REVIEW

Industrial Potential of Organic Solvent Tolerant Bacteria

Yogita N. Sardessai*,† and Saroj Bhosle‡

Goa College of Pharmacy, Panjim, Goa, India, and Department of Microbiology, Goa University, Goa, India

Most bacteria and their enzymes are destroyed or inactivated in the presence of organic solvents. Organic solvent tolerant bacteria are a relatively novel group of extremophilic microorganisms that combat these destructive effects and thrive in the presence of high concentrations of organic solvents as a result of various adaptations. These bacteria are being explored for their potential in industrial and environmental biotechnology, since their enzymes retain activity in the presence of toxic solvents. This property could be exploited to carry out bioremediation and biocatalysis in the presence of an organic phase. Because a large number of substrates used in industrial chemistry, such as steroids, are water-insoluble, their bioconversion rates are affected by poor dissolution in water. This problem can be overcome by carrying out the process in a biphasic organic-aqueous fermentation system, wherein the substrate is dissolved in the organic phase and provided to cells present in the aqueous phase. In bioprocessing of fine chemicals such as *cis*-diols and epoxides using such cultures, organic solvents can be used to extract a toxic product from the aqueous phase, thereby improving the efficiency of the process. Bacterial strains reported to grow on and utilize saturated concentrations of organic solvents such as toluene can revolutionize the removal of such pollutants. It is now known that enzymes display striking new properties in the presence of organic solvents. The role of solvent-stable enzymes in nonaqueous biocatalysis needs to be explored and could result in novel applications.

Contents

1. Introduction

1.1. History and Biodiversity. Organic solvent tolerant bacteria are a novel and unique group of extremophilic microorganisms that thrive in the presence of very high concentrations of organic solvents (*1*). In general, organic solvents are extremely toxic to bacteria, as they disrupt the cell membrane, compromising the structural and functional integrity of the cell (*2*, *3*); however, organic solvent tolerant bacteria circumvent these toxic effects by virtue of various adaptations such as solvent efflux pumps, rapid membrane repair, lower cell membrane permeability, increased membrane rigidity, decreased cell surface hydrophobicity etc. The mechanisms of solvent tolerance particularly in *Pseudomonas* and *E. coli* strains have been extensively studied (*4*−*11*).

Most of the reported organic solvent tolerant bacteria are strains of *Pseudomonas* species (i.e., *P. putida*, *P. aeruginosa*, *P. fluorescens*, etc). *Pseudomonas putida* IH-2000 is of soil origin (*1*), whereas *P. putida* DOT-T1E has been isolated from wastewater (*12*). Others are laboratory strains that have gradually adapted to solvents, e.g., *P. putida* S12 (*13*). Organic solvent tolerant mutants of *E. coli* have been constructed from solvent-sensitive parent strains (*14*). Several Gram-positive bacteria such as strains of *Bacillus*, *Rhodococcus*, and *Arthrobacter* have been reported from natural habitats (*15*−*18*). It has been reported that the numbers of organic solvent tolerant bacteria in marine habitats are much higher than in soil (*16*).

1.2. Physiological Basis of Solvent Toxicity and the Concept of Organic Solvent Tolerance. The primary site of action of organic solvents is the cell membrane. The cytoplasmic membrane of bacterial cells, a phospholipid bilayer, is a matrix in which various

* To whom correspondence should be addressed. E-mail: nnnps@sancharnet.in.
† Goa College of Pharmacy.
‡ Goa University.

10.1021/bp0200595 CCC: $27.50
Published on Web 03/10/2004

Yogita Sardessai is a lecturer in microbiology at the Goa College of Pharmacy, Panaji. She received her B.Sc. (1991) and M.Sc. (1994) degrees in Microbiology from the Goa University. For her Ph.D. thesis, she has worked on organic solvent tolerant bacteria and their role in steroid transformation. Her research interests include extremophiles such as alkalophiles and organic solvent tolerant bacteria, marine microbes, and bioactive metabolites from such sources.

Dr. Saroj Bhosle is a reader in the Department of Microbiology, Goa University. She has been in the field of microbiology research and teaching for over 19 years. Her research interests include the environmental impact and applications of marine and estuarine microflora; the biodiversity and enzymes of alkalophiles and their role in the degradation of polyphenoles, amines, and other organic compounds; and organic solvent tolerant bacteria. She is a member of several academic bodies and is actively involved in organizing coordinated training programs.

enzymes and transport proteins are embedded. It plays a vital role in solute transport, maintaining the energy status of the cell, regulation of the intracellular environment, turgor pressure, signal transduction, and energy transducing processes. Solvents partition into and disrupt the lipid bilayer, thus compromising cell viability (*3*). It has been proved that it is not the chemical structure of the solvent but the concentration to which it accumulates in the cell membrane that plays a crucial role in determining toxicity (*19*). Physiological investigation of microbes has revealed a corelation between solvent toxicity and its log *P* value (Table 1). The parameter log *P* is defined as the partition coefficient of the given solvent in an equimolar mixture of octanol and water (*1*). The greater the polarity, the lower the log *P* value and the greater the toxicity of the solvent. Generally, solvents with log *P* values between 1 and 4 are considered extremely toxic, as their degree of partitioning into the aqueous layer (which contains the cells) and from there into the bacterial lipid membrane bilayer is high. Lipophilic solvents (log $P > 4$) can show a high degree of accumulation in the membranes but will not reach a high membrane concentration owing to their low water solubility and so are not toxic to an organism. Solvents in the log *P* range of 1−4 are more water-soluble and still partition well to the membrane; as a result the actual membrane concentration of these solvents will be relatively high, thus making them very toxic to cells (*8*).

Table 1. Organic Solvents and Their log *P* Values

solvent	log *P* value	solvent	log *P* value
n-decane	5.6	styrene	3.0
decalin	4.8	octanol	2.9
diphenyl ether	4.3	carbon tetrachloride	2.7
cyclooctane	4.2	toluene	2.5
propyl benzene	3.8	heptanol	2.4
tetralin	3.8	dimethyl phthlate	2.3
methyl cyclohexane	3.7	fluorobenzene	2.2
hexane	3.5	benzene	2.0
cyclohexane	3.2	chloroform	2.0
ethyl benzene	3.1	cyclohexanol	1.5
p-xylene	3.0	*n*-butanol	0.8

Each organism has its own intrinsic tolerance level for organic solvents, which is determined genetically and is also influenced by environmental factors (*20*). Organic solvent tolerance is believed to be a strain-specific property (*21*). The tolerance level of each microorganism is represented by its index value. The index value is the log *P* value of the most toxic organic solvent among those that can be tolerated by the organism. Every bacterium can grow on agar media overlaid with any one of the organic solvents having a log *P* value greater than the index value. This empirical rule, derived from observations of microbial growth on agar media, implies the organism would also be able to grow in a two-phase liquid system consisting of the nutrient medium and an organic solvent with log *P* greater than the index value. However, under such conditions, the growth of bacteria is suppressed by organic solvents having log *P* value near the index value (*14*). Some known organic solvent tolerant bacteria and their characteristics are summed up in Table 2.

The index value of the known *Pseudomonas* strains is 2.5 (log *P* value of toluene), and that of *E. coli* xylene-tolerant mutants is 3. The reported organic solvent tolerant Gram-positive bacterial strains are tolerant to benzene (log $P = 2$). As per log *P* values, benzene is more toxic than toluene, which in turn is more toxic than xylene. The authors have isolated a strain of *Bacillus* (SB1) from a mangrove ecosystem that tolerates butanol (log $P = 0.8$), which has the lowest index value reported for any organic solvent tolerant bacterium (*18*).

2. Biphasic/Nonaqueous Systems in Biocatalysis

2.1. Advantages of Biotechnological Processes using Enzymes in the Presence of Organic Solvents. Water is not the ideal medium for the majority of organic reactions. Many reactants such as molecular oxygen, steroids, and lipids are more soluble in organic solvents than in water, and some products may be quite labile in an aqueous environment. Nonaqueous media

Table 2. Some Reported Strains of Organic Solvent Tolerant Bacteria and Their Characteristics

strain	index solvent	index value	reference	additional information
P. putida IH-2000	cyclohexane, xylene, styrene, heptanol	2.5	1	first organic solvent tolerant bacterium reported, does not metabolize toluene
P. putida DOT-T1E	90% toluene	2.5	12	degrades toluene
P. putida S12	toluene	2.3	19	degrades styrene, octanol and heptanol
P. aeruginosa PST-01	toluene	2.5	23	produces proteolytic enzyme stable in the presence of cyclohexane, toluene, ethanol, acetone
P. aeruginosa LST-03	toluene	2.5	22	produces organic solvent stable lipase active in the presence of cyclohexane toluene, ethanol, acetone
E. coli JA300 mutants of JA 300			14	solvent tolerant mutants produced from a sensitive parent
OST3408	cyclohexane	3.2		
OST3301	*n*-pentane			
OST3121	*p*-xylene	3.0		
P. putida ST-200	*p*-xylene	3	30	cholesterol degrader
Bacillus OS-1906	benzene	2	16	marine polyaromatic hydrocarbon degrader
Bacillus DS-994	benzene	2	16	utilizes sulfur compounds such as thiophene in water/petroleum 2-phase systems
Bacillus strain SB1	*n*-butanol	0.8	18	transforms cholesterol dissolved in chloroform, degrades hydrocarbons, has the lowest index value reported for any organic solvent tolerant bacterium
Bacillus strain BC1	chloroform	2	32	marine bacterium, transforms cholesterol dissolved in chloroform
Flavobacterium DS-711	benzene	2	16	degrades crude oil
Arthrobacter ST-1	benzene	2	16	marine origin, cholesterol degrader, accumulates 1,4-androstadiene-3,17-dione

allow a much increased volumetric activity to be achieved to accomplish reactions with such water-insoluble substrates (e.g., aerobic oxidation of estrogens catalyzed by fungal laccase, cholesterol transformation). Extracellular enzymes of organic solvent tolerant bacteria are stable in the presence of organic solvents. This assumption has been confirmed by the isolation of organic solvent stable lipase and protease from organic solvent tolerant bacteria. (*22*−*24*).

Microbial contamination, by contrast, is much less of a problem in solvents, and the consequent absence of microbial proteases may lead to an apparent stabilization in the biocatalyst. Some polymerizing reactions, such as the polymerization of phenols catalyzed by peroxidase, will produce a higher molecular weight product when carried out in a solution more able to dissolve the product (i.e., oligomers) initially formed (*25*). Under normal physiological conditions, hydrolytic enzymes catalyze the degradation of polymers, i.e., hydrolases are transferases normally transferring a moeity to the acceptor, water. Water is normally present in a vast molar excess over other potential acceptor molecules, so no reaction occurs other than hydrolysis. Also the normal concentration of water (about 55.5 M) is much greater than its typical K_m (about 50 mM) and the rate of hydrolysis will not be affected as the hydrolysis proceeds. By greatly reducing the water activity in these systems, they can be used to transfer to other acceptors (*26*−*28*). An example of this can be found in the transesterifiction reactions of esterases and lipases. Lipolytic enzymes are often used in nonaqueous media because these media can increase substrate solubility and facilitate product recovery and are favorable for reactions such as ester synthesis and *trans*-esterification, both of which are thermodynamically unfavorable in water. The lipolytic enzyme from *P. aeruginosa* LST-03 is very stable in the presence of organic solvents such as toluene, cyclohexane, ethanol, and acetone and therefore can be used for reactions in media containing organic solvents (*22*). Enzymatic reactions using protease in the presence of organic solvents have been extensively studied for the synthesis of peptides and esters. If organic solvents can be used as media for enzymatic reactions, the reaction equilibria of hydrolytic enzymes can be shifted toward completion of the reverse reaction of hydrolysis, which is the synthetic reaction. The stability of the proteolytic enzyme from *P. aeruginosa* PST-01 is considerably enhanced by addition of organic solvents such as cyclohexane, toluene, ethanol, and acetone (*23*). Khmelnitsky et al. (1988) have investigated various methods for stabilizing enzymes in the presence of organic solvents (*29*). However, use of extracellular enzymes from organic solvent tolerant bacteria can solve the major problem of denaturation and inactivation of the enzyme in the organic phase. Moreover, whole cells could also be used for the bioconversion as they can withstand exposure to the solvent.

2.2. Use of Organic Solvent Tolerant Bacteria in Optimizing Bioprocesses Involving Water-Insoluble Substrates/Products. Organic solvent tolerant bacteria can serve as invaluable agents in catalyzing the biotransformations of water-insoluble substances in organic−aqueous biphasic systems.

For bioconversion of organic compounds with low solubilities in water, large volumes of appropriate medium are required for solubilization of the compounds. This consumption of media and water and the inevitable treatment of wastewater constitute one of the major cost factors in bioconversion fermentation. If the water-insoluble compounds were suspended in a small volume of the medium, it would take a very long time to complete the bioconversion. The solubility of most steroid compounds as well as the products expected from the bioconversion in water is extremely low (10^{-2}−10^{-3} g/L of water). Cholesterol is usually suspended in bioconversion systems containing surfactants to affect the bioconversion rate, but this does not prevent the formation of solid particles. Some organic solvent tolerant bacteria capable of transforming cholesterol in a biphasic system containing cholesterol dissolved in the organic phase and cells suspended in aqueous phase have been discovered. *Pseudomonas* sp. strain ST-200 effectively oxidized the C3 and C6 positions of cholesterol by introduction of a hydroxyl or ketone group in the presence of a mixed organic solvent (*p*-xylene and *p*-diphenyl methane 3:7 v/v) (*30*). The cholesterol oxidase enzyme of ST-200 is constitutive and extracellular in nature. Its cholesterol

consumption rate in the presence of benzene, toluene, *p*-xylene, propyl benzene, or diphenylmethane was 3- to 3.5-fold higher than in absence of organic solvent (*31*). *Arthrobacter* ST-1 shows a very high percentage of cholesterol degradation when *n*-decane and *n*-dodecane are employed as organic phase. Androsta-1,4-diene-3,17 -dione was the product obtained (*15*). Recently, Sardessai and Bhosle have described a biphasic biotransformation system in which cholesterol was dissolved in 50% chloroform and cells were suspended in phosphate buffer. Two *Bacillus* cultures strain SB1 and BC1 were found to effectively transform cholesterol to cholest-4-ene-3,6-dione (*32*).

Another significant aspect is the use of organic solvent tolerant bacteria to scavenge toxic substrates/products from the cells using a second liquid extraction phase in the production process. An important advantage of having an organic phase in the system lies in elimination of toxic products from the fermenter. Product toxicity of fine chemicals is a problem in several biotechnological processes. In many instances, a second phase of the organic solvent can extract the toxic product from the aqueous phase during the fermentation. *Pseudomonas oleovorans* can convert 1,7-octadiene into both 7,8-epoxy-1-octene and 1,2,7,8-diepoxyoctane when grown on octane. Epoxides are very toxic to cells, and hence high concentrations of the products could not be reached in the aqueous phase. By using cyclohexane as the second phase, the production of epoxides was enhanced because the monoepoxide preferentially partitioned into the cyclohexane phase. The diepoxide, however, was more evenly distributed between the aqueous and organic phases. The solvent phase (cyclohexane, $\log P = 3.2$) extracts only the monoepoxide and not the less lipophilic diepoxide, which is the second product. For selective removal of diepoxide from the aqueous phase, an even less polar solvent would have to be used ($\log P < 3.2$), which would be extremely toxic to normal cells. The use of organisms such as *P. oleovorans*, *E. coli*, or *Nocardia corallina* in production of lipophilic compounds in 2-phase systems is limited by the range of solvents that the organism can withstand as a second phase. It is here that solvent-tolerant bacteria play a vital role by allowing a new degree of freedom in coping with toxic products. They can tolerate solvents that are much less lipophilic, and these can be used to partition out toxic products from the aqueous phase. Many important fine chemicals including catechols, phenols, aldehydes and ketones, low molecular weight epoxides and diepoxides, medium-chain alcohols, and terpenoids are in low lipophilicity range (*8*).

Over the past decade, nonaqueous enzymology has emerged as a major area of biotechnology research and development. The reason for this interest is stimulated by the fact that enzymes exhibit striking new properties in organic solvents. Enzyme selectivity in organic solvents is distinctly different from that in water and can be markedly controlled or reversed by the solvent (*33*). Many applications have been developed in chemical processing in organic solvents, particularly for the synthesis of optically active intermediates, food-related conversions and analysis (*28*), and others (*34*−*37*). Use of enzymes in stereospecific and stereoselective reactions in nonaqueous media such as *n*-hexane and acetonitrile is becoming increasingly popular as a result of high-temperature stability (*38*), the insoluble nature of the biocatalyst in nonaqueous solvents, and the shift in reaction equilibria to favor synthesis. Traditionally, most studies on enzyme activity have involved enzymes in aqueous environments. These studies have established that many environmental factors including pH, ionic strength, water activity, and temperature control enzyme activity. It is expected that the same factors may influence the enzyme activity in the organic phase also (*39*). Differences that arise out of the usual microenvironment of enzymes in organic solvents include the degree of protein hydration, influence of organic solvents on the protein structure, susceptibility to inactivation, and variations in the ionization state of the protein. Enzymes generally show a lower catalytic efficiency (up to 3−4 orders of magnitude) when employed in organic solvents than in aqueous buffer. One reason for this can be diffusional limitations. Generally, when enzymes are employed in organic solvents, they are used as a suspended powder, and the dispersion of this powder is a critical factor in expression of catalytic activity. Methods that will allow the dissolution of the enzyme in the solvent, such as enzyme complexation with ion-pair forming surfactants, synthetic amphipatic lipids that coat the enzyme molecule, or covalent linking of the protein to amphipatic polymers such as poly(ethylene glycol), are important in improving the catalytic performance. Improvements can also be obtained by incorporating carbohydrates, polymers, and organic buffers in the dry catalyst and by lyophilizing the catalyst in the presence of nonbuffer salts such as KCl (*28*).

Another major factor influencing activity and stability of enzyme suspensions in organic solvents is the amount of water activity of the enzyme in these solvents (*40*). Too little water causes loss of activity, whereas too much leads to rapid and irreversible denaturation. The polar organic solvents are more effective at stripping the water that is essential for protein hydration and conformational stability and are thus detrimental to the enzyme. Another important factor influencing the biocatalytic environment is the pH. The pH value of the enzymes environment determines the ionization state of the enzyme. However, no protonation/deprotonation can occur in an organic environment. The pH of the environment from which the enzyme is taken before being placed in the water-poor environment of the organic solvent therefore determines the ionization state of the enzyme. This pH is retained in the enzyme and this property is referred to as pH memory. Therefore, enzyme powders should be obtained from an aqueous solution of the pH affording maximal activity for optimal enzymatic performance (*28*).

Most studies on nonaqueous enzymology described above have been done on animal enzymes. It would be interesting to compare the behavior of the regular enzymes with the bacterial enzymes derived from organic solvent tolerant bacteria.

3. Role of Organic Solvent Tolerant Bacteria in Bioremediation and Wastewater Treatment

The persistance of benzene, toluene, and xylenes in contaminated sites is indicative of the lack of natural systems that can efficiently degrade these compounds (*17*). Although many aromatic hydrocarbon degrading strains have been isolated, they are usually solvent-sensitive and degradation of aromatic hydrocarbons occurs only when these compounds are supplied at low concentrations (*3*). There is considerable interest in the isolation of microbes able to thrive in high concentrations of organic solvents, because such organisms can be used as vehicles in the elimination of low molecular weight aromatics that are highly carcinogenic even in ppm amounts. Since most natural contaminated sites are saturated with solvents such as benzene, toluene, etc.,

organic solvent tolerant bacteria with the requisite catabolic potential can be of vital importance in cleanup operations. For instance, a *Rhodococcus* sp. strain 33 isolated from a contaminated site in Sydney can degrade benzene at concentrations of 200 ppm and tolerate high concentrations of benzene. This culture also grows in the presence of 6% NaCl and at temperatures from 0 to 37 °C, which are necessary characteristics for a culture if it has to be used in cleaning up marine oil spills (*17*). Kato et al. (1996) have described the isolation procedure used for the isolation of several useful organic solvent tolerant bacteria from the deep sea. Benzene was added to artificial seawater containing samples of deep sea sediment to a concentration of 50% (v/v), and the cultures were incubated for a week at room temperature on a shaker. After the plates were incubated, the benzene layer was carefully separated from the seawater layers, and a portion of each benzene layer was spread on a suitable agar medium. Colonies that grew after incubation for 2 days at 30 °C were isolated and purified. This led to the isolation of several strains such as *Flavobacterium* sp. DS-711, which degrades crude oil, *Bacillus* sp. DS-994, which utilizes sulfur compounds, *Bacillus* sp. DS-1906, which degrades polyaromatic hydrocarbons, and *Arthrobacter* ST-1, which degrades cholesterol (*16*). Abe et al. (1995) isolated an organic solvent tolerant bacterium from deep sea sediment samples after treatment with 50% v/v benzene. This strain, *Bacillus* DS-1906, showed polyaromatic hydrocarbon degrading ability in the presence of organic solvent. It degraded 48% of naphthalene solubilized in *n*-hexane and the amount degraded was more in the presence of solvent (*41*). Huertas et al. (1998) assayed the tolerance of three different toluene degraders to organic solvents in soil. The toluene-tolerant *P. putida* DOT-T1E recovered from the shock faster than *P. putida* F1 and hence became established at higher densities in the polluted sites. Their studies show that *Pseudomonas* strains are more resistant to solvents in soils than in liquid culture medium, which may explain why these microbes deal with these pollutants in soil and in biofilms. However, the level of tolerance in soil is related to the level of tolerance in liquid medium. The higher the tolerance in the liquid medium, the faster the recovery of the strain in soil after solvent shock. Therefore, in sites heavily polluted by aromatic hydrocarbons, solvent-tolerant strains would be expected to become established first, to colonize the site and become predominant in the removal of such compounds (*21*). The catabolic potential of *P. putida* DOT-T1E was expanded to include *m*- and *p*-xylene and related hydrocarbons by transfer of the TOL plasmid pWW0-Km (*12*).

4. Future Trends in Nonaqueous Biocatalysis

Present research in this field has advanced sufficiently to suggest that it is possible to make enzymes more active in organic solvents than in water. There is also an intriguing possibility of molding enzyme active centers and thus optimizing enzyme activity in organic solvents for a desired substrate by molecular imprinting (*28*). Studies are being carried out to enhance enzyme activity in organic solvents by protein engineering using random and rational mutagenesis (*42*). For instance, hydrophobic amino acids have been used to substitute charged amino acid residues, since charged residues help solvation in water and will have destabilizing effects in a nonaqueous solvent. Replacing them with hydrophobic residues prevents destabilization and enhances kinetic stability of the enzyme subtilisin. Recently, enzyme microcrystals grown from aqueous solutions and cross-linked with a bifunctional agent such as glutaraldehyde exhibit a higher kinetic tolerance to high temperatures, near anhydrous organic solvents, and mixed aqueous−organic solvents than both soluble and conventionally immobilized enzymes. This cross-linked enzyme crystal (CLEC) technology is extremely attractive for the chemical industry because of several reasons: (a) It is applicable to almost any protein irespective of size, subunit composition, and extent of glycosylation. (b) It yields a catalyst with increased stability against heat, organic solvents, and exogenous proteolysis. (c) The enzyme obtained is more stable and active than the soluble enzyme, has a higher volumetric activity, and is more uniform and pure as compared to immobilized enzymes. For example, CLECs of *C. rugosa* lipase show a 10-fold enhancement in the optical purity of the pharmaceutically important products ketoprofen, ibuprofen, and fluorbiprofen. (d) They are insoluble in water and organic solvents and can be recycled several times (*43*).

Industrial biotransformations have been revolutionizing biotechnology mainly because of their selectivity and efficiency. They catalyze production of stereospecific and regiospecific compounds without using chemical protection groups and produce pure isomers rather than racemic mixtures. Also, the level of pollution caused is much less as compared to chemical catalysts. Biological systems are capable of meeting great challenges. However, most industrial systems are not designed for fragile catalysts. This is where organic solvent tolerant bacteria prove invaluable on account of their greater flexibility of performance in biotransformations in the presence of an organic phase.

References and Notes

(1) Inoue, A.; Horikoshi, K. A *Pseudomonas putida* thrives in high concentrations of toluene. *Nature* **1989**, *338*, 264−266.

(2) Sikkema, J.; de Bont, J.; Poolman, B. Interactions of cyclic hydrocarbons with biological membranes. *J. Biol. Chem.* **1994**, *269*, 8022−8026.

(3) Sikkema, J.; de Bont, J.; Poolman, B. Mechanisms of solvent toxicity of hydrocarbons. *Microbiol. Rev.* **1995**, *59*, 201−222.

(4) Cruden, D.; Wolfram, J.; Rogers, R.; Gibson, D. Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic-aqueous) medium. *Appl. Environ. Microbiol.* **1992**, *58*, 2723−2729.

(5) Pinkart, C.; Wolfram, J. W.; Rogers, R.; White, D. Cell envelope changes in solvent tolerant and solvent sensitive *Pseudomonas putida* strains following exposure to *o*-xylene. *Appl. Environ. Microbiol.* **1996**, *62*, 1129−1132.

(6) Aono, R.; Kobayashi, K. Cell surface properties of organic solvent tolerant mutants of *E. coli* K-12. *Appl. Environ. Microbiol.* **1997**, *63*, 3637−3642.

(7) Ramos, J.; Duque, E.; Rodriguez-Herva, J.; Godoy, P.; Haidour, A.; Reyes, F.; Fernandez-Barrero, A. Mechanisms for solvent tolerance in bacteria. *J. Biol. Chem.* **1997**, *272*, 3887−3890.

(8) De Bont, J. Solvent tolerant bacteria in bio-catalysis. *Trends Biotechnol.* **1998**, *16*, 493−499.

(9) Tsukagoshi, N.; Aono, R. Entry into and release of solvents by *Escherichia coli* in an organic-aqueous two-liquid-phase system and substrate specificity of the AcrAB-tolC solvent extruding pump. *J. Bacteriol.* **2000**, *182*, 4803−4810.

(10) Sardessai, Y.; Bhosle, S. Tolerance of bacteria to organic solvents. *Res. Microbiol.* **2002**, *153*, 263−268.

(11) Ramos, J.; Duque, E.; Gallegos, M.; Godoy, P.; Ramos-Gonsalez, M.; Rojas, A.; Teran, W.; Segura, A. Mechanisms of solvent tolerance in gram-negative bacteria. *Annu. Rev. Microbiol.* **2003**, *56*, 743−768.

(12) Ramos, J.; Duque, E.; Huertas, M.; Haidour, A. Isolation and expansion of the catabolic potential of a *Pseudomonas*

strain able to grow in the presence of high concentrations of aromatic hydrocarbons. *J. Bacteriol.* **1995**, *177*, 3911−3916.
(13) Weber, F.; Ooijkaas, L.; Schemen, R.; Hartmans, S.; de Bont, J. Adaptation of *Pseudomonas putida* S12 to high concentrations of styrene and other organic solvents. *Appl. Environ. Microbiol.* **1993**, *59*, 3502−3504.
(14) Aono, R.; Aibe, K.; Inoue, A.; Horikoshi, K. Preparation of organic solvent tolerant mutants from *E. coli* K-12. *Agric. Biol. Chem.* **1991**, *55*, 1935−1938.
(15) Moriya, K.; Yanigitani, S.; Usami, R.; Horikoshi, K. Isolation and some properties of an organic solvent tolerant marine bacterium degrading cholesterol. *J. Mar. Biotechnol.* **1995**, *2*, 131−133.
(16) Kato, C.; Inoue, A.; Horikoshi, K. Isolating and characterising deep sea marine microorganisms. *Trends Biotechnol.* **1996**, *14*, 6−12.
(17) Paje, M.; Neilan, B.; Couperwhite, I. A *Rhodococcus* species that thrives on medium saturated with liquid benzene. *Microbiology* **1997**, *143*, 2975−2981.
(18) Sardessai, Y.; Bhosle, S. Organic solvent tolerant bacteria in mangrove ecosystem. *Curr. Sci.* **2002**, *82*, 622−623.
(19) Isken, S.; De Bont, J. Active efflux of organic solvents by *Pseudomonas putida* is induced by solvents. *J. Bacteriol.* **1998**, *180*, 6769−6772.
(20) Kobayashi, H.; Yamamoto, M.; Aono, R. Appearance of a stress response protein, phage shock protein A, in *Escherichia coli* exposed to hydrophobic organic solvents. *Microbiology* **1998**, *144*, 353−359.
(21) Huertas, M.; Duque, E. Survival in soil of different toluene degrading *Pseudomonas* strains after solvent shock. *Appl. Environ Microbiol.* **1998**, *64*, 38−42.
(22) Ogino, H.; Miyamoto, K.; Ishikawa, H. Organic solvent tolerant bacterium which secretes organic solvent stable lipolytic enzyme. *Appl. Environ. Microbiol.* **1994**, *60*, 3884−3886.
(23) Ogino, H.; Yasui, K.; Shiotani, T.; Ishiwara, T.; Ishikawa, H. Organic solvent tolerant bacterium which secretes an organic solvent stable proteolytic enzyme. *Appl. Environ. Microbiol.* **1995**, *61*, 4258−4262.
(24) Ogino, H.; Yokoo, J.; Watanabe, F.; Ishikawa, Y. Cloning and sequencing organic solvent stable protease secreted from *Pseudomonas aeruginosa* PST-01 and its expression. *Nature* **2000**, *5*, 191−200.
(25) Dordick, J.; Marletta, M.; Klibanov, A. Peroxidases depolymerise lignin in organic media but not in water. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 6255−6257.
(26) Bell, G.; Halling, P.; Moore, B.; Partridge, J.; Rees, D. Biocatalyst behaviour in low water systems. *Trends Biotechnol.* **1995**, *13*, 468−473.
(27) Klibanov, A. Enzymatic catalysis in anhydrous organic solvents. *Trends Biochem. Sci.* **1989**, *4*, 141−144.
(28) Klibanov, A. Why are enzymes less active in organic solvents than in water? *Trends Biotechnol.* **1997**, *15*, 97−101.
(29) Khmelnitsky, Y.; Levashov, A.; Klyachko, N.; Martinek, K. Engineering biocatalytic systems in organic media with low water content. *Enzyme Microb. Technol.* **1988**, *10*, 710−724.
(30) Aono, R.; Doukyu, N.; Kobayashi, H.; Nakajima, H.; Horikoshi, K. Oxidative bioconversion of cholesterol by *Pseudomonas* sp. strain ST-200 in a given water-organic solvent 2-phase system. *Appl. Environ. Microbiol.* **1994**, *60*, 2518−2523.
(31) Doukyu, N.; Aono, R. Purification of extracellular cholesterol oxidase with high activity in the presence of organic solvents from *Pseudomonas* sp. strain ST-200. *Appl. Environ. Microbiol.* **1998**, *64*, 1929−1932.
(32) Sardessai, Y.; Bhosle, S. Isolation of an organic solvent tolerant cholesterol transforming *Bacillus* species, BC1, from coastal sediment. *Mar. Biotechnol.* **2003**, *5*, 1−3.
(33) Wescott, C.; Klibanov, A. The solvent dependence of enzyme specificity, *Biochim. Biophys. Acta* **1994**, *1206*, 1−9.
(34) Dordick, J. Enzymatic catalysis in monophasic organic solvents. *Enzyme Microb. Technol.* **1989**, *11*, 194−211.
(35) Gupta, M. Enzyme function in organic solvents. *Eur. J. Biochem.* **1992**, *203*, 25−32.
(36) Ke, T.; Klibanov, A. On enzymatic activity in organic solvents as a function of enzyme history. *Biotechnol. Bioeng.* **1998**, *57*, 746−750.
(37) Dai, L.; Klibanov, A. Striking activation of oxidative enzymes suspended in nonaqueous media, *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9475−9478.
(38) Zaks, A.; Klibanov, A. Enzymatic catalysis in organic media at 100 degrees C. *Science* **1984**, *224*, 1249−1251.
(39) Mattiasson, B.; Adlercruetz, P. Tailoring the microenvironment of enzymes in water-poor systems. *Trends Biotechnol.* **1991**, *9*, 394−398.
(40) Zaks, A.; Klibanov, A. The effect of water on enzyme action in organic media. *J. Biol. Chem.* **1988**, *263*, 8017−8021.
(41) Abe, A.; Inoue, A.; Usami, R.; Moriya, K.; Horikoshi, K. Properties of a newly isolated marine bacterium that can degrade polyaromatic hydrocarbons in the presence of organic solvents. *J. Mar. Biotechnol.* **1995**, *2*, 182−186.
(42) Chen, K.; Robinson, A.; Van Dam, M.; Martinez, P.; Economou, C.; Arnold, F. Enzyme engineering for nonaqueous solvents. II. Additive effects of mutations on the stability and activity of subtilisin E in polar organic media. *Biotechnol. Prog.* **1991**, *7*, 125−129.
(43) St. Clair, N.; Wang, Y.; Margolin, A. Cofactor bound crosslinked enzyme crystals (CLEC) of alcohol dehydrogenase. *Angew Chem., Int. Ed.* **2000**, *39*, 380−383.

Accepted for publication December 15, 2003.

BP0200595