10	Leaching solution—pump station	Water	1	2.0 ± 0.5	8.0	Moist
536/10	Productive solution—pump station	Water	2	2.0 ± 0.5	12.0	Moist
537/10	5-11-1 (repair-restoration works)	Water	2	2.0 ± 0.5	12.0	Moist
538/10	5-7-4 borehole	Water	1	2.0 ± 0.5	10.0	Moist

2.6. Purification of Isolates from Extraneous Microflora

In order to obtain pure cultures of the isolated isolates, a series of passages were carried out using the dilution method on 9K nutrient medium. As a result, pure isolates were obtained as follows: No. 534—after the 2nd passage; No. 537—after the 1st passage; No. 536—after the 1st passage; No. 531—after the 1st passage; No. 538—after the 1st passage; and No. 535—after the 3rd passage. Pure isolates were stored in a refrigerator at a temperature of 5 ± 1 °C.

Culture No. 538 served as seed material (these strains were later submitted to the republican collection of microorganisms B-RKM 0767) the following nucleotide sequence of the 16S rRNA gene region was established according to the known protocol. Molecular identification was conducted using the polymerase chain reaction (PCR) technique, with 16S rRNA universal primers employed for DNA amplification. DNA extraction and purification were carried out using the DNeasy™ Blood & Tissue Kit (Qiagen, Manchester,

UK), in accordance with the manufacturer's protocol. The extracted DNA was diluted 1:10 with biologically sterilized water and then combined using the Mega Mix-Royal solution kit (Microzone, Stourbridge, UK). This mixture contained Taq polymerase, a 2x enhancing buffer (6 mM $MgCl_2$) with 400 µM dNTPs, and blue MiZN loading dye and stabilizer, in preparation for PCR. Stock DNA samples were stored at −20 °C for future use.

For the PCR, 12.5 µL of the Mega Mix-Royal solution was added to an equal volume comprising DNA, primers, and water, resulting in a total reaction volume of 25 µL to initiate Taq polymerase activity. The PCR was run on a G-Storm thermocycler (LabTech International, Rotherham, UK) under the following cycling conditions: initial denaturation at 95 °C for 300 s, followed by 30 cycles of denaturation at 95 °C for 60 s, annealing at 50 °C for 45 s, extension at 72 °C for 1.5 min, and a final extension step at 72 °C for 10 min. Amplified DNA fragments were separated and visualized through agarose gel electrophoresis. A 10 µL sample of the PCR product was run on a 1.5% agarose gel. A DNA ladder up to 4500 bp was included for size reference, and distilled water served as a negative control. Electrophoresis was performed using Tris-Acetic-EDTA buffer at 100 volts for three hours in a HU15 horizontal gel unit (LabTech International, Rotherham, UK).

Post-electrophoresis, gels were stained with 0.5% ethidium bromide for 15 min, rinsed in water, and then visualized and documented using the SynGene software and INGENIUS UV imaging system (Syngene Biotechnology & Life Science, Caerphilly, UK). The amplified DNA was sequenced using 25 µL of product at a concentration of 20 ng/µL, processed by Eurofins Genomics DNA sequencing service [27,28]. The obtained result sequence was TCTOTCTTITAGTGCIOGGACAACCCAGGGAAACTTGGGCTAATACCG CATGAGCCCTGAGGGGGGAAA-GCGGGGGATCTTCGGACCTCGCGCTAAGGGAGGA GGCCTACGTCTGA-TTAGCTAGTTGGCGGGGTAAAOOCCCACCAAGGCGACGATC GGTAOCTOGTCTGAGAGGACGACCAGCCACACTGGGACTGAGACACGGCCCAGA CTCCIACGGGAGGCAOCGTGGGGAATTTITCGCAATGGGGGCAACCCTGACGAAGC AATOCCOCGTGGATGAAGAAGGCCTTCGGG1TGTAAAGTCC1TTCGTGOAOGACOA A A-AGTOGGTTCLAATACAATCTGCTATTGACGTGAATCCAAOAAGAA-GCACCGGC TAACICCGTGCCAGCAGCCGCGGTAATACGGGGGGTGCAAGCGTTAATCGGAAT CACIGGGCGTAAAGGGIGCGTAGGCGGTACG7TAGGKTGTCGTGAAAG-CCCCOOGC CAACGTOGOAATGOCGGTGGAAACCGGCGCACTACTAGTATGOOAGAOGGTGGTO GAATTCCAG GTGTAGCGGGAAATGCGTAGATATCTGGAOGAACATCAGTGGCGAAG CjCGGCCACCTGGCCCAATACTGACGCTGAGGCACGAAAGCGTGGGGA.

2.7. Optimization of Bacteria Growth Culturing in Nutrient Salts

Nutrient salts were added to the leaching solution and conditions for the reproduction of bacteria were created (in terms of temperature and air supply). The experiments were conducted without adding bacteria at all. Despite this, within a few days the solution turned into a characteristic red color. This allowed us to conclude that the solution of the storage pool of leaching solutions contains strains of *Acidithiobacillus ferrooxidans* and they are capable of multiplying in the presence of aeration.

2.8. Conducting Pilot Studies

In the uranium productive solution processing shop, tanks were installed and the corresponding piping for air supply was made. Bacterial cultivation processes were carried out for use in pilot industrial tests. The grown strains were adapted and activated on the deposit's production solution at relatively low temperatures of 8–10 °C. For conducting experiments, the first batch of 15 L of culture liquid containing bacteria was produced in laboratory conditions. The second batch of 15 L of culture liquid with *Acidithiobacillus ferrooxidans* strain was accumulated at a temperature of 25 °C on a shaker with air access.

The resulting volumes were transferred to the shop for conducting large-scale studies on assembled installations with a volume of 1 m^3 each (Figure S1). During these studies it was established that the process of bacterial leaching can proceed at a temperature of 8–10 °C, but the processes are significantly slower by 300%. The experiment was conducted for 3 months with the solution being renewed once a month. In laboratory conditions at a temperature of 8–10 °C the oxidation rate of divalent iron was 3 times slower than at a temperature of 25 °C. The standard deviation of the indicators was ±10%.

3. Results and Discussion

3.1. Characterization of Samples

To carry out work on the isolation of aboriginal acidophilic microorganisms at the uranium mine, eight samples of mine water were collected (Table 2).

Table 2. Enrichment cultures obtained on 9K medium.

Sample No.	Culture No.	Cultivation Duration	Microscopy	Fe^{3+} Content (g/L)	Contaminating Microflora
531/10	531	26 days	10–15 motile small rod-shaped cells in field of view	3.15	Absent
532/10	532	-	No growth	-	-
533/10	533	-	No growth	-	-
534/10	534	9 days	Motile small rod-shaped cells, single and paired	6.6	Microscopic fungus
535/10	535	12 days	Motile small rod-shaped cells, single and paired	6.72	Microscopic fungus
536/10	536	23 days	Motile small rod-shaped cells, up to 10 per field	2.84	Microscopic fungus
537/10	537	16 days	Motile small rod-shaped cells, single and paired	7.36	Microscopic fungus
538/10	538	29 days	Up to 15 motile small rod-shaped cells in field of view	3.1	Absent

A. ferrooxidans bacteria were found in the samples, which allowed us to draw a conclusion about the possible influence of this type of bacteria on the leaching process. The data on sampling are presented, and experiments were conducted on the reproduction of bacteria on the 9K medium to identify active and adapted strains. The studies made it possible to isolate bacterial strains in laboratory conditions for subsequent reproduction and a series of experiments. An important result of the work is the presence of *A. ferrooxidans* bacteria at the uranium deposit, which affect the process of underground leaching. This type of bacteria was recorded in all samples taken, including in the leaching solution after ion exchange columns (standard CHK-3M model column, Russia) in the basin of leaching solution.

Six enrichment cultures of iron-oxidizing bacteria growing at a temperature of 8 ± 1 °C were obtained, of which no extraneous microflora was detected in isolates 531 and 538 under direct microscopy; a microscopic fungus was present in the remaining samples. A 200% higher oxidizing capacity was noted for enrichment cultures No. 534, 535, and 537 compared to other samples. Over a period of 9–16 days, the content of trivalent iron

(Table 2) exceeded the content of trivalent iron in other samples. Iron-oxidizing bacteria were not isolated from mine water samples with a pH of 5.0 (No. 532, 533).

The biochemical parameters of the working solution were determined: pH—1.5; Fe^{2+} content—3.25 g/L; and Fe^{3+} content—0.15 g/L. An experiment was conducted on the possibility of growth of the isolated isolates No. 531, 534, 535, and 537 in the working solution. Weak growth of the culture in the working solution was noted, the color of the culture liquid was yellow, which might be due to the low pH of the solution and the low content of ferrous iron. In the next experiment, 9K nutrient medium was added to the working solution in a ratio of 1:1 and 10% of the seed culture. The seed culture was not added to the control variants (K 1—working solution, K 2—working solution + 9K (1:1), K 3—9K medium). The cultivation was carried out on a shaker with a rotation speed of 180 rpm at a temperature of 8 ± 1and 18 ± 1 °C for 5 days (Table 3).

Table 3. Dynamics of Fe^{3+} accumulation by mixed culture No. 535 on 9K medium.

Day	Microscopy	pH	Fe^{3+} Content (g/L)	Comments
0	No growth	1.5	0.1	Inoculated with sample No. 535
2	Single motile small rod-shaped cells	1.4	0.36	-
4	Motile small rod-shaped cells	1.4	0.85	-
6	Motile small rod-shaped cells	1.4	2.4	-
9	Motile small rod-shaped cells	1.4	5.3	Microscopic fungi appear
12	Motile small rod-shaped cells, fungi	1.3	6.72	-

As can be seen from the results of the experiment, the isolates differ in the activity of oxidation of divalent iron. At a temperature of 18 ± 1 °C, the oxidizing capacity of the culture increases (Table 4). The highest oxidizing capacity was noted in isolates 538, 535, and 536, which oxidized almost all the divalent iron contained in the working solution (3.25 g/L), which is 80% faster than at a temperature of 12 °C. In the working solution, the native culture of iron-oxidizing bacteria begins to actively develop when salts of the 9K (K 2) medium are added to the working solution.

Table 4. Growth of isolates in a working solution with 9K medium at different temperatures.

Culture	Temperature			
	8 °C		18 °C	
	Microscopy Moving Cells in the Field of View	Content Fe^{3+}, g/L	Microscopy Moving Cells in the Field of View	Content Fe^{3+}, g/L
Isolate 531	until 12	2.8	3–4	2.9
Isolate 534	2–3	2.6	until 10	2.9
Isolate 535	5–6	3.1	5–6	3.1
Isolate 536	until 10	2.8	until 10	3.1
Isolate 537	5–6	2.9	5–7	2.97
Isolate 538	10–12	3.1	10–12	3.31
K 1—working solution	absence of growth	-	absence of growth	-
K 2—working solution + 9K	until 5	2.9	3–4	3.1
K 3—medium 9K	absence of growth	-	absence of growth	-

The effect of salts included in the 9K nutrient medium on the growth of iron-oxidizing bacteria was determined. Cultivation was carried out on a shaker with a rotation speed of 180 rpm at a temperature of 18 ± 1 °C for 5 days.

A. ferrooxidans strain culture No. 538 showed appropriate rate of oxidizing capacity (Table 5).

Table 5. Effect of various salts included in the 9K medium on the growth of iron-oxidizing bacteria.

Models	Microscopy Moving Cells in the Field of View	Parameters During Fermentation		
		pH	Fe^{2+}, g/L	Fe^{3+}, g/L
working solution + $(NH_4)_2SO_4$	2–3	2.14	0	3.72
working solution + K_2HPO_4	until 10	2.48	0	3.92
working solution + $MgSO_4$	4–3	2.07	0	3.86
working solution + KCl	10–12	2.04	0	3.98
working solution + $Ca(NO_3)_2$	5–8	2.04	0	3.78
working solution + II solution of medium 9K * without seeding	10–12; Brown solution	1.98	0	5.6
working solution without seeding	absence of growth	1.94	0	non

* composition of the 2nd solution of 9K medium: $FeSO_4 \times 7H_2O$—44.2 g/L; 10N H_2SO_4—1 mL.

The diagram helps identify optimal pH ranges for maximum solubility or precipitation of specific metal ions. Moreover, Figure 1 illustrates conditions under which bioleaching processes could be enhanced or inhibited due to ion speciation down to concentration 0.1 nM/L. The buffering behavior of phosphate in the system and the competition between sulfate and phosphate for metal complexation. Finally, we predict the importance of pH control in processes like iron oxidation, metal recovery using microbial metabolism involving *Acidithiobacillus ferrooxidans*.

As one can conclude from Figure 1, Fe^{3+} dominates at low pH as Fe^{3+} and Fe^{2+}. With increasing pH, $Fe(OH)^{2+}$, $Fe(OH)_3$, and other hydrolyzed forms become more stable. $FePO_4$ and other phosphate complexes are seen at mid pH levels. The presence of solid Fe_2O_3(cr) (brown line) and $FePO_4$(s) (precipitates) at higher pH indicates potential for precipitation (Figure S2A). It is well-known that phosphoric acid exists in various protonation states depending on pH, for example, at low pH H_3PO_4, $H_2PO_4^-$ while at neutral pH HPO_4^{2-}, PO_4^{3-}. Moreover, complexes with metals like Mg, Fe, and NH_4^+ mainly present as Mg^{2+} and $MgSO_4$, with increasing formations of $MgHPO_4$ and $MgPO_4^-$ complexes as pH increases. It exists mostly as free SO_4^{2-} and in complexes like $FeSO_4^+$, $FeHSO_4^{2+}$, and $NH_4SO_4^-$ depending on pH (Figure S2A). The log concentration of free ions decreases with increasing pH due to complexation or precipitation. Precipitates such as $FePO_4$(s) and Fe_2O_3(cr) form as pH rises, indicating reduced solubility of Fe species. Figure S2C provides a more detailed understanding of species distribution and equilibrium dynamics at strongly acidic conditions at the beginning of the process, which are highly relevant to microbial leaching and acid mine drainage systems.

Fe^{2+} cations dominate the system across the entire pH range. Minor formation of $FeHSO_4^+$, $FeSO_4$, $FeH_2PO_4^{2+}$, and $FeHPO_4$ indicates interactions with sulfate and phosphate. Complexes like $MgHPO_4^+$, $FeH_2PO_4^{2+}$, and $NH_4H_2PO_4$ are observed (Figure S2C). Precipitation of $FePO_4 \cdot 2H_2O$(s) occurs above pH ~2.4, a key marker for iron–phosphate interactions, and present as Mg^{2+}, $MgSO_4$, and phosphate complexes like $MgHPO_4^+$; $MgPO_4^-$ NH_3 and $Fe(NH_3)_2^+$ species emerge above pH 2.2.

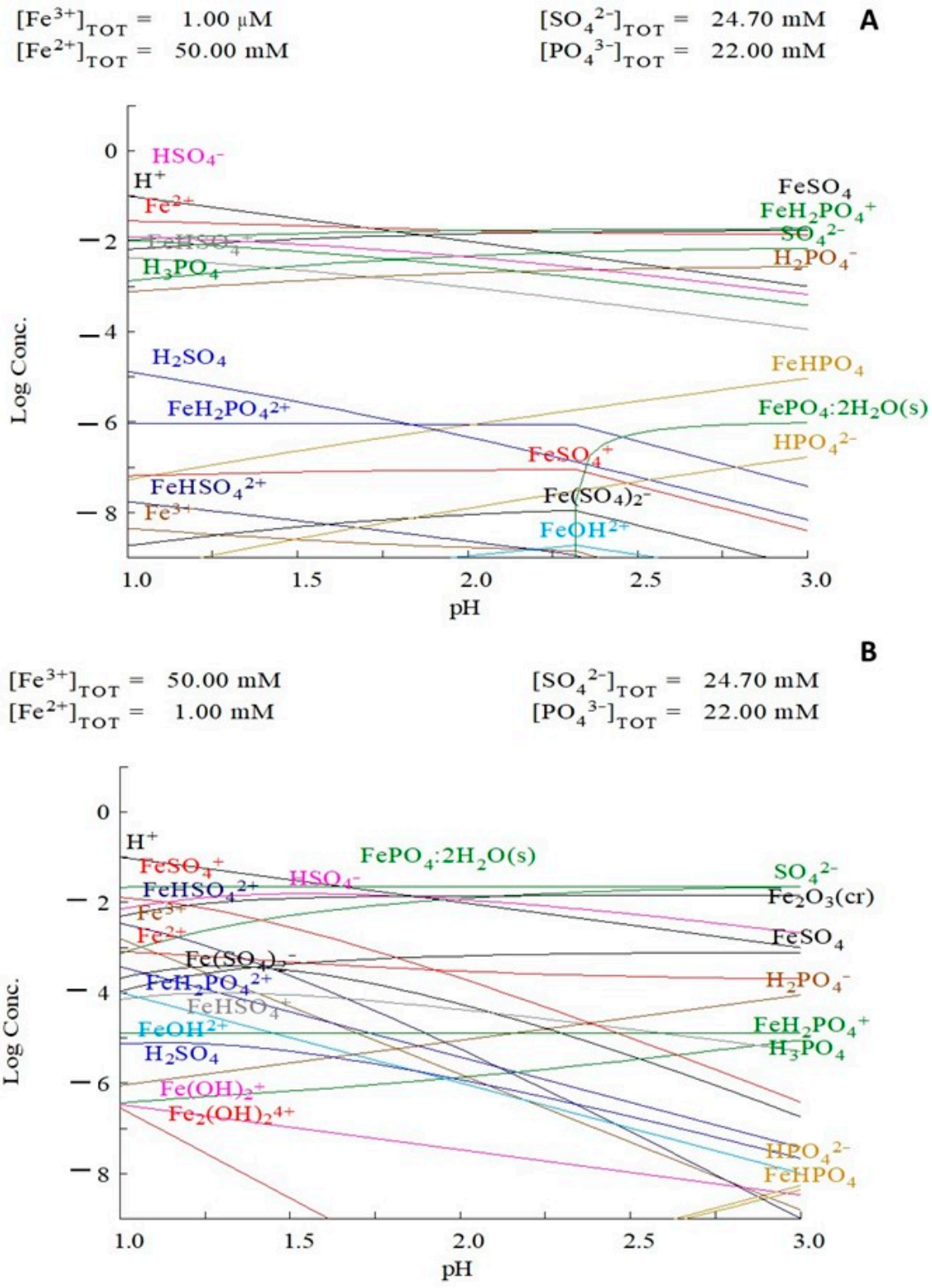

Figure 1. Speciation of iron ions in model solution: $[PO_4^{3-}]_{tot}$ = 22.0 mM; $[Fe^{3+}]_{tot}$ = 50.0 mM. $[Fe^{2+}]_{tot}$ = 0.01 mM; $[SO_4^{2-}]_{tot}$ = 24.7 mM in a pH range of 1–3.0. (**A**) State of the ion system before leaching; (**B**) the system after leaching at state of equilibrium.

This pH range is representative of acidic leaching environments or acidophilic microbial habitats (such as for *Acidithiobacillus ferrooxidans*). The dominance of Fe^{2+} supports bacterial oxidation as the main driver of Fe^{3+} production in bioleaching. Fe^{3+} solubility remains low, but phosphate complexation and precipitation as $FePO_4 \cdot 2H_2O$(s) may act as

a limiting factor for iron bioavailability (Figure 1A). Ammonium and phosphate species show strong potential for complexation, influencing microbial metabolism and nutrient availability. Precise pH control is critical to avoid unwanted precipitation (e.g., $FePO_4$ solids) that could reduce iron availability. Microbial oxidation efficiency could be affected by the availability of Fe^{2+} and phosphate complexes. Operating this modeling data helps in designing and optimizing bioleaching or bioreactor systems by identifying species behavior under acidic conditions.

It was noted that when adding the II solution of the 9K medium to the working solution, the native iron-oxidizing bacteria present in the working solution are activated, which is confirmed by a change in the color of the culture liquid from yellow to brown. In other variants, in the presence of bacterial cell growth, the color of the culture liquid is pale orange. Thus, the potential activity of iron-oxidizing bacteria was determined by the presence and amount of divalent iron ions in the medium.

Production of *Acidithiobacillus ferrooxidans* biomass culture were carried out via fermentations in Erlenmeyer flasks (Table 6). The data presented in Table 7 indicate a significant increase in Fe^{3+} concentration over the 9-day observation period, rising from an initial value of 0.2 g/L to 7.1 g/L. This significant increase suggests active biological oxidation of Fe^{2+} to Fe^{3+}, likely mediated by microbial processes following immobilization. The pH remained relatively stable around 2.0 throughout the experiment, which is conducive to the activity of acidophilic iron-oxidizing microorganisms.

Table 6. Oxidation of Fe^{2+} by *Acidithiobacillus ferrooxidans* culture No. 535.

Sample	Fe^{3+} Content (g/L)	pH	Comments
Initial solution (before inoculation)	0.2	2.1	-
After 3 days	2.9	2.1	Slight fungal presence
After 7 days	5.3	2.0	Fungi developed
After 10 days	6.7	2.0	Mycelium and spores observed

Table 7. Oxidation of uranium-bearing productive solution by culture No. 535 immobilized on porous polypropylene.

Sample	Fe^{3+} Content (g/L)	pH	Comments
Initial solution	0.2	2.1	Before immobilization
After 3 days	3.6	2.0	-
After 5 days	5.1	2.0	-
After 7 days	6.2	2.0	Slight fungal presence
After 9 days	7.1	2.0	Mycelium detected

10 L of biomass of *Acidithiobacillus ferrooxidans* culture pcs. M in the form of culture liquid were obtained. This work required long-term laboratory studies in order to isolate individual bacteria with their subsequent reproduction on the 9K medium. Isolation of a pure strain is a key step in studying the effect of bacteria on the process of underground borehole leaching of uranium by conducting experiments with the strain "B-RKM 0767" (identification of RNA analysis is shown above). For comparison, previously studied bacterial strains were presented (Figure S1 photo—B-RKM 0767 taken from previously

conducted studies of the Research Laboratory "BioGeoTechnology of gold, uranium and polymetallic ores"). For the purpose of comparing the processes, studies were conducted on a strain grown at other deposits, directly used in the laboratory. The experiments showed high activity of the strain B-RKM 0767 to leaching solutions of the uranium deposit. A decision was made to use this strain of strain B-RKM 0767 for conducting pilot industrial studies. To identify the strain B-RKM 0767, a genomic analysis was carried out, which confirmed with a probability of 99% that this strain belongs to the bacteria *A. ferrooxidans*.

3.2. Cultivation of Bacteria for Use in Industrial Conditions

At the first stage, adaptation of bacteria and cultivation of active strains of strain B-RKM 0767 were carried out. A series of experiments were conducted using bacteria on a production solution—sorption mother liquor with the addition of nutrient salts. The composition of the production solution is as follows: sorption mother liquor Fe^{2+}—2.7 g/L, H_2SO_4—3.5 g/L, and sulfates up to 20 g/L. One of the most important parameters of bacteria is the addition of nutrient salts, which ensures the growth and activity of bacteria. For these purposes, a series of experiments were conducted with the addition of salts in accordance with the calculation of the volumes of the used production solution (KH_2PO_4—3.0 g/L; $MgSO_4 \times 7H_2O$—0.5 g/L; $(NH_4)_2SO_4$—3.0 g/L; KCl—0.1 g/L; $Ca(NO_3)_2$—0.01 g/L) (according to the 9K environment). As a result, it was found that the effect of salts is minimal. It was found that the production solution contains the components necessary for the reproduction of bacteria. This is one of the key results obtained in the course of these experiments, which made it possible to simplify the task of using bacterial leaching in industrial conditions. A series of experiments were conducted on the effect of nutrients on the reproduction of bacteria directly on the production leaching solutions. It has been established that the limiting stage is the presence of divalent iron, which corresponds to the conditions of this uranium deposit, where there is a problem of converting divalent iron into trivalent iron to increase the oxidation-reduction potential of the leaching solution. In general, for leaching solutions, it has been established that the main limiting stage of bacterial reproduction directly on the leaching solutions (sorption mother liquor) is the supply of air (oxygen). The oxygen content in the initial solution was 6–7 mg/L, which corresponds to the solubility of oxygen in aqueous solutions. The supply of compressed air (by aeration) at the rate of 1 m^3 air/h/m^3 solution allowed for the conversion of all divalent iron into the trivalent form within 24 days. For the oxidation process of 112 g of divalent iron of 16 g of oxygen are required. The sorption mother liquor contains 2.7 g/L of iron. Thus, to oxidize this amount of iron, a minimum of 0.385 g/L of oxygen is required (which is almost 50 times more than dissolved oxygen).

4. Discussion

The study of uranium deposit leaching solutions showed that the average sulfuric acid content is from 3 g/L to 5 g/L, at pH 1.5–2, the content of divalent iron is 2.7 g/L. These indicators are stable due to the use of storage pools for leaching and productive solutions with a volume of over 2000 m^3 each. Thus, there is an averaging of the compositions of both productive solutions and leaching solutions. Oxidation of divalent iron is described by the main equations:

$$2FeSO_4 + ½\ O_2 + H_2SO_4 = Fe_2(SO_4)_3 + H_2O \quad (1)$$

$$UO_3(s) + H_2SO_4(l) \rightarrow UO_2SO_4(l) + H_2O(l) \quad (2)$$

$$UO_2(s) + H_2SO_4\ (l) \rightarrow UO_2SO_4(l) + 2H^+\ (l) \quad (3)$$

The reaction takes place in the presence of iron-oxidizing bacteria which act as a catalyst for this reaction. A control experiment in the laboratory in one-liter flasks on a sorption mother liquor with aeration with and without bacteria also indicated the need for the presence of iron-oxidizing bacteria in the solution. As a control experiment in the productive solution processing shop, aeration was carried out in a cube tank under similar conditions without adding iron-oxidizing bacteria for 24 h. At the same time, the content of divalent iron in the solution did not change. This confirmed that for effective oxidation of divalent iron for these production solutions, the presence of adapted iron-oxidizing bacteria is necessary.

The data support the previously proposed assumption that temperature is a critical factor in intensifying the oxidation process. The specific oxidation rate of Fe^{2+} within the studied temperature ranges is proportional to the specific growth rate of *A. ferrooxidans*. This relationship is inferred from experimental curves showing bacterial growth and iron oxidation at various temperatures. As a first approximation, the process can be described using three main variables the concentration of ferrous iron (Fe^{2+}), the concentration of ferric iron (Fe^{3+}), and the medium's acidity. Under actual conditions at the uranium deposit, the total iron content (sum of Fe^{2+} and Fe^{3+}) remains relatively stable. This allows the process to be effectively described using only two primary variables ferrous iron concentration and pH. It should be noted that temperature tends to remain relatively stable due to averaging over short periods. However, in summer, temperatures may increase from 10–12 °C to 14–16 °C. Within the studied pH range of 1.5–2.0, the relationship between acidity and sulfuric acid concentration is approximately linear. According to the oxidation reaction (Equation (1)), one molecule of sulfuric acid is required to oxidize two molecules of ferrous iron. Therefore, when oxidizing 1 g/L of Fe^{2+} to Fe^{3+}, the sulfuric acid content decreases by approximately 0.88 g/L. This leads to a corresponding increase in pH and a rise in the oxidation-reduction potential of the solution. These findings support the feasibility of using iron-oxidizing bacteria directly in situ, within the ore-bearing layer.

Experiments on growing native bacterial strains on 9K medium showed their ability to reproduce and oxidize divalent iron to trivalent iron by iron-oxidizing bacteria at temperatures corresponding to the technological mode of the uranium deposit (8–10 °C). It was found that bacteria in individual samples on the 9th day began to actively reproduce and convert divalent iron to trivalent (the solutions turned into a characteristic red color). This confirmed the fact about the potential possibility of using bacterial leaching at the uranium deposit at relatively low temperatures. A control experiment without bacteria on the sorption mother liquor showed that oxidation of divalent iron does not occur. Dissolved oxygen in the solution in an amount of up to 8 mg/L is not enough to oxidize the volume of divalent iron. A special factor is the temperature regime. It was found that the process of reproduction and manifestation of bacterial activity occurred at 80 °C. When the temperature increases to 180 °C, the oxidation process of divalent iron occurs 300% faster. Another key factor in the process is the need for aeration. Analysis of the balance of the chemical reaction of divalent iron oxidation shows the need for aeration of the solution due to the insufficiency of dissolved oxygen for the oxidation of 2.7 g/L of divalent iron. It is necessary to ensure a minimum supply of oxygen (air in terms of oxygen) at the rate of 0.385 g/L of pure oxygen per liter of solution. For production conditions, higher oxygen supply volumes will be required due to the assimilation of oxygen by bacteria.

Previously researchers used Medusa software v.2.0.5 to predict uranium species in solution in a wide pH range. Thus, at low pH, uranium is mostly present as free uranyl ion (UO_2^{2+}) and its nitrate complex. Then, as pH increases, hydrolysis of uranyl begins, leading to the formation of polymeric hydroxo species and eventually precipitation as uranium hydroxide. Ultimately, at high pH, soluble anionic hydroxo complexes dominate.

UO_2^{2+} is predominant in strongly acidic conditions (pH < 3). $UO_2NO_3^+$ is major species from approximately pH 2 to 4.5, which indicates complexation of uranyl with nitrate, common in nitric acid solutions. $(UO_2)_2(OH)_2^{2+}$ can be found in the intermediate pH range (~3.5–5.5) in model conditions. Of course, this is just for restricted conditions and in real case scenarios there are may be much more salts [29].

Previously it was stated that water soluble uranium(VI) can undergo partial reduction to uranium(IV) and/or uranium(V)-containing precipitates (such as U_3O_8 and U_4O_9) in the presence of Tamusu claystones, with this process being more favorable under acidic conditions. The reduction in U(VI) is primarily driven by the leaching of structural Fe^{2+}, followed by surface adsorption and interfacial reactions. Under alkaline conditions, organic matter may become the dominant factor in facilitating the partial reduction in U(VI). Additionally, phosphorus-rich sites on the surface of Tamusu claystones act as reactive centers for uranium accumulation, suggesting the potential formation of U(VI)- and/or U(IV)-phosphate mineral phases. Furthermore, the presence of trace amounts of Fe^{3+} in the claystones may contribute to the partial oxidation of high-purity UO_2 to form U_4O_9 and/or U_3O_8 [30].

5. Conclusions

Six isolates of chemolithotrophic iron-oxidizing bacteria capable of growing and oxidizing ferrous iron at temperatures of 8 ± 1 °C and 18 ± 1 °C were isolated from mine water samples collected from boreholes at a uranium deposit. It was observed that the isolates differ in their ferrous iron oxidation activity. At 18 ± 1 °C, the bacterial oxidation activity significantly increases. The working solution contains an active indigenous culture of iron-oxidizing bacteria, which begins to proliferate intensively upon the addition of 9K medium in a 1:1 ratio. The potential activity of iron-oxidizing bacteria is primarily determined by the presence and concentration of ferrous iron ions in the medium. For production-scale testing, 10 L of *Acidithiobacillus ferrooxidans* strain "M" biomass were prepared in the form of culture liquid with a cell titer of 10^8 CFU/mL. *A. ferrooxidans* bacteria are naturally present in the leaching solutions at the uranium deposit. A control experiment using leaching solution without added bacteria confirmed their indigenous presence and biological activity. The B-RKM 0767 strain demonstrated activity at 8 °C, though at a significantly lower rate than at 18 °C. This allows for simplification of the industrial process for bacterial application at the deposit. The production solution contains essential nutrient salts required for bacterial reproduction. The limiting factors are the availability of ferrous iron, which provides energy, and oxygen (air supply). Strain B-RKM 0767 exhibited high activity under the specific conditions of the uranium deposit. Further studies using this strain utilizing various medium was culturing medium will be considered. Other strains isolated from the leaching solutions will be utilized in future research. Genomic analysis of strain B-RKM 0767 (16S rRNA gene sequencing) confirmed with 99% probability that the strain belongs to the species *Acidithiobacillus ferrooxidans*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/min15070727/s1, Figure S1. Three cube tanks for studying bacterial growth on the sorption mother liquor; Figure S2. Speciation of iron ions in model solution: $[Mg^{2+}]_{tot}$ = 2.00 mM; $[PO_4^{3-}]_{tot}$ = 22.00 mM; $[Fe^{3+}]_{tot}$ = 50.00 mM. $[Fe^{2+}]_{tot}$ = 10.00 nM; $[NO_3^-]_{tot}$ = 0.12 mM ; $[NH_3]_{tot}$ = 45.40 mM $[SO_4^{2-}]_{tot}$ = 24.70 mM in a pH range of 1.0–2.5.

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