CHAPTER 2.

REVIEW OF LITERATURE
2.1. BACKDROP

Presence of hydrocarbons in the environment, either due to natural synthesis or due to anthropogenic activities, can be mineralized by microbial activities. Metabolic pathways involved in the degradation of hydrocarbons have been elucidated for various organisms and compounds [Gibson and Subramanian 1984; Dagley 1986; Smith 1990, 1994; Isken and de Bont, 1998].

Consequently, the persistence of benzene, toluene and xylene in contaminated sites is indicative of the lack of natural systems that can effectively degrade these compounds. Although many aromatic hydrocarbon degrading microbial strains have been isolated, as they are solvent sensitive, degradation of aromatic hydrocarbon occurs only when these compounds are present at low concentrations. Identification of potential organic solvent tolerant bacteria with the requisite catabolic potential could be of vital importance in clean up operations as these can be used as tools for elimination of low molecular weight aromatics which are highly carcinogenic at a very low concentration.

Moreover, during biotransformation processes, aromatic hydrocarbons can be converted into value-added compounds such as cisdiols, epoxides, indigo etc. [Ensley et al., 1983; Gibson et al., 1970; Mermod et al., 1986; Whited et al., 1986], however, their biological conversion is quite expensive which can be economized using biphasic fermentation processes [Hogson, 1994]. Finally, understanding the solvent tolerance mechanism will be important to create novel microorganisms having enhanced biocatalytic potential, through recombinant technology.

2.2. ORGANIC SOLVENT TOLERANT BACTERIA

A number of solvent tolerant bacteria like Acinetobacter sp.[Chen et al., 2009], Aromatoleum aromaticum [Trautwein et al., 2008], Bacillus sp. [Matsumoto et al., 2002; Sardesai and Bhosle, 2003, Shah et al., 2010], Burkholderia sp. [Zahir et al., 2006], Brevibacillus sp. [Moreno et al., 2009; Kongpol et al., 2009], Enterobacter sp. [Neumann et al., 2005; Gupta et al., 2006], Escherichia coli [Kobayashi et al., 1998; Asako et al., 1999], Moraxella sp. [Devi et al., 2006], Providencia sp. [Kadavy et al., 2000], Pseudomonas putida [Inoue and Horikoshi, 1989; Aono et al., 1992;
Heipieper et al., 1995; Ramos et al., 1997; Kobayashi et al., 2000; Chen et al., 2009; Gaur and Khare, 2009, *Rhodococcus* sp. [Na et al., 2005; Kita et al., 2009], and *Staphylococcus haemolyticus* [Nielsen et al., 2005], are reported to grow in media containing organic solvents.

### 2.2.1. Review of Organic Solvent Tolerant Bacteria

Organic solvent tolerant bacteria were classically reported by Inoue and Horikoshi [1989]. They reported *Pseudomonas putida* strain IH-2000 to thrive in various solvents up to 50% (v/v) concentration. The unusually high tolerance of strain IH-2000 for toluene and other solvents reflects the presence of cell surface components that are unique to the strain.

Inoue et al., [1991] isolated a *Pseudomonas putida* strain able to grow in the presence of more than 50% toluene from soil. The strain also exhibited tolerance to aliphatic hydrocarbons, alicyclic hydrocarbons, aromatic hydrocarbons, alcohols, and ethers. The stability of the solvent tolerance of strain IH-2000 was stimulated by addition of Mg$^{2+}$ and Ca$^{2+}$ to the medium containing toluene.

Inoue and Horikoshi [1991] determined the solvent-tolerance of Gram-negative and Gram-positive bacteria by the solvent parameter, log $P$. According to them, the solvent-tolerance of bacteria tested were in the following order, *Pseudomonas* > *Escherichia, Serratia, Klebsiella, Proteus, Achromobacter, Acinetobacter, Alteromonas, Aeromonas, Flavobacterium, Alcaligenes, Bacillus, Agrobacterium, Micrococcus, Staphylococcus, Streptococcus, Leuconostoc, Lactobacillus, Corynebacterium, Chromobacterium, Brevibacterium, Rhodococcus*.

Aono et al., [1992] isolated a novel toluene tolerant strain, *Pseudomonas aeruginosa* ST-001 that grew in solvents with log $P$ value greater than or equal to 2.4, such as n-heptanol and toluene.

Cruden et al., [1992] examined utilization of toluene, $m$-xylene, $p$-xylene, 1,2,4-trimethylbenzene, and 3-ethyltoluene by *Pseudomonas putida* strain Idaho as growth substrates when provided in two phase system at 5 to 50% (v/v). An initial lag period accompanied by cell death, followed by a period of exponential growth was observed during growth on $p$-xylene [20% (v/v)]. Electron micrographs revealed that
during growth in the presence of \( p \)-xylene, the outer cell membrane becomes convoluted and membrane fragments are shed into the culture medium. At the same time, the cytoplasmic membrane invaginates, forming vesicles, and becomes disorganized. Electron-dense intracellular inclusions were observed in cells grown with \( p \)-xylene \((20\% \text{ v/v})\) and \( p \)-xylene vapours, which are not present in cells grown with succinate.

Aono et al., [1994] attempted to clone gene \( ostA \), one of the genes determining the level of organic solvent tolerance. This gene was cloned from an \( n \)-hexane-tolerant strain of \( E. \) coli, JA300. A JA300-based \( n \)-hexane-sensitive strain, OST4251, was converted to the \( n \)-hexane-tolerant phenotype by transformation with DNA containing the \( ostA \) gene derived from JA300. Thus, the cloned \( ostA \) gene complemented the \( n \)-hexane-sensitive phenotype of OST4251.

Solvent tolerance trait with respect to end product inhibition was reviewed by Shah and Mishra [1995]. Solvent tolerance imparts tolerance to end products like ethanol, acetone, butanol, butanediol and organic acids that are produced during pentose metabolism. Employing solvent tolerant strains in fermentation shall mitigate adverse effects of end products on the performance of bioprocess, which has a practical dimension due to economic interest.

Heipieper et al., [1995] investigated the isomerization of \( cis \) to \( trans \) unsaturated fatty acid in \( Pseudomonas \) putida, a mechanism of this bacterium, to adapt its membrane to toxic environmental influences, as an indicator for toxicity of 10 organic compounds (aromatics and aliphatic alcohols). They observed direct correlation between the hydrophobicity of the compounds (log \( P \)), concentration dependent growth inhibition, and the \( trans/cis \) ratio of unsaturated fatty acids. They discussed application of this system in terms of an indicator for toxicity and environmental stress particularly during bioremediation processes.

Isken and de Bont [1996] investigated solvent resistance mechanism of strain \( Pseudomonas \) putida strain S12 by using \( ^{14}\text{C} \)-labelled toluene. They obtained evidence that an energy-dependent export system may be responsible for this resistance to toluene.
Pinkart et al., [1996] studied the changes in cell envelope of solvent-tolerant and -sensitive Pseudomonas putida strains following exposure to o-xylene. Both the strains produced trans-unsaturated fatty acids, while the tolerant strain showed modified lipopolysaccharides, an increase in total fatty acids and saturated fatty acids. These envelope modifications aid in survival at high concentrations of organic solvents.

Ramos et al., [1997] investigated short and long term responses of Pseudomonas putida strain DOT-T1 to toluene and related hydrocarbons. They reported an increase in the rigidity of the cell membrane by rapid transformation of the fatty acid cis-9,10-methylene hexadecanoic acid (C17:cyclopropane) to unsaturated 9-cis-hexadecenoic acid (C16:1,9 cis) and subsequent transformation to the trans isomer shaped short term responses. While the long term responses included changes in fatty acids, alterations in the level of the phospholipid polar head groups: cardiolipin increases and phosphatidylethanolamine decreases. The two alterations lead to increased cell membrane rigidity and should be regarded as physical mechanisms that prevent solvent entry. Biochemical mechanisms that decrease the concentration of toluene in the cell membrane also take place and involve: (i) a solvent exclusion system and (ii) metabolic removal of toluene via oxidation.

Kieboom et al., [1998a] examined the induction of the membrane-associated organic solvent efflux system SrpABC of Pseudomonas putida strain S12 by cloning a 312-bp DNA fragment, containing the srp promoter, in the broad-host-range reporter vector pKRZ-1. Aromatic and aliphatic solvents and alcohols induced expression of the srpABC genes while general stress conditions like pH, temperature, NaCl, or the presence of organic acids failed to do so. In spite that solvent efflux pump in P. putida S12 is a member of the resistance-nodulation-cell division family of transporters, the srpABC genes were not induced by antibiotics or heavy metals.

Kieboom et al., [1998b] cloned genes for solvent efflux pump from Pseudomonas putida strain S12 and determined its nucleotide sequence. The deduced amino acid sequences encoded by the three genes involved show a striking resemblance to proteins known to be involved in proton-dependent multidrug efflux systems. The genes for the efflux pump where transferred to solvent sensitive P. putida resulted in acquisition of solvent tolerance.
Ramos et al., [1998] investigated solvent tolerance mechanism in Pseudomonas putida strain DOT-T1E. The radiolabelled experiment suggested that exclusion system works specifically with certain aromatic substrates. The mutation in P. putida DOT-T1E-18 was cloned, and the knocked out gene was sequenced and found to be homologous to the drug exclusion gene mexB, which belongs to the efflux pump family of the resistant nodulator division type.

Li et al., [1998] investigated the involvement of the three known efflux systems, MexA-MexB-OprM, MexC-MexDOprJ, and MexE-MexF-OprN in solvent tolerance of genetically defined efflux pump mutants Pseudomonas aeruginosa. They reported that all three systems imparted some form of resistance towards n-hexane and p-xylene, with MexAB-OprM imparting the highest tolerance. Energy dependence of solvent tolerance is indicated by it being compromised by protonophore. These data suggest that the efflux of organic solvents is a factor in the tolerance of P. aeruginosa to these compounds and that the multidrug efflux systems of this organism can accommodate organic solvents, as well as antibiotics.

Kobayashi et al., [1998] observed that on exposure to hydrophobic organic solvents, n-hexane or cyclooctane, a 28 kDa protein associated with inner membrane was induced strongly in Escherichia coli K-12 cells. Partial amino acid sequence revealed it to be phage-shock protein PspA. PspA is induced in stress conditions in E. coli. These suggest E. coli cells are exposed to strong stress in presence of organic solvents. Introducing a multi-copy plasmid vector carrying the psp operon into E. coli improved the survival frequency of cells exposed suddenly to n-hexane, but not its growth rate in presence of n-hexane.

Kobayashi et al., [1999] isolated a toluene-sensitive mutant, No. 32, which showed unchanged antibiotic resistance levels, from toluene-tolerant Pseudomonas putida strain IH-2000 by transposon mutagenesis with Tn5. Gene cyoC, which is one of the subunits of cytochrome o, was disrupted by Tn5 insertion. They examined membrane protein, phospholipid, and lipopolysaccharide (LPS) of IH-2000 and that of mutant No. 32. The gene disrupted by insertion of Tn5 was identified as cyoC, which is one of the subunits of cytochrome o. The membrane protein, phospholipid, and lipopolysaccharide (LPS) of IH-2000 and that of mutant No. 32 were examined and compared. Some of the outer membrane proteins showed a decrease in mutant
No. 32. Change in relative proportion of fatty acid components including dodecanoic acid, 2-hydroxydodecanoic acid, 3-hydroxydodecanoic acid, and 3-hydroxydecanoic acid in both IH-2000 and No. 32 was observed. In addition No. 32 showed increased cell surface hydrophobicity. Thus mutation of cyoC decreased outer membrane proteins and altered fatty acid composition of LPS. Thereby the changes in outer membrane resulted in increased cell surface hydrophobicity, and mutant No. 32 is considered to be sensitive to toluene.

Isken et al., [1999] studied effect of various organic solvents on the growth rate of Pseudomonas putida strain S12. Reduction in biomass yield was observed for toluene, ethylbenzene, propylbenzene, xylene, hexane, and cyclohexane. Thus, energy-consuming adaptation processes as well as the uncoupling effect of the solvents reduce the yield of the tolerant cells.

Asako et al., [1999] investigated the organic solvent tolerance of Escherichia coli under conditions in which OmpF levels were controlled by various means as follows: alteration of NaCl concentration in the medium, transformation with a stress-responsive gene (marA, robA, or soxS), or disruption of the ompF gene. They observed that solvent tolerance of E. coli did not depend upon OmpF levels in the membrane.

Tsukagoshi and Aono [2000] studied solvent resistance of ΔacrAB and/or ΔtolC mutants in the presence of a large volume of solvent. Both mutants were hypersensitive to nonane (log P 5.5), where solvent entered cells rapidly. Retardation in entry of solvents with log P 4.4 was observed. The AcrAB-TolC complex likely extrudes solvents with a log PoW in the range of 3.4 to 6.0 through a first-order reaction with octane, heptane, and n-hexane as most favourable substrates for the efflux system.

Kobayashi et al., [2000] observed a toluene-tolerant bacterium Pseudomonas putida IH-2000 released membrane vesicles (MVs). MVs were composed of phospholipids, lipopolysaccharides (LPS), and very low amounts of outer membrane proteins. The MVs also contained a higher concentration of toluene molecules (0.172 ± 0.012 mol/mol of lipid) than that found in the cell membrane. The shedding of MVs is an integral part of the toluene tolerance system of IH-2000 where the toluene molecules adhering to the outer membrane are eliminated.
Helaeomyia petrolei (oil fly) larvae inhabit the asphalt seeps of Rancho La Brea in Los Angeles, California. Kadavy et al., [2000] examined the culturable microbial gut contents of larvae collected from the viscous oil where 9 of 14 strains were identified as Providencia sp. These nine strains classified as Providencia rettgeri exhibited dramatic resistance to tetracycline, vancomycin, bacitracin, erythromycin, novobiocin, polymyxin, colistin, and nitrofurantoin. All 12 isolates were sensitive to nalidixic acid, streptomycin, norfloxacin, aztreonam, cipericillin, pipericillin, and cefotaxime. On pre-exposure of 20 mg of tetracycline per ml, seven of nine oil fly bacteria tolerated overlays of 100% cyclohexane, six of nine tolerated 10% xylene, benzene, or toluene (10:90 in cyclohexane), and three of nine (OF007, OF010, and OF011) tolerated overlays of 50% xylene–50% cyclohexane. The correlation between antibiotic resistance and organic solvent tolerance can be explained by an active efflux pump maintained in oil fly bacteria owing to constant selective pressure of La Brea’s solvent-rich environment. Hence, the oil fly bacteria and their genes for solvent tolerance offer a microbial reservoir of antibiotic resistance genes.

Matsumoto et al., [2002] isolated toluene tolerant Bacillus cereus strain R1. Toluene accumulated rapidly in cells while growing on glucose, while in presence of valinomycin (K+ ionophore) and o-vanadate (ATPase inhibitor) as inhibitors of energy metabolism, the effect of glucose was counteracted. These suggest the presence of toluene efflux in the strain R1.

Fernandes et al., [2003] correlated role of efflux pump with solvent tolerance and cross resistance with antibiotics. Understanding the solvent tolerance mechanism and its relationship to cross resistance shall impact on the type and use of disinfectants and disinfecting procedures.

Recognising the importance of OSTB, Hayashi et al., [2003] proposed a cell growth assay using tetrazolium violet and image analysis for assessing tolerance. The observations from the above mentioned method coincided with conventional method using solvent overlaid media for assessing organic solvent tolerance in five microorganisms including E. coli. The assay also performed well while assessing solvent mixture tolerance in E. coli OST3410.

Sardesai and Bhosle [2003] isolated organic solvent tolerant Bacillus species capable of transforming cholesterol to its non toxic derivative, cholest-4-ene-3,6-
dione. Such solvent tolerant bacteria have application in biphasic system that is operational in steroid transformation, a multi-million dollar industry.

Shimizu et al., [2005] exposed Escherichia coli strains (OST3410, TK33, and TK31) to organic solvents and investigated gene expression profiles before and after exposure to organic solvents. They selected six genes that showed higher gene expression. Among these genes, glpC encoding the anaerobic glycerol-3-phosphate dehydrogenase subunit C remarkably increased the organic solvent tolerance.

Nielsen et al., [2005] isolated strain of Staphylococcus haemolyticus that tolerated 100% toluene, benzene and p-xylene on plate overlays and saturating levels of these solvents in monophasic liquid cultures. The proportion of anteiso fatty acids increased from 25.8 to 33.7% while the proportion of 20:0 straight-chain fatty acids decreased from 19.3 to 10.1% in membrane fatty acids of cells exposed to solvent. No changes in the membrane phospholipid composition were noted. Finally, six strains of Staphylococcus aureus and five strains of Staphylococcus epidermidis, which were not obtained by solvent selection, also exhibited exceptional solvent tolerance.

Na et al., [2005] isolated twenty-two benzene-utilizing bacteria from soil. Three highly tolerant isolates grew on benzene when liquid benzene was added to the basal salt medium at 10–90% (v/v). Among them isolate B-4, identified as Rhodococcus opacus 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 revealed the benzene dioxygenase pathway is involved in benzene catabolism in strain B-4.

Neumann et al., [2005] studied changes in cell shape of phenol-degrading solvent-tolerant bacterium Pseudomonas putida strain P8 in presence of phenol and 4-chlorophenol. The cells showed an increase in the diameter depending on the toxic effects of the applied concentrations of both the solvents. Similar effect was measured for an alkanol degrading bacterium, Enterobacter sp. VKGH12 in presence of n-butanol. Here the changes in cell size correlated till non-lethal concentrations. The cells reacted to the presence of organic solvents by decreasing the ratio between surface and volume of the cells and therefore reducing their relative surfaces.
Gupta et al., [2006] isolated solvent tolerant strain of *Enterobacter aerogenes* from soil by cyclohexane enrichment method which thrived in presence of solvents with log $P$ value above 3.2. Transmission electron micrographs showed convoluted cell membrane and accumulation of solvent in case of the cells grown in cyclohexane.

Zahir et al., [2006] isolated and characterized few organic solvent tolerant bacteria, namely *Pseudomonas* sp., *Pseudomonas citronellolis*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, *Staphylococcus* sp., *Bacillus cereus*, and *Bacillus* sp. They reported *Staphylococcus* cells grown in presence of 50 mM toluene produced an extracellular capsule.

Devi et al., [2006] isolated organic solvent-tolerant marine bacterium, *Moraxella* sp. MB1 that transformed citrinin to decarboxycitrinin in a biphasic system. This transformation was affected by intracellular enzyme decarboxylase, produced by MB1. The biotransformed product was purified and identified as decarboxycitrinin using electrospray ionization mass spectrometry (ESI-MS/MS) and nuclear magnetic resonance (NMR) spectrometry. They also reported the antibiotic activity of both citrinin and decarboxycitrinin.

Trautwein et al., [2008] investigated the response of *Aromatoleum aromaticum* strain EbN1 to several aromatic substrates at semi-inhibitory (about 50%) concentrations under two different conditions. Under all growth conditions, impaired growth was paralleled by decelerated nitrate-nitrite consumption. Furthermore, alkylbenzene-utilizing cultures accumulated poly (3-hydroxybutyrate) (PHB) up to 10% of the cell dry weight. These physiological responses were supported at the proteomic level (as determined by two-dimensional difference gel electrophoresis), e.g., up-regulation of PHB granule-associated phasins, cytochrome *cdl* nitrite reductase of denitrification, and several proteins involved in oxidative (e.g., SodB) and general (e.g., ClpB) stress responses also.

Identifying the potential of OSTB in bioremediation, Chen et al., [2009] examined solvent tolerance of four strains, two *Pseudomonas* sp. B1 and J2, and two *Acinetobacter* sp. B2 and J6, by microcalorimetry. The strains B2 and J2 showed high tolerance to organic solvent at 10% (v/v) benzene and 70% (v/v) of toluene, respectively. Strains B1 and B2 degraded about 67% and 94% of 0.1 % benzene (v/v) within 72 h in a medium with benzene as the sole carbon source, respectively. Strains
J2 and J6 degraded approximately 92% and 85% of 0.1% (v/v) toluene within 72 h, respectively. In earlier studies on these strains, genes responsible for degradation were detected, that implicate the potential of OSTB in clean-up of benzene and toluene in the environment.

The toxicity of fine chemicals to the producer organism is a problem in several biotechnological production processes. In several instances, an organic phase can be used to extract the toxic product from the aqueous phase during fermentation. With the discovery of solvent-tolerant bacteria, more solvents can now be used in such two-liquid water–solvent systems. De Bont [1998] examined the mechanisms of bacterial solvent tolerance, such as the active efflux of solvents from the cytoplasmic membrane and solvent-impermeable outer membranes. A similar investigation on molecular mechanism of solvent tolerance in *Rhodococcus opacus* strain B4 was done by Kita *et al.*, [2009].

An interesting phenomenon of acquisition of solvent tolerance in *Brevibacillus brevis* was reported by Moreno *et al.*, [2009] during an olive waste vermicomposting. Another organism, exhibiting trichloroethylene (TCE) – tolerance contained a nucleotide sequence encoding a conserved protein domain (ACR_tran) ascribable to the HAE1-RND family. *B. brevis*, isolated from natural soil, showed no DNA sequences of HAE1 transporters. A transfer of solvent-resistance genes from Gram-negative bacteria during the vermicomposting process could explain the presence of HAE1 transporters in *B. brevis* isolated from the vermicompost. Under TCE stress conditions, the acquired nucleotide sequence could be translated into proteins, and the tolerance to solvents is conferred to the bacterium. The isolate was designated as strain BEA1 (EF079071).

Kongpol *et al.*, [2009] isolated solvent tolerant *Brevibacillus agri* that tolerated solvents of a broad range of log $P$ value, up to 5% and 20% (v/v) concentration. Surface characteristics of cells exposed to organic solvent were investigated using the bacterial adhesion to hydrocarbon test, a contact angle measurement, $z$ potential determination, and fluorescence microscopy analysis and compared with that of non-exposed cells. The results showed a unique indigenous cell surface characteristic which stabilize solvent-water interface of the solvent droplets.
Gaur and Khare, [2009] investigated cellular response mechanism of *Pseudomonas putida* to solvents with different hydrophobicity, cyclohexane (log $P$ 3.2) and tetradecane (log $P$ 7.6). Cyclohexane altered the cell membrane and adaptation of organisms to solvent was due to change in morphology, size, permeability and surface hydrophobicity. No such changes were observed in the cells grown in tetradecane.

Torres *et al.*, [2011] reviewed the solvent tolerance mechanism in Gram positive bacteria. Generally various mechanisms of solvent tolerance are shared among Gram negative and Gram positive microorganisms like: 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.

2.2.2. Physiological Basis of Solvent Toxicity and the Concept of Organic Solvent Tolerance

Physiological investigation of microbes has revealed a correlation between solvent toxicity and its log $P$ value. The parameter log $P$ is defined as the partition coefficient of the given solvent in an equimolar mixture of octanol and water. Greater the polarity, lower the log $P$ value and 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 [de Bont, 1998].

Each organism has its own intrinsic tolerance level for organic solvents, which is determined genetically and is also influenced by environmental factors. Organic solvent tolerance is believed to be a strain-specific property. 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 [Sardesai and Bhosle, 2004].

Table 2. Organic solvents and their log $P$ values.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>log $P$ value</th>
<th>Solvent</th>
<th>log $P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexadecane</td>
<td>8.8</td>
<td>Octanol</td>
<td>2.9</td>
</tr>
<tr>
<td>$n$-Decane</td>
<td>5.6</td>
<td>Carbon tetrachloride</td>
<td>2.7</td>
</tr>
<tr>
<td>Decalin</td>
<td>4.8</td>
<td>Toluene</td>
<td>2.5</td>
</tr>
<tr>
<td>Cyclooctane</td>
<td>4.5</td>
<td>Heptanol</td>
<td>2.4</td>
</tr>
<tr>
<td>Diphenyl ether</td>
<td>4.3</td>
<td>Dimethyl phthalate</td>
<td>2.3</td>
</tr>
<tr>
<td>Cyclooctane</td>
<td>4.2</td>
<td>Fluorobenzene</td>
<td>2.2</td>
</tr>
<tr>
<td>Hexane</td>
<td>3.9</td>
<td>Benzene</td>
<td>2.1</td>
</tr>
<tr>
<td>Propyl benzene</td>
<td>3.8</td>
<td>Chloroform</td>
<td>2.0</td>
</tr>
<tr>
<td>Tetralin</td>
<td>3.8</td>
<td>Cyclohexanol</td>
<td>1.5</td>
</tr>
<tr>
<td>Methyl cyclohexane</td>
<td>3.7</td>
<td>Isoamyl alcohol</td>
<td>1.3</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>3.2</td>
<td>Methyl-tert-butyl ether</td>
<td>0.9</td>
</tr>
<tr>
<td>Ethyl benzene</td>
<td>3.1</td>
<td>n-Butanol</td>
<td>0.8</td>
</tr>
<tr>
<td>p-Xylene</td>
<td>3.1</td>
<td>Acetone</td>
<td>-0.23</td>
</tr>
<tr>
<td>Styrene</td>
<td>3.0</td>
<td>Ethanol</td>
<td>-0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dimethylsulfoxide</td>
<td>-1.35</td>
</tr>
</tbody>
</table>

The different techniques support the view that the actual membrane concentration of a solvent is an important parameter. Actual membrane concentrations
depend on: (1) the concentration of solvent in the water phase; and (2) the partitioning of the solvent from the water phase to the membrane.

Sikkema et al., [1994] performed experiment with $^{14}$C-labelled solvents, and found a correlation between the log $P_{O/W}$ value of a solvent and its partitioning between the membrane and water ($\log P_{M/W}$):

$$\log P_{M/W} = 0.97 \times \log P_{O/W} - 0.64$$

Using this equation, it is possible to calculate the actual concentration of a solvent in a membrane if its concentration in the water phase is known. Lipophilic solvents ($\log P_{O/W}>4$) have a high $\log P_{M/W}$ and accumulate preferentially in membranes, but they will not reach a high membrane concentration owing to their low water solubility and so are not toxic to an organism. Solvents in a $\log P_{O/W}$ 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, explaining how these solvents are toxic to cells [Sikkema et al., 1994; Sikkema et al., 1995; Weber et al., 1996].

Microorganisms have mechanisms that enable them to tolerate lethal concentrations of toxic compounds. This feature has been exploited in a wide range of bioprocesses that range from bioremediation applications to production of fine chemicals in two-phase reaction media.

Bacteria counter balance accumulation of lipophilic compounds in membranes by several ways since it affects its structural and functional properties. The mechanisms involved the headgroup composition of phospholipids [Weber et al., 1996; Ramos et al., 1997], outer-membrane proteins [Li et al., 1995; Ramos et al., 1997], modification of lipopolysaccharides [Pinkart et al., 1996], cell hydrophobicity [Weber et al., 1996], rate of turnover of membrane components [Pinkart et al., 1996], and the composition of the fatty acids of the phospholipids [Heipieper et al., 1994] have been studied.

2.2.2.1. **trans** – Unsaturated Fatty Acids

In the presence of solvents, solvent tolerant *Pseudomonas* species synthesize $trans$ acids by a direct isomerisation of $cis$- to $trans$-unsaturated fatty acids without a
shift in the position of the double bond by a \textit{cis–trans} isomerase [Heipieper \textit{et al.}, 1994; Keweloh and Heipieper, 1996]. The proportion of \textit{trans} acids formed depends on the concentration and lipophilicity of the solvent. \textit{Cis–trans} isomerization is a way of changing the fluidity of the membrane in reaction to changing environmental conditions that does not require an energy input [Heipieper and de Bont, 1994; Heipieper \textit{et al.}, 1995]. The formation of \textit{trans} acids has also been observed in response to other stress factors (such as the presence of heavy metals, high temperature, water stress and low pH) [Heipieper \textit{et al.}, 1996].

The benefit of this isomerization is based on the steric differences between \textit{cis} and \textit{trans} unsaturated fatty acids. The \textit{cis} configuration affects a strong increase in membrane fluidity by its bent steric structure, while the \textit{trans} configuration inserts into the membrane in a fashion similar to that of saturated fatty acids. Therefore, the conversion of \textit{cis} to \textit{trans} unsaturated fatty acids reduces the membrane fluidity. [MacDonald \textit{et al.}, 1985; Okuyama \textit{et al.}, 1991].

\textbf{2.2.2.2. Active Efflux of Solvents from Membranes}

The above mentioned mechanism can alter the physical properties of membranes to obviate the effect of solvent temporarily. The active removal of solvents from the cytoplasmic membrane would be a dynamic and longer-lasting mechanism for solvent tolerance.

Aono \textit{et al.}, [1995] reported cyclohexane-tolerant mutants of \textit{E. coli} that were also resistant to low levels of several antibiotics, such as ampicillin, chloramphenicol and tetracycline. Subsequently multidrug-efflux pumps can handle a wide range of structurally dissimilar compounds, is supported by relationship between organic-solvent tolerance and antibiotic resistance in \textit{E. coli} [Paulsen \textit{et al.}, 1996;].

An attempt to clone the genes responsible for a solvent-efflux pump in \textit{P. putida} S12 was undertaken [Kieboom \textit{et al.}, 1997]. Transposon mutants that had lost the toluene-tolerant phenotype were constructed, which made it possible to clone and sequence the genes for the solvent-efflux pump. The amino acid sequences encoded by the three genes isolated strongly resembled proteins involved in proton-dependent multidrug-efflux systems of the resistance-nodulation-division (RND) family [Nikaido, 1996]. These efflux pumps are composed of three proteins that are thought
to span the inner and outer membranes of Gram-negative bacteria. Members of this family are involved in such diverse activities as exporting heavy metals, oligosaccharides and antibiotics [Nikaido, 1996]. The proteins of *P. putida* S12 were termed SrpA, SrpB and SrpC, and showed the highest homology with those for the *MexAB oprM*-encoded multidrug-resistance pump in *Pseudomonas aeruginosa* [Poole *et al.*, 1993; Srikumar *et al.*, 1997]. These observations complement those made at the whole-cell level on the export of toluene and resistance to antibiotics [Isken and de Bont, 1996; Isken *et al.*, 1997]. Interestingly, a similar situation has been described in another strain of *Pseudomonas*. The genes that encoded three proteins involved in solvent transport have been isolated and they showed a significant homology with genes encoding the RND-family proteins [H. Hirayama *et al.*, unpublished].

![Fig. 1. Schematic diagram showing toluene import and efflux in Pseudomonas putida S12. For details refer text.](image)

### 2.3. SOLVENT TOLERANT ENZYMES

Due to growing demand for enantiopure pharmaceuticals [Stinson, 1998], enzymes are increasingly exploited for asymmetric synthetic transformations [Zaks and Dodds, 1997]. Unless the applications of enzymes are broadened from its natural aqueous media, its range of industrial bioconversions for production of speciality chemicals and polymers shall be limited by various factors. Majority of such compounds being water insoluble, water generates unwanted side reactions along with
degrading common organic reagents. In addition, the product recovery becomes cumbersome and thermodynamic equilibria of many processes are unfavourable in water.

Theoretically, all problems mentioned above can be alleviated by use of organic solvents as reaction media instead of water. In first instance, the idea seems to contradict with the conventional belief of denaturation of enzymes in organic solvents. This notion, however, comes from examining enzymes in aqueous–organic mixtures, not in neat (pure) organic solvents. Although it is tempting to assume that if enzymes denature in biphasic aqueous-organic system, they will certainly suffer the same fate in the pure organic solvents, this assumption has now been shown to be wrong [Griebenow and Klibanov, 1996].

2.4. ORGANIC SOLVENT TOLERANT PROTEASE

2.4.1. Backdrop

Proteases (EC 3.4.21) occupy a pivotal position with respect to their applications in both physiological and commercial fields. Proteolytic enzymes catalyze the cleavage of peptide bonds in other proteins. Proteases are degradative enzymes which catalyze the total hydrolysis of proteins. With the advent of analytical techniques, various functions of proteases have been demonstrated, that include highly specific and selective modifications of proteins such as activation of zymogenic forms of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, and processing and transport of secretory proteins across the membranes.

The current estimated value of the worldwide sales of industrial enzymes is $1 billion, in which proteases account for 60% of the total worldwide sale of enzymes [Godfrey and West, 1996]. They have weaved their own niche as an indispensable biocatalyst in industrial sectors of detergent, leather, pharmaceuticals, food, textile, silk, bakery, soy processing, meat tendering, brewery, protein processing, peptide synthesis, ultra filtration membrane cleaning, extraction of silver from used X-ray films as well as in basic research. [Anwar and Saleemuddin, 1998; Gupta et al., 2002]. Normally, proteases catalyze the hydrolysis reaction in aqueous conditions, but in water restricted media, the enzymatic action of protease reverts, leading to
synthesis of peptides and esters [Zaks and Klibanov, 1984; Gupta and Roy, 2004]. Thus, microbial proteases that can mediate catalysis in non-aqueous solvents offer new possibilities such as shifting of thermodynamic equilibrium in favor of synthesis, increasing the solubility of hydrophobic substrates and products and facilitate the product recovery and improving thermal stability of enzymes [Geok et al., 2003].

Since most of the enzymes including proteases are not stable in organic solvents, several contemporary techniques such as site-directed mutagenesis [Martinez et al., 1992], immobilization [Clark and Bailey, 1984; Kise et al., 1990], chemical modification [Takahashi et al., 1984] and directed evolution [Chen and Arnold, 1991] have been used to obtain organic solvent stable proteases [Gupta and Roy, 2004; Ogino and Ishikawa, 2001]. However, exploring naturally organic solvent stable proteases shall mitigate some of the limitations encountered by above-mentioned modified proteases.

Additionally, solvent-tolerant microorganisms catalyze biotransformation with the whole cells in two-phase solvent–water systems [Isken et al., 1999]. Many biofouling species, such as bacteria, diatoms, algal spores, and invertebrate larvae use protein and glycoprotein polymers for formation of biofilm [Cooksey and Wiggleworth-Cooksey, 1995; Callow and Callow, 2000; Kamino, 2001]. As the paints contain solvents, a solvent stable protease might be able to cleave proteins and arrest the colonizatio of fouler.

2.4.2. Review of Organic Solvent Tolerant Protease

Up till now, major work on solvent stable protease has been explored from Pseudomonas sp. [Geok et al., 2003; Ramos et al., 1995; Ogino et al., 1995; Kim et al., 1998; Ogino et al., 1999a; Gupta et al., 2005a; Gupta and Khare, 2006; Tang et al., 2008; Gaur et al., 2010] and different Bacillus sp. [Reddy et al., 2008; Gupta et al., 2005b], viz. Bacillus cereus [Ghorbel et al., 2003; Joshi et al., 2007; Shah et al., 2010; Xu et al., 2010], Bacillus pumilus [Rahman et al., 2007], Bacillus licheniformis [Shimogaki et al., 1991; Sareen and Mishra, 2008; Li et al., 2009], Bacillus sphaericus [Fang et al., 2009; Liu et al., 2010] and Bacillus subtilis [Rai and Mukherjee, 2009; Abusham et al., 2009]. Apart from these, Marinobacter [Sana et al., 2006], Salinivibrio sp. [Karbalaei-Heidari et al., 2007], Natrialba magadii [Ruiz et al., 2007], Halobacterium sp. [Akolkar et al., 2008], Aeromonas veronii [Divakar
et al., 2010], and Geomicrobium sp. [Karan et al., 2011] are also studied for the production of organic solvent proteases.

Ogino et al., [1995] isolated solvent tolerant Pseudomonas aeruginosa that secreted a proteolytic enzyme. The proteolytic activity of the supernatant of the culture was stable in the presence of various organic solvents. The stability of the enzyme was almost the same, in the presence of organic solvents with log $P$ values equal to or more than 3.2, to that in the absence of organic solvents.

Ogino et al., [1999a] purified an organic solvent-stable protease (PST-01 protease) from organic solvent-tolerant Pseudomonas aeruginosa strain PST-01 by successive hydrophobic interaction chromatography using Butyl-Toyopearl gels. The purified enzyme was homogeneous and had molecular mass of 38 kDa as determined by SDS-PAGE. The optimum temperature and pH for casein hydrolysis were 55 °C and 8.5, respectively. PST-01 protease was inhibited by EDTA, 1,10-phenanthroline, and phosphoramidon, indicating being a metalloprotease. Addition of zinc or cobalt ions reactivated PST-01 protease, inhibited by EDTA. The stability of PST-01 protease in solutions containing water-soluble organic solvents or alcohols was higher than that in the absence of organic solvent. In comparison to, commercially available proteases, namely, subtilisin Carlsberg, thermolysin, and α-chymotrypsin, PST-01 protease was more stable in the presence of water-soluble organic solvents or alcohols.

Ogino et al., [1999c] investigated the equilibrium yields of the peptide Cbz-Arg-Leu-NH$_2$ synthesized from Cbz-Arg and Leu-NH$_2$ using the PST-01 protease in the presence of organic solvents under various conditions. Nucleophile concentration determined the equilibrium yield rather than the concentration of the carboxyl component. The optimum temperature and pH were 30 °C and greater than 5.0, respectively for a high equilibrium yield. Under optimum conditions the equilibrium yields were 71.6% and 87.7% in the presence of 50% (v/v) DMF and 60% (v/v) DMSO, respectively. Furthermore, the PST-01 protease also catalyzed the syntheses of the dipeptides Cbz-Lys-Leu-NH$_2$, Cbz-Ala-Leu-NH$_2$, Cbz-Ala-Phe-NH$_2$, Cbz-Arg-Leu-NH$_2$, and Cbz-Lys-Phe-NH$_2$ with equilibrium yields of more than 60% in the presence of 50% (v/v) DMF and 50 mM sodium phosphate buffer (pH 7.0).
Khan et al., [2000] cloned a leucine aminopeptidase gene of *Aquifex aeolicus*, a hyperthermophilic bacterium, and expressed in *Escherichia coli*, and its expression product was purified and characterized. The expressed protein was purified to homogeneity by using heat to denature contaminating proteins followed by ion-exchange chromatography to purify the heat-stable product. Single band of 54 kDa was observed on SDS-PAGE. Kinetic studies on the purified enzyme confirmed that it was a leucine aminopeptidase. The optimum temperature was 80 °C and pH around 8.0 to 8.5. After heating at 115 °C for 30 min, 27% of its activity was retained. It was highly resistant to organic solvents such as methanol, ethanol, tetrahydrofuran, dimethyl sulfoxide, acetone, acetonitrile, dimethyl formamide, 1-propanol, 2-propanol, and dioxane.

Ogino et al., [2000] investigated the initial synthetic rates of peptide Cbz–Arg–Leu–NH$_2$ from Cbz–Arg and Leu–NH$_2$ using PST-01 protease in the presence and absence of organic solvents under various conditions. The synthetic rates of Cbz–Arg–Leu–NH$_2$ were 1.6-, 2.4-, and 5.1-times higher in the presence of 50% (v/v) methanol, 50% (v/v) N,N-dimethylformamide (DMF) and 60% (v/v) dimethyl sulfoxide (DMSO), respectively than that in the absence of organic solvent. The PST-01 protease was not only stable in the presence of organic solvents but also exhibited high reaction rates in the presence of methanol, DMF, and DMSO. When the Cbz–Arg concentration was lower than 60 mM or the Leu–NH$_2$ concentration was lower than 400 mM, the initial rates increased linearly with increase in their concentrations. However, the rates did not increase when the Leu–NH$_2$ concentration was more than 500 mM. The optimum temperature and pH of the reaction were 40 °C and 7.0, respectively.

Ghorbel et al., [2003] isolated an organic solvent-tolerant *B. cereus* strain from fishing industry wastewater producing organic solvent-stable protease. 95% of enzyme’s initial activity was retained after pre-incubation at 30 °C for 24 h in the presence of 25% methanol, DMSO, acetonitrile and DMF. Ca$^{2+}$ was required for enzyme activity above 37 °C, and it increased thermostability above 40 °C. The optimum temperature for the protease activity was at 60 °C in the presence of 2 mM Ca$^{2+}$ and 50 °C in the absence of Ca$^{2+}$. At 60 °C, Ca$^{2+}$ (2 mM) stimulated the protease activity by 500%. Other bivalent metal ions such as Mg$^{2+}$ and Mn$^{2+}$ also increased activity by 285 and 157%, respectively, while Zn$^{2+}$ and Cu$^{2+}$ had a strong inhibitory
effect. The pH optimum was 8.0. Enzyme activity was inhibited by EDTA, suggesting that the preparation contains metalloproteases(s).

Geok et al., [2003] isolated *Pseudomonas aeruginosa* strain K which is PAH degrader and BTEX tolerant extracellular organic solvent-tolerant protease producer. The protease tolerated up to at least 50% (v/v) of benzene, *n*-hexane, 1-decanol, iso-octane and *n*-hexadecane and was also stable in the presence of 25% (v/v) *n*-decane and *n*-dodecane. This enzyme strain K was activated 2.5, 1.5 and 1.2 times by 75% (v/v) of 1-decanol, iso-octane and *n*-dodecane, respectively. This organic solvent stable protease could be used as a biocatalyst for enzymatic synthesis in the presence of organic solvents.

Sareen et al., [2004] studied the synthesis of the analgesic dipeptide kyotorphin precursor (Bz-Tyr-Arg-NH₂) under kinetically controlled conditions in 10–90% (v/v) aqueous-acetonitrile media at −20 °C using a novel protease obtained from the cell free supernatant of a *Bacillus licheniformis* mutant strain (RSP-09-37). α-Chymotrypsin (CT) was used for comparison. The conditions for maximum yield of kyotorphin precursor synthesis were optimized using CT by varying the type of nucleophile (amide and ester), nucleophile concentration and nucleophile to acyl donor ratio. The nucleophile (Arg-NH₂) at a concentration 400 mM and nucleophile to acyl donor ratio 1:40 was found to be optimum for kyotorphin precursor synthesis. The protease from *B. licheniformis* RSP-09-37 was stable even at 90% acetonitrile concentration and allowed for a significantly higher synthesis over hydrolysis ratio (S/H ratio) of 15.6 compared to only 3.0 found for CT at −20 °C.

Rahman et al., [2005a] optimized nutritional requirements of organic solvent tolerant bacteria *Pseudomonas aeruginosa* strain K isolated from soil. Maximum protease activity was achieved with sorbitol as the sole carbon source, followed by starch and lactose at pH 7.0 and 37 °C. Dextrose, sucrose and glycerol greatly reduced the protease production. The best organic nitrogen source was casamino acid. Tryptone, soytone and yeast extract supported protease production while corn steep liquor and beef extract inhibited the protease activity. Significant protease production was observed with sodium nitrate as a sole nitrogen source however, ammonium nitrate completely inhibited the production. More than 62% drop in production
occurred in the presence of amino acids. Addition of metal ions such as K$^+$, Mg$^{2+}$ and Ca$^{2+}$ maximized the enzyme production.

Rahman et al., [2005b] investigated the physical factors affecting the production of an organic solvent-tolerant protease from *Pseudomonas aeruginosa* strain K. The optimum temperature and pH for growth and protease production was 37 °C and 7.0. Maximum enzyme activity was achieved at static conditions with 4.0% (v/v) inoculum. Shifting the culture from stationary to shaking condition decreased the protease production (6.0–10.0% v/v). Neutral media increased the protease production compared to acidic or alkaline media.

Naidu and Devi, [2005] isolated *Bacillus* sp. strain K-30 from detergent industry that produced thermostable alkaline protease utilizing rice bran. The optimum conditions for protease activity was 55 °C at pH 9 with 4% inoculum in the medium containing 1% rice bran after 96 h of incubation. Beef extract, tryptone and yeast extract proved to be good nitrogen sources while lactose, starch, and sucrose were suitable carbon sources for enzyme production. The extracellular production of the enzyme, its thermostable nature and compatibility with most commercial detergents were the features which suggested its application in the detergent industry.

Gupta et al., [2005b] reported a solvent tolerant *Pseudomonas aeruginosa* strain PseA isolated from soil that secreted a novel alkaline protease, which was stable and active in the presence of range of organic solvents. The protease was purified by combination of ion exchange and hydrophobic interaction chromatography using Q-Sepharose and Phenyl Sepharose 6 Fast Flow matrix, respectively to achieve 11.6-fold purification with 60% recovery. The apparent molecular mass based on SDS-PAGE was estimated to be 35 kDa. The enzyme showed optimum activity at pH 8.0 and temperature 60 °C. The $K_m$ and $V_{max}$ towards caseinolytic activity were found to be 2.7 mg/ml and 3 μmol min$^{-1}$, respectively. The protease was sensitive to EDTA and 1,10-phenanthroline, thus was characterized as a metalloproteases. The protease resisted range of detergents, surfactants and solvents. It was stable and active in all the solvents having log $P$ above 3.2, at least up to 72 h. These two properties made it an ideal choice for applications in detergent formulations and enzymatic peptide synthesis.
Sana et al., [2006] reported purification and characterization of a salt, solvent, detergent and bleach tolerant alkaline serine protease produced by a truly marine bacterium. A 69-fold purification (specific activity 791.7 U/mg protein, unit expressed as μmole of tyrosine liberated per minute) was achieved by a three-step purification procedure. The enzyme was active over a broad range of pH (6.0–11.0) and temperature (30 to 70 °C). This enzyme exhibited appreciable activity in presence of up to 30% NaCl. Ba$^{2+}$ and Ca$^{2+}$ enhanced the enzyme activity while heavy metals like Co$^{2+}$, Zn$^{2+}$, Hg$^{2+}$ inactivated the enzyme. The enzyme was completely stable in presence of laboratory detergents (Tween 80 and Triton X-100), oxidizing agents, reducing agents, commercial detergents and bleaches (hydrogen peroxide and sodium perborate) after 1 h of pre-incubation. Water miscible and immiscible organic solvents like ethylene glycol, ethanol, butanol, acetone, DMSO, xylene and perchloroethylene enhanced as well as stabilized the enzyme activity.

Rahman et al., [2006] purified an organic solvent-tolerant strain K protease to homogeneity by ammonium sulphate precipitation and anion exchange chromatography with 124-fold increase in specific activity. The molecular mass of the purified enzyme as revealed by SDS-PAGE electrophoresis was 51 kDa. The strain K protease was an alkaline metalloprotease with an optimum pH and temperature of 10.0 and 70 °C, respectively. The enzyme showed stability and activation in the presence of organic solvents with log \( P \) values equal or more than 4.0. After 14 days of incubation with solvents, the purified protease was activated 1.11, 1.82, 1.50, 1.75 and 1.80 times in 1-decanol, isoctane, decane, dodecane and hexadecane, respectively.

Gupta and Khare, [2006] reported solvent stable protease that retained most of the activity at least up to 10 days in the presence of hydrophobic organic solvents (log \( P \geq 2.0 \)) at 25% (v/v) concentrations. The protease was able to withstand as high as 75% concentration of solvents at least up to 48 h. \( P. \ aeruginosa \) strain and its protease, both seemed promising for solvent bioremediation, wastewater treatment and carrying out biotransformation in non-aqueous medium.

Rahman et al., [2007] reported a protease from \( B. pumilus \) strain 115b to be stable in 25% (v/v) benzene and toluene and it was activated 1.7 and 2.5-fold by \( n \)-dodecane and \( n \)-tetradecane, respectively. The gene encoding the organic solvent
tolerant protease was cloned and its nucleotide sequence determined. Sequence analysis revealed an open reading frame (ORF) of 1,149 bp that encoded a polypeptide of 383 amino acid residues. The polypeptide composed of 29 residues of a signal peptide, a propeptide of 79 residues and a mature protein of 275 amino acids with a calculated molecular mass of 27,846 Da.

Joshi et al., [2007] reported a moderately cold active, extracellular alkaline protease producing bacterium Bacillus cereus MTCC 6840 isolated from a fresh water lake. The optimum culture conditions for enzyme production were pH 9.0, temperature 25 °C and duration 24 h. Among a variety of substrates used, fructose as a carbon source and a combination of yeast extract and peptone as nitrogen source, supported the maximum protease production by the organism (120 U/ml). Fe⁺⁺ and Co⁺⁺ stimulated the enzyme activity whereas Ca⁺⁺, Cu⁺⁺, K⁺, Mg⁺⁺ and Mn⁺⁺ inhibited it to varying extents. The protease was found to be highly stable in the presence of NaCl, SDS and acetone. Treatment with EDTA and PMSF resulted in the considerable loss of enzyme activity.

Karbalaei-Heidari et al., [2007] reported a metalloprotease secreted by the moderately halophilic bacterium Salinivibrio sp. strain AF-2004 which was purified to homogeneity by acetone precipitation and subsequent Q-Sepharose anion exchange and Sephacryl S-200 gel filtration chromatography. The apparent molecular mass of the protease was 31 kDa by SDS-PAGE, whereas it was estimated at approximately 29 kDa by Sephacryl S-200 gel filtration. The purified protease had a specific activity of 116.8 mol of tyrosine/min/mg protein on casein. The optimum temperature and salinity of the enzyme were at 55 °C and 0–0.5 M NaCl. It was identified as a serine metalloprotease, since PMSF, Pefabloc SC, chymostatin and EDTA inhibited it. The protease in solutions containing water-soluble organic solvents or alcohols was more stable than that in the absence of organic solvents. These characteristics made it an ideal choice for applications in industrial processes containing organic solvents and/or salts.

Ruiz et al., [2007] reported an extracellular protease produced by the haloalkaliphilic archaeon Natrialba magadii that was active and stable in aqueous-organic solvent mixtures containing 1.5 M NaCl and glycerol, dimethylsulfoxide (DMSO), N,N-dimethyl formamide, propylene glycol, and dioxane. Among the
solvents tested, DMSO, propylene glycol, and glycerol were effective in preserving enzyme stability in suboptimal NaCl concentrations. The stabilizing effect of DMSO on this haloalkaliphilic protease was more efficient at pH 8 than at pH 10, suggesting that DMSO might not substitute for salt to allow halophilic proteins to withstand the effect of high pH values. These results showed that *N. magadii* extracellular protease was a solvent tolerant enzyme with potential application in aqueous-organic solvent biocatalysis.

Gupta and Khare, [2007] reported that glycerol (0.7%) as carbon source, casein (0.4%) and yeast extract (0.6%) as nitrogen sources, CaCl$_2$ (0.6 mM) as metal ion (pH 7.0), 2.5% inoculum, incubation time of 24 h, temperature of 30 °C with shaking at 250 rpm, were found to be the best culture conditions leading to maximum growth and protease production (1601 U ml$^{-1}$) by *Pseudomonas aeruginosa*. The optimum pH and temperature for enzyme activity were 9.0 and 55 °C, respectively. It retained 100%, 91% and 80% of its initial activity after heating for 30 min at 45, 50 and 55 °C, respectively. The enzyme exhibited remarkable stability towards range of hydrophobic organic solvents. This unique property confirmed its attractiveness for its use in non-aqueous enzymology.

Sareen and Mishra, [2008] achieved 85-fold purification of a protease from the cell-free supernatant of *Bacillus licheniformis* RSP-09-37, a mutant from a thermophilic bacterial strain, *B. licheniformis* strain RSP-09, using affinity chromatography with α-casein agarose resin. SDS-PAGE and GFC-HPLC results concurred and revealed a monomeric nature of the protein, with molecular mass of 55 kDa. The purified protease revealed temperature optima of 50 °C and pH optima of 10.0 and was classified as serine protease based on its complete inhibition with PMSF. The purified protease exhibited tolerance to both detergents and organic solvent. The synthetic activity of the protease was tested using transesterification reaction between N-acetyl-L-phenylalanine-ethyl ester and n-propanol in organic solvents varying in their log $P$ values and the kinetic parameters of the enzyme in these organic solvents were studied. The enzyme showed potential to be employed for synthetic reactions and in detergent formulations.

Reddy *et al.*, [2008] investigated the purification and characterization of a novel protease produced by *Bacillus* sp. strain RKY3. The enzyme was purified in
two steps by ammonium sulphate precipitation, followed by anion exchange chromatography. The purified protease had a molecular mass of approximately 38 kDa, which was highly active over a broad range of pH between 7.0 and 9.0 and the optimum temperature for enzyme activity was found to be 60 °C. Ca$^{2+}$ ions did not greatly enhance the activity or the stability of the enzyme. The purified protease was considered to be serine protease, since PMSF (1 mmol liter$^{-1}$) completely inhibited the protease activity. The purified protease was stable with oxidants (2% H$_2$O$_2$), reducing agents (2% SDS), and organic solvents (25%) such as benzene, hexane, and toluene. The purified protease could have potential applications in protease-based detergent and bleaching industries and for soy protein and gelatin hydrolysis in the food processing industry.

Akolkar et al., [2008] isolated halophilic archaea belonging to three different genera- Halobacterium, Haloarcula and Haloferax, from Kandla salt pans. The isolates had an optimum requirement of 25% NaCl for growth. Increase in organic solvent tolerance of isolates was observed at higher NaCl concentrations. Among the three isolates, Halobacterium sp. SP1(1) was found to be more tolerant than Haloarcula sp. SP2(2) and Haloferax sp. SP1(2a). The extracellular protease of Halobacterium sp. SP1(1) showed higher solvent tolerance as compared to the organism itself. The enzyme was highly tolerant to toluene, xylene, n-decane, n-dodecane and n-undecane, majority of which are frequently used in paints. These findings may help in understanding the mechanism of organic solvent tolerance in halophilic archaea and their application in antifouling coatings.

Mahanta et al., [2008] assessed deoiled Jatropha seed cake for its suitability as substrate for enzyme production by solid-state fermentation (SSF). Solvent tolerant Pseudomonas aeruginosa PseA strain previously reported by them was used for fermentation. The seed cake supported good bacterial growth and enzyme production (protease, 1818 U g$^{-1}$ of substrate and lipase, 625 U g$^{-1}$ of substrate). Maximum protease and lipase production was observed at 50% substrate moisture, a growth period of 72 and 120 h, and a substrate pH of 6.0 and 7.0, respectively. Enrichment with maltose as carbon source increased protease and lipase production by 6.3- and 1.6-fold, respectively. Nitrogen supplementation with peptone for protease and NaNO$_3$ for lipase production also enhanced the enzyme yield reaching 11,376 U protease activity and 1084 U lipase activity per gram of Jatropha seed cake.
Extracellular protease, isolated from PT121 isolate, identified as *Pseudomonas aeruginosa*, exhibited stability in the presence of hydrophilic organic solvents [Tang *et al.*, 2008]. The protease activity in production medium was 10876 U ml\(^{-1}\) after 72 h incubation which retained most of its activity up to at least 14 days in the presence of various organic solvents at 50% concentration. This protease showed high activity as a catalyst for aspartame precursor Cbz-Asp-Phe-NH\(_2\) synthesis in the presence of 50% dimethylsulfoxide (DMSO).

Thumar *et al.*, [2009] isolated a salt-tolerant alkaliphilic actinomycete, *Streptomyces clavuligerus* strain Mit-1 from Mithapur, coastal region of Gujarat, India. The organism could grow up to 15% salt concentration and pH 11, optimally at 5% and pH 9. It was able to tolerate and secrete alkaline protease in the presence of a number of organic solvents including xylene, ethanol, acetone, butanol, benzene and chloroform. Besides, it could also utilize these solvents as the sole source of carbon with significant enzyme production. Further, the enzyme secretion increased by 50-fold in the presence of butanol. Crude enzyme preparation was more stable at 37 °C in solvents as compared to partially purified and purified enzymes.

Rai and Mukherjee, [2009] reported an organic solvent stable, alkaline serine protease (Bsubap-I) with molecular mass of 33.1 kDa, purified from *Bacillus subtilis* strain DM-04. Protease showed optimum activity at temperature and pH range of 37–45 °C and 10.0–10.5, respectively. The enzyme activity of Bsubap-I was significantly enhanced in presence of Fe\(^{2+}\). The thermal resistance and stability of Bsubap-I in presence of surfactants, detergents, and organic solvents, and its dehairing activity supported its candidature for application in leather industry, laundry detergent formulations, ultrafiltration membrane cleaning, and peptide synthesis. The broad substrate specificity and differential antibacterial property of Bsubap-I suggested the natural ecological role of this enzyme for the producing bacterium.

According to Fang *et al.*, [2009], *Bacillus sphaericus* strain DS11 was able to sustain and grow in a wide range of organic solvents. The crude protease from this strain exhibited remarkable solvent stability and retained most of the activity at least up to 14 days at 37 °C and 200 rpm in the presence of various organic solvents at 25% (v/v) concentration. More than 80% activity was observed in the presence of organic solvents having log \(P\) value from 1.8 to 3.5, whereas in methanol, ethanol, and 1-
butanol, the residual activity was 35%, 36%, and 42%, respectively. In the presence of solvents with log $P$ values equal to or more than 4.0, like $n$-decane, octane, isooctane, and heptane, the protease activity was enhanced. This organic solvent-stable protease could be used as a biocatalyst for peptide synthesis in organic media.

Abusham et al., [2009] studied optimization of the production of organic solvent tolerant protease from *Bacillus subtilis* strain Rand, isolated from the contaminated soil found in Port Dickson, Malaysia. The production of the thermostable organic solvent tolerant protease was optimized by varying various physical culture conditions. Inoculation with 5.0% (v/v) of ($A_{600} = 0.5$) inoculum, in a culture medium (pH 7.0) with incubation time of 24 h at 37 °C with 200 rpm shaking, were the best culture conditions, resulted in the maximum growth and production of protease (444.7 U ml$^{-1}$; 4042.4 U mg$^{-1}$). The Rand protease was not only stable in the presence of organic solvents, but it also exhibited a higher activity than in the absence of organic solvent, except for pyridine which inhibited the protease activity. The enzyme retained 100, 99 and 80% of its initial activity, after the heat treatment for 30 min at 50, 55, and 60 °C, respectively.

Li et al., [2009] isolated an organic solvent-stable alkaline protease producing bacterium from the crude oil contaminant soil and identified as *Bacillus licheniformis*. The enzyme retained more than 95% of its initial activity after pre-incubation at 40 °C for 1 h in the presence of 50% (v/v) organic solvents such as DMSO, DMF, and cyclohexane. The optimum pH and temperature were 9.5 and 60 °C, respectively. This organic solvent-stable protease could be used as a biocatalyst for organic solvent-based enzymatic synthesis.

Gaur et al., [2010] purified aminopeptidase with 11.9-fold purification and 38% recovery from a solvent tolerant strain, *Pseudomonas aeruginosa* PseA, by ion-exchange chromatography resulting in a protease having molecular weight of 56 kDa. The enzyme was found to be stable over a pH range of 6.0–8.0 and appreciably thermostable up to 70 °C. It exhibited $K_m$ of 3.02 mM and $V_{max}$ of 6.71 μmol mg$^{-1}$ min$^{-1}$ towards L-Leu-p-nitroanilide. Remarkable stability in both hydrophilic and hydrophobic solvents made PseA aminopeptidase a unique protease. The enzyme could have potential applications in non-aqueous enzymology due to its marked thermostability and striking solvent stability.
Shah et al., [2010] isolated *Bacillus cereus*, strain AK1871 from crude oil contaminated samples, which produced a solvent, detergent and oxidizing agent tolerant serine alkaline protease. A 58-fold purification of protease was achieved by a three-step purification procedure. The optimum pH and temperature of protease were 8.0 and 60 °C. Presence of Li⁺, Ba²⁺, K⁺, Mg²⁺ and Mn²⁺ did not affect, while heavy metals like Cr³⁺, Hg²⁺ and Cu²⁺ inactivated the enzyme. It was stable in the presence of non-ionic detergents (Triton X-100 and Tween 80), and oxidizing and bleaching agents (hydrogen peroxide). The protease exhibited noteworthy stability and activation in the presence of organic solvents with log P values equal to or more than 2.0. This protease could be used in detergent formulations, enzymatic peptide synthesis, biotransformation reactions and in the formulation of antifouling agent.

Xu et al., [2010] isolated *Bacillus cereus*; strain WQ9-2 producing an extracellular solvent-stable protease. The protease from strain WQ9-2 was purified to homogeneity with an estimated molecular mass of 37 kDa. The purified protease showed maximum activity at 50 °C and pH 8.0. The protease was classified as a metalloprotease since it was strongly inhibited by EDTA and 1,10-phenanthroline. The protease showed extreme activity and stability in the presence of both 50% (v/v) hydrophilic or hydrophobic solvents. The synthesis of the precursor (Cbz-Ala-Phe-NH₂) of a bitter dipeptide could be catalyzed by the protease in the presence of 50% dimethylsulfoxide with the product crystals separating directly. The protease could have potential application in peptide synthesis.

Liu et al., [2010] employed response surface methodology (RSM) to enhance the production of organic solvent-stable protease by *Bacillus sphaericus* strain DS11. A significant influence of glycerol, MgSO₄·7H₂O, and pH on organic solvent-stable protease production was noted with Plackett-Burman design. Then, a three-level Box-Behnken design was employed to optimize the medium composition and culture conditions for the production of the protease in shake-flask. Using this methodology, the quadratic regression model of producing organic solvent-stable protease was built and the optimal combinations of media constituents and culture conditions for maximum protease production were determined as glycerol 12.47 g liter⁻¹, MgSO₄·7H₂O 0.73 g liter⁻¹, and pH 8.25. Protease production obtained experimentally coincident with the predicted value and the model was proven to be adequate. The
enhancement of protease from 465.06 U/mL to 1182.68 U/mL was achieved with the optimization procedure.

Divakar et al., [2010] isolated a mesophilic bacterium, *Aeromonas veronii* strain PG01, from industrial wastes, producing an extracellular thermostable organic solvent tolerant protease. The optimum condition for cell growth and protease production was pH 7.0 and 30 °C. The protease produced was purified 53-fold to homogeneity with overall yield of 32%, through ammonium sulphate precipitation, ion-exchange and gel permeation chromatography (GPC). The molecular weight, as determined by GPC–HPLC, was found to be about 67 kDa. SDS-PAGE revealed that the enzyme consisted of two subunits, with molecular weight of 33 kDa. The optimum pH and temperature for this protease was 7.5 and 60 °C. This enzyme was stable and active after incubation with benzene and it was activated 1.3- and 1.5-fold by n-hexane and n-dodecane, respectively. The protease was inhibited by 1,10-phenanthroline indicating it to be a metalloprotease.

Rai and Mukherjee, [2010] achieved optimum protease production of 518 U by *Bacillus subtilis* strain DM-04 in submerged fermentation through response surface method. They purified an alkaline protease by 23.5-fold by a combination of cation and anion exchange chromatography, ethanol precipitation followed by reverse-phase HPLC that existed in zwitterionic form at pH 7.0. The purified protease (Alzwiprase) had a subunit molecular mass of 16.9 kDa and existed as a monomer. It showed optimum activity at 45 °C and pH 10.0, respectively. The $K_m$ and $V_{max}$ values of Alzwiprase towards casein were 59 μM and 336 μg min$^{-1}$, respectively. The significant stability and compatibility towards organic solvents, urea, surfactants, commercial laundry detergents as well as excellent stain removal and dehairing properties of Alzwiprase hold a tremendous promise for its industrial application.

Singh et al., [2011] reported psychrotrophic, solvent tolerant, psychrothermoalkaline protease producing strain of *Pseudomonas putida* isolated from dairy sludge capable of growing in the presence of 30% (v/v) organic solvents. The strain exhibited resistance against heavy metals (Cr$^{6+}$, As$^{3+}$, Pb$^{2+}$, and Cs$^{1+}$) and various antibiotics. The isolate was able to grow at wide range of temperature (10 to 40 °C) with maximum growth at 25 °C. In Glucose Gelatin Yeast Extract (GGY) broth (pH 9.0 and 25 °C), the strain produced 514 U protease ml$^{-1}$. The presence of organic
solvents \(n\)-dodecane, \(n\)-decane, isooctane and \(n\)-octane, enhanced the protease production. The protease was not only stable but its activity was enhanced in the presence of 25% (v/v) solvents \(n\)-dodecane, \(n\)-decane, isooctane, \(n\)-octane, \(n\)-hexane, \(n\)-butanol, \(n\)-heptane, cyclohexane and xylene, after prolonged incubation of fourteen days. The molecular weight of purified protease was \(~53\) kDa as revealed by SDS-PAGE and activity gel analysis. The protease has pH and temperature optimum at 9.5 and 40 °C. Thus the above mentioned properties proved it a potential candidate for peptide synthesis and bioremediation.

Karan et al., [2011] isolated a moderately halophilic, mesophilic and alkaliphilic, \textit{Geomicrobium} sp. EMB2 strain, from Sambhar Salt Lake, India that produced an extracellular protease, which was remarkably stable in the presence of organic solvents, salt, surfactants, detergents and at alkaline pH. An overall 20-fold increase in protease production was achieved in the optimized medium (721 U/ml) as compared with the unoptimized medium (37 U/ml). The high production level coupled with novel properties established it a prospective industrial enzyme.

### 2.5. ORGANIC SOLVENT TOLERANT LIPASE

#### 2.5.1. Backdrop

Lipases (triacylglycerol hydrolases E.C.3.1.1.3), have proved to be better biocatalysts for performing various reactions such as esterification, transesterification, stereospecific hydrolysis of racemic esters and organic synthesis under water-restricted environment, when adsorbed to an oil-water interface due to activation. [Schlatmann \textit{et al.}, 1991; Soni and Madamwar, 2000; Berglund and Hutt, 2000; Ferrera \textit{et al.}, 2005]. Due to the ability of microbial lipases to mediate as catalyst in non-aqueous solvents, they have potential for shifting of the thermodynamic equilibria in favour of synthesis, enabling the use of hydrophobic substrates, controlling substrate specificity by solvent engineering and improving thermal stability of the enzymes [Koops \textit{et al.}, 1999]. Due to inadequacy of organic solvents to strip water molecules from enzyme surface especially into the active site, the enzyme is rendered inactive [Yang \textit{et al.}, 2004]. Such restrictions are surmounted by strategies like chemical modification of amino acids on enzyme surface [DeSantis and
Jones, 1999], protein engineering [Magnusson et al., 2005], medium engineering [Laane, 1987], use of ionic liquids [Katalin et al., 2002] (supercritical fluids) and co-lyophilization with non-buffer salts [Mine et al., 2003] for enhancing enzyme activity and stability. Alternatively, it shall be more preferable to screen naturally evolved solvent tolerant enzymes for application in non-aqueous enzymatic synthesis. Various reports on purification and characterization of solvent tolerant lipase have been reviewed here.

2.5.2. Review of Organic Solvent Tolerant Lipase

Ogino et al., [1994] reported solvent stability of lipase in presence of toluene, cyclohexane, ethanol, and acetone. Lin et al., [1996] stabilized organic solvent tolerant lipase from Pseudomonas pseudoalcaligenes strain F-111 by addition of calcium ion, which lost 68% of its initial activity during storage at 4 °C for 24 h. Ogino et al., [1999c] investigated production of lipase in presence of cyclohexane from Pseudomonas aeruginosa strain LST-03. The lipase activity was better in presence of cyclohexane as compared to its absence.

Ogino et al., [2000] purified solvent stable lipase (LST-03) from Pseudomonas aeruginosa strain LST-03 by ion-exchange and hydrophobic interaction chromatography in the presence of 2-propanol. LST-03 was stable in presence of n-decane, ethyleneglycol, DMSO, n-octane, n-heptane, isoctane, and cyclohexane than in the absence of an organic solvent.

Ito et al., [2001] investigated efficient lipase production by two-step fed-batch culture of an organic solvent-tolerant bacterium, Pseudomonas aeruginosa strain LST-03. C18 (stearic acid) and C20 (arachidic acid), were found to function as effective inducers for the production of lipase.

Leščić et al., [2001] investigated substrate specificity, regioselectivity and transesterification activity of purified extracellular lipase from Streptomyces rimosus. Lipase was stable in solvent mixtures containing 50% (v/v) ethanol, 1,4-dioxane, acetonitrile or acetone. Tetrahydrofuran and N,N-dimethylformamide (both 50%) inactivated the enzyme with $t_{1/2}$ of 5 min and $t_{1/2}$ of 2 h, respectively.
Hun *et al.*, [2003] isolated *Bacillus sphaericus* strain 205y producing solvent stable lipase with lipase activity of 0.42 U/ml/min at 36 h incubation time. Lipase activity was activated by *n*-hexane and *p*-xylene by 3.5- and 2.9-folds, respectively, while it lowered with DMSO and got inactivated with hexadecane and acetonitrile.

Rahman *et al.*, [2005] purified an organic solvent-tolerant S5 lipase which was purified by affinity chromatography and anion exchange chromatography by 387-fold to yield 60 kDa molecular mass. The optimal temperature and pH were 45 °C and 9.0, respectively. It exhibited the highest stability in the presence of various organic solvents such as *n*-dodecane, 1-pentanol, and toluene. Ca$^{2+}$ and Mg$^{2+}$ stimulated lipase activity, whereas EDTA had no effect on its activity. The S5 lipase exhibited the highest activity in the presence of palm oil as a natural oil and triolein as a synthetic triglyceride.

Rahman *et al.*, [2006] isolated organic solvent tolerant *Pseudomonas* sp. which showed high lipase activity when peptone was used as organic nitrogen source. Addition of additional carbon source resulted in reduction of enzyme production. Olive oil was preferred triglyceride. Absence of Mg$^{2+}$ and presence of Na$^+$ enhanced lipase production.

Fang *et al.*, [2006] isolated *Staphylococcus saprophyticus* producing solvent tolerant lipase. The lipase showed 42 U/ml at 24 h incubation time and was stable in the presence of 25% (v/v) *p*-xylene, benzene, toluene, and hexane.

Sulong *et al.*, [2006] expressed organic solvent tolerant (OST) lipase gene from *Bacillus sphaericus* strain 205y extracellularly. The 8-fold purification with 32% recovery was obtained by ultrafiltration and hydrophobic interaction chromatography (HIC). The purified 205y lipase revealed homogeneity on denaturing gel electrophoresis and the molecular mass was approximately 30 kDa. The optimum pH and temperature for the purified 205y lipase was 7.0–8.0 and 55 °C, respectively. Organic solvents such as dimethylsulfoxide (DMSO), methanol, *p*-xylene and *n*-decane enhanced the activity.

Gaur *et al.*, [2008a] carried out media optimization for solvent tolerant lipase by *Pseudomonas aeruginosa* strain PseA by response surface methodology which led to 5.58-fold increase in lipase production (4580 IU/ml) over un-optimized media. This
strain also was reported to be secreting alkaline and solvent stable protease. It produced an extra cellular lipase with suitable properties for detergent applications viz. (i) alkaline in nature, (ii) stability and compatibility towards bleach oxidants, surfactants and detergent formulations and (iii) resistant to proteolysis.

Gaur et al., [2008b] purified lipase from solvent tolerant strain of Pseudomonas aeruginosa PseA by gel exclusion chromatography leading to 8.6-fold purification and 51.6% recovery. SDS-PAGE revealed Mw ~60 kDa. The optimum pH and temperature for activity were found to be 8.0 and 40 °C, respectively. It was stable in presence of divalent metal ions like Ca\(^{2+}\), Mg\(^{2+}\) whereas Cu\(^{2+}\) and Zn\(^{2+}\) were found to be inhibitory. The enzyme activity was not affected significantly by 1 mM EDTA. β-Mercaptoethanol reduced the enzyme activity to 48% after 1 h whereas glutathione activated the lipase. Serine inhibitor PMSF showed no reduction in enzyme activity. Non-ionic detergents Tween-80 and Brij-35 stimulated the lipase activity. Cationic surfactant CTAB inhibited the enzyme activity whereas anionic surfactants sodium deoxycholate caused only 10% reduction in activity. Lipase preferred longer carbon chain (C16) fatty acid ester substrates over the shorter ones and showed random positional specificity for triolein hydrolysis.

Zhao et al., [2008] purified an extracellular lipase to homogeneity with a purification factor of 5.5-fold from a bacterial strain Serratia marcescens ECU1010. The purified lipase was a dimer with two homologous subunits, of which the molecular mass is 65 kDa, and the pI is 4.2. The pH and temperature optima were shown to be pH 8.0 and 45 °C, respectively. The lipase showed maximum activity on p-nitrophenyl myristate (C\(_{14}\)) and was activated by some surfactants such as Gum Arabic, polyvinyl alcohol (PVA) and Pg350me, but not by Ca\(^{2+}\). The enzyme displayed pretty high stability in many water miscible and immiscible solvents, which makes it extremely suitable for chemo-enzymatic applications in non-aqueous phase organic synthesis including enantiomeric resolution.

Mahanta et al., [2008] used deoiled Jatropha seed cake as substrate for enzyme production (protease, 1818 U/g of substrate and lipase, 625 U/g of substrate) by solvent tolerant Pseudomonas aeruginosa PseA strain. Maximum protease and lipase production was observed at 50% substrate moisture, a growth period of 72 and 120 h, and a substrate pH of 6.0 and 7.0, respectively. Enrichment with maltose as
carbon source increased protease and lipase production by 6.3- and 1.6-fold, respectively. Nitrogen supplementation with peptone for protease and NaNO₃ for lipase production also enhanced the enzyme yield reaching 11,376 U protease activity and 1084 U lipase activity per gram of Jatropha seed cake. These results demonstrated viable approach for utilization of this huge biomass by solid-state fermentation for the production of industrial enzymes. This offers significant benefit due to low cost and abundant availability of cake during biodiesel production.

Shu et al., [2009] added ampicillin and kanamycin to the TB-T medium to screen B. cepacia complex strains from rhizosphere soil samples. The selected colonies from the modified TB-T medium were then qualitatively screened for their ability to produce extracellular lipase on the rhodamine B-olive oil agar plates. 35 lipolytic pseudo-B. cepacia complex strains were isolated with 65 % positive rate of lipolytic bacteria. Among them 15 showed tolerance to benzene, n-hexane and n-heptane at concentration of 10% (V/V).

Zhang et al., [2009] cloned, sequenced, and over expressed a novel lipase gene from an organic solvent degrading strain Pseudomonas fluorescens JCM5963 as an N-terminus His-tag fusion protein in E. coli. The recombinant lipase (rPFL) was purified to homogeneity by Ni-NTA affinity chromatography and Sephacryl S-200 gel filtration chromatography. The optimum pH and temperature were 55 °C and 9.0, respectively. Its activity was found to increase in the presence of metal ions such as Ca²⁺, Sn²⁺ and some non-ionic surfactants. In addition, rPFL was activated by and remained stable in a series of water-miscible organic solvents solutions and was highly tolerant to some water-immiscible organic solvents. These features rendered this novel lipase, attractive for biotechnological applications in the field of organic synthesis and detergent additives.

Dandavate et al., [2009] isolated Burkholderia multivorans strain V2 (BMV2) from soil which was found to produce an extracellular solvent tolerant lipase (6.477 U/ml). This lipase exhibited maximum stability in n-hexane retaining about 97.8% activity for 24 h. After performing statistical optimization of medium components for lipase production, a 2.2-fold (14 U/ml) enhancement in the lipase production was observed. The crude lipase from BMV2 was partially purified by ultrafiltration and gel permeation chromatography with 24.64-fold purification. The
Km and Vmax values for partially purified BMV2 lipase were found to be 1.56 mM and 5.62 μmoles/mg/min. The metal ions Ca^{2+}, Mg^{2+} and Mn^{2+} had stimulatory effect on lipase activity, whereas Cu^{2+}, Fe^{2+} and Zn^{2+} strongly inhibited the lipase activity. EDTA and PMSF at 10 mM concentration strongly inhibited the lipase activity. Non-ionic and anionic surfactants stimulated the lipase activity. BMV2 lipase was proved to be efficient in synthesis of ethyl butyrate ester under non-aqueous environment.

Kawata et al., [2010] characterized and improved the organic solvent-stability of the LST-03 lipase from Pseudomonas aeruginosa strain LST-03 which was highly active and stable in the presence of various organic solvents. Residues in lipase that potentially provide this stability were identified and mutated to other amino acids in an effort to increase the organic solvent-stability of the protein. S155L, G157R, S164K, S194R, and D209N mutations were found to improve the organic solvent-stability of the wild-type LST-03 lipase. Such mutations were found to induce structural changes, including the formation of a salt bridge, hydrogen bonds, lead to an improved packing of the hydrophobic core, and pI shift of side chain. These changes increased the stability of the protein, thereby improving the organic solvent-stability of the wild-type LST-03 lipase. In addition, a single mutation was found to stabilize the lipase by single or multiple factors.

Ji et al., [2010] purified an organic solvent-tolerant lipase from newly isolated Pseudomonas aeruginosa strain LX1 by ammonium sulfate precipitation and ion-exchange chromatography leading to 4.3-fold purification and 41.1% recovery. The purified lipase from P. aeruginosa LX1 was homogeneous as determined by SDS-PAGE, and the molecular mass was estimated to be 56 kDa. The optimum pH and temperature for lipase activity were found to be 7.0 and 40 °C, respectively. Its hydrolytic activity was found to be highest towards p-nitrophenyl palmitate (C16) among the various p-nitrophenol esters investigated. The lipase displayed higher stability in the presence of various organic solvents, such as n-hexadecane, isoctane, n-hexane, DMSO, and DMF, than in the absence of an organic solvent. The immobilized lipase was more stable in the presence of n-hexadecane, tert-butanol, and acetonitrile. The transesterification activity of the lipase from P. aeruginosa LX1 indicated that it is a potential biocatalyst for biodiesel production.
Ahmed et al., [2010a] isolated Acinetobacter sp. strain EH28 producing a thermostable alkaline lipase from oil rich soil sample. The lipase was partially purified by ammonium sulphate precipitation followed by hydrophobic interaction chromatography with 24.2-fold purification and 57.1 U/ml specific activity. The partially purified enzyme exhibited maximum activity at pH 10.0 and at 50 °C. It was highly stable and retained more than 80% of its initial activity upon exposure to various organic solvents. The EH28 lipase was used for synthesis of the flavor ester ethyl caprylate in organic solvents, thus providing a concept of application of Acinetobacter sp. lipase in non-aqueous catalysis. Reaction parameters best suited for this esterification reaction were 40 °C reaction temperature, 1.3:1 ratio of caprylic acid to ethanol and cyclohexane as the medium.

Ahmed et al., [2010b] reported Bacillus subtilis strain EH 37 a novel thermostable alkaline lipase isolated from oil rich soil sample. Partial purification by ammonium sulfate precipitation and hydrophobic interaction chromatography yielded 17.8-fold purification and 41.9 U ml⁻¹ specific activity. The optimum pH and temperature of partially purified lipase was 8.0 and 60 °C. Ca²⁺, Mg²⁺ and Zn²⁺ exhibited stimulatory effect on lipase activity, whereas Fe³⁺ and Co²⁺ reduced its activity. On solvent exposure, enzyme retained more than 80% of its initial activity, exhibited 107% and 115% activity in the presence of 15% isopropyl alcohol and 30% n-hexane, respectively. The EH 37 lipase also proved to be an efficient catalyst in synthesis of ethyl caprylate in organic solvent, thus providing a concept of application of B. subtilis lipase in non-aqueous catalysis.

Cadirci and Yasa, [2010] reported a solvent tolerant Pseudomonas fluorescens producing lipase (3.5 U/l). The lipase production medium was optimized where olive oil acted as an inducer and peptone was selected as a nitrogen source (15 U/l). After optimization, initial lipase activity was increased to 638.3%. SDS-PAGE and zymogram analysis exhibited two lipases with a molecular weight of 15 and 38 kDa. Partially purified lipase was active at pH 7.0 and 30 and 60 °C. The lipase activity was stable in the presence of organic solvents such as cyclohexane, styrene, hexane and heptane.

Uttatree et al., [2010] isolated the benzene tolerant Acinetobacter baylyi from marine sludge in Angsila, Thailand that could constitutively secrete lipolytic enzymes.
The enzyme was successfully purified 21.89-fold to homogeneity by ammonium sulphate precipitation and gel-permeable column chromatography with a relative molecular mass as 30 kDa. The enzyme expressed maximum activity at 60 °C and pH 8.0 with p-nitrophenyl palmitate as a substrate. A study on solvent stability revealed that the enzyme was highly resistant to many organic solvents especially benzene and isoamyl alcohol, but inhibited by 40% in presence of decane, hexane, acetonitrile, and short-chain alcohols. Lipase activity was completely inhibited in the presence of Fe$^{2+}$, Mn$^{2+}$, EDTA, SDS, and Triton X-100 while it was suffered detrimentally by Tween 80. The activity was enhanced by PMSF, Na$^+$, and Mg$^{2+}$ and no significant effect was found in the presence of Ca$^{2+}$ and Li$^+$. Half of an activity was retained by Ba$^{2+}$, Ag$^+$, Hg$^+$, Ni$^{2+}$, Zn$^{2+}$, and DTT.

Ebrahimpour et al., [2011] cloned the mature ARM lipase gene into the pTrcHis expression vector and over-expressed in E. coli TOP10 host. The optimum lipase expression was obtained after 18 h post induction incubation with 1.0 mM IPTG, where the lipase activity was approximately 1623-fold higher than wild type. A rapid, high efficient, one-step purification of the His-tagged recombinant lipase was achieved using immobilized metal affinity chromatography with 63.2% recovery and purification factor of 14.6. The purified lipase was characterized as a highly active (7092 U/mg), serine-hydrolase, thermostable, organic solvent tolerant, 1,3-specific lipase with a molecular weight of about 44 kDa. The enzyme was a monomer with disulfide bond(s) in its structure, but was not a metalloenzyme. ARM lipase was active in a broad range of temperature and pH with optimum lipolytic activity at pH 8.0 and 65 °C.

### 2.6. DEGRADATION OF BENZENE AND TOLUENE

#### 2.6.1. Backdrop

Hydrocarbons and their derivatives, encompassing solid, liquid and gaseous fossil carbon deposits, compounds of biological origin like lipids and fatty acids from plants, animals and microbes and the products of their conversion in anoxic zones, are ubiquitous in the biosphere. Due to high carbon and energy contents of such highly reduced compounds, during the course of evolution, microorganisms have acquired the ability to utilize these hydrocarbons as sources of carbon and energy. It is almost a century since first hydrocarbon degrading bacteria were isolated and described, and
the current state of literature includes almost 200 bacterial, cyanobacterial, algal and fungal genera, representing more than 500 species and strains [Prince et al., 2003; Head et al., 2006].

With the first reported oil spill of more than 200,000 barrels of crude oil from the oil tanker *Exxon valdez* in Prince William Sound, Alaska, the problem of hydrocarbon contamination in the environment has been of concern. Recently, the Deepwater Horizon well which was plugged on 15<sup>th</sup> July 2010, had spewed some 750 million litres of crude oil into the Gulf of Mexico and earned the title of the biggest accidental marine oil spill ever. Much of the oil had already vanished from surface waters, and so far the most visible effects had been oiled seabirds, turtles and salt-marsh fringes [Mascarelli, 2010].

![Image of oil spill effects](image)

*Fig. 2. Work on previous spills suggests oil can harm wildlife and linger in sediments for decades [Mascarelli, 2010].*

The most serious damage done in the ocean was to the organisms that were spawned there and exposed to submerged and dispersed oil as embryos and larvae.
Exposure to PAHs early in an organism’s life cycle can also lead to infertility and developmental problems. After the 1989 Exxon Valdez spill, rare mutations in the small fraction of the population of Salmon to grow showed an extra fin or an enlarged heart sac [Mascarelli, 2010].

*Fig. 3. Toxic compounds of oil could stunt growth and cause mutations in crustaceans and fish in the Gulf [Mascarelli, 2010].*

In addition, BTEX compounds are toxic to humans and are confirmed or suspected carcinogens [Dean, 1985]; thus the U.S. Environmental Protection Agency (US EPA) classifies them as environmental priority pollutants, giving their removal from polluted environments, a particular interest [Dean, 1985].

### 2.6.2. Review of Degradation of Benzene and Toluene by Bacteria

Greene *et al.* [2000] isolated 55 genomically distinct bacteria (standards) from soil contaminated with C5+, which contained benzene (45%, wt/wt), dicyclopentadiene (DCPD) plus cyclopentadiene (together 20%), toluene (6%), styrene (3%), xylene (2%), naphthalene (2%), and smaller quantities of other compounds. Substrate utilization pattern was benzene (31 of 44 standards tested) > toluene (23 of 44) > xylenes (14 of 44) > styrene (10 of 44) > naphthalene (10 of 44). Benzene degradation by the dominant community members detected by reverse
sample genome probing (RSGP) was observed at all stages of succession. Except DPCD, all C5+ components were effectively degraded by enrichment. Overall, degradation of individual C5+ hydrocarbons followed first-order kinetics, with the highest rates of removal for benzene.

Parales et al., [2000] examined the chemotactic behaviour of five motile strains of bacteria that use five different pathways to degrade toluene for their ability to detect and swim towards this pollutant. Three of the five strains (*Pseudomonas putida* F1, *Ralstonia pickettii* PKO1, and *Burkholderia cepacia* G4) were attracted to toluene. In each case, the response was dependent on induction by growth with toluene. *Pseudomonas mendocina* KR1 and *P. putida* PaW15 did not show a convincing response.

Kim et al., [2002] isolated *Rhodococcus* sp. strain DK17 from soil capable of growth on benzene, phenol, toluene, ethyl benzene, isopropyl benzene, and other alkyl benzene isomers. A mutant strain, DK180, isolated for the inability to grow on o-xylene, retained the ability to grow on benzene but was unable to grow on alkyl benzenes due to loss of a meta-cleavage dioxygenase needed for metabolism of methyl-substituted catecholes.

Shinoda et al., [2004] isolated denitrifying bacterium, *Thauera* sp. strain DNT-1, that grew on toluene as the sole carbon and energy source under both aerobic and anaerobic conditions. Under aerobic and denitrifying conditions, toluene degradation occurred through a dioxygenase-mediated pathway and the benzyl succinate pathway, respectively. Homologous genes for toluene dioxygenase (*tod*) and benzylsuccinate synthase (*bss*), which are the key enzymes in aerobic and anaerobic toluene degradation, respectively, were cloned from genomic DNA of strain DNT-1. Northern blot analyses and real-time quantitative reverse transcriptase PCR suggested that the *tod* genes were induced under aerobic conditions, whereas the *bss* genes were induced under both aerobic and anaerobic conditions. The results suggested that strain DNT-1 modulated the expression of two different initial pathways of toluene degradation according to the availability of oxygen in the environment.

Methyl bromide (CH$_3$Br) and methyl chloride (CH$_3$Cl) are important precursors for destruction of stratospheric ozone, and oceanic uptake is an important
component of the biogeochemical cycle of these methyl halides. Goodwin et al., [2005] attempted to identify and characterize the organisms mediating halocarbon biodegradation, and studied the effect of cometabolic substrates on CH₃Br biodegradation using ¹³CH₃Br incubation technique. Toluene inhibited CH₃Br and CH₃Cl degradation in seawater samples. Moreover, Oxy6, a marine bacterium able to co-oxidize CH₃Br while growing on toluene was isolated from subtropical Western Atlantic seawater. Oxy6 also oxidized a variety of toluene (TOL) pathway intermediates such as benzyl alcohol, benzyaldehyde, benzoate, and catechol, but the inability of Pseudomonas putida strain mt-2 to degrade CH₃Br suggested that the TOL pathway might not be responsible for CH₃Br biodegradation. Based on molecular phylogenetic analysis, Oxy6 was identified to be a member of the family Sphingomonadaceae related to species within the Porphyrobacter genus. The widespread inhibitory effect of toluene on natural seawater samples and the metabolic capabilities of Oxy6 indicated a possible link between aromatic hydrocarbon utilization and the biogeochemical cycle of methyl halides.

Kim et al., 2008 isolated Pseudoxanthomonas spadix, strain BD-a59 that degraded benzene, toluene, ethylbenzene, and o-, m-, and p-xylene (BTEX) compounds on minimal salts media containing 0.01% yeast extract, with BTEX as sole carbon and energy source. In slurry systems amended with sterile soil solids, degradation occurred very quickly, while the enhancement effect was lost when soil was combusted first to remove organic matter indicating that some components of insoluble organic compounds were nutritionally beneficial for BTEX degradation. This study suggested that strain BD-a59 had the potential to assist in BTEX biodegradation at contaminated sites.

Oka et al., [2008] examined the active members in a benzene-degrading sulfidogenic consortium by stable isotope probing (SIP). SIP-terminal restriction fragment length polymorphism analysis indicated that a 270-bp peak incorporated the majority of the ¹³C label and was a sequence closely related to that of clone SB-21 (GenBank accession no. AF029045). SB-21 belonged to family Desulfobacteraceae. Bacteria similar to SB-21 were identified as one of the dominant microbes in benzene degrading enrichments established from widely dispersed environments such as a Mediterranean lagoon in France [Musat and Widdel, 2008] and an oil refinery in Oklahoma [Ulrich and Edwards, 2003]. Thus, SB-21 potentially could serve as a
biomarker for in situ biodegradation of benzene in the environment under sulfidogenic and methanogenic conditions.

Weelink et al., [2008] isolated a benzene-degrading chlorate-reducing enrichment bacterium; strain BC that degraded benzene in conjunction with chlorate reduction. The closest cultured relative is Alicycliphilus denitrificans type strain K601, a cyclohexanol-degrading nitrate-reducing betaproteobacterium. Chlorate reductase (0.4 U mg\(^{-1}\) protein) and chlorite dismutase (5.7 U mg\(^{-1}\) protein) activities in cell extracts of strain BC were determined.

Auffret et al., [2009] reported degradation of a mixture of hydrocarbons, gasoline, and diesel oil additives by two strains, identified as Rhodococcus wratislaviensis IFP 2016 and Rhodococcus aetherivorans IFP 2017, which were isolated from a microbial consortium that degraded 15 petroleum compounds or additives when provided in a mixture containing 16 compounds (benzene, toluene, ethylbenzene, \(m\)-xylene, \(p\)-xylene, \(o\)-xylene, octane, hexadecane, 2,2,4-trimethylpentane [isooctane], cyclohexane, cyclohexanol, naphthalene, methyl tert-butyl ether [MTBE], ethyl tert-butyl alcohol [TBA], and 2-ethylhexyl nitrate [2-EHN]).

Sun et al., [2010] identified aerobic toluene-degrading microorganism in soil microcosms by stable isotope probing (SIP). Several approaches (terminal restriction fragment length polymorphism, 16S rRNA gene sequencing, and quantitative PCR) provided evidence that the microorganism responsible was a member of the genus Polaromonas and could grow on toluene. This microorganism also transformed benzene, but not \(m\)-xylene or \(cis\)-dichloroethene.

Wang et al., [2008] isolated BTX (benzene, toluene and xylene) degrading bacteria from Pacific Ocean sediment and nearshore surface water. Pseudomonas, Rhodococcus, Exiguobacterium and Bacillus spp. were detected in seawater near a ferry dock, while only Bacillus sp. was detected from the deep-sea sediment. Majority of the isolates showed degradation of more than one compound, with preference for \(p\)-xylene and ethylbenzene than the other two. All the bacteria tolerated and thrived on the compounds at 5–20\% (v/v) concentration. Thus, the observations suggested that BTX degrading bacteria are widely spread in marine environments and they might be of potential in bio treatment of BTEX in saline environments.
Kim et al., [2009] isolated Acinetobacter strain B113, able to degrade benzene, toluene, and ethylbenzene compounds (BTE) from gasoline-contaminated sediment at a gas station in Geoje, Korea. Addition of yeast extract accelerated the biodegradation rates of BTE which otherwise were relatively low in MSB broth, which suggested that yeast extract might provide a factor that was necessary for its growth or BTE biodegradation activity. However, interestingly, the biodegradation of BTE compounds occurred very quickly in slurry systems amended with sterile soil as compared to combusted soil devoid of organic matters. In later situation, the enhancement effect on BTE biodegradation was lost, indicating that some insoluble organic compounds were probably beneficial for BTE degradation in contaminated sediment. This study suggested that strain B113 might play an important role in biodegradation of BTE at the contaminated sites.