CHAPTER V

DISCUSSION

Organic solvent-tolerant bacteria are a relatively novel group of extremophilic microorganisms that combat destructive effects of toxic organic solvent and thrive in the presence of high concentrations of organic solvents. These bacteria are being explored for their potential in industrial and environmental biotechnology. This property could be exploited to carry out in bioremediation and as a biocatalysis in the presence of an organic phase. The first report of an organic solvent-tolerant bacterium was by Inoue and Horikoshi in 1989 (Inoue and Horikoshi,1989). They discovered a strain of *Pseudomonas putida* IH-2000 which could actively grow and multiply in the presence of 50% (v/v) toluene. This surprising observation was confirmed by others (Hirayama *et al.*,1998; Inoue and Horikoshi,1989; Inoue *et al.*,1991; Ramos *et al.*,1995; Weber *et al.*,1993) and the search to uncover the mechanisms behind this extraordinary characteristic began. A large number of the reported organic-solvent-tolerant bacteria are *Pseudomonas* strains, especially *P. putida* (Bernal *et al.*,2007; Faizal *et al.*,2005; Hirayama *et al.*,1998). Organic solvent-tolerant bacteria can be isolated from soil. Enrichment technique was applied to increase quantity of organic solvent-tolerant bacteria (Na *et al.*,2005). Solvent tolerant bacteria have been isolated from ecological niche such as soil or deep sea. The most organic solvent-tolerant bacteria species are gram-negative such as genera *Pseudomonas* (Inoue and Horikoshi,1989; Ramos *et al.*,1995) and *E. coli* (Aono,1998). Gram positive organisms have been reported to exhibit organic solvent tolerance including species of *Bacillus* (Isken and de Bont,1998), and *Rhodococcus* (Paje *et al.*,1997).

In this investigation, thirty six organic-solvent tolerant bacteria were isolated at 45°C in the presence of either toluene or cyclohexane vapor as a sole carbon source. Of 36 isolates, there were 3 gram-negative isolates and 33 gram-positive isolates, of which the majority was the genus *Bacillus*. The results suggest that organic solvent-tolerance gram-positive bacteria can be isolated from soil more than organic-solvent tolerance gram-negative bacteria. These results were in agreement with a recent report by Zahir et al., (2006)(Zahir *et al.*,2006). From 36 isolates, four isolates were chosen for further studies according to their characteristics obtained from primary and secondary test. They could utilize and were tolerant to various organic solvents and were able to grow in the presence of organic solvent directly overlaid as a second layer onto the minimal agar and as a second phase on liquid cell culture.

Organic solvent tolerant *Bacillus pallidus* can utilize 2-propanol at 60°C, therefore it has potential of the in bioremediation of hot solvent-containing industrial waste streams (Bustard *et al.*,2002). Thermophilic saccharide fermentations have been suggested as potential novel system for industrial ethanol of which *Clostridium thermohydrosulfuricum* can grow and tolerate to various organic solvents at 60°C. Obviously thermophilic organic solvent have a potential usefor biotechnology application and remediation.

A thermo-tolerant, organic-solvent tolerance, gram-negative *Deinococcus geothermalis* T27 gram-negative bacteria exhibited an intriguing ability to tolerate a broad range of organic solvent (log P_{ow} value among 0.7 to 5.6). The log P_{ow} is a reliable for the toxicity of organic solvent (Inoue and Horikoshi,1991). Organic solvents with log P_{ow} values between 1 and 5 (Cruden *et al.*,1992) or log P_{ow} 1.5-3 (Ramos *et al.*,1997), are highly toxic to microorganisms. When exposed to organic solvent with log P_{ow} value greater than 3, *n*-decane (5.6) and diethyl phthalate (3.3) cells of *D. geothermalis* T27

slightly decreased suggesting a relatively high tolerance of cells. For log P_{ow} value ranging from 1.5-3, *D. geothermalis* T27 was able to survive in the presence of toluene and benzene, while it was impeded to do so in the presence of styrene and xylene. These results were in agreement with a report of organic solvent-tolerance *P. putida* strain IH-2000 capable of growth in a culture medium containing more than 50% (v/v) toluene (Hirayama *et al.*,1998), *P. putida* Idaho which able to utilize toluene, *m*-xylene, *p*-xylene, in a two-phase system at 5 to 50% (v/v) (Cruden *et al.*,1992), *P. putida* strain T-57 able to grow on toluene when liquid toluene was added to mineral salt basal medium at 10-90% (v/v) (Faizal *et al.*,2005), and *Flabobacterium* sp. from deep sea which could tolerate the exposure of benzene (5% v/v) and toluene (10% v/v) (Moriya and Horikoshi,1993).

Further investigation on solvent tolerance of *D. geothermalis* T27 was extended using solvent with log P_{ow} value below 2.0; butyl acetate (1.8), n-butanol (0.8), ethyl acetate (0.7). It has been known that the solvent with a log P_{ow} value below 2.0 is extremely toxic to bacterial cell. Recently, it has been reported that *Enterobacter* sp.VKGH12 exhibits tolerance to *n*-butanol up to 1.5% (v/v) (Veeranagouda *et al.*,2006). *D. geothermalis* T27 was shown to be sensitive to *n*-butanol as well; however, the survival of *D. geothermalis* T27 in the presence of *n*-butanol its demonstrated high tolerance towards butyl acetate and ethyl acetate. This is the first report of a bacterium able to tolerate solvents with such low P_{ow}. Supplementation of Ca^{2+} improved solvent tolerance of *D. geothermalis* T27, white supplementation of Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} ions could enhance the organic solvent tolerance levels of *E. coli* (Aono,1998). Different requirement was shown in other organisms for example, supplementation of Mg^{2+} ions in the culture medium improved solvent tolerance in *Pseudomonas putida* DOT-T1(Ramos *et al.*,1995) and lag period was significantly short in *Pseudomonas putida*T-57 (Faizal *et al.*,2005); the stability of the solvent tolerance of *Pseudomonas putida* IH-2000 was

stimulated by addition of Mg^{2+} and Ca^{2+} to medium containing toluene (Inoue and Horikoshi,1991); or *Haloarcula vallismortis* and two *Haloarcula* strains OHF-1 and ODF-2 showed high tolerance to organic solvent in the presence of high NaCl concentrations (Usami *et al.*,2005).

Gram-negative bacteria, namely certain strains of *Pseudomonas* and some *E. coli* mutants, have devised various novel adaptive mechanisms which enable them to thrive in presence of supersaturating amounts of toxic organic solvents. Previous reports have stated that organic solvents generally have a marked effect on bacterial cell shape and size as well as cell membrane structure (Sardessai and Bhosle,2002). Gram-negative bacteria, the mechanisms involved in solvent tolerance mechanisms have been extensively as follows (Sardessai and Bhosle,2002):

Known mechanisms:

1) modifications in cell envelope to increase cell membrane rigidity and decrease permeablility:

a) *cis-trans* isomerisation of membrane fatty acids by *cis-trans* isomerase

b) decreased cell surface hydrophobicity

c) changes in chemical composition/proportions of membrane lipids and proteins

2) increased rate of membrane repair enzymes

3) special solvent-inactivating enzymes

4) active efflux of solvents by means of solvent efflux pumps

5) release of membrane vesicles with solvent molecules adhering to them

6) production of phage shock protein (stress protein in *E. coli*)

(Sardessai and Bhosle,2002)

D. geothermalis T27 cells in a direct contact to ethyl acetate appears to have a smaller size. The alteration of bacteria cell shape and size is one of the developed mechanisms that allow the bacteria to survive when exposed to various forms of

environmental stresses. Although there are a number of reports that cell size increases when exposed to toxic organic compounds. Major factor responsible for these changes seems to be the surface-to-volume ratio of the cells. Two bacteria, *P. putida* and *Enterobacter* sp., increased in size when exposed to phenol, 4-chlorophenol, and butanol (Neumann *et al.*,2005). Presumably minimizing the surface-to-volume ratio is favourable, as it minimizes the target for toxic solvents. The enhanced surface might enhance uptake of potential growth substrates, as in glucose-grown cells (which were not solvent-adapted), the addition of butanol led to the opposite effect: a decrease in the surface–volume ratio. However, decrease in cell size has been reported for *Enterobacter* sp. when grown on 1-butanol as the sole carbon and energy source (Veeranagouda *et al.*,2006).

Furthermore, fatty acid composition analysis of cells *D. geothermalis* T27 exposed to ethyl acetate revealed that there was no significant change in the level of fatty acid composition. Organic solvent tolerant *Pseudomonas* species have the ability to rapidly synthesize trans-unsaturated fatty acids in response to organic solvents in their environment. This result demonstrated distinguished characteristic of *D. geothermalis* T27 in the presence of ethyl acetate as it did not exhibit distorted cell structure. The outer and cytoplasmic membranes were apparently well defined event after the solvent shock. Due to their relatively high impermeability, the outer membrane is an important barrier against intrusive and toxic compounds. The outer membrane consists mainly of lipopolysaccharides (LPS), a heteropolymeric layer of lipids and sugars. Changes in LPS content and composition in *P. aeruginosa* have been established for several stress factors, such as heat shock, oxygen stress, and exposure to antibiotics such as gentamicin (Makin and Beverdge,1996). *D. geothermalis* T27 could degrade ethyl acetate and induced esterase activity when exposed to ethyl acetate. In fact, solvent tolerance of could also occur without metabolizing the organic solvent (Cruden *et al.*,1992; Inoue and

Horikoshi,1989). Biodegradation is generally be part of long-term adaptive responses but is not a main mechanism leading to solvent tolerance (Isken and de Bont,1998). In addition, biodegradation is highly specific and, thus, cannot play a general role in a solvent tolerance (Isken and de Bont,1998).

Gram-positive organisms have been reported to exhibit organic solvent tolerance, including species of *Bacillus*, *Rhodococcus*, *Enterococcus*, *Staphylococcus*, and *Arthrobacter*. In this study, *Bacillus cereus* stain 4/1 has ability tolerant to styrene (3), toluene (2.5), 1-heptanol (2.3), benzene (2) and chloroform (2) (log P_{ow} between 2-3). The reports of organic solvent tolerance with log P_{ow} between 2-3 of genus *Bacillus* include *Bacillus cereus* R1 which can grow in the presence of styrene and toluene at 1% (v/v) at 30°C (Matsumoto *et al.*,2002); *B. cereus* strain ZZ2, *B. cereus* strain ZZ3, *B. cereus* strain ZZ4 tolerant to toluene and benzene 0.5 % and 1% v/v respectively (Zahir *et al.*,2006). *Bacillus* sp. strain SB-1 is tolerant to toluene, benzene and chloroform on plate overlaid (Sardessai and Bhosle,2002). *Bacillus* sp. DS-994 can grow on toluene and benzene at 10% and 5% (v/v) respectively (Moriya and Horikoshi,1993). Another bacterial species such as *Staphylococcus saprophyticus* strain M36 is tolerant to benzene and toluene up to 40% (v/v) at 37°C (Fang *et al.*,2006). *Staphylococcus* sp. strain ZZ1 is tolerant to hexane, toluene and benzene 0.5 % and 1% (v/v), respectively (Zahir *et al.*,2006), while *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* are tolerant to toluene and benzene at 37°C (Nielsen *et al.*,2005). *Rhodococcus opacus* B-4 can tolerate to styrene, toluene, and benzene with 10% (v/v) at 28°C (Na *et al.*,2005).

B. subitilis strains 45 tolerate to organic solvent with log P_{ow} more than 3 such as *n*-decane (5.6), *n*-heptane (4.7), *n*-octane (4.5), *n*-hexane (3.5) diethylphthalate (3.3) cyclohexane (3.2) at 45°C.

Previous studies have shown the ability of other *Bacillus* sp. for organic solvent tolerance as well, for example, *Bacillus subilis* Marburg tolerant to decane and octane overlaid on plate at 30°C (Hayashi *et al.*,2003); *Bacillus* sp. DS-994 can grown on *p*-xylene 10% (v/v) (Moriya and Horikoshi,1993); *Bacillus* sp. strain SB-1 tolerant to *n*-hexane, cycolohexane, xylene on plate overlaid with all of the above organic solvent (Sardessai and Bhosle,2002); *B. cereus* strain ZZ2, *B. cereus* strain ZZ3, *B. cereus* strain ZZ4 and *Staphylococcus* sp. strain ZZ1 tolerant to hexane, cyclohexane, *p*-xylene at 1.3 %, 1%, 1.2 v/v (Zahir *et al.*,2006), respectively. *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* are also tolerant to cyclohexane, *p*-xylene and cyclohexane at 37°C (Nielsen *et al.*,2005). *Rhodococcus opacus* B-4 can utilize and tolerance to *n*-decane, *n*-octane, *n*-hexane, diethylphtalate, cycolhexane, ethylbenzen, and *o-*, *m-*, and *p*-xylene at 10% (v/v) at 28°C (Na *et al.*,2005).

For *Brevibacillus agri* strain 13, it has ability to tolerate organic solvent with log P_{ow} below 2, e.g. butyl acetate (1.8), *n*-butanol (0.8) and ethyl acetate (0.7). There are a few reports on cells able to tolerate reported organic solvent with log P_{ow} below 2. For example, *Bacillus* sp. SB-1 can grow on *n*-butanol at 1% (v/v) at 48°C (Sardessai and Bhosle,2002). *Clostridium acetobutylicum* was reported to exhibit tolerance to butanol (Hermann *et al.*,1985). It appears that bacteria tolerant to a wide range of solvents with log P_{ow} values as low as 0.8 exist in nature. Solvent tolerance, though a strain-specific property, is influenced by environmental factors and availability of nutrients. *Brevibacillus agri* strain 13 could serve as a unique model in understanding the solvent

tolerance phenomenon in gram-positive bacteria and play a significant role in bioremediation and biocatalysis.

The role of alkaline earth cations is probably stabilization of membrane structure by reduction of charge repulsion between anionic molecules in membrane. We determined the effect of factors on growth and organic solvent tolerant. Supplement with Ca^{2+}, Mg^{2+}, and Na^{+} improved tolerance of *B. subtilis* strain 45 to *n*-heptane. Supplementation of Ca^{2+} improved to toluene tolerance of *B. cereus* strain 4/1. This report corresponds with the presence of Mg^{2+} ions whic is also found to have a beneficial effect on growth in the presence of *n*-butanol on *Bacillus* sp. SB-1 (Sardessai and Bhosle,2002).

Extensive work has been done on the organic solvent tolerance mechanisms of gram-negative bacteria (Aono *et al.*,1994; de Bont,1998), but very little is known about the solvent tolerance of gram-positive bacteria. The proposed of organic solvent tolerance mechanism of gram-positive are as follows:

Proposed mechanisms:

1) protection rendered by endospores
2) induction of general stress regulon, leading to the production of general stress proteins
3) solvent deactivating or emulsifying enzymes

(Yogita N. Sardessai, 2002)

Bacillus spores are known to withstand heat, ultraviolet and oxidative damage, dessication and chemical agents like acids, bases, phenols, alcohols, alkylating agents, etc (Nicholson *et al.*,2000). *Bacillus* endospores have survived in 95 % (v/v) ethanol for prolonged periods. However, the growth rate of *B. subtilis* is found to be lowered but the final yield remains unchanged when ethanol is present in the growth medium. At concentrations allowing growth at half-maximal rate, practically no spores are formed.

Sensitivity to ethanol is much greater for the sporulation process than growth, since a concentration of 0.7 M may reduce the yield of spores to the extremely low value of 10−5, although it reduces growth rate only by half (Bohin *et al.*,1976,). It is possible that similar effects on sporulation may be induced by organic solvents. However, in thes study, endospore formation was not detected in gram-positive *Bacillus subtilis* strain 45, *Bacillus cereus* strain 4/1, and *Brevibacillus agri* strain 13 when exposed to organic solvent.

It is believed that organic solvent deactivating enzymes or organic solvent emulsifying substances could play an important role in diminishing solvent toxicity in gram-positive bacteria (Abe *et al.*,1995). Transformation or degradation of solvents was postulated to be a major adaptive response for a benzene-tolerant *Rhodococcus* strain (Paje *et al.*,1997). Bacteria *Bacillus cereus* strain 4/1 could degrade toluene, *Bacillus subtilis* strain 45 could degrade *n*-decane and cyclohexane, while *Brevibacillus agri* strain 13 could degrade *n*-butanol and induced enzyme alcohol dehydrogenase when exposured with *n*-butanol. However, many solvent-tolerant bacterial strains can grow abundantly in the presence of solvents without degrading or modifying them. Biodegradation is highly specific and, thus, cannot play a general role in a solvent tolerance (Weber and de Bont 1996; Isken and de Bont 1998; Isken and Heipieper 2002).

There is a difference between the cell membranes of gram-positive and gram-negative bacteria, and the additional outer membrane is missing in gram-positive bacteria, which however possess a more extensively linked peptidoglycan (Sikkema *et al.*,1995). Furthermore, fatty acid composition analysis of cells exposed to organic solvent revealed that there was no significant change in the level of fatty acid composition. The observation on cells adaptation of *Bacillus cereus* strain R1 in the presence of organic solvent showed that no increase in the concentration of unsaturated fatty acids was

observed in toluene-adapted cells (Matsumoto et al. 2002). Gram-positive *Bacillus subtilis* strain 45, *Bacillus cereus* strain 4/1, and *Brevibacillus agri* strain 13 cells did not change cell size after exposure organic solvent significance of statistics at confidence 95% (t test analysis).

Although solvent-tolerant *Pseudomonas* species have the ability to rapidly synthesize trans-unsaturated fatty acids in response to solvents in their environment, it is differently occurred in *Bacillus cereus* strain R1. Where change in the concentration of unsaturated fatty acids was not observed in toluene-adapted cells. Furthermore, under no growth condition could trans-unsaturated fatty acids be detected. These relults correspond with the results obtained from solvent-exposed *Bacillus subtilis* strain 45 and *Bacillus cereus* strain 4/1. Nonetheless, there was change in fatty acid composition of *Brevibacillus agri* strain 13 in that C16:1 was increased 2.65 time.