

Effect of organic solvents on solvent-tolerant *Aeromonas hydrophila* IBB_{P08} and *Pseudomonas aeruginosa* IBB_{P010}

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Alkanes (*n*-hexane, *n*-heptane) with logarithm of partition coefficient between *n*-octanol and water ($\log P_{OW}$) 3.86 to 4.39 were less toxic to *Aeromonas hydrophila* IBB_{P08} and *Pseudomonas aeruginosa* IBB_{P010} as compared to aromatics (toluene, styrene, xylene isomers, ethylbenzene & propylbenzene) with $\log P_{OW}$ 2.64 to 3.69. The toxicity of 0.5% (v/v) second phase of organic solvents to these bacteria could be predictable on the basis of the solvents' $\log P_{OW}$. The tolerance, viability, adhesion and β -galactosidase activity of *A. hydrophila* IBB_{P08} and *P. aeruginosa* IBB_{P010} cells in the presence of 0.5% (v/v) organic solvents varied significantly. The results indicated that *A. hydrophila* IBB_{P08} was more susceptible to organic solvents than *P. aeruginosa* IBB_{P010}, whereas both the bacterial strains harbour plasmids. *A. hydrophila* IBB_{P08} did not possess hydrophobe/amphiphile efflux 1 (HAE1) transporter genes, while *P. aeruginosa* IBB_{P010} did possess these genes. The adaptation mechanisms (modification of cell hydrophobicity, induction of β -galactosidase activity and changes in the membrane's lipid and protein content) of bacterial cells, underlying solvent tolerance, in *A. hydrophila* IBB_{P08} and *P. aeruginosa* IBB_{P010} showed a complex response to the presence of 0.5% (v/v) organic solvents in the culture medium. Bacterial strains able to survive in the presence of organic solvents could be used in two-phase biotransformation systems with whole cells for adequate bioremediation of heavily contaminated sites and could be a source for new solvent-stable enzymes with different applications.

Keywords: Adaptation mechanisms, bacteria, organic solvents, tolerance

Introduction

Spills of petroleum and petroleum products occur frequently and they are a major cause of environment pollution. These spills contain relatively high concentrations of various types of organic solvents, such as, alkanes, alkenes, cycloalkanes, cycloalkenes and aromatics, which are highly toxic to the majority of living organisms and can be tolerated only by relatively few species; thus, causing serious environmental problems. For a long time, toxic effects of otherwise suitable organic solvents constituted a major drawback for their application in biotechnology and for the production of fine chemicals by whole-cell biotransformations¹.

It is generally thought that organic solvents exhibit extreme toxicity toward living microorganisms because of their accumulation in hydrophobic biological membranes¹⁻⁴. By virtue of this toxicity, organic solvents were used in the past as permeabilization agents, disinfectants, food

preservatives and industrial solvents⁵. This toxicity correlates with the hydrophobic character of the organic solvent, expressed by the logarithm of its partition coefficient between *n*-octanol and water ($\log P_{OW}$). Organic solvents with a $\log P_{OW}$ value 1 to 5 are highly toxic to whole cells^{2,3,6}. For this toxic effect, the choice of organic solvents for whole-cell biotransformations in two-phase solvent-water systems is limited. Only less-toxic solvents with higher hydrophobicities can be used for the purpose^{1,6-9}. Despite the extreme toxicity, organic solvent-tolerant bacteria that are capable of growth in two-phase solvent-water systems have been isolated. Many of these tolerant bacterial species, including the first strain isolated, were Gram-negative, such as, *Pseudomonas aeruginosa*, *P. fluorescens*, *P. putida* and closely related *Pseudomonas* sp.¹. Recently, Gram-positive bacteria that are capable to grow in the presence of organic solvents have also been isolated^{5,9}. Such solvent-tolerant microorganisms provided key for the use of otherwise toxic solvents in whole-cell two-phase biotransformations by overcoming the toxic effects of substrates and

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products. Many efforts have been made to uncover the mechanisms behind the organic solvent tolerance of these bacterial strains. Up to now different adaptation mechanisms have been found, such as, rigidification of the cell membrane, change in the membrane's protein content or composition, active excretion of the solvent, adaptation of the energetic status, changes in cell wall and outer membrane composition, modification of the cell surface properties, morphological changes and metabolization or transformation of the solvent¹.

Isolation and characterization of two new Gram-negative bacteria, *A. hydrophila* IBB_{Po8} and *P. aeruginosa* IBB_{Po10}, those are tolerant to alkanes (*n*-hexane, *n*-heptane) and aromatics (toluene, styrene, xylene isomers, ethylbenzene, propylbenzene) were made in the present study. The toxic effects of 0.5% (v/v) alkanes (*n*-hexane, *n*-heptane) and aromatics (toluene, styrene, xylene isomers, ethylbenzene, propylbenzene) on *A. hydrophila* IBB_{Po8} and *P. aeruginosa* IBB_{Po10} and the adaptation mechanisms behind their resistance were also studied. Bacterial strains capable to survive in the presence of organic solvents have great application in bioremediation of heavily contaminated sites, and they may become a source for new solvent-stable enzymes with different applications.

Materials and Methods

Isolation and Characterization of Solvent-tolerant Bacterial Strains.

The number of viable bacterial strains in Poeni oily sludge (Teleorman County, Romania) was estimated by a modified most probable number (MPN) procedure¹⁰. Growth in 96-microwell plates was identified using INT [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride], which forms an insoluble red precipitate in wells containing bacteria that can use Poeni crude oil or organic solvents, as sole carbon source. Isolation of IBB_{Po8} and IBB_{Po10} bacterial strains from Poeni oily sludge was carried out on minimal medium¹¹, using the enriched cultures method, with 1% (v/v) organic solvents as sole carbon source. For further characterization of IBB_{Po8} and IBB_{Po10} bacterial strains, various necessary physiological and biochemical tests were performed. The taxonomic affiliation of IBB_{Po8} and IBB_{Po10} bacterial strains was determined based on their phenotypic characteristics and also based on the G+C content of the bacterial chromosome¹².

Cellular and Molecular Modifications Induced by Organic Solvents on *A. hydrophila* IBB_{Po8} and *P. aeruginosa* IBB_{Po10}.

Bacterial cells (10⁶ CFU mL⁻¹) were cultivated on minimal¹¹ medium added with 0.25% (w/v) protein hydrolysate amicase and protease peptone (control), and on mineral medium in the presence of 0.5% (v/v) organic solvents (alkanes: *n*-hexane & *n*-heptane; aromatics: toluene, styrene, xylene isomers, ethylbenzene & propylbenzene). Flasks were sealed and incubated for 24 h at 28°C on a rotary shaker (150-200 rpm). Petri plates were also sealed and incubated for 24 h at 28°C. Here, as elsewhere, in this study, experiments were repeated at least three times.

Bacterial Cell Growth in Presence of Organic Solvents

Growth of the bacterial cells in liquid medium in the presence of different 0.5% (v/v) organic solvents was assessed by measuring turbidity (OD_{660nm}) after 24 h. Growth on solid medium overlaid with 5 mm layer of organic solvents was assessed as described by Nielsen *et al*¹³.

Bacterial Cell Viability in Presence of Organic Solvents

Serial dilutions of culture liquid were spread on agar LB-Mg⁷ medium using the method of Ramos *et al*¹⁴ and the number of viable cells (CFU mL⁻¹) was determined.

Bacterial Cell Adhesion to Organic Solvents

Bacterial adhesion to organic solvents was determined by using the method of Rosenberg *et al*¹⁵. The bacterial adhesion to organic solvents was also studied on wet mount under the optical microscope.

β-Galactosidase Activity in Presence of Organic Solvents

β-Galactosidase activity was determined in triplicate by measuring the hydrolysis of the chromogenic substrate, *o*-nitrophenyl-β-D-galactoside, as described by Miller¹⁶.

Phospholipids Modification in Presence of Organic Solvents

Lipids were extracted with chloroform-methanol (2:1) mixture using the method of Benning and Somerville¹⁷. The samples were spotted onto 20×20 cm² Silica gel 60 TLC aluminium sheets (Merck), and the separation was performed using chloroform-methanol-acetic acid-water (85:22.5:10:4 v/v/v/v) mixture as mobile phase, as described by Stancu & Grifoll¹⁸. The identification of the phospholipids was performed based on their mobilities (R_f) as compared with those of standard phospholipids.

Protein Profile Modification in Presence of Organic Solvents

Membrane and periplasmic protein fractions were extracted with HE buffer (10 mM HEPES-NaOH, pH 7.6, 10 mM EDTA, 10 mM MgCl_2), dissolved in Laemmli buffer and denatured at 95°C for 5 min. 30 μg of protein per lane were loaded onto a 10% (w/v) polyacrylamide gel¹⁹. Gels were stained with Coomassie brilliant blue and destained in ethanol-glacial acetic acid-water (4.5:1:4.5 v/v/v) mixture. Protein content was measured by the method of Bradford²⁰.

Rhodamine 6G Accumulation in Presence of Organic Solvents

Bacterial cells were spotted on agar LB-Mg⁷ medium added with 1-1000 $\mu\text{g mL}^{-1}$ rhodamine 6G. MIC_{90} of rhodamine 6G was determined as the concentrations that inhibited the growth of 90% of the bacterial cell. Accumulation of rhodamine 6G in bacterial cells was observed under UV light after 24 h incubation at 28°C. The medium without rhodamine 6G served as control.

Specific Amplification of HAE1 (Hydrophobe/Amphiphile Efflux 1) Gene Fragment by PCR

Template DNA for PCR was obtained using the method of Whyte *et al*²¹. For PCR amplification, 5 μL of DNA extract was added to a final volume of 50 μL reaction mixture, containing: 5 $\times$ GoTaq flexi buffer, MgCl_2 , dNTP mix, primers (A24f2, 5'-CCSRTITTYGCITGGGT-3'; A577r2, 5'-SAICC ARAIRCGCATSGC-3'²²) and GoTaq DNA polymerase (Promega). PCR was performed with a C1000 thermal cycler (Bio-Rad). PCR program consisted of initial denaturation for 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50° and 52°C for 1 min, and extension step at 72°C for 1 min, and a final extension at 72°C for 5 min. After separation on 1.6% (w/v) TBE agarose gel and staining with fast blast DNA stain (Bio-Rad), the amplified fragments were analyzed.

Lipase and Protease Production in Presence of Organic Solvents

Bacterial cells were spotted on tributyrin agar medium²³ and wheat meal agar medium²⁴ for lipolytic and proteolytic enzyme production, respectively, as described by Ogino *et al*^{23,24}.

Reagents

n-Hexane (96% pure), toluene (99% pure), styrene (99% pure), *o*-xylene (99% pure), *p*-xylene (99% pure) and propylbenzene (98% pure) were obtained from Merck; *n*-heptane (99% pure), *m*-xylene (98%

pure), ethylbenzene (98% pure), were obtained from Sigma-Aldrich. Other reagents used were procured from Merck, Sigma-Aldrich, Difco Laboratories, Promega, bioMérieux or Bio-Rad Laboratories.

Results and Discussion

Isolation and Characterization of Solvent-tolerant Bacterial Strains.

Bacteria with specific metabolic capabilities, such as, oil degradation, can be enumerated based on their ability to grow on selective media. The MPN procedure is particularly well suited for bacteria that grow on insoluble substrates, due to the difficulties in preparation of solid media containing a homogenous distribution of the appropriate carbon source. Also, many agar based solid media contain impurities that allow growth of organisms that cannot degrade the target substrate, leading to overestimation of the size of the population of interest. Haines *et al*¹⁰ developed a 96-well microtiter plate MPN procedure to enumerate hydrocarbon-degrading bacteria. Selective minimal medium¹¹ with Poeni crude oil or organic solvents, as sole carbon source, was used to enumerate the total hydrocarbon-degrading bacteria and solvent-degrading bacteria in Poeni oily sludge (Fig. 1). The number of viable total hydrocarbon-degrading bacteria per g of Poeni oily sludge was between 10^{8-11} g^{-1} , while the number of viable solvent-degrading bacteria was between 10^{2-5} g^{-1} .

Isolation of IBB_{Po8} and IBB_{Po10} bacterial strains from Poeni oily sludge was carried out on minimal medium, using the enriched culture method with 1% (v/v) organic solvents. The use of minimal medium with 1%

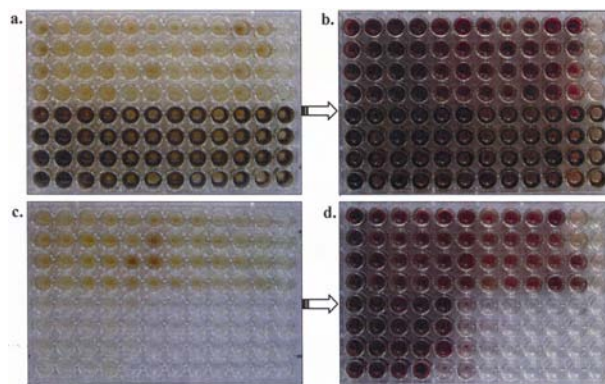


Fig. 1—MPN determination of total hydrocarbon-degrading and solvent-degrading bacteria in 96-microwell plates. After 2 wk incubation at 28°C (panel a & c), each well was added with 0.3% (w/v) INT (panel b & d) which forms an insoluble red precipitate in wells containing bacteria that can use crude oil (panel b) or organic solvents (panel d) as a sole carbon source.

(v/v) organic solvents as sole carbon source allowed the selective development of IBB_{Po8} and IBB_{Po10} solvent-tolerant bacterial strains. The taxonomic affiliation of bacterial strains was determined based on their phenotypic characteristics and also on the G+C content of the bacterial chromosome. The identification result for IBB_{Po8} and IBB_{Po10} strain with API profile 3457754 and 1354575, and with G+C content of the DNA 61.5 and 66.6 mol%, respectively corresponded to *A. hydrophila* and *P. aeruginosa*. Both isolated solvent-tolerant bacteria belong to Gamma-Proteobacteria. Previous studies have reported that the dominance of Gamma-Proteobacteria is a characteristic of bacterial communities inhabiting environments highly contaminated with petroleum and petroleum products²⁵.

Cellular and Molecular Modifications Induced by Organic Solvents on *A. hydrophila* IBB_{Po8} and *P. aeruginosa* IBB_{Po10}

Bacterial Cell Growth

The bacterial cell growth depends not only on the inherent toxicity of the solvent, but also on the intrinsic tolerance of the bacterial strain, and this tolerance varies significantly from one species to another. Alkanes (*n*-hexane, *n*-heptane) with log P_{OW} 3.86 to 4.39 were less toxic for bacterial strains as compared with aromatics (toluene, styrene, xylene isomers, ethylbenzene, propylbenzene) with log P_{OW} 2.64 to 3.69 (Table 1). The growth of the bacterial

strains on liquid medium in the presence of 0.5% (v/v) organic solvents was 25-75% for *A. hydrophila* IBB_{Po8}, and 50-100% for *P. aeruginosa* IBB_{Po10}. Thus, the toxicity of hydrocarbons in supersaturating concentrations, added as a second phase, is generally in inverse correlation with log P_{OW} , which is in accordance with previous studies^{2,3,6}.

The growth of the bacterial strains on solid medium overlaid with organic solvents was 0-50% for *A. hydrophila* IBB_{Po8} and 10-100% for *P. aeruginosa* IBB_{Po10} (Table 1). In plate overlay assays (30 μ L, 10⁶ CFU mL⁻¹), *A. hydrophila* IBB_{Po8} and *P. aeruginosa* IBB_{Po10} tolerated all organic solvents for 6 h, except toluene and styrene in the case of *A. hydrophila* IBB_{Po8}. After removal of the organic solvents and subsequent incubation at 28°C, confluent cell growth was seen after 24 h. Although *A. hydrophila* IBB_{Po8} was able to use 0.5% (v/v) toluene and styrene in liquid medium, it was not able to grow on solid medium overlaid with these organic solvents. This can be due to the fragmentation of liquid medium surface film in small droplets, being more available to bacterial degradation due to the stirring (150-200 rpm).

Bacterial Cell Viability

The viability of *A. hydrophila* IBB_{Po8} and *P. aeruginosa* IBB_{Po10} cells to organic solvents differed with the strains and also with the hydrophobic substrate (Table 1). The bacterial cells presented a higher viability (CFU mL⁻¹ = 10⁷⁻⁹) when growth was

Table 1—*A. hydrophila* IBB_{Po8} and *P. aeruginosa* IBB_{Po10} cells tolerance, viability and adhesion in the presence of organic solvents

Variant	log P_{OW} ^a	Growth in the presence of organic solvents (%) ^b				Cells viability in the presence of organic solvents (CFU mL ⁻¹) ^c		Adhesion to organic solvents (% BATS) ^d	
		<i>A. hydrophila</i> IBB _{Po8}		<i>P. aeruginosa</i> IBB _{Po10}		<i>A. hydrophila</i> IBB _{Po8}	<i>P. aeruginosa</i> IBB _{Po10}	<i>A. hydrophila</i> IBB _{Po8}	<i>P. aeruginosa</i> IBB _{Po10}
		Liquid medium	Solid medium	Liquid medium	Solid medium				
Control	-	100	100	100	100	3.3×10 ⁹	3.4×10 ⁹	-	-
<i>n</i> -Hexane	3.86	75	10	100	50	3.1×10 ⁷	2.2×10 ⁹	39.3	49.0
<i>n</i> -Heptane	4.39	75	50	100	50	2.4×10 ⁷	2.3×10 ⁹	36.8	53.3
Toluene	2.64	25	0	50	10	2.3×10 ²	2.0×10 ⁵	7.8	15.6
Styrene	2.86	25	0	50	10	1.9×10 ²	1.5×10 ⁴	1.3	6.1
<i>o</i> -Xylene	3.09	50	10	75	100	2.2×10 ³	1.4×10 ⁶	16.2	22.5
<i>m</i> -Xylene	3.14	75	10	100	100	4.0×10 ⁶	1.6×10 ⁶	17.1	29.6
<i>p</i> -Xylene	3.14	50	10	75	75	1.8×10 ²	2.3×10 ⁴	16.3	25.6
Ethylbenzene	3.17	50	25	75	100	5.2×10 ³	6.0×10 ⁴	6.3	17.3
Propylbenzene	3.69	75	25	100	100	3.6×10 ⁴	3.0×10 ⁵	13.8	26.4

^aLogarithm of partition coefficient between *n*-octanol and water; ^bGrowth on liquid and solid media in the presence of organic solvents was estimated by measuring turbidity and by determining the formation of resistant bacterial colonies, respectively and the tolerance is represented by the frequency of growth, with that observed in the absence of any organic solvent taken as 100%; ^cSerial dilutions of culture liquid were spread on agar LB-Mg medium and the number of viable cells (CFU mL⁻¹) was determined; ^dDecrease of the turbidity in aqueous phase in the presence of organic solvents (% BATS).

made in the presence of alkanes (*n*-hexane, *n*-heptane) with log P_{OW} 3.86 to 4.39 as compared to the bacterial cells (CFU mL⁻¹ = 10²⁻⁶) grown in the presence of aromatics (toluene, styrene, xylene isomers, ethylbenzene, propylbenzene) with log P_{OW} 2.64 to 3.69. This is in agreement with previous studies where alkanes inhibited cellular activity only partially compared to aromatics². Alkanes bind less abundantly to viable bacterial cells and are less toxic to them, while aromatics bind more abundantly to viable bacterial cells and are more toxic to them. *A. hydrophila* IBB_{Po8} cells showed lower viability (CFU mL⁻¹ = 10²⁻⁷) as compared to *P. aeruginosa* IBB_{Po10} cells (CFU mL⁻¹ = 10⁴⁻⁹) when growth was made in the presence of organic solvents. Alkanes (*n*-hexane, *n*-heptane) had no effect on the viability of *P. aeruginosa* IBB_{Po10} cells in comparison to the control (CFU mL⁻¹ = 10⁹).

Bacterial Cell Adhesion

The affinity of microbial cells for hydrophobic interfaces is an important property that directly affects the efficiency of various bioprocesses, such as, bioremediation, using whole cell²⁶. This affinity for hydrophobic surfaces, i.e., cell surface hydrophobicity, has been measured using microbial-adhesion-to-hydrocarbon (MATH) assay¹⁵. During the MATH test, hydrophobic cells immediately adhere to droplets formed by fission of the organic phase during vigorous mixing. This prevents the droplets from coalescing and stabilizes the emulsion. Since bacterial cells only hinder coalescence and do not cause droplet fission, an excessive volume of organic phase causes inefficient breakdown and reduces the efficiency of emulsion formation²⁶.

In this assay the *A. hydrophila* IBB_{Po8} and *P. aeruginosa* IBB_{Po10} cells adhered to 0.5% (v/v) organic solvents microdroplets, formed as a result of mechanical dispersion, which were stable, causing the decrease of the turbidity in the aqueous phase. Bacterial cells presented higher (36.8-53.3%) hydrophobicity when the growth was made in the presence of alkanes (*n*-hexane, *n*-heptane) as compared with the cell hydrophobicity (1.3-29.6%) when the growth was done in the presence of aromatics (toluene, styrene, xylene isomers, ethylbenzene, propylbenzene) (Table 1), which was further confirmed by the optical microscope observations (data not shown). *A. hydrophila* IBB_{Po8} cells presented lower value of cell hydrophobicity (1.3-39.3%) as compared with *P. aeruginosa* IBB_{Po10}

cells (6.1-53.3%). Earlier studies have shown that²⁷, after adaptation to toluene, *P. putida* S12 cells become less hydrophobic. The low hydrophobicity of the cell wall represents a defensive mechanism, which keeps away the organic solvents molecules from the cell surface, preventing accumulation of the toxic compounds in high concentrations in the bacterial cell membranes²⁻⁴.

β-Galactosidase Activity

After bacterial cells permeabilization with chloroform and sodium dodecyl sulfate, *o*-nitrophenyl-β-D-galactoside diffused into the cells and it was hydrolyzed by the β-galactosidase enzyme entrapped within the cell. *A. hydrophila* IBB_{Po8} and *P. aeruginosa* IBB_{Po10} were found β-galactosidase-positive. Under appropriate physiological conditions, any compound that can be used by bacteria as carbon source and energy may repress or induce constitutive β-galactosidase. To check this fact, the level of β-galactosidase activity was measured for bacterial cells incubated for 24 h in the presence of 0.5% (v/v) organic solvents and without them (Fig. 2). β-galactosidase activity measurements revealed that the *lacZ* gene was induced in *A. hydrophila* IBB_{Po8} and *P. aeruginosa* IBB_{Po10} grown in the presence of organic solvents. Organic solvents induced β-galactosidase activity in *A. hydrophila* IBB_{Po8} and *P. aeruginosa* IBB_{Po10}, respectively to a range of 1.7-3.8 and 3.3-6.0 times greater than the control. Similar results were previously obtained by Kieboom *et al*²⁸ for *P. putida* S12.

Phospholipids Modification

The TLC studies revealed the differences in phospholipids (motilities, phospholipid headgroups composition) extracted from bacterial cells incubated in the absence (control) and presence of 0.5% (v/v) organic solvents (Fig. 3a). The phospholipids identified, based on their motilities (R_f), in *A. hydrophila* IBB_{Po8} and *P. aeruginosa* IBB_{Po10} cells were phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI) and cardiolipin (CL). The phospholipids found in *A. hydrophila* IBB_{Po8} cells were PE (R_f = 0.58), PG (R_f = 0.87) and CL (R_f = 0.98), while PE (R_f = 0.53-0.58), PG (R_f = 0.84-0.88), CL (R_f = 0.94-0.96) were present in cells incubated in the presence of organic solvents. The phospholipids found in *P. aeruginosa* IBB_{Po10} cells were PE (R_f = 0.52), PG (R_f = 0.78) and CL (R_f = 0.97), while PE (R_f = 0.47-0.56), PG (R_f = 0.71-0.80), PI (R_f = 0.84-0.91), CL (R_f = 0.93-0.97) were present in cells incubated in

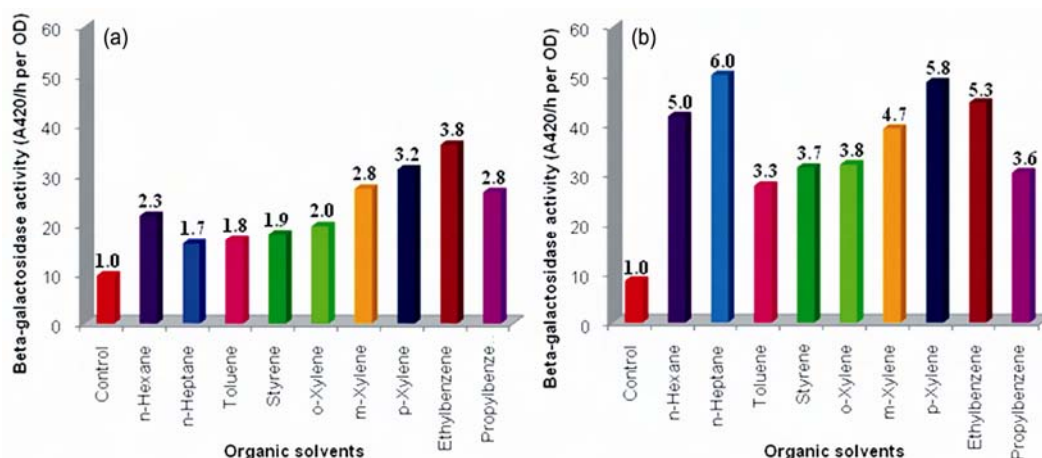


Fig. 2— β -galactosidase activity in the presence of solvents: *A. hydrophila* IBB_{Po8} (a) and *P. aeruginosa* IBB_{Po10} (b); β -galactosidase activity, expressed as the change in absorbance at 420 nm min⁻¹ mL⁻¹ of cells/optical density units at 650 nm, was measured in permeabilized cells and induction fold was calculated (values above each column).

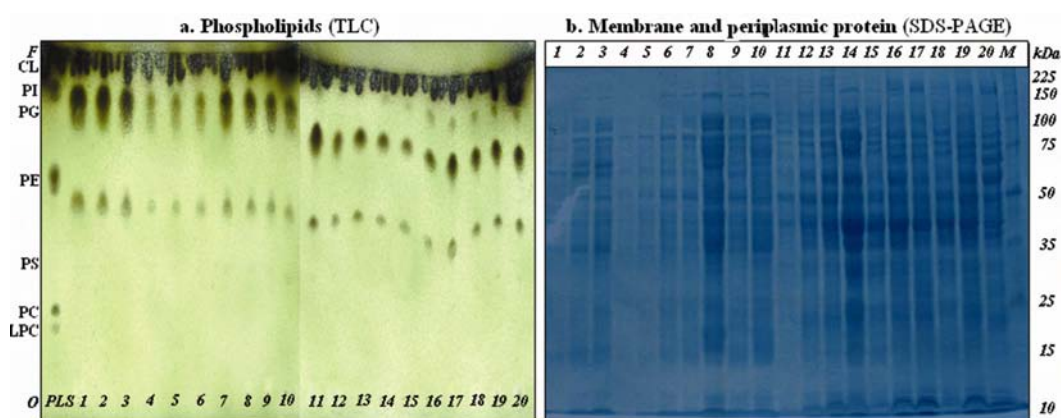


Fig. 3—Phospholipid (a) and protein (b) profile modification of solvent-tolerant bacterial strains in the presence of organic solvents: *A. hydrophila* IBB_{Po8} (lanes 1-10) and *P. aeruginosa* IBB_{Po10} (lanes 11-20); bacterial strains cultivated onto minimal medium added with 0.25% (w/v) protein hydrolysate ampicase and protease peptone (control; lanes 1, 11) and on minimal medium with 0.5% (v/v) *n*-hexane (lanes 2, 12), *n*-heptane (lanes 3, 13), toluene (lanes 4, 14), styrene (lanes 5, 15), *o*-xylene (lanes 6, 16), *m*-xylene (lanes 7, 17), *p*-xylene (lanes 8, 18), ethylbenzene (lanes 9, 19) and propylbenzene (lanes 10, 20). a. Phospholipids standards, Sigma-Aldrich, Supelco (lane PLS); origin (O), solvent front (F), lysophosphatidylcholine (LPC), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), cardiolipin (CL); b. Broad range protein molecular weight marker, Promega (lane M).

the presence of organic solvents. Thus, a decrease in the level of PE and PG were observed in the bacterial cells incubated in the presence of alkanes (except strain IBB_{Po8}) and aromatics, compared with the control. Previous studies have shown that, when bacterial cells are exposed to organic solvents, the initial stages of the damage involved are binding and penetration into the lipid bilayer^{1,2,4}. As a consequence, membrane fluidity is affected and bacteria launch appropriate responses to diminish the disruptive effects. Membrane fluidity is re-adjusted primarily by altering the composition of the lipid bilayer through compensatory mechanisms that

resemble some of those observed in response to physical and chemical stresses imposed by the environment⁴. Organic solvents can modify the ratios of saturated and unsaturated fatty acids or induce *cis-trans* isomerization and can induce changes in phospholipids. Alterations of the phospholipid headgroups had an additional effect on the physico-chemical properties of the membrane^{1,2,4,8}. As a long-term response to toluene in *P. putida* DOT-T1E, changes in the phospholipid polar headgroups were reported. Further, an enrichment of CL up to 22% of the total phospholipids and a decrease in PE was observed^{4,14}. Such alterations lead to an increase in

membrane viscosity counteracting the fluidizing effect of the solvent¹. In mutants of *P. putida* P8 lacking the CL synthesis, the *cis-trans* isomerisation took place on similar levels in wild-type and mutant strains (measured as response to 4-chlorophenol exposure), but such adaptive reaction was not able to compensate the lack of CL production²⁹.

Protein Profile Modification

Apart from changes in the composition of the cytoplasmic membrane and in the dynamics of the formation of phospholipids, alterations in the protein content have been observed as a response to organic solvents. These adaptations reestablish the stability and fluidity of the membrane once it is disturbed by the organic solvents^{3,27,30}.

To investigate the modifications induced by 0.5% (v/v) organic solvents to membrane and periplasmic protein profile of *A. hydrophila* IBB_{Po8} and *P. aeruginosa* IBB_{Po10} cells, one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed. The electrophoresis studies showed differences between protein profiles of bacterial cells incubated in the absence (control) and presence of 0.5% (v/v) organic solvents (Fig. 3b). Induction of proteins synthesis in the membrane and periplasmic protein profile was observed in *A. hydrophila* IBB_{Po8} cells grown in the presence of *n*-hexane, *n*-heptane, *p*-xylene, ethylbenzene and propylbenzene; while in the cells grown in presence of toluene, styrene, *o*-xylene, *m*-xylene, the proteins were synthesized in barely detectable quantities. On the other hand, strong induction of the synthesis of proteins in the membrane and periplasmic protein profile was observed in *P. aeruginosa* IBB_{Po10} cells grown in the presence of alkanes (*n*-hexane, *n*-heptane) and aromatics (toluene, styrene, xylene isomers, ethylbenzene, propylbenzene).

The induction of a large number of proteins was also demonstrated in *Escherichia coli* in the presence of pollutants, leading to the induction of 53 different proteins³¹. When *Clostridium acetobutylicum* initiated the solvent transformation, various known heat-shock proteins are expressed³². In *P. putida* KT2442, the expression of approximately 100 proteins was reported to be affected by the presence of 2-chlorophenol³³.

Rhodamine 6G Accumulation.

In this study, a fluorescent dye rhodamine 6G, a substrate of multidrug resistance (MDR) protein in bacterial cells to identify MDR activity, was used. Rhodamine 6G is a P-glycoprotein substrate, which

mediates the energy-dependent efflux of certain toxic compounds, such as, antibiotics, dyes, organic solvents with no apparent structural or functional similarities from the bacterial cells^{2,34}.

A. hydrophila IBB_{Po8} and *P. aeruginosa* IBB_{Po10} cells grown in the absence (control) and presence of 0.5% (v/v) organic solvents tolerate the presence of rhodamine 6G in culture medium and they also accumulate this toxic compound in bacterial cells (Fig. 4). *A. hydrophila* IBB_{Po8} (MIC₉₀ = 100-500 µg mL⁻¹) was more sensitive to rhodamine 6G as compared to *P. aeruginosa* IBB_{Po10} (MIC₉₀ > 1000 µg mL⁻¹). Accumulation of rhodamine 6G in both the strains of bacterial cells differ with strain, as well as with the organic solvents. The accumulation of rhodamine 6G in *A. hydrophila* IBB_{Po8} cells was lower (0-75%) as compared in *P. aeruginosa* IBB_{Po10} cells (100%).

Specific Amplification of HAE1 (Hydrophobe/Amphiphile Efflux 1) Gene Fragment by PCR

Although the mechanisms underlying organic solvent tolerance are not yet fully understood, but a number of factors involved in the process have been characterized. Several workers have identified constitutive and inducible efflux pumps belonging to the RND (resistance, nodulation, and cell division) family as being involved in organic solvents tolerance^{1,4,13}. To determine whether efflux pumps of RND family were present in *A. hydrophila* IBB_{Po8} and *P. aeruginosa* IBB_{Po10} cells incubated with or without 0.5% (v/v) organic solvents (alkanes: *n*-hexane, *n*-heptane; aromatics: toluene, *m*-xylene, ethylbenzene), template DNA was amplified in a PCR with oligonucleotides A24f2 and A577r2 (Fig. 5). These primers were specifically designed²² to amplify the HAE1 family of transporters, which includes all the known drug- or solvent-resistant RND transporters plus 60 hypothetical transporters⁹. Despite the fact that these transporters are widespread among Gram-negative bacterial strains, only *P. aeruginosa* IBB_{Po10} amplified the expected 550 bp fragment, while *A. hydrophila* IBB_{Po8} failed to amplify bands of the predicted size (Fig. 5). In case of *P. aeruginosa* IBB_{Po10}, unspecific amplification of other fragments was also obtained. Similar results were obtained when PCR was performed by using the same program, but with annealing at 52°C (data not shown).

Lipase and Protease Production

Most bacteria and their enzymes are denatured and inactivated in the presence of organic solvents.

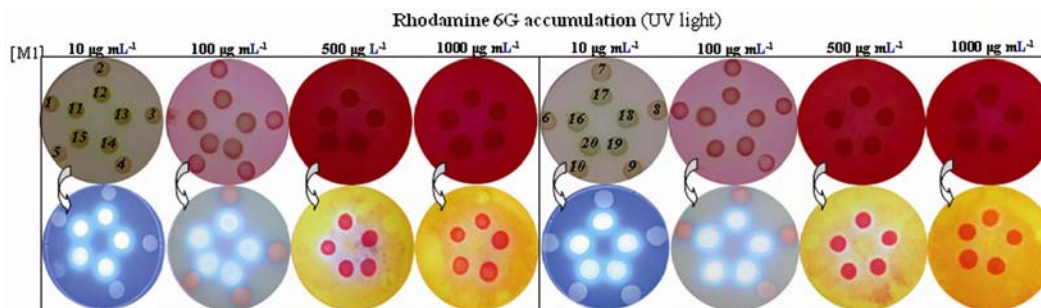


Fig. 4—Rhodamine 6G accumulation in solvent-tolerant bacterial strains in the presence of organic solvents: *A. hydrophila* IBB_{Po8} (spots 1-10) and *P. aeruginosa* IBB_{Po10} (spots 11-20); bacterial strains cultivated onto minimal medium added with 0.25% (w/v) protein hydrolysate amicase and protease peptone (control; spots 1, 11) and on minimal medium with 0.5% (v/v) *n*-hexane (spots 2, 12), *n*-heptane (spots 3, 13), toluene (spots 4, 14), styrene (spots 5, 15), *o*-xylene (spots 6, 16), *m*-xylene (spots 7, 17), *p*-xylene (spots 8, 18), ethylbenzene (spots 9, 19) and propylbenzene (spots 10, 20); accumulation of rhodamine 6G in bacterial cells was observed by the fluorescence of rhodamine 6G under UV light.

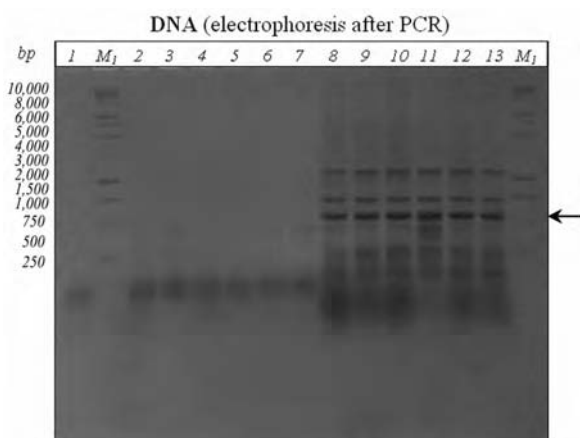


Fig. 5—PCR products of solvent-tolerant bacterial strains using primers that amplify HAE1 fragment: 1 kb DNA ladder, Promega (lane M₁); negative control DNA (lane 1); *A. hydrophila* IBB_{Po8} (lanes 2-7); *P. aeruginosa* IBB_{Po10} (lanes 8-13); bacterial strains cultivated onto minimal medium added with 0.25% (w/v) protein hydrolysate amicase and protease peptone (control; lanes 2, 8) and on minimal medium with 0.5% (v/v) *n*-hexane (lanes 3, 9), *n*-heptane (lanes 4, 10), toluene (lanes 5, 11), *m*-xylene (lanes 6, 12) and ethylbenzene (lanes 7, 13); annealing at 50°C; the positions of the expected HAE1 fragment are indicated with arrow.

Organic solvent-tolerant bacteria combat these destructive effects and thrive in the presence of high concentrations of organic solvents as a result of various adaptations mechanisms^{23,24}. The capacity of *A. hydrophila* IBB_{Po8} and *P. aeruginosa* IBB_{Po10} to produce lipolytic and proteolytic enzymes in the presence and absence of 0.5% (v/v) organic solvents was tested (data not shown). Lipolytic enzymes were produced by *A. hydrophila* IBB_{Po8} and *P. aeruginosa* IBB_{Po10} cells incubated in both the presence and absence of organic solvents. However, proteolytic enzymes were produced only by *P. aeruginosa* IBB_{Po10} cells both in the presence and absence of

organic solvents. Further studies are in progress on the stability of these enzymes in the presence of organic solvents.

Although both bacterial strains harbour plasmids, *A. hydrophila* IBB_{Po8} was more sensitive to organic solvents and other toxic compound (ampicillin, kanamycin, rhodamine 6G) as compared to *P. aeruginosa* IBB_{Po10} (data not shown). Catabolic plasmids are non-essential genetic elements for bacteria, but they do provide metabolic versatility not normally present in bacterial cell³⁵. It is obvious from the above results that a combination of different mechanisms allows the survival of *A. hydrophila* IBB_{Po8} and *P. aeruginosa* IBB_{Po10} in the presence of 0.5% (v/v) alkanes (*n*-hexane, *n*-heptane) and aromatics (toluene, styrene, xylene isomers, ethylbenzene, propylbenzene).

In natural environments, microorganisms are exposed to changing conditions and have, therefore, developed a series of mechanisms to cope with these stressors³⁶. According to Isken and de Bont³, a cascade of short-term and long-term mechanisms acts jointly to reach a complete adaptation of solvent-tolerant bacteria. The regulation of such diverse response system may be connected to a general stress response. Hence, studying solvent-tolerant bacteria and, particularly, the molecular mechanisms enabling them to withstand such rather hostile environmental conditions will not only contribute to further developing efficient two-phase biotransformation systems with whole cells but will also provide deep new insights into the general stress response of bacteria¹. The use of solvent-tolerant strains offers new perspectives in environmental biotechnology, and this will simplify the application of organic

solvents in bioremediation of contaminated sites, “end of the pipe” biotransformation of industrial wastes and may have potential for use in biphasic bioconversion systems. Apart from the application of the solvent-tolerant strains in whole-cell systems, these strains may become a source for new solvent-stable enzymes with different applications^{3,8,23,24}.

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