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THE ROLE OF GLYCINE-BETAINE IN THE GROWTH AND HYDROGENASES ACTIVITY OF *RALSTONIA EUTROPHA* H16

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Glycine-betaine (GB) is an abundant organic material in soil, and the chemo-lithoautotrophic soil bacterium *R. eutropha* H16, one of the best bio-technological research models, may involve it in metabolism. The effect of different concentrations of GB (7–300 *μmol/mL*) on bacterial growth parameters and H_2-oxidizing activity of hydrogenases (Hyd) was studied. Stimulation of bacterial growth was registered at GB low concentrations, however, high concentrations have a partial inhibitory effect, in contrast to control (Fructose-Nitrogen medium). Upon GB supplementation, the min and max H_2-oxidizing Hyd activity of *R. eutropha* whole cells were 3.4±0.01 *U* (*g* CDW)$^{-1}$ and 16.4±0.01 *U* (*g* CDW)$^{-1}$, respectively at concentrations of 7 *μmol/mL* and 300 *μmol/mL*, while it is absent in the control. These results might be used to develop new approaches to produce oxygen-tolerant Hyd.

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***Keywords*:** glycine-betaine, *Ralstonia eutropha* H16, [NiFe]-Hydrogenases.

Introduction. Glycine-Betaine (GB; *N*,*N*,*N*-trimethylglicyne) is one of the abundant soil organic materials that can be freely utilized by a wide range of soil organisms, such as plants and microorganisms [1]. Various pathways of GB transport and metabolism in bacterial cells have been identified. According to the literature data, the uptake of GB is predominantly an ATP-dependent process, and the transport activity depends on the microbe's environmental conditions or growth medium composition. In case of osmotic stress and cold conditions, GB uptake by the several bacterial cells occurs by the Betaine-choline-carnitine transport family transporters and specific ATP binding cassette family transporters [2–4]. GB is not only utilized from the environment, but also synthesized from the choline via a two-stage reaction catalyzed by O_2-dependent choline dehydrogenase: choline is oxidized into betaine aldehyde, and then into GB. In turn, GB can be catabolized to dimethylglycine, sarcosine, or glycine, depending on cellular demands. It was shown, that these processes are regulated by bet group genes [1, 4, 5].

According to our previous studies, glycine (Gly) is the best stimulant of hydrogenases (Hyd) activity among the studied 9 L-Amino acids. Therefore, Gly is one of the key components of Hyd synthesis or maturation [6].

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Nevertheless, GB performs various functions in the bacterial cell:

i) GB is important for bacterial osmoadaptation, it acts as an osmoprotectant in the salt mediums;

ii) GB products can function as a nutrient;

iii) GB and its catabolites are involved in the detoxification of the catabolic byproducts and ROS, such as hydrogen peroxide and formaldehyde;

iv) in the biotechnological industry, GB is used as a protein stabilizer and a stabilization agent for single-stranded nucleic acids [1, 4]. Some authors also show that GB can also act as a cryoprotectant, preventing cold-induced protein aggregation and maintaining membrane fluidity at low temperatures [2].

Ralstonia eutropha (*Wautersia eutropha* or *Cupriviadus necator*) is a Gram-negative facultative chemolithoautotrophic, nonpathogenic, soil bacterium belonging to the β-subclass from Proteobacteria. This bacterium was previously called *Hydrogenomonas eutropha*, because of the possibility of using molecular hydrogen (H_2) and carbon dioxide (CO_2) as the only sources of energy and carbon, however, it is also able to grow heterotrophically using fructose, gluconic acid, various other organic acids, and even aromatic compounds as a carbon and energy sources [7].

Ralstonia eutropha H16 is a bacterium with high biotechnological potential: it has O_2-tolerant Hyd and might help the future H_2-based biotechnology for the production of various commercially, environmentally, and medically valuable components.

Enzymatic biofuel cells (EFC) or microbial fuel cells (MFC) are involved in the physiological coupling of H_2 oxidation and O_2 reduction, they may be effectively used for electricity generation. Hyd of *R. eutropha* can be considered promising candidates as anodic biocatalysts in EFC.

Four different oxygen-tolerant [NiFe]-Hyd were identified in the chemolitho-autotrophic *R. eutropha*: a membrane-bound Hyd (MBH), cytoplasmic soluble Hyd (SH), a regulatory Hyd (RH) and an actinobacterial type Hyd (AH) [8, 9]. The MBH has involved in H_2 uptake-driven respiration with O_2 as the terminal electron acceptor, while SH is a bidirectional Hyd that directly reduces NAD^+ to NADH with usage of H_2, and thus generates reducing equivalents. RH controls Hyd gene transcription according to the availability of H_2. AH belongs to the group of high-affinity Hyd and has a high tolerance towards O_2 [9].

Since *R. eutropha* H16 can grow both autotrophic and heterotrophic, it is important to investigate and optimize the Hyd synthesis conditions. Moreover, it has been demonstrated that energy limitation, e.g., starvation and O_2 limitation favors the catabolic de-repression of Hyd gene expression. It was shown that organic wastes, such as glycerol, lignocellulose-containing brewery spent grain hydrolysate and dairy waste products are promising carbon and energy sources for the formation of biomass harboring significant amounts of the biotechnologically relevant Hyd [10, 11]. In addition, recently we have shown that some L-amino acids induce Hyd activity during heterotrophic growth on fructose [6, 12].

It is a promising perspective to identify all biochemical substances necessary for the production of O_2-tolerant Hyd, avoiding an autotrophic fermentation process, when is required to use of dangerous gas mixtures of H_2, O_2, and CO_2.

In this context, it is important to find the optimal conditions when using GB different concentrations will be possible to obtain biomass with active Hyd as a biocatalyst of BioFC.

Materials and Methods.

Growth Media and Cultivation Conditions of Bacteria. *R. eutropha* H16 (DSM 428) was kindly provided by Dr. Oliver Lenz (Technical University Berlin, Germany) and it is cultivated heterotrophically in fructose–nitrogen (FN) minimal mineral medium by the addition of 7–300 *μmol* GB. The basic FN consisted of 100 *mL* 10×H16 buffer, 85 *mL* water, and the following sterilized solutions: 1 *mL* NH_4Cl (20% w/v), 100 *μL* $MgSO_4 \times 7H_2O$ (20% w/v), 100 *μL* $CaCl_2 \times 2H_2O$ (1% w/v), 100 *μL* $FeCl_3 \times 6H_2O$ (0.5% w/v), 1 *mL* $NiCl_2 \times 6H_2O$ (0.001% w/v) and 1 *mL* fructose (40% w/v). The 10×H16 buffer contained 90 *g* $Na_2HPO_4 \times 12\ H_2O$ and 15 *g* KH_2PO_4 added to 1000 *mL* H_2O (final pH of 7.0) [10, 13].

Aerobic conditions for cultivation experiments were conducted by using 250 *mL* baffled flasks with 100 *mL* solutions. 1.5% of bacterial pre-culture was added to the FN growth medium Bacteria were cultivated aerobically on a shaker at 130 *rpm* and 30°C, 72 *h*, and pre-cultivation was done on a shaker at 130 *rpm* and 37°C [10, 13].

Bacterial Growth Parameters Investigation. Bacterial growth was determined by using a spectrophotometer 600 *nm* (Spectro UV-VIS Auto, "LaboMed", Los Angeles CA, USA).

Cell dry weight (CDW) of bacteria was applied to estimate bacterial biomass yield and expressed in gL^{-1} [14].

The medium's pH was measured by using a pH electrode of HJ1131B pH-meter ("Hanna Instruments", Portugal). pH of mediums was regulated by using 0.1 *M* NaOH or 0.1 *N* HCl solutions depending on starting mediums pH.

Oxidation–Reduction Potential (ORP) Determination. Bacterial cultivation medium ORP was determined by using a couple of glass oxidation-reduction platinum (Pt) (EPB-1, Measuring Instruments Enterprise, Gomel, Belarus, or PT42BNC, "Hanna Instruments", Portugal) and titanium-silicate (Ti–Si) (EO–O_2, "Measuring Instruments Enterprise", Gomel, Belarus) electrodes. The Pt electrode is sensitive to O_2 and H_2 in the medium, while Ti–Si electrode is not affected by the presence of O_2 or H_2 and it measures the overall ORP [15]. Before performing the analysis both electrode readings were tested in a control solution. It is a combination of 0.049 *M* potassium ferricyanide ($K_3[Fe(CN)_6]$) and 0.05 *M* potassium ferrocyanide ($K_4[Fe(CN)_6] \cdot 3H_2O$) (pH 6.86). The readings of both electrodes in the solution at 25°C were +245±10 *mV* [10, 16, 17].

Preparation of Cells Extracts and Determination of Hyd Activity. Biomass formation of *R. eutropha* H16 was followed by measuring the optical density at 600 *nm* (OD_{600}). During 72 *h* by 24 *h* periods cells were harvested by centrifugation (5000 *rpm*, 4°C for 10 *min*). For the preparation of wet cell extracts, all steps were carried out at 4°C. Harvested cells were washed with K–PO_4 buffer (50 *mM* K–PO_4 at pH 7.0).

H_2-oxidizing total activity of whole cells was quantified by monitoring H_2-dependent methylene blue reduction at 570 *nm* and 30°C with a Cary 50 UV-Vis spectrophotometer. Aliquots of cell extracts (15 to 20 *mL*) or bacterial whole cells,

were added to anaerobic cuvettes containing 1.9 *mL* reaction mixture (50 *mM* H_2-saturated K–PO_4 buffer (pH 7.0)) and methylene blue as the artificial electron acceptor [9].

Data Processing and Reagents Used in the Research. Microsoft Excel 2016 was used for data processing. The data show values determined from 3 independent measurements; the standard average of the mean data with standard errors was determined using the corresponding Microsoft Excel 2016 function, and Student criteria (p) were considered to approve the difference in average data among different series of measurements [16, 18]. The difference was valid when $p < 0.05$.

Glycine-Betaine, Fructose, NH_4Cl, $K_3[Fe(CN)_6]$ ("Carl Roth GmbH", Germany) and all other reagents used in the study were of analytical grade.

Results and Discussion.

Aerobic Growth of R. eutropha H16 by the Presence of GB Different Concentrations. Many soil bacteria use GB not only as an osmoprotectant, but also may use it as a sole source of carbon and nitrogen [1, 4], *R. eutropha* is one of the soil bacteria and may involve GB in cell metabolism. To cultivate *R. eutropha* H16 different concentrations of GB were used, *μmol*/*mL*: 7, 15, 25, 50, 75, 100, 150, 200, 250, 300. GB was supplemented to the FN standard mineral medium as was presented in "Materials and Methods". The effect of GB on the aerobic growth parameters (OD_{600}, ORP, and pH variations) and Hyd activity of *R. eutropha* H16 was detected by comparison with a control experiment, which was the growth of *R. eutropha* on the standard FN medium (see methods).

Biomass formation was evaluated by measuring OD at 600 *nm*. Bacterial growth at low concentrations of GB was stimulated ~30–40% after 48 *h*, in contrast to control: the maximal growth stimulation was detected at 15 *μmol*/*mL* and 25 *μmol*/*mL* concentrations (OD_{600} ~4.4±0.02). However, at high concentrations, the growth activity of *R. eutropha* was partially inhibited (OD_{600} ~2.7±0.02) compared with control by ~10–20% (Fig.1).

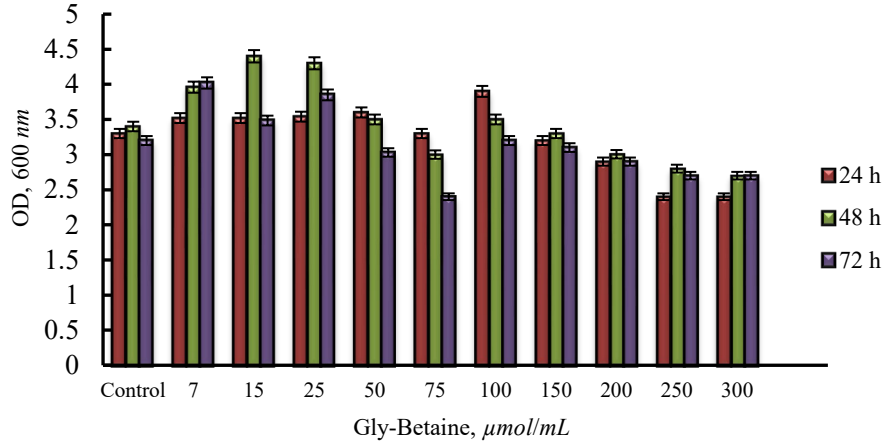

Fig. 1. Growth activity of *R. eutropha* H16 on FN medium by supplementation of GB different concentrations (7–300 *μmol*/*mL*), during 72 *h* with 24 *h* intervals ($n = 5$, $p < 0.05$).

R. eutropha was grown overnight in the FN medium (at 37°C) after 1.5% of bacterial pre-cultures were added into GB containing FN mediums (at 30°C). Bacterial growth led to the decrease of culture medium pH from pH 7.0 up to pH 6.5, after 24 *h* in all studied samples (Fig. 2).

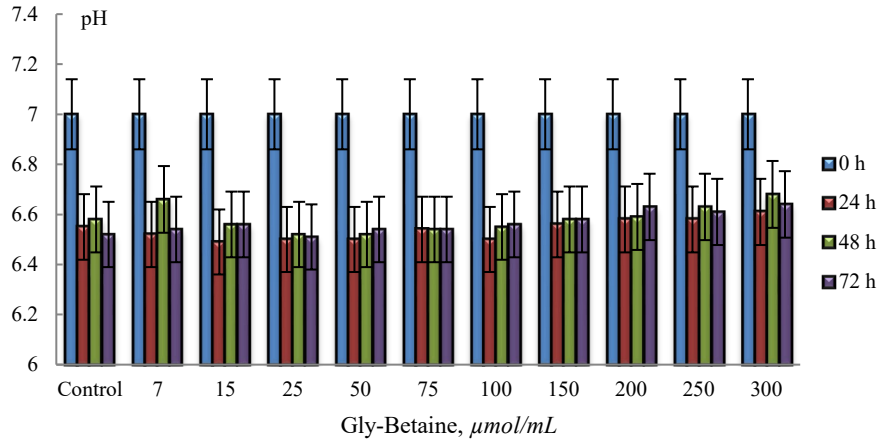

Fig. 2. pH changes during bacterial growth of *R. eutropha* on FN medium by supplementation of GB in different concentrations (7–300 *µmol/mL*), 72 *h* ($n = 5$, $p < 0.05$).

In parallel with a decrease in extracellular pH, a slight reduction in ORP was observed (Fig. 2, Fig. 3 a, b). The reduction in the values of both parameters is associated with the adaptation of microorganisms to environmental conditions and with the production of different metabolites [15, 19]. The maximal decrease of ORP was detected in the case of concentrations 75 *µmol/mL* and 100 *µmol/mL*. According to Pt and Ti–Si electrode records ORP decreased from +320±10 *mV* to +120±10 *mV* and from +230±10 *mV* to +40±10 *mV*, respectively (Fig. 3, a, b).

GB, as an osmoprotectant, is a highly soluble substance that does not have a charge at physiological pH values and is non-toxic regardless of concentration [20]. A variety of electrolytes such as NaCl, KCl, NaH_2PO_4, K_2HPO_4, and nonelectrolytes like sucrose, raffinose, and inositol triggered the uptake of GB. The transport of GB was energy-dependent and occurred against a concentration gradient, GB transport is driven by the electrochemical proton gradient [21]. Therefore, active transport of a large amount of GB may lead to energy depletion of cells, which is the main reason for growth inhibition and stimulation of Hyds activity.

The influence of various GB concentrations on the total H_2-oxidizing Hyd activity was tested by cultivating *R. eutropha* H16 for 24, 48, and 72 *h*. Compared to the control, where the H_2-oxidizing Hyd activity was absent, the addition of GB induced Hyd activity. Basically, at concentrations of 250 and 300 *µmol/mL*, the maximum activity of 16.4 ± 0.05 *U* (*g* CDW)$^{-1}$ was observed after 24 *h* of growth (Fig. 4). Interestingly the total H_2-oxidizing Hyd activity of *R. eutropha* H16 was more significant in the case of high GB concentrations, however, partial inhibition of bacterial growth was observed at the same concentrations. The minimum Hyd activity was respectively observed at low concentrations (7 *µmol/mL* – 4.0±0.05 *U* (*g* CDW)$^{-1}$) (Fig. 4), where growth stimulation was recorded, in contrast to the control.

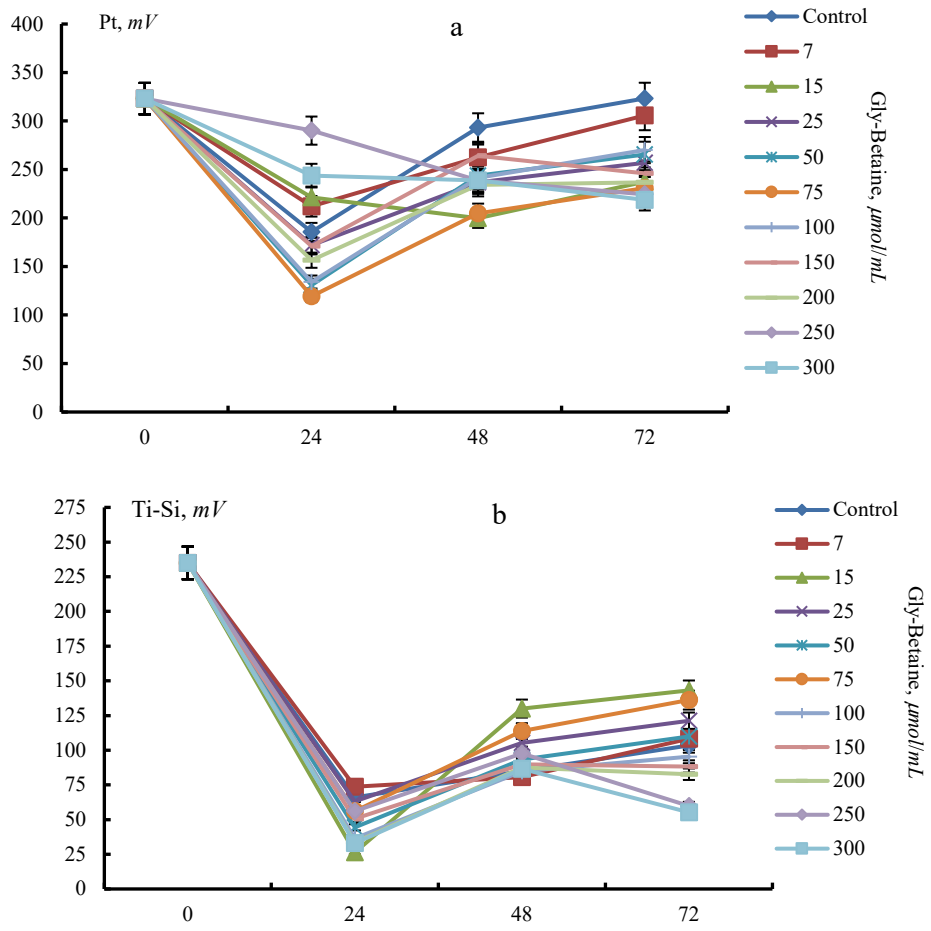

Fig. 3. Effects of GB different concentrations (7–300 *μmol/mL* on the kinetics of ORP during bacterial growth of *R. eutropha* H16 72 *h* ($n = 5$, $p < 0.05$). The ORP measured using Pt (a) and Ti–Si (b) electrodes was expressed in *mV* [vs Ag/AgCl (saturated by KCl)]. For details, see the "Materials and Methods".

The reason for the stimulation of Hyd activity might be caused by the osmoprotective features of GB. The physicochemical basis of this striking effect is not fully understood, but there is strong evidence that it is partly due to the exclusion of osmoprotectant molecules of GB from the water layer in contact with protein surfaces. In this situation, native (i.e. folded) protein structures are thermodynamically favorable, because they present the least possible surface area to the water, hence GB might promote folding of proteins, such as Hyds [19].

In addition, one of the GB catabolism pathways promotes the synthesis of Gly, and Gly is one of the identified L-amino acids responsible for the induction of H_2-oxidizing activity during the heterotrophic growth of *R. eutropha* H16 [12].

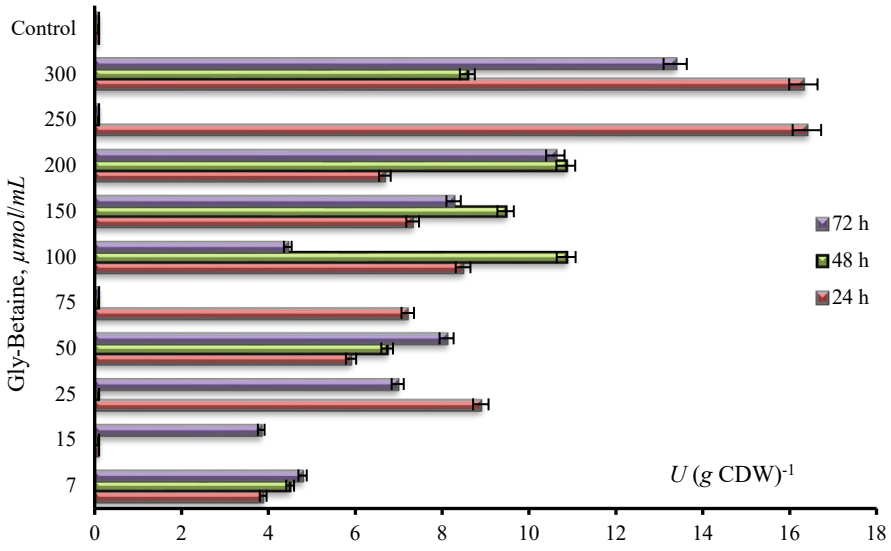

Fig. 4. H_2-oxidizing total Hyd activity during bacterial growth of *R. eutropha* H16 on FN medium by supplementation of GB different concentrations (7–300 *μmol/mL*). Bacteria were grown for 72 *h* and activity expressed as *U* (*g* CDW)$^{-1}$, *n*=5, p < 0.05. For details, see the "Materials and Methods".

Conclusion. GB is an important organic material for many soil bacteria and can be involved in various metabolic chains of *R. eutropha*, which leads to changes in the parameters of growth and activity of Hyds. There are much evidence and hypothesis about how this might be involved and affect bacterial metabolism, but there is no deep explanation of the mechanisms.

GB may be metabolized into Gly, and Gly leads to the synthesis or maturation of Hyds. In the case of uptake high concentrations of GB occurs energy depletion, which in turn promotes Hyd synthesis and high activity of this enzyme. GB also may act as a protein stabilizer and is involved in the folding process of Hyds. Future studies are required to suggest a mechanism regarding to such changes in *R. eutropha* metabolism and stimulation of Hyd activity.

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Մ. Կ. ԻՍԿԱՆԴԱՐՅԱՆ

ԳԼԻՑԻՆ-ԲԵՏԱԻՆԻ ԴԵՐԸ *RALSTONIA EUTROPHA* H16-Ի ՋՐԱԾՆԻ ՆՅՈՒԹԱՓՈԽԱՆԱԿԱՅԻՆ ԳՈՐԾԸՆԹԱՑՈՒՄ

Գլիցին-բետաինը (ԳԲ) հողում ամենատարածված օրգանական միացություններից է, և հողի քիմոլիտոավտոտրոֆ բակտերիան *R. eutropha* H16, որը կենսատեխնոլոգիական հետազոտության լավագույն մոդելներից է, կարող է ներգրավել այն նյութափոխանակային գործընթացում: Ուսումնասիրվել է ԳԲ-ի տարբեր կոնցենտրացիաների (7 *մկմոլ/մլ*–300 *մկմոլ/մլ*) ազդեցությունը բակտերիաների աճի ցուցանիշների և Հիդրոգենազային (Հիդ) ջրածին-օքսիդացնող ակտիվության վրա: Բակտերիաների աճի խթանումը գրանցվել է ԳԲ-ի ցածր կոնցենտրացիաների դեպքում, սակայն բարձր կոնցենտրացիաներն ունեցել են մասնակի արգելակող ազդեցություն, ի տարբերություն ստուգիչի (ֆրուկտոզ–ազոտային միջավայր): Երբ ԳԲ հավելվել է միջավայր, Հիդ-ային H_2-օքսիդացնող նվազագույն և առավելագույն ակտիվությունը *R. eutropha*-ի ամբողջական բջիջներում կազմել է՝ 3.4±0.01 U (q ԲՉՔ)$^{-1}$ և 16.4±0.01 U (q ԲՉՔ)$^{-1}$, համապատասխանաբար 7 *մկմոլ/մլ* և 300 *մկմոլ/մլ* կոնցենտրացիաների դեպքում, սակայն ստուգիչում ակտիվություն չի գրանցվել: Այս արդյունքները կարող են կիրառվել թթվածին-կայուն Հիդ-ների ստացման նոր մոտեցումների մշակման նպատակով:

М. К. ИСКАНДАРЯН

РОЛЬ ГЛИЦИНА-БЕТАИНА В МЕТАБОЛИЗМЕ ВОДОРОДА *RALSTONIA EUTROPHA* H16

Глицин-бетаин (ГБ) является распространенным органическим веществом в почве, и хемолитоавтотрофная почвенная бактерия *R. eutropha* H16 – одна из лучших моделей биотехнологических исследований – может вовлекать его в метаболизм. Исследовано влияние различных концентраций ГБ (7–300 *мкмоль*/*мл*) на параметры роста бактерий и водород-окисляющую активность гидрогеназы. Стимуляция роста бактерий регистрировалась при низких концентрациях ГБ, однако высокие концентрации оказали частичное ингибирующее действие, в отличие от контроля (фруктоза–азотная среда). При добавлении ГБ минимальная и максимальная H_2-окисляющая активность гидрогеназы целых клеток *R. eutropha* составляла 3.4±0.01 *U* (*г* СВК)$^{-1}$ и 16.4±0.01 *U* (*г* СВК)$^{-1}$ соответственно при концентрациях 7 и 300 *мкмоль*/*мл*, а в контроле – отсутствует. Эти результаты могут привести к разработке новых подходов к получению толерантных к кислороду Гид.