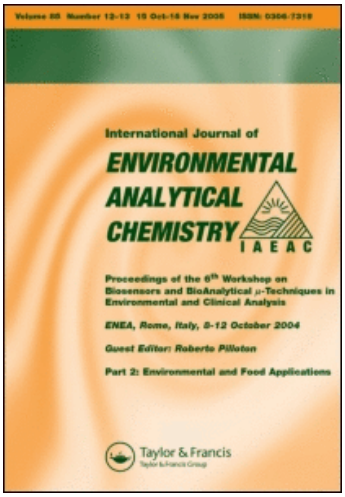

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Electrophysical analysis of microbial cells and biosensor technology

Oleg V. Ignatov[a]; Olga I. Guliy[a]; Victor D. Bunin[b]; Vladimir V. Ignatov[a]
[a] Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov 410049, Russia [b] State Research Center for Applied Microbiology, Obolensk, Moscow Region, Russia

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Electrophysical analysis of microbial cells and biosensor technology

OLEG V. IGNATOV*†, OLGA I. GULIY†,
VICTOR D. BUNIN‡ and VLADIMIR V. IGNATOV†

†Institute of Biochemistry and Physiology of Plants and Microorganisms,
Russian Academy of Sciences, 13 Entuziastov av., Saratov 410049, Russia
‡State Research Center for Applied Microbiology, Obolensk, Moscow Region, Russia

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A new kind of biosensor system may be developed by using an electrophysical technique for measurement of electrophysical properties of microbial cells. The electro-optical (EO) analysis of cell suspensions was used as the basis for our work. It is based on the recording of changes in the optical characteristics of cell suspensions under the orienting effect of an electric field. Promising fields of use of microbial cell suspensions for EO analysis were developed. One of the promising fields is the development of biosensor systems for analysis of low-molecular-weight substances. Another field is the investigation of cellular metabolism. The most recent direction of our work is the application of EO analysis to cell detection. We showed that the determination of bacteria may be achieved by selection and matching of antibodies specific to individual bacterium types and by comparing spectra of bacteria in the presence and absence of specific antibodies. The same principles were used for investigations of the bacteria–phage interaction. Thus electro-optical analysis of cell suspensions opens new opportunities for creation of new biosensor methods in biotechnology, environmental control and medicine.

Keywords: Electro-orientation; Toxic compounds; Cell detection; Antibody; Phage

1. Introduction

A new kind of microbial biosensor system may be developed by using an electrophysical technique for measurement of electrophysical properties of microbial cells. The electro-optical analysis of cell suspensions was used as the basis for our work. It is based on the recording of changes in optical characteristics of cell suspensions under the orienting effect of an electric field. The electro-optical technique includes a number of interrelated processes [1]: (1) action of electric field on the suspended bioparticles; (2) generation of induced charges on the boundaries of cell structures and cell surface; (3) creation of the driving torque; (4) cell transition into an oriented state, which leads to the anisotropy of optical properties of a cell suspension. Such effects induced the fluctuations of light scattering and relevant variations in optical density.

*Corresponding author. Fax: +7-8452-970383. Email: oignatov@ibppm.sgu.ru; oignatov@mail.ru

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In this article, promising areas of application of EO analysis of microbial suspensions are suggested to solve some biotechnology problems.

2. Measurements of cellular orientational spectra with an electro-optical analyzer

An electro-optical analyzer (ELUS EO) has been developed at the State Research Center for Applied Microbiology, Obolensk, Russia and was used as the basic instrument for electro-optical measurements. The analyser consists of the following modules: a unit for sample preparation, a mixer, an AC field generator, an EO-flow cell, a microcontroller of liquid stream transactions, a thermal system, an operator interface, and an image processor. The analyzer was used for determination of electro-physical and morphometric parameters of microorganisms. It operates at multiple software programmable frequencies of 0.4 kHz to 20 MHz. The software program was used for collection, calculation and data processing.

The orientational spectra (OS) of the cells were measured with an EO ELUS analyzer at a wavelength of 670 nm. An OS is given as the frequency dependence of the difference (δD) between the suspension-optical-density values (D_a and D_b) measured during the propagation of a beam of non-polarized light along (D_a) and across (D_b) the orienting-field direction. This difference was normalized to the optical density value (D) measured for cells at random orientation. There is reason to assume that the general view of the OS under the chosen experimental conditions (wavelength, relatively small amplitude of the orienting-electric-field strength, etc.) is essentially determined by the frequency dependence of the anisotropy of the cell polarizability $\Delta\gamma = \gamma_a - \gamma_b$ [2, 3]:

$$\delta D(\omega) = \frac{D_a - D_b}{D} = \Delta\gamma(\omega) E^2 F \tag{1}$$

where E is the orienting-field strength, ω is the orienting-field frequency, and F is a coefficient including an 'optical factor', which depends, among other things, on the cell size and refractive index [4].

For rod-shaped axisymmetric particles, which are good models for the cell types used by us, the polarizability tensor has only two distinct components: a longitudinal one, γ_a (corresponding to the direction of the long axis of the particle) and a transverse one, γ_b (corresponding to the orthogonal direction). The preferred direction (along or across the strength-vector direction of the orienting electric field) and the degree of particle orientation (characterized by the width of the Boltzmann function of particle distribution by orientational angle) depend on the sign of $\Delta\gamma$ and on the value of the parameter:

$$q = \frac{\Delta\gamma E^2}{2kT} \tag{2}$$

where k is the Boltzmann constant and T is the absolute temperature. In our experiment, at a relatively weak cell-orientation degree $q \ll 1$.

The design of the ELBIC apparatus makes it possible to represent the OS as the frequency dependence of the relation:

$$\delta \mathrm{OD}_{\text{relative units}} = \frac{\delta D}{E^2 F \kappa} \approx A \Delta\gamma \tag{3}$$

which was used in our experiments. In this equation, $\kappa = I/I_0$ is the transmittance coefficient (I and I_0 are the intensities of transmitted and incident light, respectively). The constant A contains a scale multiplier that makes for easy reading of the measured results for $\delta OD_{\text{relative units}}$, the magnitude of which in this case had values of the order 10^2–10^3. At sufficiently low degrees of cell orientation, such normalization ensures the independence of $\delta OD_{\text{relative units}}$ from the cell concentration, the orienting-field strength E, and the attenuation of the light beam during its passage through the scattering medium.

Thus, the dependence of $\delta OD_{\text{relative units}}$ on the orienting-field frequency ω coincides with the frequency dispersion of the anisotropy of the particle-polarizability tensor $\Delta\gamma(\omega)$ with an accuracy of the constant. Depending on the orienting-field frequency, the frequency dispersion $\Delta\gamma(\omega)$ mirrors the effects of various cell structural elements. These elements are: (a) cell-surface biopolymers and associated high- and low-molecular-weight compounds (coming from the environment) that form a double electric layer directly at the cell–environment interface (ω of the order of unities and tens of Hz); (b) the components of the cell wall and cytoplasmic membrane (ω of the order of tens and hundreds of kHz); and (c) the elements of the cell inner structure (cell organelles) (ω of the order of unities and tens of MHz).

This makes it possible to obtain information on the various physical–chemical and physiological–biochemical processes occurring on the surface of and inside the cell, including substrate-induced enzymatic processes taking place in microbial cells.

The arrangement of nine electrodes placed in the measuring cell with respect to the incident light beam is shown schematically in figure 1. The two outer rows of electrodes are parallel-connected to a terminal of an orienting-field generator. The middle row is parallel-connected to the other terminal. This design provides for a high degree of spatial homogeneity of the orienting field in the probed bulk of cell suspension. This

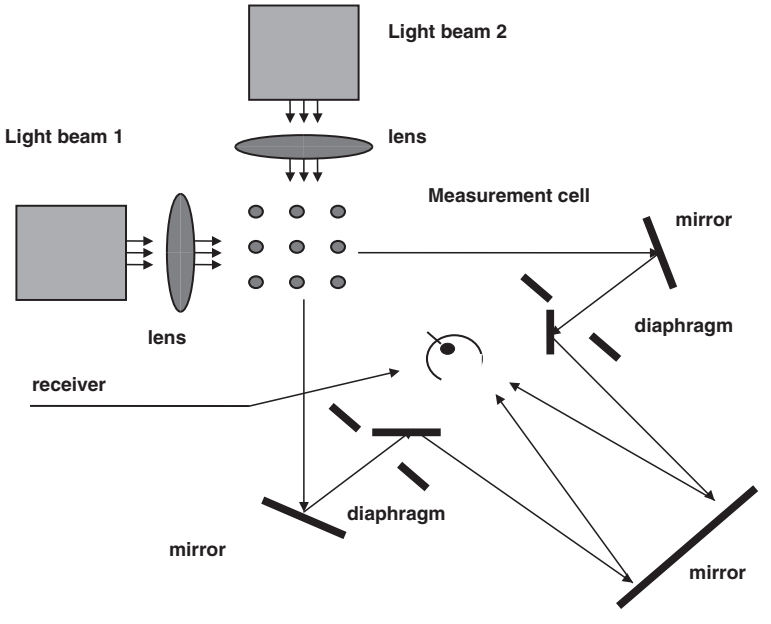

Figure 1. Schematic arrangement of the electro-optical part.

decreases substantially the probability of dielectrophoresis (the latter effect was observed only when the pulse length of the orienting field was increased five- to ten-fold as compared with the value used in this work).

3. Quantitative determination of low-molecular-weight substances by using electric-field cell orientation in microbial suspensions

One possibility is the use of EO analysis to develop biosensor systems for analysis of low-molecular-weight substances in aqueous media. We investigated cells of several strains having enzyme systems for the initial metabolism of toxic compounds. These processes lead to the redistribution of charges in the microbial cells and may be recorded by electro-optical methods. Redistribution of the charges in the microbial cells may be used for determination of substrates of the enzyme reaction and, finally, for determination of substrate concentration.

We showed that there occur changes in the EO parameters of microbial cells during metabolism of low-molecular-weight substances, used as a model system. We investigated the possibility of determining the organophosphorus aromatic nitro insecticides metaphos and sumithion by measuring orientational spectra (OS) of cell suspensions during the cellular metabolism of these insecticides [5]. In this article we examined the effect of cellular metabolism of the organophosphorus aromatic nitro insecticides metaphos and sumithion on the electrophysical properties of *Pseudomonas putida* C-11, *P. putida* BA-11, and *Acinetobacter calcoaceticum* A-122 suspensions. We used the dependences of cell-suspension optical-density changes induced by electric-field orientation on the orienting-field frequency in the range of 10–10 000 kHz. Substantial orientational-spectrum changes, caused by insecticide action, occurred at frequencies of 10–1000 kHz. The plots of electro-optical effect *vs*. insecticide concentration were linear over the following concentration ranges: 0.5–3.0 mM metaphos (figure 2) and 0.5–3.5 mM sumithion for *P. putida* C-11. For cells of *P. putida* BA-11 and *A. calcoaceticum* A-122, similar results were obtained. We showed the possibility of developing a novel method for determining the organophosphorus aromatic nitro insecticides metaphos and sumithion by measuring the OS of suspensions of the strains that can metabolize the substrates being determined.

Thus, we showed the possibility of developing a novel method (a biosensor system) for determining low-molecular-weight substances with the help of EO analysis of microbial cells.

4. Investigating cellular metabolism by EO analysis of cell suspensions

Another approach to the possibility of using EO analysis of microbial cells is the use of its results for studies of cellular metabolism. When microorganisms possessing specialized enzyme-systems metabolize low-molecular-weight compounds, the EO characteristics of cell suspensions change.

So, for example, we used *p*-nitrophenol (PNP) to study metabolic processes in microbial cells.

We found that when microorganisms possessing specialized enzyme-systems metabolize the toxic low-molecular-weight compound PNP, the electro-optic (EO)

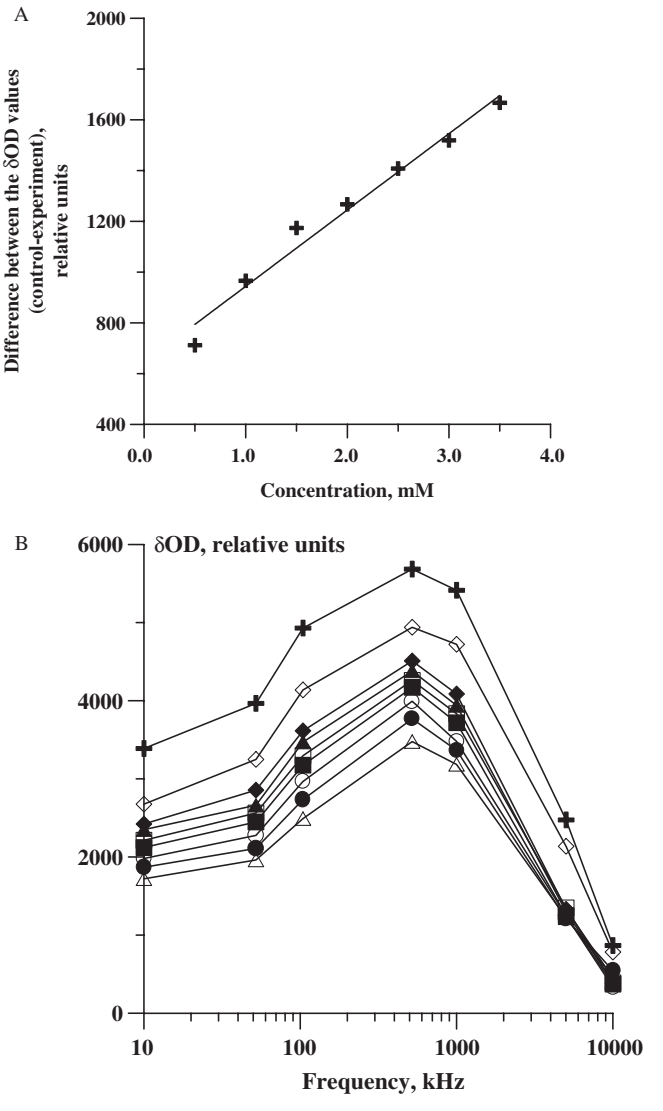

Figure 2. (A) The difference between control and experimental δOD values at 10 kHz *vs.* sumithion concentration. (B) OS of *P. putida* C-11 cells incubated with sumithion at 0.5 (◇), 1.0 (◆), 1.5 (□), 2.0 (■), 2.5 (○), 3.0 (●), 3.5 (△), and 5.0 (▲) mM; (+) control [cells incubated in substrate-free deionized water].

characteristics of cell suspensions change [6]. This fact is associated with the change occurring in the electrophysical properties of cellular structures during metabolism. In different strains, the metabolism of the same compound may give rise to different intermediates.

As is well known, two PNP-metabolic pathways are possible in microorganisms (figure 3): (a) via a direct hydrolytic detachment of the nitro group and the production of hydroquinone [7], or (b) via the preliminary reduction of the nitro group to an amino group, followed by a detachment of ammonium ions and the formation of nitropyrocatechin [8].

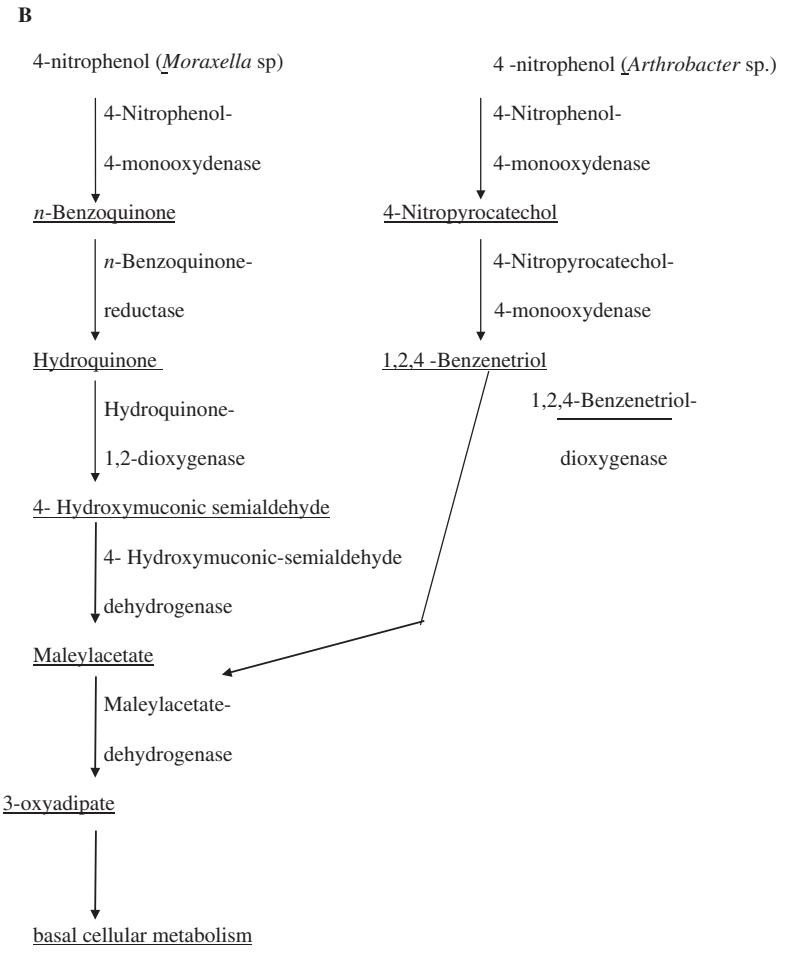

Figure 3. Schematic of the initial metabolism of PNP by a *Moraxella* sp. (A) and an *Arthrobacter* sp. (B).

We used two *P. putida* strains (C-11 and BA-11) and *A. calcoaceticum* strain (A-122), which can utilize PNP as their sole carbon and energy source [9]. The effect of cellular *p*-nitrophenol (PNP) metabolism on the electrophysical properties of *P. putida* C-11, *P. putida* BA-11, and *A. calcoaceticum* A-122 was studied. We found that when *P. putida* C-11, *P. putida* BA-11, and *A. calcoaceticum* A-122 were incubated with various PNP-concentrations, there occurred substantial changes in the OS of suspensions of all the strains [10].

Because the hydrolytic elimination of the nitro group gives rise to hydroquinone, we examined the effect of hydroquinone metabolism on the EO characteristics of the cell suspensions. To this end, *P. putida* C-11, *P. putida* BA-11, and *A. calcoaceticum* A-122 cells, prepared for OS measurements, were incubated with 1.0 and 2.5 mM hydroquinone at 25°C. There were pronounced changes in the OS of the suspension of hydroquinone-incubated *P. putida* C-11; however, hydroquinone did not affect the EO characteristics of the *P. putida* BA-11 and *A. calcoaceticum* A-122 suspensions (figure 4).

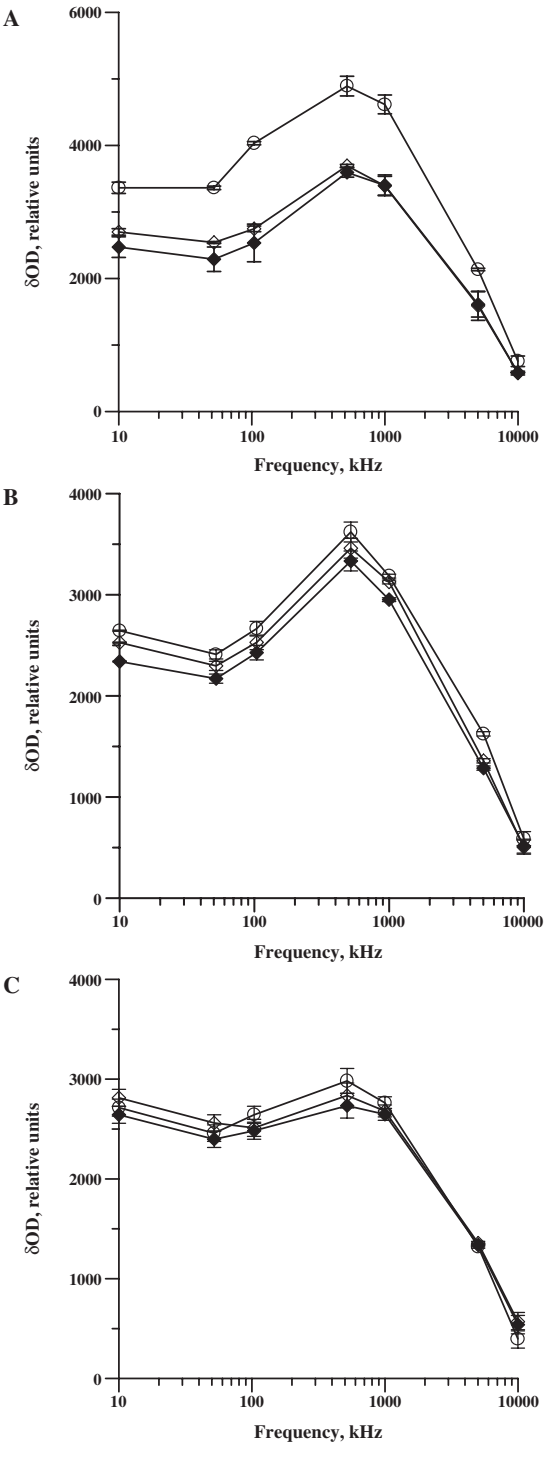

Figure 4. OS of hydroquinone-incubated *P. putida* C-11 (A), *P. putida* BA-11 (B), and *A. calcoaceticum* A-122 (C). (○) Control [cells incubated in substrate-free deionized water]. (◇) Cells incubated in deionized water with 1.0 mM hydroquinone. (◆) Cells incubated in deionized water with 2.5 mM hydroquinone.

The fact that when *P. putida* C-11 is incubated with hydroquinone there occur substantial OS-changes suggests that the PNP-metabolic pathway in this strain (as in the *Moraxella* sp. [7]) is via the production of hydroquinone. No wide OS-changes were found when *P. putida* BA-11 and *A. calcoaceticum* A-122 were incubated with hydroquinone. Possibly, in *P. putida* BA-11 and *A. calcoaceticum* A-122 (as in *Arthrobacter* sp. strain JS443 [8]), the metabolism of PNP occurs via the formation of 4-nitropyrocatechin, followed by a fission of the aromatic ring.

To confirm the results obtained, we decided to investigate the respiratory activity (RA) of the strains toward hydroquinone and PNP. The analyte concentrations were 1.0 mM for *A. calcoaceticum* A-122 and 2.5 mM for *P. putida* C-11 and BA-11. The hydroquinone concentrations used were chosen with account taken of the data obtained previously for the RA of the three strains toward PNP. In *P. putida* C-11 and BA-11, the RA toward PNP was maximal at 2.5 mM PNP; in *A. calcoaceticum* A-122, at 1.0 mM PNP. *A. calcoaceticum* A-122 and *P. putida* BA-11 did not have RA toward hydroquinone, whereas *P. putida* C-11 did. All the strains tested had RA toward PNP. Thus, the data obtained confirm the interrelationship between changes in cellular EO-properties and enzymatic processes taking place in microbial cells under the action of the toxic substrates.

We speculate that, in *P. putida* C-11, the initial metabolism of PNP is effected via the production of hydroquinone, an intermediate of PNP metabolism; and in *P. putida* BA-11 and *A. calcoaceticum* A-122, via the formation of 4-nitropyrocatechin, followed by a fission of the aromatic ring. The data obtained make it likely that EO analysis can be used for investigating cellular metabolism [10].

Thus, we showed the possibility of developing a novel method for studies of cellular metabolism by measuring the OS of suspensions of the strains that can metabolize the substrates.

5. Detection of microbial cells with the help of electro-optical analysis

We recently reported on an EO analyzer for detection and identification of cells. Detection and identification of pathogenic bacteria in the environment present multiple challenges [11, 12]. The electro-optical technique includes a number of interrelated processes [1]: (1) action of an electric field on the suspended bioparticles; (2) generation of induced charges on the boundaries of cell structures and cell surface; (3) creation of the driving torque; (4) cell transition into an oriented state, which leads to the anisotropy of optical properties of a cell suspension. Such effect induces the fluctuations of light scattering and relevant variations in optical density. The optical density changes can be detected by our optical technique, thus allowing rapid and accurate detection of target bacteria in aqueous solutions.

5.1 *Detection of microbial cells with the help of specific-antibody binding*

During biospecific interactions an antibody is bound to the microorganism causing a change in the dielectric properties of the microorganism-antibody complex and the electro-optical signal. The changes in optical density can be detected by our optical technique, thus allowing rapid and accurate detection of target bacteria in aqueous solutions. We suggest that electro-orientational spectral analysis of cell suspensions

may be used for discrimination of different types of bacteria with the help of selective binding agents (antibodies). An electro-optical approach has been used for studies of *Listeria monocytogenes*-antibody binding. Electro-orientational spectra were used for discrimination of bacteria before and after selective binding to antibodies [13].

For identification of bacteria with the help of electro-optical techniques, the electro-optical approach should be integrated with immunoassay technologies. Figure 5A demonstrates the variation in amplitudes of electro-optical signals for *L. monocytogenes* cells as a result of formation of *Listeria monocytogenes*-monoclonal antibodies bio-specific complexes. The electro-orientation properties, e.g. the rate of *L. monocytogenes*

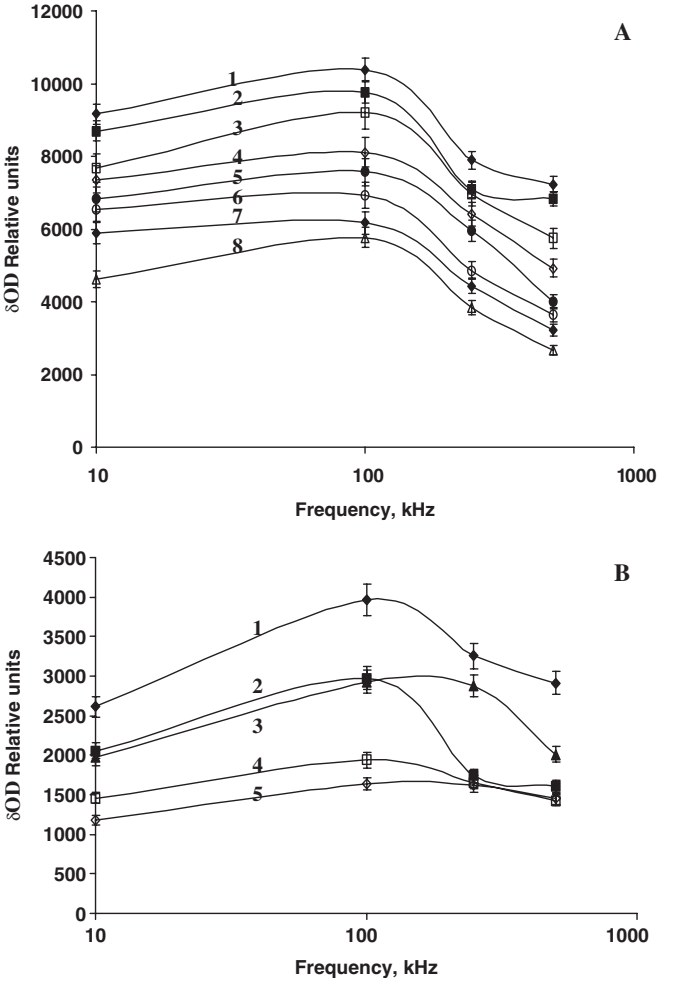

Figure 5. (A) The electro-orientation spectra of *L. monocytogenes* cells suspended in distilled water, obtained after incubation with different concentrations of monoclonal anti-*L. monocytogenes* antibodies. (1) Control (without mAb); (2) 0.475 μg mL^{-1} mAb; (3) 1.425 μg mL^{-1} mAb; (4) 1.9 μg mL^{-1} mAb; (5) 2.3 μg mL^{-1} mAb; (6) 4.75 μg mL^{-1} mAb; (7) 7.1 μg mL^{-1} mAb; (8) 9.5 μg mL^{-1} mAb. (B) Electro-optical properties of mixed cells (*A. brasilense*, *E. coli* K-12, *Listeria monocytogenes*) suspended in distilled water, obtained after incubation with different concentrations of monoclonal anti-*L. monocytogenes* antibodies. (1) Control (without mAb); (2) 0.475 μg mL^{-1} mAb; (3) 1.425 μg mL^{-1} mAb; (4) 1.9 μg mL^{-1} mAb; (5) 2.3 μg mL^{-1} mAb.

cell orientation observed at a given field orientation frequency is altered sufficiently. During biospecific interaction an antibody is bound to the microorganism, causing a change in the dielectric properties of the microorganism-antibody complex and the electrooptic signal reaches its maximum at 100–200 kHz. The experimental data from EO signals at different frequencies have indicated that EO spectral analysis should be conducted preferably at low frequencies (10–500 kHz), and the time of the measurement should be between 20 and 25 min.

One of the possible potential unfavorable cases of pathogen monitoring in the environment might be the case when targeted and debris cells present in suspension are of equal size. Therefore, we investigated the possibility of recording changes in EO spectra of the *L. monocytogenes* in a mixed culture suspension containing *E. coli* K-12 and *A. brasilense* Sp7 as a result of specific interaction with monoclonal anti-*Listeria monocytogenes* antibodies. We found that the changes in the electro-optical signal *L. monocytogenes*-anti-*Listeria* antibody in the mixture of *E. coli* K-12 and *A. brasilense* Sp7 cells were significant (figure 5B). At the same time, control experiments in the absence of *L. monocytogenes* cells have shown that there are no non-specific changes in electro-optical properties in cell suspensions of *E. coli* K-12 and *A. brasilense* Sp7 in the presence of anti-*Listeria* antibodies. Thus, the determination of the presence of particular bacteria within a mixed sample is achievable by use of antibodies specific to individual bacterium types and by comparing spectra of a bacterium in the presence and absence of an antibody. The results indicate that electro-orientational spectra can be used for discrimination of bacteria before and after interaction with selective antibodies [13].

5.2 *Detection of microbial cells with the help of specific bacteriophage binding*

The current focus of our research group is to apply the electro-optical analyzer for direct detection and discrimination of bacteria based on biospecific binding. More specifically, the purpose of this article is to demonstrate the potential of using the electro-optical technique in combination with bacteriophage amplification for direct monitoring of *E. coli*. Bacteriophages are virus particles that generally attach to and infect a narrow range of host cells [14, 15]. Infection of bacteria by a bacteriophage starts by recognition of the host through binding to an outer membrane receptor. Like human viruses, they inject their genetic material into the bacterial cell, replicate by the hundreds per cell, then burst out before moving on to the next host cell. In the case of tailed phage, this binding triggers conformational changes that are transmitted along the tail to the capsid, allowing its opening and the release of the viral genome, which causes a change in the dielectric properties of cells. Advantages of the phage technology include its simplicity, ease of use, low cost, safety and immunogenicity (for vaccine studies). The bacteriophage specificity has been demonstrated at both species and strain levels in the literature. Specific bacteriophages are very good indicators for determining the species and type of bacteria. That is why they have found a wide application in medical practice for the fast identification of viable bacteria [14–16]. The phage–cell interaction process is fairly complex and depends on the structure of the phage itself [17, 18] and the presence of F-pili in bacteria [19]. In our work, *Escherichia coli* XL-1 and bacteriophage M13K07 were chosen as a model system [20]. The bacteriophage M13 is an *E. coli*-specific filamentous phage. It is a long, thin bacterial virus that infects *E. coli* cells without cell lysis [21, 22]. The phage M13 infection of *E. coli* cells containing

F-pili has been extensively studied [23, 24]. Phage action on a bacterial cell may follow different paths: a lytic reaction, lysis from the outside, and a lysogenic reaction. Whatever the path, due to entry of viral DNA into *E. coli* cell cytoplasm, with the capsid protein (g8p) integrated into the inner cytoplasmic membrane, the phage infection leads to considerable changes in the electrophysical parameters of the cells, including changes in their dielectric properties and various kinds of damage to the intracellular structures [5, 23]. Therefore, discrimination of microorganisms might be done on the basis of measurement of the electro-optical spectra of the microorganisms in the absence and presence of phage.

Our preliminary studies have shown that the following electro-optical conditions are optimal for measurement of the electro-optical spectra of *E. coli*: electric-field strength, $17\,V\,cm^{-1}$; application time, 16 s; and discrete frequencies 250, 500, 750, 1000, and 2000 kHz.

For controlling phage transfection to the bacteria, we grew the cells in LB nutrient medium containing kanamycin, because phage M13K07 confers resistance to this antibiotic [25]. The cells grew well with kanamycin, indicative of phage transfection. Figure 6 shows electro-optical spectra of *E. coli* after incubation with various numbers of M13K07 phage. As a result of these studies, we showed that the maximum OS changes occurred when the cells were infected at a rate of twenty (20) phage per bacterium. To record cell infection, we added 20 phage per bacterium in the suspension in subsequent experiments. In fact, we found that even a rate of one phage per bacterium leads to changes in the EO-spectrum. But the best resolution was received at a rate of 20 phage per bacterium. We found that considerable changes in the magnitude of the EO signal were detected in the first 5 min after the phage injection. This can be explained by the formation of a bacteria–phage complex.

Since many pathogenic organisms differ little from a normal flora, it is important to test the specificity of the electro-optical system in the presence of interfering factors, first and foremost in the presence of a foreign microflora. Therefore, in our next experiments the electro-optical measurements were done in the absence and presence of a foreign microflora which would not be infected by phage M13K07 (figure 7). As control, we used cells of *Azospirillum brasilense*. *A. brasilense* Sp7 was chosen because it occupies a different taxonomic position and has a cell size similar to that of *E. coli* XL-1. To this end, phage was added to a mixed suspension containing *E. coli* XL-1 and *A. brasilense* Sp7 (figure 7A) (OD_{665} 0.42–0.44). The cell–phage interaction conditions were analogous to those used in the experiments with XL-1 alone. We found that during the *E. coli* XL-1-phage M13K07 complex formation in the presence of the foreign microflora (*A. brasilense* Sp7) there occurred a substantial decrease in the magnitude of the EO signal. Control experiments were run in parallel to explore the possibility of non-specific interaction of phage M13K07 with *A. brasilense* Sp7. When *A. brasilense* Sp7 was incubated with the phage, the cell OS did not change either; that is, the phage did not infect the cells (figure 7B). Thus, we found that specific changes in the EO parameters of cell suspensions under the influence of M13K07 occur only in *E. coli* XL-1 and do not occur in *A. brasilense* Sp7.

This preliminary study has shown that a combination of the EO approach with phage as a recognition element has an excellent potential for mediator-less detection of phage–bacteria complex formation. The interaction of *E. coli* with phage M13K07 induces a strong EO-signal as a result of substantial changes in the EO properties of the *E. coli* XL-1 suspension infected by phage M13K07. This approach has the following

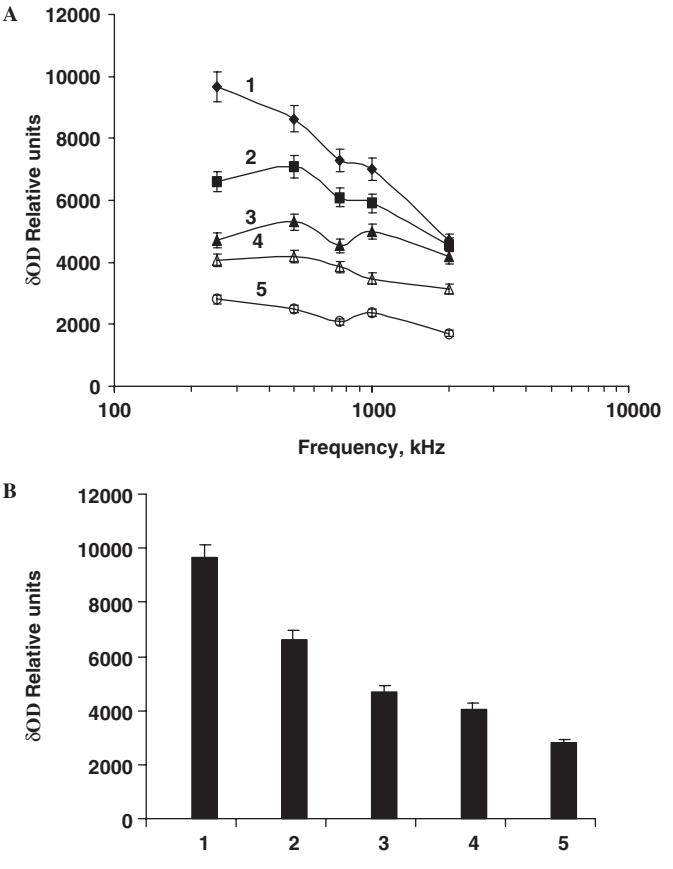

Figure 6. (A) The electro-orientation spectra and (B) relative units at 250 kHz frequency of viable *E. coli* XL-1 cells suspended in distilled water, obtained after incubation with a M13K07 phage. (1) Control (without phages); (2) 1 phage per bacterium; (3) 5 phages per bacterium; (4) 10 phages per bacterium; (5) 20 phage per bacterium.

advantages: (1) bacteria from biological samples need not be purified; (2) the infection of phage to bacteria is specific; (3) exogenous substrates and mediators are not required for detection; and (4) it is suitable for any phage–bacterium system when bacteria-specific phages are available.

Thus, we showed the possibility of developing a novel method for detection of microbial cells by measuring the OS of cell suspensions with the help of interaction with specific biological agents (antibodies and phages) [20].

6. Conclusion

Thus, we showed the possibility of developing a novel method for determining low-molecular-weight substances by measuring the OS of suspensions of the strains that can metabolize the substrates being determined. Also, EO analysis of microbial cells may be used for determination of substrates of an enzyme reaction and, finally, for determination of substrate concentration. The same approach may be used for

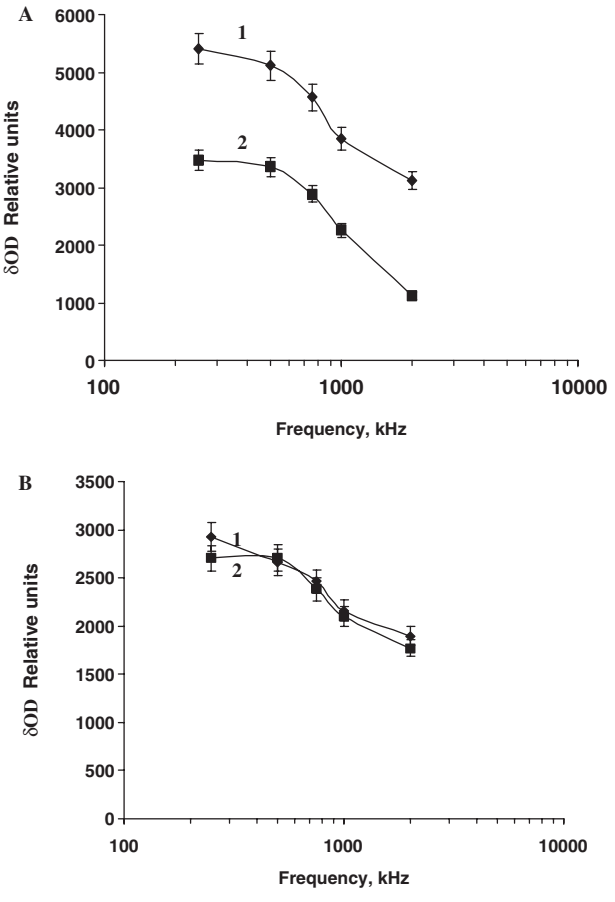

Figure 7. The electro-orientation spectra of mixed cells (*E. coli* XL-1, *A. brasilense* Sp7) (A) and cells *A. brasilense* Sp7 (B) suspended in distilled water, obtained after incubation with 20 M13K07 phage. (1) Control (without phage); (2) 20 phage per bacterium.

investigating cellular metabolism. We show that the results of EO analysis can be used for discrimination of bacteria before and after interaction with selective antibodies. A combination of the EO approach with phage technology is a generic technology that enables rapid and specific detection of viable bacteria and might be a basis for development of portable biosensor systems for detection of pathogens in medical research, food processing and environmental analysis. Thus, electro-optical analysis of cell suspensions opens new possibilities for development of new biosensor methods in biotechnology, environmental control, and medicine.

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