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THE CONTENTS OF NEUTRAL AND POLAR LIPIDS IN *Clostridia* CELLS UNDER CULTIVATION IN THE PRESENCE OF BUTANOL

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The aim of the study was to evaluate changes in the portion of polar and neutral lipids in the cells of *Clostridium* during their cultivation in the presence of butanol. Four natural isolates of *Clostridium* genus were studied with flow cytometry approaches. Under the optimal culture conditions, the polar lipids prevailed over neutral ones in bacterial cells; the content of neutral lipids doubles in spores of these microorganisms, while the content of polar ones was reduced. Strains No 1 and No 2 were able to grow at 1% butanol in the medium, and the strain No 4 was at 1.5%. When cultivated in the presence of different concentrations of butanol, the bacterial strains did not differ in such cytomorphological features as granularity and cell size. The quantitative content of polar and neutral lipids in the presence of butanol varied depending on the content of butanol in the medium, but this effect had a strain-specific character and did not show a correlation with the resistance of these bacteria to butanol. So, the content of polar and neutral lipids varied depending on butanol content in the medium. However this effect was strain-specific independently of resistance of these bacteria to butanol. The use of bacterial biomass as a source of lipids for the production of biofuels requires further optimization of the process to increase the content of the neutral lipid fraction in bacterial cells.

Key words: *Clostridium*, polar and neutral lipids, butanol, flow cytometry.

In recent years an interest to renewable energy sources, including biofuels such as bioethanol, biobutanol, biogas, biodiesel was greatly increased. Advances of genetic engineering in creation of new strains, and the use of cheaper raw materials, can substantially increase the competitiveness of biofuels in comparison to their classic analogues that are produced by refining or direct extraction from natural deposits. During biological synthesis of the products the large quantities of microbial biomass are produced, which are only partially involved into further fermentation, while the rests can be used as a source of useful biological molecules, such as proteins, lipids, vitamins, sugars and so on.

An important aspect of studying polar lipids of clostridia is connected to the ability of some of their psychrophilic species (*Clostridium psychrophilum*) to grow at low temperatures [1]. The composition of polar lipids in clostridia varies depending on cultivation conditions, accumulation of end products, etc. [2, 3]. There was paid much less attention to neutral lipids of clostridia and, thus, information about them is extremely limited, although there were discovered several molecules among them with unique molecular structure [4]. The content of neutral lipids in membranes of clostridia (species *Clostridium saccharoperbutylacetonicum*) can reach 26%, while glycolipids and phospholipids are 26% and 48%, correspondingly [5].

Analysis of content of neutral and polar lipids in cells is an important indicator that provides a fast assessment of the strain potential to be used in the process of biodiesel production [6]. There are no data on accumulation of neutral lipids in clostridia cells during aceto-butylic (ABE) fermentation. Perhaps this lack happened because it is generally thought that the accumulation of neutral lipids is stimulated in the presence of oxygen [7] and thus this process should be restricted under anaerobiosis. However, lipids serve not only as structural components, but

also as a source of energy [8] and therefore the accumulation of neutral lipids is of great importance under anaerobic conditions too.

Among the methods that in recent years are increasingly used for monitoring of the ABE-fermentation is a method of flow cytometry (FC), which allows analyzing the microbial populations at the level of individual cells. Earlier FC method was applied to determine the quantity and measure the size of cells of *Clostridum acetobtylicum*, and there was noticed a correlation between the yield of solvent and the colonies morphology, the number of cells within them and their average size [9]. There was developed a method for studying the cell cycle of clostridia by staining them with fluorescent dyes (propidium iodide and Syto9), and cell sorting was performed by their forward (FS) and side scattered (SS) light values [10, 11]. It was found that the cells that synthesize solvents (acetone and butanol) are typical not swollen, but, instead, resemble the cells in the active phase of division. The FC was used in the analysis of the impact of sigma factors (σF, σE and σG) on the sporogenesis and solvents synthesis by *C. acetobutylicum* [12, 13]. It was shown a possibility to distinguish the *C. pasteurianum* cells in the phase of formation of acid from the ones in the solvents synthesis stage with a help of double labeling with hexidium iodide and Syto13. Color changes detected with FC related to the metabolic changes that occur in the cells during periodic pH-uncontrolled fermentation.

Clostridia are spore-forming bacteria that complicate a determination of their viability, which is an important parameter to control fermentation. The portion of metabolically active cells in a population of *C. pasteurianum* was determined with FC using bis-oksonol. Analysis of cells labeled with bis-oksonol was linked to the changes in the physiological state of population during periodic fermentation and can be used to facilitate the control over fermentation and provide it almost in real time [14].

Thus, to establish multi-product production on the basement of ABE-fermentation: butanol, acetone and ethanol, which released into the environment, and biodiesel — from the biomass of clostridia cells, it is necessary to determine the effect of butanol, that accumulates in the medium during the continuous cultivation, on the content of polar and neutral lipids in cells. The purpose of the work was to evaluate the changes in the neutral and polar lipids in clostridia cells under cultivation with butanol by using the flow cytometry facility. Experiments were carried out with natural clostridia isolates that let to assess the prospects of searching of new strains in the natural cenosis.

Materials and Methods

Objects

The objects of study were four strains of bacteria of the genus *Clostridium* isolated from the natural econishes (peat and field soil) and that showed the highest volumes of acetone production [15]. Strains of bacteria were kindly presented for researches by S. Skrotskiy at the Zabolotny Institute of Microbiology and Virology of NAS of Ukraine. There were given next numerical designation (No1, 2, 3 and 4) to the strains.

To maintain the bacterial cells in the active growth phase, they were cultivated in thioglycolate broth (HiMedia Laboratories Pvt. Ltd., India) without shaking under a layer of vaseline oil at 37 °C. The aliquots were transferred to fresh portion of broth every 24 h. This approach allowed to minimize sporogenesis, but did not prevent it completely and thus a part of population was represented as spores. We used cells that were grown for 24 h.

Model

Butanol is a limiting factor and it maximum concentration in the environment reaches 1–1.5% under continuous cultivation. Thus it was reasonable to study the content of polar and neutral lipids in cells just under these concentrations. In order to eliminate the impact of some factors, such as the natural resistance of studied strains to butanol, temporal differences in fermentation, sporogenesis, etc., we excluded bacterial fermentation stage and added butanol and actively grown cells directly into the broth. This methodical approach allowed simultaneously evaluate the effect of different concentrations of butanol on the neutral and polar lipids in bacterial cells before initiation of sporogenesis and determine the concentration of butanol, which studied strains of bacteria were resistance, which is a significant indicator of the prospects of using of the strains for industrial ABE-fermentation.

Cultivation of bacteria with butanol

Working concentrations of butanol were 0.25, 0.5, 1.0, 1.5 and 2.0%. Butanol (Sigma-Aldrich Chemie GmbH, Germany) was added to the broth just before bacterial cells inoculation. Aliquots of bacterial suspensions were added

into the prepared broth and cultivated without shaking in incubation containers with gas-generating system packages Anaerocult-A (Merck KGaA, Germany) at 28 °C for 72 h. Sampling, for determination of polar and neutral lipids, was performed on 0, 4, 24 and 48 h of cultivation. Resistance of bacteria to butanol was evaluated by their ability to increase biomass and was monitored with optical density measurements at 600 nm using a photometer Tablet Multiscan FC Microplate Photometer (Thermo Scientific, USA).

Fixation of bacterial cells

Aliquots (1 ml) of bacterial suspensions were transferred into microtubes and centrifuged at 10,000 rpm, 5 min. Pellet was resuspended in 950 mkl phosphate buffer saline (PBS, pH 7.2) and 50 mkl of glutaraldehyde (25% stock solution) was added. Samples were kept at room temperature for 15 min and then they were washed twice by centrifugation with 15 min interval in PBS. Finally sedimented samples were resuspended in 70% methanol in PBS and stored at 4 °C.

Detection of bacterial fluorescence with flow cytometry

Fluorescent dye nile red (1 mg/ml in methanol) was added 1:100 to the samples just 5 min before the measurements with flow cytometry. Samples were thoroughly mixed. Measuring optical parameters was performed with flow cytometer COULTER EPICS XL (Beckman, USA) equipped with 488 nm laser and software SYSTEM II. There were determined forward scattering (FS) and side scattering (SS) values of light beam and optical values at filters FL1–FL4. The fluorescence values were presented in optical units (o.u.) with indication of an appropriate filter. Fluorescence in the yellow region of the spectrum detected with filter FL2 (575 nm) is relevant to the content of neutral lipids in cells, while fluorescence in the red region of the spectrum, which corresponds to filter FL4 (675 nm), represents the content of polar lipids [6]. The data presented in the form of dot plots, in which each dot corresponds to one object (cell). Data were collected for 10,000 objects in each sample.

Statistical analysis

The values of the mean and median, from the data obtained with flow cytometry, were determined with a help of Flowing Software version 2.5.1 (Turku Centre for Biotechnology, University of Turku, Finland). Statistical data processing and graphical presentation of results was performed using the software packages Microsoft Excel and Statistica v.10 (StatSoft, Inc., 2011). The difference between mean values was evaluated with *t*-test and was considered as significant at $p \leq 0.05$. Cluster analysis of data was performed with Ward method and a percent of disagreement as a distance measure. Impact of factors (multiple linear and nonlinear quadratic effects) was determined with factorial ANOVA and non-factorial surface design (central composite).

Results and Discussion

Optical features of bacterial population stained with nile red

Flow cytometry makes possible to determine a size of the objects by the values of forward scattering of light (FS). It is known that clostridia can form spores, which are smaller than cells and thus FS data measurements can help to track the sporogenesis in bacteria. Objects within each bacterial population were differentiated into three fractions according to their FS values (Fig. 1*A*): the first one containing most of objects had a mean FS value 301.2±6.1 o.u. (Table), and the second one, presumably was represented with dividing cells, because their mean FS value was 623.4±29.2 o.u. that was twice over the value in the first fraction; the third one was, presumably, the bacterial spores since their FS value was the smallest and 155.1±4.1 o.u. only. Staining with nile red and measurement of optical characteristics of objects in each of these fractions can add an additional approval for this conclusion, since cells in fractions 1 and 2 possessed low fluorescence intensity in the yellow-green range (filters FL1 and FL2) and active fluorescence in orange and red spectrum (filters FL3 and FL4, respectively), while representatives in fraction 3 showed active fluorescence on all filters. This feature comes from the fluorescence properties of the dye, because when the dye binds to the lipids in cytoplasm and membranes then a maximum of its emission is in red range of spectrum, which detected with filter FL4. That is why a normal cell has high values of fluorescence in orange and red spectral regions. In case when nile red is dissolved in lipid droplets an yellow-green fluorescence appears with the emission maximum in the green zone of about 530 nm, which correspond to filters FL1 (525 nm) and FL2 (575 nm) used in our flow cytometer. At the same time, the fluorescence at 585 nm is more relate to the content of neutral lipids within cells, while fluorescence at 650 nm and above – to polar lipids [6]. Therefore, the optical data collected with all filters are

suitable for the comprehensive analysis of the content of lipids in the cells and in addition can assist in spores' distinction.

The quantity of objects in the fraction 3 showed a high positive correlation with the level of fluorescence on filters FL1 ($R = 0.96$, $R^2 = 0.93$ at $p \leq 0.001$) and FL2 ($R = 0.95$, $R^2 = 0.90$ at $p \leq 0.001$) (Fig. 1*B*). However, this correlation was marked only in populations grown under optimal conditions, and was broken or utterly disappeared in cells grown in the presence of butanol.

Comparison of strains by their optical characteristics showed that strains No1 and №2 had more similarity to each other, and therefore formed a separate cluster (Fig. 1*C*), while strains No3 and No4 formed another cluster. The main reason of this difference was higher (at least 2 times) fluorescence in orange and red spectra (filters FL3 and FL4, respectively) detected in the strains No3 and No4. This, in turn, may indicate a twice higher content of polar lipids in the cells of these strains. At the same time, the neutral lipid content (filter FL2) was similar in all strains and was within a range 12–14 o.u.

Obviously, that the fluorescence intensity of bacterial population depends directly on the content of polar ($R^2 = 0.86$, $R^2_{Adjusted} = 0.84$, $F_{(3, 27)} = 54.5$ at $p \leq 0.02$) and neutral lipids ($R^2 = 0.84$, $R^2_{\text{Adjusted}} = 0.82$, $F_{(3, 27)} = 46.9$ at $p \leq 0.001$) in separate fractions. During cultivation of bacteria under optimal conditions the polar lipids prevailed over neutral and the content of polar lipids was much higher in cells of clostridia in comparison to spores, while the content of neutral lipids was increased almost twice in spores (Fig. 2). Therefore, carrying out researches with spore forming bacteria it is important to keep in mind that an increment of fluorescence at FL2 can be a result of sporogenesis as well

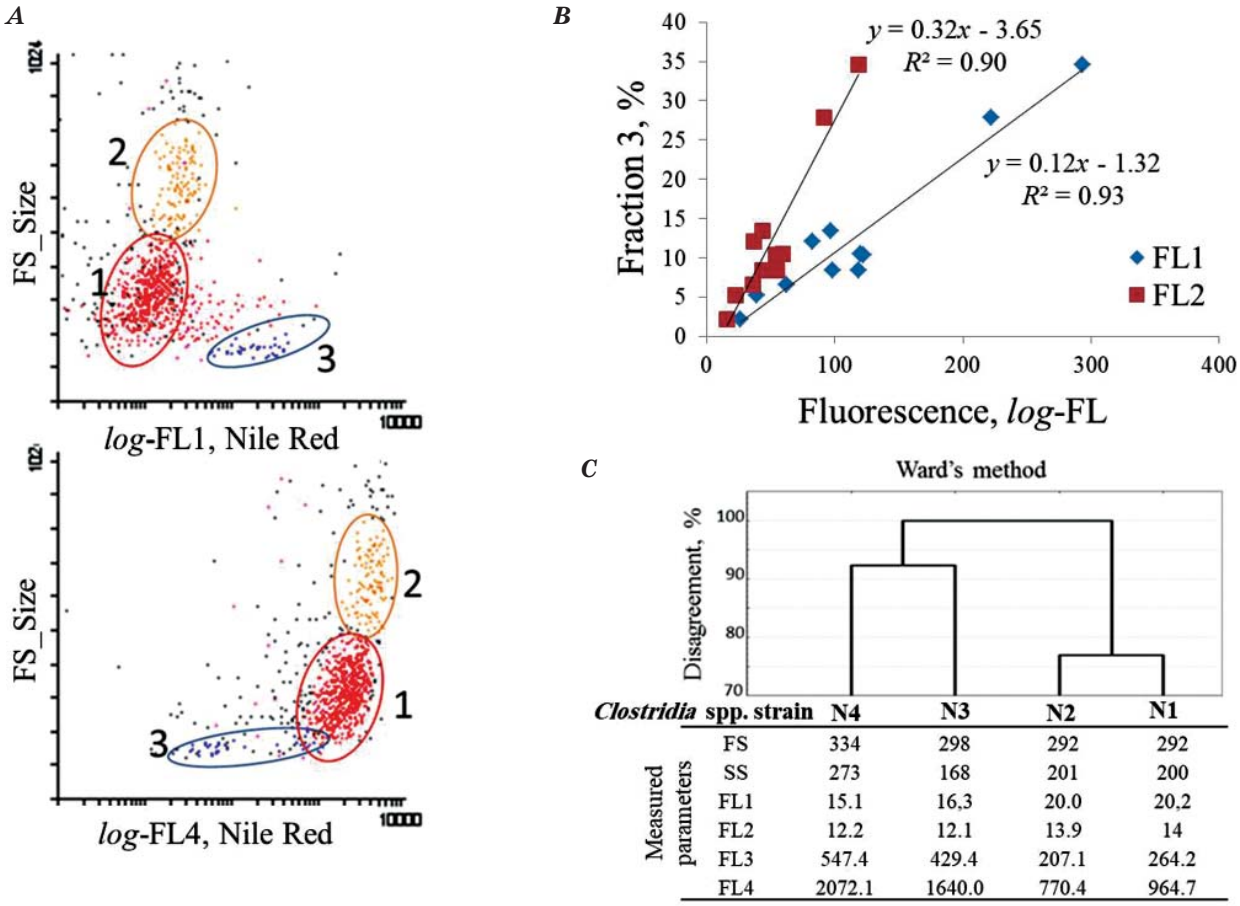


Fig. 1. **The main spectral characteristics of strains of *Clostridia* spp.:**
A — dot plots of cells stained with nile red and separated into three main fractions according to their forward light scattering (FS) values and fluorescence on filters FL1 and FL4; *B* — correlation between the values of fluorescence on FL1 and FL2 (neutral lipids) and the quantity of cells in the fraction 3 (bacterial spores); *C* — clustering of strains according to the optical characteristics obtained by flow cytometry

as accumulation of neutral lipids that is not associated with sporogenesis.

The growth of bacteria in the presence of butanol

Bacteria grown in broth with butanol (0.25–2%) showed a dose-dependent reduction of biomass yield with increasing concentrations of butanol. Strain No3 was the most sensitive to butanol and at 0.5% of butanol content in broth the growth of this strain was substantially reduced, while at higher concentrations it was suppressed. Other strains were able to grow at concentrations of butanol 1.0% and the strain No4 showed a slight increase in biomass even at 1.5% of butanol. None of the strains was able to grow at 2% butanol.

Butanol is a major limiting factor of optimization of the process of ABE-fermentation and it negative effects are evident at concentration 1% [16]. However, the resistance of strain No4 to 1.5% of butanol suggests that there are may exist strains with an increased tolerance to butanol among natural isolates.

Optical features of bacterial populations grown with butanol

Cultivation of bacteria with butanol resulted in some cellular changes, which reflected on their optical features. The main mathematical parameters that can be taken into account in this case are the mean and median values. Median better reflects the structure of a population in case if there are several cellular fractions in it. However, the mean values are more relevant to the content of chemical compounds such as neutral and polar lipids [6].

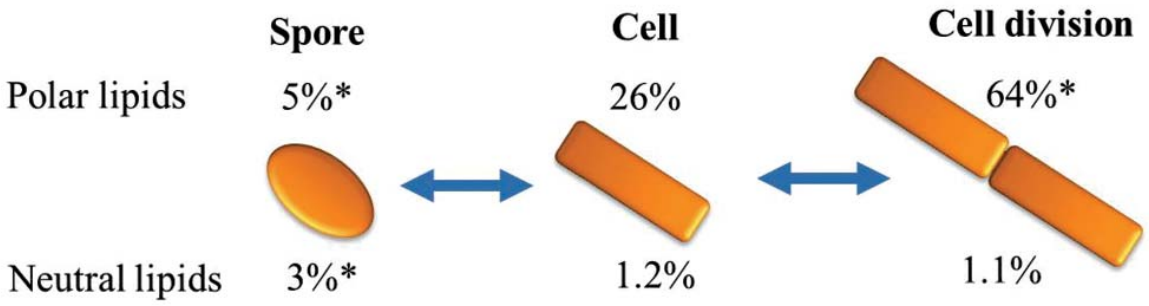
Assessing the impact of butanol on the structure of population, it was found non-linear relationship between the concentration of butanol and the median of fluorescence at FL1–FL4. It was marked a decrease in fluorescence of cells with an increase of butanol portion to 1–1.5% and recovery of fluorescence with a further increase of butanol to 2%. This effect was associated with the formation of a new fraction of cells (data not shown) characterized by low fluorescence and considered as a group of dead cells, which occurred after butanol degradation of cellular membranes.

Analysis of populations by their mean values showed that butanol decreased the degree of correlation between the quantity of objects in the fraction 3 and fluorescence at FL1 and FL2 to 9% ($p \leq 0.78$) and 65% ($p \leq 0.02$), respectively. In addition, butanol showed a positive strain-specific effect on FL2 and FL4 values and the ratio of FL4/FL2 (Fig. 3). The changes of the mean values of other parameters (FS, SS, FL1 and FL3) did not depend on the concentration of butanol instead were strain-specific and time dependent only.

Simultaneous reduction of fluorescence at FL2 and FL4 indicated a dose-dependent decrease in the content of neutral and polar lipids in bacterial cells during cultivation with butanol. The most stable content of neutral and polar lipids was observed in strain No1 in which a lipids pool declined slightly in the presence of butanol in comparison to other strains (Fig. 3*C*). Strain No2 characterized with the highest content of neutral lipids in the absence of butanol, but this number decreased almost 2.5 times in the presence of butanol. Strain No4 had a low content of neutral lipids, but in the

The values of forward light scattering (FS) for cell fractions detected by flow cytometry

Strain №	Fraction 1			Fraction 2			Fraction 3		
	Mean	Median	CV	Mean	Median	CV	Mean	Median	CV
1	296.3	287.0	22.7	590.3	544.0	8.4	155.9	154.0	13.0
2	297.4	292.0	24.3	651.9	611.0	12.5	156.7	155.0	12.5
3	301.5	299.0	21.9	607.7	582.0	14.0	158.5	156.0	12.3
4	309.6	309.0	22.9	643.5	630.0	13.2	149.1	143.0	11.0
Mean	301.2	296.8	23.0	623.4	591.8	12.0	155.1	152.0	12.2
Sd	6.1	9.5	1.0	29.2	37.5	2.5	4.1	6.1	0.9



Strain	Spore		Cell		Cell division	
	Polar	Neutral	Polar	Neutral	Polar	Neutral
N1	245.6	180.8	839.4	29.8	2786.0	26.6
N2	214.6	183.9	642.7	163.1	2852.0	33.2
N3	301.1	153.2	1615.2	28.1	3103.8	126.5
N4	219.4	87.6	2004.9	15.9	3844.3	24.9
Mean	**245.2**	**151.4**	**1275.5**	**59.2**	**3146.5**	**52.8**
Sd	39.7	44.7	642.4	69.5	484.9	49.3

Fig. 2. **The content of polar and neutral lipids in cells and spores of *Clostridia* spp. expressed as a percentage of the total fluorescence at filters FL4 (polar lipids) and FL2 (neutral lipids) presented in the table**

presence of butanol their quantity grew up in 2 times on average. The increase of the content of neutral lipids in the strain No4 occurred in the cells and spores of this bacterium. However, there was marked a proportional increase of the portion of polar lipids in spores, while the polar lipids in the cells decreased.

In none of the cases the quantity of neutral lipids exceeded polar lipids, and therefore it is not possible to talk about an accumulation of neutral lipids in the cells or spores of these bacteria.

Thus, the cell size and granularity of the studied bacterial strains were not affected during cultivation with butanol (0.25–2%). The quantity of polar and neutral lipids as well as their ratio were strain-specific. The results indicate that there are may occur bacterial strains with increased tolerance to butanol among natural isolates of *Clostridium* (such as strains No2 and No4); however, their resistance to butanol, probably, do not relate to the synthesis or accumulation of polar or neutral lipids.

The results of the current study were obtained in model that considered butanol variations only, which is far from reality of ABE-fermentation, but these data indicate the prospects of searching the new strains among natural isolates, which will possess not only resistance to butanol, but will synthesize the neutral lipids in adequate quantities. In this case a method of flow cytometry and staining of cells with fluorescent dye nile red let quickly and accurately evaluate the strains and optimize the cultivation conditions to increase yield of microbial biomass with optimal content of neutral and polar lipids in it, that will be sufficient for biofuels production or extraction of specific lipids for technical, medical or biological applications.

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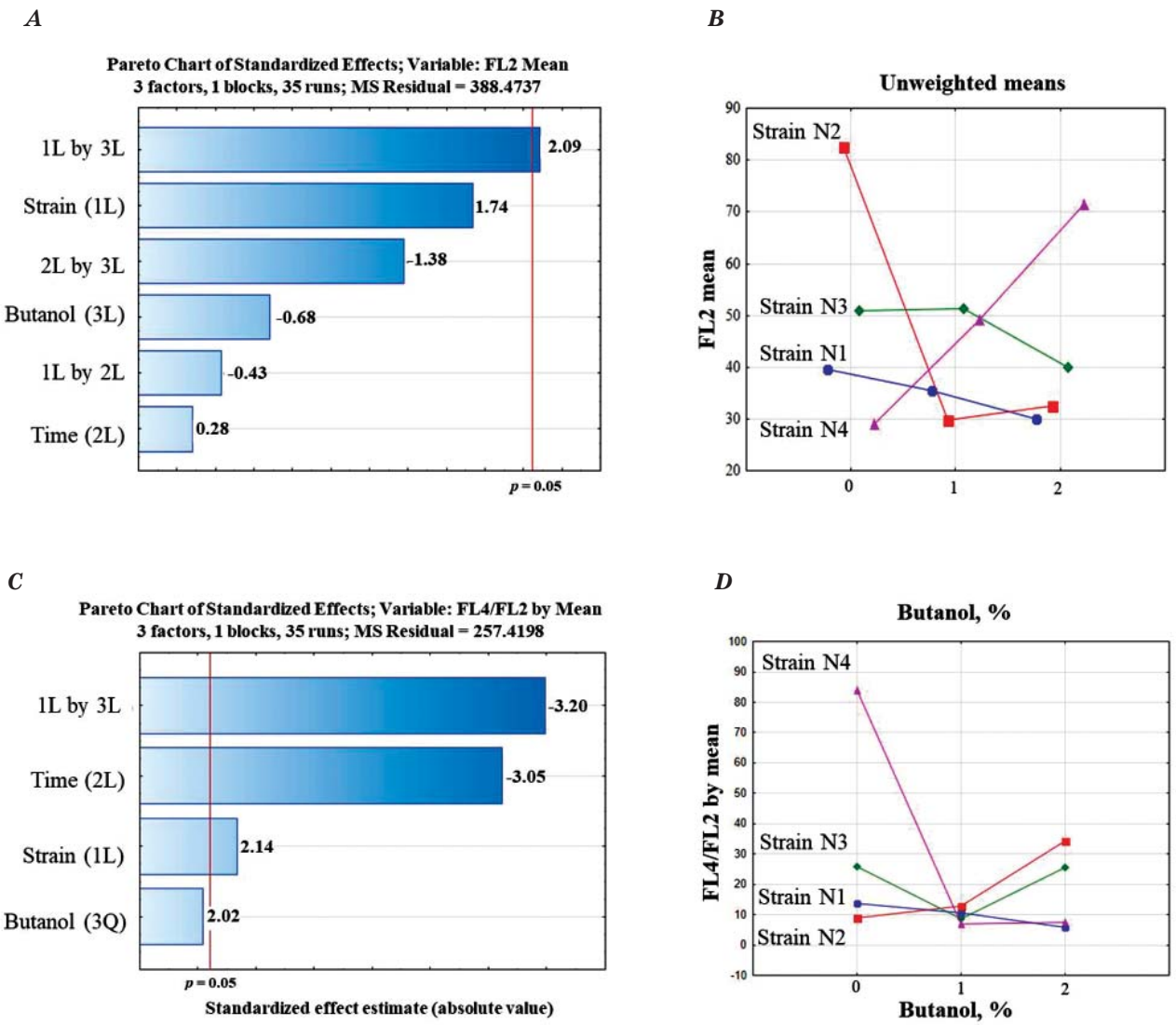

Fig. 3. **Influence of butanol on the main optical parameters of bacterial cells determined with flow cytometry after staining with nile red:**
(*A* and *B*) — the Pareto charts that demonstrate negative and positive, linear (L) and quadratic (Q) effects of the studied variables (strain — variable 1, the duration of cultivation — variable 2, the concentration of butanol — variable 3) onto the mean values of fluorescence at FL2 (*A*) and the ratio of fluorescence at FL4 and FL2 (*B*). (*C* and *D*) — Strain-specific dependence of fluorescence at FL2 and ratio of FL4/FL2 on the concentration of butanol in the broth

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ВМІСТ НЕЙТРАЛЬНИХ І ПОЛЯРНИХ ЛІПІДІВ У КЛІТИНАХ КЛОСТРИДІЙ ЗА УМОВ КУЛЬТИВУВАННЯ У ПРИСУТНОСТІ БУТАНОЛУ

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Метою роботи було оцінити зміни у складі нейтральних і полярних ліпідів у клітинах клостридій за культивування у присутності бутанолу. Дослідження проведено на чотирьох природних ізолятах клостридій методом проточної цитофлуориметрії. За оптимальних умов культивування у клітинах бактерій полярні ліпіди превалювали над нейтральними; вміст нейтральних ліпідів збільшувався вдвічі у спорах цих мікроорганізмів і водночас знижувався вміст полярних. Штами №1 і №2 були здатні рости за 1% бутанолу в середовищі, а штам №4 — 1,5%. Під час культивування за різних концентрацій бутанолу досліджені штами бактерій не відрізнялись за такими цитоморфологічними ознаками, як гранулярність і розмір клітин. Зміст полярних і нейтральних ліпідів у присутності бутанолу змінювалося істотно, проте зміни носили штаммоспеціфіческій характер і не дозволили виявити загальні закономірності. Таким чином, вміст полярних і нейтральних ліпідів змінювався залежно від вмісту бутанолу у середовищі, однак цей ефект мав штаммоспецифічний характер і не виявляв зв'язку зі стійкістю цих бактерій до бутанолу. Використання відпрацьованої біомаси бактерій як джерела ліпідів для виробництва біопалива потребує подальшої оптимізації процесу для збільшення вмісту фракції нейтральних ліпідів у клітинах бактерій.

Ключові слова: *Clostridium*, полярні і нейтральні ліпіди, бутанол, проточна цитофлуориметрія.

СОДЕРЖАНИЕ НЕЙТРАЛЬНЫХ И ПОЛЯРНЫХ ЛИПИДОВ В КЛЕТКАХ КЛОСТРИДИЙ ПРИ КУЛЬТИВИРОВАНИИ В ПРИСУТСТВИИ БУТАНОЛА

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Целью работы было оценить изменения в составе нейтральных и полярных липидов в клетках клостридий при культивировании в присутствии бутанола. Исследование проведено на четырех природных изолятах клостридий методом проточной цитофлуориметрии. При оптимальных условиях культивирования в клетках бактерий полярные липиды превалировали над нейтральными; содержание нейтральных липидов увеличивалось вдвое в спорах этих микроорганизмов и одновременно снижалось содержание полярных. Штаммы №1 и №2 были способны расти при 1% бутанола, а штамм №4 — при 1,5%. В условиях культивирования при различных концентрациях бутанола исследованные штаммы бактерий не отличались по таким цитоморфологическим признакам, как гранулярность и размер клеток. Содержание полярных и нейтральных липидов в присутствии бутанола менялось существенным образом, однако изменения носили штаммоспецифический характер и не позволили выявить общие закономерности. Таким образом, содержание полярных и нейтральных липидов изменялось в зависимости от содержания бутанола в среде, однако этот эффект был штаммоспецифическим вне связи с устойчивостью этих бактерий к бутанолу. Использование отработанной биомассы бактерий как источника липидов для производства биотоплива требует дальнейшей оптимизации процесса для увеличения содержания в клетках бактерий фракций нейтральных липидов.

Ключевые слова: *Clostridium*, липиды, бутанол, проточная цитофлуориметрия.