UDC: 579.846.2:22

ACTIVITY OF PERIPLASMIC HYDROGENASE OF THE INTESTINAL SULFATE-REDUCING BACTERIA

I. V. Kushkevych[1], H. L. Antonyak[2]
ivan.kushkevych@gmail.com

[1]Institute of Animal Biology of NAAS, V. Stus St., 38, Lviv, 79034, Ukraine
[2]Ivan Franko National University of Lviv, Hrushevsky St., 4, 79005, Lviv, Ukraine

In this paper we present a periplasmic hydrogenase activity of the sulfate-reducing bacteria Desulfovibrio piger Vib-7 and Desulfomicrobium sp. Rod-9 isolated from the human large intestine. The effect of temperature and pH as well as substrate concentration on the hydrogenase activity in the cell-free extracts of the D. piger Vib-7 and Desulfomicrobium sp. Rod-9 was studied. The optimal temperature for hydrogenase reaction is +35 ºC and pH 8.0 for extracts of both bacterial strains. Based on experimental data, the analysis of the kinetic properties of the periplasmic hydrogenase by the studied bacteria was carried out. Our experimental data have shown that the kinetic curves of the periplasmic hydrogenase reaction have tendency to saturation. The kinetics of the activity in cell-free extracts of the studied bacteria was consistent to the zero-order reaction in the range of 0–20 min. A monotonic increase in the activity of the enzyme was observed under the influence of hydrogen in concentrations range from 100 until 2000 μM. The periplasmic hydrogenase activity, initial (instantaneous) reaction rate (205.67±18.91 μmol H_2/min×mg^{-1} protein) and maximum rate of the hydrogenase reaction (2500±219 μmol H_2/min×mg^{-1} protein) were significantly higher in the cell-free extracts of the D. piger Vib-7 strain than Desulfomicrobium sp. Rod-9. Michaelis constants (K_m) of the activity were similar to each other: 864±73 and 669±62 μM for D. piger Vib-7 and Desulfomicrobium sp. Rod-9, respectively. Although values of K_m of both enzymes were similar and were not statistically different, they cross the vertical axis at different points.

Keywords: SULFATE-REDUCING BACTERIA, HYDROGENASE ACTIVITY, TOXICITY, INTESTINAL MICROBIOCENOSIS, BOWEL DISEASES

АКТИВНІСТЬ ПЕРИПЛАЗМАТИЧНОЇ ГІДРОГЕНАЗИ СУЛЬФАТВІДНОВЛЮВАЛЬНИХ БАКТЕРІЙ КИШЕЧНИКА

І. В. Кушкевич[1], Г. Л. Антоняк[2]
ivan.kushkevych@gmail.com

[1]Інститут біології тварин НААН, вул. В. Стуса, 38, Львів, 79034, Україна
[2]Львівський національний університет імені Івана Франка,
вул. Грушевського, 4, Львів, 79005, Україна

Досліджено активність периплазматичної гідрогенази сульфатвідновлювальних бактерій Desulfovibrio piger Vib-7 та Desulfomicrobium sp. Rod-9, виділених з товстого кишечника людини. Вивчено вплив температури, pH та концентрації субстрату на гідрогеназну активність безклітинних екстрактів бактерій D. piger Vib-7 і Desulfomicrobium sp. Rod-9. Встановлено оптимальну температуру (+35 ºС) та pH 8,0 для гідрогеназної реакції екстрактів обох штамів бактерій. На основі експериментальних даних, проаналізовано кінетичні властивості периплазматичної гідрогенази досліджуваних бактерій. Наші експериментальні дані показали, що кінетичні криві гідрогеназної реакції мають тенденцію до насичення. Кінетичні активності безклітинних екстрактів сульфатвідновлювальних бактерій підпорядковуються до реакції нульового порядку в інтервалі 0–20 хв. За впливу гідрогену у діапазоні концентрацій від 100 до 2000 мкМ виявлено монотонне збільшення активності ферменту. Активність периплазматичної гідрогенази, початкова (миттєва) швидкість реакції (205,67±18,91 мкмоль H_2/хв×$мг^{-1}$ білка) і максимальна

швидкість гідрогеназної реакції (2500±219 мкмоль H_2/хв×мг$^{-1}$ білка) є значно вища у безклітинних екстрактах штаму D. piger Vib-7, ніж Desulfomicrobium sp. Rod-9. Константи Міхаеліса (K_m) гідрогеназної активності є близькі: 864±73 та 669±62 мкМ для D. piger Vib-7 і Desulfomicrobium sp. Rod-9, відповідно. Хоча значення K_m обох ферментів близькі і статистично не відрізняються, вони перетинають вісь ординат у різних точках.

Ключові слова: СУЛЬФАТВІДНОВЛЮВАЛЬНІ БАКТЕРІЇ, ГІДРОГЕНАЗНА АКТИВНІСТЬ, ТОКСИЧНІСТЬ, КИШКОВІ МІКРОБІОЦЕНОЗИ, ЗАХВОРЮВАННЯ КИШЕЧНИКА

АКТИВНОСТЬ ПЕРИПЛАЗМАТИЧЕСКОЙ ГИДРОГЕНАЗЫ СУЛЬФАТВОССТАНОВИТЕЛЬНЫХ БАКТЕРИЙ КИШЕЧНИКА

И. В. Кушкевич[1], Г. Л. Антоняк[2]
ivan.kushkevych@gmail.com

[1]Институт биологии животных НААН, ул. В. Стуса, 38, Львов, 79034, Украина
[2]Львовский национальный университет имени Ивана Франко,
ул. Грушевского, 4, Львов, 79005, Украина

Исследовано активность периплазматической гидрогеназы сульфатвосстановительных бактерий Desulfovibrio piger Vib-7 и Desulfomicrobium sp. Rod-9, выделенных из толстого кишечника человека. Изучено влияние температуры, pH и концентрации субстрата на гидрогеназну активность бесклеточных экстрактов бактерий D. piger Vib-7 и Desulfomicrobium sp. Rod-9. Установлено оптимальную температуру (+35 ºС) и pH 8,0 для гидрогеназной реакции экстрактов обоих штаммов бактерий. На основании экспериментальных данных, проанализированы кинетические свойства периплазматической гидрогеназы исследуемых бактерий. Наши экспериментальные данные показали, что кинетические кривые гидрогеназной реакции имеют тенденцию к насыщению. Кинетические активности бесклеточных экстрактов сульфат-восстановительных бактерий подчиняются реакции нулевого порядка в интервале 0–20 мин. Под влиянием водорода в диапазоне концентраций от 100 до 2000 мкМ обнаружено монотонное увеличение активности фермента. Активность периплазматической гидрогеназы, начальная (мгновенная) скорость реакции (205,67±18,91 мкмоль H_2/мин×мг$^{-1}$ белка) и максимальная скорость гидрогеназнои реакции (2500±219 мкмоль H_2/мин×мг$^{-1}$ белка) значительно выше в бесклеточных экстрактах штамма D. piger Vib-7, чем Desulfomicrobium sp. Rod-9. Константы Михаэлиса (K_m) гидрогеназной активности близкие: 864±73 и 669±62 мкМ для D. piger Vib-7 и Desulfomicrobium sp. Rod-9, соответственно. Хотя значение K_m обоих ферментов близки и статистически не отличаются, они пересекают ось ординат в разных точках.

Ключевые слова: СУЛЬФАТВОССТАНОВИТЕЛЬНЫЕ БАКТЕРИИ, ГИДРОГЕНАЗНАЯ АКТИВНОСТЬ, ТОКСИЧНОСТЬ, КИШЕЧНЫЕ МИКРОБИОЦЕНОЗЫ, ЗАБОЛЕВАНИЯ КИШЕЧНИКА

Hydrogenases are enzymes capable to catalyze the oxidation of molecular hydrogen or its production from protons and electrons according to the reversible reaction: $H_2 \leftrightarrow 2H^+ + 2e^-$. Most of the enzymes fall into to major classes: NiFe and Fe-only hydrogenases [1, 2]. Fe-hydrogenase from *Desulfovibrio desulfuricans* ATCC 7757 has a single hydrophobic channel that runs from the molecular surface to the buried active site and points at the vacant coordination site of one of the two Fe centers at the active site [3]. NiFe hydrogenases from *Desulfovibrio gigas*, *D. fructosovorans* and *Desulfomicrobium baculatum* have also hydrophobic chanels and cavities. Furthermore, one of the channels points at a vacant site on the Ni center, suggesting that, by analogy with the Fe hydrogenases, this metal ion may be the primary substrate binding site [1, 4, 5].

The fully sequenced *Desulfovibrio vulgaris* Hildenborough has a total of six hydrogenases [2, 6]. Four of them are periplasmic and therefore presumably are involved in hydrogen oxidation, including a soluble iron-only hydrogenase, two membrane-associated nickel-iron hydrogenase isozymes, and a membrane-associated nickel-iron-selenium hydrogenase [1, 4, 3]. The [NiFe] hydrogenases are widely distributed in the sulfate-reducing bacteria, but many *Desulfovibrio* spp. are exceptional in also possessing a soluble [Fe] hydrogenase [4]. Despite the importance of these enzymes, it remains unclear why *D. vulgaris* possesses four periplasmic hydrogenases when a single enzyme could be sufficient [1, 2, 7].

There are a lot of data about hydrogenase of the sulfate-reducing bacteria. However, the data about hydrogenase activity of the sulfate-reducing bacteria *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 isolated from the human large intestine has not been yet studied.

The aim of our work was to study periplasmic hydrogenase activity of the sulfate-reducing bacteria *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 isolated from the human large intestine and to carry out the kinetic analysis of hydrogenase reaction.

Materials and Methods

Objects of the study were the sulfate-reducing bacteria *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 isolated from the human large intestine [8, 9].

The bacteria were grown for 72 hours at +37 °C under anaerobic conditions in nutrition modified Kravtsov-Sorokin's liquid medium [8]. The tubes were brim-filled with medium and closed to provide anaerobic conditions.

Cell-free extracts were prepared from stationary phase cultures. The bacteria were washed from the medium with 0.9 % NaCl solution. The bacterial cells were homogenized using the ultrasonic disintegrator at 22 kHz for 5 minutes at 0 ºC to obtain the cell-free extracts. The suspension was displaced into centrifugal tubes and cell-free extract was separated from the cells fragments by centrifugation during 30 minutes at 15000 rpm at +4 ºC.

Protein concentration in the cell-free extracts was determined by the Lowry method.

Hydrogenase activity was measured by the oxidation of photochemically reduced methyl viologen (*MP Biomedicals, California, USA*) as described in paper [10]. The standard assay system for measuring the oxidation of photochemically reduced methyl viologen contained 4 mm methyl viologen, 0.04 mm proflavine, and 40 mm EDTA (pH 5.0) in 3 ml in a 1 cm light path cuvette with a Thunberg side arm. 0.2 ml of cell-free extracts was added in system. The same assay system without added cell-free extracts was used as control. The reaction was initiated by tipping enzyme into the cuvette. All spectrophotometric assays were done at room temperature and the activity was determined by measuring the absorbancy decrease at 600 nm [10]. Enzyme activity was expressed as $U \times mg^{-1}$ protein. The unit equal to 1 μmol H_2/min (or 2 μmol/min of oxidized methyl viologen).

Kinetic analysis of the enzyme reaction was performed in a standard incubation system (as described above) with modified physical and chemical characteristics or the respective components (the incubation time, substrate concentration, temperature and pH). The kinetic parameters characterizing the hydrogenase reaction are the initial (instantaneous) reaction rate (V_0), maximum rate of the reaction (V_{max}), maximum amount of the reaction product (P_{max}) and characteristic reaction time (time half saturation) τ were determined. The amount of the reaction product was calculated stoichiometrically. The kinetic parameters characterizing hydrogenase reactions are Michaelis constant (K_m) and maximum reaction rate of substrate consumption were determined by Lineweaver-Burk plot [11].

Kinetic and statistical calculations of the results were carried out using the software MS Office and Origin computer programs. The research results were treated by methods of variation statistics using Student *t*-test.

The equation of the straight line that the best approximates the experimental data was calculated by method of least squares. The absolute value of the correlation coefficient *r* was from 0.90 to 0.99. The significance of the calculated parameters of line was tested by the Fisher's *F*-test. The accurate approximation was when P≤0.05 [12].

Results and Discussion

The activity of periplasmic hydrogenase in various cellular fractions including cell-free extract, soluble, and sedimentary was studied (table 1). Our results showed that the highest of the activity of the periplasmic hydrogenase in the cell-free extract (1421.4±123.7 U×mg^{-1} protein) for *D. piger* Vib-7 compared to the *Desulfomicrobium* sp. Rod-9 strain where the enzyme is 568.7±45.6 U×mg^{-1} protein. A similar activity of the enzyme is measured in the soluble fraction for both bacterial strains. However, hydrogenase activity was not found in sedimentary fraction. That is why for all our manipulations, we have used the cell-free extract in next experiments.

Table 1

Hydrogenase activity in the *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 stains

Fractions	Hydrogenase activity (U×mg^{-1} protein)	
	Desulfovibrio piger Vib-7	*Desulfomicrobium* sp. Rod-9
Cell-free extract	1421.4±123.7	568.7±45.6***
Individual fractions:		
soluble	1409.7±137.6	553.9±52.4***
sedimentary	nd	nd

Note: Catalytic activities were measured in the in the hydrogen consumption assays. "nd": not determined. Significance of the values M±m, n=5; * — P<0.05, ** — P<0.01, *** — P<0.001, compared to the *Desulfovibrio piger* Vib-7 strain

The activity of the studied enzyme in the cell-free extracts of the obtained sulfate-reducing bacteria under the effect of temperature and pH in the incubation medium was studied (fig. 1).

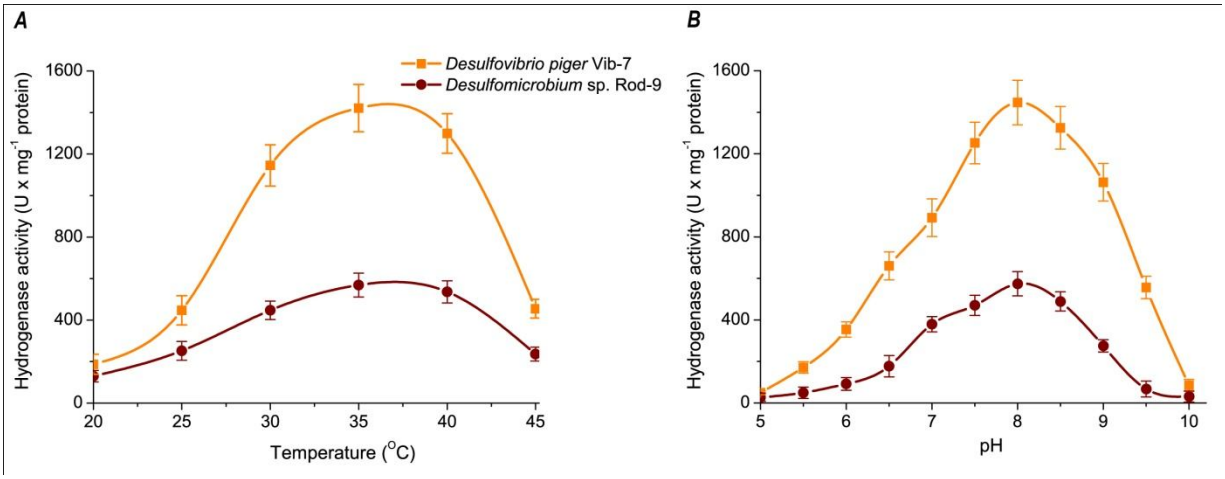

Fig. 1. Effect of various temperature (***A***) and pH (***B***) on the periplasmic hydrogenase activity in the cell-free extracts of the *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9. Catalytic activities were measured in the hydrogen consumption assays

The maximum hydrogenase activity for both bacterial strains was determined at +35 ºC. An increase or decrease in temperature of incubation led to a decrease of the activity of the enzyme in the cell-free bacterial extracts. The most pick of the enzyme activity

was determined in the cell-free extracts of the *D, piger* Vib-7 and the *Desulfomicrobium* sp. Rod-9 at pH 8.0. To study the characteristics and mechanism of the periplasmic hydrogenase reaction, the initial (instantaneous) reaction rate (V_0), maximum consumption of hydrogen (P_{max}) and reaction time (τ) was defined. The dynamics of the hydrogen consumption by the cell-free extracts of the *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 to establishment the kinetic of the periplasmic hydrogenase parameters was studied (fig. 2).

Our experimental data showed that the kinetic curves of the periplasmic hydrogenase activity have tendency to saturation (fig. 2*A*). Analysis of the results allows to reach the conclusion that the kinetics of the activity in cell-free extracts of the studied bacteria was consistent to the zero-order reaction in the range of 0–20 min (the graph of the dependence of the product on the incubation time was almost linear in this interval of time). Therefore the duration of the incubation of bacterial cells extracts was 20 min in subsequent experiments.

The amount of H_2 consumed in the hydrogenase reaction was higher in the cell-free extracts of the *D. piger* Vib-7 compared to the *Desulfomicrobium* sp. Rod-9 in the entire range of time factor. The basic kinetic properties of the hydrogenase reaction in the extracts of the sulfate-reducing bacteria were calculated by linearization of the data in the {P/t; P} coordinates (fig. 2*B*, table 2).

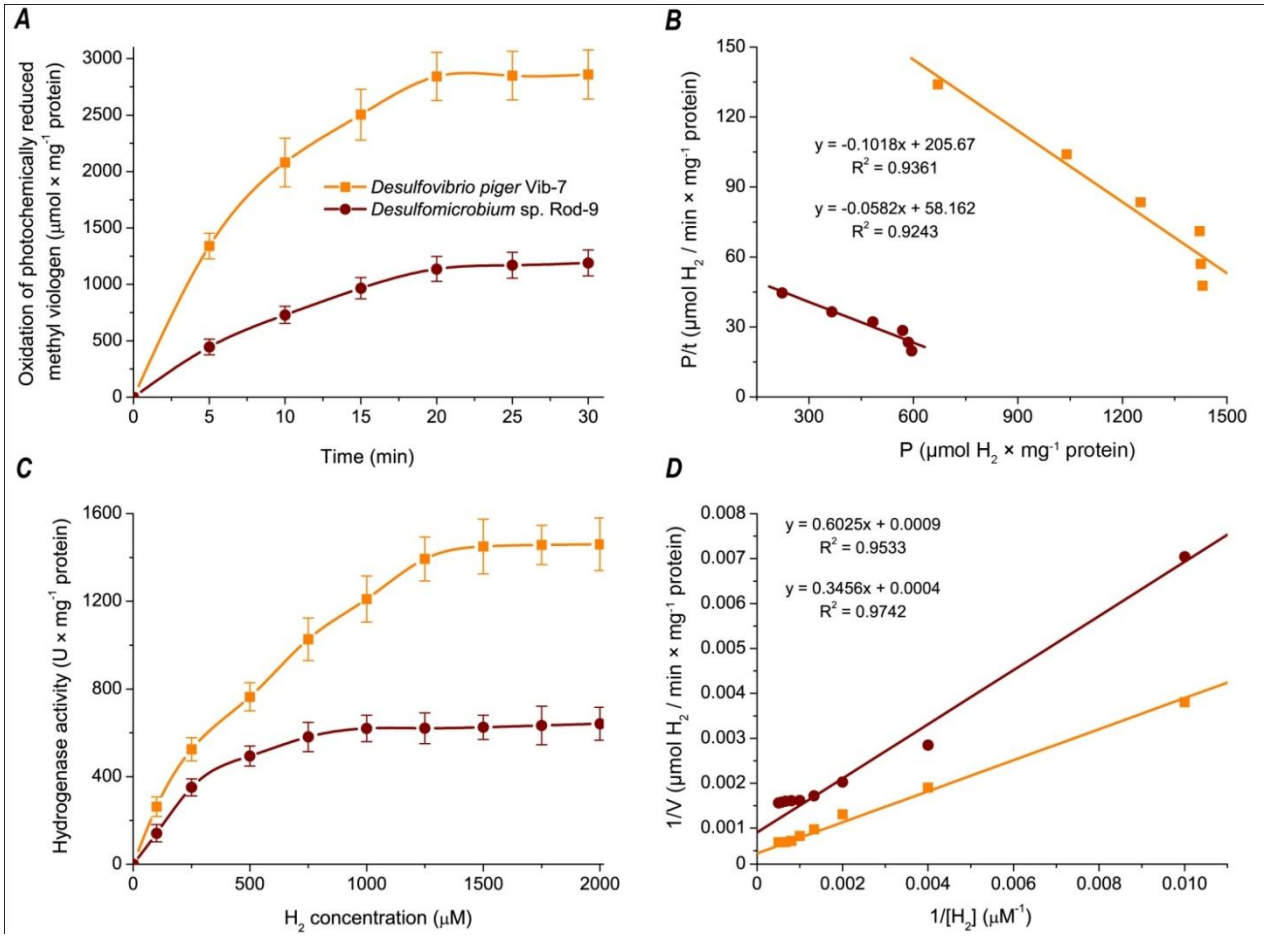

Fig. 2. Kinetic parameters of the periplasmic hydrogenase activity in cell-free extracts of *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9: ***A*** — the dynamics of hydrogen consumption (M±m, n=5); ***B*** — the linearization of the curves of the product reaction in {P/t; P} coordinates (n=5; R^2>0.9; F<0.02); ***C*** — the effect of various substrate (H_2) concentration on the periplasmic hydrogenase activity (M±m, n=5); ***D*** — linearization of concentration curves, which are shown in fig. 2***C***, in the Lineweaver-Burk plot, where *V* is rate of hydrogenase reaction and [H_2] is substrate concentration (n=5; R^2>0.95; F<0.005)

The kinetic hydrogenase parameters of the *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 cell-free extracts were significantly differ from each other. Values of the initial (instantaneous) reaction rate (V_0) for the activity of the cell-free extracts of both bacterial strains by the maximum amount of the product reaction (P_{max}) was calculated. As shown in table 2, V_0 for hydrogenase activity was higher in the cell-free extracts of the *D. piger* Vib-7 (205.67±18.91 μmol H_2/min×mg^{-1} protein) compared to *Desulfomicrobium* sp. Rod-9 (58.16±5.38 μmol H_2/min×mg^{-1} protein).

Table 2

Kinetic parameters of hydrogen consumption in the cell-free extracts of the *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9

Kinetic parameters	Sulfate-reducing bacteria	
	Desulfovibrio piger Vib-7	*Desulfomicrobium* sp. Rod-9
V_0 (μmol H_2/min × mg^{-1} protein)	205.67±18.91	58.16±5.38***
P_{max} (μmol H_2 × mg^{-1} protein)	2020.33±198.44	999.35±95.27***
τ (min)	9.82±0.96	17.18±1.58**

Note: V_0 is initial (instantaneous) reaction rate; P_{max} is maximum amount of the product of reaction; τ is the reaction time (half saturation period). Significance of the values M±m, n=5; ** — P<0.01, *** — P<0.001, compared to the *Desulfovibrio piger* Vib-7 strain

Next task of our study was to carry out a kinetic analysis of the hydrogenase activity depending on the substrate concentration. According to the obtained results, hydrogen increasing in concentrations range from 100 until 2000 μM causes a monotonic increase in enzymatic activity of the enzyme and after that activity was maintained on an unchanged level (plateau) (fig. 2*C*).

Curves of the dependence {1/V; 1/[H_2]} were different by tangent slope and intersect the vertical axis in one point (fig. 2*D*). The basic kinetic parameters of the hydrogenase activity in the *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 cell-free extracts was identified by linearization of the data in the Lineweaver-Burk plot (table 3).

Table 3

Kinetic parameters of hydrogen consumption in the *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 depending on H_2 concentration

Kinetic parameters	Sulfate-reducing bacteria	
	Desulfovibrio piger Vib-7	*Desulfomicrobium* sp. Rod-9
V_{max} (μmol H_2/min×mg^{-1} protein)	2500±219	1111±107***
K_m (μM)	864±73	669±62

Note: V_{max} is maximum rate of the hydrogenase reaction; K_m is Michaelis constant which was determined by H_2 consumption. Significance of the values M±m, n=5; *** — P<0.001, compared to the *Desulfovibrio piger* Vib-7

The K_m values were micromole concentration range which is consistent with similar constants from the literature data [2]. Calculation of the kinetic parameters of the hydrogenase activity indicates that the maximum rate (V_{max}) of hydrogen consumption in the cell-free extracts of the *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 was differ from each other. However, the Michaelis constants (K_m) of the periplasmic hydrogenase activity in the extracts for both bacterial strains were similar to each other: 864±73 and 669±62 μM for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively. Although values of K_m of both enzymes were similar and were not statistically different, they cross the vertical axis at different points.

The results of our study were consistent to previous literature data characterizing the activity of hydrogenase [1–

3, 5, 6]. Van der Westen H.M. and co-authors have described that the K_m value for benzyl viologen in the hydrogen consumption assay was 3.0 mM and V_{max}, was 50000 U/mg. Comparison of these data with values in the literature for previous preparations of hydrogenase from *Desulfovibrio* is not straight-forward because the assays were not all the same. However these researchers have obtained similar data to our results of the hydrogenase activity. There hydrogenase activity is 1600 U/mg protein in bacterial cell extract described in the paper [7], what is very close to the enzyme activity in the cell-free extracts of the *D. piger* Vib-7.

Conclusions

The periplasmic hydrogenase activity, initial (instantaneous) reaction rate (V_0), and the maximum rate of the hydrogenase reaction (V_{max}) was significantly higher in the cell-free extracts of the *D. piger* Vib-7 strain than *Desulfomicrobium* sp. Rod-9. The optimal temperature for hydrogenase reaction was +35 ºC and pH 8.0 for both bacterial strains. Michaelis constants (K_m) of the activity were similar to each other: 864±73 and 669±62 μM for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively. The obtained results might be promising for further study, in particular for more detail understanding physiological and biochemical properties of the studied sulfate-reducing bacteria, prediction and prevention of human inflammatory bowel disease.

Prospects for further research. The studies of the physiological and biochemical properties of the sulfate-reducing bacteria, the process of the dissimilatory sulfate reduction, in particular participate of hydrogenase in the process, the activity and kinetic properties of this enzyme in the *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 bacterial strains, their production of hydrogen sulfide in detail can be prospects to clarify the etiological role of these bacteria in the development of various diseases. The data on the concentration of hydrogen sulfide and acetate, produced by the isolates is supposed to help in establishing and assessing a toxicity effect of these substances on the epithelial cells of the human and animal intestine. Such studies might help in predicting the development of diseases in the gastrointestinal tract, by providing further details on the etiology of bowel diseases which are very important for the clinical diagnosis of these disease types.

1. Adams M. W. The structure and mechanism of ironhydrogenases. *Biochim. Biophys. Acta*, 1990, vol. 1020, pp. 115–145.

2. Caffrey S. M., Park H. S., Voordouw J. K. He Z., Zhou J., Voordouw G. Function of Periplasmic Hydrogenases in the Sulfate-Reducing Bacterium *Desulfovibrio vulgaris* Hildenborough. *J. Bacteriol.*, 2007, vol. 189, no. 17, pp. 6159–6167.

3. Nicolet Y., Piras C., Legrand P., Hatchikian C. E., Fontecilla-Camps J. C. *Desulfovibrio desulfuricans* iron hydrogenase: the structure shows unusual coordination to an active site Fe binuclear center. *Structure with Folding & Design,* 1999, vol. 7, no. 1, pp. 13–23.

4. Barton L. L., Hamilton W. A. *Sulphate-reducing Bacteria. Environmental and Engineered*. Cambridge University Pres, 2007. 553 p.

5. Volbeda A., Fontecilla-Camps J. C. Crystal structure of the nickel–iron hydrogenase from *Desulfovibrio gigas*. *Nature*, 1995, vol. 373, pp. 580–587.

6. Nicolet Y., Cavazza C., Fontecilla-Camps J. C. Fe-only hydrogenases: structure, function and evolution. *J. Inorg. Biochem*., 2002, vol. 91, pp. 1–8.

7. Van der Westen H. M., Mayhew S. G., Veeger C. Separation of hydrogenase from intact cells of *Desulfovibrio vulgaris*. Purification and properties. *FEBS Lett*., 1978, vol. 86, no. 1, pp. 122–126.

8. Kushkevych I. V. Identification of sulfate-reducing bacteria strains of human large intestine. Sci. *Int. Journ. Biological studies/Studia Biologica*, 2013, vol. 7, no. 3, pp. 115–124.

9. Kushkevych I. V., Bartoš M., Bartošová L. Sequence analysis of the 16S rRNA gene of sulfate-reducing bacteria isolated from human intestine. *Int. Jour. Curr. Microbiol. Appl. Sci*, 2014, vol. 3, no. 2, pp. 239–248.

10.Yu L., Wolin M. J. Hydrogenase Measurement with Photochemically Reduced Methyl Viologen. *J. Bacteriol.*, 1969, pp. 51–55.

11.Keleti T. *Basic Enzyme Kinetics*. Akademiai Kiado. 1988. 422 p.

12.Bailey N. T. J. *Statistical Methods in Biology*. Cambridge University Press, 1995. 252 p.