American Journal of Microbiology and Biotechnology
2014; 1(2): 49-55
Published online October 20, 2014 (http://www.aascit.org/journal/ajmb)

Keywords
Sulfate-Reducing Bacteria,
Desulfovibrio piger,
Sulfates,
Hydrogen Sulfide,
Acetate,
Inflammatory Bowel Diseases,
Ulcerative Colitis

Received: September 15, 2014
Revised: October 11, 2014
Accepted: October 12, 2014

Dissimilatory sulfate reduction in bacterium *Desulfovibrio piger* Vib-7 under the effect of medium with differential acidity

Ivan Kushkevych

Laboratory of Molecular Biology and Clinical Biochemistry, Institute of Animal Biology of NAAS of Ukraine

Email address
ivan.kushkevych@gmail.com

Citation
Ivan Kushkevych. Dissimilatory Sulfate Reduction in Bacterium *Desulfovibrio Piger* Vib-7 under the Effect of Medium with Differential Acidity. *American Journal of Microbiology and Biotechnology*. Vol. 1, No. 2, 2014, pp. 49-55.

Abstract
In this paper, the effect of cultivation medium with differential acidity on the dissimilatory sulfate reduction process in bacterium *Desulfovibrio piger* Vib-7 isolated from the human intestine was studied. Microbiological, biochemical, and biophysical methods of the studies, and statistical processing of the results were used; the obtained data were compared with those from literature. The most intensive growth of intestinal bacterium *D. piger* Vib-7, sulfate and lactate dissimilation, and sulfide and acetate production was determined at the optimum pH 7.0–8.0. A correlation and cross-correlation analysis of these processes under the effect of differential acidity of the culture medium was carried out. The described results of these studies can be the prospects to clarify the etiological role of these bacteria in the development of inflammatory bowel diseases humans and animals.

1. Introduction

Sulfate-reducing bacteria carry out the dissimilatory sulfate reduction to hydrogen sulfide [1,2]. They use sulfate as an electron acceptor and lactate as the electron donor. Lactate is incompletely oxidized by these bacteria, to acetate [2]. The final metabolism products of these microorganisms can be accumulated in toxic concentrations in the human and animal intestine, causing many inflammatory bowel diseases and cancer [3,4,5,6,7,8]. The toxic effect of hydrogen sulfide on the cells of the intestine blocks their metabolism through inhibition of cytochrome oxidase, and the destruction of disulfide bridges of the organic compounds of the intestine [9,10]. This may disrupt the structure of the protective epithelial layer. Hydrogen sulfide can penetrate through the cell membrane without the need for specific receptors. Its affect on adenosine-5'-triphosphate-dependent potassium channels, cytochrome *c* oxidase, DNA damage and inactivate enzymes in intestinal cells and, consequently, causes disease. In addition, it was found that increasing the acetate concentration leads to three-fold decrease of butyrate in the gut [2,4,11,12]. There is evidence that the *Desulfovibrio* genus is dominant among intestinal sulfate-reducing bacteria [5,7,13]. The fact that influence of cultivation media of differential acidity on the dissimilatory sulfate reduction by the *Desulfovibrio* genus has been insufficiently studied provides relevance for this work.

The aim of this work was to study the process of dissimilatory sulfate reduction by the *Desulfovibrio piger* Vib-7 of the human large intestine under the influence of differential acidity of cultivation medium, and to carry out correlation and cross-correlation analysis of this process as well as comparison with data from literature.

2. Materials and Methods

The objects of the study were the sulfate-reducing bacteria of the *Desulfovibrio piger* strain Vib-7 obtained from the human large intestine [14,15].

The bacteria were grown in nutrition modified Kravtsov-Sorokin's liquid medium of such composition (g/l): Na_2SO_4 – 0.5; KH_2PO_4 – 0.3; K_2HPO_4 – 0.5; $(NH_4)_2SO_4$ – 0.2; NH_4Cl – 1.0; $CaCl_2 \times 6H_2O$ – 0.06; $MgSO_4 \times 7H_2O$ – 0.1; $C_3H_5O_3Na$ – 2.0; yeast extract – 1.0; $FeSO_4 \times 7H_2O$ – 0.004; sodium citrate$\times 2H_2O$ – 0.3. Before passaging microbial cultures in the medium, 0.05 ml/l of sterile solution of $Na_2S \times 9H_2O$ (1%) was added. The medium was heated in boiling water for 30 min in order to obtain an oxygen-free medium, and cooled to +30°C. The bacteria were grown for 72 hours at +37°C under anaerobic conditions. The tubes were brim-filled with medium and closed to provide anaerobic conditions.

To study the effect of pH on the growth of bacteria *D. piger* Vib-7 and their process of dissimilatory sulfate reduction, 10% HCl or NaOH were added in the Kravtsov-Sorokin's liquid medium to adjust the final pH (3.0; 4.0; 5.0; 6.0; 7.0; 8.0; 9.0 and 10.0) of the cultivation medium.

Accumulation biomass of sulphate-reducing bacteria in liquid medium (without Mohr's salt) was determined by turbidity of dilute suspension of cells by photometric method [16].

The sulfate ions concentration in the medium was determined by turbidymetric method after precipitation by barium chloride. To stabilize the suspension, glycerol was used [17].

Hydrogen sulfide concentration in the culture medium was photometrically determined using spectrophotometer (λ=665 nm, cuvette with optical path 30 mm). The reaction mixture had the following composition: zinc citrate (27.3 mM) – 10 ml; distilled water – 1.98 ml; *n*-aminodimethylaniline solution (5.5 mM) – 4 ml, and 20 µl of test solution. After 5 min, 1 ml of ferric chloride (0.125 M) was added and methylene blue formation was observed. The concentration of hydrogen sulfide was established by calibration curve [18].

Determination of lactate concentration was carried out through a dehydrogenation reaction of lactate by lactate dehydrogenase in the presence of NAD^+, with formation of pyruvate and NADH. For determination of lactate content the following reagents were used: hydrazine-glycine buffer pH 9.0 (glycine – 0.1 M solution containing 0.1 M hydrazine); NAD^+ – 0.03 M solution, pH 6.0; lactate dehydrogenase solution (protein content was about 2 mg/ml). The samples of glycine and hydrazine were dissolved in a small amount of distilled water, a pH of 9.0 was maintained by a 2 N NaOH solution, then the mixture was diluted by distilled water to 100 ml. Solutions of NAD^+ and lactate dehydrogenase were kept on ice. The content of the tubes was thoroughly mixed and placed inside a thermostat at +25°C for 60 min. After incubation, samples were cooled and the optical density of the samples was measured at 340 nm. The quantity of the lactic acid was subsequently calculated [19].

Accumulation of acetate ions in the bacterial cultures during their growth in the medium was determined by titration [20].

Using the experimental data, the basic statistical parameters (M – mean, m – standard error, M±m) have been calculated. For the estimation of the reliability between the statistical characteristics, Student's *t*-test was used. The difference was significant when $P \geq 0.95$ [21]. The Pearson's correlation coefficient (*r*) was calculated using Excel program [22]. Statistical processing of the results was performed using Excel, Origin and Statistica computer programs.

3. Results and Discussion

The results of this study have shown that the isolated strain *D. piger* Vib-7 during growth consumes sulfate as an electron acceptor and accumulates hydrogen sulfide in the medium with pH from 4.0 to 10.

Under these conditions the bacterial strain consumed lactate as the electron donor which actively incompletely oxidized to acetate. Different growth rates of the *D. piger* Vib-7 in the modified Kravtsov-Sorokin's medium depending on the pH of the medium have been studied (Figure 1).

The *D. piger* Vib-7 strain has accumulated different biomasses during the 72 hours of cultivation under influence of various acidity of the medium; while bacteria have consumed sulfate and lactate, producing hydrogen sulfide and acetate at varying rates. The growth rate of these bacteria depends on the consummation of sulfate and lactate, and the accumulation of hydrogen sulfide and acetate as well as it depended on the pH of the medium.

The stationary growth phase of the *D. piger* Vib-7 began after 48^{th} hours of cultivation. The highest biomass (4.18 mg/ml) was accumulated by the strain *D. piger* Vib-7 at pH 8 at the 60^{th} hour of cultivation. The lowest bacterial biomass (1.09 mg/ml) was accumulated at pH 4 at the same time of cultivation. The bacterial growth and sulfate reduction process in acidic medium (pH 3) was not observed.

The studied bacteria actively reduced the sulfate ions and produced hydrogen sulfide. The highest concentration of hydrogen sulfide (3.21 mM) was produced by bacteria *D. piger* Vib-7 at pH 8 on the 72^{th} hour of cultivation; while the bacteria used about 100% of the sulfate in the medium compared to the initial sulfate concentration. The strain *D. piger* Vib-7 produced hydrogen sulfide in the lowest concentration at pH 4 compared to other pHs.

The presence of lactate as an electron donor in the medium during dissimilatory sulfate reduction allowed production of acetate. The highest concentration of acetate ions (15.92 mM) by the strain *D. piger* Vib-7 was achieved on 72^{th} hour of cultivation at pH 7–8; while the bacteria used about 100% of lactate in the medium, compared to its initial lactate concentration.

Thus, the studied bacteria *D. piger* Vib-7 consumes sulfate as terminal electron acceptor and lactate as electron donor respiration. In addition, the strain intensely produces hydrogen sulfide and acetate in the medium. This process of dissimilatory sulfate reduction is influenced by the acidity of the medium.

Correlation analysis is used to determine whether the values of two variables are associated. Other words the correlation – a statistical relation between two or more variables such that systematic changes in the value of one variable are

accompanied by systematic changes in the other. The correlation coefficient ranges from -1.0 to +1.0. The closer *r* is to +1 or -1, the more closely the two variables are related. If *r* is close to 0, it means there is no relationship between the variables. If *r* is positive, it means that as one variable gets larger the other gets larger. If *r* is negative it means that as one gets larger, the other gets smaller (often called an «inverse» correlation). While correlation coefficients are normally reported as *r* = (a value between -1 and +1), squaring them makes then easier to understand. Values between 0.7 and 1.0 (-0.7 and -1.0) indicate a strong positive (negative) linear relationship via a firm linear rule [4].

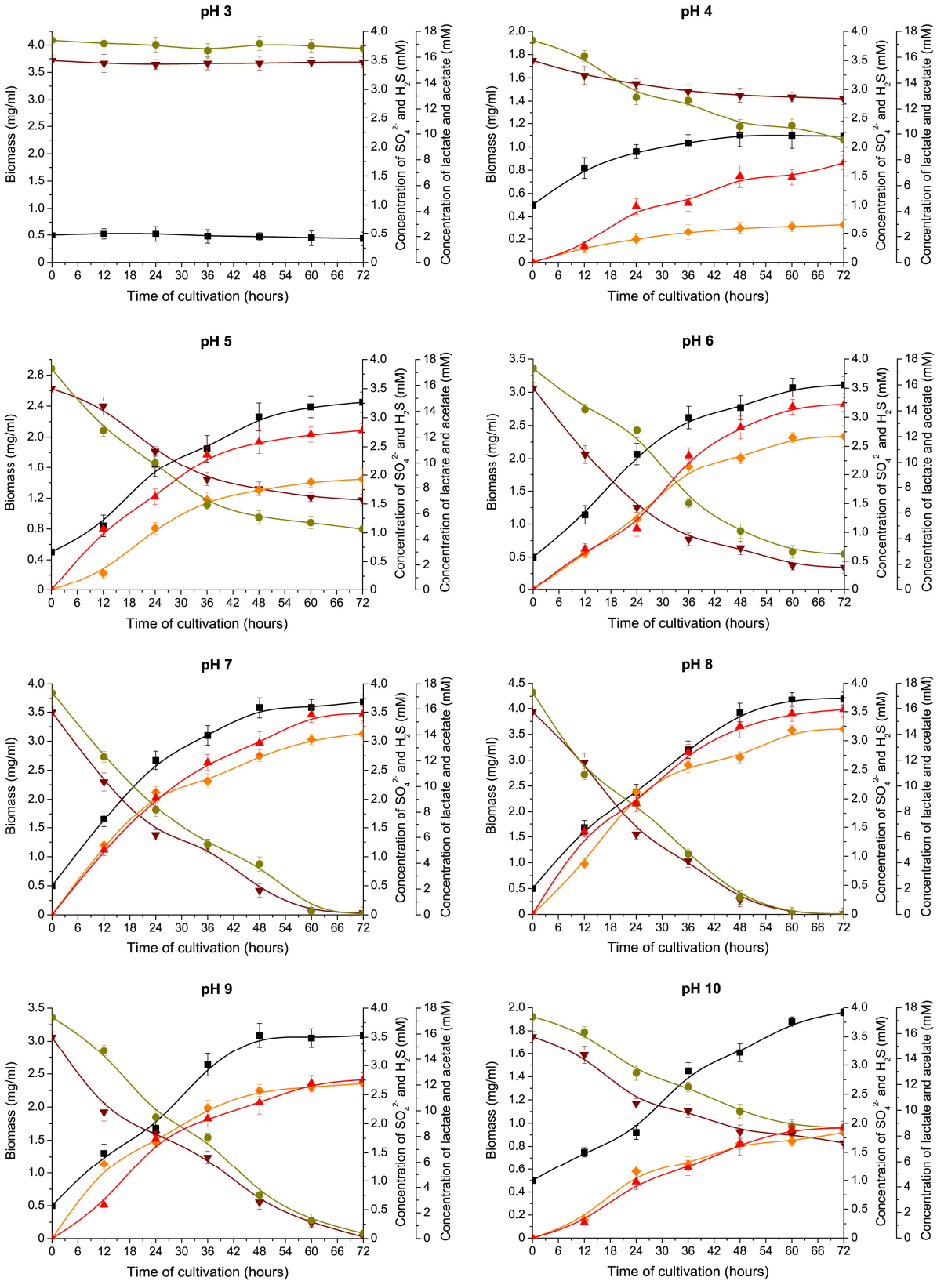

Figure 1. *The growth of Desulfovibrio piger Vib-7 and their process of dissimilatory sulfate reduction under influence of the cultivation medium with differential acidity: –■– biomass; –▼– sulfate; –♦– sulfide; –●– lactate; –▲– acetate*

The correlation coefficients (*r*) between parameters of dissimilatory sulfate reduction by the *D. piger* Vib-7 strains were defined (Table 1).

The strong inversely negative correlation was observed between biomass and sulfate; biomass and lactate; sulfate and sulfide; sulfate and acetate; lactate and acetate; and lactate and sulfide.

The strong positive correlation was determined between biomass and sulfide; biomass and acetate; lactate and sulfate; acetate and sulfide.

Table 1. *Correlation coefficients (r) between dissimilatory sulfate reduction parameters by the Desulfovibrio piger Vib-7 under influence of differential acidity of the medium*

	pH 4					**pH 5**				
	Biomass	**Sulfate**	**Sulfide**	**Lactate**	**Acetate**	**Biomass**	**Sulfate**	**Sulfide**	**Lactate**	**Acetate**
Biomass	1	-0.983	0.983	-0.926	0.926	1	-0.993	0.993	-0.974	0.975
Sulfate	-0.983	1	-1	0.908	-0.908	-0.993	1	-1	0.981	-0.981
Sulfide	0.983	-1	1	-0.908	0.908	0.993	-1	1	-0.981	0.981
Lactate	-0.926	0.908	-0.908	1	-1	-0.974	0.981	-0.981	1	-1
Acetate	0.926	-0.908	0.908	-1	1	0.975	-0.981	0.981	-1	1
	pH 6							**pH 7**		
	Biomass	Sulfate	Sulfide	Lactate	Acetate	Biomass	Sulfate	Sulfide	Lactate	Acetate
Biomass	1	-0.994	0.991	-0.968	0.968	1	-0.986	0.994	-0.974	0.99
Sulfate	-0.994	1	-0.98	0.951	-0.951	-0.986	1	-0.995	0.991	-0.993
Sulfide	0.991	-0.98	1	-0.992	0.992	0.994	-0.995	1	-0.985	0.993
Lactate	-0.968	0.951	-0.992	1	-1	-0.974	0.991	-0.985	1	-0.996
Acetate	0.968	-0.951	0.992	-1	1	0.99	-0.993	0.993	-0.996	1
	pH 8							**pH 9**		
	Biomass	Sulfate	Sulfide	Lactate	Acetate	Biomass	Sulfate	Sulfide	Lactate	Acetate
Biomass	1	-0.992	0.979	-0.999	0.995	1	-0.966	0.977	-0.962	0.967
Sulfate	-0.992	1	-0.992	0.986	-0.985	-0.966	1	-0.971	0.98	-0.962
Sulfide	0.979	-0.992	1	-0.972	0.979	0.977	-0.971	1	-0.938	0.965
Lactate	-0.999	0.986	-0.972	1	-0.997	-0.962	0.98	-0.938	1	-0.978
Acetate	0.995	-0.985	0.979	-0.997	1	0.967	-0.962	0.965	-0.978	1

Cross-correlation analysis is the tool most commonly used in the analysis of multiple time series. Its application to biological objects merits special attention. The next stage of this research project was to carry out a cross-correlation analysis between the sulfate reduction parameters by the strain *D. piger* Vib-7. The cross-correlation analysis is basically a generalization of standard linear correlation analysis. A measure of the strength of the correlation is given by the correlation coefficient. This analysis provides a correlation between two time series or two waveforms. The observations of one series are correlated with the observations of another series at various lags and leads.

The results of the cross-correlation analysis showed a strong correlation in pairs of the following parameters: biomass and sulfate, biomass and lactate, sulfate and sulfide, sulfate and acetate, lactate and acetate, lactate and sulfide, biomass and sulfide, biomass and acetate, lactate and sulfate, acetate and sulfide. The processes that describe the changes in these parameters closely conjugate in the bacteria the *D. piger* Vib-7 (Figure 2).

The cross-correlation test of two time-series data sets involves many calculations of the coefficient *r* by time-shifting one data set relative to the other data set. Each shift is called a «lag» and the lag time is simply the sampling period of the two time-series data sets. A typical cross-correlation shows enough lags in both negative and positive directions to show the cyclical relationship of the two sets of data. In cross-correlation analysis, waveforms that alternate are out-of-phase from each other and will have a negative relationship, whereas waveforms that are synchronous will be in-phase and have a positive relationship. Based on experimental data, surface models of dissimilatory sulfate reduction parameters were constructed by the *D. piger* Vib-7 under the influence of media of different acidity (Figure 3).

The obtained surface models show that strain *D. piger* Vib-7 grows intensively in the medium with pH 7–8. The most intense sulfate reduction process occurs under these conditions. This pH is optimum in particular for the production of hydrogen sulfide and acetate as well as consuming sulfate and lactate. Decreasing the pH to acidic conditions (pH<7) or increasing to alkaline (pH>8) conditions inhibited these processes.

Thus, the constructed surface models have provided an opportunity to confirm and to establish the optimum growing point for the studied bacteria, their sulfate and lactate consumption as well as the production of sulfide and acetate.

The human large intestine contains mucus and small amount of enzymes (peptidase, lipase, amylase, phosphatase, and nuclease) which have a lower level of enzymatic activity than those in the small intestine. The pH of the large intestine is in the range of 8.5–9. Based on obtained data it can be argued that isolated srtain *D. piger* Vib-7 can actively grow under these conditions as well as intensive accumulate sulfide and acetate in the human intestine. This may be the cause of ulcerative colitis and in turn bowel cancer. Hydrogen sulfide has negative affects on the intestinal mucosa, it is toxic to epithelial cells, specifically inhibits the growth of the colonocytes, phagocytosis, causes the death of intestinal bacteria, and induces hyperproliferation and metabolic abnormalities of epithelial cells [2,5,6,10].

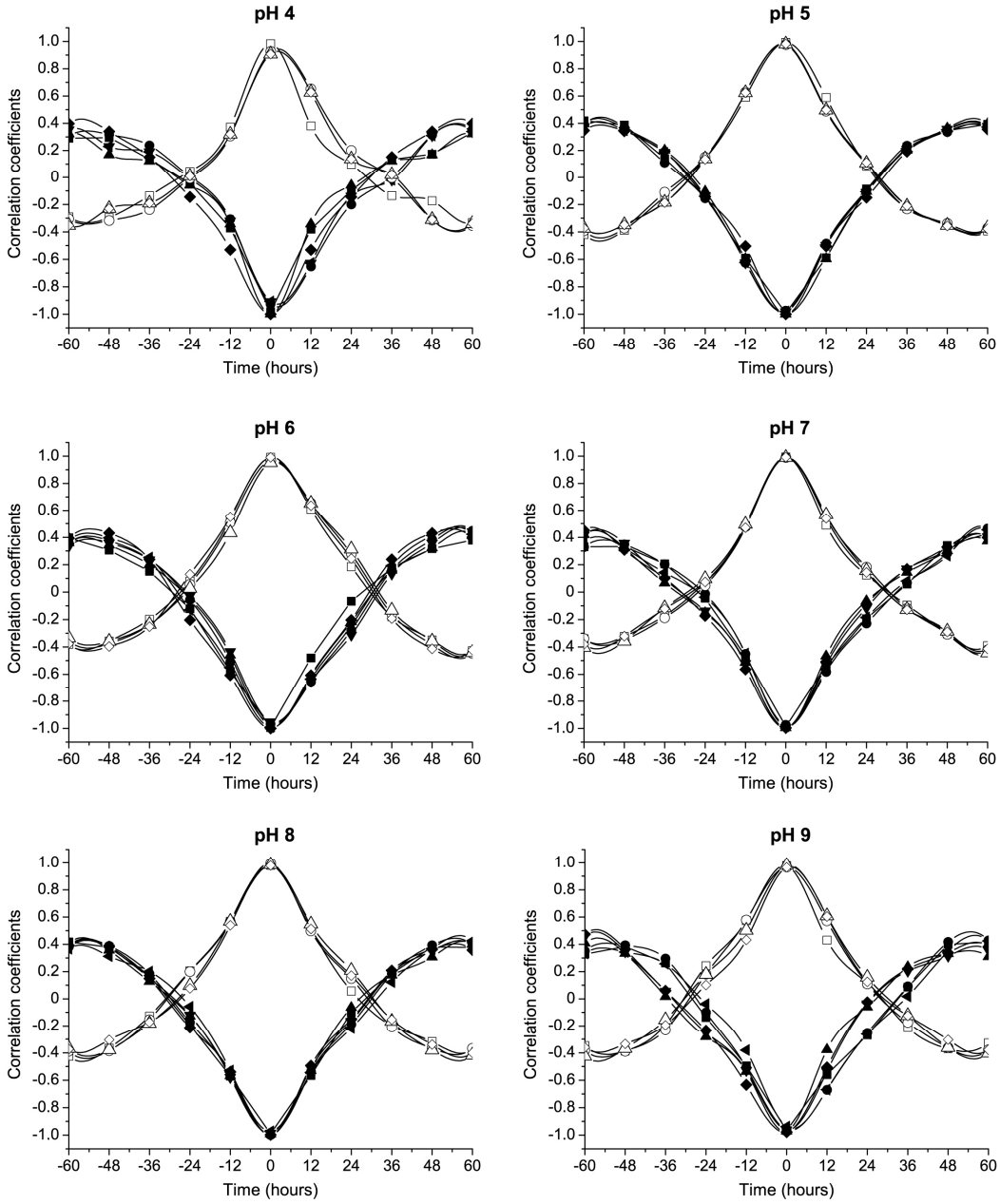

Figure 2. *Cross-correlation between dissimilatory sulfate reduction parameters by the bacteria Desulfovibrio piger Vib-7 under influence of the medium with differential acidity: –■– biomass and sulfate; –●– biomass and lactate; –▲– sulfate and sulfide; –▼– sulfate and acetate; –♦– lactate and acetate; –◀– lactate and sulfide; –□– biomass and sulfide; –○– biomass and acetate; –Δ– lactate and sulfate; –◊– acetate and sulfide*

Dissimilatory sulfate reduction by the strain *D. piger* Vib-7 and the formation of hydrogen sulfide and acetate in the intestinal lumen could cause a variety of inflammatory processes. The sulfate-reducing bacteria which produce the largest amount of hydrogen sulfide were isolated from the feces of the human distal colon. These results were probably obtained because the proximal part of the colon is acidic (pH<5.5) while the dist section of the colon has a neutral pH which is a more ideal environment for the growth of these bacteria [11,12].

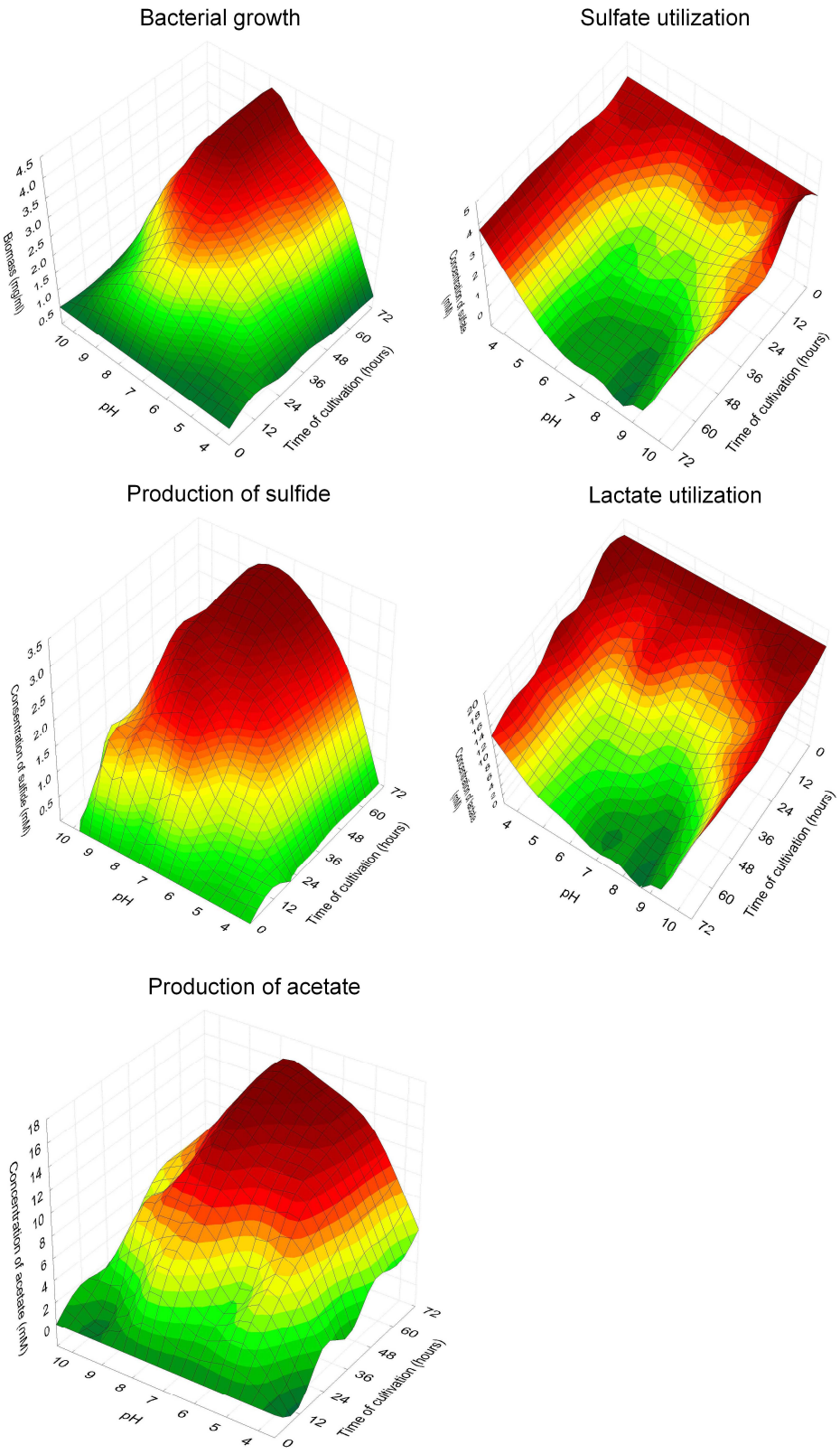

Figure 3. *The surface models of dissimilatory sulfate reduction parameters by the bacteria Desulfovibrio piger Vib-7 under influence of medium with differential acidity (3D surface)*

4. Conclusions

The intensive growth of the *D. piger* Vib-7 and their dissimilatory sulfate reduction depends on the acidity of the cultivation medium. The most intensive growth and sulfate and lactate consumption as well as the accumulation of hydrogen sulfide and acetate were established at pH 7–8. Having used all of the sulfate and lactate in the medium, the stationary growth phase began. Cross-correlations of dissimilatory sulfate reduction parameters can be selected to construct models of intensive sulfate reduction by the studied *D. piger* Vib-7 as well as a more detailed understanding of

the role these strains have in the development of bowel diseases.

Taking into consideration all of the obtained results: studies of the strain *D. piger* Vib-7 growth in the medium of differential acidity, their sulfate and lactate consumption, the accumulation of hydrogen sulfide and acetate by the bacteria, and correlation analyses of the parameters of dissimilatory sulfate reduction by the *D. piger* Vib-7, the isolated bacteria may cause various human intestinal diseases and inflammatory bowel processes. Therefore these bacteria are very interesting and promising for further studies.

Acknowledgements

The authors express their gratitude to Mark Scott from University of Mary Washington (Fredericksburg, Virginia, USA) for his assistance and critical reading of the manuscript. The authors also express their gratitude to Dr. Yurij J. Beno from Biophysics Department, Faculty of Biology, Ivan Franko National University of Lviv (Ukraine) for his assistance in perform the correlation and statistical analysis.

References

[1] Barton LL and Hamilton WA (2010) Sulphate-Reducing Bacteria. Environmental and Engineered Systems. Cambridge University Press. 552 p.

[2] Kushkevych IV (2012) Sulfate-reducing bacteria of the human intestine. I. Dissimilatory sulfate reduction. Studia Biologica. Vol. 6(1). P. 149–180.

[3] Kushkevych IV (2012) Sulfate-reducing bacteria of the human intestine. II. The role in the diseases development. Studia Biologica. Vol. 6(2). P. 221–250.

[4] Cummings JH, Macfarlane GT, Macfarlane S (2003) Intestinal Bacteria and Ulcerative Colitis. Curr Issues Intest Microbiol. Vol. 4. P. 9–20.

[5] Gibson GR, Cummings JH, Macfarlane GT (1991) Growth and activities of sulphate-reducing bacteria in gut contents of health subjects and patients with ulcerative colitis. FEMS Microbiol Ecol. Vol. 86. P. 103–112.

[6] Gibson GR, Macfarlane GT, Cummings JH (1993) Sulphate-reducing bacteria and hydrogen metabolism in the human large intestine. Gut. Vol. 34. P. 437–439.

[7] Gibson GR, Macfarlane S, Macfarlane GT (1993) Metabolic interactions involving sulphate-reducing and methanogenic bacteria in the human large intestine. FEMS Microbiol Ecol. Vol. 12. P. 117–125.

[8] Loubinoux J, Mory F, Pereira IA, Le Faou AE (2000) Bacteremia caused by a strain of *Desulfovibrio* related to the provisionally named *Desulfovibrio fairfieldensis.* J Clin Microbiol. Vol. 38. P. 931–934.

[9] Loubinoux J, Bronowicji JP, Pereira IA (2002) Sulphate-reducing bacteria in human feces and their association with inflammatory diseases. FEMS Microbiol Ecol. Vol. 40. P. 107–112.

[10] Rowan FE, Docherty NG, Coffey JC et al. (2009) Sulphate-reducing bacteria and hydrogen sulphide in the aetiology of ulcerative colitis. British J Surgery. Vol. 96. P. 151–158.

[11] Pitcher MC, Cummings JH (1996) Hydrogen sulphide: a bacterial toxin in ulcerative colitis? Gut. Vol. 39. P. 1–4.

[12] Levine J, Ellis CJ, Furne JK, Springfield J, Levitt MD (1998) Fecal Hydrogen Sulfide Production in Ulcerative Colitis. The American J Gastroenterol. Vol. 93(1). 83–87.

[13] Kushkevych IV, Moroz OM (2012) Growth of various strains of sulfate-reducing bacteria of human large intestine. Studia Biologica. Vol. 6(3). P. 115–124.

[14] Kushkevych IV (2013) Identification of sulfate-reducing bacteria strains of human large intestine. Studia Biologica. Vol. 7(3). P. 115–124.

[15] Kushkevych IV, Bartos M, Bartosova L (2014) Sequence analysis of the 16S rRNA gene of sulfate-reducing bacteria isolated from human intestine. Int J Curr Microbiol Appl Sci. Vol. 3(2). P. 239–248.

[16] Sutton S (2011) Measurement of Microbial Cells by Optical Density. *J Valid Tech* Vol. 17(1). P. 46–49.

[17] Kolmert A, Wikstrom P, Hallberg KB (2000) A fast and simple turbidimetric method for the determination of sulfate in sulfate-reducing bacterial cultures. J Microbiol Methods. Vol. 41. P. 179–184.

[18] Sugiyama M (2002) Reagent composition for measuring hydrogen sulfide and method for measuring hydrogen / U.S. Pat. 6340596 B1 USA, Int. Cl. G 01 N 33/00.

[19] Vlizlo VV, Fedoruk RS, Makar IA et al. (2004) Physiological and biochemical methods of researches in biology, stockbreeding and veterinary medicine. Handbook Institute of Animal Biology. Third Edition: revised and enlarged Lviv. 402 p.

[20] Campbell MK, Farrell SO (2008) Biochemistry. Sixth Edition. Publisher: Cengage Learning. 800 p.

[21] Bailey NTJ (1995) Statistical Methods in Biology. Cambridge University Press. 252 p.

[22] Chen PY, Popovich PM (2002) Correlation: Parametric and Nonparametric Measures. Sage University Papers Series on Quantitative Applications in the Social Sciences. 104 p.