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THE REGULARITIES OF IRON COMPOUNDS TRANSFORMATION BY *CITROBACTER FREUNDII* Ml-31.1/1

The purpose of the work was to study the regularities of Fe(III) compounds reduction under the conditions of limited aeration by the strain Citrobacter freundii Ml-31.1/1, and to assess the role of Fe(III)-reducing bacteria (FRB) in iron compounds transformation in ecosystems. The following methods were used: cultivation of microorganisms under the conditions of limited aeration, colorimetric determination of biomass and Fe(III) and Fe(II) compounds concentration, the potentiometric measuring of pH and Eh, gas chromatography. Results: Thermodynamic calculations of microbial interaction with iron compounds were experimentally verified. The regularities of Fe(III) and Fe(II) compounds transformation by the model strain Citrobacter freundii Ml-31.1/1, isolated from the typical mid-latitude ecosystem – river sludge were obtained. The balance of iron compounds redistribution in cells and in the extracellular fraction was established. Conclusions: The results obtained are the basis to assume that non-specific interaction of microorganisms with iron may significantly influence transformation of iron. Thermodynamic prognosis allows to carry out targeted regulation of microbial metabolism.

K e y w o r d s: thermodynamic prognosis, microbial Fe(III) reduction, precipitation of iron compounds, biogeochemical cycles of iron transformation.

The participation of microorganisms in the processes of iron compounds transformation is already known for about a century [1, 2]. Although microbial iron reduction has been studied for a long time, the regularities of iron compounds transformation by microorganisms, as well as their contribution to biogeochemical cycles of iron transformation in ecosystems are still poorly understood.

It is considered that the leading role in the reduction of Fe(III) to Fe(II) belongs to dissimilatory Fe(III)-reducing bacteria. However, a limited number of microbial species (for example, *Shewanella putrefaciens, Geobacter metallireducens*) is known to specifically reduce Fe(III) in dissimilatory metabolism producing energy [3, 4, 5]. On the other hand, there are many microorganisms that reduce Fe(III) compound as well as oxygen, nitrate, sulfate and other terminal electron acceptors (for example, *Bacillus, Clostridium, Desulfovibrio, Escherichia, Pseudomonas,* etc.) [2, 6].

Thermodynamic evaluation of pathways of iron compounds transformation confirms that Fe(III) may be reduced to Fe(II) by a wide range of microorganisms. We assume that these microorganisms can reduce Fe(III) compounds non-specifically, i.e, by production of exometabolites-reducing agents, by lowering of redox-potential of nutrient medium, as well as by low-potential redox-enzymes functioning.

These organisms are widespread in natural ecosystems, and microbial reduction of Fe(III) to Fe (II) is thermodynamically feasible. So, we assume that the non-specific Fe(III) reduction should significantly influence the biogeochemical cycles of iron compounds transformation in ecosystems.

Therefore, the purpose of our work was to study the regularities of Fe(III) compounds reduction under the conditions of limited aeration by the strain *Citrobacter freundii* Ml-31.1/1, and to assess the role of Fe(III)-reducing bacteria in iron compounds transformation in ecosystems.

Materials and methods. The regularities of microbial iron reduction were studied using a model strain *Citrobacter freundii* Ml-31.1/1. We isolated as the result of natural ecosystems screening for the presence of Fe(III)-reducing bacteria. We isolated the strain from a widespread natural ecosystem of middle latitudes – river sludge (river Grun, Synivka village, Sumy region, Ukraine). The strain reduced Fe(III) compounds the most effectively among the dominant strains of this ecosystem. Basing on the totality of morphological, physiological and biochemical characteristics as well as phylogenetic analysis results the strain was identified as *Citrobacter freundii*. The strain *Citrobacter freundii* Ml-31.1/1 is deposited in the collection of Department of Extremophilic Microorganisms Biology of Zabolotny Institute of Microbiology and Virology of NASU.

River sludge is a mixture of organic compounds that are degradation products of aquatic organisms. Therefore, cultivation of microorganisms was carried out in protein nutrient medium "Nutrient broth" (NB) (HiMedia Laboratories Pvt. Ltd., India), and in the NB with glucose (NB+gl) (glucose concentration was 10 g/l). Glucose was used as the additional carbon and energy source stimulating the functioning of low-potential redox-enzymes. The concentration Fe(III) citrate in the nutrient medium was 0.2 g/l of iron cations.

Nutrient medium (200 ml) and 3 ml of microbial suspension in physiological solution (McFarland standard 6 – $1.8 \cdot 10^9$ CFU/ml of cell suspension) were added in flasks (volume 250 ml). The flasks were sealed by rubber stoppers and metal clamps. Cultivation was carried out under the conditions of limited aeration at 30 °C during 48 hours.

Following parameters were the criteria of microbial metabolic activity. They were change of pH and Eh value, oxygen consumption, hydrogen and carbon dioxide synthesis by microorganisms, biomass increase, decrease of Fe(III) concentration and increase of Fe(II) concentration.

The pH and Eh of the culture liquid was measured by the potentiometric method with the pH-meter-millivoltmeter "pH-150 MA". The measuring electrode ЭСК-10603/4 was used to determine the pH. Redox-potential was measured using platinum measuring electrode ЭПВ-1. The silver chloride electrode ЭВЛ-1М3 was used as reference.

The gas phase composition in the flasks was determined by the standard method using a gas chromatograph ЛХМ-8-МД [7]. The chromatograph is equipped with two steel columns – first (I) for analysis of H_2, O_2, N_2 and CH_4, second (II) – for analysis of CO_2.

Detector-katharometer column parameters: I – l = 3 m, d = 3 mm, with a molecular sieve 13X (NaX); II – l = 2 m, d = 3 mm, with Porapak-Q carrier. Column temperature – +60 °C, evaporator temperature – +75 °C, detector temperature – +60 °C. The detector current – 50 mA. Carrier gas – argon, the gas flow rate – 30 cm^3/min.

The percentage of primary gases – H_2, CO_2, N_2 and O_2 – in the gas phase was determined by the standard procedure calculating the peak square of the gas phase components.

The concentration of Fe(III) and Fe(II) was determined in cells and in the extracellular fraction. The concentration of dissolved and precipitated Fe(III) and Fe(II) compounds was also calculated in the extracellular fraction.

The contents of Fe(III) and Fe(II) was measured by colorimetric methods. The concentration of Fe(II) was determined using *o*-phenanthroline [8]. 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 (КФК-2МП) at $\lambda = 490$ nm and an optical path length of 0.5 cm.

The concentration of Fe(III) was determined by the formation of red-colored compounds of Fe(III) with potassium rhodanide in acidic conditions [8]. 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 on photoelectric colorimeter (КФК-2МП) at $\lambda = 490$ nm and optical path length of 1 cm.

The concentration of iron compounds was measured according to the following scheme. First of all, 6 ml of culture liquid was centrifuged at 2655 g for 15 minutes. The supernatant was decanted and used for the measurement of dissolved iron compounds concentrations by the method described above.

The precipitate consisted of bacterial biomass and precipitated iron compounds. It was suspended in the same volume (6 ml) of citric acid solution (3 g/l). Citric acid solution of was used as the chelating compound to transform the precipitated iron compound into dissolved form. which is available for the colorimetric measuring. The obtained solution was centrifuged at 2655 g for 15 minutes again. The supernatant was decanted and used to determine the concentration of the precipitated iron compounds.

The precipitate consisting of bacterial biomass was suspended in 6 ml of physiological solution to measure its optical density. Optical density of the cell suspension was measure by photoelectric colorimeter (КФК-2МП) at $\lambda = 540$ nm in 0.5 cm cuvette. Biomass growth was estimated by increase of its concentration in the medium. For this purpose optical density of biomass suspension was recalculated to the concentration of biomass according to the calibration graph.

The cell suspension was centrifuged one more time at 2655 g for 15 minutes. The supernatant was decanted. The precipitated biomass of microorganisms was used to determine the concentration of iron compounds in cells. The precipitate was suspended in 6 ml of 10 % HCl. Microbial cells were lysed in strongly acidic conditions at pH = 1.0. Iron compounds became available for measurement. The color of Fe(II) and *o*-phenanthroline complex develops slowly and is weakly expressed at pH < 2.0 [8]. Therefore, the obtained acidic solution was neutralized by adding dry Na_2CO_3 to accurately measure the concentration of Fe(II) compounds in the cell fraction.

Results and discussion. Only a limited number of microorganisms (for example, *Shewanella putrefaciens, Geobacter metallireducens*) were thought to reduce Fe(III) compounds and to use it as a terminal electron acceptor in dissimilatory metabolism [3, 4].

Thermodynamic evaluation of iron compounds transformation pathways showed that microorganisms may reduce Fe(III) to Fe(II), as well as mobilize

and immobilize iron compounds. The transformation of iron compounds is determined by the values of pH and redox-potential, that are influenced by microorganisms. The fields of iron thermodynamic stability were designed by us according to Pourbaix equations [9].

Fe(III)-reducing bacteria were shown to be widespread in natural ecosystems according to our previous screening. These ecosystems differ in geographical location (Antarctic, Arctic, the Dead and the Black Sea, South America (Ecuador), mid-latitudes (Ukraine, Abkhazia)) and in a range of extreme factors affecting microorganisms (organic and inorganic xenobiotics, temperature, light conditions, etc.) [10].

Iron compounds are present in ecosystems at a concentration from several milligrams to grams per 1 kg of soil [11]. The number Fe(III)-reducing bacteria, according to our researches, ranges from $1.1 \cdot 10^2$ to $2.8 \cdot 10^7$ cells/g of absolutely dry sample [12]. Therefore, microorganisms capable to reduce Fe(III) to Fe(II) can be assumed to play an important role in the biogeochemical cycles of iron compounds transformation in ecosystems.

Microbial iron reduction efficiency depends on the difference of potentials between electron donor and acceptor systems. Acceptor system potential, i.e. Fe(III) compounds, doesn't change. Therefore, the efficiency of microbial iron reduction can be increased by changing the potential of donor system [9, 13]. In this connection, to study the regularities of iron compounds transformation model strain *Citrobacter freundii* Ml-31.1/1 was inoculated in two variants of the liquid medium. The first one was a protein medium (nutrient broth – NB). The second one was a protein medium with glucose at the concentration of 10 g/l. Glucose was used as an additional low-potential electron donor for microorganisms.

Comparison of microbial growth parameters indicated that the presence of glucose in protein culture medium increased the activity of the strain growth (Fig. 1).

This is manifested in the biomass increase. Culture reaches the stationary growth phase after 5 hours of cultivation in protein culture medium, as well as in the medium with glucose. However, the optical density at this point is 1.4 times higher in the medium with glucose. Decrease of pH and redox-potential of the medium was proportional to the growth of biomass during 5 hours of cultivation. The pH of the protein medium decreased to a minimum value of 6.9, and than remained within 7.0-7.2. The pH of the medium with glucose decreased to 6.4 in 5 hours of growth, and further – to 5.3 gradually over the

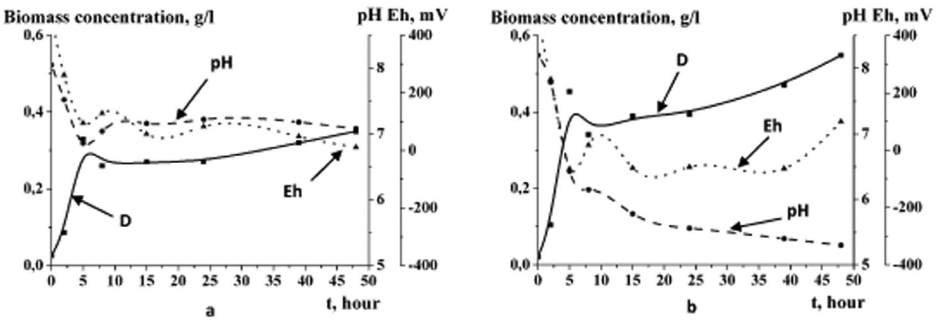

Fig. 1. Dynamics of changes in growth metabolic parameters of the strain *Citrobacter freundii* **Ml-31.1/1: a – in protein medium (NB); b – in protein medium with glucose (NB+gl)**

next 43 hours. The redox-potential of the medium with glucose was decreased from +450 mV to –65 mV, and of the protein medium – only to +95 mV. Consequently, during the strain growth redox-potential of the medium with glucose decreased to 160 mV lower than of the protein medium.

Oxygen consumption occurred proportionally to the biomass growth in the protein medium (Fig. 2). The concentration of O_2 in the gas phase decreased from 21 % to 13.9 % during 8 hours of growth. After microorganisms reached the stationary growth phase the rate of its use was significantly decreased. At the same time the concentration of O_2 on glucose medium was decreased up to 11.3 %. After 15 hours of microbial growth the concentration of O_2 on glucose medium was 4 %.

The concentration of carbon dioxide after 8 hours of cultivation in the medium with glucose was 1.8 times higher than in the protein medium. The maximum concentration of CO_2 in the protein medium was 7.6 % after 48 hours of the strain growth. In the medium with glucose maximum concentration of carbon dioxide (23.8 %) was noted after 39 hours.

The maximum hydrogen concentration after 8 hours of cultivation in protein medium was 1.3 %, that is 5 times lower than in the medium with glucose. Maximum concentration of H_2 on the medium with glucose was after 15 hours of cultivation (21.6 %) and then remained within 15.7-14.5 %. It is 18 times higher than for the protein medium.

Thermodynamic evaluation allows to prognose the pathways of iron compounds transformation by microorganisms. The data obtained suggest that the strain can quickly and effectively reduce Fe(III) to Fe(II) [9, 10]. This is facilitated by low value of the redox-potential of culture liquid, intensive decrease of oxygen concentration in the gas phase that may inhibit Fe(III) reduction as the competitive terminal electron acceptor [13]. Active hydrogen synthesis may indicate the functioning of low potential redox-enzymes that contribute to the effective reduction of Fe(III) compounds.

Comparison of metabolic parameters of the strain growth gives reason to suppose that the efficiency of Fe(III) reduction by microorganisms will be higher on the medium with glucose. This is evidenced by the following: the redox-potential of the medium with glucose was 160 mV lower than that of the protein medium, concentration of O_2 after 15 hours of growth was 2.8 times lower and H_2 concentration was 19.1 times higher.

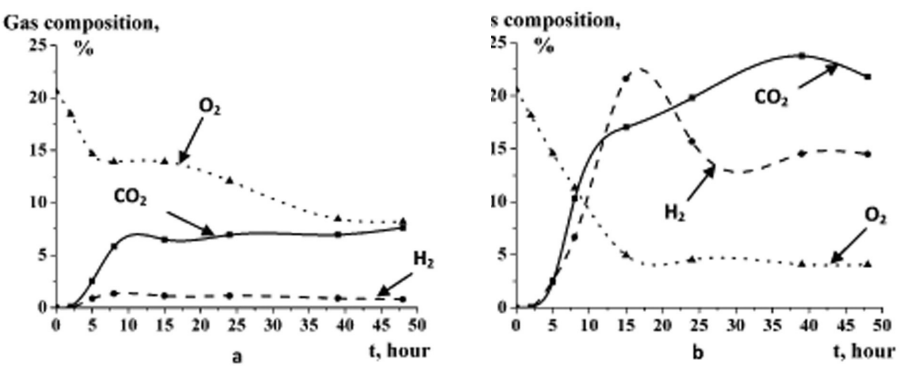

Fig. 2. Dynamics of changes in the concentration of O_2, H_2, CO_2 during the growth of the strain *Citrobacter freundii* **Ml-31.1/1: a – in protein medium (NB); b – in protein medium with glucose (NB+gl)**

We can prognose Fe(III) reduction, as well as the effectiveness of iron compounds precipitation. Basing on thermodynamic calculations the efficiency of iron precipitation on the protein medium may be higher than on the medium with glucose.

Ions of Fe^{3+} are not stable at the pH above 1.6, and Fe^{2+} ions – at the pH above 6.6 [9]. The pH values of the protein medium did not fall below 6.9. So it is likely that Fe(III) and Fe(II) may precipitate if microorganisms degrade the chelating compound – citrate. At the same time, organic acids are often degradation products of glucose. They may chelate iron and keep it in solution in stable form.

Furthermore, CO_2 concentration increasing in the protein medium at the pH values close to neutral promotes the formation of insoluble iron carbonate ($FeCO_3$). Despite the fact that CO_2 concentration in the medium with glucose was by 3.1 times higher than in the protein medium its pH was significantly lower (up to 5.3). Formation of insoluble iron carbonate is impossible in acidic conditions.

Formation of sulfide during the degradation of sulfur containing amino acids by microorganisms is another factor contributing to the precipitation of iron compounds. Well-soluble form of sulfide – sulfhydryl anion (HS^-) – dominates at the pH close to neutral, as it is in the protein medium. Consequently, HS^- precipitates Fe(II) compounds in the form of FeS. Additionally, sulfhydryl group reduces Fe(III) to Fe(II) and then forms an insoluble iron sulfide (FeS). At the same time, microorganisms acidified the medium with glucose. Under such conditions, the H2S form is predominant. Hydrogen sulfide is gas that has low solubility in water. That is why iron sulfide is not formed [14].

Obtained results of the strain *Citrobacter freundii* Ml-31.1/1 interaction with Fe(III) confirm our thermodynamic calculations [10]. Microorganisms reduced Fe(III) to Fe(II), precipitated iron compounds and accumulated them in cells.

As expected, Fe(III) reduction efficiency was twice higher in the medium with glucose than in the protein medium. The effectiveness of Fe(III) and Fe(II) precipitation was by 6.1 times higher in the protein medium. The efficiency of iron compounds accumulation was by 3.4 times higher in the protein medium too.

It is known that microorganisms can dissimilatory reduce Fe(III) by iron specific reductases with energy obtaining (for example, *Shewanella putrefaciens, Geobacter metallireducens* [3, 4, 5]). In this case, iron reduction correlates with biomass increase, that is its reduction occurs in logarithmic growth phase. This process is carried out by enzymes specialized, namely specific, for Fe(III). We call this type of Fe(III) reduction as specific iron reduction.

Another way is to reduce Fe(III) in late logarithmic and stationary growth phases, and presence of Fe(III) doesn't increase biomass yield. In this case, iron reduction may occur due to non-specific redox-enzymes as well as exometabolites-reducing agents (for example, cysteine). This type of Fe(III) reduction we call non-specific iron reduction.

Comparison of metabolic parameters of the strain growth gives reason to suppose that it reduced Fe(III) to Fe(II) non-specifically. Active reduction of Fe(III) began after 2 hours of microbial growth. Intensive Fe(III) reduction occurred in the late logarithmic growth phase, and also continued in early station-

ary growth phase in the medium with glucose (Fig. 3). This may indicate non-specific Fe(III) reduction due to the accumulation of exometabolites-reducing agents in the culture medium.

The efficiency of iron compounds precipitation was higher in the protein medium than in the medium with glucose. The most intensive Fe(III) compounds precipitation was observed when microorganisms reached the stationary growth phase after 10 hours of growth. The most intensive precipitation of insoluble Fe(II) compounds was noted after 15 hours of growth (Fig. 4).

The efficiency of iron compounds accumulation in cells was also higher in the protein medium. The rate of their accumulation does not correlate with the biomass increase. We assume that the accumulation of iron can be attributed to its sorption by dead cells. Substrate concentration in the protein medium is lower than in the protein medium with glucose, where glucose is an additional source of carbon and energy. Since microorganisms in the protein medium are limited in the substrate, the cell death and biomass sorption of iron compounds may occur more intensively. Fig. 5 illustrates the concentration of iron compounds absorbed by microbial cells.

The efficiency of Fe(III) reduction to Fe(II) twice increased when glucose was used as the additional electron donor. Concentration of Fe(II) was 89 mg/l

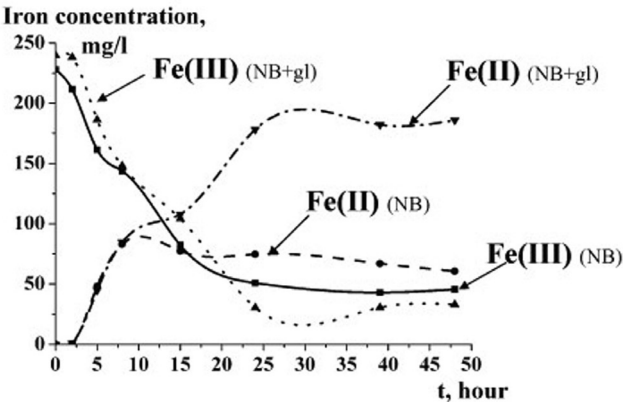

Fig. 3. Dynamics of changes in the concentration of dissolved Fe(III) and Fe(II) compounds during the growth of the strain *Citrobacter freundii* Ml-31.1/1 in protein medium (NB) and in protein medium with glucose (NB+gl)

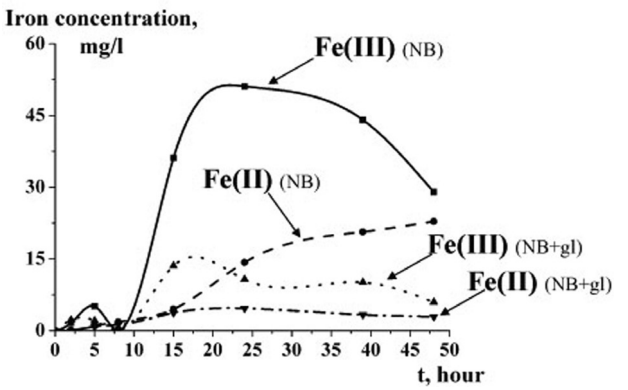

Fig. 4. Dynamics of changes in the concentration of precipitated Fe(III) and Fe(II) compounds during the growth of the strain *Citrobacter freundii* Ml-31.1/1 in protein medium (NB) and in protein medium with glucose (NB+gl)

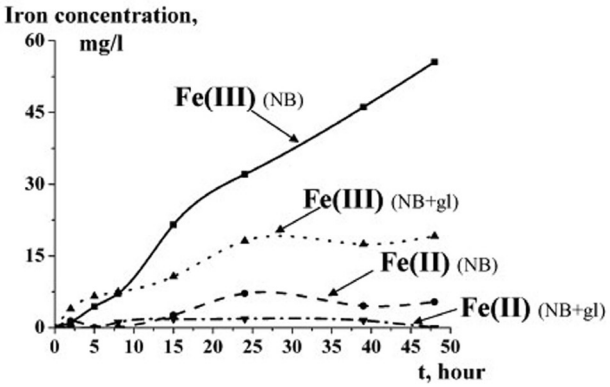

Fig. 5. Regularities of Fe(III) and Fe(II) compounds accumulation by cells of the strain *Citrobacter freundii* **Ml-31.1/1 in protein medium (NB) and in protein medium with glucose (NB+gl)**

in the protein medium after 48 hours of the strain growth, and 188 mg/l in the medium with glucose.

The balance of iron compounds redistribution in cells and the extracellular fraction after 48 hours of growth is shown on Fig. 6.

The efficiency of Fe(III) reduction to Fe(II) by *Citrobacter freundii* Ml-31.1/1 was 2 times higher in the medium with glucose than in the protein medium. The efficiency of Fe(III) and Fe(II) precipitation in the medium with glucose was by 5 and by 8 times lower respectively. The efficiency of Fe(III) and Fe(II) accumulation was also 3 times lower in the medium with glucose.

Thus, we obtained the regularities of Fe(III) and Fe(II) compounds transformation by the model strain *Citrobacter freundii* Ml-31.1/1, isolated

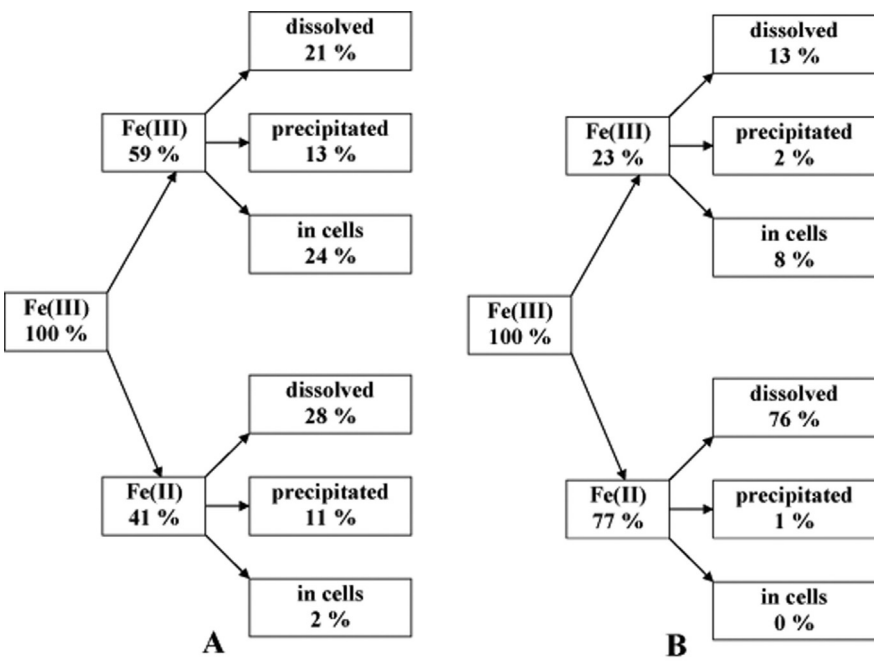

Fig. 6. The balance of redistribution of precipitated and dissolved iron compounds in cells and the extracellular fraction by the strain *Citrobacter freundii* **Ml-31.1/1: a – in protein medium in protein medium (NB) and in protein medium with glucose (NB); b – in protein medium with glucose in protein medium (NB) and in protein medium with glucose (NB+gl)**

from the typical mid-latitude ecosystem – river sludge. Thermodynamic calculations of microbial interaction with iron compounds were experimentally verified. It was shown that the reduction of Fe(III) to Fe(II) by microorganisms occurs non-specifically in late logarithmic and early stationary growth phase. It is demonstrated that efficiency of Fe(III)-reduction increased in the presence of low-potential electron donor. The efficiency of Fe(III) and Fe(II) compounds precipitation increased at the pH value close to neutral, and when carbon dioxide and sulfhydryl groups were accumulated.

Conclusions.

The results obtained are the basis to assume that non-specific interaction of microorganisms with iron may significantly influence transformation of iron.

Thermodynamic prognosis allows to carry out targeted regulation of microbial metabolism. It makes possible to prognose and control the intensity and efficiency of iron compounds transformation by microorganisms.

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ЗАКОНОМІРНОСТІ ТРАНСФОРМАЦІЇ СПОЛУК ЗАЛІЗА *CITROBACTER FREUNDII* Ml-31.1/1

Р е з ю м е

Метою роботи було дослідження закономірностей відновлення сполук Fe(III) в умовах обмеженої аерації штамом *Citrobacter freundii* Ml-31.1/1, а також оцінка ролі залізовідновлювальних бактерій у трансформації сполук заліза в екосистемах. Були використані наступні методи: культивування мікроорганізмів в умовах обмеженої аерації, колориметричне визначення біомаси і концентрації сполук Fe(III) і Fe(II), потенціометричне вимірювання pH і Eh, газова хроматографія. Результати. Експериментально підтверджено термодинамічні розрахунки взаємодії мікроорганізмів зі сполуками заліза. Отримано закономірності трансформації сполук Fe(III) і Fe(II) модельним штамом *Citrobacter freundii* Ml-31.1/1, виділеним із типової екосистеми середніх широт України – річкового мулу. Встановлено баланс перерозподілу сполук заліза у клітинах, а також розчинених і осаджених Fe(III) і Fe(II) у позаклітинній фракції. Висновки. Отримані результати дозволяють припускати, що неспецифічна взаємодія мікроорганізмів із залізом може суттєво впливати на трансформацію сполук заліза. Термодинамічне прогнозування дозволяє здійснювати спрямовану регуляцію мікробного метаболізму.

К л ю ч о в і с л о в а: термодинамічний прогноз, мікробне відновлення Fe(III), осадження сполук заліза, біогеохімічні цикли трансформації заліза.

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ЗАКОНОМЕРНОСТИ ТРАНСФОРМАЦИИ СОЕДИНЕНИЙ ЖЕЛЕЗА *CITROBACTER FREUNDII* Ml-31.1/1

Р е з ю м е

Целью работы было исследование закономерностей восстановления соединений Fe(III) в условиях ограниченной аэрации штаммом *Citrobacter freundii* Ml-31.1/1, а также оценка роли железовосстанавливающих бактерий в трансформации соединений железа в экосистемах. Были использованы следующие методы: культивирование микроорганизмов в условиях ограниченной аэрации, колориметрическое определение биомассы и концентрации соединений Fe(III) и Fe(II), потенциометрическое измерение pH и Eh, газовая хроматография. Результаты. Экспериментально подтверждены термодинамические расчеты взаимодействия микроорганизмов с соединениями железа. Получены закономерности трансформации соединений Fe(III) и Fe(II) модельным штаммом *Citrobacter freundii* Ml-31.1/1, выделенным из типовой экосистемы средних широт Украины – речного ила. Установлен баланс перераспределения соединений железа в клетках, а также растворенных и осажденных Fe(III) и Fe(II) во внеклеточной фракции. Выводы. Полученные результаты дают основания предполагать, что неспецифическое взаимодействие микроорганизмов с железом может существенно влиять на трансформацию соединений железа. Термодинамическое прогнозирование позволяет осуществлять направленную регуляцию микробного метаболизма.

К л ю ч е в ы е с л о в а: термодинамический прогноз, микробное восстановление Fe(III), осаждение соединений железа, биогеохимические циклы трансформации железа.

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