



The effect of organic solvents on selected microorganisms and model liposome membrane

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Abstract

The effect of methanol, ethanol, acetone, *N,N*-dimethylformamide (DMF), dimethyl sulfoxide and Nujol on the growth of *Escherichia coli* DH5 α , *Bacillus subtilis* and *Saccharomyces cerevisiae* D273 was investigated. All of the tested cultures appeared susceptible to the organic media they were treated with, which evinced in apparent hindering of cell development. The observed diverse solvent tolerance, except from their different biochemical activity, may also be related to the changes in cell membrane fluidity induced by the solvent species. Parallel electron paramagnetic resonance investigations using egg yolk lecithin model liposomes revealed that the fluidity of the phospholipid system in cell membranes may either be considerably decreased (Nujol, DMF, ethanol) or increased (acetone), thus rendering difficult the intracellular nutrient supply. Hence, even the chemically neutral Nujol produced a distinct cell-growth inhibitory effect. These results are fairly consistent with the outcome of the survival tests, particularly for the bacteria strains.

Keywords Organic solvents · Bacteria · Yeast · Liposomes · Electron paramagnetic resonance (EPR)

Introduction

Organic solvents, such as dimethyl sulfoxide (DMSO), *N,N*-Dimethylformamide (DMF) or alcohols are frequently used in deactivation of microorganisms [1–7]. They usually serve as a liquid medium when introducing a specified dopant (drug), however they may themselves prove toxic for a number of bacteria or fungi. Once accumulated in a cell membrane,

organic solvent molecules may severely affect its function and finally cause cell death [3]. As a measure of solvent toxicity, the log *P* parameter is used, where *P* is the partition coefficient of a given solvent in an equimolar mixture of octanol and water. Generally, solvents with log *P* between 1 and 5 are considered highly toxic for microorganisms.

There are many bacteria and fungi which are capable of growing even at high concentrations of organic solvents. In fact, the natural intrinsic immunity of a microorganism seems to be crucial to its tolerance for toxic media [1, 8]. Thus, Gram-negative bacteria are slightly more tolerant than Gram-positive bacteria, since the outer membrane of Gram-negative bacteria acts as a barrier, preventing cell penetration by hydrophobic chemicals. Some strains of *Pseudomonas putida* can actively grow and multiply in the presence of very toxic toluene (50% v/v) [1, 9, 10] and a mutant *Escherichia coli* strain does tolerate cyclohexane [11]. Several mechanisms elucidating solvent tolerance of Gram-negative bacteria have been proposed. One involves modification of the cell membrane components, such as *cis*–*trans* isomerisation of membrane fatty acids by *cis*-isomerase and decreased cell surface hydrophobicity, which may reduce the solvent permeability. Other mechanisms postulate diminishing of the cell energy status [6, 7, 12] and/or minimizing accumulation of solvent molecules inside of the

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membrane by discarding them from the lipid bilayer using active efflux pumps [13, 14]. In the case of Gram-positive bacteria, which also display some tolerance, for example some strain of *Rhodococcus* were found to use alkanes and aromatic compounds as sole carbon and energy source. It was previously shown that the incubation of *Rhodococcus* in the presence of *n*-hexadecane led to an increase in the content of total lipids (particularly, of saturated fatty acids) in the cells. It is known that an increase in the amount of saturated and trans-unsaturated fatty acids is associated with a decrease in cell membrane fluidity and, hence, with an increase in the bacterial resistance to organic solvents [5, 7, 15–17].

Saccharomyces cerevisiae KK-211 was the first yeast diploid strain which tolerated isooctane [18]. At the time, only few organic solvent-tolerant eukaryotes had been isolated [19]. The *Saccharomyces cerevisiae* KK-211 strain currently plays an important role in the investigations of molecular mechanisms related to the resistance of microorganisms to organic solvents [20].

DMF, DMSO and methanol are solvents commonly used in in vitro experiments, in particular when trying to introduce low-soluble compounds into a cellular system. This is the case *i.a.* of metal phthalocyanines or porphyrins, many of them showing biochemical activity, anticipated to be applied in photosensitized deactivation of diverse microorganisms and anti-tumor treatment [21–24]. The protective function of a cell membrane may be reduced due to a solvent effect, and consequently the cell growth suppressed. Therefore, we felt essential to explore also natural liposomes in contact with selected organic solvents (all with $\log P < 1$), and a hydrophobic but lipophilic mineral oil Nujol. For this reason we used a spin label EPR method, successfully applied in our previous investigations of diversely doped phospholipid bilayers [25–27]. In that we were able to relate the effects found in liposome membranes with the solvent tolerance demonstrated particularly by *Escherichia coli* DH5 α and *Bacillus subtilis*.

Materials and methods

Microorganisms and media

(1) *Saccharomyces cerevisiae* wild strain D273-10B/A₁ *met* MAT α , was grown in YPD medium (1% yeast extract, 1% bactopectone, 2% glucose) at 30 °C. (2) *Escherichia coli* Gram(–) bacteria, strain DH5 α *supE44*, Δ *lacU169* *hsdR17* *recA1* *endA1* *thi1* *relA1*, was grown in LB medium (1% yeast extract, 1% bactopectone, 0.1% glucose, 0.5% NaCl) at 37 °C (3) *Bacillus subtilis* Gram(+) bacteria, was grown in broth (0.2 g yeast extract, 0.2 g beef extract, 0.4 g NaCl)

at 37 °C. For plating the media were supplemented with 2% agar.

Solvents

DMF, DMSO, methanol, ethanol, acetone and Nujol, all analytical grade, were purchased from Sigma Aldrich Poland, and used as supplied.

DMF has proved inhibitory activity to many strains of bacteria, nevertheless it can be tolerated by strains of *Staphylococcus*, which are able to grow and multiply in the presence of 10% (v/v) DMF [28] and in some cases even up to 50% (v/v) [29]. Interestingly, some bacteria, e.g. *Bacillus subtilis*, can simply use DMF as a source of carbon and nitrogen [15]. On the other hand DMF was applied as solvent in algal growth inhibition tests [30].

DMSO is often used to dissolve hydrophobic compounds used in biological research [22, 23, 31]. However, it may strongly affect the structure and properties of cell membranes even at low concentrations, as a result of dehydration of the membrane surface, as reported elsewhere [32].

Methanol and ethanol are known to be toxic to bacterial cells but only at high concentrations (several % v/v) [9, 33]. The membranes of cells cultured in the presence of alcohols are more rigid than those of normally grown ones, due to a decrease in the lipid to protein ratio [34]. However, in bacteria the fluidity of their membranes may also increase in the presence of alcohols [35].

Acetone is considered relatively less toxic, and e.g. in *E. coli* [9] or *Bacillus aquimaris* the cells produce more lipids containing unsaturated fatty acids in the cellular membrane [36], thus providing a kind of protection against this solvent.

Nujol is a mineral oil, non-polar and chemically inert. Since it does not mix with water, the studied microorganisms grew in a two-phase Nujol-water system. Its choice was reasoned by the search for non-toxic lipophilic media appropriate for so-called intelligent drug delivery systems. Thus far, the impact of Nujol on phospholipid membranes as well as on the growth of bacteria and yeast has not been reported elsewhere.

Culturing of microorganisms in the presence of organic solvents

Tests were performed in liquid medium at different concentration of the organic solvents (in the range 0–20% v/v). The particular cultures were inoculated with 8×10^2 c. f. u. (colony forming unit) and incubated 24 h at 30 °C (yeast) and 37 °C (bacteria). Next, samples of 10 μ l per culture were taken and inoculated in solid media and incubated for 24 h at appropriate temperature.

Cell survival test

Survival tests of the cells treated with organic solvents were carried out in liquid medium after 24 h of incubation. Samples (0.1 ml) were taken periodically and plated on a solid medium, and the number of living cells in the culture was determined by the viable count method [37, 38]. In this work it was assessed for the solvent concentration of 4.8% v/v, at which practically all of the tested microorganisms were still able to grow. The counts of colony forming units (CFU ml⁻¹) in all survival tests were compared to control samples.

EPR method and liposome testing

Fresh hen eggs were purchased from a local commercial egg farm (Ferma Jaj Spożywczych Andrzej Tatar, Wróbleńska 31, 46-022 Kępa, Poland). The lecithin (EYL) was prepared from egg yolks, at the Faculty of Chemistry, University of Opole, according to a standard procedure [39].

Liposomes were produced in a sonication process (ultrasonic disintegrator TECHPAN UD-20) in a quartz vessel cooled with an ice-water mixture, and each sample contained 60 μM of EYL and 2 ml of water. The procedure included alternating cycles of 30 s of sonication followed by 60 s of cooling, and the total preparation time was 150 s.

The appropriate spin probe was introduced into the water dispersion of liposomes in a quantity of 1% against EYL (i.e. its molar ratio to lipid particles was 0.001). After 15 min of incubating, the sample was divided into batches (0.25 ml each) which were doped with the tested solvents, gradually increasing their concentration from 0% up to 4% v/v (ref. to water).

Two different spin probes, TEMPO and 16-DOXYL, were used to investigate the solvent impact upon the fluidity of the membrane's phospholipid system, Fig. 1. Since the performance of a spin probe in a liquid medium is strongly related to its viscosity, this is reflected in its EPR spectrum. The

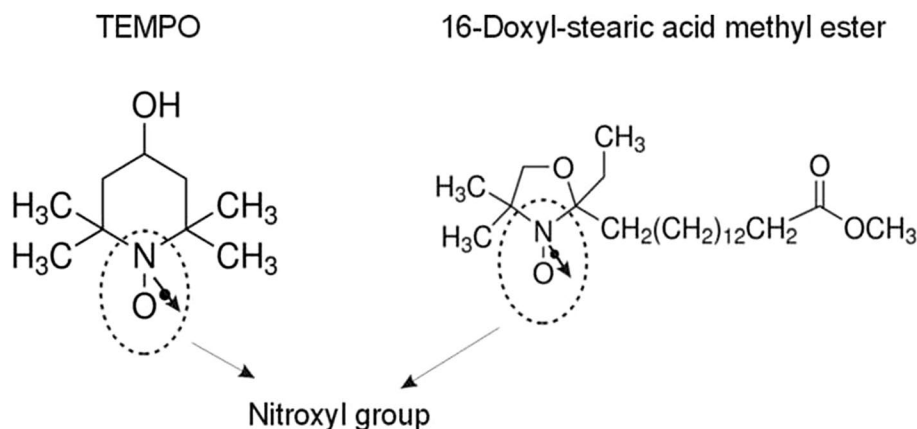
TEMPO spin label is amphiphilic and hence used to explore the membrane's interface zone, whereas the 16-DOXYL one is hydrophobic and thus deeply penetrates the bilayer's center. From the EPR spectrum of the TEMPO spin label the partition parameter *F* was derived (Fig. 2a), which allowed estimating the distribution of the probe between the water phase and the lipid part, expressed by the particular components *P* and *H*, respectively. As reported elsewhere [40], *F* depends on the fluidity of the phospholipid setup at the peripheral part of the membrane, and increase in fluidity corresponds to its greater value. In the case of the 16-DOXYL spin label, its rotation velocity within the system also depends on the membrane's fluidity, and based on the EPR spectrum, the τ parameter was derived and defined as the rotational correlation time (Fig. 2b, see caption) [40, 41]. In a rigid ambient rotation of the spin probe is hindered, hence increase in τ implies decrease in fluidity.

The measurements were repeated 10 times each and the reported values of the spectroscopic parameters *F* (Fig. 3) and τ (Fig. 4) refer to their arithmetic mean. Estimated measurement errors were 5% and 6% for the TEMPO and 16-DOXYL probe, respectively. In the discussion of results, normalized (relative) values F/F_0 and τ/τ_0 have been used (F_0 and τ_0 apply to the reference liposome sample containing no extra solvent).

Results and discussion

Effect of the applied solvents upon the development of the particular microorganisms has been visualized in the micrographs reported in Table 1. Even though these results represent only some general view of the cultures' condition, one may assume, that the growth of the individual species was apparently inhibited when exceeding the solvent concentration of 4.8%. For this reason, the survival tests were performed just for this amount of the solvent (Table 2).

Fig. 1 Chemical structure of the spin probes, TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) and 16-DOXYL (2-ethyl-2-(15-methoxy-15-oxopentadecyl)-4,4-dimethyl-3-oxazolidinyloxy) stearic acid methyl ester); position of the spin active nitroxyl groups was indicated



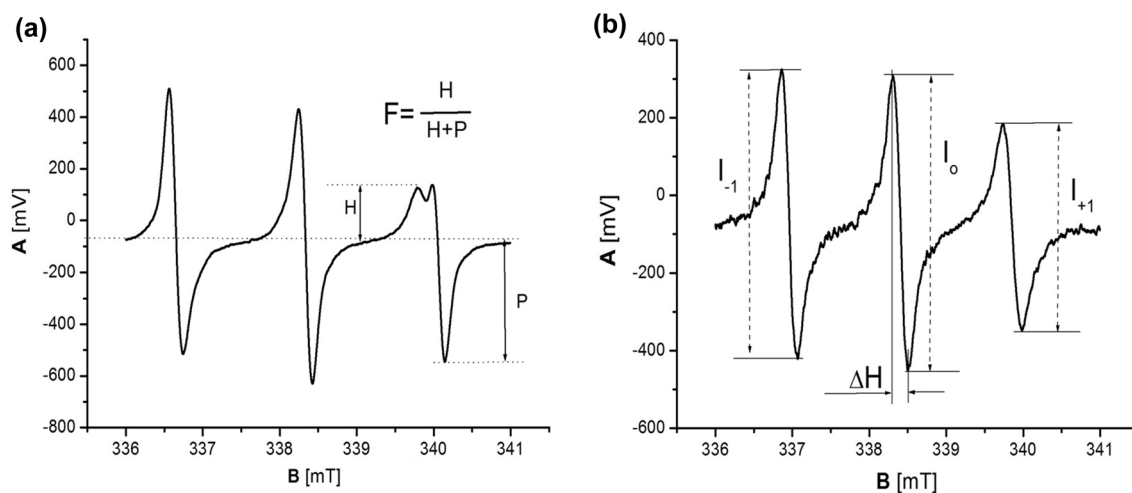


Fig. 2 EPR spectra of spin probes placed in a liposome membrane **a** TEMPO spin label and the partition parameter F ; **b** 16-DOXYL spin label; the τ parameter (rotational correlation time) was calculated as follows: $\tau = 5.95 \Delta H ((I_0/I_{+1})^{1/2} + (I_0/I_{-1})^{1/2} - 2) 10^{-10}$ [s]

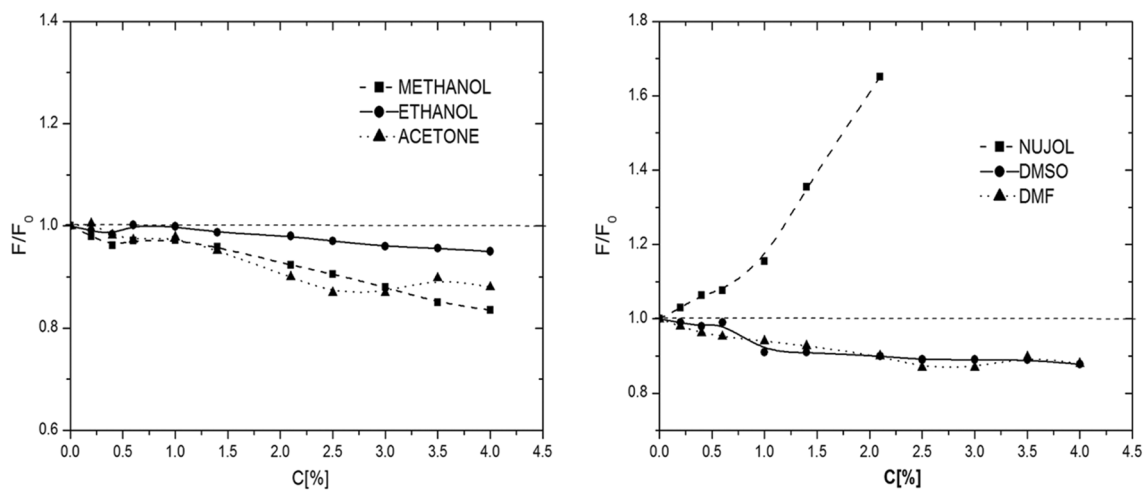


Fig. 3 Effect of the solvent upon the water–lipid interface as featured by EPR measurements involving the TEMPO spin label. F/F_0 represents the relative partition parameter; values > 1 indicate for increase and < 1 for decrease in fluidity

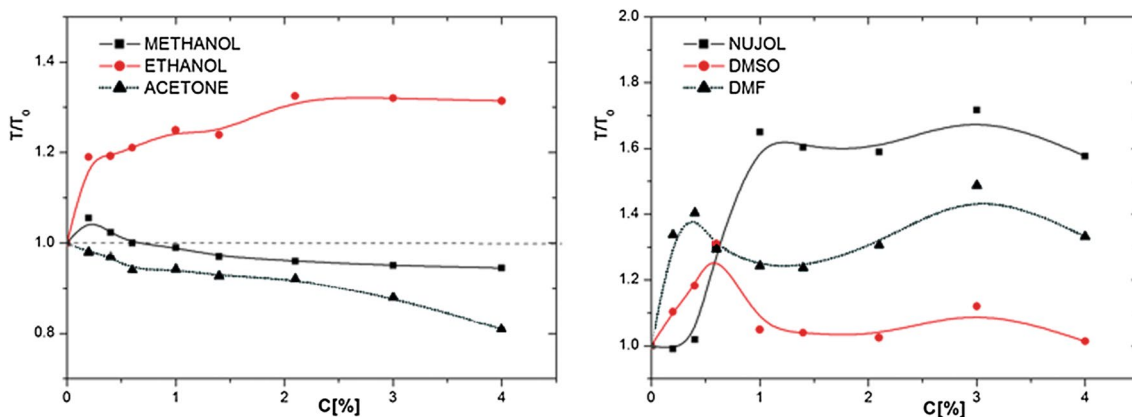


Fig. 4 Effect of the solvent upon the central part of the liposome membrane as featured by EPR measurements involving the 16-DOXYL spin label. τ/τ_0 represents the relative rotational correlation time parameter; values < 1 indicate for increase and > 1 for decrease in fluidity

Table 1 Comparison of the solvent effect in the investigated cultures; c—solvent concentration (% v/v), Ref.—reference sample (no solvent added)

Solvent	Microorganism											
	<i>Saccharomyces cerevisiae</i>				<i>Bacillus subtilis</i>				<i>Escherichia coli</i>			
c, % v/v	Ref.	4,8	9,1	20,0	Ref.	4,8	9,1	20,0	Ref.	4,8	9,1	20,0
DMF												
DMSO												
Methanol												
Ethanol												
Acetone												
Nujol												

Table 2 The viable count in cultures, (%), treated with 4.8% v/v of organic solvents

Microorganism			
Solvent	<i>S. cerevisiae</i>	<i>B. subtilis</i>	<i>E. coli</i>
DMF	0.1	8.9	6.5
DMSO	0.1	4.7	26.3
Methanol	0.7	63.2	52.3
Ethanol	1.2	1.3	28.9
Acetone	2.1	5.3	5.4
Nujol	73.0	62.6	93.8

All of the explored microorganisms demonstrated an apparent susceptibility to the applied organic media. Generally, the mineral oil Nujol revealed the weakest lethal effect, as follows from Table 1, nevertheless in the case of *Bacillus subtilis* the viable count appeared comparable to that demonstrated by methanol, Table 2. Although this finding was fairly surprising since this liquid is considered chemically inert, however the results obtained for the other microorganisms clearly indicate that Nujol in some cases may inhibit their proper development.

DMF and acetone were found the most toxic ones, as follows from Table 2, and the result for DMF is consistent with the data reported elsewhere [28, 29]. On the other hand, behavior of the cultures doped with acetone, which toxicity is generally considered lower compared to other organic media, proved somewhat far from the effect expected based on the published data [36]. The viable count determined for the strains of *B. subtilis* and *E. coli* is much below the records of the potentially more toxic methanol and even that found for DMF. However, the effect of the remaining solvents on

the tested cultures was found rather typical for the applied microorganisms. As anticipated, the both bacteria strains appeared definitely better solvent-tolerant than the yeast *Saccharomyces cerevisiae*.

The susceptibility and/or tolerance to organic solvents may be explained in terms of natural immunity mechanisms triggered once the microorganisms have been exposed to a stress caused by these solvents. Particularly, when the microbes have been fit with enzymes which do not lose their activity in the presence of organic media. For instance, *B. cereus* produces a solvent-resistant protease, and the enzyme was reported to have retained at least 95% of its initial activity when the bacteria was treated with methanol, DMSO, acetonitrile and DMF [42]. It is possible, that also *B. subtilis* and *E. coli* have developed a similar adaptive mechanism, and hence proved generally less susceptible to the applied solvents than demonstrated by *S. cerevisiae* (Table 2). On the other hand, biochemical activity of organic solvents is critical regarding their transfer and partition in the microbes, and consequently their chemical interaction with enzymes. This may lead to structural deformations and blocking of the enzyme's active sites, which eventually would suppress the cell's immunity under stressful conditions [43–47].

EPR studies

The effect of the studied solvents upon the membrane's condition at the water–lipid interface was estimated from the behavior of the TEMPO spin label. As follows from Fig. 3, only Nujol exhibited a notable fluidizing effect. However, such pronounced changes in fluidity may be confusing, since most probably they resulted due to the formation of a 2- or 3-phase system at the lipid bilayer surface

involving Nujol, water and the lipid heads. Hence, it seems the TEMPO results refer only to the Nujol phase, rather than to the water–lipid interface, because the spin probe readily dissolves in this particular liquid. On the other hand, the remaining solvents easily mix with water and distribute well within the interface layer, so the EPR results presented in Fig. 3 seem plausible. Generally, they demonstrate stiffening of the water–lipid interface (at the bilayer surface), slightly increasing with the solvent concentration. The least effect was observed for ethanol.

Fluidity of the phospholipid system within the non-polar central part of the membrane was particularly affected by Nujol yielding a 60% increase of the τ parameter (Fig. 4), which implies considerable stiffening of the bilayer's interior. Somewhat less increase was demonstrated by DMF (40%) and ethanol (30%), whereas DMSO showed a 25% rise in τ at concentration about 0.5%, however no effect at higher contents of this solvent was observed. Slight increase in membrane fluidity was produced by methanol and some more pronounced fluidization was caused by acetone.

The outer cell membrane is the main barrier the solvent molecules have to pass through. In *E. coli*, the membrane includes phosphatidylethanolamine, phosphatidylglycerol and cardiolipin, whereas in *B. subtilis* it contains also lysyl-phosphatidylglycerol and glycolipids [48, 49]. On the other hand, in *S. cerevisiae* the plasma membrane is rich in phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine [50].

The response of the microbe's cellular system facing a solvent-stress basically results from changes both in membrane lipid composition as well as in protein, sterol, hopanoid, and carotenoid content, which modify the plasma membrane properties (fluidity, membrane permeability, rigidity) [9]. Generally, the Gram-negative bacteria *E. coli* compared to the Gram-positive *B. subtilis* is considered less susceptible to organic solvents because its outer membrane is likely a more effective permeability barrier [51]. Presumably, in *E. coli* under solvent-stress conditions, the process of the membrane's structure modification runs faster than in the case of *B. subtilis*, thus making the lipid bilayer more tight and compact [12]. Nevertheless, the solvent-tolerance mechanism in the both bacteria strains proved comparable and the most important modifications which were identified included cis–trans isomerization of fatty acids, changes in the ratio of saturated and unsaturated fatty acids, and changes in the phospholipids headgroups [4, 6].

Despite differences in the membrane structure, the fatty acid compositions of *S. cerevisiae* and *E. coli* are quite similar, and also the changes induced in these microorganisms by ethanol were found alike [12]. In both cases, the proportion of the unsaturated oleic acid proved increased at the expense of the saturated palmitic acid as a function of increasing alcohol concentration. In *B. subtilis*, the fatty acid moiety of

the phospholipids was affected differently when treated with methanol and ethanol, however in both cases the synthesis of phosphatidylglycerol was strongly inhibited. According to literature sources, these alcohols may reduce the total cell phospholipid contents even by 50%. Nevertheless, the composition of fatty acids (e.g. 12-methyltetradecanoic acid) was modified only by ethanol, and in the presence of methanol the changes were negligible [52]. These findings and conclusions are consistent with the results reported in Table 2.

More dramatic changes resulting in breaking of the membrane structure were found when the microbes were left to grow in the presence of acetone. This may be attributed to the enhanced accumulation of acetone within the lipid bilayer of the cell membranes [53]. Similar conclusions follow from the results presented in Fig. 4, which confirm the fluidizing effect of acetone on the phospholipid system. This fact has also been supported by the low survival outcomes in acetone-treated samples (Table 2).

As follows from the literature, the effect of DMSO may be connected with the DMSO-responsive genes, which are believed to control a variety of cellular functions, e.g. carbohydrate, amino acid and lipid metabolism, cellular stress responses, and energy transfer [54].

Concluding remarks

Experiments carried out in this work essentially reflect the complexity of the solvent-tolerance problem. Two effects are important here, a chemical one, which follows from the biochemical activity of solvent species, and a physical one resulting from the non-chemical intermolecular interactions involving the cell membrane system and solvent molecules. Obviously, the feedback of the particular microorganisms when treated with organic solvents strongly depends on their individual cellular features. Hence, the same solvent may completely inhibit the growth of one organism while being more or less tolerated by another one. It could be speculated on the diversified behavior of microorganisms by considering the possibility of the solvents being used (metabolized) by the growing cultures as a carbon and/or nitrogen source (nutrient) [15]. The results presented in Table 2 for the both bacteria strains and methanol (and even DMF), and also *E. coli* when exposed to DMSO and ethanol, seem to confirm this suggestion. Besides, the solvent-generated modifications of intracellular structures postulated elsewhere [3] may also explain the differences in immunity demonstrated by the studied strains (Table 2). On the other hand, EPR data generally indicate for stiffening of the phospholipid system in most of the studied cases. This might have hindered to some extent the intracellular transport of nutrients crucial for the cells to grow. Whether this was a compelling reason for the growth-inhibition effect demonstrated by DMF (Fig. 4,

Table 2) is not quite clear, nevertheless it should be considered possible in this case. Incidentally, the surprisingly low viable count revealed by acetone one may eventually relate to the apparent fluidizing effect produced by this solvent (Fig. 4), which also could have prevented the proper cell development. Even the chemically passive Nujol exhibited a quite pronounced effect upon the cell growth, which may be referred to the considerable decrease in membrane's fluidity. These results evidently support the important correlation between EPR data and those of the survival tests reported in Table 2 and Figs. 3 and 4. Moreover, they show how important is the selection of a proper solvent for a biotechnological assay.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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