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LACTATE DEHYDROGENASE ACTIVITY IN CELL-FREE EXTRACTS OF SULFATE-REDUCING BACTERIA *DESULFOVIBRIO PIGER* VIB-7 AND *DESULFOMICROBIUM* SP. ROD-9

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Lactate dehydrogenase activity in cell-free extracts of the sulfate-reducing bacteria *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 isolated from human intestine was studied. The optimal temperature (+35ºC) and pH 8.0 for lactate dehydrogenase reaction in the extracts of both bacterial strains were defined. Analysis of the kinetic properties of the bacterial lactate dehydrogenase was carried out. The lactate dehydrogenase activity, initial (instantaneous) reaction rate (V_0) and maximum rate of the lactate dehydrogenase reaction (V_{max}) was significantly higher in the *D. piger* Vib-7 cells than in the *Desulfomicrobium* sp. Rod-9. The kinetic parameters of lactate dehydrogenase activity depended on the substrate concentration. According to the obtained results, lactate increase in concentrations range from 1.0 to 9.0 mM causes a increase in enzymatic activity of the studied enzyme and after that activity was maintained on an unchanged (plateau) level. Michaelis constants (K_m) of the studied enzyme reaction (0.83±0.07 mM for *D. piger* Vib-7 and 1.54±0.14 mM for *Desulfomicrobium* sp. Rod-9) were calculated.

Keywords: sulfate-reducing bacteria, lactate dehydrogenase, intestinal microbiocenosis, inflammatory bowel diseases.

Sulfate-reducing bacteria reduce inorganic sulfate or other oxidized sulfur forms to sulfide [2, 3]. They consume lactate or other organic compounds as electron donor. Sulfate ion is electron acceptor in process of the dissimilatory sulfate reduction [2, 7]. Some sulfate-reducing bacteria can oxidize organic compounds completely to carbon dioxide and water as well as incompletely to acetate [7, 18].

One of the more important carbon and energy substrates for sulfate-reducers is lactate [8]. Lactate in *Desulfovibrio* sp. is oxidized to pyruvate, which is then oxidized to acetate via acetyl CoA. The oxidation of lactate to pyruvate in a species of *Desulfovibrio (D. vulgaris* Marburg) has recently been found to require energy [2, 15, 18].

In previous researches, it was presented growth of isolated sulfate-reducing bacteria *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 consuming lactate ions as electron donor. The studied strains had oxidized lactate incompletely with forming acetate ions in the medium of cultivation [9].

Oxidation of lactate in the cells of sulfate-reducing bacteria is due to enzymatic activity of a membrane-bound lactate dehydrogenase [17, 18]. This enzyme is an important in carbon and energy metabolism in sulfate-reducing bacteria [4]. It has received great attention because of its important metabolic role and because of its existence in several isozymic forms. Lactate dehydrogenase had been assumed to be the starting enzyme for carbon and energy metabolism in dissimilatory sulfate-reducing bacteria, *Desulfovibrio* [15, 18].

From literature data, it is known that lactate dehydrogenase is very difficult to study because this enzyme is unstable, especially when membrane-bound [16]. Consequently very little

work has been done on studying this enzyme, its interaction with other enzymes, and electron transfer proteins in the lactate/sulfate metabolism pathway [14, 17, 18]. A review on bacterial lactate dehydrogenase did not comment on the *D. piger* and *Desulfomicrobium* enzyme. As far as it is aware, lactate dehydrogenase from intestinal sulfate-reducing bacteria *D. piger* and *Desulfomicrobium* has never been well-characterized.

The aim of our work was to study lactate dehydrogenase activity of sulfate-reducing bacteria *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 isolated from the human large intestine and to carry out the kinetic analysis of lactate dehydrogenase 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 [9, 10]. The strains are kept in the collection of microorganisms at the Biotechnology laboratory of Pharmacy Faculty at the University of Veterinary and Pharmaceutical Sciences Brno (Czech Republic).

The bacteria were grown for 72 hours at +37°C under anaerobic conditions in nutrition modified Kravtsov-Sorokin's liquid medium [9]. The tubes were brim-filled with medium and closed to provide anaerobic conditions.

The cell-free extracts were prepared from exponential-phase cultures. The bacterial cells were homogenized using the ultrasonic disintegrator at 22 kHz for 5 minutes at 0ºC 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 g at +4ºC. Protein concentration in the cell-free extracts was determined by the Lowry method [13].

Lactate dehydrogenase activity in cell-free extracts was measured with use of a commercial Cytotoxity Assay Kit (Roche Diagnostics GmbH, Germany) [21]. When the plasma membrane is damaged, lactate dehydrogenase is released into cell culture media. The released lactate dehydrogenase can be quantified by a coupled enzymatic reaction. First, lactate dehydrogenase catalyzes the conversion of lactate to pyruvate via reduction of NAD^+ to NADH. Second, diaphorase uses NADH to reduce a tetrazolium salt to a red formazan product. Therefore, the level of formazan formation is directly proportional to the amount of released lactate dehydrogenase in the medium. Enzyme activity was expressed as $U \times mg^{-1}$ protein. Without lactate in the reaction mixture there was no formation of formazan. The activity of the studied enzyme in the cell-free extracts of both bacterial strains under the effect of different temperature (+20, +25, +30, +35, +40, +45°C) and pH (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0) in process of the enzymatic reaction in the incubation medium was measured.

Kinetic analysis of the enzyme reaction was performed in a standard incubation medium, which was modified by physical and chemical characteristics or the respective components (the incubation time, substrate concentration (sodium DL-lactate solution (Sigma-Aldrich)), temperature and pH). The kinetic parameters characterizing the lactate dehydrogenase reaction are the initial (instantaneous) reaction rate (V_0), maximum rate of the lactate dehydrogenase 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 lactate dehydrogenase reactions are Michaelis constant (K_m) and maximum reaction rate of substrate decomposition *were determined by Lineweaver-Burk plot* [6].

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 0.98. The significance of the calculated parameters of line was tested by the Fisher's *F*-test. The accurate approximation was when P≤0.05 [1].

Results and discussion

A membrane-bound lactate dehydrogenase, an important enzyme in carbon and energy metabolism in sulfate-reducing bacteria of the *Desulfovibrio* and *Desulfomicrobium* genera, was solubilized from the membrane fraction of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9. Activity of lactate dehydrogenase in different fractions including cell-free extract, soluble, and sedimentary has been studied (Table 1). Our results have shown that the highest activity of the enzyme was measured in cell-free extracts (0.472±0.037 and 0.153±0.014 U×mg^{-1} protein for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively). Similar activity of the lactate dehydrogenase was detected in the soluble fraction compared to cell-free extracts. The lowest lactate dehydrogenase activity was found in sedimentary fraction, it values equal 0.227±0.018 and 0.073±0.005 U×mg^{-1} protein for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively.

Table 1

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

Fractions	Lactate dehydrogenase activity (U×mg^{-1} protein)	
	Desulfovibrio piger Vib-7	*Desulfomicrobium* sp. Rod-9
Cell-free extract	0.472±0.037	0.153±0.014***
Individual fractions:		
soluble	0.462±0.041	0.147±0.012***
sedimentary	0.227±0.018	0.073±0.005***

Note. Enzyme activity has been determined at fifteen-minute incubation. Significance of the values M±m, n=5; ***P<0.001, compared to *Desulfovibrio piger* Vib-7 strain.

From literature data, it is known that the enzyme activity depends on temperature and pH [4, 17]. The effect of temperature and pH of the incubation medium on the lactate dehydrogenase activity in the cell-free extracts of the obtained sulfate-reducing bacteria was studied (Fig. 1). The maximum lactate dehydrogenase activity for both bacterial strains was determined at +35ºC. An increase or decrease in temperature of incubation leads to a decrease of the activity of studied enzyme in the cell-free bacterial extracts. The highest activity of lactate dehydrogenase was determined in the cell-free extracts of the *D. piger* Vib-7 and the *Desulfomicrobium* sp. Rod-9 at pH 7.5–8.5.

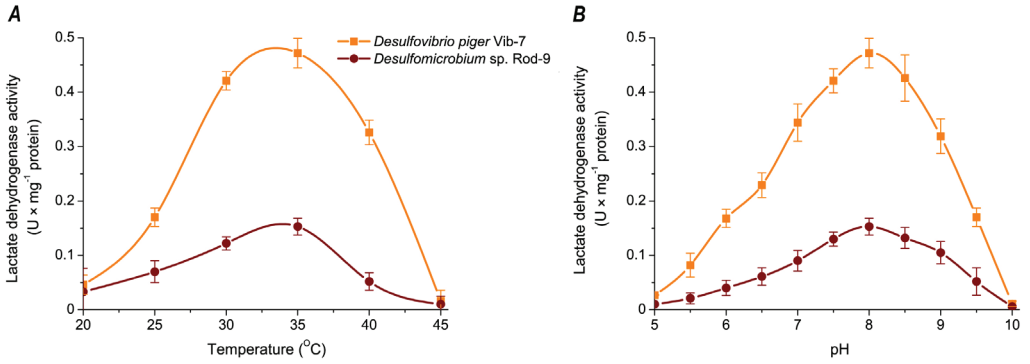

Fig. 1. Effect of various temperature (***A***) and pH (***B***) on the lactate dehydrogenase activity in the cell-free extracts of *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9.

Thus, temperature and pH optimum of this enzyme with lactate as a substrate was +35ºC and pH 8.0, respectively. The enzyme activity exhibited typical bell-shaped curves as a function of temperature and pH.

To study the characteristics and mechanism of lactate dehydrogenase reaction, the initial (instantaneous) reaction rate (V_0), maximum amount (plateau) of the product of reaction (P_{max}) and reaction time (τ) was defined. Dynamics of formazan formation in the cell-free extracts of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 to investigate the kinetic of lactate dehydrogenase parameters was studied (Fig. 2).

Experimental data showed that the kinetic curves of lactate dehydrogenase reaction have tendency to saturation (Fig. 2, *A*). Analysis of the results allows to reach the conclusion that the kinetics of lactate dehydrogenase activity in cell-free extracts of the studied bacteria was consistent to the zero-order reaction in the range of 2–3 min (the graph of the dependence of product formation on the incubation time was almost linear in this interval of time). Therefore the duration of the incubation of bacterial cells extracts was 3 min in subsequent experiments.

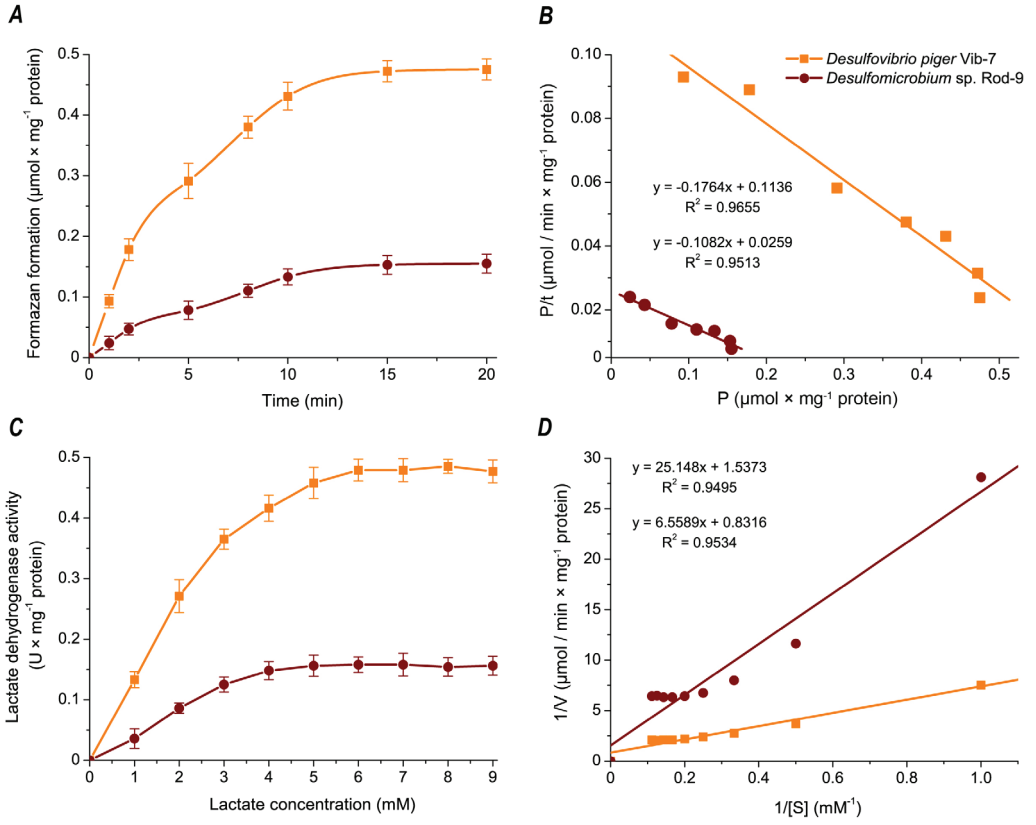

Fig. 2. Kinetic parameters of lactate dehydrogenase activity in the cell-free extracts of *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9: ***A*** – dynamics of product formation (M±m, n=5); ***B*** – linearization of curves of lactate product formation in {P/t; P} coordinates (n=5; R^2>0.95; F<0.02); ***C*** – effect of different lactate concentration on lactate dehydrogenase 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 lactate dehydrogenase reaction and S is substrate concentration (n=5; R^2>0.9; F<0.005).

Amount of product formation in lactate dehydrogenase reaction from the *D. piger* Vib-7 is higher compared to the *Desulfomicrobium* sp. Rod-9 in the entire range of time factor. The basic kinetic properties of the reaction in the cell-free extracts of the sulfate-reducing bacteria are calculated by linearization of the data in the {P/t; P} coordinates (Fig. 2, *B*, Table 2).

Table 2

Kinetic parameters of the formazan formation in the cell-free extracts of *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/min×mg^{-1} protein)	0.114±0.012	0.026±0.022***
P_{max} (μmol×mg^{-1} protein)	0.644±0.044	0.239±0.018***
τ (min)	5.669±0.532	9.242±0.863**

Note. V_0 is initial (instantaneous) reaction rate; P_{max} is maximum amount (plateau) 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 *Desulfovibrio piger* Vib-7 strain.

The kinetic parameters of lactate dehydrogenase in *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 cell-free extracts are significantly different. Values of initial (instantaneous) reaction rate (V_0) for lactate dehydrogenase reaction in the cell-free extracts of both bacterial strains was calculated by the maximum amount of the product reaction (P_{max}). As shown in Table 2, V_0 for lactate dehydrogenase reaction is higher in the cell-free extracts of *D. piger* Vib-7 (0.114±0.012 μmol/min×mg^{-1} protein) compared to *Desulfomicrobium* sp. Rod-9 (0.026±0.022 μmol/min×mg^{-1} protein). Based on these data, there is suppose that the *D. piger* Vib-7 can consume and oxidize lactate ion more faster in their cells than *Desulfomicrobium* sp. Rod-9 strain.

The kinetic analysis of lactate dehydrogenase activity depending on the substrate concentration was studied. According to the obtained results, lactate increase in concentrations range from 1.0 to 9.0 mM causes a monotonic increase in enzymatic activity of the studied enzyme and after that the activity was maintained on an unchanged (plateau) level (Fig. 2, *C*). Curves of the dependence {1/V; 1/[S]} were different by tangent slope and intersect the vertical axis in one point (Fig. 2, *D*). The basic kinetic parameters of lactate dehydrogenase activity in *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 lactate dehydrogenase in the cell extracts of *Desulfovibrio piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 depending on lactate concentration

Kinetic parameters	Sulfate-reducing bacteria	
	Desulfovibrio piger Vib-7	*Desulfomicrobium* sp. Rod-9
V_{max} (μmol/min×mg^{-1} protein)	1.20±0.11	0.65±1.73***
K_m (mM)	0.83±0.07	1.54±0.14***

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

The K_m values are milimolar concentration range which is consistent with similar constants from the literature data [4, 16]. Calculation of the kinetic parameters of lactate dehydrogenase reaction indicates that the maximum rate (V_{max}) of formazan formation in the cell-free extracts of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 was different from each other. Michaelis constants (K_m) of lactate dehydrogenase activity for both bacterial strains were also significantly different: 0.83±0.07 and 1.54±0.14 mM for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively.

Thus based on the obtained studies results and according to the kinetic parameters of lactate dehydrogenase activity determined by formazan formation for both bacterial strains, we have concluded that the *D. piger* Vib-7 can oxidize lactate more faster compared to the *Desulfomicrobium* sp. Rod-9. The activity of lactate dehydrogenase, V_0 and V_{max} were significantly higher in the *D. piger* Vib-7 cells than *Desulfomicrobium* sp. Rod-9. According to these data, process of the dissimilatory sulfate reduction and consummation of sulfate can be faster in *D. piger* Vib-7 strain than *Desulfomicrobium* sp. Rod-9.

From literature data, it is known that *Desulfovibrio* genus was often isolated from healthy and sick humans and animals [5, 11]. This bacterial genus plays a more pathogenic role than other genera of sulfate-reducing bacteria [3, 19, 20]. Bacteria *Desulfovibrio fairfieldensis* was isolated in mono- as well as polymicrobial infections of the gastrointestinal tract [11, 12]. Twelve out of 100 samples of purulent peritoneal and pleural cavities in humans contained *Desulfovibrio piger*, *D. fairfieldensis* or *D. desulfuricans* [11]. Bacteria *D. desulfuricans* have also been isolated from the colon during bleeding microvilli, causing bacteremia [12]. Probably, the *D. piger* Vib-7 strain can also be more dangerous and have some pathogenic significance in inflammatory bowel diseases development, producing hydrogen sulfide at a higher rate of lactate and sulfate consumption.

The obtained results of lactate dehydrogenase reaction in the cell-free extracts of *D. piger* Vib-7 are consistent to data Ogata M. (1981) and coautors. D-Lactate dehydrogenase, the starting enzyme for carbon and energy metabolism in dissimilatory sulfate-reducing bacteria, was obtained from the soluble fraction of the sonicate of *Desulfovibrio vulgaris*. There was calculated Michaelis constant (K_m equal 0.8 mM) for enzymatic reaction of D-lactate as the electron donor substrate [16].

A membrane-bound lactate dehydrogenase was solubilized from the membrane fraction of *Desulfovibrio desulfurieans* (ATCC 7757). The enzyme activity of *D. desulfurieans* had close values to lactate dehydrogenase activity in the cell-free extracts of *D. piger* Vib-7. The lactate dehydrogenase activity was specific for D(-)-lactate and had a K_m for D(-)-lactate of 0.43 mM. The pH optimum was between 6.5 and 8.5 [4].

The highest activity of lactate dehydrogenase in the cell-free extract of the bacterial strains was detected (0.472±0.037 and 0.153±0.014 $U \times mg^{-1}$ protein for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively). The maximum lactate dehydrogenase activity for both strains was determined at +35ºC and at pH 8.0.

The kinetic parameters of formazan formation in the cell-free extracts of *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9 were studied. The initial (instantaneous) reaction rate (V_0) was 0.114±0.012 $\mu mol/min \times mg^{-1}$ protein for *D. piger* Vib-7 and 0.026±0.022 $\mu mol/min \times mg^{-1}$ protein for *Desulfomicrobium* sp. Rod-9. These kinetic parameters of lactate dehydrogenase activity depended on the substrate concentration. According to the obtained results, lactate increase in concentrations range from 1.0 to 9.0 mM causes a monotonic increase in enzymatic activity of the studied enzyme and after that activity was maintained on an unchanged (plateau) level.

The lactate dehydrogenase activity, V_0 and V_{max} was significantly higher in the *D. piger* Vib-7 cells than *Desulfomicrobium* sp. Rod-9. Michaelis constants (K_m) of the lactate dehydrogenase reaction were 0.83±0.07 and 1.54±0.14 mM for *D. piger* Vib-7 and *Desulfomicrobium* sp. Rod-9, respectively.

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АКТИВНІСТЬ ЛАКТАТДЕГІДРОГЕНАЗИ БЕЗКЛІТИННИХ ЕКСТРАКТІВ СУЛЬФАТВІДНОВЛЮВАЛЬНИХ БАКТЕРІЙ *DESULFOVIBRIO PIGER* VIB-7 ТА *DESULFOMICROBIUM* SP. ROD-9

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Досліджено активність лактатдегідрогенази безклітинних екстрактів сульфатвідновлю-вальних бактерій *Desulfovibrio piger* Vib-7 і *Desulfomicrobium* sp. Rod-9, ізольованих з кишечника людини. Визначено оптимальну температуру (+35ºС) і pH 8.0 для лактатдегідрогеназної реакції у екстрактах обох штамів. Проведено аналіз кінетичних властивостей лактатдегідрогенази досліджуваних бактерій. Встановлено, що активність лактатдегідрогенази, початкова (миттєва) швидкість реакції (V_0) і максимальна швидкість лактатдегідрогеназної реакції (V_{max}) є значно вища у клітинах бактерій *D. piger* Vib-7, а ніж у *Desulfomicrobium* sp. Rod-9. Кінетичні параметри активності лактатдегідрогенази залежали від концентрації субстрату. Відповідно до отриманих результатів, збільшення концентрації лактату у діапазоні від 1,0 до 9,0 мМ спричиняло наростання активності досліджуваного ензиму, після чого активність підтримується на незмінному (плато) рівні. Розраховано константи Міхаеліса (K_m) досліджуваної ферментативної реакції (0,83±0,07 мМ для *D. piger* Vib-7 та 1,54±0,14 мМ для *Desulfomicrobium* sp. Rod-9).

Ключові слова: сульфатвідновлювальні бактерії, лактатдегідрогеназа, мікробіоценоз кишечника, запальні захворювання кишечника.

АКТИВНОСТЬ ЛАКТАТДЕГИДРОГЕНАЗЫ БЕСКЛЕТОЧНЫХ ЭКСТРАКТОВ СУЛЬФАТВОССТАНАВЛИВАЮЩИХ БАКТЕРИЙ *DESULFOVIBRIO PIGER* VIB-7 И *DESULFOMICROBIUM* SP. ROD-9

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Исследовано активность лактатдегидрогеназы бесклеточных экстрактов сульфат-восстановительных бактерий *Desulfovibrio piger* Vib-7 и *Desulfomicro-*

bium sp. Rod-9, изолированных из кишечника человека. Определены оптимальные температура (+35ºС) и pH 8,0 для лактатдегидрогеназной реакции в экстрактах обоих штаммов. Проведено анализ кинетических свойств лактатдегидрогеназы исследуемых бактерий. Установлено, что активность лактатдегидрогеназы, начальная (мгновенная) скорость реакции (V_0) и максимальная скорость лактатдегидрогеназной реакции (V_{max}) значительно выше в клетках бактерий *D. piger* Vib-7, чем в *Desulfomicrobium* sp. Rod-9. Кинетические параметры активности лактатдегидрогеназы зависели от концентрации субстрата. Согласно полученным результатам, увеличение концентрации лактата в диапазоне от 1,0 до 9,0 мМ вызывало нарастание активности исследуемого фермента, после чего активность поддерживается на неизменном (плато) уровне. Рассчитаны константы Михаэлиса (K_m) исследуемой ферментативной реакции (0,83±0,07 мМ для *D. piger* Vib-7 и 1,54±0,14 мМ для *Desulfomicrobium* sp. Rod-9).

Ключевые слова: сульфатвосстанавливающие бактерии, лактатдегидрогеназа, микробиоценоз кишечника, воспалительные заболевания кишечника.