DISCUSSION

The verities of pesticides are being used world wide to control the damage caused to crops by different pests. The pesticides are designed to be persistent in environment so as to achieve effective control of pests for longer periods. However, their easy availability and reckless use during last few decades has resulted in their build up in ecosystem to carcinogenic levels (Newcombe et al. 1994). The organochlorine pesticides widely used before 1970s were banned as they were reported to be toxic to flora and fauna (Morgan et al. 1980; Nakata et al. 2002). The organophosphorous (OP) insecticides despite of their higher cost have emerged as safe alternative to organochlorinated compounds as they were known to have short half life (Gupta 2006). However, over the years extensive use of organophosphate compounds has resulted in their accumulation to levels which are toxic to flora, fauna and also human population. (Baker and Richards 2000; Solomon et al. 1996). This may be attributed to (a) their low aqueous phase solubility leading to absorption/adsorption on soil matrix and (b) co-metabolism of the parent compound to more toxic intermediates, accumulating in environment.

The low water solubility of these xenobiotic compounds limits their bioavailability to resident microbial populations capable of transforming them (Volkering et al. 1998; Angelova and Schamander 1999). The problem is being addressed by using chemical surfactants viz. Tween 80, Triton X-100, Tergitol, NPX, Brij 35 etc. to improve their apparent aqueous phase solubility. These chemical surfactants are of petrochemical origin and being complex molecules tend to persist, at the site of application, even after purpose of their applications has been attained hence themselves act as a pollutant at the site (Edwards et al. 1991).

Thus, there is a need to develop environment friendly option for achieving desired levels of clean-up of polluted sites. The potential of biosurfactant preparations from different microbes have been explored over the time for their emulsification potential in diverse applications ranging from enhanced oil recovery to components of cosmetics (Ron and Rosenberg 2002). The putative application of these surface active molecules in bioremediation studies has mostly been explored in lab scale studies related to pesticides, polychlorinated biphenyls (PCB) and polycyclic aromatic hydrocarbons (PAHs) (Banat et al. 2000; Noordman et al. 2002; Cameotra and Bollag
However, the basic deterrents for their field scale applications are their high cost of production, lack of data regarding their interactions with other microbial populations and stability in the environment. Thus, there is a need to make persistent efforts to lower the cost of production of these surface active molecules for field scale applications, to ascertain their biological safety in environmental applications and to determine level of their supplement to maintain a critical balance between bioavailability, toxicity and degradation of pollutants.

The results of the present study are discussed in light of information already available in literature so as to outline protocols to achieve bioremediation of sites polluted with nerve agents.

5.1 Isolation and screening

5.1.1 Enrichment and isolation

The soil samples were collected from agriculture fields situated in and around city of Amritsar, Punjab, India used for cultivation of wheat, rice, brinjal and potato crops having history of repeated spray of organophosphate pesticides over the years. The possibility of isolating efficient degraders in samples collected from sites polluted with target compounds is higher as the microbial populations might have adapted to higher levels of pesticides. The exchange of genes among different microbial cultures might have resulted in evolution of microbes those can efficiently transform such compounds (Seffernick and Wackett 2001; Ghigo 2001; Johnson and Spain 2003; Fux 2005).

5.1.2 Screening of chlorpyryrifos degraders

The initial screening of isolates to grow in presence 100 mg l\(^{-1}\) chlorpyryrifos was carried out on medium supplemented with glucose and yeast extract to ensure growth of isolates in growth limiting concentrations of chlorpyryrifos. However, for selecting isolates capable of carrying out effective transformation of chlorpyryrifos, without accumulation of TCP, the selected isolates were evaluated for their ability to grow on medium without any glucose supplement. The isolates ChlD, F-3, CH-y, 13.9 and C-2 selected for further studies were found to be efficient degraders of chlorpyryrifos as over the enrichment period they were adapted to grow in high concentration of chlorpyryrifos. It has been reported in literature that co-metabolic transformations of chlorpyryrifos result in synthesis of lethal intermediate TCP, a potent antimicrobial compound (Racke et al. 1990b). Thus, the targeted enrichment and isolation strategy followed during the study helped to select those populations which
were capable of using chlorpyrifos as sole source of carbon and energy without accumulation of TCP. Vidya Lakshmi et al. 2008 reported that naturally adapted microbes retain metabolic activity under natural conditions and can be used for field scale applications. Li et al. (2008) reported isolation of chlorpyrifos-degraders belonging to different genera viz. *Sphingomonas* sp., *Stenotrophomonas* sp., *Bacillus* sp., *Brevundimonas* sp. and *Pseudomonas* sp. from chlorpyrifos-contaminated samples.

5.1.3 Screening of biosurfactant producers

The microbial populations, in samples collected from agricultural fields with high concentrations of chlorpyrifos, might have developed ability to improve the aqueous phase partitioning of chlorpyrifos by producing surface active compounds. Thus, enriched microbial populations from these sites were screened for their ability to produce surface active agents. In the present study three different methods were used for screening of isolates as no single method can be employed for screening of microbial diversity (Youssef 2004). The agar plate assay methods i.e. blood agar plate assay and CTAB methylene blue agar plate assay were used for high throughput screening of all the isolates to identify potential biosurfactant processes (Mulligan et al. 1993; Siegmund and Wagner 1991). The blood agar plate assay method is based on hemolysis due to lowering of surface tension and the isolates ChlD, C-4, MC-1 and M-3 showed clear zone of hemolysis on blood agar plates. The CTAB-MB plate assay is selective for identification of anionic biosurfactant producers and the isolates ChlD, C-4, MC-1, M-3 including M-2 showed blue coloration with halo around their colonies. There are reports in literature regarding use of these methods for screening of microbial diversity (Pinzon and Ju 2009).

The third method used was to evaluate ability of isolates to lower surface tension of the growth medium (Magaritis et al. 1979). It is most reliable and standard method to detect the biosurfactant production by the microbes usually grown with glucose as a carbon source of choice (Willumsen and Karlson 1997; Bonilla et al. 2005; Joshi et al. 2008).

Nine (9) different isolates ChlD, F-3, CH-y, 13.9, C-2, C-4, MC-1, M-3 and M-2 were selected for further studies based on their ability to produce biosurfactant and degrade chlorpyrifos.
5.2 Identification

The isolates selected after screening were identified by polyphasic approaches based on evaluation of biochemical characteristics (Pallaroni, 1986) and amplification of 16S rRNA coding DNA (Lane, 1991).

Based upon percentage homology with the known database, chlorpyrifos degrading isolates were identified as ChlD (*Pseudomonas aeruginosa*), F-3 (*Klebsiella pneumoniae*), CH-y (*Stenotrophomonas maltophilia*), 13.9 (*Ochrobactrum intermedium*) and C-2 (*Bacillus cereus*). There are reports available in literature regarding chlorpyrifos degradation by different isolates including *Stenotrophomonas* sp., *Ochrobactrum* sp., *Pseudomonas* sp., *Klebsiella* sp. and *Bacillus* sp. (Yang et al. 2006; Vidya Lakshmi et al. 2008).

The isolates C-4 (*Pseudomonas* sp.), MC-1 (*Aerococcus viridans*), M-2 (*Ochrobactrum* sp.), an un-identified isolate M-3 and ChlD were identified having ability to produce biosurfactant. Different strains of *Pseudomonas* spp. have been reported in literature for their potential to produce rhamnolipid biosurfactant with two L-rhamnose molecules linked to a chain of β-hydroxydecanoyl β-hydroxydecanoate (Jarvis and Johnson 1949; Sylfadt et al. 1985). There are few reports regarding biosurfactant production potential of *Aerococcus* sp. and *Ochrobactrum* sp. Thus, their further exploitation would open new potential applications by using their surface active molecules.

The PCR based screening method is a fast and reliable method for detecting presence of desired gene or functional property from large number of samples/isolates. The use of *opd* primers, specific for amplification of gene coding for organophosphate hydrolase (OPH) an enzyme involved in degradation of organophosphate pesticides are reported (Singh et al. 2003). Out of the five isolates three cultures namely ChlD, CH-y and F-3 were positive for presence of organophosphate degrading gene showing an amplified product of 900 bp.

The other two chlorpyrifos degrading strains C-2 and 13.9 didn’t show presence of amplified product indicating that they might have some other organophosphate hydrolyzing gene. There are reports available that some of the OP-compounds hydrolyzing cultures have the potential to hydrolyze such compounds but do not contain *opd* gene (Dave et al. 1993; Cheng et al. 1993).

The DNA of five selected biosurfactant producers was amplified using *kpd* primers for detecting presence of *rhlB* gene coding for rhamnosyl transferase reported
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by Boudour et al. 2003. Rhamnosyl transferase (Rt1) is an enzyme known to catalyze the final step of joining the fatty acids moiety to sugar moiety resulting in formation of rhamnolipid molecule (Rahim et al. 2001). Three cultures ChlD, M-2, M-3 and C-4 were positive for rhlB gene showing amplified product of 221 bp with kpd primers which indicate that the strains have ability to produce anionic rhamnolipid based surface active compounds.

5.3 Biosurfactant production by selected strains

The five selected biosurfactant producers reduced the surface tension of growth medium from initial 65 mN M⁻¹ to less than 30 mN M⁻¹ after 120 h of incubation. Biosurfactants with ability to lower the surface tension of growth medium to 35 mN M⁻¹ are considered as good surfactants (Syldatk et al. 1985; Mulligan 2005).

Further isolate ChlD attained higher biosurfactant production (3.12 g l⁻¹) as compared to other isolates using glucose as carbon source. The biosurfactant production potential of all the isolates was evaluated in medium supplemented with molasses, a by-product of sugar industry, so as to explore strategies to lower the cost of production. The field scale application of biosurfactants is hampered because of high cost of production and purification and they have to compete with the low cost chemical surfactants which are generally used for bioremediation protocols. Thus, ChlD and other isolates are potential candidates for their use in bioremediation protocol, where generally molasses and other nutrient supplements are added to stimulate the activity of resident population. The “in-situ” production of biosurfactant by these isolates at required levels may enhance the process of bioremediation. Molasses is a complex substrate consists of 48-56% total sugar (mainly sucrose), 9-12% non-sugar organic matter, 2-4% protein, 1.5-5% potassium, 0.4-0.8% calcium, 0.06% magnesium, 0.6-2.0% phosphorus, 1.0-3.0 mg kg⁻¹ biotin, 15-55 mg kg⁻¹ pantothenic acid, 2500-6000 mg kg⁻¹ inositol and 1.8 mg kg⁻¹ thiamine (Makkar and Cameotra, 1997a). The cells of isolates supported 1.96 g l⁻¹ of biosurfactant using molasses which is higher than the yield reported in literature. Patel and Desai 1997 reported that Pseudomonas aeruginosa GS3 produced rhamnolipid biosurfactant with 0.25 g l⁻¹ of rhamnose concentration using 7% (w/v) molasses and 0.5% (w/v) cornsteep liquor as the primary carbon and nitrogen source. The other studies on ability of Pseudomonas aeruginosa to produce rhamnolipids from wide range of substrates such as alkanes, pyruvate, fructose, succinate, citrate, glycerol, mannitol, glucose and olive oil are reported in literature (Robert et al. 1989; Lynd et al. 1999).
5.4 Optimization of biosurfactant production by ChlD

Based on these initial findings further optimization studies were carried out using isolate ChlD so as to understand effect of different physico-chemical conditions and nutrient supplements to enhance its biosurfactant production efficiency. The studies in the literature suggest that physico-chemical conditions found to be significantly affecting the biosurfactant production by pseudomonads (Wei et al. 2005).

The isolate ChlD was found to be versatile in its ability to grow under wide range of pH, temperature and oxygen availability. The maximum production of biosurfactant was attained around neutral pH (6.5-7.5). However, it was able to support biosurfactant production above CMC levels of 200 mg l\(^{-1}\) at pH as low as 5.0 to high pH of 9.0 indicating to its potential for field scale applications.

The different studies reported in literature suggests that growth and biosurfactant production potential of different Pseudomonas spp. was best supported in the pH range of 6.5-8.0 (Healy et al. 1996). Pruthi and Cameotra (2003) reported pH of the growth medium in the range of 6.4-7.2 supported optimum biosurfactant production by Pseudomonas putida.

The cells of ChlD supported maximum reduction in surface tension of 28 mN M\(^{-1}\) and biosurfactant production of 3.12 g l\(^{-1}\) at 30\(^\circ\)C in comparison to 0.8 g l\(^{-1}\) and 2.13 g l\(^{-1}\) at 25\(^\circ\)C and 40\(^\circ\)C respectively. Similarly, Pruthi and Cameotra (2003) shown that optimum temperature range for biosurfactant production by Pseudomonas putida was 30\(^\circ\)C-40\(^\circ\)C with maximum production at 30\(^\circ\)C.

The yield of biosurfactant was found to be directly proportional to rpm at which flasks were incubated, with optimum production supported at 150 rpm. The ChlD cells under static conditions were able to support 0.6 g l\(^{-1}\) yield of biosurfactant. This ability of ChlD can be exploited for “in-situ” biosurfactant production under microcosm and mesocosm studies. The overall yield of biosurfactant increased with increase in rpm with a maximum of 3.24 g l\(^{-1}\) at 200 rpm. This may be attributed to optimum aeration & continuous mixing of nutrients supporting better growth and higher biosurfactant yield (Guerra-Santos et al. 1986).

A wide range of carbon sources such as different oils, glucose and glycerol have been reported to support biosurfactant production (Lin, 1996; Bonilla et al. 2005). The isolate ChlD supported maximum biosurfactant production of 7.25 g l\(^{-1}\) when grown on glycerol as carbon source followed by glucose and mannitol with
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3.31, 3.1 and 2.5 g l⁻¹ of yield. Thus, it provides a wide range of selection from molasses to glycerol and as per the desired application suitable carbon source could be used. *Pseudomonas aeruginosa* can produce rhamnolipids from wide range of substrates including glucose and glycerol (Robert *et al.* 1989). Ciapina *et al.* 2006 reported a yield of 1.7 g l⁻¹ by *Rhodococcus erythropolis* cells grown on glycerol as sole carbon source.

The optimum concentrations of both NaNO₃ as an inorganic nitrogen supplement and tryptone as organic nutrient supplement to growth medium was found to be 0.25% (w/v) for biosurfactant production by ChlD. Further increase in these levels resulted in lowering of biosurfactant production. This is in line with literature suggesting that nitrogen although is required for initial biomass generation and biosurfactant production starts after depletion of available nitrogen (Zhang and Miller 1994 and 1995). Silva *et al.* 2010 reported the production of 8.0 g l⁻¹ biosurfactant by *Pseudomonas aeruginosa* UCP0992 when grown in presence of glycerol 3% (w/v) along with 0.6% (w/v) NaNO₃ after 120 h incubation. Similarly, Pacheco *et al.* 2010 reported maximal biosurfactant production of 285 mg l⁻¹ by *Rhodococcus erythropolis* using glycerol as carbon source and sodium nitrate and yeast extract as inorganic and organic nitrogen sources along with potassium phosphate (150 mmol l⁻¹) as a source of phosphorus.

The biosurfactant production potential of ChlD reduced to 0.5 and 0.2 g l⁻¹ in presence of NH₄Cl and (NH₄)₂SO₄ respectively. There are reports that ammonium salts added as nitrogen supplement could support growth but biosurfactant production was less. This may be due to lowering of pH of growth medium leading to decrease in biosurfactant production (Abouseoud *et al.* 2008).

Production medium (pH 7.0) supplemented with 2% (w/v) glycerol and 0.25% (w/v) tryptone supported higher biosurfactant production of 7.25 g l⁻¹ after 120 h of incubation as compared to 3.9 g l⁻¹ achieved by MSM grown cells. The PM having contains sodium nitrate as inorganic nitrogen source has approximately 5.6 times higher phosphate concentration as compared to MSM. The higher phosphate concentration was found to support higher glycolipid production in *Rhodococcus* sp. 51T7 (Espuny *et al.* 1996). Kim *et al.* (1997) reported a positive influence of phosphate on biosurfactant production by *Bacillus subtilis* C9. The overall concentration of phosphate also effect the biosurfactant production as Pacheco *et al.* (2010) observed that with increase in potassium phosphate buffer concentration from
30 to 150 mmol l$^{-1}$ cells of *Rhodococcus erythropolis* achieved higher biomass and biosurfactant yield.

### 5.5 Analysis of biosurfactant produced by ChlD

The crude biosurfactant preparation resolved into two spots corresponding to mono-rhamnolipids and di-rhamnolipids on silica gel with characteristic Rf value of 0.72 and 0.42 for mono-rhamnolipids and di-rhamnolipids respectively reported in literature (Zhang and Miller 1994; Nitschke *et al.* 2006; Joshi *et al.* 2008).

The selection of method employed for extraction and purification of biosurfactant depends on final application of the preparation. The biosurfactants used as constituents of cosmetic/analytical kits need to be of highest purity whereas, crude or partially purified preparations might be suitable for bioremediation applications. Different methods are viz. acid precipitation, ammonium sulphate precipitation, foam fractionation, adsorption on polystyrene resins, organic solvent extraction followed by column chromatography have been reported to achieve purification of surface active molecules (Sen and Swaminathan 2005; Muthusamy *et al.* 2008). Among these methods column chromatography supports high purity, fast and effective recovery/purification desired compounds from crude biosurfactant extract.

The biosurfactant of ChlD was efficiently purified to mono-rhamnolipid and di-rhamnolipid fractions by column chromatography. The CMC (critical micellar concentration) of the pure mono-rhamnolipid and di-rhamnolipid produced by ChlD were determined to be 66 mg l$^{-1}$ and 500 mg l$^{-1}$ respectively as compared to 200 mg l$^{-1}$ of crude biosurfactant preparation. Torrens *et al.* 1998 reported CMC of the mono-rhamnolipid to be 50 mg l$^{-1}$, where as CMC of the pure di-rhamnolipid in this study was observed to be 500 mg l$^{-1}$ which is relatively high, suggesting it to be a weak surfactant as compared to mono-rhamnolipid.

### 5.6 Emulsification activity of biosurfactant

The research related to application of biosurfactant as emulsifiers to improve the dispersion of water insoluble pollutants into water has gained importance being an environmentally safe option as compared to use of chemical surfactant and emulsifiers.

The cell free supernatant of growth medium or crude biosurfactant produced by ChlD was checked for its ability to emulsify non-aqueous phase soluble hydrocarbons i.e. n-Hexadecane, kerosene oil and mobil oil in aqueous phase. It was observed that it improved their aqueous phase emulsification by 3.56, 6.56 and 6.55
times. The $E_{24}$ index of the suspension thus formed was found to be significantly higher 16.6, 80.0 and 101 folds as compared to the control without biosurfactant. The good $E_{24}$ index, the measure of the stability of the emulsion after 24 h indicates to versatile nature of biosurfactant produced by ChlD. The literature shows that the emulsification activity of biosurfactant can be used for many different purposes those includes release of oil trapped by capillaries in the oil wells, flushing of soil to remove certain hydrocarbons and also as wetting and lubricating agent at industrial front (Harvey et al. 1990; Providenti et al. 1995; Banat et al. 2000; Cameotra and Bollag 2003). The crude biosurfactant extract of ChlD had ability to partition chlorpyrifos to aqueous phase. However, the partially purified surfactant preparation was used so as to avoid batch to batch variation in further studies on assessing the biodegradation potential of chlorpyrifos. The partially purified biosurfactant preparation efficiently partition 5% to 20% (w/v) chlorpyrifos to aqueous phase at sub CMC levels of 20 to 100 mg l$^{-1}$.

5.7 Chlorpyrifos degradation by bacterial isolates

There has always been an interest in isolating the microbes capable of degrading chlorpyrifos as out of different OPs used in agricultural fields, chlorpyrifos is not prone to accelerated degradation in environment. There are reports in literature regarding microbial conversion of chlorpyrifos to DETP, which is further used as a source of carbon and phosphorous. Singh et al. (2003) isolated six chlorpyrifos degrading bacteria capable of degrading chlorpyrifos in both liquid medium and soil. Wang et al. (2006) reported degradation of 1 and 10 mg l$^{-1}$ of chlorpyrifos in 1.48 and 5 days respectively in mineral salts medium at pH 7.0 and 25 °C by pure culture Bacillus laterosporus DSP.

The five potential isolates used in this study belonging to Pseudomonas sp., Klebsiella sp., Ochrobactrum sp., Stenotrophomonas sp. and Bacillus sp. are potent chlorpyrifos degraders as they degrade chlorpyrifos both in presence of glucose as a co-substrate and in absence of glucose. Although, presence of glucose reduced the overall degradation rate of chlorpyrifos however, they can achieve similar levels of degradation after extended period of 20 days incubation. This indicates that presence of glucose might be catabolically repressing the enzymes involved in chlorpyrifos degradation. However, presence of glucose might be a help in improving the cell numbers and possible biosurfactant production especially in case of ChlD to improve availability of chlorpyrifos.
5.8 Chlorpyrifos degradation by substrate-induced cells of selected isolates

It was observed that induction of cells with 2 mg l\(^{-1}\) of chlorpyrifos during their activation phase improved the degradation potential of these isolates from 0.9 folds to 2.43 folds as compared to their respective controls with un-induced cells. This indicates that the chlorpyrifos degrading hydrolases/phosphotriesterases may be inducible. Similar reports had been reported earlier in literature where induction of cells with substrate or key intermediates of degradative pathway may improve their efficiency of degradation.

Armenante et al. (1994) also observed that the ability of *Phanerochaete chrysosporium* to degrade 2,4,6-TCP can be improved by induction with its substrate TCP. Higson 1998 reported inducible degradative system where degradation of polycyclic aromatic hydrocarbons by white-rot fungi was faster by fungi when exposed to PAHs prior to its inoculation in liquid medium. Similarly, Palanisami et al. (2009) reported that chlorpyrifos degradation by marine cyanobacterium *Phormidium valderianum* BDU 20041 increased after induction with chlorpyrifos.

5.9 Optimization studies for chlorpyrifos degradation by ChlD

The optimization studies were carried out with ChlD isolate it was able to support maximum degradation of chlorpyrifos. The effect of different physicochemical conditions and nutrient supplements on its degradation efficiency was evaluated by two approaches (a) one variable at a time and (b) response surface methodology using Box-Behnken design which predicts the effect of interaction between the individual variables on degradation efficiency.

The overall degradation of chlorpyrifos showed a peculiar pattern with almost 45.0% degradation in just two days and after that a lag of 24 h was observed. In the next 24 h the overall degradation achieved increase to almost 80.0%. This might be due to induction of TCP metabolism after its accumulation to threshold values in growth medium. Further studies on this would give a better insight as cells of isolate ChlD and other four isolates were found to be efficient degraders of TCP. Cho et al. 2009 reported 83.3% degradation of 30 mg l\(^{-1}\) chlorpyrifos up to 3\(^{rd}\) day by lactic acid bacteria and after that degradation process slows down and it took overall nine (9) days to achieve complete degradation.

Cells grown in M-9 medium of initial pH 8.0 supported maximum degradation. As per available reports, efficient chlorpyrifos degradation was observed in pH range of 7.5 to 8.5 (Greenhalgh et al. 1980; Singh et al. 2003) Thus, it can be
assume that the alkaline conditions may be better suited for effective degradation of OP compounds by their microbial degraders.

The cells of ChlD could achieve almost 50.0% degradation of chlorpyrifos when incubated at 50°C thus, indicating its potential for “in-situ” bioremediation protocols in tropical environments. However, cells were able to maintain the 80.0% degradation rate in the temperature range of 25-40 °C. The ability of the cells to maintain high level of chlorpyrifos degradation at higher temperature indicate that cells might be constitutively metabolizing chlorpyrifos, as plasmid borne characters may lost at high temperatures (Deshpande et al. 2001). Liu et al. (2003) had also reported a low temperature of 28°C as the optimum temperature for chlorpyrifos degradation to Aspergillus sp. during lab studies in liquid medium.

The cells of ChlD added at initial O.D₅₄₀≈0.75 (equivalent 9.2 x 10⁷ C.F.U ml⁻¹) supported best degradation potential. However, to a range of O.D₅₄₀≈0.25 to 1.25 was found to support more than 70% degradation. This indicates to ability of the ChlD cells to carry out efficient transformation even at low initial inoculum levels. A suitable initial inoculum level can counterbalance the initial population decline due to certain environmental factors and toxic substrate concentrations to degrade the pesticides (Comeau et al. 1993; Duquenne et al. 1996). Singh et al. 2006 also reported that optimum inoculum size has a noticeable effect on biodegradation of chlorpyrifos by Enterobacter sp. as no chlorpyrifos degradation was observed below an inoculum density of 10⁷ cells g⁻¹. Awasthi et al. 2000 reported that addition of inoculum of 2 x 10⁶ C.F.U ml⁻¹ was optimal for endosulfan (organochlorine pesticide) degradation and further increase in inoculum size had no effect on overall degradation. Karpouzas et al. 2005 observed that Sphingomonas paucimobilis, at inoculum level 4.3 x 10⁸ C.F.U ml⁻¹ was able to completely degrade 10 mg l⁻¹ of organophosphorous compounds ethoprophos and cadusafos. Thus, it is desirable to use optimum level of inoculum which could survive the initial exposure and achieve the desired levels of pesticide degradation as low inoculum levels were generally not able to support effective degradation of target pollutants (Ramadan et al. 1990).

The chlorpyrifos degradation potential of ChlD was affected by presence of glucose in the medium. The overall degradation of chlorpyrifos in first 5 days of incubation in presence of glucose although was significantly lowered, however after 20 days a comparable degradation efficiency was achieved by cells grown in absence of 2% (w/v) glucose. The 1% (w/v) supplement of glucose to medium had a
significant effect on overall chlorpyrifos degradation achieved by ChlD cells after 5 and 20 days. The reason for this would be investigated further. The yeast extract supplement to medium resulted in lowering of chlorpyrifos degradation potential of ChlD.

There are reports in the literature regarding co-metabolism of chlorpyrifos by *Pseudomonas diminuta* (Serdar *et al.* 1982). However, ChlD was able to degrade chlorpyrifos without any co-metabolite addition during lab scale studies and presence of glucose and yeast extract lowered its degradation ability. Cook (1987) reported that easily bioavailable carbon and nitrogen components can reduce the utilization of pesticides as an energy source for bacteria.

The ChlD cells continuously sub-cultured 2-3 times in absence of Zn$^{++}$ supplement in the growth medium supported only 70.0% degradation of chlorpyrifos after 120 h of incubation as compared to 86.5% degradation achieved by cells grown in Zn$^{++}$ supplement. The reports available in the literature show that removal of zinc metal ion from enzyme phosphotriesterase of *Pseudomonas diminuta* results in loss of organophosphate hydrolyzing activity (Dumas *et al.* 1989). Two zinc metal ions present in bi-nuclear metal centre are vital for maximal catalytic activity of the enzyme (Vanhooke *et al.* 1996; Benning *et al.* 2001). Further it was observed that in presence of high concentration of Zn$^{++}$ a significant decrease in chlorpyrifos degradation was evidenced as only 42% of degradation in presence of 0.5 mM of Zn$^{++}$ concentration was observed. Similarly, Kong *et al.* (1992) reported that addition of high concentrations (0.5 mM) of Cd (II) or Cr (VI) as metal ions resulting in low microbial growth and reduced dechlorination for various chlorophenols in freshwater sediment slurries.

The cells of ChlD supported 84.2% degradation of chlorpyrifos supplemented to M-9 medium at a rate of 10 mg l$^{-1}$ and with increase in concentration up to 120 mg l$^{-1}$ 65.1% degradation was achieved. Thus, it is evident that degradation potential of ChlD was not significantly decreased even after 12 folds increase of chlorpyrifos in medium. Hua *et al.* in 2009 observed that with increase in chlorpyrifos concentrations from 4 to 12 mg kg$^{-1}$ the overall degradation decrease from 83.8% to 79.5% of respective supplement after 35 days of incubation. On the other hand Shan *et al.* (2006) reported suppressed growth of bacterial, fungal and actinomycete populations in the presence of chlorpyrifos (10 mg kg$^{-1}$). Vischetti *et al.* (2007) reported that
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treatment of an Italian field soil with 10 mg kg\(^{-1}\) and 50 mg kg\(^{-1}\) chlorpyrifos resulted in reduction of soil microbial biomass by 25% and 50% respectively.

In light of the reports in literature ChlD is a potent natural soil isolate which could survive in presence of significantly higher chlorpyrifos concentrations.

The ability of ChlD cells to use chlorpyrifos as sole source of carbon and energy in M-9 medium shows that at 50 mg l\(^{-1}\) chlorpyrifos supplement maximum increase in number of colony from initial 1.0 x 10\(^8\) CFU ml\(^{-1}\) to 6.19 x 10\(^8\) CFU ml\(^{-1}\) after 120 h of incubation was observed as compared to the control without chlorpyrifos whereas, further increase in chlorpyrifos supplement decreases the number of C.F.U. However, Similar, reports in the literature indicate that presence of concentrations higher than 50 mg kg\(^{-1}\) may results in lowering of biomass (Vischetti et al. 2007). In this concern, there are reports that DETP and other related phosphorothionate or phosphorodithioate molecules produced during the transformation of organophosphate pesticides can also be utilized as a sole source of carbon and phosphorus by bacterial Psuedomonas spp. isolated from pesticide contaminated soils for their proliferation (Cook et al. 1978; Rosenberg and Alexander, 1979). In addition Ohshiro et al. (1996) reported that chlorpyrifos could be hydrolyzed by Arthrobacter sp. strain B-5 as a carbon and energy source rather than by co-metabolism. Similarly, Yali et al. (2002) reported isolation of a Pseudomonas sp. from polluted soils of a pesticide factory capable of complete degradation of methyl parathion and using it as a sole source of carbon and nitrogen.

5.10 Degradation of intermediate 3,5,6-trichloropyridinol (TCP) by ChlD under optimized conditions

The accumulation of the TCP after action of phosphatases on chlorpyrifos is of ecological significance as it inhibits proliferation of the microbial populations. Thus, it is desirable that cells transforming chlorpyrifos must not accumulate TCP. The cells of ChlD and other four isolates CH-y, F-3, C-2 and 13.9 were able to metabolize TCP (5 mg l\(^{-1}\)) to different levels. Li et al. 2007 reported that addition of TCP at higher concentration reduced the degradation rate of chlorpyrifos by Sphingomonas sp.

The studies suggest that solubility of TCP in aqueous phase is more as compared to chlorpyrifos hence it is more bioavailable for microbes those have the potential to degrade TCP hence TCP may not be a rate limiting intermediate in presence of its degraders ((Barrett et al. 2000; Caceres et al. 2007).
5.11 Optimization of chlorpyrifos degradation by response surface methodology

The statistical model studies gave insights into the interactions of different variables so as to control the physico-chemical and nutritive conditions “in-situ” to sustain higher degradation rates of chlorpyrifos. The optimization studies with one variable at a time indicated that chlorpyrifos concentration, pH and Zn\(^{++}\) are the main factors affecting the efficiency of chlorpyrifos degradation. The interaction of these three independent variables affecting the overall degradation efficiency of ChlID was studied using Box-Bhenken based RSM. The coefficients of determination (R\(^2\)) and R\(^2\) adjusted for chlorpyrifos degradation as function of independent variables was calculated to be 98.7% and 97.0% respectively. The value of R\(^2\) adj is closer to unity indicate effective fit of the model in the experimental data. The R\(^2\) Adj value of 97.0% in our observations indicated to a high level of significance for the model. In addition, smaller the difference between the predicted and observed values more will be the fitness of the model (Filiz et al. 2007), which showed that the model correlated well with measured data and was statistically significant at P ≤ 0.005.

The contour plots suggest that at low chlorpyrifos concentrations of 8 mg l\(^{-1}\) to 10 mg l\(^{-1}\) efficient degradation of 92% and 86.4% (± 1.07 of standard deviation) could be achieved. Similarly, during optimization using conventional optimization techniques also indicate that with increasing chlorpyrifos concentration degradation potential of the strain ChlID decreases and vice versa. Pandey and Singh (2004) reported that a dose of 4 L/hm\(^2\) chlorpyrifos showed a short-term inhibitory effect on the total microbial population in agricultural field soils. Shan et al. (2006) also indicated that with increasing chlorpyrifos concentration (2, 4, and 10 mg kg\(^{-1}\)) a decrease in bacterial, fungal and actinomycete populations and there was an enhanced inhibitory effect of chlorpyrifos on microbial population.

The results further suggested that among the three variables used in the statistical studies addition of metal ions (Zn) increases the degradation potential of ChlID. The phosphotriesterase from *Pseudomonas diminuta* is a highly efficient zinc metalloenzyme catalyst for the hydrolysis of variety of organophosphorus nerve agents (Donarski *et al.* 1989). Omburo *et al.* 1992 isolated an *opd* gene encodes a homodimer parathion hydrolase, which contains divalent zinc ions as a cofactor.

The contour plot further explains the better degradation efficiency of ChlID at pH 7.8. Wang *et al.* (2006) had also reported that chlorpyrifos degradation rate by
Bacillus latersprorus DSP was increased with increase in pH by order of pH 7.0 to pH 9.0. Thus a narrow range of alkaline pH supports the efficient degradation of chlorpyrifos below or above that range a drastic decrease in degradation efficiency was observed, which reflect the observation obtained by conventional optimization techniques. This further suggests that the model effectively fits the experimental data. These complex interactions of different variables affect enzyme activities that in turn may cause metabolite inhibition/stimulation during degradation process.

5.12 Chlorpyrifos degradation by ChlD in soil

The successful transfer of bioremediation technology from lab to land requires strategies to reproduce results obtained under defined laboratory conditions in unpredictable environment where controlling multiple parameters is very difficult. Another basic difference is that “in-situ” degradation studies in soil are having multiphasic environment. The soil matrix plays important role as it compete with microbial cells for binding to pollutants to be degraded. The physical adsorption/absorption of pesticides on soil particles, pH of clay particles and availability of oxygen and water is different than those prevalent in aqueous phase. In light of this, further studies with axenic cultures and their mixed consortia were taken up in soil slurry. In this soil study amended with 50 mg kg⁻¹ of soil was used.

The degradation potential of ChlD and its 15 different combinations with other four isolates ranging from 2 to all 5 together was evaluated in soil slurry studies. As expected the overall degradation potential in soil slurry was lower than that achieved in liquid medium studies as chlorpyrifos may tend to interact with soil matrix and other components. On the other hand soil matrix might be providing some protection to bacterial cells as for the mixed bacterial populations overall concentration of chlorpyrifos used in soil slurry was 5 times more than in aqueous phase studies.

The microbial consortium of adapted microbial populations has better chances of success rate in biodegradation. This might be due to their better adaptability toward the environmental stresses and potential to achieve complete mineralization of the target pollutant. The basic stability of association between components of consortium is main factor and to achieve this it is important to use compatible cultures. Further the synergy believes that cultures would improve the efficiency of degradation. It was observed that out of 15 total consortia combinations 6 combinations were able to achieve more than 70% degradation of chlorpyrifos.
The consortium designated C3 based on three cultures namely ChlD, CH-y and F-3 achieved more than 84.0% degradation and consortium designated C5 comprising of all five isolates was able to degrade 79.6% of chlorpyrifos (10 mg l\(^{-1}\)) within 120 h of incubation. The potential of the consortium to degrade chlorpyrifos in soil slurry studies was also evaluated. The consortium C5 achieved 78% degradation and consortium C3 supported 70.3% degradation of chlorpyrifos after 10 days of incubation. Similarly, Munnecke and Hsieh (1974) reported the bacterial consortium consisting of *Pseudomonas* sp., *Xanthomonas* sp., *Azotomonas* sp. and a *Brevibacterium* sp. can rapidly hydrolyze parathion as compared to individual cultures. The biodegradation of pesticides, such as endosulfan (Awasthi *et al.* 2000), 1,3-dichloropropene (Ou *et al.* 2001) and diazonin degradation (Cycon *et al.* 2009) by a microbial consortium has been reported. The population of microbial consortium can complement each other’s metabolic potential to effectively achieve the desired transformation of target pollutant as compared to individual culture (Macek *et al.* 2000; Kuiper *et al.* 2004; Chaudhry *et al.* 2005). Furthermore the microbial consortia can be beneficial to plants by synthesizing the compounds that protect the plants against plant pathogens, degradation of toxicants before putting any negative impact on plants and providing key nutrients (Dams *et al.* 2007; Liu *et al.* 2007). Vidya Lakshmi *et al.* (2008) also developed a microbial consortium consisting of *Pseudomonas fluorescens*, *Brucella melitensis*, *Bacillus subtilis*, *Bacillus cereus*, *Klebsiella* sp., *Serratia marcescens* and *Pseudomonas aeruginosa* achieved 75–87% degradation of chlorpyrifos (50 mg kg\(^{-1}\)) after 20 days of incubation.

### 5.13 Role of biosurfactant produced by ChlD in enhanced solubilization of chlorpyrifos

Pesticides, which have low water solubility can adsorb to humic material thus, are not available to degrading populations in soil (Alexander, 1999). The field experiments showed that solid phase concentrations of OP pesticide are higher than soluble concentrations by up to 3 orders (Detmer and Purpus 2007). The use of chemical surfactants for bioremediation purposes has been prevalent, that has some associated concern regarding their toxicity to microbial populations their low efficiency necessitating their use at high concentrations resulting in toxicity to flora and fauna.

Kanga *et al.* 1997 reported that glycolipid of *Rhodococcus* species 413A was observed to be 50% less toxic than a synthetic surfactant Tween 80 in capable of
solubilizing naphthalene. A biosurfactant produced by *P. aeruginosa* was compared with Marlon A-350 a synthetic surfactant widely used in the industry and it was observed that biosurfactant was comparatively less toxic and mutagenic (Flasz *et al.* 1998).

The biosurfactant although are environmentally friendly and are effective at low concentrations of usually sub-CMC levels, however concern regarding their biological safety and high cost are big drawbacks. In the present study it was evident that addition of biosurfactant at even low concentrations of 10 mg l⁻¹ or 0.01% (w/v) can achieve comparable emulsification activity to 400 mg l⁻¹ of chemical surfactants like Tween 80 and SDS and 6% (w/v) DMSO. Lafrance and Lapointe 1998 reported that rhamnolipid biosurfactant by a *Pseudomonas* sp. UG2 at 0.25% was much more efficient in mobilization and co-transport of pyrene than SDS and had less impact on the soil. Page *et al.* (1999) reported that biosurfactant from *Rhodococcus* strain H13-A was 35-fold more effective than the synthetic surfactant Tween 80 in increasing the mass transfer of PAHs into the aqueous phase. Biosurfactants being biodegradable can be added ex-situ or produced in-situ at the polluted site to improve the aqueous phase solubility of non-aqueous compounds resulting in efficient bioremediation.

Further there is less need for product purity and in-situ production of microbial surfactant by indigenous or introduced microbial populations is also possible (Ivshina *et al.* 1998, 2001). Warranaphon *et al.* (2008) isolated a biosurfactant-producing bacterium *Burkholderia cenocepacia* BSP3 with CMC of 316 mg l⁻¹ have the ability to emulsify methyl parathion, ethyl parathion and trifluralin hence potential strain to be used to increase the bioavailability of pesticide for bioremediation of contaminated soil.

5.14 Effect of enhanced solubility on chlorpyrifos biodegradation

Bintein and Deviller 1994 reported that hydrophobic organic compounds like lindane are less bioavailable in the soil relative to the aqueous solution hence need more time to degrade efficiently. The supplement of biosurfactant produced by ChlD to medium invariably resulted in improved degradation efficiency of individual cultures and their consortia C5 and C3. A significant and interesting observation from the study was that different cultures and their consortia response to presence of different concentrations of biosurfactant. The consortium C5 achieved higher degradation efficiency in soil slurry supplemented with 100 mg l⁻¹ biosurfactant whereas consortium C3 supported good degradation activity at 40 mg l⁻¹ biosurfactant
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supplement. Similarly in case of pure culture studies with isolates F-3 and 13.9 the addition of biosurfactant lower the chlorpyrifos degradation efficiency. However, at high concentration of biosurfactant supplement all the cultures showed lower degradation potential which may be due to the micellerization of chlorpyrifos toxic concentration of emulsified chlorpyrifos in growth medium which significantly affects the degradation potential or may be due to biological activity of biosurfactant which are known to other microbial species.

During the course of these studies no accumulation of TCP was detected by GC and HPLC analysis. The cells were grown in presence of TCP were able to degrade it indicating to their potential to transform TCP in the growth medium as it is being formed in the medium after degradation of chlorpyrifos.

The biosurfactant used in the study is an anionic rhamnolipid produced by ChlID. The reports indicated that anionic surfactants proved to be more effective in biodegradation of the non aqueous phase soluble polychlorinated compounds (Billingsley et al. 2002). Such compounds have lower affinity for the interior of anionic surfactants due to presence of the similar non-polar chain length (Javert and Heath, 1991) which promoted slow and continues release of the hydrophobic pollutants from the micelles, bringing them in contact with the degrading bacteria hence enhances the degradation process without putting inhibitory effect on the microbial growth due to the toxic concentration of the pollutants.

The surfactants addition may also alter the hydrophobicity of cell which allows the direct contact of the microbial cells and the pollutant molecules for their efficient degradation (Tang et al. 1998). Providenti et al. 1995 observed enhanced degradation of phenanthrene (50 mg l\(^{-1}\)) to 6.5%, 8.2%, and 9.8%, respectively using rhamnolipid biosurfactant by a *Pseudomonas* sp. UG2 at 0, 25, and 250 mg l\(^{-1}\) rhamnose equivalents after 35 days of incubation. Makkar and Cameotra 1997 also studied the effect of surfactin produced by *B. subtilis* on enhanced biodegradation of the hydrophobic pesticide endosulfan. Similarly Fiebig et al. 1997 has shown that in the presence of a glycolipid (GL-K12) biosurfactant from *Pseudomonas cepacia* at the concentration of 200 mg l\(^{-1}\) enhanced degradation of Arochlor 1242 by mixed cultures obtained from the soil was observed. Awasthi et al. 1999 showed enhanced microbial degradation of endosulfan a hydrophobic organic pesticide by 30%–45% in presence of biosurfactant produced by *Bacillus subtilis* MTCC1427. The literature evidences that surfactant can enhance the process of microbial degradation of
hydrophobic organic compounds by solubilizing them from solid phase of the soil into the aqueous phase (Kim et al. 2001).

5.15 Biochemical studies on the enzymes responsible for degradation

As per the reports available in literature the enzyme for degradation/transformation of chlorpyrifos were found to be intracellular (Hung and Liao, 1996; Richins et al. 1997; Elashvili et al. 1998). The crude extracts of cells of all the isolates showed ability to hydrolyze phosphoester bond as evident from release of p-nitrophenol from the substrate p-nitrophenylphosphate whereas, no activity was observed in cell free supernatant. The enzyme extract of ChlD, CH-y, F-3, C-2 and 13.9 supported more than 80% degradation of 2.0 mM chlorpyrifos and 3.0 mM TCP within 2 h of incubation at 25°C.

The interesting observation of the study is the identification of a protein of almost 338 kDa which has chlorpyrifos hydrolyzing ability in the substrate gel. The protein having a pI of 4.9 showed activity both in native and re-natured SDS PAGE. Horne et al. (2002) also suggest that phosphotriesterase is a 384-amino-acid protein with a molecular mass of approximately 35 kDa when it is cleaved from its signal peptide. Further, studies will be carried out to determine its nature that whether chlorpyrifos hydrolyzing protein associated with ChlD is a single protein or attached to transporter proteins which might be involved in flux of chlorpyrifos across cell membrane in ChlD.