Effect of cations on the activity of NADP$^+$-dependent glutamate dehydrogenase in *Acinetobacter calcoaceticus* IMV B-7241, *Rhodococcus erythropolis* IMV Ac-5017 and *Nocardia vaccinii* IMV B-7405 grown on industrial waste

Tetiana Pirog[1,2], Olesya Paliichuk[1], Daria Lutsai[1], Liliia Kliuchka[1], Tetiana Shevchuk[2]

1 – National University of Food Technologies, Kyiv, Ukraine
2 – Institute of Microbiology and Virology of National Academy of Sciences of Ukraine Kyiv, Ukraine

Keywords:

Glutamate dehydrogenase
Activators
Waste
Biosurfactants

Article history:

Received 01.08.2020
Received in revised form 23.12.2020
Accepted 25.03.2021

Corresponding author:

Tatiana Pirog
E-mail: tapirog@nuft.edu.ua

DOI:
10.24263/2304-974X-2021-10-1-17

Abstract

Introduction. It is studied the activity of NADP$^+$-dependent glutamate dehydrogenase in the presence of mono- and divalent cations (potential activators of this key enzyme of surface-active aminolipids biosynthesis) in *A. calcoaceticus* IMV B-7241, *R. erythropolis* IMV Ac-5017 and *N. vaccinii* IMV B-7405 during cultivation on waste of biodiesel production and sunflower oil waste.

Materials and methods. Cultivation of strains was performed in liquid mineral media using as substrates: refined and waste (after frying potato) sunflower oil, refined glycerol and waste of biodiesel production. NADP$^+$-dependent (EC 1.4.1.4) glutamate dehydrogenase activity in cell-free extracts was analyzed for glutamate formation during oxidation of NADPH at 340 nm. Monovalent (Na^+, K^+) and divalent (Mg^{2+}, Ca^{2+}, Zn^{2+}) cations in the form of salts of NaCl, KCl, $MgSO_4 \times 7H_2O$, $CaCl_2$ and $ZnSO_4 \times 7H_2O$ were added to the reaction mixture, as well as into the medium for strains cultivation.

Results and discussion. Calcium cations were found to be activators of NADP$^+$-dependent glutamate dehydrogenase activity in *R. erythropolis* IMV Ac-5017 and *N. vaccinii* IMV B-7405 grown on refined and waste sunflower oil: in the presence of 1–5 mmol Ca^{2+} in the mixture, the activity of the enzyme increased 1.3–2 times compared with that without these cations. The increase in the concentration of $CaCl_2$ to 0.2−0.4 g/l in oil-containing medium of strains IMV Ac-5017 and IMV B-7405 cultivation was accompanied by an increase in NADP$^+$-dependent glutamate dehydrogenase activity by 1.3–1.5 times compared with that on basic medium. When additional quantity of $CaCl_2$ (0.1−0.2 g/l) was introduced into the medium with purified glycerol for the cultivation of *A. calcoaceticus* IMV B-7241, an increase in NADP$^+$-dependent glutamate dehydrogenase activity was observed by almost 2.5−3 times compared with those for strain IMV B-7241 on the basic medium. There was no impact of activating cations magnesium, zinc, potassium and sodium on NADP$^+$-dependent glutamate dehydrogenase activity of all strains grown on oil-containing substrates and glycerol of different degrees of purification.

Conclusion. The results demonstrate the possibility to increase activity of key enzymes of the biosynthesis of the desired product: the composition of the medium should be modified by changing the content of enzymes' activators.

Introduction

Microbial surfactants are products of multifunctional purpose because they not only reduce the surface tension at the interface and emulsify different substrates, but also exhibit antimicrobial and antiadhesive activity (e.g. ability to destruct biofilms) [1–4]. However, under different conditions of producers cultivation, the composition of surfactants and their properties may change. This is due to the fact that microbial surfactants are secondary metabolites that are synthesized as a complex of similar compounds, the composition and ratio of which may vary depending on the growing conditions of the producer [5], which in turn will change the properties of the final product.

Earlier [5] we showed that the detection of potential activators and / or inhibitors of key enzymes (defining biosynthesis of components of the microbial surfactant complex responsible for certain properties) allows to regulate the composition and therefore the properties of the final product. This could be achieved by following modification of the nutrient composition.

A key enzyme of biosynthesis of surface-active aminolipid, responsible for the antimicrobial activity in *Acinetobacter calcoaceticus* IMV B-7241, *Nocardia vaccinii* IMV B-7405 and *Rhodococcus erythropolis* IMV Ac-5017 is $NADP^+$-dependent glutamate dehydrogenase. Its activators in IMV B-7241 strain are cations of calcium, magnesium and zinc, in IMV Ac-5017 – calcium, in IMV B-7405 – calcium, sodium and potassium [6]. Further increase of the content of enzyme's activators in the medium cultivation was accompanied by an increase of $NADP^+$ dependent glutamate dehydrogenase by 1.5–3 times compared to a basic medium.

Further experiments showed that the additional introduction of $CaCl_2$ (0.1 g/l) into the cultivation medium of *R. erythropolis* IMV Ac-5017, increasing the concentration of this salt to 0.4 g/l in the medium for growing *N. vaccinii* IMV -7405, as well as the adding of $CaCl_2$ (0.1 g/l), increasing the content of $MgSO_4 \cdot 7H_2O$ to 0.2 g/l or the adding of Zn^{2+} (38 μm) in the medium of *A. calcoaceticus* IMV B-7241 cultivation was accompanied by the synthesis of surfactants, which minimal inhibitory concentrations (MICs)relative to bacterial and yeast test cultures were 1.2–13 times lower, their adhesion on abiotic surfaces treated with the surfactants was on average 10–40% lower, and the degree of biofilm destruction was 7 −20% higher compared to the indicators for surfactants obtained on the base medium [6].

In publication [6] *A. calcoaceticus* IMV B-7241 and *R. erythropolis* IMV Ac-5017 were grown on ethanol, while *N. vaccinii* IMV B-7405 – on purified glycerol. One of the approaches to reducing the cost of the final product is the use of industrial waste as substrates for their production. In our previous studies [7], we have established the possibility of surfactant synthesis under cultivation of strains IMV B-7241, IMV Ac-5017 and IMV B-7405 on waste oil and waste of biodiesel production. However, the antimicrobial activity of surfactants synthesized by *N. vaccinii* IMV B-7405 on the oil-based substrates depended on the quality of waste oil [8]. The biological activity of surfactants synthesized by *A. calcoaceticus* IMV B-7241 on waste of biodiesel production was lower than the surfactants obtained on purified glycerol [9].

We assumed that antimicrobial and antiadhesive activity of surfactants synthesized on industrial waste can be increased by adding of activators of $NADP^+$-dependent glutamate dehydrogenase into the medium cultivation. However, the presence of toxic substances in such substrates [7-9] may cause inhibition of the activity of this key enzyme.

The aim of this work was to determine activity of $NADP^+$-dependent glutamate dehydrogenase in the presence of mono- and divalent cations (potential activators of this key enzyme of surface-active aminolipids biosynthesis) in *A. calcoaceticus* IMV B-7241, *R. erythropolis* IMV Ac-5017 and *N. vaccinii* IMV B-7405 during cultivation on waste of biodiesel production and sunflower oil waste.

Materials and methods

Object of research

The objects of research were strains of oil-oxidizing bacteria, identified as *Nocardia vaccinii* K-8, *Acinetobacter calcoaceticus* K-4 and *Rhodococcus erythropolis* EK-1. Strains K-8, K-4 and EK-1 are registered in the Depository of Microorganisms of D.K. Zabolotnyi Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine with the numbers IMV B-7405, IMV B-7241 and IMV Ac-5017, respectively.

Medium composition and conditions of

A liquid medium of the following composition (g/l) was used for the cultivation of *N. vaccinii* IMV B-7405: $NaNO_3$ – 0.5; $MgSO_4 \times 7H_2O$ – 0.1; $CaCl_2$ – 0.1; KH_2PO_4 – 0.1; $FeSO_4 \times 7H_2O$ – 0.01; yeast autolysate – 0.5% (v/v).

Modifications of basic medium:

1 – increasing the content of $CaCl_2$ to 0.2 g/l;

2 – increasing the content of $CaCl_2$to 0.4 g/l;

3 – adding NaCl (0.5 g/L);

4 – adding KCl (0.5 g/l);

5 – adding NaCl (0.5 g/l) and KCl (0.5 g/l).

A. calcoaceticus IMV B-7241 strain was cultivated in the following medium (g/l): $(NH_2)_2CO$ – 0.35; $MgSO_4 \times 7H_2O$ – 0.1; NaCl – 1.0; Na_2HPO_4 – 0.6; KH_2PO_4 – 0.14; pH 6.8-7.0. Yeast autolysate – 0.5% (v/v) and a solution of microelements – 0.1% (v/v) were additionally included into the medium. The solution of microelements contained (g/100 ml): $ZnSO_4 \times 7H_2O$ – 1,1; $MnSO_4 \times H_2O$ – 0.6; $FeSO_4 \times 7H_2O$ – 0.1; $CuSO_4 \times 5H_2O$ – 0.004; $CoSO_4 \times 7H_2O$ – 0.03; H_3BO_3 – 0.006; KI – 0.0001; EDTA (Trilon B) – 0.5.

Modifications of the basic medium: 1) adding $CaCl_2$ (0.1 g/l), 2) adding $CaCl_2$ (0.2 g/l), 3) adding Zn^{2+} (38 μmol), 4) adding $CaCl_2$ (0.1 g/l) and Zn^{2+} (38 μmol), 5) $CaCl_2$ (0.2 g/l) and Zn^{2+} (38 μmol).

R. erythropolis IMV Ac-5017 strain was grown in the following medium (g/l): $NaNO_3$ – 1,3; NaCl – 1.0; $Na_2HPO_4 \times 12H_2O$ – 0,6; KH_2PO_4 – 0.14; $MgSO_4 \times 7H_2O$ – 0.1; $FeSO_4 \times 7H_2O$ – 0.001.

Modifications of the basic medium: 1) adding $CaCl_2$ (0.1 g/l).

Refined and waste sunflower oil (after frying potato at McDonald's restaurant chain, Kyiv, Ukraine), purified glycerol and waste of biodiesel production (biofuel plant, Poltava region, Ukraine) were used as carbon sources. The concentration of substrates was 1% (v/v).

Cultures in the exponential growth phase, grown in media of the above mentioned composition, containing 0.5% (v/v) of the corresponding substrate were used as the inoculum. The amount of inoculum (10^{-4}–10^{-5} cells/ml) was 5−10% from the volume of the nutrient medium. Cultivation of bacteria was carried out in 750 ml flasks with the volume of medium equal to 100 ml on a shaker (220 rpm) at 28-30º C until mid exponential growth phase (24-48 hours).

Enzymatic analyses

Preparation of cell-free extracts. To obtain cell-free extracts, the culture liquid was centrifuged (5000 g, 20 min, 4° C). The resulting cell precipitate was washed twice from medium residues with 0.05 mol of K^+-phosphate buffer (pH 7.0), centrifuged (4000 g, 15

min, 4° C). The washed cells were resuspended in 0.05 mol of K^+-phosphate buffer (pH 7.0) and destroyed by ultrasound (22 kHz) 3 times for 20 s at 4° C on an UZDN-1 device. The resulting disintegrate was centrifuged (12000 g, 30 min, 4° C), the precipitate was separated, and the supernatant was used for further studies as a cell-free extract.

Analysis of NADP$^+$-dependent glutamate dehydrogenase activity. NAD$^+$-dependent (EC 1.4.1.2), NAD (P)$^+$-dependent (EC 1.4.1.3) and NADP$^+$-dependent (EC 1.4.1.4) glutamate dehydrogenase activity was analyzed by glutamate formation during oxidation of NAD and NADPH at 340 nm [10]. During the study of the effect of cations on the activity of glutamate dehydrogenase 0.001 and 0.005 mmol Zn^{2+}, 1−10 mmol Ca^{2+}, 5 and 10 mmol Mg^{2+}, 25−100 mmol Na^+, K^+ in the form of solutions of salts of $ZnSO_4 \times 7H_2O$, $CaCl_2$, $MgSO_4 \times 7\ H_2O$, NaCl and KCl, respectively, were added to the reaction mixture.

Activity of the enzyme was expressed in nmol of the product obtained per minute of the reaction calculated per 1 mg of a protein. The protein content in the cell-free extracts was determined by Bradford [11]. Glutamate dehydrogenase activity was assayed at 28-30° C – a temperature optimal for the growth of *R. erythropolis* IMV Ac-5017, *A. calcoaceticus* IMV B-7241 and *N. vaccinii* IMV B-7405.

Statistical analysis

All experiments were performed in 3 replicates, the number of parallel determination in the experiments was 3-5. Statistical processing of experimental data was carried out as described in previous papers [6, 7]. The differences in averages were considered reliable at the significance level $p < 0.05$.

Results and discussion

Glutamate dehydrogenase is responsible for reductive amination of 2-oxoglutarate with the formation of glutamate (donor of amino groups in the subsequent biosynthesis of amino lipids) [10]. Therefore, the higher the activity of this enzyme in the cells producing surfactants, the higher the content in the surfactant complex of amino lipids responsible for the antimicrobial activity of the target product.

Activity of NADP $^+$-glutamate dehydrogenase depending on the concentration of cations in the reaction mixture.

Table 1 presents the data on NADP$^+$-dependent glutamate dehydrogenase activity in the presence of different concentrations of monovalent cations in the reaction mixture in *A. calcoaceticus* IMV B-7241, *R. erythropolis* IMV Ac-5017 and *N. vaccinii* IMV B-7405 grown on refined and waste sunflower oil.

As shown in Table 1 calcium cations at a concentration of 1-5 mmol triggered NADP$^+$-dependent glutamate dehydrogenase activity in *R. erythropolis* IMV Ac-5017 and *N. vaccinii* IMV B-7405 cells grown on both oil containing substrates. Other divalent cations (magnesium and zinc) either inhibited the activity of this enzyme in both strains, or did not influence the activity of glutamate dehydrogenase in the mixture (e.g. the same as in the control without cations). At the same time, all studied divalent cations did not have any effect on the NADP$^+$-dependent glutamate dehydrogenase activity in *A. calcoaceticus* IMVB-7241. Such trends were observed during the cultivation of the strain on both refined and spent sunflower oil.

Table 1

Effect of cations on NADP$^+$-dependent glutamate dehydrogenase activity in cell-free extracts *A. calcoaceticus* IMV B-7241, *R. erythropolis* IMV Ac-5017 and *N. vaccinii* IMV B-7405

Cation	Concentration in the reaction mixture, mmol	NADP$^+$-glutamate dehydrogenase activity (nmol·min^{-1}·mg^{-1}of protein) in cells of strains		
		IMV B-7241	IMV B-7405	IMV Ac-5017
Substrate – refined sunflower oil				
No cations	0	**486±24**	**5 73±29**	**308±15**
Ca^{2+}	1	486±24	N.d.	**615±30**
	5	486±24	**769±38**	259±13
	10	340±17	385±19	259±13
Mg^{2+}	5	486±24	145±7	154±8
	10	486±24	148±7	154±8
Zn^{2+}	0.001	470±23	192±9	259±13
	0.005	470±23	192±9	259±13
Na^+	25	486±24	473±23	154±8
	50	486±24	473±23	154±8
	100	486±24	473±23	154±8
K^+	25	486±24	473±23	154±8
	50	486±24	473±23	154±8
	100	486±24	473±23	154±8
Substrate – fried sunflower oil				
No cations	0	**579±29**	**377±19**	**555±28**
Ca^{2+}	1	579±29	N. d .	**763±38**
	5	579±29	**756±37**	555±28
	10	579±29	377±18	555±28
Mg^{2+}	5	579±29	230±11	370±18
	10	579±29	230±11	370±18
Zn^{2+}	0.001	579±29	377±18	370±18
	0.005	579±29	377±18	370±18
Na^+	25	380±19	377±18	**741±37**
	50	260±13	377±18	**741±37**
	100	198±10	189±9	**741±37**
K^+	25	N.d.	377±18	**741±37**
	50	275±14	189±9	**741±37**
	100	180±9	189±9	555±28

Note: N.d. – not determined

Monovalent cations did not affect the activity of NADP$^+$-dependent glutamate dehydrogenase during of *A. calcoaceticus* IMV B-7241 cultivation on refined oil and inhibited the activity when culturing the strain in waste oil. In the presence of potassium and sodium cations, a decrease in the activity of this enzyme was observed in *R. erythropolis* IMV Ac-5017 and *N. vaccinii* IMV-7405 cells grown on refined oil. However, underg the cultivation on waste oil monovalent cations activated NADP$^+$-dependent glutamate dehydrogenase in *R. erythropolis* IMV Ac-5017 (see Table 1).

NADP$^+$-glutamate dehydrogenase activity depending on the concentration of cations in the culture medium of the strains.

At the next step, the activity of NADP$^+$-dependent glutamate dehydrogenase was determined during cultivation of *A. calcoaceticus* IMV B-7241, *R. erythropolis* IMV Ac-5017 and *N. vaccinii* IMV B-7405 cells in basic and modified oil containing media. Mono- and divalent cations – potential enzyme activators (Table 2) – were added to the media. The choice of cations for the modification of the cultivation media of the studied strains was based on their effect on the activity of the enzyme according to the table. 1. Also the results of previous studies [6] were considered: triggers of NADP$^+$-dependent glutamate dehydrogenase were identified during cultivation of *A. calcoaceticus* IMV B-7241 and *R. erythropolis* IMV Ac-5017 on ethanol, *N. vaccinii* IMV B-7405 – on purified glycerol.

The research results given in the Table 2, confirmed the data in Table 1: there are no positive effects of calcium and zinc cations on the enzyme activity of strain *A. calcoaceticus* IMV B-7241; calcium cations triggered NADP$^+$-dependent glutamate dehydrogenase activity in *R. erythropolis* IMV Ac-5017 and *N. vaccinii* IMV-7405 cells grown on both refined and waste oil.

At the same time adding of potassium chloride and sodium to the oil containing medium of *R. erythropolis* IMV Ac-5017 did not increase NADP$^+$-dependent glutamate dehydrogenase activity (see the Table 2). The lack of correlation between the monovalent cations' effect on the activity of the enzyme in the reaction mixture (see the Table 1) and in the culture medium (see the Table 2) can be explained as follows. Actual content of cations in bacterial cells and culture medium differs. Values of enzymatic activity in cell-free extracts do not always correspond to the speed of real process in intact cells. This speed depends not only on the content of the enzyme but also on the pool of substrates, enzyme regulations and other factors.

Previously, [6] we showed that cations of calcium and zinc were activators of NADP-dependent glutamate dehydrogenase when *A. calcoaceticus* IMV B-7241 cultivated on ethanol. While sodium and potassium cations were activators when *N. vaccinii* IMV-7405 cultivated on purified glycerol

There were no activating effects observed if oil-containing media used (see Table1 and 2). We can assume that *A. calcoaceticus* IMV B-7241 and *N. vaccinii* IMV B-7405 have several glutamate dehydrogenases, which operate during cultivation on various substrates.

For example, two NAD$^+$-dependent glutamate dehydrogenases were found in the extremely halophilic strain *Salinibacter ruber* M31 (DSM 13855T) [12]. The activity of one of the enzymes increased 1.4 and 67 times in the presence of 3 M sodium chloride and 3 M potassium chloride, respectively, and the activity of the other, under similar conditions, decreased 8.8 and 4 times, respectively.

It is known from the literature that monovalent and divalent cations can be both inhibitors and activators of glutamate dehydrogenase in microorganisms [13−24].

The activity of this enzyme in the archaea *Thermococcus*sp. increased by 135, 104 and 250% in the presence of 5 mmol $CaCl_2$, $MgCl_2$ and $MnCl_2$, respectively [15]. Later [18] it was found that calcium and magnesium cations are also activators of NADP$^+$-dependent glutamate dehydrogenase in archaea *Thermococcus waiotapuensis*: the presence of 10 mmol $CaCl_2$ and 10 mmol $MgSO_4$ increased an activity by 1.3 times compared to the control (without cations of metals). Divalent iron cations at a concentration of 1 mmol increased the activity of NADP$^+$-glutamate dehydrogenase in *Klebsiella pneumoniae* F-5-2 by 10% [19]. It was found in [19] that silver cations were potent inhibitors of this enzyme in the *K. pneumoniae* F-5-2 strain.

Table 2

Influence of cations in media for cultivation of *A. calcoaceticus* IMV B-7241, *R. erythropolis* IMV AC-5017 and *N. vaccinii* IMV B-7405 on $NADP^+$-dependent glutamate dehydrogenase activity

Strain	Type of oil in medium	Cultivation medium	$NADP^+$-glutamate-dehydrogenase activity ($nmol \cdot min^{-1} \cdot mg^{-1}$protein)
N. vaccinii IMV B-7405	Refined	Basic	**515±25**
		+ 0.2 g/l $CaCl_2$	**690±34**
		+ 0.4 g/l $CaCl_2$	538±26
	Fried (waste)	Basic	**329±16**
		+ 0.2 g/l $CaCl_2$	**425±21**
		+ 0.4 g/l $CaCl_2$	**488±24**
R. erythropolis IMV Ac-5017	Refined	Basic	308±15
		+ 0.1 g/l $CaCl_2$	**618**±30
		+ 0.5 g/l KCl	308±15
		+ 0.5 g/l NaCl	308±15
		+ (0.5 g/l KCl +0.5 g/l NaCl)	308±15
	Fried (waste)	Basic	**555±28**
		+ 0.1 g/l $CaCl_2$	**741±37**
		+ 0.5 g/l KCl	555±28
		+ 0.5 g/l NaCl	555±28
		+ (0.5 g/l KCl +0.5 g/l NaCl)	555±28
A. calcoaceticus IMV B-7241	Refined	Basic	**459±23**
		+ 0.1 g/l $CaCl_2$	464±23
		+ 0.2 g/l $CaCl_2$	464±23
		+ 38 μmol Zn^{2+}	464±23
		+ (0.1 g/l $CaCl_2$+38 μmol Zn^{2+})	475±24
		+ (0.2 g/l $CaCl_2$+38 μmol Zn^{2+})	471±24
	Fried (waste)	Basic	**538±27**
		+ 0.1 g/l $CaCl_2$	533±27
		+ 0.2 g/l $CaCl_2$	538±27
		+ 38 μmol Zn^{2+}	469±23
		+ (0.1 g/l $CaCl_2$+38 μmol Zn^{2+})	494±25
		+ (0.2 g/l $CaCl_2$+38 μmol Zn^{2+})	400±20

The activity of NAD^+-dependent glutamate dehydrogenase in *Laccaria bicolor* fungi increased by 50% in the presence of 1 mM calcium sulfate and magnesium chloride, but decreased by 60–70% when 0.1 mmol Cu^{2+} was added to the reaction mixture [16].

Cations of potassium and sodium at the concentration 50–200 mM are enzyme activators in aerobic hyperthermophile archaea *Aeropyrum pernix* K1 [17]. At the same time, the activity of purified $NADP^+$-dependent glutamate dehydrogenase *Pyrobaculum calidifontis* was inhibited by 50% in the presence of 100–200 mmol of potassium chloride and 100–300 mmol of sodium chloride [22].

Alba-Lois et al. [20] found that the additional of 1 M NaCl into the culture medium of halotolerant yeast *Debaryomyces hansenii* was accompanied by a fivefold increase in the activity of $NADP^+$-dependent glutamate dehydrogenase compared to the activity of the enzyme when grown on a medium without sodium chloride. At the same time, the activity of the purified enzyme did not increase in the presence of this salt. The researchers explained such unexpected results by the fact that the increased activity of $NADP^+$-dependent glutamate dehydrogenase in *D. hansenii* is a kind of defence mechanism against the inhibitory effect of high ionic strength.

It was shown in [16] that the stability of *Escherichia coli* glutamate dehydrogenase increased in the presence of lithium cations at a concentration of 1 to 10 mM, 1 M sodium phosphate, or 1 M ammonium sulfate.

Data on the effect of zinc cations on the activity of $NADP^+$-dependent glutamate dehydrogenase inmicroorganisms appeared in 1980 [13], but so far there are only a few such publications. In [13] it is reported that depending on the concentration of Zn^{2+} can be either an activator or inhibitor of this enzyme: at a concentration of less than 0.1 mmol activity of glutamate dehydrogenase of *Mycobacterium smegmatis* raised, while at concentrations above 0.1 mmol an inhibition of enzyme activity was observed. It was observed that the activity of $NADP^+$-dependent glutamate dehydrogenase *E. coli* in the presence of 1 mM Zn^{2+} decreased by 40% [14]; at a concentration of 5 mmol $ZnCl_2$ – the activity of this enzyme reduced in *Aspergillus terreus* [21].

Geotrichum sandidum S12 glutamate dehydrogenase is unique because it has substrate specificity towards glutamate, 2-oxoglutarate, hexanol and isoamyl alcohol [23]. In the presence of ADP, Fe^{2+}, K^+ and Zn^{2+} an increase in enzymatic activity towards hexanol was observed; and in the presence of EDTA, Mn^{2+} and ATF – its inhibition.

Influence of divalent cations in the culture medium of *A. calcoaceticus* IMV B-7241 with glycerol of different purification degree on the activity of $NADP^+$- glutamate dehydrogenase.

At the next step it was analyzed how activators impact $NADP^+$-dependent glutamate dehydrogenase during the cultivation of *A. calcoaceticus* IMV B-7241 on purified glycerol and waste of biodiesel production. Data in Table 3 show that after adding calcium cations into the medium with refined glycerol for *A. calcoaceticus* IMV B-7241 cultivation activity of $NADP^+$-glutamate dehydrogenase increased almost 2.5-3 times comparing to the basic medium.

However, after adding $CaCl_2$ into the media with waste of biodiesel production $NADP^+$-dependent glutamate dehydrogenase activity remained almost the same as in the basic medium. Also under cultivating *A. calcoaceticus* IMV B-7241 on oil containing substrates adding zinc cations into glycerol media did not increase activity of the enzyme (see Table 2 and 3).

Table 3

$NADP^+$-dependent glutamate dehydrogenase activity during cultivation of *A. calcoaceticus* IMV B-7241 on glycerol of various purity

Growth substrate	Cultivation media	$NADP^+$-glutamate-dehydrogenase activity (nmol · min^{-1} · mg^{-1}protein)
Purified glycerol	Basic	**159±8**
	+ 0.1 g/l $CaCl_2$	**401±20**
	+ 0.2 g/l $CaCl_2$	**476±24**
	+ 38 μmol Zn^{2+}	160±8
	+ (0.1 g/l $CaCl_2$+38 μmol Zn^{2+})	239±12
	+ (0.2 g/l $CaCl_2$+38 μmol Zn^{2+})	154±8
Wastes of biodiesel production	Basic	**526±26**
	+ 0.1 g/l $CaCl_2$	541±27
	+ 0.2 g/l $CaCl_2$	541±27
	+ 38 μmol Zn^{2+}	532±26
	+ (0.1 g/l $CaCl_2$+38 μmol Zn^{2+})	532±26
	+ (0.2 g/l $CaCl_2$+38 μmol Zn^{2+})	532±26

In our opinion, one of the reasons why Zn^{2+} activates $NADP^+$-dependent glutamate dehydrogenase [6] on ethanol media but does not on oil and glycerol may be the presence of several glutamate dehydrogenases in a strain IMV B-7241. However, in the next studies we could not identify NAD^+- or $NAD(P)^+$-dependent glutamate dehydrogenase activity in cells *A. calcoaceticus* IMV B-7241 grown on oil-containing substrates, purified glycerol and waste of biodiesel production. Probably, the strain IMV B-7241 has several $NADP^+$-dependent enzymes that function when grown on different substrates. However, the final conclusion in favor of such an assumption can be made only after the isolation of the relevant enzymes and the study of their physicochemical properties.

In addition, it is possible that in the *A. calcoaceticus* IMV B-7241 strain during cultivation on oil and glycerol, glutamate formation is not involved in glutamate dehydrogenase, but glutamine synthetase and glutamate synthase [24]. These enzymes carry out (like glutamate dehydrogenase) reductive amination of 2-oxoglutarate with the formation of glutamate as a result of two successive reactions that occur with the participation of ATP. van Heeswijk et al. [24] note that in *E. coli* and *Salmonella typhimurium* cells, glutamate dehydrogenase was characterized by high activity during exponential bacterial growth, however, as the sources of carbon and nitrogen nutrition in the culture medium were exhausted, it was decomposed by ATP-dependent proteases.

It was found in [19] that during the cultivation of *K. pneumoniae* F-5-2 under aerobic conditions, both glutamate dehydrogenase and glutamine synthetase functioned simultaneously in bacterial cells. $NADP^+$-dependent glutamate dehydrogenase catalyzed the not only amination of 2-oxoglutarate, but also 2-oxovalerate and 2-oxobutyrate and was stable in the pH range 5.5-11.5. The optimum pH of glutamine synthetase was 8.0, this enzyme was stable at pH 6.0-7.0. Unlike glutamate dehydrogenase, the activity of glutamine synthetase was strongly suppressed by ferrous iron cations. In addition to iron cations, mercury and cuprum cations were found to be inhibitors of this enzyme *in K. pneumoniae* F-5-2.

Note that the final conclusion about the functioning of or several NADP + dependent glutamate dehydrogenase, or glutamate dehydrogenase, glutamine synthetase and glutamate synthase in the *A. calcoaceticus* IMV B-7241 strain grown on oil and glycerol can be done only after the isolation of the corresponding enzymes and the study of their physicochemical properties.

Conclusion

The results of this study confirm the earlier data on possibility to regulate the activity of key enzymes of the biosynthesis of the final product. This can be achieved by modifying the composition of the medium, e.g. by changing a content of activators (inhibitors) of these enzymes.

The obtained data suggest that adding $CaCl_2$ to the oil-containing media of *R. erythropolis* IMV Ac-5017 and *N. vaccinii* IMV B-7405, as well as to the medium with purified glycerol for growing *A. calcoaceticus* IMV B-7241 will result in synthesis of surfactants with increased antimicrobial and antiadhesive activity.

References

1. Jimoh A.A., Lin J. (2019), Biosurfactant: A new frontier for greener technology and environmental sustainability, *Ecotoxicology and Environmental Safety*, 184, 109607, DOI: 10.1016/j.ecoenv.2019.109607.
2. Mazeyrat-Gourbeyre F., Baillieul F., Clément C., Ongena M., Dorey S. (2020), Biosurfactants in Plant Protection Against Diseases: Rhamnolipids and Lipopeptides Case Study, *Frontiers in Bioengineering and Biotechnology*, 8, 1014, DOI: 10.3389/fbioe.2020.01014.
3. Adu S.A., Naughton P.J., Marchant R., Banat I.M. (2020), Microbial Biosurfactants in Cosmetic and Personal Skincare Pharmaceutical Formulations, *Pharmaceutics*, 12(11), pp. 1099, DOI: 10.3390/pharmaceutics12111099.
4. Ribeiro B.G., Guerra J.M.C., Sarubbo L.A. (2020), Biosurfactants: Production and application prospects in the food industry. *Biotechnology Progress*, 36(5), e3030, DOI: 10.1002/btpr.3030.
5. Pirog T.P., Kliuchka L.V., Shevchuk T.A., Muchnyk F.V. (2019), Interrelation of Chemical Composition and Biological Properties of Microbial Surfactants, *Mikrobiologichny Zhurnal*, 81(3), pp. 84−104, DOI: https://doi.org/10.15407/microbiolj81.03.084.
6. Pirog T.P., Shevchuk T.A., Nikituk L.V., Lutsai D.A., Paliichuk O.I. (2018), Influence of cultivation conditions on antimicrobial and anti-adhesive activity of surfactants of bacteria of *Acinetobacter*, *Rhodococcus* and *Nocardia* genera. *Proceedings of the National Academy of Sciences of Belarus. Biological series*, 63(3), pp. 307–315. https://doi.org/10.29235/1029-8940-2018-63-3-307-315.
7. Pirog T., Sofilkanych A., Konon A., Shevchuk T., Ivanov S. (2013), Intensification of surfactants' synthesis by *Rhodococcus erythropolis* IMV Ac-5017, *Acinetobacter calcoaceticus* IMV B-7241 and *Nocardia vaccinii* K-8 on fried oil and glycerol containing medium, *Food and Bioproducts Processing*, 91(2), pp. 149−157. http://dx.doi.org/10.1016/j.fbp.2013.01.001.
8. Pirog T.P., Nikituk L.V., Antonuk S.I., Shevchuk T.A., Iutynska G.O. (2017), Peculiarities of *Nocardia vaccinii* IMV B-7405 surfactants synthesis on waste oil of different quality and their antimicrobial properties. *Mikrobiologichny Zhurnal*, 79(2), pp. 13−22, DOI: https://doi.org/10.15407/microbiolj79.02.013.
9. Pirog T.P., Lutsay D.A., Shevchuk T.A., Iutynska G.O., Elperin I.V. (2018) Antimicrobial and antiadhesive activity of surfactants synthesized by *Acinetobacter calcoaceticus* IIB B-

7241 on technical glycerin. *Mikrobiologichny Zhurnal*, 80(2), pp. 14−27, DOI: https://doi.org/10.15407/microbiolj80.02.014 .
10. Smith E.L., Austen B.M., Blumenthal K.M., Nyc J.F. (1975), Glutamate dehydrogenases. In: *The Enzymes*, 3rd ed., vol. 11, New York: Academic Press, pp. 293−367.
11. Bradford M. (1976), A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Analytical Biochemistry*, 72 (3), pp. 248–254.
12. Bonete M.J., Pérez-Pomares F., Díaz S., Ferrer J., Oren A. (2003), Occurrence of two different glutamate dehydrogenase activities in the halophilic bacterium *Salinibacter ruber*. *FEMS Microbiology Letters*, 226(1), pp. 181−186, DOI: 10.1016/S0378-1097(03)00592-5.
13. Sarada K.V., Rao N.A., Venkitasubramanian T.A. (1980), Isolation and characterisation of glutamate dehydrogenase from *Mycobacterium smegmatis* CDC 46. *Biochimica et Biophysica Acta*, 615(2), pp. 299–308.
14. Lin H.P.P., Reeves H.C. (1991), Purification and characterization of $NADP^+$-specific glutamate dehydrogenase from *Escherichia coli*. *Current Microbiology*, 22(6), pp. 371–376.
15. Hudson R.C., Ruttersmith L.D., Daniel R.M. (1993), Glutamate dehydrogenase from the extremely thermophilic archaebacterial isolate AN1. *Biochimica et Biophysica Acta*, 1202(2), pp. 244–250.
16. Garnier A., Berredjem A., Botton B. (1997), Purification and characterization of the NAD-dependent glutamate dehydrogenase in the ectomycorrhizal fungus *Laccaria bicolor* (Maire) Orton. *Fungal Genetics and Biology*, 22(3), pp.168−176, DOI: 10.1006/fgbi.1997.1004.
17. Bhuiya M.W., Sakuraba H., Kujo C., Nunoura-Kominato N., Kawarabayasi Y., Kikuchi H. et al. (2000), Glutamate dehydrogenase from the aerobic hyperthermophilic archaeon *Aeropyrum pernix* K1: enzymatic characterization, identification of the encoding gene, and phylogenetic implications, *Extremophiles*, 4(6), pp. 333−341, DOI: 10.1007/s007920070002.
18. Lee M.K., González J.M., Robb F.T. (2002), Extremely thermostable glutamate dehydrogenase (GDH) from the freshwater archaeon *Thermococcus waiotapuensis*: cloning and comparison with two marine hyperthermophilic GDHs, *Extremophiles*, 6(2), pp. 151−159, DOI: 10.1007/s007920100238.
19. Kim Y.J., Yoshizawa M., Takenaka S., Murakami S., Aoki K. (2002), Ammonia assimilation in *Klebsiella pneumoniae* F-5-2 that can utilize ammonium and nitrate ions simultaneously: purification and characterization of glutamate dehydrogenase and glutamine synthetase, *The Journal of Bioscience and Bioengineering*, 93(6), pp. 584−588, DOI: 10.1016/s1389-1723(02)80241-9.
20. Alba-Lois L., Segal C., Rodarte B., Valdés-López V., DeLuna A., Cárdenas R. (2004), NADP-glutamate dehydrogenase activity is increased under hyperosmotic conditions in the halotolerant yeast *Debaryomyces hansenii*, *Current Microbiology*, 48(1), pp. 68−72, DOI: 10.1007/s00284-003-4076-7.
21. Choudhury R., Punekar N.S. (2009), *Aspergillus terreus* NADP-glutamate dehydrogenase is kinetically distinct from the allosteric enzyme of other *Aspergilli*. *Mycological Research*, 113(10), pp. 1121–1126, DOI: 10.1016/j.mycres.2009.07.009.
22. Wakamatsu T., Higashi C., Ohmori T., Doi K., Ohshima T. (2013), Biochemical characterization of two glutamate dehydrogenases with different cofactor specificities from a hyperthermophilic archaeon *Pyrobaculum calidifontis*, *Extremophiles*, 17(3), 379−389, DOI: 10.1007/s00792-013-0527-7.
23. Zhu J., Lu K., Xu X., Wang X., Shi J. (2017), Purification and characterization of a novel glutamate dehydrogenase from *Geotrichum candidum* with higher alcohol and amino acid activity, *AMB Express*, 7(1), 9, DOI: 10.1186/s13568-016-0307-8.
24. van Heeswijk W.C., Westerhoff H.V., Boogerd F.C. (2013), Nitrogen assimilation in *Escherichia coli*: putting molecular data into a systems perspective. *Microbiology and Molecular Biology Reviews*, 77(4), pp. 628−695, DOI: 10.1128/MMBR.00025-13.