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REGULATION OF THE STRAIN *Citrobacter freundii* ML-31.1/1 INTERACTION WITH IRON COMPOUNDS

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The aim of the work was to show the possibility for the strain *Citrobacter freundii* Ml-31.1/1 on the base of its metabolism regulation to carry out two opposite processes: mobilization of iron compounds and their immobilization. To do this investigation thermodynamic calculations were used. It was also used potentiometric measuring of pH and Eh values, colorimetric determination of microbial biomass increase and changes of iron compounds concentration and gas chromatography. The possibility of theoretically grounded regulation of iron transformation pathways was experimentally confirmed. Taking as an example the culture *C. freundii* Ml-31.1/1, we have shown its ability to provide both immobilization and mobilization of iron compounds under specific conditions for microbial growth in nutrient medium. Obtained regularities are the basis to estimate the role of microorganisms in iron biogeochemical cycles. These data could be used as a base for the development of effective biotechnological approaches such as iron extraction from depleted deposits and water purification from iron compounds.

Key words: thermodynamic prognosis, microbial interaction with iron, mobilization, immobilization, iron compounds, iron biogeochemical cycles, biotechnology of environment protection.

Iron is the fourth abundant element in the earth's crust [1, 2]. This element makes significant influence on the biogeochemical cycles in ecosystems due to its widespread, the ability to change the valence and to form complex compounds with organic matter [3–6].

Transformation of iron compounds is a multistage process. It includes a complex of reactions [2]. Microorganisms possess a significant role in the process of iron cycling in the biosphere. Redox potential, pH, water regime, structure of iron minerals and microbial activity determine the speed and intensity of iron transformation in ecosystems.

Microbial technologies have a set of advantages for both water purification from iron compounds and iron extraction from depleted deposits. Microbial biotechnologies have low operating cost. They are also environmentally friendly. That is why investigation of microbial metabolism regulation is actual for development of novel environmental biotechnologies.

Thermodynamic prognosis allows to calculate theoretically feasible pathways of iron compounds transformation by microorganisms [7, 8]. It is well known that microorganisms can reduce and oxidize iron compounds as well as mobilize and immobilize them [1, 3]. However, the conditions of iron transformation are still poorly studied [4].

We showed that the values of pH and redox potential of the medium are the key parameters to estimate the direction of iron transformation pathways [7] (Fig. 1).

Chelated compounds of Fe(III) and Fe(II) are stable in the pH range from 0 to 12.0. Ions of Fe^{3+} are stable at pH < 1.6 (Fig. 1, reaction № 6), and Fe^{2+} ions — at pH < 6.6 (Fig. 1, reaction № 8).

Reactions № 1–3 are within the zone of water thermodynamic stability (from −414 mV to +814 mV), that is why microorganisms can reduce Fe(III) to Fe(II). The reduction reactions of Fe(II) to Fe^0 are outside of this zone (reactions № 4, 5). Consequently, microbial reduction of Fe(II) compounds is impossible.

Creation of the appropriate conditions for microorganisms allows to regulate their interaction with iron compounds and achieve

the desired effect: Fe(III) reduction to Fe(II), mobilization and immobilization of iron compounds.

As the process of microbial transformation of iron compounds can be predicted and regulated, it can be used in both biotechnological approaches: water purification from iron and iron extraction from depleted deposits.

In this regard, the target of the study was the regulation of metabolism of *C. freundii* Ml-31.1/1 on the basis of thermodynamic calculations. The essence of metabolism regulation was in the ability of the microorganism to perform two opposite processes, namely mobilization and immobilization of iron compounds, depending on the culture conditions of the strain *C. freundii* Ml-31.1/1.

Materials and Methods

The object of the study was the strain Ml-31.1/1. According to cultural, morphological, physiological and biochemical properties as well as the results of phylogenetic analysis, the strain Ml-31.1/1 was identified as *Citrobacter freundii*. It is one of the dominant

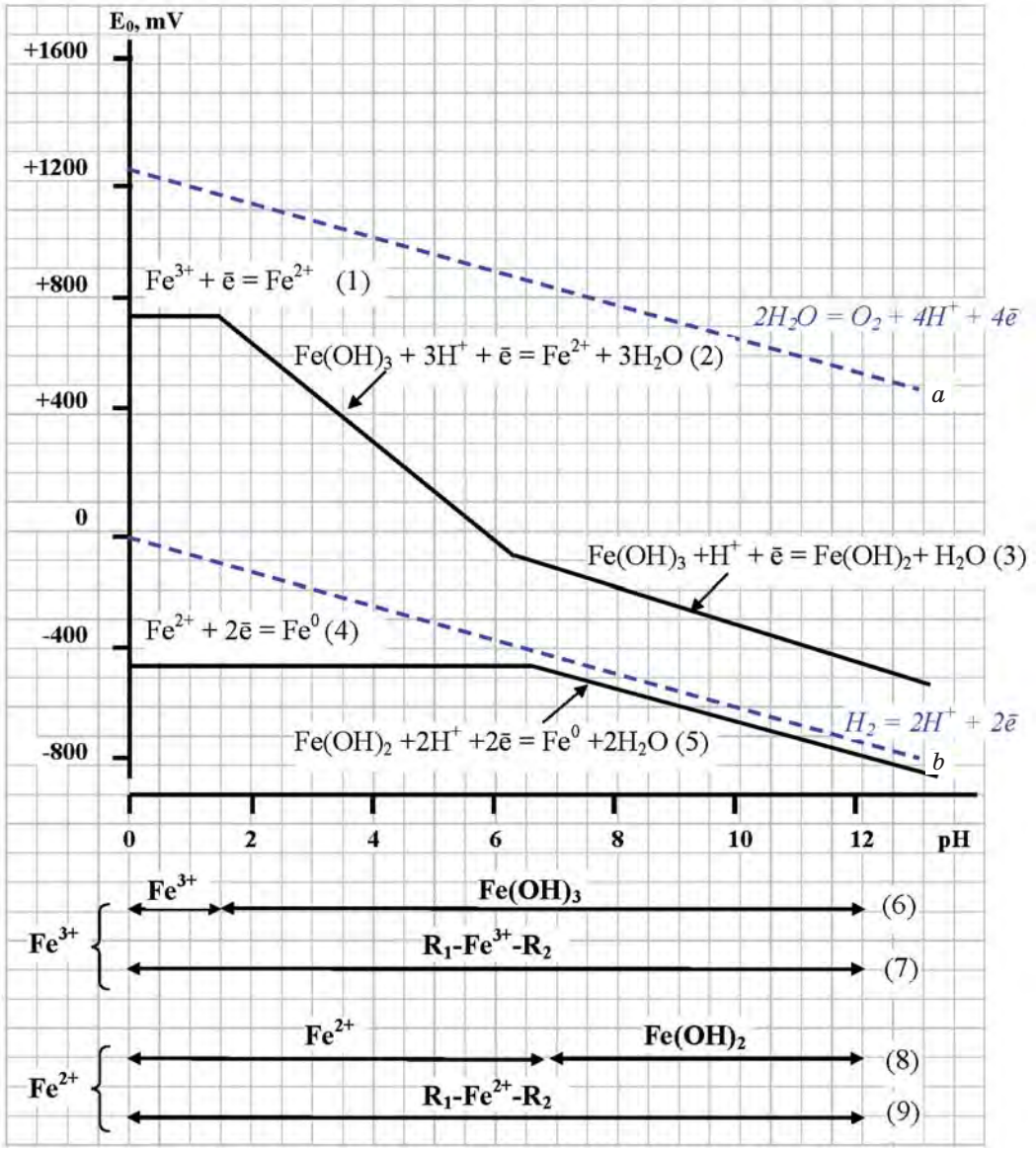

Fig. 1. **Zones of redox-stability of Fe(II) and Fe(III) compounds:**
a (top) and *b* (lower) — limit of thermodynamic stability of water:
a — is described by the equation: $O_2+4H^+ = 2H_2O$ and $Eh = 1.228 - 0.0591 \cdot pH - 0.0295 \cdot \lg PH_2$;
b — is described by the equation: $2H^+ + 2e = H_2$ and $Eh = 0.000 - 0.0591 \cdot pH - 0.0591 \cdot \lg PH_2$;
R_1–, R_2– — organic radicals (organic acids or amino acids)

microorganisms in the river sludge (a widespread natural ecosystem of middle latitude, river Grun, Synivka village, Sumy region, Ukraine). The strain was able to transform iron compounds effectively.

The strain can grow under aerobic and anaerobic conditions. Therefore, *C. freundii* Ml-31.1/1 can realize all theoretically feasible types of microbial interaction with iron compounds (Fig. 1).

Since the foci of the study were two opposite processes of microbial interaction with iron compounds, *C. freundii* Ml-31.1/1 was cultivated in two types of nutrient media. The first nutrient medium was designed for microbial immobilization of iron compounds and the second one was for mobilization of iron compounds. The composition of mineral salts and growth factors was common for both types of the medium. Nutrient medium differed according to carbon and energy sources and iron compounds.

The composition of basic culture medium, g/l:

- NH_4Cl — 1.0;
- K_2HPO_4 — 2.0;
- Na_2SO_4 — 0.5;
- yeast extract — 0.1;
- distilled water — 1 l.

Sodium citrate ($C_6H_6O_7Na_2 \cdot 1.5H_2O$) at the concentration of 0.7 g/l was used as the carbon and energy source in the first variant of nutrient medium. As this medium was supplied for microbial immobilization of iron, it contained soluble compound of Fe(III), namely Fe(III) citrate. Its concentration in the medium was 0.22 g/l.

Citrate of Fe(III) was prepared in the following way. Iron powder (3.0 g) was added to 100 ml flask. Iron powder was dissolved in the concentrated HCl. The acid was added fractionally (by the portions of 2 ml) to the flask with iron under the continuous argon flow. Argon was used to prevent oxidation of low-potential Fe(II) by oxygen of air. To accelerate the reaction, the flask was heated in the boiling water bath. The dissolution of iron took 2 hours. The chelator solution, sodium citrate ($C_6H_6O_7Na_2 \cdot 1.5H_2O$), was added to the flask after iron dissolution. Sodium citrate (15.0 g) was dissolved in 70 ml of distilled water and the solution was added to the flask with iron. Obtained acidic solution of Fe(II) citrate (pH 1.0) was neutralized by addition of 6.0 g of dry sodium hydrogen carbonate ($NaHCO_3$). The neutralized solution of Fe(II) citrate was adjusted to the volume of 100 ml. Further, Fe(II) was oxidized to Fe(III) by oxygen of air. To accelerate the reaction the solution was boiled for 20 min. To complete oxidation Fe(II) to Fe (III) 5.0 ml of 50% hydrogen peroxide solution was fractionally (0.5 ml) added. The absence of red color after addition of *o*-phenanthroline, the specific indicator for Fe(II), indicated complete oxidation of Fe(II) [9]. Solution of Fe(III) citrate was sterilized for 10 min in a sealed flask in the boiling water bath.

Glucose (10.0 g/l) was used as the carbon and energy source in the second variant of the nutrient medium designed to the mobilization of iron. Iron was used in the form of hydroxide $Fe(OH)_3$ at the concentration of 0.16 g/l.

To prepare iron hydroxide (concentration 3.0 g/l of Fe^{3+}) 8.7 g of $FeCl_3$ was dissolved in 100 ml of distilled water. Dry sodium carbonate (Na_2CO_3) (6.0 g) was added with stirring to obtained Fe(III) solution to precipitate iron (pH 7.0). Obtained $Fe(OH)_3$ precipitate was washed three times in distilled water by centrifugation and supernatant decantation. Washed $Fe(OH)_3$ precipitate was suspended in 100 ml of distilled water. Iron hydroxide suspension was sterilized in a sealed flask for10 min in the boiling water bath.

Nutrient medium (100 ml) and 3 ml of microbial suspension in 0.9% saline solution (McFarland standard 6 — $1.8 \cdot 10^9$ CFU/ml of cell suspension) were added to the flasks (volume 500 ml). Daily *C. freundii* Ml-31.1/1 culture grown on meat-peptone agar with Fe(III) citrate (0.5 g/l of Fe^{3+}) was used as the inoculum. The flasks with the medium for iron immobilization (first variant) were hermetically sealed in atmosphere of air. In aerobic conditions microorganisms consumed citrate since it was the only source of carbon and energy in the medium as well as chelator in the complex [Fe(III)-citrate]. That naturally led to the destruction of the complex and Fe(III) precipitation.

To ensure iron mobilization, microorganisms were cultured under the conditions of limited aeration. Flasks with medium for iron mobilization (second variant) were purged by argon. Partial replacement of air by argon (O_2 concentration was 10%) shifted microbial metabolism to glucose fermentation and Fe(III) mobilization by metabolic products (organic acids). Cultivation was performed at 30 °C during 16 days. Nutrient medium without inoculum was used as a sterility control. The experiment was performed in triplicate. Statistic analysis of experimental data was carried out using OriginPro 8.5.1, $P \leq 0.05$.

Metabolic activity of the strain was evaluated by such parameters: changing of pH and Eh values, oxygen consumption, synthesis of hydrogen and carbon dioxide, biomass growth, quantity of mobilized and immobilized Fe(III) and Fe(II) compounds.

The pH and Eh value of culture liquid was measured potentiometrically (pH-meter-millivoltmeter "pH-150 MA"). To measure the pH value, porous glass electrode ESK-10603/4 was used. Redox potential was measured by platinum electrode EPV-1. Silver chloride electrode EVL-1M3 served as the reference one.

The composition of the gas phase in the flask was determined by the standard method by gas chromatograph LHM-8-MD [10]. Gas phase composition (N_2, CO_2, N_2 and O_2) was calculated by peak square of the gas phase components.

The content of Fe(III) and Fe(II) was determined by colorimetric methods. The concentration of Fe(II) was determined using *o*-phenanthroline [9]. For this purpose 0.75 ml of 0.25% *o*-phenanthroline solution was added to 1.5 ml of the sample. The presence of Fe(II) was indicated by the appearance of red-orange color due to the interaction of Fe(II) compounds with *o*-phenanthroline. The measurement of Fe(II) was conducted using the photoelectric colorimeter (KFK-2MP) at λ = 490 nm and an optical path length of 0.5 cm.

The presence of Fe(III) was evidenced by red-colored complex of Fe(III) with potassium rhodanide under acidic conditions [9]. Rhodanide (0.25 ml of 1.5 M KSCN) and 0.75 ml of concentrated HCl were added to 1.5 ml of the sample. The presence of Fe(III) was indicated by the appearance of the red color. Concentration of Fe(II) was measured by photoelectric colorimeter (KFK-2MP) at λ = 490 nm and optical path length of 1 cm.

Concentration of mobilized and immobilized iron compounds was measured as follows. Culture liquid (6 ml) was centrifuged at 2655 g during 15 min. The supernatant was decanted and used to quantify dissolved iron compounds by the method described above.

The precipitate consisting of bacterial biomass and insoluble iron compounds was suspended in the same volume (6 ml) of citric acid solution (concentration 3 g/l). The solution of citric acid was used as a chelating compound for dissolving the precipitated iron compounds and their colorimetric determination. To precipitate microbial cells obtained solution was again centrifuged at 2655 g for 15 minutes. The supernatant was decanted and used to determine the concentration of the iron compounds chelated by citric acid.

Washed microbial biomass was suspended in 6 ml of 0.9% saline solution. Optical density of suspended biomass was measured by photoelectric colorimeter KFK-2MP at λ = 540 nm, cuvette 0.5 cm.

Microbial suspension was centrifuged one more time at 2655 for 15 min. The supernatant was decanted and precipitated biomass of microorganisms was used to determine the concentration of intracellular iron compounds. For this purpose precipitate was suspended in 6 ml of 10% solution of HCl for 15 min. Microbial cells were lysed under strong acidic conditions. Iron compounds became available for measurement. Acidic conditions complicate measuring of Fe(II) concentration with o-phenanthroline. Colored complex of Fe(II) with *o*-phenanthroline at the pH < 2.0 develops slowly and is weakly expressed [9]. Therefore, to accurately measure Fe(II) concentration in the cell fraction, the obtained acidic solution was neutralized by adding of dry Na_2CO_3.

Results and Discussion

Thermodynamic evaluation of iron compounds stability fields is the tool for prediction of iron transformation by microorganisms. Microorganisms can oxidize and reduce iron compounds, as well as mobilize and immobilize them. The values of pH and redox potential of medium are determining factors that make influence on the redox state of iron (Fig. 1) [7, 8].

Based on the composition of culture medium and conditions of microbial growth, we can control and regulate the pathways of iron compounds transformation.

To verify this hypothesis we cultivated the strain *C. freundii* Ml-31.1/1 in two types of nutrient medium.

Cultivation of *Citrobacter freundii* Ml-31.1/1 in the nutrient medium of the first type (in the air atmosphere) facilitated immobilization of iron compounds. In this case Fe(III) citrate was used as soluble iron compound, and dibasic sodium citrate was the only donor of electrons. Therefore, the mechanism of iron precipitation is in microbial consumption of the citrate that is both source of electrons and chelator in [Fe(III)-citrate] complex. That should lead to degradation of [Fe(III)-citrate] and iron precipitation.

Destruction of Fe(III)-citrate complex by microorganisms causes immediate Fe(III) precipitation as Fe^{3+} cations are stable only

under acidic conditions at the pH < 1.6 (Fig. 1, reaction № 6).

Conditions of cultivation in the second variant of medium supplied iron mobilization. Microorganisms grew under lowered concentration of oxygen (10%). Glucose was used as a single source of electron and energy. Iron was in the form of an insoluble hydroxide $Fe(OH)_3$. Organic acids (chelators) that mobilize iron compounds were accumulated in the medium during glucose degradation (Fig. 1, reactions № 7, 9).

The strain carried out theoretically grounded types of iron compounds transformation: mobilization (reduction and chelation) and immobilization.

Fig. 2 shows increase of microbial biomass, decrease of pH and redox potential of the medium in the presence of Fe(III) citrate (*a*) and ferric hydroxide $Fe(OH)_3$ (*b*). Microorganisms reached the stationary growth phase after two days of cultivation. However, biomass growth in glucose medium was 4.4 times higher than in the medium with citrate since glucose is energetically more benefitial for microorganisms.

The pH of the medium with citrate remained within the range 7.1–7.5 (Fig. 2, *a*). Whereas values of pH was 2.1 times lower in the medium with glucose.

Redox potential of the medium with citrate was in the range of +307...+369 mV (Fig. 2, *a*). Microorganisms lowered redox potential to –105 mV on glucose medium (Fig. 2, *b*).

Thermodynamic calculations indicated that high values of pH and Eh provided immobilization of iron compounds when cultivation of microorganisms were carried out in the medium with citrate. On the other hand, low pH and Eh of the medium with glucose predetermined iron compounds mobilization, and the possibility of reduction of Fe(III) to Fe (II) (Fig. 1).

Fig. 3 presents the data of the gas phase changes during the growth of microorganisms in the presence of Fe(III) compounds.

Oxygen concentration decreased per day from 21% to 15% in the medium with citrate. The minimal concentration of O_2 (16 days) was 14.2%. Oxygen concentration decreased from 8.1 to 0% in 8 days when glucose was used as the medium. In the medium with citrate, hydrogen concentration did not exceed 0.07%, and in the medium with glucose it was 2.6% during the first day of cultivation. Maximal concentration of carbon dioxide in the medium with citrate was 6.1% after 2 days of cultivation. In glucose medium maximal CO_2 concentration reached 26.5% after 8 days of growth.

Regulation of microbial metabolism allows carrying out mobilization and immobilization of iron compounds. As theoretically calculated (Fig. 1) microorganisms immobilized Fe(III) in the medium with citrate, and mobilized it in glucose medium.

Fig. 4 shows immobilization and mobilization of iron compounds by the strain *C. freundii* Ml-31.1/1.

Iron immobilization correlated with biomass growth in the medium with citrate. During the growth microorganisms consumed citrate (single carbon and energy source) that was chelating agent for iron. Cations of Fe^{3+} precipitate at the pH > 1.6 [7, 8] (Fig. 1,

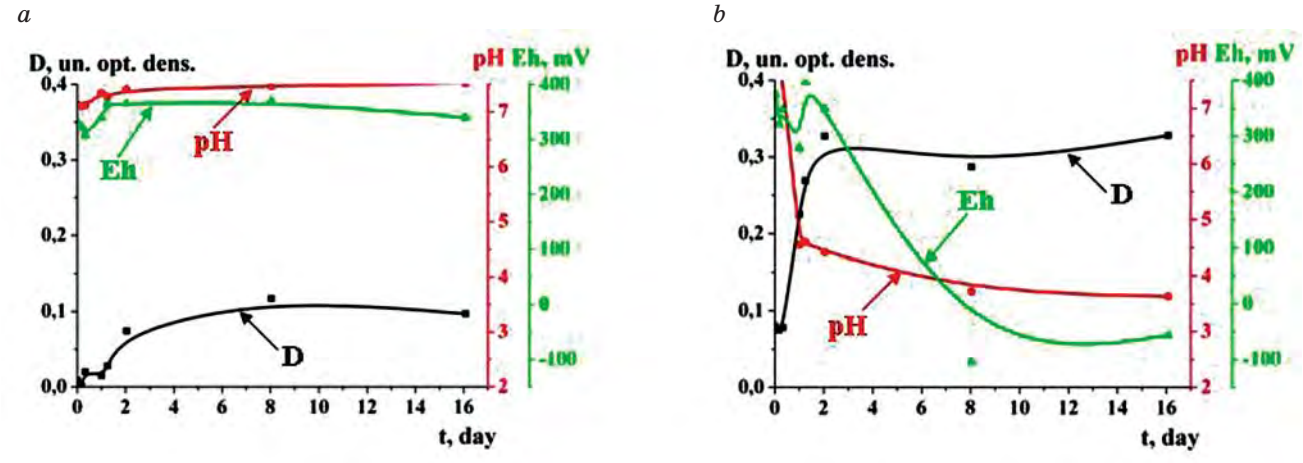

Fig. 2. **Regularities of the strain *Citrobacter freundii* Ml-31.1/1 growth:**
a — medium with citrate; *b* — medium with glucose (* $P \leq 0.05$)

reaction № 6). Therefore microbial destruction of chelator (citrate) leads to inevitable Fe(III) precipitation as the pH of the culture medium is 7.1 (Fig. 4, *a*).

In glucose medium iron compounds mobilization coincided with the stationary growth phase of microorganisms. During this period, organic acids (products of glucose fermentation) must have been accumulated, which was evidenced by pH decrease. Insoluble iron compounds presumably were chelated by organic acids and transformed into soluble complexes (Fig. 4, *b*) under these conditions (Fig. 1, reactions № 7, 9).

Substantial difference of potentials between the donor and acceptor systems is the necessary condition for Fe(III) reduction. Metabolically active microorganisms (donor system) reduced redox potential of the medium from +320 mV to −105 mV in the medium with glucose. These conditions promote reduction of Fe(III) to Fe(II) (Fig. 5). Fe(III) reduction logically took place at low values of redox potential. At the same time, Fe(III) reduction didn't occur in the medium with citrate, as redox potential of the medium was +304 mV.

The strain reduced insoluble $Fe(OH)_3$ to soluble Fe(II) in the stationary growth phase.

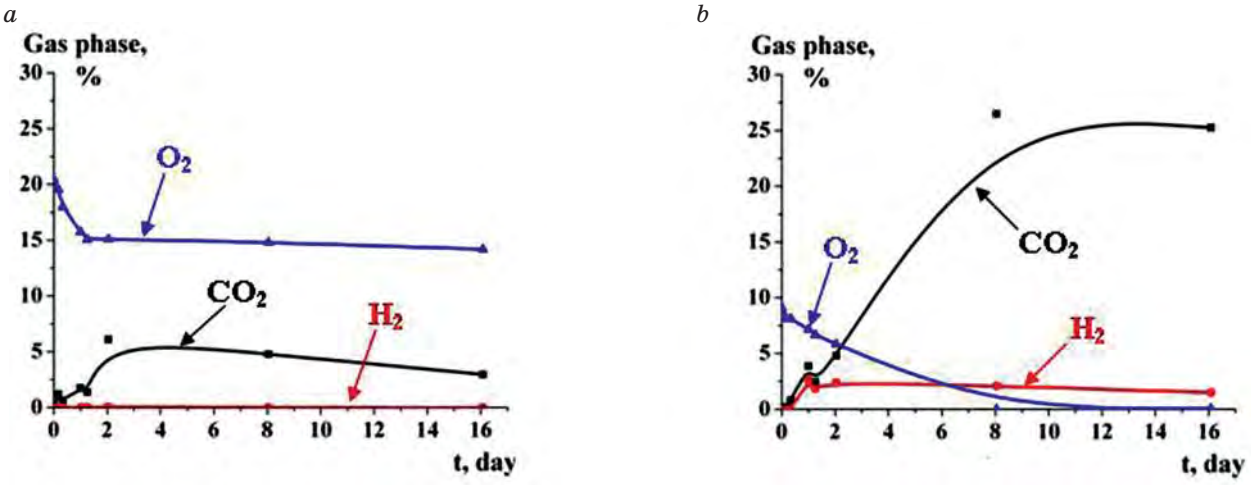

Fig. 3. **Regularities of the gas phase change during the strain *Citrobacter freundii* Ml-31.1/1 growth:**
a — medium with citrate; *b* — medium with glucose (* $P \leq 0.05$)

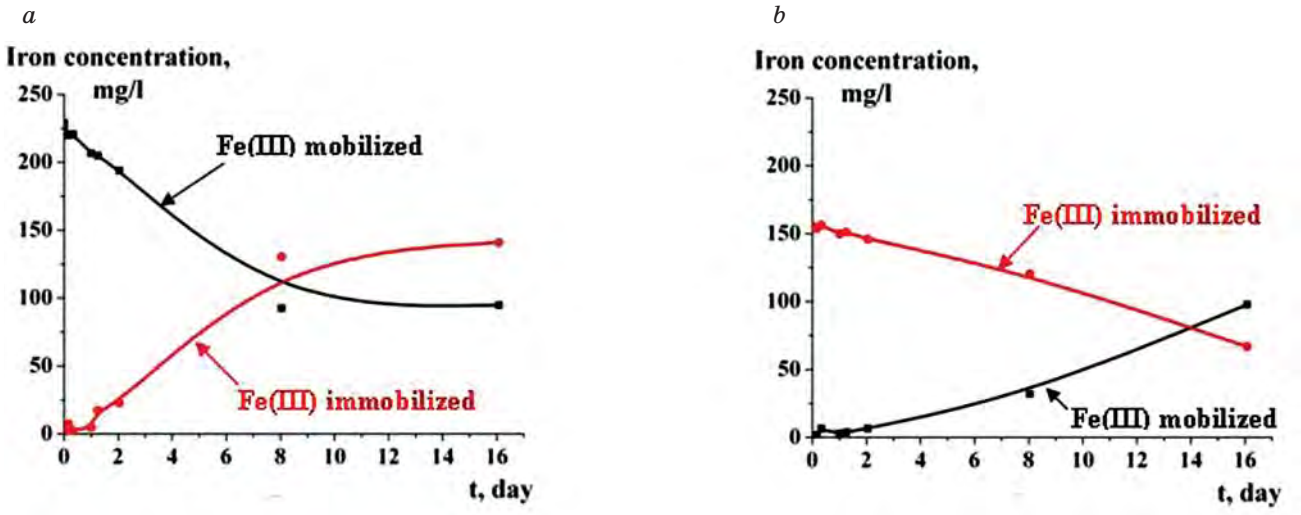

Fig. 4. **Regularities of iron compounds mobilization and immobilization during the strain *Citrobacter freundii* Ml-31.1/1 growth:**
a — medium with citrate; *b* — medium with glucose (* $P \leq 0.05$)

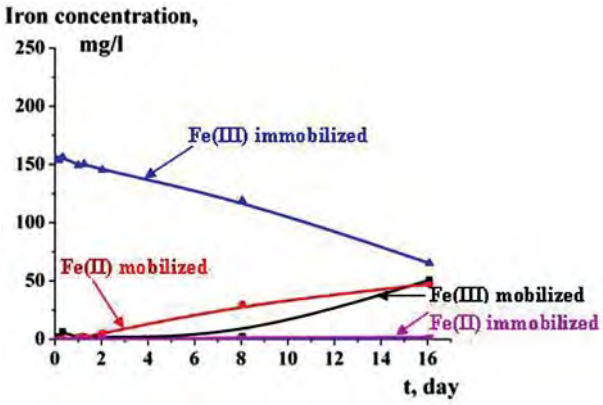

Fig. 5. **Reallocation of mobilized and immobilized compounds of Fe(III) and Fe(II) during the strain *Citrobacter freundii* Ml-31.1/1 growth in the medium with glucose (* $P \leq 0.05$)**

Soluble Fe(II) compounds were accumulated in the culture medium. The concentration of insoluble Fe(II) was only 1.6 mg/l. Soluble Fe(II) concentration reached 47 mg/l. The efficiency of Fe(III) reduction was 30%.

Compounds of Fe(II) are stable in soluble form at the pH < 6.6, and Fe(III) — at the pH < 1.6 [7, 8]. So Fe(II) will remain soluble even if chelating complexes are destructed under the conditions of medium with glucose (pH 3.6–4.6) (Fig. 1). Therefore, such mobilization of iron is more effective than mobilization of Fe(III) compounds only by organic acid chelation.

Efficiency of iron compounds immobilization and mobilization process as well as their mass-balance are presented in Fig. 6.

Efficiency of Fe(III) compounds immobilization was 60% in the medium with citrate.

Efficiency of iron compounds mobilization also was 60% in the medium with glucose. Thus, 30% of iron was mobilized in the form

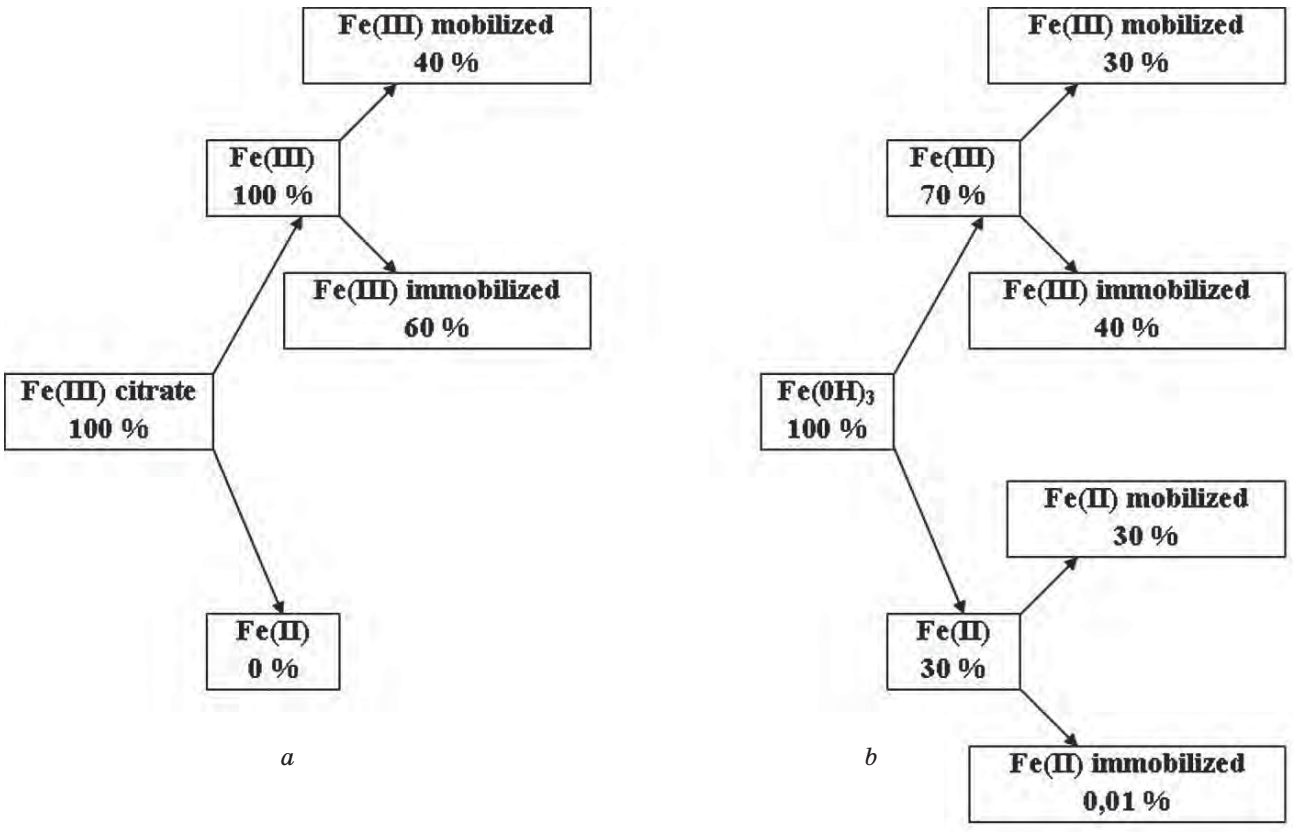

Fig. 6. **Dispersion of mobilized and immobilized iron compounds during the strain *Citrobacter freundii* Ml-31.1/1 growth:**
a — medium with citrate; *b* — medium with glucose (* $P \leq 0.05$)

of Fe(III), and another 30% — in the form of Fe(II).

Microbial transformation of iron compounds occurs during destruction of organic matter in freshwater and marine sediments, aromatic hydrocarbons in contaminated aquifers, accumulation of iron minerals, corrosion, etc. [11].

Microbial iron reduction influence water quality, since it leads to the increase of the concentration of soluble iron compounds phosphates and toxic metals in surface and ground waters [12–17]. Therefore, the possibility of regulation of iron compounds transformation pathways is necessary to assess the interaction of microorganisms with iron and to make influence on biogeochemical cycles in ecosystems.

Thus, the possibility of theoretically (thermodynamic prognosis) grounded regulation of iron transformation pathways was experimentally confirmed. Single strain (*Citrobacter freundii* Ml-31.1/1) was able to immobilize soluble iron compounds and mobilize insoluble iron compounds with efficiency 60% depending on specific conditions of microbial growth. Therefore, microorganisms can be suggested to significantly affect the redistribution of soluble and insoluble iron compounds in ecosystems. The possibility to control and regulate the processes of iron compound transformation by microorganisms can be used in effective biotechnologies of water purification from iron and iron extraction from depleted fields.

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РЕГУЛЯЦІЯ ВЗАЄМОДІЇ ШТАМУ *Citrobacter freundii* ML-31.1/1 ЗІ СПОЛУКАМИ ЗАЛІЗА

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Метою роботи було на основі термодинамічних розрахунків показати можливість регуляції метаболізму культури *Citrobacter freundii* Ml-31.1/1 для здійснення двох взаємно протилежних процесів: мобілізації та іммобілізації сполук заліза. Використано такі методи: потенціометричне вимірювання показників pH і Eh, колориметричне визначення приросту мікробної біомаси і зміни концентрації сполук заліза, газова хроматографія. Експериментально підтверджено можливість теоретично обґрунтованої регуляції шляхів трансформації заліза. На прикладі однієї культури (*C. freundii* Ml-31.1/1) продемонстровано можливість як іммобілізації, так і мобілізації сполук заліза шляхом створення специфічних умов розвитку мікроорганізмів у живильному середовищі. Отримані закономірності є основою для оцінки ролі мікроорганізмів у біогеохімічних циклах трансформації сполук заліза і підґрунтям для розробки біотехнологій як підвищення ефективності вилучення заліза зі збіднених родовищ, так і очищення води від заліза.

Ключові слова: термодинамічний прогноз, регуляція взаємодії мікроорганізмів із залізом, мобілізація, іммобілізація, сполуки заліза, біогеохімічні цикли, природоохоронні біотехнології.

РЕГУЛЯЦИЯ ВЗАИМОДЕЙСТВИЯ ШТАММА *Citrobacter freundii* ML-31.1/1 С СОЕДИНЕНИЯМИ ЖЕЛЕЗА

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Цель работы — на основании термодинамических расчетов показать возможность регуляции метаболизма культуры *Citrobacter freundii* Ml-31.1/1 для осуществления двух взаимно противоположных процессов: мобилизации и иммобилизации соединений железа. Использованы следующие методы: потенциометрическое измерение показателей pH и Eh, колориметрическое определение прироста микробной биомассы и изменения концентрации соединений железа, газовая хроматография. Экспериментально подтверждена возможность теоретически обоснованной регуляции путей трансформации железа. На примере одной культуры (*C. freundii* Ml-31.1/1) продемонстрирована возможность как иммобилизации, так и мобилизации соединений железа путем создания специфических условий развития микроорганизмов в питательной среде. Установленные закономерности являются основой для оценки роли микроорганизмов в биогеохимических циклах трансформации соединений железа и предпосылкой для разработки биотехнологий как повышения эффективности извлечения железа из истощенных месторождений, так и очищения воды от железа.

Ключевые слова: термодинамический прогноз, регуляция взаимодействия микроорганизмов с железом, мобилизация, иммобилизация, соединения железа, биогеохимические циклы, природоохранные биотехнологии.