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Organic solvent adaptation of Gram positive bacteria: Applications and biotechnological potentials

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ABSTRACT

Organic-solvent-tolerant bacteria are considered extremophiles with different tolerance levels that change among species and strains, but also depend on the inherent toxicity of the solvent. Extensive studies to understand the mechanisms of organic solvent tolerance have been done in Gram-negative bacteria. On the contrary, the information on the solvent tolerance mechanisms in Gram-positive bacteria remains scarce. Possible shared mechanisms among Gram-(−) and Gram-(+) microorganisms include: energy-dependent active efflux pumps that export toxic organic solvents to the external medium; *cis*-to-*trans* isomerization of unsaturated membrane fatty acids and modifications in the membrane phospholipid headgroups; formation of vesicles loaded with toxic compounds; and changes in the biosynthesis rate of phospholipids to accelerate repair processes. However, additional physiological responses of Gram-(+) bacteria to organic solvents seem to be specific. The aim of the present work is to review the state of the art of responsible mechanisms for organic solvent tolerance in Gram-positive bacteria, and their industrial and environmental biotechnology potential.

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1. Introduction

Organic solvents can be extremely toxic to all life forms because they are able to bind to the cell membrane affecting its integrity. Disruption of membrane functions implies loss of the permeability barrier and the energy transducer; concomitantly leading to cellular metabolism damages, growth inhibition, and, finally cell death (Sardessai and Bhosle, 2002a; Heipieper et al., 2007). Despite this, for almost two decades, organic solvent-tolerant bacteria capable of thriving in the presence of these toxic compounds have been reported (Inoue and Horikoshi, 1989; Zahir et al., 2006). The first report of an organic-solvent-tolerant bacterium was described in 1989 on a *Pseudomonas putida* IH-2000 able to grow in the presence of very toxic toluene (Inoue and Horikoshi, 1989). Since that time, solvent-tolerant bacteria are being explored for their potential in industrial and environmental biotechnology (Sardessai and Bhosle, 2004). Their enzymes are expected to be stable and active in the presence of toxic solvents, representing one of the most promising tools for biocatalysis in non-aqueous systems (Castro et al., 1992; Ogino and Ishikawa, 2001; Fang et al., 2006; Takeda et al., 2006; Gupta and Khare, 2009).

Most of the studies on solvent-tolerant microorganisms were focused on Gram-(−) bacteria, which display a cascade of adaptive mechanisms used to acclimatize in the presence of toxic organic solvents. Two major mechanisms have been extensively described particularly in *Pseudomonas* sp. and *E. coli* species as typical models. The first one involves alterations of the cellular membrane composition in order to decrease solvent permeability (Pinkart et al., 1996; Aono and Kobayashi, 1997; Ramos et al., 1997; Tsubata et al., 1997; Heipieper et al., 2003). The second type reduces the accumulation of organic solvents in the inner membrane by transporting solvent molecules out of the lipid bilayer (Isken and de Bont, 1996). Likewise, solvent utilization at high rates or solvent biotransformation to a less toxic product was observed in some tolerant bacteria (Vangnai et al., 2002). In addition, modifications in the overall morphology of cells were reported in Gram-(−) microorganisms in response to organic solvents and other stressful environments (Shi and Xia, 2003; Neumann et al., 2005). However, limited studies have been done in order to understand the effects of organic solvents in Gram-(+) bacteria. Although microorganisms belonging to *Bacillus*, *Rhodococcus*, *Staphylococcus* and *Arthrobacter* species tolerant to very toxic organic solvents have been reported (Abe et al., 1995; Moriya et al., 1995; Baigorí et al., 1996; Kato et al., 1996; Paje et al., 1997; Torres and Castro, 2003; Na et al., 2005; Nielsen et al., 2005; Zahir et al., 2006).

In order to test solvent toxicity on cells and cellular components, a solvent hydrophobicity (log P) parameter was established. The log P is defined as the logarithm of the solvent partitioning coefficient between octan-1-ol and water (Laane et al., 1987). It is generally accepted that solvents with log P values below 5 are considered extremely toxic because of their high degree of partitioning into the aqueous layer surrounding the cells, and from there into the lipid membrane bilayer (Inoue and Horikoshi, 1991). Toxicity of organic solvents appears to be, at first instance, elated to their ability to dissolve into biological membranes, causing an increase of the cell membrane fluidity compromising the physiological functions of critical cell components (Sikkema et al., 1995; de Bont, 1998).

Additionally, solvent toxicity is directly related to the accumulation of solvent molecules inside the cell membrane. Each organism has its own intrinsic solvent tolerance level, which is genetically determined and environmentally influenced. Therefore, organic solvent tolerance is believed to be a strain-specific property (Kobayashi et al., 1998; Huertas and Duque, 1998).

The aim of the present work is to review the state of the art of the responsible mechanisms for organic solvent tolerance of Gram-(+) bacteria, and their industrial and environmental biotechnology potential.

2. Mechanisms of organic solvent tolerance in Gram-(+) bacteria

Unlike Gram-(−) bacteria, in which the mechanisms of tolerance to organic solvents have been extensively studied and reviewed, very little information regarding what makes Gram-(+) bacteria tolerant to toxic solvents is available. Due to the differences between the cell envelopes of Gram-(+) and Gram-(−) bacteria, one would expect

Table 1
Mechanisms of organic solvent-tolerance proposed in Gram-positive bacteria.

OS-tolerance mechanism	Microorganism (OS)	References
General stress response		
Sigma β genes: multidrug efflux proteins (proposed)	*B. subtilis* (ethanol)	Petersohn et al., 1999
Hsp33 stress protein	*B. psychrosaccharolyticus* (2-propanol)	Kang et al., 2007
Deactivation of organic solvents		
Biodegradation	*Bacillus* sp., *Rhodococcus* sp. (benzene, toluene, xylene)	Paje et al., 1997; Wang et al., 2008
	B. pallidus ST3 (2-propanol)	Bustard et al., 2002
Esterefication	*B. licheniformis* S-86 (3-methylbutan-1-ol)	Torres et al., 2009a
Changes in cell morphology		
Decrease in cell surface-to-volume ratio (filamentous growth)	*B. licheniformis* S-86 (3-methylbutan-1-ol)	Torres et al., 2009a
Unusual extracellular capsule	*Staphylococcus* sp. ZZ1 (toluene)	Zahir et al., 2006
Phenotypic adaptation: change in colonies' color	*R. erythropolis* (wáter-inmiscible solvents)	de Carvalho et al., 2004
Cell surface modifications		
Decreased cell surface hydrophobicity	*B. licheniformis* S-86 (3-methylbutan-1-ol)	Torres et al., 2009a
Increased cell surface hydrophobicity	*Mycobacterium frederiksbergense* (anthracene)	Wick et al. 2002
Cell membrane adaptations		
Increased membrane fluidity (changes in fatty acid)	*Staphylococcus haemolyticus* (toluene); *Rhodococcus erythropolis* DCL14 (short-chain alcohols)	Nielsen et al., 2005; Pepi et al., 2008
Increased membrane fluidity (changes in fatty acid)	*Bacillus* sp. ORAs2 (toluene); *Rhodococcus erythropolis* DCL14 (alkanes and long-chain alcohols)	de Carvalho et al. 2005; Pepi et al., 2008
Changes in membrane proteins	*Clostridium thermocellum* 27405 (ethanol)	Williams, et al., 2007
Solvent excretion		
Energy-dependent toluene efflux pump	*B. cereus* R1 (toluene)	Matsumoto et al., 2002

OS, organic solvent.

that the response to toxic organic solvents is not the same for these two types of microorganisms. Some organic solvent tolerance mechanisms in Gram-(+) bacteria have been proposed such as induction of general stress regulon (Sardessai and Bhosle, 2002a); production of organic solvent emulsifying or deactivating enzymes (Moriya et al., 1995) and, a process seen in Gram-(−) bacteria, active solvent efflux pumps (Inoue et al., 1991; Moriya et al., 1995) (Table 1). Also, cell morphology alterations and filamentous growth were observed in solvent resistant bacteria in response to environmental stress, including organic solvents (Torres et al, 2009b). Perturbation on cell morphology could implicate major changes in the composition of cell membrane and metabolism (Maier et al., 1999; Nielsen et al., 2005). However, the variety of mechanisms that could confer adaptation to organic solvents implies that bacterial solvent tolerance cannot only possibly be provided by a single one type mechanism (Heipieper et al., 2007). It is very likely that the combination of different metabolic strategies leads to cellular solvent-tolerance.

2.1. General stress response

There have been some reports in Gram-(+) bacteria describing that general stress stimuli which threaten the cell membrane and compromising cell viability, induce a large number of stress proteins with protective function (Petersohn et al. 2001; Van Schaik et al. 2004). In particular, sigma β regulon is activated under stress or energy limitations and inducing approximately 100 genes in *B. subtilis*. Initially it was postulated that *B. subtilis* sigma β genes are induced only in the stationary phase. However, more recently it was already demonstrated that sigma β activity is induced both upon entry into the stationary phase and under environmental stress such as salt, heat stress and/or ethanol shock during exponential growth phase (Hecker and Völker, 2001, Sardessai and Bhosle, 2002a). General stress proteins are proven to play a crucial role in protecting Gram-(−) bacteria from organic solvent shock, which strengthens the hypothesis that stress proteins could be involved also in the solvent tolerance mechanisms in Gram-(+) bacteria (Aono et al., 1994, 1998; Nakajima et al., 1995; Asako et al., 1997). A typical example is the *bmrUR* operon in *B. subtilis*, which is under sigma β control and encodes multidrug efflux proteins that will most likely contribute to solvent tolerance (Petersohn et al., 1999). In *Bacillus psychrosaccharolyticus* the Hsp33 stress protein is activated by oxidative stress but also was induced and activated under propan-2-ol and other solvents stress condition (Kang et al., 2007). Furthermore, when *B. psychrosaccharolyticus* Hsp33 protein was over-expressed in *E. coli*, improved stress tolerance to the organic solvent compared with the parental strain was observed.

2.2. Sporulation

One of the most distinctive characteristic in some Gram-(+) bacteria allowing them to survive under adverse environmental conditions is their ability to form endospores (Wipat and Harwood, 1999). Spores from *Clostridium* and *Bacillus spp.* are recognized as hard resistant life-forms able to survive under extreme environmental conditions such as heat, oxidative damage, desiccation and harsh chemicals including phenols and others. However, an almost full inhibition of the sporulation was demonstrated in different strains of *B. subtilis* by ethanol, but the growth was not abolished, only diminished (Bohin et al., 1976, Abate et al., 1999). Similar behavior was observed in the organic solvent-tolerant strain *B. licheniformis* S-86 in the presence of very toxic 3-methylbutan-1-ol (Torres et al., 2005). 0.4% Ethanol strongly depleted *B. licheniformis* S-86 sporulation by 10^4-fold, whereas its growth rate was only decreased by half (Torres et al., 2009a). Those results show that the sporulation process is highly sensitive to alcohols, and also probably to other organic solvents, and therefore seems not to intervene in solvent tolerance of *Bacillus* spp. growing cells.

The sporulation process of *Bacillus* and *Clostridum* species has some similarities but many differences too (Paredes et al., 2005). The best studied solventogenic *Clostridium* species are *C. acetobutylicum* and *C. beijerinckii* which are able to synthesize acetone, butanol and ethanol. Solvent production in *Clostridium acetobutylicum* is associated to the reconversion of acids and correlated with the starting point of the sporulation process. The key genes for solvent production in solventogenic *Clostridium* spp. are localized in the megaplasmid pSOL1 but it is activated by SpoOA, a gene located in the genome, that trigger the sporulation process (Cornillot et al., 1997). Also, it is well-known that asporogenous mutants of *C. acetobutylicum* are unable to synthesize solvents (Papoutsakis, 2008). Based on the present knowledge, the relationship between solvent toxicity and sporulation in the strictly anerobic solventogenic *Clostridum* species remains unclear.

2.3. Deactivation of organic solvents

Deactivation of solvent molecules through their emulsification, biodegradation or transformation was postulated to play a relevant role in diminishing solvent toxicity in Gram-positive bacteria (Abe et al., 1995; Sardessai and Bhosle, 2002a,b). At first, solvent biodegradation was considered to play a major adaptive role for some solvent-tolerant *Rhodococcus* and *Bacillus* strains (Paje et al. 1997; Sardessai and Bhosle 2002a; Wang et al., 2008). An example was described in the co-cultivation of starch-degrading *B. amyloliquefaciens* MIR-41 with the ethanol-producer Gram-(−) bacteria *Zymomonas mobilis*, wherein ethanol was assimilated by *B. amyloliquefaciens* concomitantly with the simultaneous depletion of cell growth and α-amylase production (Abate et al., 1999). Similarly, a 30% reduction of initial 3-methylbutan-1-ol concentration in the medium was detected in *Bacillus licheniformis* S-86 cultures supplemented with the alcohol, indicating the presence of metabolic pathways used for solvent bioconversion (Torres et al., 2009a). In addition, several organic solvents, including the 3-methylbutan-1-ol, increased the production of esterases in *B. licheniformis* S-86, suggesting the involvement of hydrolases in the mechanisms of organic solvent tolerance (Torres et al., 2005). Indeed, some of the 3-methylbutan-1-ol (1.1%) present in the culture medium, was transformed in a more hydrophobic and less toxic isoamyl acetate ester, suggesting that *B. licheniformis* S-86 esterases could contribute somehow in the tolerance to organic solvents (Torres et al., 2009a). Similar results were reported in *Bacillus pallidus* ST3 which was able to degrade high concentrations of isopropanol (Bustard et al., 2002). Additionally, some hydrolases from *Bacillus* species were reported very active in aqueous restricted environments, e.g. non-aqueous media, indicating that those microbes are armored genetically to challenge the effects of harsh organic solvents (Castro et al., 1992; Baigorí et al., 1996, Castro and Knubovets, 2003; Costas et al., 2008; Sana et al., 2007; Sareen and Mishra 2008; Gupta and Khare, 2009). However, considering the complexity and diversity of Gram-(+) microbial metabolism, solvent bioconversion could be just one of the mechanisms intervening in solvent tolerance, and probably combined with other adaptive physiological responses.

2.4. Changes in cell morphology

Modifications in the overall cell morphology were reported in Gram-(−) microorganisms in response to stressful environments including organic solvents (Shi and Xia, 2003; Neumann et al., 2005). This behavior was observed as well in some solvent-tolerant Gram-(+) bacteria (Maier et al., 1999; Nielsen et al., 2005). *B. licheniformis* S-86 showed an extensive filamentous growth in the presence of 0.6% 3-methylbutan-1-ol supplemented cultures (Fig. 1) (Torres et al., 2009a). The response observed in *B. licheniformis* S-86 when grown in the presence of toxic 3-methylbutan-1-ol, was a cell morphology change with an increase in cell volume (4.7-fold). Previous studies have already described changes in cell size as an adaptive mechanism for several

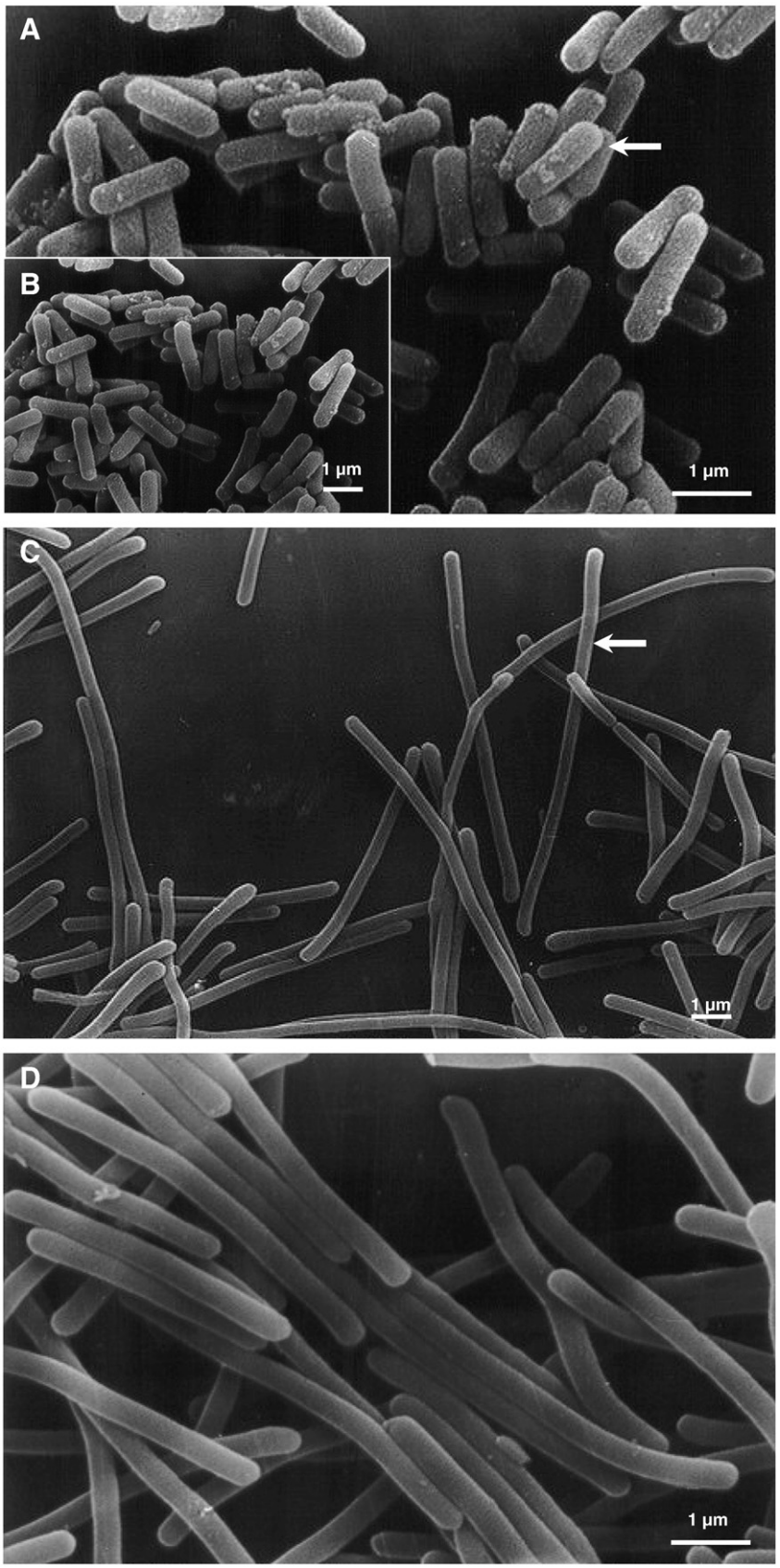

Fig. 1. SEM photographs of exponentially growing cells of *B. licheniformis* S-86 in control medium (A; magnification: 12,000 times, B; magnification: 6000 times); in the presence of 0.6% (v/v) 3-methlbutan-1-ol (C; magnification: 6000 times, D; magnification: 12,000 times) (Torres et al., 2009a). Arrows: A) Rod-shaped cell with a rough surface. C) Elongated cell, an almost smooth surface was observed compared to control cells.

bacteria under stress conditions (Neumann et al., 2005; Chakravarty and Banerjee, 2008). In *B. licheniformis* S-86, the decrease in cell surface-to-volume ratio renders it a minor attachable surface exposition to toxic compounds and could contribute to increase the effectiveness of other solvent tolerance mechanisms, such as solvent efflux pumps (Volker et al., 1999). Transmission electron microscope examination of *B. licheniformis* S-86 structures indicated the presence of septa and frequent cellular division in control cells. However, in the presence of 3-methylbutan-1-ol septa formation was not frequent and cytoplasmic membrane division was not accompanied by cell wall partition, which appears extremely thick at this site (Fig. 2) (Torres et al., 2009a). Recently, cell filamentation was suggested as a possible physiological mechanism for bacteria, including *Bacillus* spp., exposed to stress environments. These mechanisms could be summarized by interference of cell division gene expression, and possible SOS response (Mattick et al., 2000). Coincidently, induction of heat shock proteins, which could inhibit cell division, was reported in response to primary alcohols in *Acinetobacter* sp. (Benndorf et al., 1999). From another point of view, filamentation is an alternative to reduce the exposition of cell surface to environmental stressing agents, and could also possibly be a mechanism to exchange valuable information among the cell community.

Another kind of morphological change was observed in *Staphylococcus* sp. ZZ1 in response to toluene, which produced an unusual extracellular capsule (Zahir et al., 2006). The hydrophilic carbohydrate capsule repels organic solvents and prevents them from reaching the cell membrane. However, *Staphylococcus* sp. ZZ1 exhibits tolerance to toxic organic solvents under solvent shock conditions without previous adaptation (e.g. capsule synthesis) which suggests the presence of other physiological mechanisms that make these Gram-(+) bacteria solvent tolerant. Also, an increased production of the capsular polymer emulsan was observed in cultures of Gram-(−) bacteria *Acinetobacter venetianus* Rag-1 supplemented with ethanol as sole carbon source (Panilaitis et al., 2007). Those similar findings in morphology and capsule production among a Gram-(+) and Gram-(−) bacteria are indicative of common survival strategies.

2.5. Cell surface modification (hydrophobic shift)

Hydrophobicity of bacterial cell envelope has great influence on the cell's ability to adhere to surfaces or substrates, including the binding to toxic compounds. A change in the surface hydrophobicity of Gram-(−) bacteria exposed to toxic organic solvents was already observed in detail (Aono and Kobayashi, 1997; Sardessai and Bhosle, 2004, Neumann et al., 2005). Moreover, this adaptive response was described in Gram-(+) bacteria to challenge toxic effects of organic solvents. Gram-(+) *B. licheniformis* S-86 is not able to grow in the presence of organic solvents naturally and show little affinity toward an entirely non-polar solvent like hexane (log P 3.9), preventing this hydrophobic organic solvent from binding abundantly to the cell surface (Torres et al., 2009a). However, in the presence of a less hydrophobic organic solvent like 3-methylbutan-1-ol (log P 1.3), a small, but significantly higher affinity for the alkanol was observed in control cells (Torres et al., 2009a). This was in agreement with a previous study which found that organic solvents with lower log P (more hydrophilic) bound more abundantly to cells surfaces (Aono and Kobayashi, 1997). When *B. licheniformis* S-86 was cultured in the presence of organic solvents, cell surfaces became less hydrophobic compared to that of the control cells, and exhibited little affinity towards 3-methylbutan-1-ol. A minor hydrophobicity motif in the cell wall shall prevent the alcohol from reaching and binding to the cell membrane, allowing the cell wall to act as a permeation barrier which repels hydrophobic compounds. This response to 3-methylbutan-1-ol is consistent with a previous finding in *E. coli* K-12 mutants, which had a cell surface less hydrophobic compared to the parental cells, and displayed higher tolerance to organic solvents (Aono and Kobayashi, 1997). On the contrary, an increased hydrophobicity in the Gram-positive *Mycobacterium frederiksbergense* growing in the presence of anthracene instead of glucose was reported. The cell high affinity for anthracene as a unique carbon source involves an augmented surface hydrophobicity induced by a degradative aromatic pathway to metabolize it (Wick et al. 2002; Yamashita et al., 2007). On the other side, bacteria harboring quite hydrophobic envelopes, such as solvent-tolerant *Mycobacterium* sp. and *R. erythropolis*, showed cell aggregates in the presence of butan-1-ol. In this case, the hydrophobic character of both strains promote cellular aggregation as a way to diminish the binding of toxic hydrophilic compounds (de Carvalho et al., 2004).

2.6. Cell membrane adaptations

The main mechanism is known as "homeoviscous adaptation" which involves the increase the degree of membrane fatty acid saturation and consequently enhancing its rigidity in the presence of solvents. The homeoviscous adaptation mechanism was commonly reported in Gram-(−) bacteria in the presence of toxic organic solvents (Sinensky 1974; Heipieper et al. 1994; Heipieper and de Bont 1994). However, different physiological responses were observed in Gram-(+) bacteria exposed to toxic solvents. For example, *Staphylococcus haemolyticus*

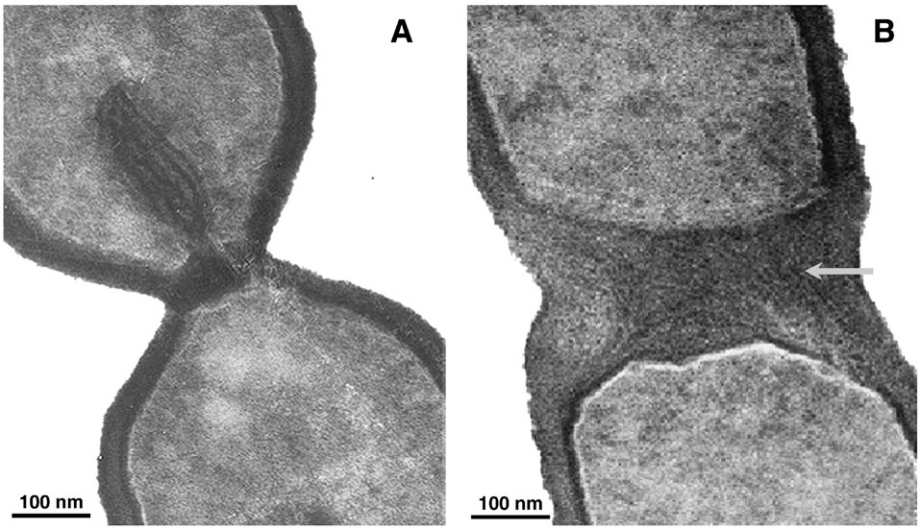

Fig. 2. TEM photographs (magnification: 140,600 times) of exponentially growing cells of *B. licheniformis* S-86 in control medium (A), and in the presence of 0.6% (v/v) 3-methlbutan-1-ol (B) (Torres et al., 2009a). Arrow: B) Extremely thick cell wall in the cell division site, the division of cytoplasmic membrane was not accompanied by cell wall partition.

cultivated in the presence of toluene, benzene or ciclohexane increased its membrane fluidity by the changing fatty acid composition (Nielsen et al., 2005). Regarding other Gram-(+) bacteria, in *Bacillus* sp. ORAs2 and *Rhodococcus erythropolis* DCL14, addition of toluene in the first one, and alkanes, alkanols and terpenes in the second one, caused a dose-dependent increase in the degree of saturation of the membrane fatty acids as usually described in Gram-negative bacteria (de Carvalho et al. 2005; Pepi et al., 2008). On the contrary, *R. erythropolis* DCL14 cultivated in the presence of short-chain alcohols, like ethanol, caused a concentration-dependent decrease in the membrane degree of saturation. These differences in *R. erythropolis* response are in agreement with those formerly published for some Gram-(−) bacteria. It has been observed that *Escherichia coli* and *P. putida* react to the presence of long-chain alcohols by increasing their degree of saturation (Ingram 1976), meanwhile in the presence of short-chain alcohols, e.g. ethanol, the degree of saturation decreases (Ingram 1976; Heipieper and de Bont 1994). According to Kabelitz et al. (2003) difference in the adaptive response towards alcohols is related to the physico-chemical properties of short-chain alcohols, which can only penetrate slightly into the hydrophobic center of the phospholipid bilayer, causing a swelling effect on the hydrophilic headgroups (Weber and de Bont 1996). To counteract this effect, the insertion of unsaturated fatty acids seems to be a better reaction against those short-chain alcohols. In contrast, long-chain alcohols and aromatic solvents, which are more hydrophobic, penetrate deeply into the membrane, thus causing an increase in the degree of saturation (Ingram 1976; Weber and de Bont 1996).

Additionally, changes in membrane protein composition were reported in Gram-(+) bacteria under solvent stress. Membrane proteomes of wild-type and ethanol-adapted *Clostridium thermocellum* 27405 were quite dissimilar and some of the specific changes may provide an advantage to deal with the alcohol (Islam et al., 2009). Approximately 60% of the proteins identified from *C. thermocellum* 27405 purified membrane fractions were observed to be differentially expressed regarding to the wild-type strain. The majority (73%) of differentially expressed proteins were down-regulated in the ethanol-adapted strain. Many of these down-regulated proteins were involved in the carbohydrate transport and metabolism. These membrane-associated proteins in the ethanol-adapted strain could either be synthesized in lower quantities or not properly incorporated into the cell membrane. Between the few up-regulated proteins in ethanol-adapted cells, several magnesium transporters were identified. Enhancement of transporter expression possibly increases the rate of incorporation of magnesium and probably others, in order to compensate a large leak of ions caused by ethanol (Islam et al., 2009).

2.7. Solvent excretion through efflux pumps

Many solvent-efflux pumps involved in solvent tolerance in Gram-(−) bacteria have been identified in the last decade, mainly in *Pseudomonas* spp. and *E. coli* (Asako et al., 1997; Kieboom et al., 1998; Li et al., 1998; Ramos et al., 1998; Kobayashi et al., 2001). These proton motive force-driven efflux systems are able to export molecules across the membrane diminishing solvent concentration in the cytoplasm (Heipieper et al., 2007). The possibility that these efflux pumps play an important role in organic solvent tolerance in Gram-(+) bacteria also cannot be neglected. For example, a hydrocarbon-pumping activity was reported in *B. cereus* R1 (Matsumoto et al., 2002). This energy-dependent mechanism, either secondary transporters or ATP-binding cassette type transporters, possibly prevent the intracellular accumulation of toluene in *B. cereus* R1 cells and could be responsible in part of the organic solvent tolerance observed in this strain (Matsumoto et al., 2002).

3. Isolation of organic-solvent-tolerant Gram-positive bacteria

From the historic point of view, solvent-tolerant bacteria were predominantly Gram-(−). Due to the additional outer membrane present in these bacteria, they might be assumed as better armored to thrive toxic organic solvents than Gram-(+) ones (Isken and de Bont, 1998). Nevertheless, numerous strains of Gram-(+) bacteria showing excellent tolerance to highly toxic organic solvents have been reported up to the present (Segura et al., 2008). Most of these reported microorganisms have been isolated from natural and pristine soil samples such as forest, and cultivation soils (Baigorí et al., 1996; Huertas and Duque, 1998) or deep sea and coastal mud and water samples (Kato et al., 1996). Most of these strains were identified as belonging to the genera *Bacillus*, *Rhodococcus*, *Clostridium*, *Enterococcus*, *Arthrobacter*, *Lactobacillus* and *Staphylococcus*. Isken and de Bont (1998) isolated five *Bacillus* strains that were able to withstand a second phase of toluene from bank-soil. Chloroform-tolerant *Bacillus* sp. BC1, able to degrade cholesterol, was isolated from Arabian Sea sediment (Sardessai and Bhosle, 2003). Segura et al. (2008) isolated three Gram-(+) toluene-tolerant marine bacteria (*Exiguobacterium* sp. EEZMo-1, *Bacillus* sp. EEZMo-2 and *Bacillus* sp. EEZMo-3) from harbor and beach waters of Granada (southern Spain). *Bacillus* sp. EEZMo-3 was more tolerant to different organic solvents than some Gram-(−) marine strains, and was also able to survive after the addition of 100% benzene, a compound that killed the highly solvent-tolerant Gram-(−) *P. putida* DOT-T1E (de Carvalho et al., 2005).

Soil and aquatic environments have been contaminated for decades with several organic solvents, particularly hydrocarbons. Taking advantage of this situation several resistant strains, including *Rhodococcus spp*., were isolated from contaminated areas (Zahir et al., 2006). A typical example is the benzene-tolerant *Rhodococcus* sp. strain 33 isolated from a contaminated site in Australia on benzene (Paje et al., 1997). *Rhodococcus opacus* B-4, B-9 and B-10 strains, also tolerant to high benzene concentrations, were isolated from a gasoline contaminated soil sample (Na et al., 2005). *R. opacus* B-4 was highly tolerant to a variety of organic solvents including *n*-alkanes and mono-aromatics, and able to survive with remarkable metabolic activity in neat organic solvents (Sameshima et al., 2008). *Bacillus* sp. ORAs2, an arsenic-resistant bacterium was isolated from polluted sediments of the Orbetello Lagoon (Tuscany, Italy), and was able to grow in the presence of toluene and benzene (Pepi et al., 2008).

Other solvent-tolerant bacteria were isolated from unique ecological niches like oil fly larval guts (Nielsen et al., 2005). Larvae of the oil fly, *Helaeomyia petrolei*, are found exclusively submerged in oil, where they ingest large quantities of oil and asphalt. Thus, any bacteria isolated from oil fly larval guts have been naturally selected for solvent tolerance. Using positive solvent selection three Gram-(+) strains were isolated from larval guts. *Enterococcus faecalis* and *Clostridium sporogenes* were isolated in media supplemented with 5% (vol/vol) acetone or butanol, and *S. haemolyticus* in media overlaid with 15% benzene/85% cyclohexane. *S. haemolyticus* were able to grow in the presence of cyclohexane, benzene, and toluene, both in monophasic liquid cultures and in organic solvent plate overlays (Nielsen et al., 2005). Another solvent-tolerant bacterium isolated *via* solvent selection was *B. licheniformis* S-86 (Torres and Castro, 2003). *B. licheniformis* S-86 showed the rare ability to grow at 55 °C in the presence of C_2–C_5 alkanols (log P = −0.86 to 2.39), and salinity concentrations up to 15% (w/v), all typical characteristics of extremophiles (Torres et al., 2005 and 2009b). Unlike *B. licheniformis* S-86, toluene-tolerant *Bacillus cereus* R1, isolated in an atmosphere saturated with toluene, grew in the presence of hydrocarbons but not when aliphatic alcohols were present (Matsumoto et al., 2002). Other authors used the solvent selection procedure mixing samples with organic solvents before cultivation. Kato et al. (1996) added 50% (v/v) benzene to samples of deep sea sediments. After seven days of incubation at room temperature a portion of each benzene layer was spread on a suitable agar medium. Using this procedure several strains of Gram-(+) organic solvent-tolerant bacteria were isolated, such as benzene-tolerant *Arthrobacter* ST-1, which degrades cholesterol, *Bacillus* sp. DS-994, which utilizes sulfur compounds, and *Bacillus* sp. DS-1906, able to degrade polyaromatic hydrocarbons (Moriya and Horikoshi,

1993; Abe et al., 1995, Kato et al., 1996). For the isolation of solvent-tolerant *Bacillus* sp. SB1, Sardessai and Bhosle (2002b) kept soaked mangrove sediment with *n*-butanol for a month. A portion of this sediment was transferred to sea water supplemented with 20% (v/v) *n*-butanol during one week after which the organic layer was transferred to a medium overlaid with 50% (v/v) of the solvent.

Some strains of Gram-positive bacteria were found to be solvent-tolerant after adaptation to the stress stimuli. *C. thermocellum* strain 27405 was able to gradually adapt to alcohol when sequentially transferred into medium containing increasing concentrations of ethanol (Islam et al., 2009). This stepwise adaptation eventually gave rise to cells that tolerated up to 8% (w/v) ethanol. This adaptation to ethanol is apparently a stable strait for this strain because adapted cultures retain tolerance even after the growth of more than 2000 generations in the absence of ethanol. Other microorganisms, such as *R. erythropolis* DCL14, were found to be solvent-tolerant after their isolation (de Carvalho et al., 2005). This strain was able to grow on alcohols ranging from C_1 to C_{12}, hydrocarbons and terpenes, as sole carbon and energy sources. In certain species of *Lactobacillus*, lactic acid is produced by fermentation in the presence of several alchohols and alkanes (Matsumoto et al., 2004).

4. Biotechnological potential of organic solvent-tolerant Gram-positive bacteria

The main application of solvent-tolerant Gram-(+) microorganisms is non-aqueous biocatalysis (Ogino and Ishikawa, 2001; Gupta and Khare, 2009). Since their enzymes are stable, some of them are extracellular, and retain a high level of catalytic activity in non-aqueous environments, these enzymes and, even whole-cells, can be used as biocatalysts in organic solvents for industrial relevant biotransformations (Castro et al., 1992; Baigorí et al., 1996; Tang et al., 2008; Pera et al., 2008; Torres et al., 2010). Furthermore, solvent-tolerant bacteria can be an alternative tool for bioremediation of solvent polluted ecosystems (Gupta and Khare, 2009).

4.1. Enzymes from solvent-tolerant Gram-positive bacteria

Enzymes synthesized by extremophile microorganisms offer new opportunities for the Green Chemistry arena wherein biocatalysis and biotransformations play a central role. Table 2 showed some organic solvent-stable enzymes from solvent-tolerant Gram-positive microorganisms. The vast majority of synthetic enzyme reactions are performed in organic media. Biotransformation in organic solvents offer unique industrially attractive advantages compared to traditional aqueous enzymology, that can be summarized as: (*i*) increased solubility of non-polar substrates and products, which markedly speeds up overall reaction rates; (*ii*) reversal of thermodynamic equilibrium in favor of synthesis over hydrolysis, allowing reactions usually not favored in aqueous solutions to occur (e.g. transesterification, thioesterification, aminolysis); (*iii*) drastic changes in the enantioselectivity of the reaction when one organic solvent is changed to another; (*iv*) suppression of unwanted water-dependent side reactions, which often degrade common organic reagents; and (*v*) elimination of microbial contamination in the reaction mixture (Torres and Castro, 2004). By this, it is obvious that the enzymes produced by solvent-tolerant bacteria that have been most studied are mainly those wherein the reverse reactions are of industrial interest or the substrates are barely soluble in aqueous systems (Gupta and Khare, 2009). Amongst these enzymes, hydrolases are the most studied especially carboxylesterases, lipases and proteases. Useful reactions performed by hydrolases include resolution of racemic mixtures by transesterification, enantio- and regioselective hydrolysis and synthesis of natural and non-natural pro-drugs, detergents, polyesters, peptides, and additives (Gupta and Roy, 2004, Illanes, 2008).

Conversion of R,S-naproxen esters to S-naproxen, the synthesis of cephalosporin-derived antibiotics, and selective conversion of heroin into morphine are typical examples of industrial applications of carboxylesterases in non-aqueous media (Bornscheuer, 2002). However, most of these reactions are restricted to commercially available sources from fungi, yeasts, and bacteria. Carboxylesterase from solvent-tolerant *B. licheniformis* S-86 was tolerant to a wide variety of organic solvents, but also was moderately thermostable, stable under extreme pH values, high salt concentrations, and ionic and non-ionic detergents (Torres et al., 2008, 2009b, 2010). These properties make this enzyme suitable as biocatalyst in non-aqueous processes wherein enzyme stability is a must. This enzyme was able to synthesize isoamyl acetate from 3-methylbutan-1-ol (isoamyl alcohol) and *p*-nitrophenyl acetate (acyl donor) in *n*-hexane (Torres et al., 2010). The resulting ester yield, obtained at a low temperature (28 °C) and with a very low amount of enzyme, indicates a high potential for this esterase in synthesis of a valuable flavor compound with a great application in food industries.

Lipases are being used in non-aqueous media for the production large amounts of compounds from fine chemicals to bulk fuels like flavor esters, pharmaceutical drugs, structured lipids, and biodiesel (Bosley, 1997; Gaur et al., 2008; Jaeger and Reetz, 1998; Khare et al., 2000). Amongst Gram-(+) bacteria, most solvent-stable lipases have been isolated from *Bacillus* genera. Benzene and toluene-tolerant *B. sphaericus* strain 205y produces an organic solvent-stable lipase (Hun et al., 2003). This lipase activity was enhanced 2.9- and 3.5-folds in *p*-xylene and *n*-hexane respectively and therefore could be useful in catalyzing esterification and transesterification reactions in organic media. Another benzene and toluene-tolerant strain, *Staphylococcus saprophyticus* M36, also produces a solvent-stable lipase (Fang et al., 2006). The lipase of strain M36 was stable in the presence of hydrophobic solvents, but also in the presence of hydrophilic ones such as methanol and ethanol, which could make this enzyme useful for biodiesel production.

Lipase from *Brevibacillus agri* 52 was found stable in up to 90% diethylenglycol, glycerol, and 1,2 propanediol at 37 °C for at least 1 h and the stability was reduced only approximately 20% after 12 h incubation, but in 40% dimethylsulfoxide, lipase activity was stable only for 1 h. In water immiscible systems like n-hexane, n-tetradecane and n-heptane, the stability of lipase resembles the water activity. Additionally, enzyme activity can be enhanced in organic solvents systems by encapsulation of *B. agri* 52 lipase in pectin gels which brings about three to four times more enzymatic activity in 70% water-miscible organic solvents compared to aqueous systems

Table 2
Organic solvent-stable enzymes from some solvent-tolerant Gram-positive strains.

Enzyme	Microorganism	Solvent stability/applications	References
Type II esterase	*B. licheniformis* S-86	*n*-Hexane, glycerol, ethylenglycol, propylenglycol/isoamyl acetate synthesis in *n*-hexane	Torres et al., 2008, 2009a
Lipase	*S. saprophyticus* M36	Benzene, toluene, *p*-xylene, *n*-hexane	Fang et al., 2006
Lipase	*B. sphaericus* 205y	*n*-Hexane, *p*-xylene	Hun et al., 2003
Metalloprotease	*B. cereus* BG1	DMSO, DMF, methanol, ethanol, isopropanol	Ghorbel et al., 2003
Protease	*B. licheniformis* RSP-09-37	Acetonitrile/dipeptide kyotorphin precursor synthesis in acetonitrile	Sareen et al., 2004
Protease	*B. pumilus* 115b	Benzene, toluene, *n*-hexane, 1-decanol, isooctane, *n*-dodecane, *n*-tetradecane	Rahman et al., 2007
Glucanotransferase	*Paenibacillus illinoisensis* ST-12 K	Toluene, benzene, cyclohexane, *p*-xylene, alcohols/cyclodextrin synthesis in ethanol	Doukyu et al., 2003

(Costas et al., 2008). These properties are making the *B. agri* 52 lipase a "multipurpose" biocatalyst type able to work under diverse non-aqueous scenarios.

Microbial proteases have attracted considerable attention due to commercial application of peptide and ester synthesis in non-aqueous environments. *Bacillus* strains have been found to produce solvent-stable proteases with potential industrial applications (Ferrero et al., 1996; Castro, 1999; Ghorbel et al., 2003; Sareen et al., 2004; Rahman et al., 2007). Some proteases exhibit stability towards hydrophobic solvents (Rahman et al., 2007); whereas, other showed stability towards hydrophilic solvents such as alcohols (Ghorbel et al., 2003) and neat glycerol (Castro, 1999). Sareen et al. (2004) have reported a psychrophilic and acetonitrile-stable protease secreted by the organic solvent-tolerant *B. licheniformis* RSP-09-37 strain. This protease was stable even at 90% acetonitrile and showed significantly better performance in the synthesis in this media of the analgesic dipeptide kyotorphin precursor compared to the commercially available mammalian α-chymotrypsin.

The organic solvent-tolerant *Paenibacillus illinoisensis* strain ST-12 K synthesizes a cyclodextrin glucanotransferase (CGTase) stable and active in the presence of large amounts of toluene, benzene, cyclohexane, *p*-xylene, and some alcohols (Doukyu et al., 2003). CGTase produces cyclodextrins from starch *via* intramolecular transglycosylation. The yield of cyclodextrin production was increased 1.4-fold by the addition of ethanol (Doukyu et al., 2003). This solvent-stable CGTase should be useful for technological applications in organic solvents since improvement of the cyclodextrins production is a must for large scale.

4.2. Whole-cell biocatalysis in organic solvent systems

In some cases the use of whole-cells as biocatalysts may have advantages over the use of enzymes. In Table 3 many organic solvent-tolerant Gram-(+) microorganisms and their applications in whole-cell biotransformations are shown. The cells can withstand exposure to the solvent, acting as a protective barrier for the enzymes. Besides, the use of cells may be easier and economically favorable when the synthesis reaction requires expensive co-enzymes or co-factors and multiple enzymatic reactions are involved (Heipieper et al., 2007). In such cases, the maintenance of the required biocatalytic activity is as relevant as the survival of the cell.

Organic solvent-tolerant Gram-positive bacterium could be successfully used to solve the problem of low solubility of steroids in aqueous media. Steroid transformation is of great importance in the pharmaceutical industry. Steroids like cholesterol are completely soluble in some organic solvents such as chloroform and 1-butanol. Marine *Bacillus* sp. BC1 were capable of transforming cholesterol in a biphasic chloroform–water (1:1) system (Sardessai and Bhosle, 2003). In addition, *Arthrobacter* sp. ST-1 was able to convert cholesterol into androsta-1,4-diene-3,17-dione, in a biphasic organic–aqueous system with n-decane or n-dodecane (Moriya et al., 1995). Recently, another *Bacillus* sp. strain SB1 was found effective to biotransform cholesterol into cholest-4-ene-3,6-dione (Sardessai and Bhosle, 2003). In this biphasic biotransformation system cholesterol was dissolved in 50% chloroform and cells were suspended in phosphate buffer.

R. erythropolis DCL14 cells were successfully used for the bioconversion of (−)-carveol to (−)-carvone, a terpene with multiple applications in the food and flavor industry (de Carvalho and da Fonseca, 2002). The conversion was carried out in *n*-dodecane-water (1:1 and 1:5) systems. In these reaction systems, cell viability could be maintained for nearly 1 month by fed-batch operation (de Carvalho and da Fonseca, 2002). Torres et al. (2009a) showed the potential utility of *B. licheniformis* S-86 for the production of banana flavor compound widely used in the food industry. *B. licheniformis* S-86 was able to produce isoamyl acetate from isoamyl alcohol with a similar or higher yield than other microorganisms (Torres et al., 2009a).

4.3. Organic solvent-tolerant Gram-positive bacteria in bioremediation

The existence of contaminated areas with highly toxic solvents, such as benzene and toluene and others is a clear indicative of the lack of biological systems that can efficiently degrade these compounds (Paje et al., 1997; Sardessai and Bhosle, 2004; Kongpol et al., 2009). This situation has stimulated in recent years the search for solvent-tolerant microorganisms that have the catabolic potential necessary to remove at high rates these toxic compounds (Sardessai and Bhosle, 2004). Table 3 also shows organic solvent-tolerant Gram-(+) microorganisms with the capacity to biodegrade toxic compounds.

Amongst Gram-(+) bacteria, strains belonging to the *Rhodococcus* genus are known for their excellent tolerance to organic solvents and its ability to degrade hydrocarbons, and hence its potential capacity for soil or water remediation and waste-stream purification. For instance, *Rhodococcus* sp. strain 33 can tolerate and degrade high concentrations of benzene (Paje et al., 1997). 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. *R. erythropolis* DCL14 was able to biodegrade a wide variety of hydrocarbons and alcohols as sole carbon and energy sources. Almost complete degradation of *n*-, *iso*- and

Table 3
Organic solvent-tolerant Gram-positive microorganisms and their applications.

Microorganism	OS tolerance	Applications	References
Bacillus sp. DS-994	Benzene	Utilizes sulfur compounds (dibenzothiophene and thiophene)	Moriya and Horikoshi, 1993
Bacillus sp. DS-1906	Benzene	Degrades naphthalene	Abe et al., 1995
Arthrobacter sp. ST-1	Benzene	Converts cholesterol to androsta-1,4-diene-3,17-dione	Kato et al., 1996
Rhodococcus sp. 33	Benzene	Degrades benzene	Paje et al., 1997
Bacillus pallidus ST3	2-propanol	Utilizes 2-propanol and acetone	Bustard et al., 2002
Rhodococcus erythropolis DCL14	Alcohols	Converts (−)-carveol to (−)-carvone. Degrades hydrocarbons and alcohols	de Carvalho and da Fonseca, 2002; de Carvalho and da Fonseca, 2005
Bacillus sp. BC-1	Chloroform	Transforms cholesterol	Sardessai and Bhosle, 2003
Bacillus sp. SB-1	*n*-butanol	Transform cholesterol to cholest-4-ene-3,6-dione. Utilizes *n*-butanol, benzene and toluene.	Sardessai and Bhosle, 2003
Clostridium acetobutylicum	*n*-butanol	Degrades *n*-butanol	Tomas et al., 2004
Bacillus licheniformis S-86	Ethanol, propanol, *n*-butanol, 3-methylbutan-1-ol	Isoamyl acetate synthesis	Torres et al., 2005; 2009a
Staphylococcus haemolyticus	Toluene, benzene, *p*-xylene	–	Nielsen et al., 2005
R. opacus B-4	Benzene	Utilizes many aromatic and aliphatic hydrocarbons	Na et al., 2005

OS, organic solvent.

cyclo-alkanes and aromatic compounds present in fuel oil was achieved after 9 months, more than half being consumed in the first three months (de Carvalho and da Fonseca, 2005). Na et al. (2005) isolated a benzene-tolerant *R. opacus* B-4, which could utilize many aromatic and aliphatic hydrocarbons including benzene, toluene, styrene, xylene, ethylbenzene, propylbenzene, *n*-octane and *n*-decane as sole sources of carbon and energy. Genetic analysis made to strain B-4 revealed that a benzene dioxygenase pathway is involved in benzene catabolism.

Many *Bacillus* strains were also able to degrade toxic organic solvents. *Bacillus* sp. DS-1906 can biodegrade polyaromatic hydrocarbon in the presence of organic solvent, being able to convert almost 50% of the naphthalene solubilized in *n*-hexane (Abe et al., 1995). Solvent-tolerant and desulfurizing *Bacillus* sp. DS-994 was able to utilize organic sulfur compounds present in petroleum such as dibenzothiophene and thiophene in biphasic water–petroleum systems (Kato et al., 1996). The aerobic biodegradation of a high-concentration of hydroxylic solvents was achieved by thermophile and solvent-tolerant *B. pallidus* ST3 (Bustard et al., 2002). *B. pallidus* ST3 utilize high concentrations of 2-propanol and acetone at 60 °C, with no further additional carbon supplementation, indicating its potential for the bioremediation of hot solvent-containing industrial waste streams. Hydrocarbon degrader *Bacillus* sp. SB-1 was capable of growth with 1-butanol, benzene or toluene serving as the sole carbon source (Sardessai and Bhosle, 2002b). Also, solvent-tolerant *Clostridium* species were recognized for their ability to biodegrade *n*-butanol and toxic nitro-aromatics such as 2,4,6-trinitrotoluene (Spain, 1995; Hughes et al., 1998; Watrous et al., 2003; Tomas et al., 2004). Because of the potential health risks and its influence on photochemical smog, the removal of 1-butanol and other volatile organic compounds from industrial waste streams is highly desirable (Gupta and Khare, 2009).

5. Concluding remarks

Over the last twenty years, biocatalysis in organic solvents has emerged as an area of systematic research and industrial development, stimulated mainly by chemical and pharmaceutical interest. However, the stability and efficiency of enzymes in organic solvents, remains a necessary requisite for such applications. Organic solvent-tolerant bacteria could represent a solution to this problem, producing enzymes capable of catalyzing under very extreme physicochemical conditions, like in organic solvents. In addition, solvent-tolerant microorganisms are also useful in biotransformations and/or bioremediation processes with the participation of whole cells.

In this context, high-enzyme producer Gram-(+) bacteria were underestimated in regard to organic solvent tolerance and capabilities. Some Gram-(+) bacteria, such as *Bacillus* and *Lactobacillus* species have the advantages of extracellular enzyme production and lack of toxicity (most of them are considered GRAS, Generally Regarded as Safe by FDA, USA). Despite this, the majority of studies done on the solvent tolerance were conducted in Gram-(−) bacteria. Nevertheless, numerous studies concerning solvent tolerance mechanisms of Gram-positive bacteria and the properties of their enzymes were recently performed. But more studies need to be undertaken to elucidate what makes Gram-positive bacteria and their enzymes withstand such severe stress.

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