CHAPTER 3.

MATERIALS AND METHODS
3.1. SCREENING, ISOLATION, PURIFICATION AND IDENTIFICATION OF OSTB

3.1.1. Site selection

Soil, mud and sediment samples were collected from different hydrocarbon contaminated and non – contaminated sites like sea shore, salt farm, petrol pump and garden soil in Gujarat, India.

Alang – Sosiya ship breaking yard (ASSBY), located on the Bhavnagar Gopnath segment of the Saurashtra coast which forms the western flank of the Gulf of Khambhat was selected as a site. The ship-breaking process produces a lot of wastes including oil, asbestos, paint chips, heavy metals, plastic, glass and ceramics. Heavy load of pollution has greatly influenced the ecology of this area. Another site was hydrocarbon contaminated soil from Veraval, which is a parking area for heavy motor vehicles on the high way. The third site was experimental salt farms of CSMCRI, Bhavnagar. The OSTB were isolated from evaporation ponds of different salinity. Fourth and fifth sites were local petrol pumps of the Bhavnagar city. The soil was scrapped from the floor of the petrol pumps. The last sample was of rhizospheric soil from garden of CSMCRI, Bhavnagar.

3.1.2. Isolation based on solvent tolerance screening

The samples were collected by sterile spatula in sterile glass vials and transported to the lab in ice, within 2 h of collection. All the samples were inoculated in modified Luria-Bertani (MLB) medium containing (g liter\(^{-1}\)): tryptone, 10.0; yeast extract, 5.0; sodium chloride, 10; and magnesium sulphate, 0.5 [Tang et al., 2008] and over layered by toluene {10% (v/v)}. The system was incubated for 72 h at 120 rpm and the flask was sealed throughout the incubation to avoid solvent loss due to evaporation. The microorganisms were acclimatized by repeated transfer in the same culture conditions. The grown microorganisms in the enrichment medium were diluted and spread on MLB plates and isolated colonies were further purified by repeated streaking. Twenty five OSTB were obtained by above mentioned toluene enrichment in MLB.
3.1.3. Identification of OSTB through various techniques

Taxonomic characterization of 25 OSTB was conducted using polyphasic approach encompassing morphological and biochemical characteristics, fatty acid profiling, and 16S rDNA sequencing.

3.1.3.1. Biochemical characterization

Morphological and biochemical tests included Gram staining, motility tests, catalase, oxidase, methyl red, Voges-Proskauer, indole production, nitrate reduction, citrate utilization, urease activity, malonate utilization, phenylalanine deamination, starch hydrolysis, tributyrin hydrolysis, bile esculin hydrolysis, decarboxylation of different amino acids (arginine, lysine and ornithine). Carbohydrate utilization of lactose, xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, melibiose, sucrose, l-arabinose, mannose, inulin, sodium gluconate, glycerol, salicin, glucosamine, dulcitol, inositol, sorbitol, mannitol, adonitol, α-methyl-D-glucoside, ribose, rhamnose, cellobiose, melezitose, α-methyl-D-mannoside, xylitol, ONPG, D-arabinose, and sorbose was tested. For details refer Appendix I.

3.1.3.2. Fatty Acid Methyl Esters (FAME)

Cellular fatty acid analysis by Gas Chromatography (FAME) has been used for more than 50 years as a rapid and easy-to-use method for routine microbial identification. FAME identification is a dynamic test of the organism. It is specific to growth conditions, including the media and temperature used. Different libraries are available for different growth conditions and custom libraries can be created based on the customer's unique environment. In addition to identification, FAME analysis is often sensitive enough to give strain level information for a sample. For details refer Appendix II.

For this analysis, each strain was streaked on tryptone soy agar (Soyabean Casein Digest Medium; M011, HiMedia) ([g liter\(^{-1}\): pancreatic digest of casein, 17.0; papaic digest of soyabean meal, 3.0; sodium chloride, 5.0; dibasic potassium phosphate, 2.5; dextrose, 2.5; final pH at 25 °C: 7.3 ± 0.2], incubated at 30 °C for 24 h and 20 - 30 mg of cells were harvested from the plate. The fatty acid methyl ester was extracted for taxonomic purposes according to the standardised protocol of the Microbial Identification System (MIDI) as mentioned in Appendix II. The extracted
samples were analyzed with Agilent GC6850 and the profiles were compared with the Sherlock TSBA library 6.0 version (Microbial ID, MIDI Inc.). A Neighbour Joining (NJ) tree was prepared based on FAME analysis. Cluster analysis using MIDI’s tools – Dendrogram and 2D plot was performed.

3.1.3.3. Chromosomal DNA extraction and purification

All the isolates were grown in 10 ml of Zobell Marine Broth 2216 (M385; HiMedia, India) [g liter⁻¹: peptone 5.0, yeast extract 1.0, ferric citrate 0.10, sodium chloride 19.45, magnesium chloride 8.80, sodium sulphate 3.24, calcium chloride 1.80, potassium chloride 0.55, sodium bicarbonate 0.16, potassium bromide 0.08, strontium chloride 0.034, boric acid 0.022, sodium silicate 0.004, sodium fluoride 0.0024, ammonium nitrate 0.0016, disodium phosphate 0.008; final pH at 25 °C: 7.6 ± 0.2] for 24 h at 35 °C. The biomass was harvested by centrifugation at 10,000 rpm for 10 min and washed twice in sterile Tris–EDTA buffer (10:1 molar ratio, pH 8.0). The wet biomass obtained was used for DNA extraction. The extraction and purification of DNA was carried out by the phenol–chloroform extraction [Marmur, 1961].

3.1.3.4. DNA amplification and sequencing

Two primers were selected for PCR amplification experiments, forward primer 50-AGA GTT TGA TCC TGG CTC AG-30 and reverse primer 50-AAG GAG GTG ATC CAG CC-30 (Sigma) [Weisburg, 1991]. The reaction mixture for PCR amplification (BioRad) contained 10× PCR buffer 5 μl, each dNTP 200 μM, Taq DNA polymerase 1.25 U, 0.5 μM of each primers and 50–100 ng of bulk DNA, 50 μl reaction mixer, 2 mM MgCl₂. Amplification was performed in a thermal cycler for an initial denaturation at 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min and 72 °C for 2 min and a final extension at 72 °C for 10 min.

All purified DNA products were sequenced in both the directions using ABI PRISM 3100 Avant genetic analyzer (Applied Biosystems, M/S, Macrogen, South Korea). All the sequences from forward and reverse directions were aligned in MEGA version 4 [Tamura et al., 2007]. They were trimmed manually and the resulting data matrix encompassed approx. 1200 base pairs. Assembled sequence from each isolates was compared with NCBI gene bank and highest match with maximum score were considered for species identification.
3.1.3.5. Construction of phylogenetic tree

A phylogenetic tree was constructed using 16S rRNA gene sequences of OSTB along with some published sequences of various bacteria used as reference strains by Neighbour Joining (NJ) program of MEGA version 4 [Tamura et al., 2007].

3.1.3.6. Sequence diversity among OSTB

Complete 16S rRNA gene sequences of all the 25 OSTB were aligned using MEGA version 4 [Tamura et al., 2007] and sequence diversity among each species was evaluated.

3.2. SOLVENT TOLERANCE AND ANTIBIOTIC RESISTANCE STUDY OF OSTB

3.2.1. Solvent tolerance

The solvent tolerance range of OSTB was studied in connection with nine organic solvents [Table 3] across the log P range from -0.23 to 8.8 and comprised of aliphatic, alicyclic, aromatic, ether, alcohol, ketone, and chlorinated class of hydrocarbons. Zobell Marine broth (25 ml) was over layered with 25 ml organic solvent, i.e. 50% (v/v), and inoculated by cultures grown overnight.

*Table 3. Classification of selected organic solvents and their log P values.*

<table>
<thead>
<tr>
<th>Aliphatic hydrocarbons</th>
<th>Alicyclic hydrocarbons</th>
<th>Aromatic hydrocarbons</th>
<th>Others (ethers, alcohols, chlorinated compounds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexadecane (8.8)</td>
<td>Cyclooctane (4.5)</td>
<td>xylene (3.1)</td>
<td>Carbon tetrachloride (2.7)</td>
</tr>
<tr>
<td>n–hexane (3.9)</td>
<td></td>
<td>Benzene (2.1)</td>
<td>Methyl-tert-butyl ether (0.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>n–butanol (0.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Acetone (-0.23)</td>
</tr>
</tbody>
</table>
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The system was incubated in 250 ml Erlenmeyer flask with interchangeable stopper to prevent loss of solvent through evaporation. These flasks were incubated at 30 °C on a rotary shaker at 120 rpm for 7 days. The solvent tolerance was tested for purity and viability of the isolate by streaking on a Zobell Marine agar plate every alternate day and observing after 24-48 h. The total viable count was checked at the end of incubation.

The scanning electron micrographs of *B. cereus* AK1871 grown in presence and absence of toluene at 50% (v/v) concentration were taken by SEM model LEO 1430VP.

3.2.2. Antibiotic resistance pattern of OSTB

The antibiotic resistance for each OSTB was tested using the disc diffusion method. Discs (HiMedia IC006, Icosa Universal - 2) containing the following antibacterial agents were placed on the inoculated plates and incubated over night. This test was performed against 20 common antibiotics with recommended doses such as; Amikacin (Ak, 30 μg), Ampicillin (A, 10 μg), Amoxicillin (Am, 10 μg), Cefadroxil (Cq, 30 μg), Cefoperazone (Cs, 75μg), Cefazidime (Ca, 30 μg), Ceftriaxone (Ci, 30 μg), Chloramphenicol (C, 30 μg), Ciprofloxacin (Cf, 5 μg), Cloxacillin (Cx, 1 μg), Co–Trimoxazole (Co, 25 μg), Erythromcin (E, 15 μg), Gentamicin (G, 10 μg), Nalidixic acid (Na, 10 μg), Netilmycin (Nt, 10 μg), Nitrofurantoin (Nf, 300 μg), Norfloxacin (Nx, 10 μg), Penicillin (P, 10 μg), Tobramycin (Tb, 10 μg), Vancomycin (Va, 30 μg). The zone of inhibition was measured after 24 h of incubation at 30 °C.

3.2.2.1. Antibiotic resistance index (ARI)

Antibacterial resistance index (ARI) of each sampling site was determined using the formula ARI = \(\frac{y}{nx}\), where \(y\) was the actual number of resistance determinants recorded in a population of size \(n\) and \(x\) was the total number of antibacterial agents tested for, in the sensitivity test. Based on the occurrence of resistance to more than three antibiotics the isolates of each sampling sites were also grouped as multiple antibiotic resistant isolates.
3.3. STUDIES ON SOLVENT TOLERANT PROTEASE

3.3.1. Screening of organic solvent tolerant protease producer

The OSTB obtained as described earlier were further screened for their protease production on skim-milk agar (SMA) plate, which contained (g liter⁻¹): tryptone, 5.0; yeast extract, 3.0; skimmed milk powder, 25.0; and bacteriological agar 15.0 [Tang et al., 2008]. The plates were incubated at 30 °C for 24–48 h.

3.3.2. Enzyme production

The isolate *Bacillus cereus* AK1871 was grown in Erlenmeyer flask (1000 ml) containing 500 ml of Zobell Marine broth for 48 h at 35 °C on a shaker at 120 rpm. Zobell Marine broth was used as basal media for studying effect of nutritional and physical factors on protease production. The culture was centrifuged at 4 °C for 10000 rpm and 10 min. The supernatant was used as crude protease for further study.

3.3.3. Protease assay

Protease activity was assayed by following method of Sana et al., [2006] using casein as a substrate. The reaction mixture containing, 100 μl Glycine-NaOH buffer (100 mM, pH 8.5), 100 μl of 0.6% casein solution and 200 μl of enzyme solution, was incubated at 40 °C for 30 min. The reaction was terminated by addition of 400 μl of 10% trichloroacetic acid solution and the non-hydrolyzed casein was removed by centrifugation at 10000 rpm for 10 min. Peptide concentration of the supernatant was determined by Lowry’s method [1951] using tyrosine as a standard. One unit of enzyme is defined as the amount of enzyme required to produce colour equivalent to 1.0 μg tyrosine min⁻¹ at pH 8.6 at 40 °C.

Enzyme protein was also measured by using the same method with bovine serum albumin as standard. During chromatographic purification, protein concentration of each fraction was estimated by measuring its absorbance at 280 nm.
3.3.4. Nutritional factors affecting protease production

The optimization of nutritional requirements for the production of the organic solvent stable protease by an organic solvent tolerant bacterium *Bacillus cereus* AK1871 has been studied. 1% (v/v) of 24 h bacterial inoculum was inoculated into 50 ml of culture media and incubated at 35 °C and 150 rpm for 48 h. Two types of media were used in this study: minimal medium (MM) [g liter⁻¹: (NH₄)₂SO₄, 1.0; KH₂PO₄, 0.5; MgSO₄·7H₂O, 0.3; CaCl₂·2H₂O, 1.0; NaCl, 5.0] and basal medium (BM was Zobell Marine broth). The minimal media was supplemented with different nutritional factors to be studied. The culture was harvested from the medium by centrifugation at 10000 rpm and 4 °C for 10 min. The supernatant was assayed for protease activity by standard assay conditions as mentioned in section 3.3.3. The protease activity was compared with relative activity of basal media as 1.00.

3.3.4.1. Effect of carbon source on protease production

The effect of different carbon source on the protease production was studied by adding 1% (w/v) of arabinose, dextrose, fructose, glycerol, lactose, maltose, mannose, sorbitol, starch and sucrose in minimal media supplemented by 1% peptone. All carbon sources were sterilized separately at 100 °C for 20 min.

3.3.4.2. Effect of organic nitrogen source on protease production

The effect of different organic nitrogen source on the protease production was studied by adding 1% (w/v) of peptone, tryptone and yeast extract in the minimal media.

3.3.4.3. Effect of inorganic nitrogen source on protease production

The effect of different inorganic nitrogen source on the protease production was studied by adding ammonium nitrate and ammonium chloride instead of 0.1% (w/v) ammonium sulphate in the minimal media. Starch was added at 1% (w/v) as carbon source.

3.3.4.4. Effect of amino acid on protease production

The effect of amino acid on protease production was studied by replacing inorganic nitrogen source in the minimal medium with 5.0 mM amino acids such as arginine, glutamic acid, glycine, lysine and ornithine.
3.3.4.5. **Effect of metal ion on protease production**

The effect of metal ion on the protease production was studied by replacing calcium chloride in the minimal medium with 2.0 mM of each barium chloride, cobalt chloride, ferric chloride, magnesium chloride, manganous chloride, potassium chloride and strontium chloride.

3.3.5. **Physical factors affecting protease production**

The effect of inoculum size on the protease production was studied by varying inoculum size, i.e. 1.0%, 2.0%, 4.0%, 6.0%, 8.0% and 10.0% (v/v). The cultures were incubated at 35 °C, 150 rpm for 48 h. The effect of pH on protease production was studied by adjusting the initial pH of the medium to the pH values of 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 and incubated at 35 °C, 150 rpm for 48 h.

3.3.6. **Purification of solvent stable protease**

3.3.6.1. **Ammonium sulphate precipitation**

To the crude enzyme, ammonium sulphate was added slowly to 60% saturation with gentle stirring and left overnight at 4 °C for complete precipitation. The precipitate was collected after centrifugation at 12,000 rpm for 15 min at 4 °C, dissolved in minimum amount of Glycine-NaOH buffer (pH 8.6) and dialysed against the same buffer.

3.3.6.2. **Anion exchange chromatography**

The dialysed enzyme was loaded on DEAE-Cellulose pre-equilibrated with Glycine-NaOH buffer of pH 8.6. The proteins were separated based on their charge by eluting them with a concentration gradient of sodium chloride (0.1-0.5 M) in the same buffer as fractions of 5 ml each at a flow-rate of 1 ml min⁻¹. Each fraction was subjected to determination of protease activity and protein estimation by measuring OD at 280 nm. Fractions with high protease activity were pooled and concentrated by lyophilisation.
3.3.6.3. Sephadex gel filtration

For molecular weight based separation, the pooled enzyme obtained from ion exchange column chromatography was loaded to a Sephadex-G-200 (Sigma) column (size) pre-equilibrated with 0.2 M of Glycine-NaOH buffer (pH 8.6) and then eluted with the same buffer. Fractions of 3.0 ml each were collected at a flow-rate of 16 ml h⁻¹. Each fraction was analyzed for protease activity and protein by determining OD at 280 nm. The active fractions were pooled, concentrated by lyophilization and purity was checked by PAGE.

3.3.7. Electrophoresis and zymography

SDS-PAGE was performed by the method of Laemmli [1970] using 5% stacking gel and 10% resolving gel with Tris-glycine buffer, pH 6.8 and pH 8.8 respectively, containing 0.1% sodium dodecyl sulfate at 4 °C. The samples loaded were heated at 80 °C for 5 min. Following SDS-PAGE, the gel was cut into two to prevent over staining of markers by silver staining method to visualize the protein bands on the gel.

Substrate polyacrylamide gel electrophoresis (Substrate-PAGE) was performed by incorporating casein {0.1% (w/v)} into the separating gel before polymerization. After the electrophoresis, the gels were rinsed twice in 2.5% Triton X-100 for 30 min to remove SDS. The gel was then incubated in 0.2% (w/v) casein solution (0.05 M Tris, pH 7.6, 0.1 M NaCl). The gels were then stained with 0.1% Coomasie blue R-250 in methanol-acetic acid-water (40:10:50) followed by destaining with methanol-acetic acid-water (5:10:85).

3.3.8. Characterization of protease

3.3.8.1. Molecular weight determination by SDS-PAGE

SDS-PAGE was performed to determine the homogeneity and molecular mass of the purified protease using 5% stacking gel and 10% resolving gel according to the method of Laemmli [1970] and electrophoresis was performed with 15 mA constant
current. A standard molecular weight markers (100, 80, 70, 50, 40, 20, 15 and 10 kDa) (Genei; Bangalore, India) were used.

3.3.8.2. Effect of pH on activity of purified protease

The effect of pH on protease activity was studied by assaying purified protease at different pH. The pH of the reaction mixture was adjusted using one of the following buffers: 0.1 M citrate buffer (pH 5.0-6.0), 0.2 M Tris-HCl buffer (pH 7.0-9.0) and 0.2 M Glycine-NaOH buffer (pH 10.0-11.0).

3.3.8.3. Effect of temperature on activity of purified protease

To study the effect of temperature on activity of the purified enzyme, the reaction mixture was incubated at different temperatures ranging from 10 ºC to 80 ºC for 30 min and the activity was determined.

3.3.8.4. Effect of sodium chloride on activity of purified protease

To study the effect of NaCl on the protease activity, the purified enzyme was assayed in the presence of different concentrations of NaCl ranging from 0-6 M.

3.3.8.5. Effect of metal ions on activity of purified enzyme

Effect of different metal ions on purified protease was studied using different metal ions like Ba$^{2+}$, Ca$^{2+}$, Cd$^{2+}$, Co$^{2+}$, Cr$^{3+}$, Cu$^{2+}$, Hg$^{2+}$, K$^{+}$, Li$^{+}$, Mg$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, Pb$^{2+}$ and Zn$^{2+}$ (all ions in chloride form,) at final concentration of 5 mM in the assay system, pre-incubated at 60 ºC for 30 min and the relative activity was measured.

3.3.8.6. Effect of inhibitors on activity of purified protease

To study the effect of different inhibitors on the purified enzyme, aliquots were pre-incubated with different inhibitors, ethylenediaminetetraacetic acid disodium salt (EDTA, 5 mM), 1,10-Phenanthroline (o-Phe, 5 mM), urea (5 mM), iodoacetamide (5 mM), phenylmethanesulfonylfluoride (PMSF, 5 mM) and protease inhibitor cocktail {AEBSF, Bestatin hydrochloride, E-64, Leupeptin hemisulphate, Pepstatin A, o-Phe (1% (v/v))} (Sigma-Aldrich P9599) at 60 ºC for 30 min. Relative protease activity was compared with control (without inhibitors).
3.3.8.7. Effect of detergents, oxidizing, reducing and bleaching agents on activity of purified protease

Effect of detergents was studied with non-ionic detergent {Triton X-100, Tween 80, 1% (v/v)}, anionic detergent {sodium dodecyl sulphate (SDS), 5 mM}, cationic detergent {cetyl trimethyl ammonium bromide (CTAB), 5 mM}, and commercial detergents {Tide, Surf excel and Ariel; 1% (w/v)}. Influence of oxidizing - bleaching agent {hydrogen peroxide, 1% (v/v)}, reducing agent (glutathione, 5 mM) and disinfectant (sodium tetraborate, 5 mM) was also studied on protease activity. All the additives were mixed in the assay system at prescribed concentration and activity was checked.

3.3.8.8. Effect of different organic solvents on stability of purified enzyme

1.0 ml of the purified enzyme solution in 0.2 M Glycine-NaOH buffer (pH 8.6) was mixed with 0.5 ml of different organic solvents (with 0.3% Tween 80 as emulsifier) and left for 144 h at 30 °C with constant shaking. Relative protease activity, with respect to control, was measured at different time intervals of 1 h, 48 h, 96 h and 144 h.

3.3.8.9. Substrate specificity

Substrate specificity of the enzyme was tested for substrates such as casein, BSA and wheat gluten at 1% (w/v) concentration.

3.4. STUDIES ON SOLVENT TOLERANT LIPASE

3.4.1. Screening of organic solvent tolerant lipase producer

The OSTB obtained as described earlier were further screened for their lipase production on tributyrin agar plate (M157; HiMedia, India) [g liter⁻¹: peptone, 5.0; yeast extract, 3.0; agar, 25.0; tributyrin, 10 ml]. The plates were incubated at 30 °C for 24–48 h.
3.4.2. Lipase assay

Lipase activity was determined using \( p \)-nitrophenyl palmitate (pNPP) as substrate according to the method described by Winkler and Stuckmann [1979] with some modifications. 4-Nitrophenol was used as a standard. The substrate was prepared by dissolving 0.5 g of pNPP in 100 ml ethanol. The assay mixture consisted of 50 μl of suitably diluted enzyme, 2875 μl of 20 mM Tris–Cl buffer (pH 8.0) and 75 μl of substrate. The assay was performed at 50 °C for 30 min and release of \( p \)-nitrophenol was measured spectrophotometrically at 410 nm. One unit of lipase activity was defined as the amount of enzyme required to release 1 μmole of pNP ml\(^{-1}\) min\(^{-1}\) at 50 °C and pH 8.0. Enzyme protein was estimated by the Folin phenol method [Lowry et al., 1951] using bovine serum albumin as the standard.

3.4.3. Optimum lipase production

Eight OSTB, out of 25 showed lipase production when screened on tributyrin agar. The eight OSTB (\( T. \) halophilus AK39315, \( B. \) pumilus AK39651, \( B. \) licheniformis AK39762, \( B. \) flexus AK39763, \( B. \) subtilis AK39765, \( M. \) indicus AK39766, \( B. \) megaterium AK39883, \( B. \) pumilus AK39885) were grown in 50 ml Zobell marine broth containing 1% (w/v) olive oil on shaker at 150 rpm and 35 °C for 96 h. The lipase production was checked every 24 h.

3.4.4. Effect of different oils on lipase production

Four OSTB (\( B. \) licheniformis AK39762, \( B. \) flexus AK39763, \( B. \) subtilis AK39765 and \( M. \) indicus AK39766) from eight showing high lipase activity were further studied for effect of different oil on lipase production. To test the effect of oil supplement on lipase production, the Zobell Marine broth was supplemented with different oils [1% (w/v)] and cultivated at 35 °C, 150 rpm for 72 h. The oils added in the nutrient medium were castor oil, coconut oil, cottonseed oil, groundnut oil, jatropha oil, jojoba oil, and olive oil. Nutrient medium without the addition of oils was used as control. The culture broth was assayed for lipase activity every 24 h for 72 h. 1.0 ml sample drawn for lipase activity was centrifuged at 10,000 rpm and 4 °C for 10 min.
3.4.5. Effect of organic solvent on lipase stability

The stability of lipase in organic solvents was investigated by mixing 10 ml of culture supernatant (crude lipase) and 10 ml of solvent (dimethyl sulfoxide) i.e. 50% (v/v) in 250 ml Erlenmeyer flask with interchangeable stopper to prevent loss of solvent through evaporation. The system was incubated in orbital shaker at 150 rpm and 35 °C for 144 h. OSTB (B. licheniformis AK39762, B. flexus AK39763, B. subtilis AK39765 and M. indicus AK39766) showing high lipase activities were studied for solvent tolerant lipase in highly toxic solvent, DMSO (log P value -1.35) at 50 % (v/v) concentration. Relative activity of an aliquot was measured by the standard assay at regular intervals.

B. flexus AK39763 showed solvent stability compared to other three isolates, hence was chosen for further studies. The isolate AK39763 was grown in Erlenmeyer flasks (1000 ml) containing 500 ml of Zobell Marine broth containing 1% (w/v) olive oil for 72 h at 35 °C on a shaker at 150 rpm. The culture was centrifuged at 10,000 rpm and 4 °C for 10 min and the supernatant was used as crude lipase for further study. The stability of AK39763 lipase in organic solvents was further investigated by mixing 10 ml of culture supernatant (crude lipase) and 10 ml of solvent (log P value) [Hexadecane (8.8), Cyclooctane (4.5), Hexane (3.9), Xylene (3.1), Carbon tetrachloride (2.7), Benzene (2.1), Butanol (0.8), MTBE (0.9), Acetone (-0.23), and DMSO (-1.35)] in 250 ml Erlenmeyer flask with interchangeable stopper to prevent loss of solvent through evaporation. The solvent tolerance of lipase was comprehensively studied across the log P range comprising aliphatic, alicyclic, aromatic, ether, alcohol, ketone, and chlorinated class of hydrocarbons. Each solvent was used at 50 % (v/v) concentration. Relative activity of an aliquot was measured with respect to control at zero hour as 1.00 by the standard assay at regular intervals.

3.4.6. Effect of pH on lipase activity

Optimum pH of the extracellular lipase from B. licheniformis AK39762, B. flexus AK39763, B. subtilis AK39765 and M. indicus AK39766 was determined by measuring the enzyme activity over a pH value ranging from 5.0 to 9.0 by standard assay method. The pH of the reaction mixture was adjusted using one of the following buffers: 0.1 M citrate buffer (pH 5.0-6.0) and 0.2 M Tris-HCl buffer (pH 7.0-9.0).
3.5. STUDIES ON POTENTIAL OF OSTB FOR BIOREMEDICATION

3.5.1. BT transformation in liquid medium

The biodegradation ability of OSTB was evaluated with mixture of BT (Benzene and Toluene = 1:1; 1740 mg liter\(^{-1}\) of each component) in 250-ml Erlenmeyer flask with stopper containing 50 ml of biodegradation media [g liter\(^{-1}\): Magnesium sulphate, 0.2; Calcium chloride, 0.02; Monopotassium phosphate, 1.0; Dipotassium phosphate, 1.0; Ammonium nitrate, 1.0; Ferric chloride, 0.05; Yeast extract, 0.1, trace element solution, 1.0 ml]. Trace element solution (g liter\(^{-1}\)) [Cupric sulphate (CuSO\(_4\).5H\(_2\)O), 10; Boric acid (H\(_3\)BO\(_3\)), 10; Manganous sulphate (MnSO\(_4\).5H\(_2\)O), 10; Zinc sulphate (ZnSO\(_4\).7H\(_2\)O), 70; Molybdic acid (MoO\(_3\)), 10].

Each OSTB was grown on preinoculum media [g liter\(^{-1}\): Magnesium sulphate, 0.2; Calcium chloride, 0.02; Monopotassium phosphate, 1.0; Dipotassium phosphate, 1.0; Ammonium nitrate, 1.0; Ferric chloride, 0.05; NaCl, 5.0; Peptone, 1.0; Yeast extract, 1.0] and incubated on orbital shaker at 140 rpm and 35 °C for 24 ± 2 h. Biodegradation media was inoculated by 2% preinoculum and incubated on orbital shaker at 140 rpm and 35 °C for appropriate duration. Control was kept to eliminate solvent loss by evaporation.

3.5.2. BT analysis

The initial experiments were performed with analysis by GC-MS, but due to ease and reproducibility of analysis, further experiments were done using HPLC.

3.5.2.1. GC-MS

OSTB *B. licheniformis* AK1872, *B. oceanisediminis* AK39313, *M. luteus* AK39532, and *S. arlettae* AK39675 were evaluated for their biodegradation capability by this method. After 48 h of incubation in biodegradation media, 50 µl ethyl benzene was added as internal standard and extracted with 5 ml dichloromethane [DCM: CH\(_2\)Cl\(_2\), M.W. 84.93] thrice. Sodium sulphate was added to remove water molecules from DCM extract. The remaining DCM was made up to 25 ml in volumetric flask.
Benzene, toluene and ethyl benzene were quantified by gas chromatography with a Shimadzu gas chromatograph (GC-2010, Shimadzu Corporation, Kyoto Japan) fitted with a MS detector (GC-MS-QP2010, Shimadzu Corporation, Kyoto Japan) and equipped with a RTX-5 capillary column (0.25 mm by 30 m) by holding temperature at 40 °C for 3 min followed by increasing temperature to 240 °C at a rate of 15 °C/min. Helium (1.0 ml/min) was used as the carrier gas. MS detector was operated at +70 eV with electron impact mode. Reduction in benzene and toluene concentration was calculated with respect to control.

3.5.2.2. HPLC

Biodegradation ability of OSTB isolates viz. AK1871, AK1872, AK1882, VK1901, AK2641, AK39313, AK39315, AK39423, AK39427, AK39532, AK39651, AK39762, AK39763, AK39765, AK39766, AK39673, AK39674, AK39881, AK39883, AK39885 and AK39887 were analysed by HPLC analysis method. After 96 h of incubation, 50 µl ethyl benzene was added as internal standard to the biodegradation media, and extracted with 5 ml dichloromethane [CH₂Cl₂, M.W. 84.93] thrice. Sodium sulphate was added to remove water molecules from DCM. The remaining DCM was made up to 25 ml in volumetric flask.

Benzene, toluene and ethyl benzene were quantified by using a high-performance liquid chromatography (HPLC) (Waters 2695 Separations Module) equipped with Phenomenex [Luna C18(2), length 25 cm, internal diameter 4.6 mm, particle size 5.0 µm] (type: anion column) column and a Waters 2996 Photodiode Array Detector (type: eg conductometric). Mobile phase comprised of methanol: water (80:20). Column flow was 1.0 ml min⁻¹. Injection volume was 20.0 µl (auto injector). Software: Empower 2. Oven temperature: 30 °C min⁻¹. Reduction in benzene and toluene concentration was calculated with respect to control. All samples were analysed twice, and standard curve of benzene and toluene was plotted with varying concentration.