Organophosphorus compounds (OP) are ester or thiol derivatives of phosphoric acid. Tributyl phosphate (TBP) is one such organophosphorus compound; which is an alkyl (tri-n-butyl) ester of phosphoric acid with molecular formula \( \text{C}_{12}\text{H}_{27}\text{O}_{4}\text{P} \). TBP has variety of applications in different industries like nuclear industry, aviation industry, mine industry, paper industry, pesticide industry etc., which have led to its worldwide production of 3000-5000 tons per annum. Consequently, the diverse applications and high production of TBP constitutes the risk of release into the surrounding environment. The toxicology database for tributyl phosphate (TBP) is large and well documented. There are adequate studies on animal models for toxicity of TBP on different organ systems and a few studies on the effect of TBP in humans. Moreover, TBP poses a serious problem of pollution and health hazards due to its longer persistence in the environment as a result of its relative stability. As a consequence, studies on the biodegradation of TBP are becoming increasingly important.

In the present study, after rigorous two step enrichment of seven different soil and water samples, 22 morphologically different bacteria capable of growing on minimal medium containing TBP as a sole source of carbon and phosphorus were isolated. Enrichment cultures were obtained from seven different soil and water samples after around nine weeks of enrichment with TBP as an only phosphorus source. The acclimatization of these cultures to utilize TBP as a sole carbon and phosphorus source resulted in further enrichment of potent TBP degrading cultures. After rigorous enrichment, 22 morphologically different bacteria capable of growing on minimal medium containing TBP were isolated. Fifteen out of 22 isolates, which showed growth more than 0.5 in terms of OD\(_{600}\) in the minimal medium containing TBP were isolated. Fifteen out of 22 isolates, which showed growth more than 0.5 in terms of OD\(_{600}\) in the minimal medium containing TBP were considered efficient TBP degraders, which were used for further studies.

The cultural, biochemical and morphological characteristics as well as plasmid profile of these TBP degrading isolates were studied. Out of fifteen, only two isolates (PTP1 and PTP2) were Gram positive, non-motile rods whereas other thirteen isolates were Gram negative, motile, non spore forming rods. Analysis of plasmid DNA profile revealed that all the fifteen isolates harbored one to three plasmids of
molecular size ranging from 4.9 to 18.4 kb. Plasmids of size 6.7 and 4.9 kb were most frequent and were found in 11 and 8 out of 15 cultures respectively; whereas plasmid of size 18.4 kb was seen in only one isolate.

The BLAST analysis of 16S rRNA gene sequences revealed that nine out of 15 isolates belonged to subclass β-Proteobacteria, four to γ-Proteobacteria and two to bacilli. All isolates showed 95 to 100% similarity to the sequences of *Alcaligenes*, *Providencia*, *Delftia*, *Ralstonia*, and *Bacillus* genera, available in the GenBank database. Amongst all, isolate PTP1 showed the least homology with *Bacillus subtilis* (95%), whereas isolate BGW1 showed the highest homology with *Delftia* sp. (100%). The phylogenetic analysis revealed that the seven isolates viz., SBW1, SBW2, BGW3, STP11, STP12, STP13 and ACS1 clustered together and found to be closely related to genus *Alcaligenes*. The isolate BGW1 and BGS2 were closely related to *Delftia* sp. and *Ralstonia* sp. respectively. Moreover, members of γ-Proteobacteria found to be clustered with *Providencia* sp. The isolates PTP1 and PTP2 from class bacilli showed clustering with *Bacillus subtilis* and *Bacillus cereus* respectively. Although the isolates were obtained from different environmental samples, their phylogenetic analysis demonstrated that some isolates such as ACS1, BGW3 and STP12 were very closely related irrespective of their sampling site.

All the 15 isolates showed degradation of TBP in the range of 21 to 61% during 96 h of growth in the minimal medium containing 5 mM TBP; with concurrent increase in both the biomass and extracellular inorganic phosphate. These isolates were able to tolerate and degrade up to 5 mM TBP, the highest concentration reported to date (2 mM). Two isolates, *Providencia* sp. BGW4 and *Delftia* sp. BGW1 showed 61.0 ± 2.8% and 57.0 ± 2.0% TBP degradation respectively at the end of 96 h, which was highest among all the isolates. The degradation rate constants (k), calculated by first order kinetic model were between 2.35 to 9.65 µmoles ml⁻¹h⁻¹. The TBP degradation rate constants (k) for all isolates, calculated using first-order kinetic equation of the form $C_t = C_0e^{-kt}$; were in the range of 0.0024 to 0.0099 h⁻¹, moreover, the half-life of TBP in presence of cultures was in the range of 70 to 288 h.
Degradation of TBP in the presence of added carbon source and phosphorus source was studied in the minimal medium containing TBP using selected isolate *Providencia* sp. BGW4. Glucose significantly enhanced the growth and TBP degradation e.g. in the presence of 50 mM glucose, residual level of TBP was 0.95 mM after 96 h of incubation; in contrast the TBP level in the culture grown without glucose was 1.95 mM. Added inorganic phosphate showed significant inhibition of TBP degradation by *Providencia* sp. BGW4. The culture grown in the medium containing 5mM TBP and without KH$_2$PO$_4$ grew upto 0.85 OD$_{600}$ and depleted TBP from 5 mM to 1.95 mM, after 96 h of incubation. However, when the medium was supplemented with 50 mM KH$_2$PO$_4$, the same isolate grew upto 0.41 OD$_{600}$ and depleted TBP from 5 mM to 3.1 mM.

The effect of pH in the range of 4.0 to 8.0 as well as the effect of temperature in the range of 20.0 to 45.0 °C, on the TBP degradation and growth of selected cultures of *Providencia* sp. BGW4 and *Delftia* sp. BGW1 was studied. Significantly less growth and degradation of TBP was observed at pH 4.0 and pH 5.0, however, the rate of degradation was comparable for pH 7.0 and pH 8.0 for both the cultures. The optimal pH for growth of *Providencia* sp. BGW4 was 7.0 and that for TBP biodegradation was 8.0. The optimum temperature for TBP degradation and the growth of *Providencia* sp. BGW4 was 30 °C, while significant reduction in growth and degradation was observed at 20 °C. At 45 °C, *Providencia* sp. BGW4 failed to grow using TBP.

Since nitrate and sulphate are common impurities in the nuclear fuel reprocessing wastes, the effect of additional nitrate and sulphate (in the range of 0.1 mM to 100 mM) in the TBP containing media was tested using *Providencia* sp. BGW4 and *Delftia* sp. BGW1 cultures. Lower concentrations (0.1 mM) of sulphate and nitrate did not show any significant effect on the growth and TBP degradation; however, both nitrate and sulphate at 10 mM and 100 mM concentrations in the TBP containing minimal media significantly retarded the growth as well as TBP degradation by *Providencia* sp. BGW4 and *Delftia* sp. BGW1.
The effect of shaking versus static conditions on the growth and TBP degradation was tested on the isolates Providencia sp. BGW4, Delfia sp. BGW1, Ralstonia pickettii BGS2 and Providencia sp. ACS2. There was substantial reduction in the growth and TBP degradation under the static conditions.

The efforts were made to develop new GC-MS method for the analysis of TBP. The calibration curve of TBP versus peak area obtained with developed GC-MS method showed excellent linearity in the concentration range of 50-500 ppb ($r^2 = 0.995$). Although, other than dilution no offline sample preparation was carried out, the matrix effects caused by salts and other biomolecules were least. The developed GC-MS method demonstrated its feasibility and utility for quantitative estimation of TBP.

All the TBP degrading isolates were checked for the growth on the postulated intermediates of TBP i.e. dibutyl phosphate (DBP), monobutyl phosphate (MBP) and Butanol. Delfia sp. BGW1 showed maximum growth among the isolate tested on TBP and DBP substrates i.e. optical density at 600 nm was 0.968 and 1.429 while Providencia sp. BGW4 showed maximum growth on MBP with OD at 600 nm 1.327. Providencia sp. BGW4 and Providencia sp. SBS1 showed maximum growth on butanol (OD at 600 nm 1.131 and 1.078 respectively. The kinetics of butanol degradation was also studied for selected isolate, Providencia sp. SBS1 which showed 73.34% butanol degradation (estimated using GC-FID) after 96 h at 30 °C temperature.

In order to find out the pathway of TBP degradation, the isolates were investigated for phosphotriesterase (PTE), phosphodiesterase (PDE) and alkaline phosphatase (AP) enzymes using whole cell assays and cell free extract assays. In whole cell assays, the maximum specific activity of phosphotriesterase (26.85±0.9 μmoles.h$^{-1}$. g[dry weight] cells$^{-1}$) and phosphodiesterase (77.07±4.9 μmoles.h$^{-1}$. g[dry weight] cells$^{-1}$) was exhibited by Providencia sp. BGW4 and Delfia sp. BGW1 respectively. Providencia sp. BGW4 also showed maximum specific activity of alkaline phosphatase (42.41±1.2 μmoles.h$^{-1}$. g[dry weight] cells$^{-1}$). Alcaligenes sp.

Ph.D. Thesis, 2012                                    Kedar Ahire                                    University of Pune
SBW1 and *Delftia* sp. BGW1 cell extracts showed respectively 1.79±0.04 and 1.78±0.13 μmoles min⁻¹ mg protein⁻¹ PTE activities; the highest among all TBP degrading isolates. The kinetic parameters (*K_m* and *k_cat/K_m*) of PTE, PDE and AP were determined by fitting the collected data to Lineweaver-Burk double reciprocal plots for *Providencia* sp. BGW4, *Delftia* sp. BGW1 and *Providencia* sp. SBS1. The catalytic efficiency (*k_cat/K_m*) values of PTE, PDE and AP with paraoxon, bis(p-nitrophenyl) phosphate and p-nitrophenyl phosphate were 5.35 x 10⁴, 6.27 x 10² and 1.24 x 10⁴ M⁻¹ s⁻¹ respectively.

The selected isolates i.e. *Providencia* sp. BGW4, *Delftia* sp. BGW1 and *Alcaligenes* sp. SBW1 were used to find the effect of TBP on the induction of cell proteins. Specific protein of approx. 35 kDa was induced by TBP as revealed from the SDS-PAGE gels of protein samples of the cells grown in TBP containing medium (5mM). Moreover, this 35 kDa protein band could not be observed in the protein samples from the cells grown without TBP. Interestingly, it was also observed that, the proteins of approx. 50 kDa were underexpressed in the cells grown with TBP as compared to those in cells grown without TBP.