ISOLATION OF PESTICIDE DEGRADING BACTERIAL CONSORTIUM AND ITS APPLICATION FOR INDUSTRIAL WASTEWATER TREATMENT

by

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DEDICATION

This work is dedicated to

*"**My beloved parents***

Mr. and Mrs. Raja Khalid Mehmood"

It is their support and love that enabled

me to complete this task.

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SAIRA KHALID

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LIST OF ABBREVIATIONS

ACS	Assimilable Carbon Substrate
ANOVA	Analysis of Variance
APHA	American Public Health Organization
BLAST	Basic Local Alignment Search Tool
CAM	Calcium Alginate Microsphere
COD	Chemical Oxygen Demand
CP	Chlorpyrifos
DETP	Diethyl-thiophosphate
DI	Deionized
DNA	Deoxyribonucleic acid
DO	Dissolved Oxygen
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
GC-ECD	Gas Chromatography Electron Capture Detector
GCMS	Gas Chromatography Mass Spectrometry
HRT	Hydraulic Retention System
MC	Macrocapsules
MEGA	Molecular Evolutionary Genetic Analysis
MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
MSM	Mineral Salt Medium

NB	Nutrient Broth
NCBI	National Centre for Biotechnology Information
NO_3-N	Nitrate Nitrogen
OCP	Organochlorine Pesticides
OD	Optical Density
OPP	Organophosphate Pesticides
PCR	Polymerase Chain Reaction
PO_4-P	Phosphate Phosphorous
PSF	Polysulfone
SA	Sodium Alginate
SEM	Scanning Electron Microscopy
SWW	Simulated Pesticide Wastewater
TCP	3, 5, 6-Trichloro-2-pyridinol
TMP	3, 5, 6-Trichloro-2-methoxypyridine
TSS	Total Suspended Solids
USEPA	United States Environmental Protection Agency
V1	Vigor Index

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ABSTRACT

Wastewater from agrochemical industries is disposed off without any treatment into nearby water bodies; hence there is a dire need to improve remediation approaches for its removal from environment. It is hypothesized that bacterial strains present in wastewater, after acclimatization, could use Chlorpyrifos (CP) as a sole source of carbon and energy and convert it into less toxic substances, both in free as well as immobilized form. Current study aimed at isolation of bacterial consortium capable of efficient CP biodegradation in mineral salt media (MSM), simulated pesticide wastewater (SWW) and real industrial wastewater (WW) in free and immobilized form. In order to produce environmentally stable immobilization matrix for bacterial consortium, potential of calcium alginate matrix coupled with polysulfone was investigated. Biodegradation potential of bacterial consortium isolated from wastewater and agricultural soil, for CP in MSM, SWW and WW was investigated. Bacterial consortium was immobilized in Calcium Alginate Microspheres (CAMs) and coated with polysulfone to produce environmentally stable macrocapsules (MCs). Bacterial strains were identified using 16S rRNA nucleotide sequence analysis as *Pseudomonas kilonensis* SRK1 (KT013088), *Serratia marcescens* SRK2 (KT013089), *Bacillus pumilus* SRK4 (KT013091), *Achromobacter xylosoxidans* SRK5 (KT013092) and *Klebsiella sp*. T13 (KT013093). About 98% CP removal was observed at initial CP concentration of 400 mg/L in 48 h in MSM when free cells were used as consortium. In WW bacterial consortium achieved ~29% removal efficiency of initial CP concentration (545 mg/L). After pH adjustment and addition of glucose in WW >97% CP removal efficiency was achieved in WW. MCs have high thermal, pH and chemical stability than CAMs. Complete biodegradation of CP (100-600 mg/L) was achieved using MCs within 18 h much less than free cells. CAMs and MCs retain >96% residual activity in MSM upto 5 and 13 cycles respectively. In WW >90 residual activity was maintained upto 11batches by MCs. MCs have shown unaltered biomass retention and residual activity (95%) over 16 weeks of storage. GCMS analysis has shown 3, 5, 6-trichloro-2-pyridinol (TCP), 3, 5, 6-trichloro-2-methoxypyridine (TMP) and diethyl-thiophosphate (DETP) as metabolites. MCs have shown considerable benefits over free cells i.e. tolerance for higher CP concentration, complete removal in short duration, reusability, stability, protecting bacterial cells against nontargeted compounds present in wastewater. Study advances potential for field application of immobilized bacteria for biotreatment of pesticide contaminated wastewater.

Chapter 1

1. INTRODUCTION

Pesticides are used in agriculture to save crops from preharvest and postharvest losses. Use of pesticides is considered most convenient and cost-effective method to control pests. On the basis of difference in structure, pesticides are classified as organophosphate pesticides (OPPs), organochlorine pesticides (OCP), carbamate and pyrethroids (USEPA, 2014). Among all these groups OPPs are widely used because of their efficiency and comparatively short half-life. It is estimated that among all the pesticides used 38% are OPPs (Singh *et al*., 2006; Zhang *et al*., 2008; Cycon *et al*., 2013). Availability of wide variety of OPPs at low cost has also increased its use.

In Pakistan there are pesticide manufacturing, formulating, packaging and filling units. Concentration of pesticides in agrochemical industry wastewater may be above 500 mg/L. Wastewater from pesticide industries need to meet stringent discharge standards before their disposal. Pesticides have been found in water bodies of Pakistan and are harmful even at very low concentrations (ng/L). Wastewater released from agrochemical industries contains pesticides and their residues. Therefore, treatment of pesticide industry wastewater before disposal is obligatory (Affam *et al.,* 2014). According to one assessment, pesticides are cause for approximately 200,000 mortalities annually, therefore they are considered as main risk for human health globally by WHO (Karalliedde and Senanayake, 1999; Isbister *et al*., 2007). OPPs stay in the environment for longer period of time; hence there is a dire need to improve remediation approaches to remove them from environment.

Among various organophosphate pesticides including monochrotophos, profenophos and chlorpyrifos are widely used in developing countries like India and Pakistan. These pesticides are persistent and they stay in the environment for longer period of time. Only a minute amount (1%) reach targeted area/pest and rest (99%) is released into nearby environment (Pimentel, 1995).

Chlorpyrifos was selected as model pesticide for current study because of its recalcitrant nature and potential health impacts. Chlorpyrifos (CP) is a broad spectrum organophosphate pesticide extensively used to control domestic and agronomic pests. CP is stable, persistent and stays in the environment for extended period of time. Half-life of CP is variable depending upon surrounding environment and is usually from 10-120 days (Briceno *et al*., 2012).

Pesticides are damaging for human health even in very minute concentration. Environmental concentration of CP has gradually increased due to its continuous use, thus human health concerns have increased (Rauh *et al*., 2011). CP act as inhibitor of acerylcholineesterase which results in abnormal functioning of central nervous system (CNS) and ultimately death occurs. Depending upon degree of exposure to CP, health effects could be vomiting, irregular working of central nervous system, abrupt unbalanced movement, paralysis and may cause death of insects, humans and other mammals (Yadav *et al*., 2014).

CP is introduced into environment through agricultural runoff and/or industrial effluents. There is a dire need to study the treatment of CP contaminated wastewater to meet Environmental Protection Authority (EPA) standards for industrial effluents. Biodegradation could be one of the possible solutions for elimination of CP from environmental compartments. Biodegradation could be carried out onsite and in engineered confined environments such as bioreactors.

Biodegradation of pesticides have been studied widely. Bacterial species such as *Enterobacter* sp., *Stenotrophomonas* sp., *Klebsiella* sp., *Pseudomonas fluorescence, Pseudomonas aeroginosa, Bacillus subtilis, Agrobacterium* sp., *Serratia marcescens, Sphingobacterium* sp., *Cupriavividus* sp., *Pseudomonas* sp. have been reported for having CP biodegradation capability (Singh *et al.*, 2004, Yang *et al.*, 2006, Ghanem *et al*., 2007; Lakshmi *et al*., 2008; Maya *et al*., 2011, Cycon *et al*. 2013, Abraham and Silambarasan, 2013, Lu *et al*., 2013). Bacterial species carry out biodegradation of CP proficiently at laboratory scale in controlled environment. Bacterial species breakdown OPPs e.g. CP using an enzyme organophosphate hydrolase (OPH) produced by them (Singh *et al*., 2006).

Most of the studies have reported biodegradation of CP using single specie for particular pesticide. Different strains have variable resistance to pesticides this could be attributed to diverse metabolic mechanism; therefore, co-inoculation may result in higher removal and resilience to environmental changes. To completely mineralize pesticide, different bacterial species could be used collectively. Metabolically adaptable/versatile bacteria may degrade pesticides completely in short period of time (Herrero and Stuckey, 2015).

In current study bacteria capable of CP biodegradation were isolated. Efficiency of bacterial monoculture for pesticide degradation was compared to bacterial consortium. Environmental factors play important role in biodegradation process. Effect of various process parameters on biodegradation kinetics of consortium was also investigated.

Bacterial species capable of biodegradation in the presence of single carbon source, under controlled conditions may not be that effective in the existence of additional carbon sources, solvents and inorganic substances. For understanding the efficiency of CP degrading bacterial consortium in wastewater having complex composition, bacterial consortium capable of efficient

CP degradation in synthetic wastewater was introduced into real industrial wastewater (WW) collected from a local agrochemical industry. Performance of microbes in terms of CP degradation was investigated in laboratory scale batch reactors using various process parameters. Along with CP degradation, formation of metabolic intermediates like 3, 5, 6-trichloro-2-pyridinol (TCP), 3, 5, 6-trichloro-2-methoxypyridine (TMP) and diethyl-thiophosphate (DETP) was also monitored. Change in concentration of chloride ions was used as an indicator of dechlorination. Understanding these factors may significantly enhance the vitality of biodegradation system. However, separation of cells for reuse has been a major problem associated with the process. Freely suspended cells used in these studies have some other disadvantages such as cell washout, stability loss and non-reusability making biotreatment costly (Kourkoutas *et al*., 2004; Chen *et al*., 2013). Thus, there is a need to investigate methods that make the bacterial application more cost-effective and durable.

Among many methods, bacterial immobilization could be a potential solution as it stops washout, allows reuse and improves stability. One of the processes used for bacterial immobilization is the use of calcium alginate matrix. Immobilization in Calcium Alginate matrix mimics the natural mode of existence, provides nutrients without opposition from other microbes and protects cells from harsh environment. Calcium alginate has broader application as it is easy to use and biocompatible. Use of calcium alginate matrix for bacterial immobilization and its application for biodegradation of pesticides and xenobiotic has been reported (Ha *et al*., 2009; Chen *et al*., 2013; Parasad and Suresh, 2015; Bergero and Lucchesi, 2015). However, this natural polysaccharide is vulnerable to biodegradation, has low mechanical stability, and is sensitive to chelating agents. Moreover, it may be softened quickly by citric acid and EDTA. This renders calcium alginate method unsuitable for most real wastewater treatments. Calcium alginate matrix

may be strengthened by mixing it with other polymers. Some efforts have been made to make it more stable using polylysine, polyvinyl alcohol and chitosan. However complex preparation and high cost are the problems associated with their use.

In current study five bacterial strains capable of CP degradation were immobilized into Calcium Alginate Microsphere coated with polysulfone (PSf) through phase inversion to make more stable macrocapsules. Characteristics (physical, chemical strength, reuseability, shelf life) of macrocapsules and kinetics of chlorpyrifos degradation were investigated. For comparison experiments were conducted with Calcium Alginate Microsphere without coating. In addition metabolic pathway was also postulated using GCMS analysis. Study demonstrated potential of microcapsules real field application in terms of extent of biodegradation, effluent quality, reusability and shelf life.

1.1. Objectives

1. Isolation, characterization and identification of Chlorpyrifos degrading bacterial consortium.
2. Investigate potential of isolated bacteria for Chlorpyrifos biodegradation.
3. Application for real industrial effluents treatment at lab scale.
4. Investigate potential of immobilized bacteria for Chlorpyrifos biodegradation.

Chapter 2

2. LITERATURE REVIEW

Pesticides are chemicals used to control or destroy pests. Based on the chemical composition, major pesticide groups are Neonicotinoids, organophosphate pesticides (OP), organochlorine pesticides (OC), carbamate, Pyrethrin and pyrethroid**.** Based on the organism they control, pesticides are classified as insecticide, rodenticide, fungicide, nematicide, larvicides, herbicide, Acasicide and bactericide. Out of total production cost upto 22% per hectare is used for pesticides (Hattab and Ghaly, 2012). Pesticides may be in the form of solid, liquid, i.e. emulsifiable concentrates, granules or gaseous formulation. Benefits of pesticide use range from decrease in pre-harvest losses, higher marketable yields, lower cost of production per unit output, increased revenue and ample supply of food.

2.1. Wastewater of Pesticide Manufacturing Units

Major pesticide producing companies include Bayer, Syngenta, Dow Ago Science, Monsanto. Non-targeted applications in fields results in accumulation of pesticides and their residues in soil, air and water. Wastewater from pesticide manufacturing and formulating units is released into nearby water bodies or land. Unsafe disposal of pesticides usually practiced has negative impact of environmental and human health (Hattab and Ghaly, 2012).

2.2. Risk to Non-targeted Species

This results in biomagnification of some pesticides and increased risk to non-target species (Kim and Ahn, 2009; Singh *et al*., 2011). Therefore, precautions should be applied during application, discarding, dumping and handling of pesticides. When pesticides are applied or disposed off

they will mix up with surface water and leach down this ends in ground water resources. It will increase risk for non-targeted species.

2.3. Organophosphate Pesticides

Organochlorine and carbamate pesticides are replaced by organophosphate pesticide because of wide variety, less cost, efficiency and shorter half-life (Yang *et al*., 2005). Organophosphate pesticides are usually broad spectrum pesticides. Among various pesticides are dichlorvos, chlorpyrifos, diazinon, paraoxon, parathion, methyl parathion, which are extensively used all over the world.

2.4. Chlorpyrifos

Chlorpyrifos (CP) was first introduced by Germans in 1930s. Dow chemical company in 1965 has introduced a non-systematic insecticide named as Chlorpyrifos (Worthing, 1979). Chemical formula for CP is $C_9H_{11}Cl_3NO_3PS$ and chemical structure is shown in Figure 2.1. According to IUPAC the chemical name of CP is O,O,-diethyl-O-(3,5,6,-tricholoro-2-pyridinyl) phosphorothionate. Among its outdoor uses is its application for controlling agricultural pests for variety of crops like maize, fruits (citrus, peach), vegetables etc. to reduce pre-harvest and post-harvest losses. It is also used to control household pest including mosquitoes, flies, ants, termites and cockroaches (Gabaldon *et al*., 2007; Bicker *et al*., 2005).

Table 2.1: Physical and chemical characteristics of Chlorpyrifos

Characteristic of Chlorpyrifos		References
Chemical Formula	$C_9H_{11}Cl_3NO_3PS$	Simon *et al*. (1998)
IUPAC Name	O,O,-diethyl-O-(3,5,6,-tricholoro-2-pyridinyl) phosphorothioate.	EPA (2006)
Trade name	Dursban® Lorsban ®	Yadav *et al*. (2015)
Appearance	White to light yellowish crystalline solid	Dow Agro Sciences, (2010)
Melting point	42-43.5˚C	Worthing, (1979)
Vapor Pressure	$1.8x10^{-5}$ mm Hg at 25˚C	
Molecular weight	350.6 a.m.u	EPA (2006)
Water solubility (at 25˚C)	2.0mg/L	
Half Life (at 25˚C)	pH 4-5 77days pH 6 49 days pH 8 19days	Chapman and Cole, (1982)

Out of total pesticides used in USA, 11% is CP (EPA, 2004). Out of all the OP insecticides, the sales volume of CP is ranked first (USEPA, 2011). Physical and Chemical properties of CP are reported in Table 2.1.

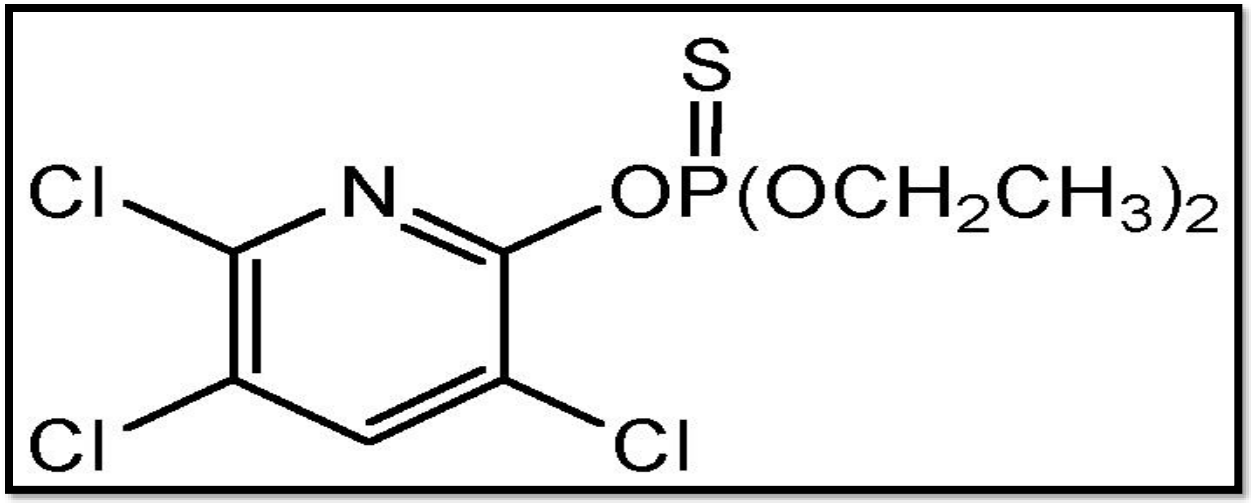


Figure 2.1: Chemical structure of Chlorpyrifos (CP)

2.5. Fate of CP in Environment

Fate of CP in the environment depends upon its chemical and physical properties and environmental factors. Biodegradation depends upon various environmental factors such as concentration, pH and temperature. When CP is introduced in environment it is found to have less potential for volatilization, chemical degradation, photolysis, resistance to leaching (Wu *et al*., 2002). CP is persistent and stays in the environmental compartments for longer period. In anaerobic CP is more persistent than aerobic environment (Lu *et al*., 2006). Half-life of CP in anaerobic environment is 6-7 times more than aerobic environment. Different studies have mentioned different half-life for CP. CP applied for controlling termites has been found effective even after 5 to 17 years. CP was found stable even after 12 months of application in soil (Chapman and Harris, 1980; Gilani *et al*., 2010). Half-life of CP at 25˚C was estimated to be 77, 49, 19 days at pH 4, 6, 8 respectively (Table 2.1). CP is marginally soluble in water but mixes well with organic solvents like acetone, ethyl acetate and methanol. Therefore, it stays in water bodies for longer period of time. It has a melting and boiling point of 41-42˚C and 170-180˚C respectively. It is highly soluble in organic solvents such as hexane, methanol, ethyl acetate and

acetone (Chishti *et al*., 2013; Yadav *et al*., 2015). First it was thought to degrade under alkaline conditions but later studies revealed the involvement of microorganisms. Depending on environmental conditions, initial concentration, biodegradation and volatilization rate CP may stay in the environment for variable time period (Singh *et al*., 2003; Surekha *et al*., 2008). Other than biodegradation, CP is also subjected to chemical degradation through process of hydrolysis and photolysis at neutral pH (Chisti *et al*., 2013).

2.6. Effect of Chlorpyrifos on Environment and Human Health

CP is introduced into environment through agricultural runoff and/or industrial effluents. CP buildup in the environment has increased public health concerns (Bicker *et al*., 2005; Rauh *et al*., 2011). There are several point and non-point sources of CP contamination. Among point sources agricultural runoff and industrial runoff fare most important. CP is proved to be extremely toxic for non-target organisms both in aquatic and terrestrial ecosystem (Yadav *et al*., 2014) due to presence of acetyl cholinesterase. Chlorpyrifos can be absorbed by fish and other aquatic organisms at moderate to high levels (USEPA, 1992; Chisti *et al*., 2013). CP and other OPs accumulate in the soil and effect activities of microrganisms such as nitrogen fixation (Kulshrestha and Kumari, 2011). Inhibition of acetyl cholinesterase in human leads to accumulation of acetylcholine in synapses and neuromuscular junction; which results in confusion, convulsion, and paralysis. In severe cases failure of central nervous system or respiratory system may lead to death because of this public concerns on safety have been increased (Sogorb *et al*., 2004; Bicker *et al*., 2005; Rauh *et al*., 2011). According to WHO OPPs are reason for about 3 million poisonings annually (Isbister *et al*., 2007). Wastewater from pesticide industries need to meet stringent discharge standards before their disposal. Thus there is a necessity for development of treatment to remove CP from environmental compartments.

2.7. Treatment of Chlorpyrifos Contaminated Sites

Current approaches practiced for treating pesticide contaminated wastewater includes chemical and physical processes, this includes incineration, open pit burning, deep-ocean dumping and advanced oxidation processes (Megharaj *et al*., 2011; Ismail *et al*., 2013). However, this treatment require land, take long time, expensive, un-ecofriendly, technically tough and suffer from variability of effectiveness. This may produce more toxic and persistent metabolites and non-degradable residues (Gao *et al*., 2012; Hattab and Ghaly, 2012) thus requiring secondary treatment. Thus it is essential to develop an effective treatment system.

2.8. Biodegradation; A Viable Option for Cleanup of Environment

One of the possible options for cleanup of CP from contaminated environment is biodegradation using microorganisms as it is cost effective and has less damage for other organisms. Removal of CP may occur due to adsorption instead of metabolism, leading to transfer of pollutant from one environment to another, thus requiring further investigation. Ex-situ treatments have attained more consideration, as they offer many benefits over in-situ treatment in terms of effective control over various process parameters (Yadav *et al.*, 2014).

Biodegradation has achieved researcher's attention for removal of organic pollutants as it is cost effective and has less damage for other indigenous organisms. Environmental factors plays very important role in CP biodegradation reaction. Most of the studies reported pH 6.0- 8.5 as optimal for bacterial biodegradation (Yang *et al*.,2005; Lu *et al*.,2013; Jabeen *et al*.,2014). pH effect biodegradation function in different ways. Enzymes are usually pH specific and perform their activity under specific pH. In the presence of CP, OPH is expressed by bacterial cells (Chishti *et al*., 2013; Tiwari and Guha, 2014). Approaches for bioremediation through environmental modification like nutrient application (additional carbon sources), aeration, or addition of

degrader microorganism(s) are gaining importance (Cycon *et al*., 2009). Half-life of CP depends upon number of environmental factors like substrate concentration, temperature and pH (Lu *et al*., 2013). At 25˚C CP has half-life of 77, 49 and 19 days for pH 4, 6 and 8 respectively (Chapman and Cole, 1982). According to Briceno *et al*. (2012) half-life of CP varies from 10-120 days. From these and number of other studies it is evident that CP degradation is strongly dependent upon environmental factors.

2.9. Bacterial Species for Chlorpyrifos Biodegradation

Microorganisms present in the environment have capability to use CP as a source of carbon and energy. Microorganisms such as bacteria, fungi and algae have been studied for biodegradation of CP. Bacterial species have catabolic genes and capability to survive under wide range of oxygen, pH and temperature. They survive in the presence of other contaminants such as heavy metals (Yadav *et al*., 2015). Bacterial degradation of CP have been studied extensively through catabolic as well as metabolic processes and TCP is found to be its primary metabolite (Chisthti *et al*., 2013, Xu *et al*., 2007). First bacterial strain i.e. *Enterobacter* sp., B-14 that was capable to hydrolyze CP was isolated by Singh *et al*. (2004). *Klebsiells* sp. isolated from activated sludge was capable of degrading CP (Ghanem *et al*., 2007). Bacterial strains belonging to different genera are found capable of CP biodegradation. For CP biodegradation bacterial species that belongs to *Sphingomonas, Pseudomonas, Bacillus subtilis, Agrobacterium, Serratia*. have been reported earlier (Yang *et al*., 2006; Li *et al*., 2007; Xu *et al*., 2007; Lakshmi, 2008; Maya *et al*. 2011; Cycon *et al.*, 2013; Abraham and Silambarasan, 2013; Yadav *et al*. 2014). Mineralization of metabolite TCP has been reported by Feng *et al*. (1997) and Yang *et al*. (2005) using *Pseudomonas* sp., and *Alcaligenes* sp. Chishti *et al*. (2013) has reported effectiveness bacterial consortium for bioremediation of CP polluted sites as compared to monoculture.

Bacterial species: *Arthrobacter* sp. *Enterobacter* strain B-14, *Alcaligenes faecalis*, *Bacillus pumilus* and *Pseudomonas aeruginosa* are founf capable of catabolizition and co-metabolization of CP and its metabolites (Mallick *et al*., 1999; Singh *et al*., 2004; Yang *et al.,* 2005; Li *et al*., 2007; Lakshmi *et al*., 2008; Maya *et al.,* 2011). Bacterial strain *Pseudomonas* sp., isolated from agriculture soil was capable of degrading CP (Maya *et al*., 2011).

Table 2.2: Summary of work carried out on biodegradation of Chlorpyrifos (CP)

Sample/Location	Bacteria	Degradation of CP	References
Agricultural soil, Australia	*Enterobacter strain B-14*	100% of 250 mg/L of CP in 2 days	Singh *et al.* (2004)
Wastewater sludge, China	*Stenotrophomonas sp.*	100% of 100 mg/L of CP in 24 h	Yang *et al.* (2006)
Activated sludge/pesticide manufacturers, Syria	*Klebsiella sp.*	92% CP in 4 days	Ghanem *et al.* (2007)
Soil samples agricultural land, Pakistan	*Bacillus pumilus* C2A1	89% of 100 mg/L CP in 15 days	Anwar *et al.* (2009)
Farmed Soil, India	*Pseudomonas sp., Agrobacterium sp., Bacillus sp.*	84 – 52 mg/L for 100-50 CP	Maya *et al.* (2011)
Sludge/ Chlorpyrifos manufacturing factory, , China	*Cupriavidus sp.* DT-1	100% of 100 $mgkg^{-1}$ CP in 20 days	Lu *et al.* (2013)
Soil / Vellore , Tamilnadu, India	*Alcaligenes sp.* JAS1	100% of 300 mg/L of CP in 12h	Silambarasan and Abraham (2013)
Chlorpyrifos contaminated agricultural soil, India	*Alcaligenes sp.* JAS1 *Ochrobactrum* sp. JAS2	100% of 300 mg/L in 24 h	Abraham and Silambarasan (2014)
CP contaminated agricultural soil, India	*Pseudomonas sp.*	60.375% of 480 mg/L of CP in 24 h	Yadav *et al.* (2014)
Agricultural land/ Kanpur, India	Mixed culture	82% of 2.48 µM CP in 15 days	Tiwari and Guha (2014)
Soil sample, Pakistan	*Mesorhizobium sp.* HN3	100% of 400 mg/L	Jabeen *et al.* (2015)
Soil sample, Pakistan	*Pseudomonas* sp.	90% in 24 h	Gilani *et al.* (2016)
Apple Orchard, Shimla, India	*Pseudomonas resinovarans*	43.90% of 400 mg/L in 96 h	Sharma *et al.* (2016)

2.10. Enzymes Responsible for Chlorpyrifos Biodegradation

Samples collected from diverse locations were used for purification of Oop degrading enzymes i.e. phosphotriesterase (PTE), methyl parathion hydrolase (MPH) and organophosphorus acid anhydrolase (OPAA) using various microorganisms in different studies (Dumas *et al.*, 1989; Defrank *et al*., 1991; Cheng *et al*., 1993; Cui *et al*., 2001; Horne *et al*., 2002; Singh *et al*., 2004; Dong *et al*., 2005). Bacterial enzyme organophosphate hydrolase is responsible for biodegradation of pesticides of organophosphate group (Singh *et al*., 2006). Microorganisms responsible for biodegradation are capable of producing OPH (Organophosphate hydrolase) that degrade OPs (Gao *et al*., 2012). OPH/ PTE act as catalyst in the first step of biodegradation (Singh *et al*., 2006). Metabolites of CP after biodegradation could be Chlorpyrifos- oxon, 3, 5, 6-trichloro-2-pyridinol (TCP), 3, 5, 6-trichloro-2-methoxypyridine (TMP) and diethyl-thiophosphate (DETP) (Kim and Ahn, 2009; Bootharaju and Pradeep, 2012; Silambarasan and Abraham, 2013; Tiwari and Guha, 2014). Production of TCP could be an indicator of intracellular as well as extracellular degradation of CP by microbe (Chishti *et al.*, 2013). Phosphatase is another enzyme which participates in biodegradation of CP (Thengodkar and Sivakami, 2010). *opd* and *mpd* gene are responsible for production of OPH enzyme (Seibert and Raushel, 2005). These enzymes are capable of hydrolysis of organophosphate pesticides, this reduces their toxicity but its efficiency vary depending upon carbon substrate. Microbes break OP by cleavage of P-S and P-O bond (Liu *et al*., 2004). Isolation of two other enzymes A-OPH and P-OPH were reported by Liu *et al*. (2001).

2.11. Metabolites Generated after Chlorpyrifos Degradation

One of the possible options for cleanup of CP from contaminated environment is biodegradation using native microorganisms. It is of utmost importance to study degradation by-products along with primary compound. In aerobic environment CP is hydrolyzed to metabolites like TCP (3, 5, 6,-tricholoro-2-pyridinol) and DETP (Diethylthiophosphate) depending upon specie involved in degradation process. TCP is intermediate compound commonly observed during CP degradation by *Enterobacte*r (Singh *et al.,* 2004) *Cupriavidus* sp. (Lu *et al.,* 2013), *Alcaligenes* sp. (Silambarasan and Abraham, 2013). TCP is more soluble in water then its parent compound which increases its mobility and ultimately causes widespread contamination. TCP has strong antimicrobial effect which prevents its own and CP degradation (Armbrust, 2001; Chen *et al*., 2012; Liu *et al*., 2012). Degradation to TCP and DETP are detectable but once they are degraded to TMP or DEP by bacterial specie, could not be detected which indicates that they are not stable and are completely mineralized (Tiwari and Guha, 2014).

2.12. Treatment of Chlorpyrifos

Biodegradation may be carried out on site and in confined environment such as bioreactors. CP Biodegradation studies have been done in bioreactors to evaluate effect of environmental and operating conditions on the process. Bioreactors could be operated in batch as well as continuous mode. The use of ex-situ treatment offers considerable benefits over in situ treatment in terms of effective control over various process parameters (Yadav *et al*., 2014). Investigating range of optimal growth conditions in which bacterial growth is not inhibited has immense importance for successful completion of biodegradation, especially under field conditions (Swissa *et al.,* 2014). Biodegradation of CP has been reported in number of studies using flask culture experiments. Less number of detailed studies has reported biodegradation of

CP using bioreactors. Most of the studies using bioreactors reported longer HRT for CP removal. However for field applications main aim of degradation studies should be efficiency enhancement and reduced HRT. Use of potential bacterial cultures in bioreactors capable of biodegrading specific compounds present in wastewater is gaining importance, since it avoids the use of conventional sources for biomass production and reduces startup time of biotreatment units (Padoley *et al.*, 2005).

2.13. Effect of Organic and Inorganic Contaminants on Biodegradation Process

Bacterial species perform efficiently at laboratory scale under controlled conditions. However, field applications of laboratory scale studies usually fails because of various reasons such as

1. Harsh environmental conditions (extreme pH, temperature).
2. Presence of supplementary chemicals used as organic solvents, stabilizers, emulsifiers and trace metals.

Wastewaters of agrochemical industries contain chemicals used as organic solvents, stabilizers, emulsifiers, trace metals along with active ingredients (Kolpin *et al*., 2000a; Swissa *et al*., 2014). Several trace metals such as Copper (Cu), Manganese (Mn), Zinc (Zn), Lead (Pb), Cadmium (Cd), Iron (Fe) and Arsenic (As) were detected in pesticide samples (Shomar, 2006). Pesticides with trace metals in their chemical structure have been detected in contaminated groundwater globally. Trace metals are not shown in chemical structures and labels of pesticides; therefore they have not been focused during biodegradation studies so far (Kolpin *et al.*, 2000b). Presence of other pesticides, their residues, additional carbon sources and solvents may have either positive or negative effect on extent of biodegradation (Kauffmann and Mandelbaum, 1998)

Microbe's affinity for pesticide in presence of these contaminants presents a unique challenge and need to be explored (Helbling, 2015).

Biodegradation studies so far have reported removal of a particular pesticide. No work has been reported yet on the concentration dependent effect of organic solvents and inorganic trace metals on CP biodegradation using bacterial consortium. Bacterial strains are capable of degrading pesticide alone but may not be equally effective in the presence of other carbon sources, solvents and inorganic contaminants like trace metals. Different strains have variable resistance to pesticides this could be attributed to diverse metabolic mechanism; therefore co-inoculation may result in higher removal and resilience to environmental changes.

2.14. Bacterial Immobilization Technique for Chlorpyrifos Biodegradation

Separation of cells for reuse has been a major problem associated with the process. Freely suspended cells used in these studies have some other disadvantages such as cell washout, stability loss and non-reusability making biotreatment costly (Kourkoutas *et al*., 2004; Chen *et al*., 2013). Thus there is a need to investigate methods that make bacterial application more cost-effective and durable.

Among many methods bacterial immobilization could be potential solution as it prevents washout, allows reuse and improves stability. One of the processes used for bacterial immobilization is use of calcium alginate matrix. Immobilization in calcium alginate microsphere mimics the natural mode of existence, provides nutrients without opposition from other microbes and protects cells from harsh environment. Calcium alginate has broader application as it is easy to use and biocompatible. Use of calcium alginate matrix for bacterial immobilization and its application for biodegradation of pesticides and xenobiotic has been

reported (Ha *et al*., 2009; Chen *et al.*, 2013; Parasad and Suresh, 2015; Bergero and Lucchesi, 2015). However this natural polysaccharide is vulnerable to biodegradation, has low mechanical stability, and is sensitive to chelating agents. Moreover it may be softened quickly by citric acid and EDTA. This renders calcium alginate method unsuitable for most WW treatments. Calcium alginate matrix may be strengthened by mixing it with other polymers. Some efforts have been made to make it more stable using clay, polylysine, polyvinyl alcohol and chitosan (Orive *et al*., 2006; Siripattanakul *et al*., 2009; Wong *et al*., 2011; Lin *et al*., 2013,). However complex preparation and high cost are the problems associated with their use.

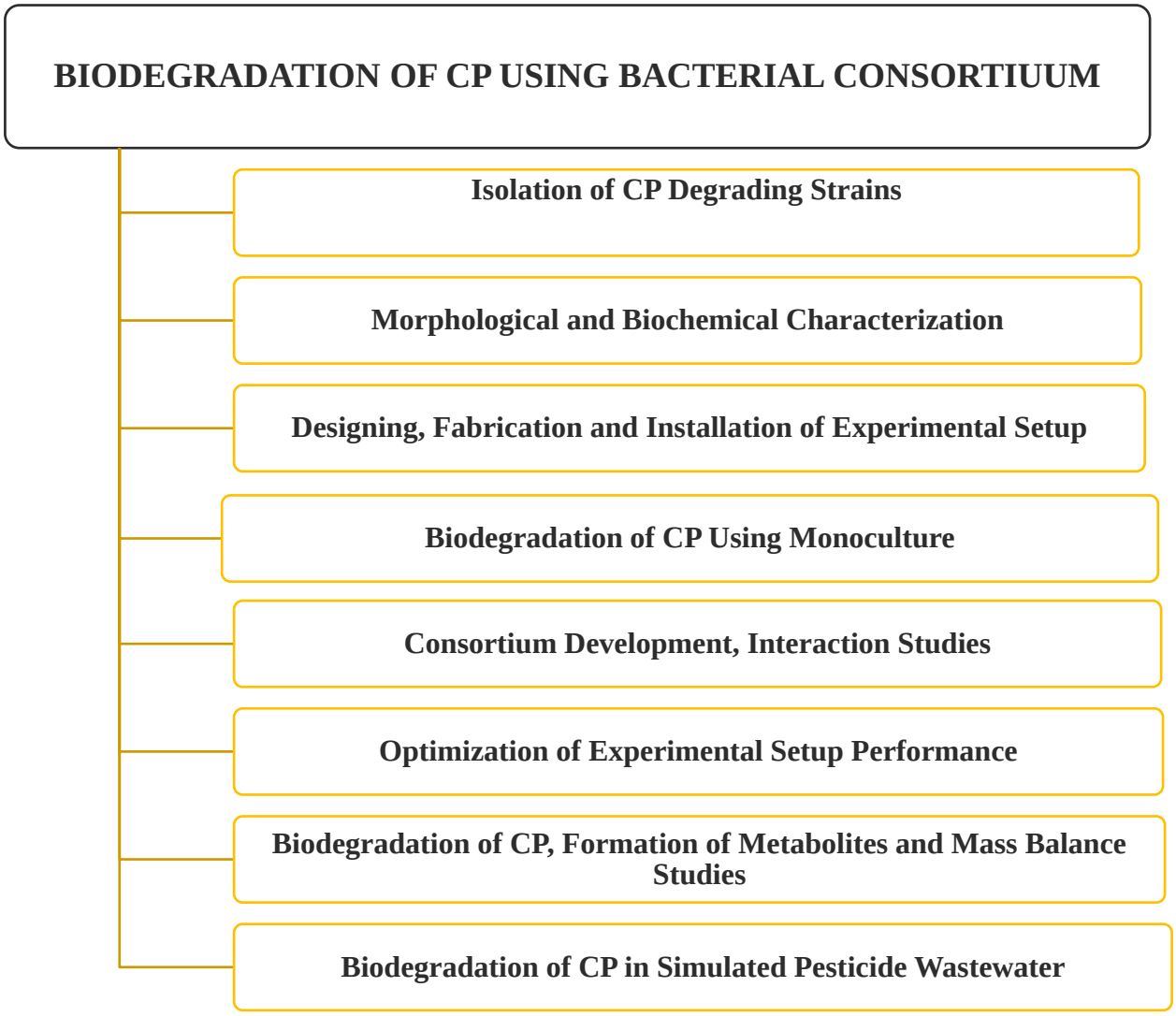
Chapter 3

3. MATERIALS AND METHODS

3.1. CHEMICALS AND MEDIA

Analytical grade standards of Chlorpyrifos (CP), 3, 5, 6-trichloro-2-pyridinol (TCP), Diethyl thiophoshate (DETP) were purchased from Sigma Aldrich Corporation, USA. 3, 5, 6-trichloro-2-methoxypyridine (TMP) was purchased from Tokyo Chemical Industry Co. Ltd., Tokyo, Japan. Stock solutions of CP were prepared in ethyl acetate and TCP/TMP/DETP in acetone. Solutions of different concentrations were prepared for calibration curve. Stock solution of technical grade chlorpyrifos was prepared using ethyl acetate as solvent. Commercial grade CP was purchased from local market. Mineral salt medium (g) was used MSM (gL^{-1}) K_2HPO_4 (1.5); KH_2PO_4 (0.5); NaCl (0.5); $MgSO_4$ (0.2); NH_4NO_3 (1.0), spiked with CP as source of carbon.

PHASE I



3.2. SAMPLE COLLECTION AND ISOLATION OF CP DEGRADING STRAINS

Soil samples were collected from agricultural field of District Chakwal, Punjab, Pakistan that has received CP application for decades. Wastewater samples were collected from wastewater drain of National University of Sciences and Technology, Islamabad, Pakistan. Pesticide industry

wastewater samples were collected from agrochemical industry located in Multan industrial estate, Pakistan hereafter named as industry "A".

For isolation of CP degrading bacteria MSM was prepared in the laboratory. pH of the medium was adjusted to 7 using I M H_2SO_4 or 1M NaOH. MSM and all glassware was sterilized at 121˚C for 15 min and cooled down to room temperature. 90 ml of MSM was taken in 250 ml Erlenmeyer flask. 10 ml of wastewater and 10 g of soil was added to MSM in separate flasks and incubated for 7 days at 35˚C in incubator. After incubation period 10 ml sample was transferred to freshly prepared MSM incubated for same period. It was repeated for several times. After several successive transfers serial dilutions were prepared and spread on MSM agar plates. Based on morphological distinction potential bacterial strains were streaked on MSM plates and incubated at 30˚C for 18-24 h. After repeated streaking on medium pure colonies were obtained.

3.3. ACCLIMATIZATION AND SCREENING OF BACTERIAL STRAINS

In process of acclimatization bacterial strains adjust themselves according to environmental alterations, allowing them to perform their activities across a wide range of environments. For acclimatization all strains were grown in MSM with gradually increasing concentration of CP from 50-400 mg/L. Flask containing 100 ml of MSM was amended with bacterial culture (OD=1 at 600 nm). Flasks were incubated at 35˚C for 48 h. After 24 h concentration of CP was increased by 50 mg/L. All strains were acclimatized using this method upto 400 mg/L. MSM (pH7) was prepared and sterilized at 121˚C for 15 min. MSM was amended with CP and was inoculated with individual bacterial strains, incubated at 35˚C for 24 h in an incubator. After 24 h samples were collected and analyzed for remaining CP. Bacterial strains showing higher CP removal efficiency were selected for biodegradation studies.

3.4. BACTERIAL CHARACTERIZATION

3.4.1. Morphological Characterization

Observing cell and colony morphology plays an important role in identification of bacterial strains. Size, shape, edge, margins, texture, elevation, pigmentation were observed using magnifying glass and microscope following standard protocols.

3.5. MOLECULAR IDENTIFICATION

Selected bacterial strains were identified using 16S rRNA analysis. 16S rRNA analysis was performed at M/S Macrogen Inc., Seoul, South Korea. Briefly, DNA was removed from bacterial strains by use of instance matrix (Bio-Rad USA) following manufacturers protocol. For amplification two universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492 R (5'- TACGGYTACCTTGTTACGACTT-5') were used (Table 3.1). Montage PCR cleanup kit (Millipore) was used for purification of amplification products. For sequencing big dye terminator cycle sequencing kit (Applied Biosystems, USA) was used. 518F and 800R were used as sequencing primers (Table 3.1). Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) was used for resolving sequencing products. Sequences were line up and matched with already published sequences at National Centre for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST). Construction of Phylogenetic tree was accomplished using software Molecular Evolutionary Genetic Analysis (MEGA-4) on the basis of evolutionary descent. Sequences were submitted at NCBI Genbank using BankIt and accession numbers were obtained.

Table 3.1: Primers for Amplification and Sequencing

S.No.	Primer	Amplification	Sequencing
1	27F AgA gTT TgA TCM TGG CTC Ag	●	
2	1492R TAC ggY TAC CTT gTT ACg ACT T	●	
3	518F CCA gCA gCC gCg gTA ATA Cg		●
4	800R TAC CAg ggT ATC TAA TCC		●

3.6. BIODEGRADATION OF CHLORPYRIFOS

3.6.1. Designing, Fabrication and Installation of Experimental Setup

Experimental setup was designed, fabricated and installed in the laboratory. Bench scale bioreactor comprised of inlet, aeration and effluent tank. Schematic arrangement of SBR is shown in Figure 3.1. Dimensions for bioreactor were total volume; 10L, working volume; 8L, internal diameter; 20 cm. Air pumps, pneumatic tubes and diffusors were used for air supply. Inlet and outlet ports were used for feeding and removal. Experiments were performed at ambient temperature. pH of wastewater was adjusted when required by addition of 1M H2SO4 or 1M NaOH. Working volume was separated in two parts i.e. 4L effluent volumes and 4L sludge volume. There were five stages in whole operation which includes fill, react, settle, decant and idle. Hydraulic retention time (HRT) 48 h was divided into fill;1 h, aeration;45 h, settlle;1 h, decant;45 min and idle;15 min. HRT 24 h cycle was divided into fill; 1 h, aeration; 23 h, settle;1h, decant; 0.75 h, idle; 0.25 h. All stages were controlled through time switches. The

influent was MSM, simulated pesticide wastewater (SWW) or real industrial wastewater (WW). Biomass used for startup was developed in laboratory using bacterial consortium or monoculture. Supernatant collected after settling was filtered and used for determination of remaining CP concentration.

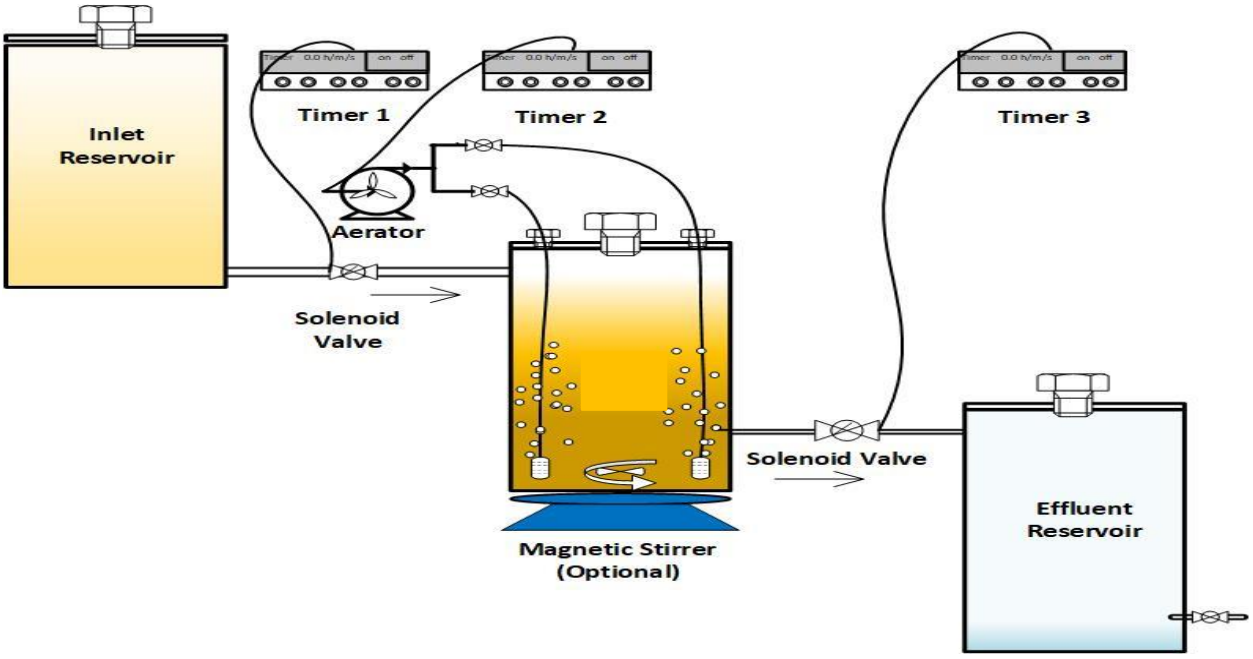


Figure 3.1: Schematic layout of laboratory scale batch reactor (1) inlet reservoir (2) air tank (3) effluent reservoir

3.6.2. Biodegradation of CP Using Monoculture

Biodegradation proficient of selected bacterial strains was investigated in bioreactors. Seeding inoculum was prepared in laboratory by growing isolated bacteria in MSM upto 24 h. After centrifugation and removal of supernatant cell pellet was obtained. NaCl 0.85% was used for resuspension of cell pellet and cell density of 2.5 at 600 nm was obtained (Silambarasan and Abraham, 2013). Cell density was measured using spectrophotometer (DR2010, HACH, USA). MSM was prepared autoclaved, cooled down to room temperature and supplemented with CP 400 mg/L. pH of MSM was adjusted to 7. The resuspended culture was used for inoculation at

10% inoculum level. MSM without bacterial inoculation was used as abiotic control. MSM without addition of CP was set biotic control. Sampling was performed on regular intervals and used for analysis.

3.6.3. Consortium Development

Seven proficient bacterial strains capable of CP biodegradation were selected. In order to estimate level of compatibility among selected strains interaction studies were performed.

3.6.4. Interaction Studies

To investigate the effect of intermediate compounds and secretion of one bacterial strain on the other, interaction studies were performed using nutrient broth (NB) as growth medium. NB was prepared and inoculated with seven selected bacterial strains individually and incubated at 37˚C, 120 rpm for 96 h. Cell density was measured using spectrophotometer (DR2010, HACH, USA) after 96 h. NB was filtered and bacterial biomass was removed. In spent filtrate containing metabolites, medium components were added and sterilized, inoculated with other strains and incubated for 96 h. Total 42 combinations were prepared. Growth at initial and end of experiment was measured. Relative growth conversions were calculated using following expression (equation1) (Mishra and Malik *et al*., 2014):

$$\text{Relative growth conversion } (\%) = \frac{\text{B2} - \text{B1}}{\text{B1}} * 100 \qquad (\mathbf{1})$$

Where B1 is biomass of individual bacterial strain and B2 is growth of same bacteria in spent filtrate of other bacteria.

3.6.5. Consortium Development for Inoculation

Mineral salt medium (MSM) was prepared as mentioned in section 3.1, pH was adjusted to 7 and sterilized at 121˚C for 15 min. CP was added as a sole source of carbon and energy. Bacterial

strains (SRK1, SRK2, SRK4, S4K5 and T13) were inoculated individually in medium and incubated at 37˚C for 24 h. After 24 h optical density of medium was adjusted to 2.5 at 600 nm (in order to get approximately equal proportion of each bacterial strain). These suspension of each bacterial strain was mixed in equal proportion (1:1:1:1:1 of SRK1, SRK2, SRK4, SRK5 and T13) to obtain bacterial consortium having approximately equal proportion of each bacterial strain. Bacterial suspension obtained after mixing was centrifuged at12000g (to remove medium components), supernatant was removed and cell pellet was obtained. For purpose of inoculation this pellet was resuspended in 0.85% sodium chloride (saline) in distilled water and adjusted to initial optical density of 2.5 at 600 nm using spectrophotometer (DR2010, HACH, USA) (Silambarasan and Abraham, 2013). 10 ml of this suspension was inoculated to obtain final volume of 100 ml of aqueous medium.

3.6.6. Optimization of Experimental Setup Performance

Effect of inoculum concentration on CP degradation

Biodegradation of CP was investigated at 5, 10 and 15% inoculum concentration. 5% inoculum concentration means, 5 ml culture having cell density 2.5 at 600 nm was used for inoculating per 100 ml of medium. Inoculum was prepared and optical density of consortium was adjusted to 2.5 at 600 nm. MSM amended with CP (100 mg/L) was inoculated with bacterial consortium. Biodegradation experiments were performed using MSM having CP 100 mg/L at pH 7 , HRT 48 h and inoculum level 10%. Sampling was carried out after fixed intervals (48 h) and collected samples were used for examination of CP removal and cell density. Optimum value (ml) of inoculum level observed in this experiment was used in forthcoming experiments.

Effect of growth medium pH on CP degradation

To examine effect of growth medium pH on CP biodegradation experiments were performed. Biodegradation of CP was investigated at pH 5, 6, 7 and 8. Inoculum was prepared and optical density of consortium was adjusted to 2.5 at 600 nm. Biodegradation experiments were performed using MSM having CP 100 mg/L at HRT 48 h and inoculum concentration 10%. Sampling was carried out after fixed intervals and collected samples were used for examination. Optimum value of inoculum level observed in this experiment was used in upcoming experiments.

Effect of initial concentration on CP degradation

Inoculum was prepared and optical density of consortium was adjusted to 2.5 at 600 nm. Biodegradation experiments were performed using MSM having CP 100-500 mg/L at pH 7, HRT 48 h and inoculum concentration 10%. Sampling was carried out after fixed intervals and collected samples were used for examination. Optimum value of inoculum level observed in this experiment was used in forthcoming experiments.

Effect of hydraulic retention time (HRT) on CP degradation

Effect of hydraulic retention time (24, 48 and 72 h) on CP removal experiments were performed at optimum conditions. Biodegradation experiments were performed using MSM amended with CP at pH 7 and inoculum concentration 10%. Sampling was carried out after fixed intervals and collected samples were used for examination. Optimum value of inoculum level observed in this experiment was used in forthcoming experiments.

3.7. BIODEGRADATION OF CP, FORMATION OF METABOLITES AND MASS BALANCE STUDY

After optimization of environmental conditions i.e. inoculum level, pH initial CP concentration and hydraulic retention time a biodegradation experiments were performed to assess formation

and degradation of metabolites in addition to CP biodegradation. Biodegradation experiments were performed using liquid medium having supplemented with CP 400 mg/L at pH 7, HRT 48 h and inoculum concentration 10%. Periodic sampling was carried out after fixed intervals and collected samples were used for analysis of remaining CP (3, 5, 6-trichloro-2-pyridinol) and TMP (3, 5, 6-trichloro-2-methoxypyridine) concentration. After collected and extraction steps samples were analyzed for remaining CP and metabolite concentration using GC-ECD. Cell density was examined using spectrophotometer (DR2010, HACH, USA).

For mass balance study experiment was conducted using initial CP concentration 400 mg/L in MSM. At 0 h and at the 48h HRT liquid medium was collected in order to determine initial and remaining CP concentration. Difference in the initial concentration of CP in liquid medium and after 48 h was considered to be either biodegraded or bioaccumulated. CP concentration bioaccumulated/adsorbed on bacterial cells was determined following method of Briceno *et al*. (2012). Sample was collected, centrifuged and supernatant was removed. Cell pellet was resuspended in ethyl acetate and mixed thoroughly followed by vortex (10 sec) and shaking for 10 min. Organic layer was collected and CP concentration was determined using GC-ECD. This gives initial CP concentration in MSM and CP accumulated/adsorbed on bacterial cells. Biodegraded fraction was calculated by subtracting final CP concentration and bioaccumulated concentration from initial concentration in liquid medium.

3.7.1. Phytotoxicity Assay

To access the toxicity level of bioremediated effluents a toxicity test was performed using seeds of *Triticum aestivu*m obtained from National Agricultural Research Center (NARC) Islamabad,, Pakistan. Seeds were grown in petri plates containing filter paper was placed petri dish with seeds on top treated with 5 ml of tap water (TI), bioremediated effluents (TII) and abiotic control

(TIII) separately (Bergero and Lucchesi, 2015). Each petri plate has 20 seeds. Petri dishes were placed in dark for a week. Number of germinated seeds was noticed and germination percentage was calculated using equation 2. Radical length and shoot length were measured. Vigor index of seedling was calculated using equation (3)

Germination percentage (G)=(Number of germinated seeds/Total number of seeds)*100 (2)

After seven days root length (LR) and shoot length (LT) were measured using centimeter scale. Seed vigor index is indicator of overall plant health, it was calculated using equation 3 (Saez *et al.*, 2014)

$$\text{Vigour index (VI)} = (\text{LR} + \text{LT}) * \frac{\text{G}}{10} \quad (3)$$

3.8. EFFECT OF OTHER CHEMICALS PRESENT IN WASTEWATER STREAM ON BIODEGRADATION OF CP

3.8.1. Effect of Organic Solvents (Petrochemicals)

Petrochemicals are widely used as solvents to make liquid formulations of pesticide. Their presence in wastewater stream is obvious, which may affect process of biodegradation. Therefore effect of presence of petrochemicals on biodegradation of CP was investigated. MSM was supplemented with different concentrations (10, 20, 100 mg/L) of benzene, toluene, xylene individually and inoculated (10%) with consortium, at pH 7, CP 400 mg/L for HRT 48h. MSM without any petrochemical and/or consortium served as control. Samples were collected after 48h for analysis of remaining CP concentration.

3.8.2. Effect of Metal Ions on CP Biodegradation

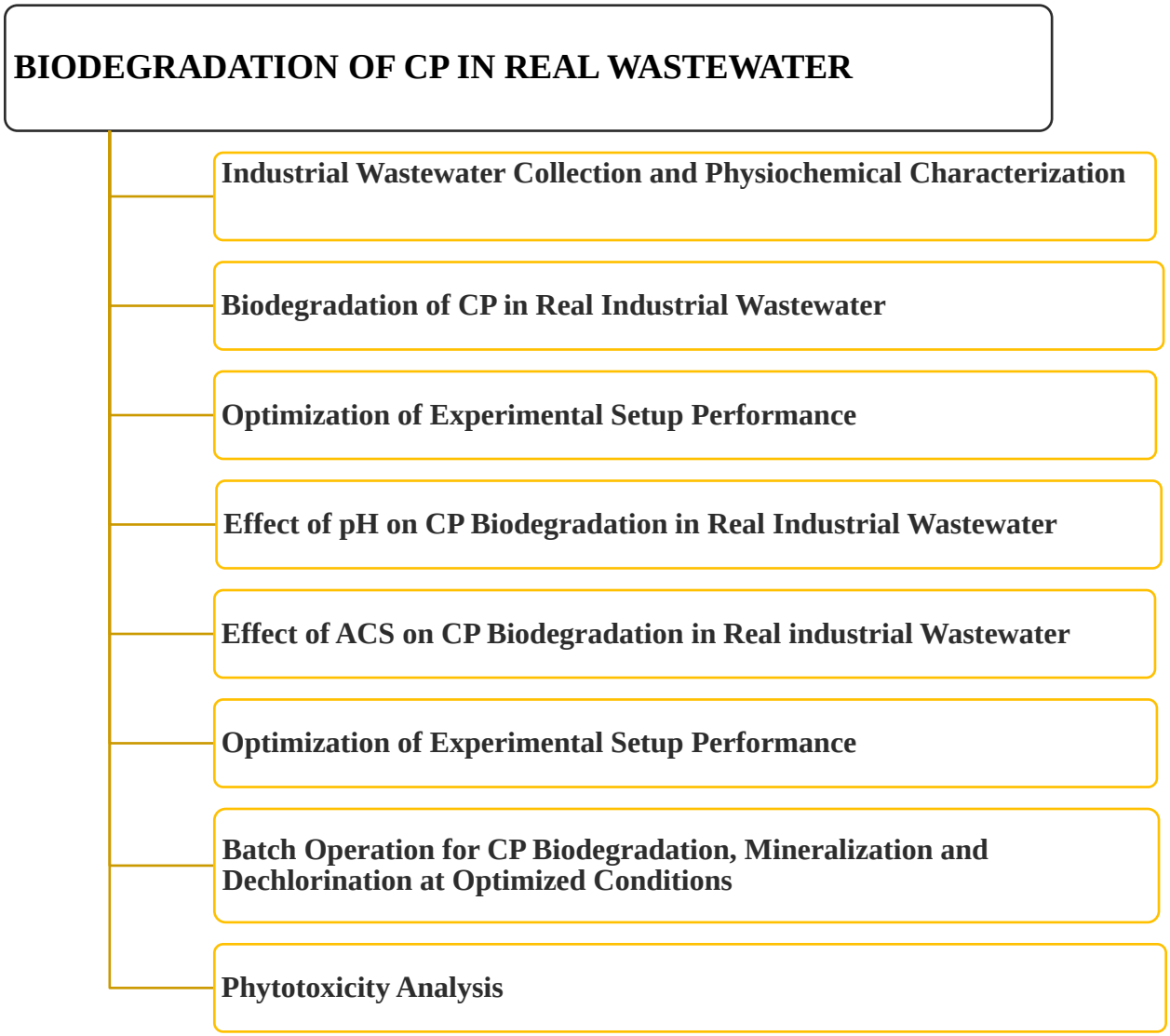
Research on pesticides mostly focuses on organic dimensions but there is a dearth in literature regarding inorganic dimensions. Inorganics which are found in commercial formulations

includes metal ions. These metal ions may take part in biochemical reactions. Therefore effect of different concentrations of metal ions on biodegradation of CP was investigated. MSM was amended with different concentration (1, 5, 10 mg/L) of Cu (II), Hg (II) and Zn(II) individually. All other conditions were same as for petrochemicals. MSM without these metal ions served as control. Samples were collected periodically for CP analysis.

3.8.3. Biodegradation of CP in Simulated Pesticide Wastewater (SWW)

Simulated pesticide wastewater (SWW) composition in (gL^{-1}) NH_4NO_3 (1); KH_2PO_4 (0.5); K_2HPO_4 (1.5); NaCl (0.5); KCl (0.5); $MgSO_4$ (0.2); glucose (6) and Chlorpyrifos; 100- 400 mg/L. 50 mg/L each of xylene, toluene and benzene was added. All ingredients were added in distilled water. Stock solution of trace metals ($CuSO_4$, $ZnSO_4$, $HgSO_4$) was prepared and 2 ml was added (Swissa *et al*. 2014). For analysis of remaining pesticide concentration samples were collected, extracted, and analyzed using GC-ECD (Shimadzu 2010).

PHASE II



3.9. BIODEGRADATION OF CP IN REAL INDUSTRIAL WASTEWATER

Five membered bacterial consortium comprised of SRK1, SRK2, SRK4, SRK5 and T13, that has proven its efficiency in synthetic wastewater for degradation of Chlorpyrifos (CP) was used for current study. Real industrial wastewater (WW) samples were collected from an agrochemical

industry (here after named as industry "A") producing pesticides and fertilizers located in Multan, Pakistan. WW was transported to the laboratory and stored at 4˚C for further use.

Initial characterization of WW was performed following standard protocols (APHA, 2012). Chemical oxygen demand (COD) was analyzed using close reflux titrimetric method following standard protocols (APHA, 2012). pH, DO and temperature was determined using portable pH meter (HACH). Electrical conductivity was measured using EC meter (WTW series 720, USA). Optical density, nitrate-nitrogen (NO_3^-—N), nitrite-nitrogen (NO_2^-—N), phosphate-phosphorous (PO_4^{+3}—P) were measured using spectrophotometer (DR2010, HACH, USA). MLSS/MLVSS was measured using standard protocol (APHA, 2012). Pesticides in WW sample were measured using Gas Chromatograph with Electron capture detector (Model: Shimadzu, 2010).

3.9.1. Biodegradation of CP

CP 545 mg/L (measured with GCECD) was already present in WW collected from industry A. When CP concentration was below 545 mg/L in WW, it was spiked with technical grade CP to get equal concentration for all experiments. WW from industry "A" was fed to bioreactor in batch mode. WW usually has higher COD and lower DO level, therefore, aeration was started to support microbial community in order to start degradation of CP. Batch experiment without provision of DO served as control. Immediately after application and at regular intervals samples were collected, extracted and analyzed using GC.

3.9.2. Biodegradation of CP in Real Industrial Wastewater

For evaluating the biodegradation capability of microbial consortium in WW experiments were performed. WW was bioaugmented with bacterial consortium and biomass was developed. It was very difficult to develop higher MLSS in WW, therefore longer start up time was given. For acclimatization of bacterial consortium initially WW was diluted using sterilized MS

(micronutrient solution) KH_2PO_4 (0.5 g/L), K_2HPO_4 (0.5 g/L), $MgSO_4$ (0.5 g/L), NH_4Cl (0.5 g/L) and fed to bioreactor. WW to MS dilution ratio from 1st to 7th cycle was 0.5:3.5, 1:3, 1.5:2.5, 2:2, 2.5:1.5, 3:1, 3.5:0.5, 4:0. Repeatedly With each coming cycle WW volume was gradually increased. Total 4 L WW was fed to bioreactor in batch mode and aeration was started. After acclimation period WW containing 545 mg/L of CP was fed to bioreactor and aeration was started. Samples for 0 h were collected. After reaction time sludge was allowed to settle. Samples were collected, filtered, extracted and analyzed for remaining CP concentration.

3.9.3. Effect of Environmental Conditions on Biodegradation of CP in Real Industrial Wastewater

Effect of pH on CP biodegradation in real industrial wastewater

In order to investigate effect of growth medium pH on biodegradation of CP in WW experiments were performed. Biodegradation of CP was investigated at acidic to basic range of pH i.e. 5, 6, 7 and 8. Seeding inoculum for bioreactor was five membered bacterial consortium. WW (4 L) having definite pH was fed to bioreactor and HRT was 48 h. Sampling was carried out after fixed intervals and collected samples were used for examination. Optimum value of pH observed in this experiment was used in forthcoming experiments.

Effect of ACS on CP biodegradation in real industrial wastewater

Influence of assimilable carbon substrates (ACS) in high concentration on CP biodegradation in WW was investigated. Experiments were performed in similar manner as before with addition of ACS (glucose, sucrose or yeast extract), individually. pH of WW was adjusted to 7 and one of the three selected ACS i.e. glucose, sucrose or yeast extract was added to WW at the rate of 2 g/L (2000 mg/L), separately. Seeding inoculum for bioreactor was five membered bacterial

consortium. WW (4 L) was fed to bioreactor and HRT was 36 h. Collected samples were used for analysis of remaining CP concentration.

3.10. BATCH OPERATION FOR CHLORPYRIFOS BIODEGRADATION MINERALIZATION AND DECHLORINATION AT OPTIMIZED CONDITIONS

Untreated WW was adjusted to pH 7 and glucose (2 g/L) was added. WW was fed to bioreactor in batch mode using inlet valve. Air was sparged in order to maintain desired DO level. Reactor was operated at HRT 24 h. Initial pH was adjusted to optimum value afterwards the system worked without temperature and pH control, but their values were monitored. After 23 h aeration was stopped and allowed to settle for 1 h. Half of the reactor volume was removed through effluent valves using controlled timers (Figure 3.1). Remaining concentration of parent compound and metabolites was analyzed using GC-ECD (Shimadzu 2010). Intermediate compounds generated after degradation of CP were also monitored. Change in their concentration was determined through continuous (0, 3, 6, 9, 12, 24 h) sample collection (0, 3, 6, 9, 12, 24 h) and analysis.

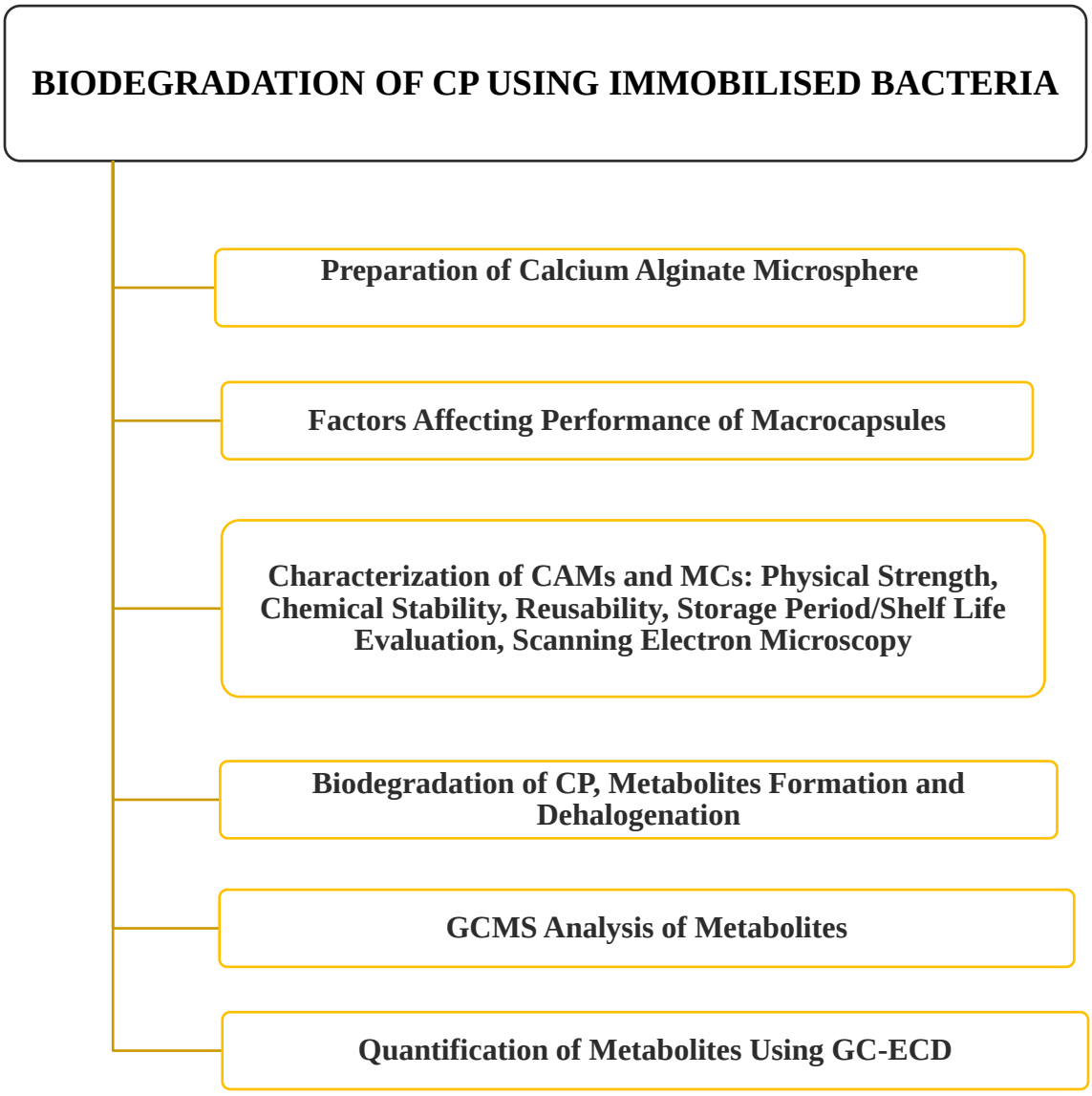
In order to determine the extent of mineralization, COD was determined following standard method (APHA, 2012). Nitrate-nitrogen (NO_3^-—N), phosphate-phosphorous (PO_4^{+3}—P) was determined using spectrophotometer (DR2010, HACH, USA). For chloride ions determination method mentioned by Bidlan and Manonmani (2002) and Saez *et al*. (2015) was followed. Briefly sample was collected, centrifuged and 1µl of supernatant was collected. Supernatant was mixed in two steps with 50 µL each of HNO_3 (0.15N) and $AgNO_3$ (0.1N). Left at room temperature for 20 min. Absorbance was measured at 600 nm using spectrophotometer (DR2010,

HACH, USA). In order to determine the concentration standard curve was developed using sodium chloride in similar manner.

3.10.1. Phytotoxicity Analysis

Current study aimed at removal of CP in WW, however wastewater coming out of pesticide industries contain pesticides and pollutants other than CP. Reduction in toxicity level is an indicator that pesticides other than CP were also degraded during the biodegradation process. To evaluate the toxicity level of WW after biotreatment phytotoxicity tests were performed following the protocol mentioned in section 3.7.1. Plants were irrigated with 5 ml of tap water (TI), bioremediated effluents (TII) and, abiotic control (TIII) individually. Number of germinated seeds was noticed and germination percentage and vigor index was calculated using equation 2 and 3.

PHASE III


BIODEGRADATION OF CP USING IMMOBILISED BACTERIA
Preparation of Calcium Alginate Microsphere
Factors Affecting Performance of Macrocapsules
Characterization of CAMs and MCs: Physical Strength, Chemical Stability, Reusability, Storage Period/Shelf Life Evaluation, Scanning Electron Microscopy
Biodegradation of CP, Metabolites Formation and Dehalogenation
GCMS Analysis of Metabolites
Quantification of Metabolites Using GC-ECD

3.11. BIODEGRADATION OF CHLORPYRIFOS USING IMMOBILIZED BACTERIA

3.11.1. Preparation of Calcium Alginate Microspheres (CAMs)

CP degrading bacterial consortium consisting of *Pseudomonas kilonensis* (KT013088), *Serratia marcescens* (KT013089), *Bacillus pumilus* (KT013091), *Acromobacter xylosoxidans* (KT013092) and *Klebsiella sp.* (KT013093) was used. Bacterial consortium was grown upto late exponential growth phase in nutrient broth (NB) containing CP. After 24 h medium was centrifuged at 8000 xg for 15 min, supernatant was removed and cell pellet was resuspended in sterilized deionized (DI) water. CAMs were prepared following the method of Suresh *et al.* (2015).

Sodium alginate (SA) was dissolved in 100 ml of boiling (100 ˚C) distilled water to prepare 2 % (w/v) solution. Bacteria were grown in NB upto exponential growth phase at 35˚C. Medium was centrifuged and supernatant was discarded. Cell pellet was resuspended in DI water and thoroughly mixed with SA solution. This mixture was stirred magnetically in order to achieve homogenous solution. 4% w/v solution of calcium chloride ($CaCl_2$) was prepared in sterilized DI water. Sodium alginate mixture was dropped in $CaCl_2$ solution. In 30 sec nearly spherical beads were formed. These CAMs were then dipped in 5% KH_2PO_4 solution for hardening and removal of excessive $CaCl_2$. After phosphorylation CAMs were transferred to DI water and stirred magnetically to remove extra $CaCl_2$ and phosphate (Tsai *et al*., 2013). CAMs were stored after repeated washing and drying at 4˚C until use.

For polymeric coating 10 % (w/v) polysulfone (PSf) solution was prepared in N-methyl-2-pyrrolidone (NMP). This solution was left overnight at magnetic stirrer at 80˚C for complete mixing. SA bead was dipped into PSf solution for 30 sec. After 30 sec a white polymer coating

was appeared on bead surface. CAMs were removed and dipped into water for 1 h. After 1 h it was again dipped into PSf solution for second coating. It was removed after 30 seconds. Macrocapsules were washed repeatedly with DI water, dried for removal of toxic organic solution and stored at 4˚C until use (Kim *et al*., 2015).

3.11.2. Factors Affecting Performance of Macrocapsules

Various factors effect performance of macrocapsules. Effect of concentration of sodium alginate (SA) was examined using 1, 1.5, 2, 2.5, 3, 3.5, 4 % (w/v). Concentration of $CaCl_2$ and varied from 2, 3, 4, 5, 6 %. Biomass concentration 300, 600, 900, 1200 mg/100ml was used to notice its effect on cell immobilization and its activity. Polysulfone concentration was varied from 5, 10 and 15 %.

Residual activity of MCs in terms of CP biodegradation was investigated in MSM. pH of the medium was adjusted to 7 and supplemented with CP 100 mg/L as a source of carbon and inoculated with MC. Incubation time was 12 h at 150 rpm and 35˚C. Samples were collected for analysis of residual CP concentration.

3.11.3. Characterization of CAM and MC

Physical strength

In order to investigate effect of physically harsh environment such as temperature and pH on CAMs and MCs experiments were performed. Effect of temperature was investigated by incubating CAMs and MCs at a temperature range (-70, -20, 4, 5, 15, 25, 35, 45, 55 and 65˚C) for 24 h. pH stability was examined by incubating CAMs and MCs in succinic acid buffer at a wide pH range of 2-12. Incubation time was 24 h. After incubation period cell leakage was estimated at 600 nm using spectrophotometer. After incubation at various pH and temperature

residual activity in terms of CP biodegradation was investigated as mentioned in section 3.11.2. Samples were collected and analyzed for residual CP concentration.

Chemical stability

Chemical stability of CAMs is of vital importance. CAMs and MCs were exposed to chemically harsh medium to examine their chemical stability. Citrate buffer (30 mM EDTA, 55 mM sodium citrate, and 0.15 mM sodium chloride) was prepared (Kim *et al*., 2015). In a flask containing citrate buffer CAMs were added and incubated at 35˚C for 1 h. Samples were collected on regular basis after 10 min interval and analyzed for cell leakage at 600 nm using spectrophotometer. Residual CP concentration was also examined as mentioned in section 3.11.2.

Reusability

For cost effectiveness of this method, reusability of CAMs or MCs is of utmost importance. MSM was prepared; pH was adjusted to 7 and augmented with CP (600 mg/L). Medium was incubated with CAMs and MC at 35˚C for 24 h. Residual CP concentration was also examined after repeated sampling and analysis. CAMs and MCs were washed with autoclaved DI water and reused for biodegradation of CP repeatedly (14 times).

Storage period/Shelf life Evaluation

For shelf life evaluation CAMs and MCs were stored at 4˚C after preparation. CAMs and MCs were used for biodegradation assay in MSM (30ml) at pH 7 and CP 600 mg/L. Medium was incubated at 35˚C for 24 h. After sampling residual CP concentration was also examined using GC-ECD. After every week (upto 20 weeks of storage) biodegradation assay was performed using similar procedure with stored CAMs and MCs.

Scanning Electron Microscopy

For SEM examination CAMs were fixed using 2.5% glutaraldehyde solution for 3 h at room temperature and washed with distilled water. Dipped in acetone (50, 60,70, 80, 90%) for 10 min each. Dipped in absolute acetone for 15 min. Immersed in isoamylacetate (2 h) and afterwards incubated at 30˚C for dehydration (2 h). After coating with platinum CAMs were examined with Scanning Electron Microscope (Tsai *et al*., 2013; Lin *et al*., 2013).

3.12. BIODEGRADATION OF CP, METABOLITES FORMATION AND DEHALOGENATION

CP (100-700 mg/L) was added in MSM of pH 7 for biodegradation assay and inoculated with MCs. Incubation period was 24 h at 35˚C. Residual CP concentration was also examined after repeated sampling and analysis. After biodegradation of toxic compounds like CP some intermediate compounds were formed. These intermediate compounds may be more toxic than parent compounds. In Immobilization technique metabolites produced after biodegradation of parent compound stays in structure and inhibit further degradation. Thus, extent of biodegradation and formation of secondary compounds formed were analyzed using GC-MS following conditions mentioned in Tiwari and Guha (2014). Samples were extracted for analysis of metabolites using sample to acetone ratio 1:3. To find out concentration of these metabolites GC-ECD was used.

Nitrate nitrogen (NO_3^-—N), phosphate phosphorous (PO_4^{+3}—P) was analyzed at 0 h and after completion of cycle using spectrophotometer. For determining extent of mineralization COD was determined following standard method (APHA, 2012). Chloride ions release was measured following method of Bidlan and Manonmani, 2002 and Saez *et al*., 2015. Samples were centrifuged (8000xg), supernatant was collected and mixed with 50 µL each of HNO_3 (0.15 N) and $AgNO_3$ (0.1 N). After 20 min of incubation at room temperature absorbance was measured

at 600 nm using spectrophotometer (DR2010, HACH, USA). NaCl was used to plot standard curve for concentration measurement.

3.10.5 Biodegradation of CP in Real Wastewater Using MCs and CAMs

Residual activity of MCs in terms of CP biodegradation was investigated in real wastewater (WW). Real wastewater was collected from agrochemical industry located in Multan industrial zone, Pakistan. pH of the wastewater was very low so it was adjusted to 7 and it has CP concentration 583 mg L^{-1}. Wastewater was added in a flask and inoculated with MCs and CAMs. Incubation time was 32-24 h at 150 rpm and 35˚C. Samples were collected for analysis of residual CP concentration.

3.13. ANALYSIS

3.13.1. Kinetics Analysis

Kinetic analysis is convenient method to understand overall progress of degradation process. Therefore successful operation of experimental setup required kinetic evaluation. Several methods for kinetic evaluation includes first order kinetic model. By plotting concentration of substrate (*In*CP) against time "t" straight line and regression equation is obtained. Slope of the straight line interpret first order rate constant k. In current study In(CP) was plotted against time t. First order rate constant, half-life, and correlation coefficient were calculated using equation (4, 5) (Yang *et al*., 2014)

$$ln\, C = a + kt \qquad (4)$$

$$t_{1/2} = \frac{In2}{k_1} \qquad (5)$$

Removal efficiency (%) was calculated using equation 6 after Jiang *et al.* (2015)

$$\frac{C\ influent\_C\ effluent}{C\ influent} * 100 \qquad (6)$$

Following algorithms were used for rate constant determination

$$\ln C = a + k1t \quad (7)$$

$$C = b + k0t \quad (8)$$

Where substrate concentration is represented by *C*, *t* is degradation period and *k*1 is first-order rate constant and *k*0 is zero-order rate constant. *t*1/2 values for batch systems were calculated using data obtained from kinetic calculations by the following expression Denga *et al.* (2015)

$$t_{1/2} = \ln 2/k \quad (9)$$

Kinetics equation obtained by plotting ln *C* against degradation *t*, *R*2, first-order rate constant *k*1 (h−1), *t*1/2(days) for all batch systems are presented in tabular form in (Table 4.4).

3.13.2. GC-ECD Analysis

Sample (1ml) was collected to determine concentration of CP medium. Mixed with ethyl acetate twice in volume and vortexed briefly. For TCP/TMP determination sample was mixed with acetone thrice in volume, vortex and supernatant was collected. TCP and TMP were analyzed using Gas Chromatogram with Electron capture detector (GC-ECD) (Shimadzu 2010) with fused silica capillary column TRB-1. Samples were analyzed for remaining CP concentration at GC conditions described previously (Zhang *et al.*, 2012).

Ethyl acetate was used as a solvent for extraction of CP at a sample to solvent ratio 1:2 (v/v) and TCP/TMP was extracted in acetone at 1:3 (v/v). For CP analysis 1 µl samples after extraction was injected in GC-ECD with fused silica capillary column TRB-1 at conditions described by Zhang *et al*. (2012). Injection port and detector temperature for CP analysis was 300 and 330 ˚C. Initial column temperature for CP analysis was 150 ˚C and increased to 270 ˚C @8 ˚C/min. Held at 270 ˚C for 5 min.

For TCP and TMP detection injection port and detector temperature were 200 and 250 °C, respectively. Initial oven temperature was 70 °C (for 1min) finally increased to 220 (10 °C min^{-1}) (Yang *et al*., 2014). Standard solutions of various concentrations were prepared and observed peak area was plotted against known concentrations to get a standard curve. This curve was used for concentration determination of injected samples.

3.13.3. GC-MS Analysis

For GCMS analysis method defined by Tiwari and Guha (2014) was followed. Sample (1ml) was collected, mixed with ethyl acetate twice in volume and vortexed briefly. For GC-MS, carrier gas was helium @ 1ml min^{1}. Temperature of injection port was 250 °C. Ion source and auxiliary line temperatures were 280 and 240 °C, and ionization was at 70 eV. Column temperature was initially 140 °C, rise to 180 °C @ 8 °C per min, held to 1 min, rise to 250 °C @ 4 °C per min, held at this temperature for a min.

3.13.4. Calculations and Statistical Analysis

Removal efficiency (%) was calculated using equation 6. Arithmetic mean and standard deviation from three replications were calculated. Results were presented in graphical form using Microsoft Excel 2010 with error bars showing standard deviation. One way analysis of variance (ANOVA) and Students t-test was used for statistical analysis.

Chapter 4

4. RESULTS AND DISCUSSION

4.1. ISOLATION, ACCLIMATIZATION, CHARACTERIZATION AND IDENTIFICATION OF CP DEGRADING BACTERIA

4.1.1. Isolation of CP Degrading Bacteria

Wastewater also contains bacteria that are mostly resistant to harsh conditions. Agricultural field where OPPs are sprayed may serve as a source for OPP degrading bacteria (Masahito *et al*., 2000). Samples for isolation were collected from three locations. 26 isolates were obtained from wastewater drain of NUST, Islamabad Pakistan and 14 isolates from Agricultural soil sample, District Chakwal, Pakistan (Table 4.1). All isolated strains demonstrate varying ability to grow with CP concentration (100 mg/L) and use it as carbon source.

Table 4.1: Bacterial isolates from various sources

S.No	Source	Isolated Strains	Total Isolates
1	Wastewater from drain 1 NUST, Islamabad Pakistan	SRK1 to SRK16	16
2	Wastewater from drain 2 NUST, Islamabad Pakistan	SK1 to SK10	10
3	Agricultural soil from District Chakwal, Pakistan	T1 to T14	14

4.1.2. Acclimatization and Screening of Bacterial Strains

All bacterial strains were tested for CP biodegradation capability. All strains were grown in MSM containing 100 mg/L of Chlorpyrifos, and incubated at 35˚C for 24 h. Seven strains demonstrated more than 50% degradation of initial CP concentration. After initial screening seven strains SRK1, SRK2, SRK3, SRK4, SRK5, T13 and T14 were selected for biodegradation studies. Selected strains were acclimatized with gradually increasing concentration of CP from 50 to 400 mg/L.

4.1.3. Morphological Characterization

Colony morphology of isolates is mentioned in Table 4.2. Majority of strains were found to be large colony size except SRK2 and T13. Shape, margins/edges, opacity, shine, color, elevation, texture for the strains SRK1-SRK5, T13 and T14 are presented in Table (4.2).

Table 4.2: Colony morphology of selected bacterial strains

Morphological Characters	**Bacterial Isolates**						
	SRK1	**SRK2**	**SRK3**	**SRK4**	**SRK5**	**T13**	**T14**
Size	L	M	L	L	L	M	L
Shape	IR	Round	IR	IR	Round	IR	Round
Edge	Lobate	Entire	Lobate	Undulate	Entire	Entire	Entire
Opacity	TL	TL	TL	OP	OP	TL	TL
Shine	Dull	Shiny	Dull	Shiny	Dull	Shiny	Shiny
Color	Creamy	Creamy	Light yellow	Off-White	Creamy	Off-White	Off-White
Elevation	Convex	Raised	Flat	Convex	Flat	Flat	Convex
Texture	Mucoid	Sticky	Mucoid	Buttery	Buttery	Buttery	Sticky

L: Large M: Medium IR : Irregular TL: Translucent OP: Opaque

4.1.4. Molecular Identification

16s rRNA gene sequence analysis for identification of chlorpyrifos (CP) degrading strain were performed. Sequences were obtained as mentioned in section 3.5. These sequences were submitted at National Center for Biotechnology Information (NCBI) GenBank through BankIt and accession numbers were obtained. Obtained sequences were compared with already reported sequences at NCB1using Basic Local Alignment Search Tool (BLAST). Accession numbers, query cover and homology (%) are mentioned in Table 4.3.

Table 4.3: Taxonomic name, accession numbers and homology% of selected bacterial strains

S. No	Strain Name	Organism Name	Query Cover %	Homology %	Accession Number
1	SRK1	*Pseudomonas kilonensis*	100	99	KT013088
2	SRK2	*Serratia marcescens*	100	99	KT013089
3	SRK3	*Bacillus sp.*	100	99	KT013090
4	SRK4	*Bacillucs pumilus*	100	99	KT013091
5	SRK5	*Achromobacter xylosoxidans*	100	97	KT013092
6	T13	*Klebsiella sp.*	100	100	KT013093
7	T14	*Psychrobacter alimentarius*	100	100	KT013087

A phylogenetic tree showing interrelationship of strains was constructed using Molecular Evolutionary Genetic Analysis 4 and presented in Figure 4.1. Neighbors-joining method was used for tree construction (Bootstrap values 50%, expressed as percentage of 500 replications. Strains SRK1- SRK5 T11 and T13 showed 97-100% similarity to *Pseudomonas kilonensis*,

Serratia marcescens, *Bacillus sp.*, *Bacillus pumilus*, *Acromobacter xylosoxidans*, *Klebsiella sp.* and *Psychrobacter alimentarius* respectively (Figure 4.1).

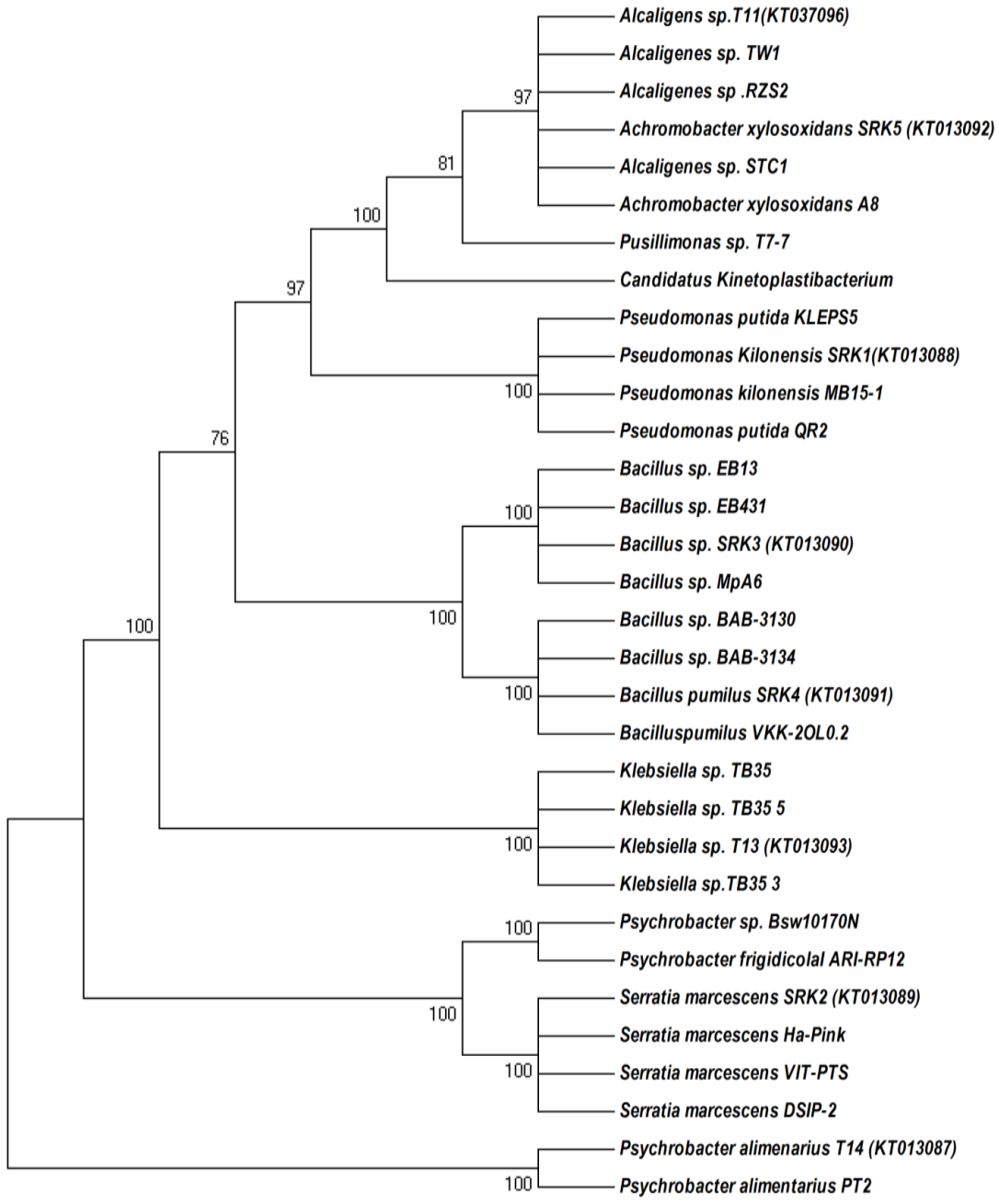


Figure 4.1: Phylogenetic relationship among isolated bacterial strains and reference sequences at NCBI on the basis of 16 S rRNA nucleotides sequences. Accession numbers (SRK1-T13) from (KT013088 to KT013093).

PHASE I

4.2. BIODEGRADATION OF CHLORPYRIFOS IN MINERAL SALT MEDIUM

4.2.1. Biodegradation of CP Using Monoculture

Biodegradation of CP was investigated in mineral salt medium (MSM). CP was the only carbon source present in medium. All selected bacterial strains vary in their CP biodegradation capability. Initial CP concentration (400 mg/L) was reduced to 66.25% (265 mg/L) when SRK1 was used as inoculum for CP biodegradation. With SRK2, SRK3, SRK4 and SRK5 63.1, 50.6, 59.1, 63.55 % degradation of CP was observed (Figure 4.2). T14 has achieved 54% removal of CP. Maximum biodegradation (68.5%) of CP was observed with T13 followed by SRK1 (Figure 4.2). With abiotic control negligible (0.5%) CP removal was observed.

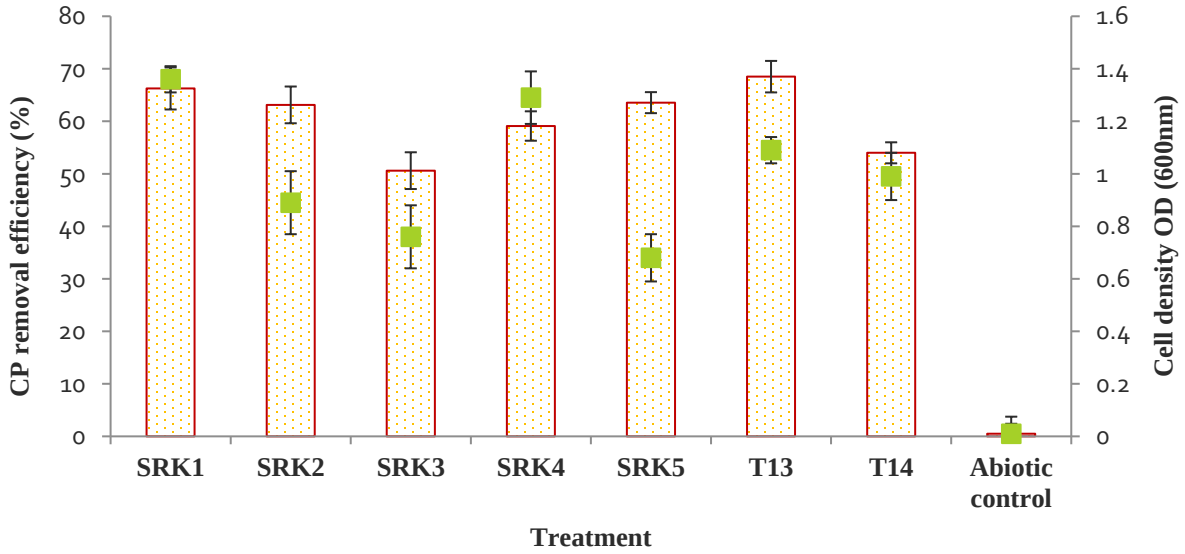


Figure 4.2: Biodegradation of CP (%) and changes in cell density (600nm) using monoculture at initial CP concentration; 400 mg/L, inoculum level; 10%, pH; 7, HRT 48h.

4.2.2. Biodegradation of Chlorpyrifos Using Bacterial Consortium

Interaction Studies

Studies on synergistic action among microorganisms could be useful for enhanced bioremediation of polluted sites. During microbial metabolism various intermediate compounds and secretions were produced (Mishra and Malik, 2014). For investigating the effect of intermediate compounds and secretions produced by one strain, on the other, interaction studies were conducted. In spent filtrate of SRK1 growth of SRK2 was reduced by 1 time. In contrast growth of SRK4, SRK5 and T13 has shown an increasing trend in spent filtrate of SRK1. In spent filtrate of SRK2 a decrease in growth of SRK1 was observed by 1.5 times, similar to growth of SRK2 in the presence of spent filtrate of SRK1 (Figure 4.3).

Figure 4.3: Interaction between bacteria i.e. relative growth of a strain in spent filtrate of another bacteria after 96 h (SRK1/SRK2 means growth of SRK2 in spent filtrate of SRK1 after 96 h) all other are in similar manner

While growth of SRK4 and SRK5 increased with spent filtrate of SRK2 as growth medium. However there was a slight increase in growth of T13 in spent filtrate of SRK2. Growth of SRK2 and T13 was reduced in the spent filtrate of SRK4 by 1.26 and 1.34 times, respectively. On the other hand growth of SRK1 and SRK5 was enhanced with SRK4 spent filtrate. All selected strains have shown an increasing trend in their growth in spent filtrate of SRK5 and T13 (Figure 4.3). T14 and SRK3 have shown antagonistic effect with all other bacterial strains because secretions and metabolites produced by these strains were toxic for other strains.

4.2.3. Optimization of Experimental Setup Performance

Effect of inoculum concentration on CP degradation

Effect of inoculum level on biodegradation of CP was investigated. At 5% (5ml culture/100ml of media) level of inoculum 72% degradation of initial CP concentration 100 mg/L was observed after 48 h at pH 7. When level of inoculum was doubled from 5 to 10% an increase in CP removal efficiency was observed. After 36 h HRT 100 mg/L CP was reduced to 98% with 10% inoculum (Figure 4.4). In order to speed up biodegradation process and achieve fast removal of CP 15% inoculum was used but results were not significant and complete removal was achieved after same duration as for 10% inoculum (Figure 4.4). 10% inoculum was selected as optimum and used in all other experiments. Anwar *et al.* (2009) reported longer lag phase with low initial inoculum density and 50% removal of initial CP concentration (50 mg/L) was achieved after 5 days. Tastan and Donmez (2015) reported increase in biodegradation of triclosan with increase in initial biomass concentration. Maximum inoculum density (1.24 at 600 nm) was observed when 10% inoculum level was used for CP biodegradation after 36 h. Figure 4.4 represents an association between increase in optical density and decrease in CP concentration. Rate constant was increased from 0.0273 to 0.0909 when initial inoculum level was doubled from 5 to 10%.

Rate constant k_{15} was 0.0865. Another study reported first order rate constant (*k*) of 1.364d^{-1} (Silambarasan and Abraham, 2013) Half-life is an indication how long CP exists in a system. Shortest half-life (0.31 days) was observed when 10% inoculum level was used, which exhibits short half-life and fast degradation of CP. With 5 and 15% initial inoculum level half-life of CP in system was 1.02 and 0.33 days respectively. Silambarasan and Abraham (2013) reported half-life of 0.5 days for CP using *Alcaligenes* sp.

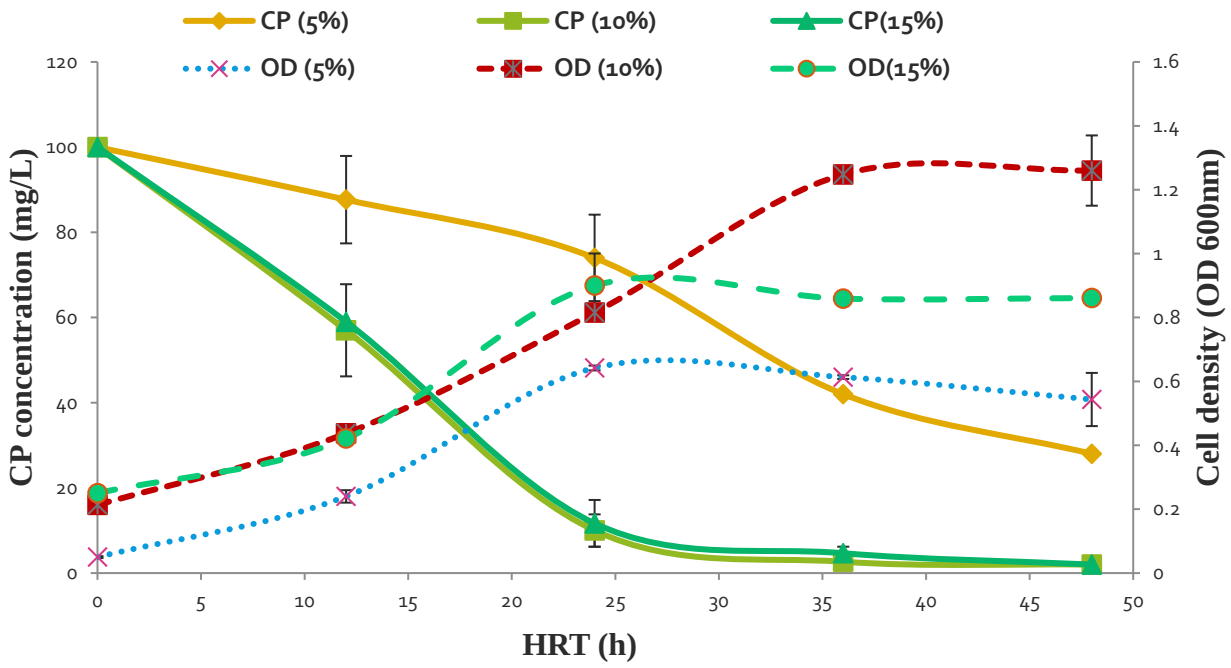


Figure 4.4: Biodegradation of Chlorpyrifos and cell density at different initial inoculum level i.e. 5, 10, 15% in mineral salt medium (initial pH 7; initial CP concentrations; 100 mg/L and HRT; 48 h)

Obtained results have shown that 10% level of inoculum was optimum for CP biodegradation. Results indicated CP biodegradation was strongly associated with level of initial bacterial inoculum. Large inoculum size means faster acclimation of bacterial population to initiate biodegradation. Acclimatization period is interval required for bacterial strain to reproduce to a level adequate to start degradation (Anwar *et al*., 2009) and smaller populations generally require

extended period. Outcomes of current experiment indicate rapid acclimation at higher initial inoculum level.

Effect of growth medium pH on CP biodegradation

In biodegradation process pH of growth medium is an important parameter which may affect enzymatic activity of bacterial strains. In order to investigate effect of growth medium pH on biodegradation of CP experiments were performed. At very low pH (2-4) and high pH (>9) biodegradation was negligible. 62% degradation of CP was observed at pH 5 after 48 h HRT in MSM with initial CP concentration (100mg/L). At pH 6 biodegradation was slightly increased to 66% (Figure 4.5). With increase in medium pH to 7 a sharp increase in CP removal was observed and 98% removal was achieved after 48 h of treatment. When pH was increased to 8 no significant increase in CP removal was detected (Figure 4.5). Results indicated medium pH from 7-8 was optimal for biodegradation of CP using bacterial consortium. Yang *et al*. (2005) reported efficient degradation of CP (250 mg/L) at pH 8 by *Alcaligenes faecalis*. Lu *et al*. (2013) reported efficient degradation of CP at pH 7 by *Cupriavidus* sp. Complete biodegradation of CP (100mg/L) in 5 days was observed at pH 7 by Jabeen *et al*. (2014), however at pH 8 complete degradation was achieved in 7 days. Optical density for pH 8 was higher than pH 7 during biodegradation period. Contrary to higher growth at pH 8, higher CP removal was observed at pH 7. Possible reason for higher CP removal at basic pH (7) could be optimum expression of enzymes responsible for biodegradation. Enzymes have active site and their activity depends upon its ionization which is strongly affected by pH changes and therefore enzymes activity stops outside a specific pH range (Kim *et al*., 2013). With increase in pH upto 7, an increase in rate constant was observed. k_5, k_6, k_7 and k_8 were 0.0192, 0.0235, 0.0865 and 0.059 h^{-1} respectively (k_5 denotes rate constant at initial pH 5, other of subscripts denotes pH in similar

manner). Half-life of CP was 1.50, 1.22, 0.33, 0.48 days at pH 5, 6, 7 and 8 (Table 4.4). Shortest half-life of CP was observed at pH 7. Kinetic analysis has shown pH 7 was optimum for CP degradation. Selected optimum pH was used in subsequent experiment.

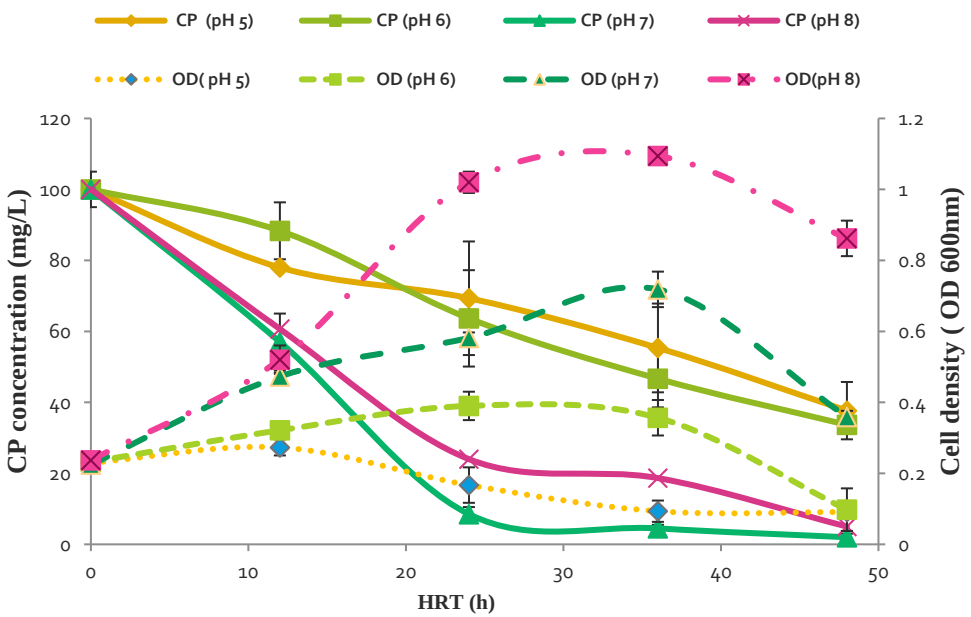


Figure 4.5: Biodegradation of chlorpyrifos and cell density at initial pH 5, 6, 7 , 8 of mineral salt medium (initial inoculum level 10 % ; initial CP concentrations 100 mg/L; HRT; 48 h)

Effect of initial concentration on CP degradation

Initial concentration of carbon substrate plays a vital role to start biodegradation process. In order to investigate effect of initial concentration on performance of bacterial consortium experiments were performed at varying initial CP concentration (100-500 mg/L). At 100 and 200 mg/L almost complete CP removal was observed at pH 7 and 10% level of inoculum after 24 h. Complete CP removal was observed at 300 mg/L after 48h (Figure 4.6). At 400 mg/L 98% CP removal was observed after 36 h and complete removal (~97%) was observed after 36 h. When initial CP concentration was 500 mg/L a lag phase was observed. At high concentration i.e. 500

mg/L statistically significant decrease in degradation rate was observed ($p<0.05$). Toxic effects of CP were not significant upto 400 mg/L; however at higher concentrations (500 mg/L) increased toxic effects were observed (Figure 4.6).

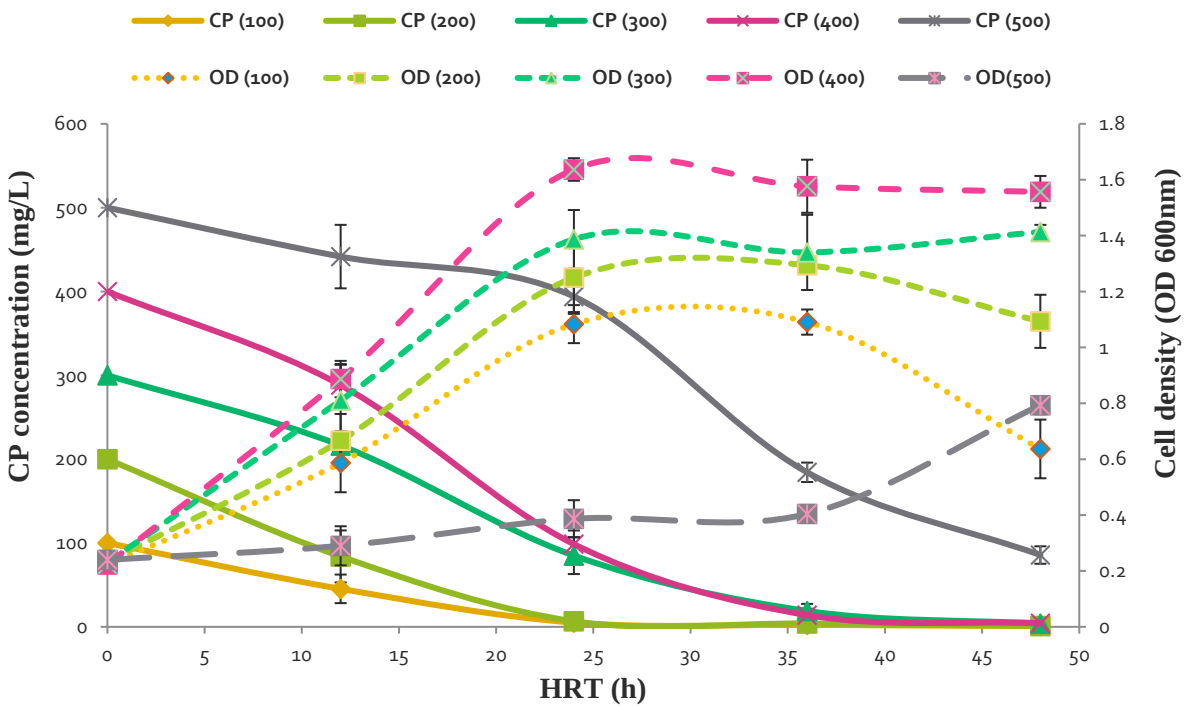


Figure 4.6: Figure 4.6: Biodegradation of Chlorpyrifos (primary y-axis) and cell density (secondary y-axis) in mineral salt medium supplemented with various CP concentrations from 100 to 500 mg/L of mineral salt medium (initial inoculum level 10 %; initial pH 7; HRT 48h)

Higher initial concentration of CP reduces biodegradation efficiency of microorganisms at initial stage (Yadav *et al*., 2015). Jabeen *et al*. (2014) reported complete biodegradation of CP in three days when initial concentration was 50 mg/L, however with increase in concentration from 100-400 mg/L only 85- 15% biodegradation was observed in same duration. In contrast to our results, Anwar *et al.* (2009) reported significant decrease in removal efficiency at lower concentrations possibly because enzymes were only expressed at higher CP concentration. But in our study

enzymes were expressed even at lowest tested concentration (10 mg/L, data not shown). k_{100}, k_{200}, k_{300} and k_{400} was 0.10, 0.11, 0.09, 0.10 and 0.0367 h^{-1} respectively (k_{100} denotes rate constant at initial CP concentration 100, all other denotes CP concentration similarly). At high concentration like 500 mg/L degradation rate was decreased to 0.0367 and half-life was increased to 0.78 days. Half-life of CP was 0.28, 0.25, 0.31and 0.28 days at 100, 200, 300 and 400 mg/L (Table 4.4). Kinetic analysis revealed pH 7 was optimum for CP degradation. Possible reason for decline in growth at higher CP concentration may be substrate inhibition (Yadav *et al*., 2014). This might be inhibition of anionic transportation, cell acidification and undesirable substrate binding to cell parts (Olson *et al*., 2003).

Effect of hydraulic retention time on CP degradation

For biodegradation in closed reactors HRT needs to be optimized. At HRT 24 h approximately 75 % CP removal was observed. Doubling HRT to 48 h degradation rate was enhanced and 98% removal was observed (Figure 4.7). Longer incubation time is required for complete mineralization of organic xenobiotics (Vijayalakshmidevi and Muthukuma, 2015). k_{24}, k_{48} and k_{72} was 0.051, 0.1004, and 0.626 h^{-1} respectively. Half-life of CP was 0.558, 0.28 and 0.437 days at HRT 24, 48 and 72 h (Table 4.4).

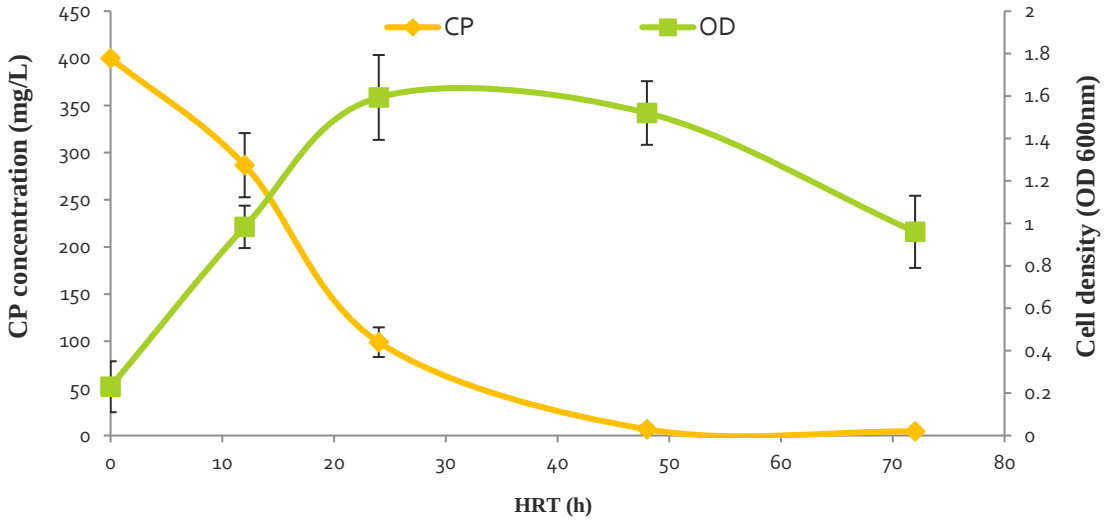


Figure 4.7: Figure 4.7: Biodegradation of Chlorpyrifos (primary y-axis) and cell density (secondary y-axis) in mineral salt medium supplemented with CP concentrations 400 mg/L; initial inoculum level 10 %; initial pH 7 after variable hydraulic retention time 24, 48, 72h.

Table 4.4: Kinetics parameter for Chlorpyrifos biodegradation by bacterial consortium

Parameters	Value	Kinetic equation	K (hrs^{-1})	R^2	$t_{1/2}$	RE (%)
Inoculum level (%)	5	InCP=4.742+0.0273t	0.0273	0.9039	1.05762	72
	10	InCP=4.698+0.0909t	0.0909	0.9523	0.31767	98
	15	InCP=4.7428+0.0865t	0.0865	0.9793	0.33385	98
pH	5	InCP=4.622+0.0192t	0.0192	0.9641		62.4
	6	InCP=4.68+0.0235t	0.0235	0.9802	1.50396	66.4
	7	InCP=4.678+0.0865t	0.0865	0.9674	1.22873	98
	8	InCP=4.674+0.0598t	0.0598	0.9598	0.33385	95
CP (mg/L)	100		0.1015	0.9618	0.48947	99
	200	InCP=4.604+0.1015t	0.1136	0.96	0.28443	99.5
	300	InCP=5.322+0.1136t	0.0919	0.9502	0.25411	98.7
	400	InCP=6.168+0.0919t	0.1004	0.9511	0.3142	98.9
	500	InCP=6.48+0.1004t	0.037	0.867	0.2876	82.8
HRT (h)	24	InCP=6.462+0.037t			0.78675	
	48	InCP=5.99+0.0517t	0.0517	0.99		75.25
	72	InCP=6.49+0.1004t	0.1004	0.98	0.55851	98.35
		InCP=6.479+0.066t	0.066	0.996	0.2876	98.9
					0.437	

4.2.4. Biodegradation of CP, Formation of Metabolites and Mass Balance Study

Biodegradation of CP

After optimization of environmental parameters i.e. inoculum concentration, pH, initial CP concentration and HRT, a final biodegradation experiment was performed to examine CP biodegradation kinetics, cell growth, formation of metabolites and pH changes. CP removal ability of bacterial consortium was determined by analyzing remaining CP concentration in liquid medium. Biodegradation of CP was enhanced when bacterial strains were used in the form of consortium. Bacterial consortium was able to mineralize 96 % of initial CP concentration in MSM at pH7 and 10% inoculum concentration within 36 h. Almost complete removal of CP was achieved in MSM after 48 h (Figure 4.8a). Singh and Walker (2006) concluded that mixed consortium will be more efficient in bioremediation of environmental compartments polluted with CP. In current study None of the individual culture has obtained complete biodegradation of CP (400 mg/L), maximum biodegradation 68.5% was observed T13 followed by SRK1(66%).

Biodegradation of CP was investigated in mineral salt medium (MSM). However with bacterial consortium complete biodegradation of CP (400 mg/L), was achieved after 48 hours. Biotreatment system comprising of mixed bacterial consortium shows more efficient and high degree of biomineralization than pure culture because of synergistic effect on metabolism of the microbial community (Khehra *et al*., 2005). Synergistic effect includes combined enzymatic mechanism, exchange of cofactors, and mineralization of metabolites produced by other members of consortium.

First order model has shown excellent fit (R^2>0.96) to the experimental data. First order growth model is applicable when degradation is dependent upon only one reactant, in our study biodegradation was depend upon CP. First order rate constant was 0.1018 (h^{-1}) this confirms fast

degradation. CP has half-life ($t_{1/2}$) of several weeks which is reduced to 0.284 day only (Figure 4.8b). Kinetic analysis revealed fast degradation of CP using selected bacterial consortium.

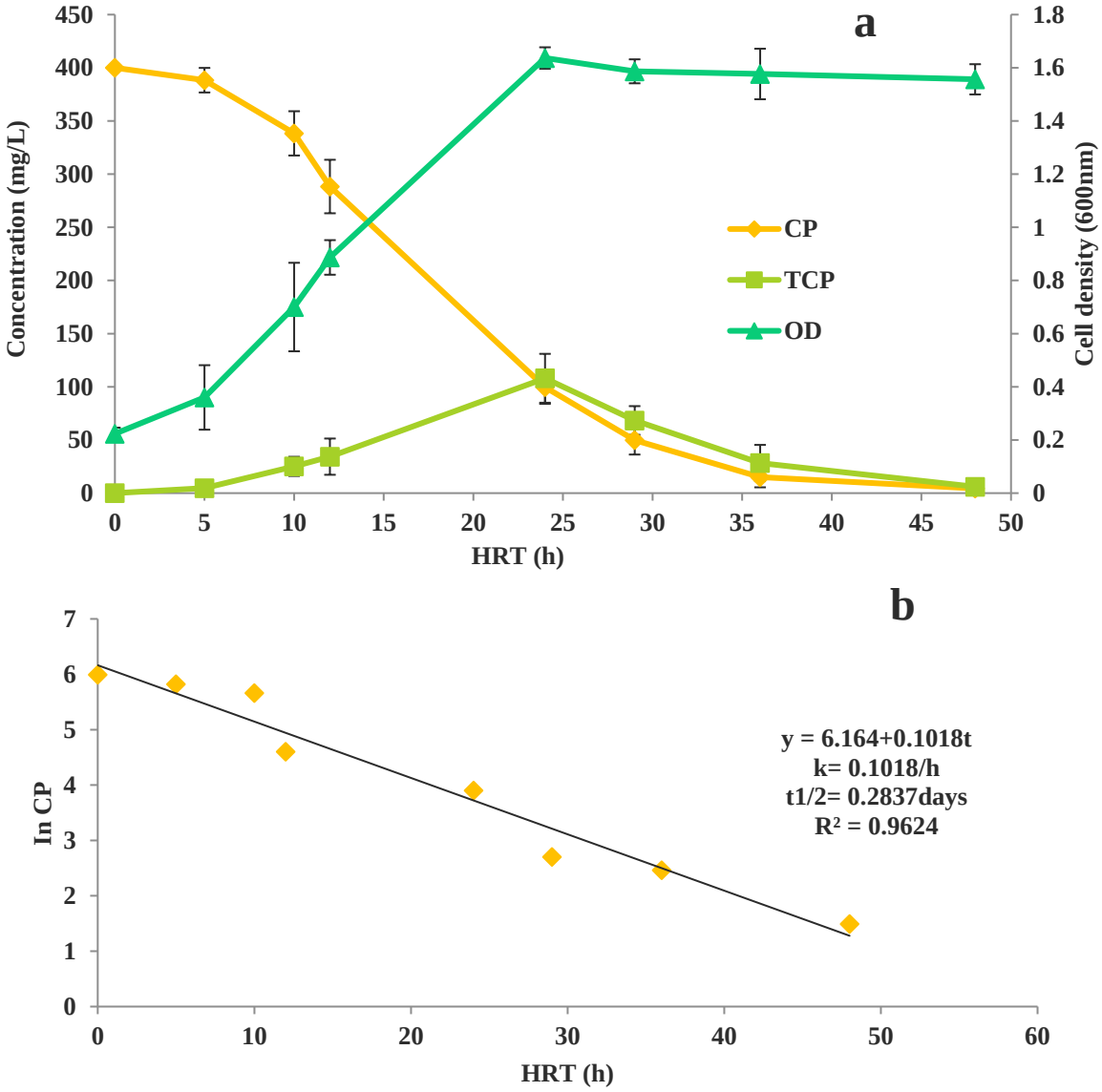


Figure 4.8: Figure 4.8: Degradation dynamics of CP concentration and TCP formation (mgL-1), cell density (600nm) by bacterial consortium (b) relationship of In(CP) and time (Initial CP concentration: 400 mg/L, initial pH: 7, HRT;48 h).

Organophosphate pesticide degradation involves expression of enzymes like OPH (organophosphorous hydrolase) and OPAA (organophosphorous acid anhydrolase) by microorganism (Gao *et al.*, 2012; Abraham *et al.*, 2014; Gilani *et al.*, 2015). Biodegradation of

CP by bacterial consortium could be attributed to expression of OPH and/or OPAA enzymes (Supreeth and Raju, 2017). Results indicated significant application potential of bacterial consortium for management of pesticide contaminated wastewater. However further understanding of synergistic action between microorganisms, enzyme mechanism may help in large scale applications. Understanding catalytic activity of microbes will help in manipulation of enzymes for achieving enzymatic degradation of CP to achieve desired output.

Formation of metabolites

Biodegradation of CP results in the formation of intermediate compounds. GC-ECD analysis has shown presence of TCP in liquid medium after 5 h of batch operation. Biomineralization of CP results in formation of metabolite TCP (Xu *et al*., 2007). With the passage of time TCP concentration increased as a result of CP breakdown. 23 mg/L TCP was observed after 24 h (Figure 4.8a). Accumulation of TCP in medium may hinder growth of CP degrading microbes (Chen *et al.*, 2012; Liu *et al*., 2012). In current study a decrease in concentration of TCP was observed after 24h, this indicates ability of bacterial consortium to tolerate and further degrade this toxic metabolite. Microbial consortium is more efficient than pure culture as they stop accumulation of metabolites produces after biodegradation (Kumar and Philip, 2006), it is very beneficial for a biodegradation system and leads to complete removal of harmful compound from contaminated environment. Abraham *et al*., (2014) reported TCP as CP metabolite after biodegradation of CP in aerobic conditions. Tiwari and Guha (2014) reported presence of metabolites TCP and TMP after aerobic degradation of CP. TMP could not be detected in GCECD analysis possibly because it was unstable and/or completely mineralized to water soluble products (Tiwari and Guha, 2014).

Fluctuation in pH during bioreactor operation

Initial pH of the liquid medium was adjusted to 7 after this system worked without pH control. pH values were monitored throughout the degradation period. There was a very slight fluctuation in medium pH from 7 – 7.14 (Figure 4.9). This pH range has supported bacterial growth which resulted in fast removal of CP. As observed while studying bacterial growth behavior, bacterial strains were able to survive and perform activity in wide range of pH. Fluctuation in medium pH with degradation may affect activity of enzyme responsible for biodegradation of CP. Contrary to this Kontro *et al*. (2005) reported drop in pH after addition of CP in medium initially. One potential reason for decline in pH could be the consequence of microbial glucose metabolism (Cycon *et al*., 2009) usually present in the medium, which results in release of organic acids in extra cellular medium, it has inhibitory effect on microbial activity. However no significant pH drop was observed in current study.

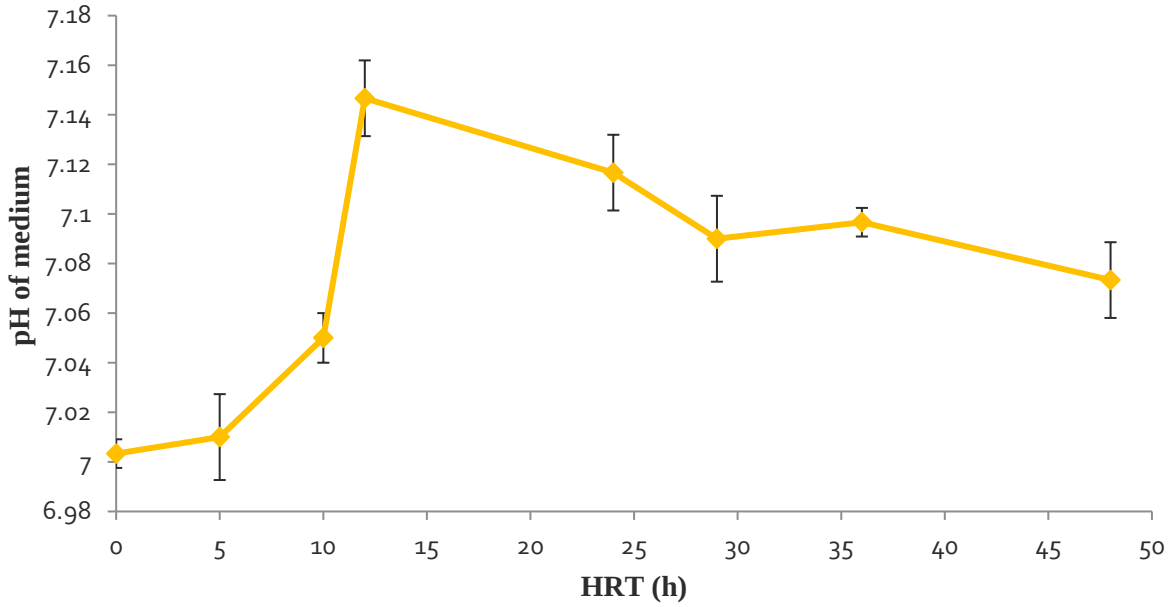


Figure 4.9: Figure 4.9: Fluctuation of pH during CP biodegradation in mineral salt medium supplemented with initial CP concentration; 400 mg/L, initial pH; 7, HRT; 48 h.

Figure represents mass balance of system with 400 mg/L initial CP concentration. 1.1% of initial CP concentration (400 mg/L) was present in the medium (Figure 4.10). For mass balance study CP on the surface of bacterial biomass (bioaccumulation) and remaining concentration in liquid medium was determined. 98.99% of initial concentration (400 mg/L) was removed from the liquid medium after 48 h, this means CP was either bioassimilated/biodegraded or adsrorbed on bacterial biomass (Figure 4.10). CP adsorbed on the surface of biomass (bioaccumulated) was 1%. Briceno *et al.* (2012) reported 9-12% adsorption on cells. Remaining CP concentration (98.99%) was considered biodegraded or bioassimilated. However, CP metabolites were detected in the medium; this indicates successful biodegradation of CP. These results confer that biodegradation was cause of CP removal not bioadsorption.

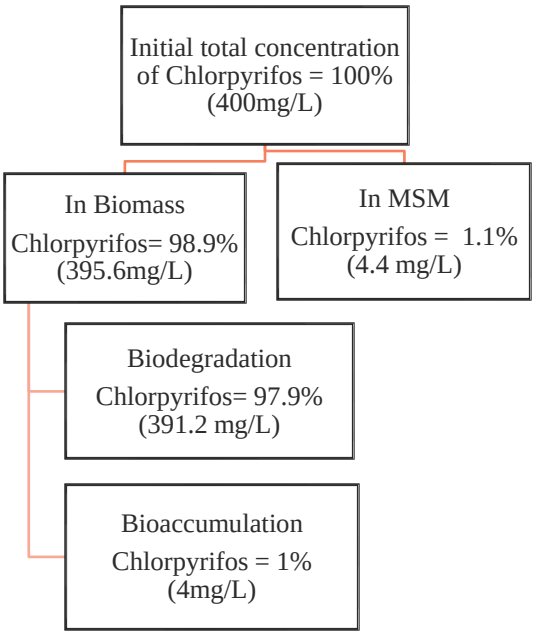


Figure 4.10: Removal mechanism and mass balance of CP biodegradation in mineral salt medium supplemented with initial CP concentration 400 mg/L initial pH; 7; HRT 48 h.

Phytotoxicity assay is valuable measure for analyzing the extent of biodegradation. To evaluate toxicity level of bioremediated effluents *Triticum aestivum* was used as indicator crop. There

were three treatments i.e. TI (irrigated with tap water), TII (irrigated with bioremediated effluents), TIII (irrigated with untreated effluents). 100 and 78.3% seeds of *T. aestivum* seeds were germinated with TI and TII (Figure 4.11). Whereas, only 55% germination was observed with TIII. Results indicated that germinated % was not much affected with TIII. Reason for this could be that germination is not sensitive to CP. Plumule and radical length with TII was 5.26 and 7.81cm respectively. 2.56 and 1.99 cm were plumule and radical length with TIII respectively (Figure 4.11). Saez *et al*. (2014) observed that seeds of *Lactuca sativa* were adversely affected by untreated effluents in term of germination, root and hypocotyl length in slurry contaminated with lindane as compared to uncontaminated slurry. Lower germination could be result of imbalance in biochemical process because of poisonous pollutants (Bidlan *et al*., 2004; Fuentes *et al*., 2013). Some microorganisms such as bacteria have capability to produce IAA (Indole acetic acid) that may promote growth of plants (Silambarasan and Abraham, 2013). However higher values in germination percentage, radical length, plumule length and over all vigour index after biotreatment with bacterial consortium are in agreement with higher CP removal suggesting that CP was effectively removed by bacterial consortium. Higher Vigour index with TII as compared to TIII (untreated control) suggested that CP and its toxic intermediate compounds were effectively degraded by bacterial consortium.

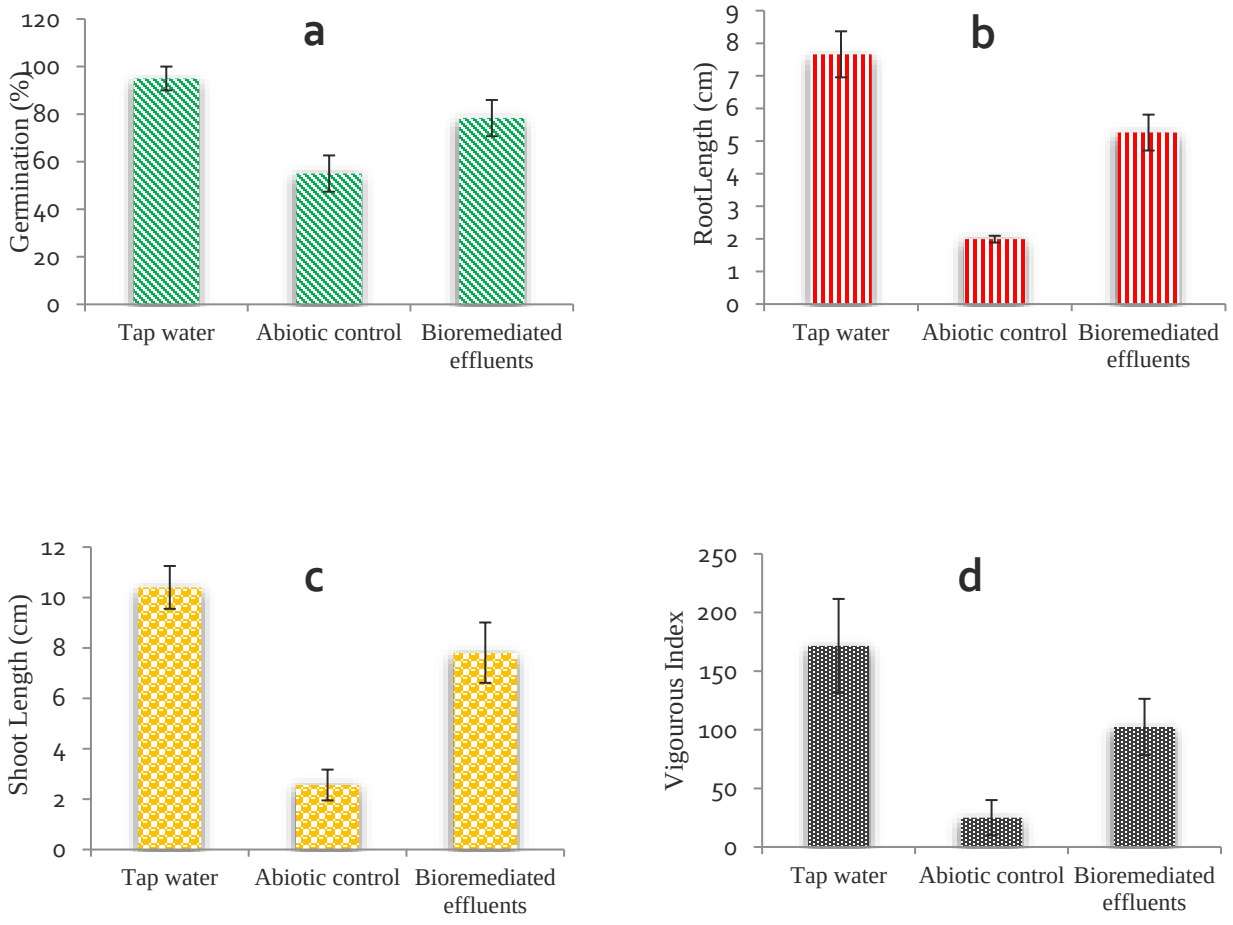


Figure 4.11: Development of Triticum aestivum seedlings irrigated with tap water, abiotic control and bioremediated effluents separately (a) germination % (b) root length (c) shoot length (d) Vigour index

4.2.5. Effect of petrochemicals (organic solvents) on biodegradation of Chlorpyrifos

Some petrochemicals are used as solvents for dissolving pesticides in commercial formulation, therefore these petrochemicals are often found in effluents of pesticide industry. The effect of presence of petrochemicals (benzene, toluene and xylene) on biodegradation of chlorpyrifos was examined. In biotic control i.e. without addition petrochemicals 98 % CP degradation was achieved. Treatment without addition of consortium served as abiotic control and CP removal didn't exceed 2%. Petrochemical free batch system achieved 98.9% CP removal. At low

concentration of petrochemicals (10 mg/L) 91, 99.8 and 88.75% of CP degradation was achieved with addition of benzene, toluene and xylene respectively (Figure 4.12 a). Degradation rate was enhanced with toluene addition. In contrast 10 mg/L of benzene and xylene has slightly decreased CP degradation. Enhanced CP removal with toluene could be result of large microbial population supported by extra carbon source. With the increase in concentration to20 mg/L of benzene, toluene and xylene, CP removal was decreased to 87.8, 98.3 and 85.3% respectively. It was interesting to observe that there was no effect on degradation rate when 20 mg/L toluene was added. Benzene and xylene inhibited degradation rate. When concentration was increased to 100 mg/L degradation was inhibited in presence of benzene, toluene and xylene and 79, 81.5 and 56 % removal efficiency was achieved (Figure 4.12 a). However in our study significant reduction in degradation efficiency was observed at 100mg/L of benzene, toluene and xylene. Although petrochemicals are a carbon source but are also reported to be toxic for microbial activity. Reason for reduction of CP removal efficiency could be lack of enzyme expression, non-targeted binding to cell components in the presence of additional carbon source (Olson *et al*. 2003; Anwar *et al*. 2009; Reddy *et al*. 2014). It can be concluded that degradation of CP continued in presence of petrochemicals but slowed down and is strongly concentration dependent.

4.2.6. Effect of metal ions on biodegradation of Chlorpyrifos

Studies reported presence of metal ions in commercial formulations. Considerable amount of trace metals are added in pesticide formulation without any scientific reason. Pesticides may be considered as source of metals ions which may take part in biochemical reaction. Therefore effect of presence of metal ions on biodegradation of CP was examined. Results indicated type and concentration of metal ions strongly effected consortium efficiency to degrade CP. About

99% CP was degraded in presence of 1 and 5 mg/L of Cu(II) after 48h (Figure 4.12 b).When concentration of Cu(II) was increased to 10 mg/L CP degradation was inhibited. Addition of other metals such as 1mg/L of Hg(II) slightly reduced degradation and 96.25% removal was achieved after 48h HRT (Figure 4.12b). Like Cu(II) low concentration of Zn(II) i.e. 1 mg/L enhanced CP degradation rate and 99.2% degradation was achieved after 48h HRT (Fig 4.12b). In case of Zn(II) contrary to strong effect at high concentration there was no effect on degradation of CP at low concentration (1-5 mg/L). Contrary to no effect at low concentration significant decrease in removal efficiency (75.5%) was observed at 10 mg/L of Zn(II). Hg(II) at 5 and 10 mg/L strongly effected CP degradation and 80.25 and 59.5% removal was achieved. For Hg(II) there was an inverse relationship among metal ion concentration and CP degradation. When concentration of metal ions was increased to 5 and 10 mg/L CP degradation was significantly decreased ($p<0.05$). Sarkouhi *et al*. (2012) reported effective degradation of organophosphate pesticides i.e. chlorpyrifos and phoxim in the presence of Ag^{+} ions. Some cations such as Cu(II) and Zn(II) may be a cofactor for CP degrading enzyme, this increased degradation rate at low concentration (Pointing *et al*. 2000; Chu *et al*. 2006). In contrast Hg(II) has inhibitory effect, possibly because metal ions competed with cofactor of CP degrading enzyme on the active site. Another reason for inhibitory effect could be formation of complexes with released enzymes which results in retardation of enzyme activity (Mahmood *et al*. 2015; Shomar *et al*. 2006). MSM with addition of CP and metal ions without bacterial inoculation served as abiotic control. In chlorpyrifos hydrolysis reaction, metal ions in a solution act as catalyst (Sarkouhi *et al*. 2012). However, no CP hydrolysis was observed in uninoculated controls in current study (data not shown). This demonstrates effect of metal ions on CP degradation was result of their interaction with bacterial enzymes/activity. According to Pointing

et al. (2000) metal ions may act as cofactor of enzymes in low concentrations. It could be concluded that all metal ions have concentration based effect on CP degradation.

4.2.7. Biodegradation of Chlorpyrifos in Simulated Pesticide Wastewater (SWW)

Figure 4.12 depicts a gradual decrease in biodegradation rate with increase in concentration from 100 to 400 mg/L. However >95% CP removal was observed upto 200 mg/L (Figure 4.12c). With increase in initial CP concentration to 300 mg/L degradation was decreased and 85.7% removal was achieved at HRT 48h (Figure 4.12c). Almost 77.5% CP removal was achieved at 400 mg/L. When medium was changed from MSM to SWW degradation continued but slowed down. Decrease in total CP removal could be related to presence of other chemicals (usually present in wastewater of pesticide industry) in SWW. Results indicated bacterial consortium has potential for application in real field application at low concentration and extended HRT. Similar to our findings, Tastan and Donmez (2015) observed biodegradation yield of 71.91% and 46.12% in synthetic medium and SWW respectively. Most probable reason for decreased in removal efficiency could be presence of multiple metals ions and petrochemicals in SWW.

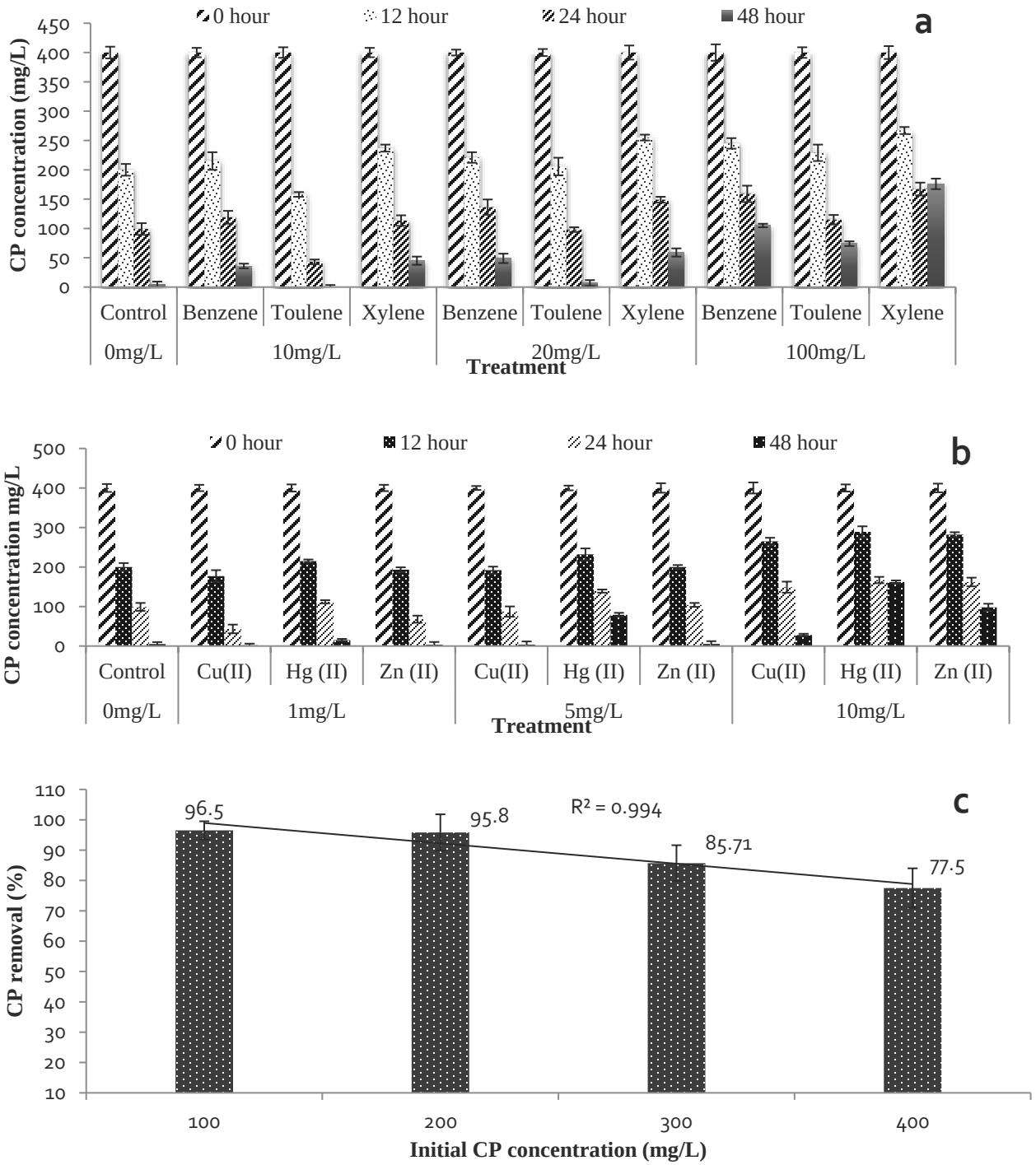


Figure 4.12: Biodegradation of chlorpyrifos in mineral salt medium at different concentrations of (a) petrochemicals; 10, 20, 100 mg/L (b) trace metals;1, 5, 10 mg/L (c) in simulated pesticide wastewater at varying CP concentration; 100, 200, 300,400 mg/L using bacterial consortium

PHASE II

4.3. CHLORPYRIFOS BIODEGRADATION IN REAL INDUSTRIAL WASTEWATER (WW)

Selected bacterial consortium was capable of efficient CP biodegradation in synthetic medium and simulated pesticide wastewater. Biodegradation of toxic compounds may be improved in industrial wastewater if microorganism is previously adapted to toxic conditions. Therefore, performance efficiency of selected bacterial consortium to biodegrade CP in WW was examined in this phase.

4.3.1. Real Industrial Wastewater (WW) Collection and Physiochemical Characterization

Wastewater was collected from industry in sterilized containers and brought to laboratory. Physiochemical analysis was performed. Results obtained from preliminary analysis are summarized in Table 4.5. Initial temperature, pH and EC was 32.5˚C , 2.92 and 2.86 mS/cm respectively. Values for nitrate-nitrogen (NO_3^-—N), nitrite-nitrogen (NO_2^-—N), phosphate-phosphorous (PO_4^{+3}—P) were 40, 60 and 825 mg/L respectively. Results revealed 8000 mg/L of COD and 140 mg/L of TSS. XRF analysis revealed presence of trace metals as mentioned in Table 4.5, these metals include K, Cr, Zn, Hg, Fe and many other. It could be inferred from the observations made during GCECD analysis that 545 mg/L of CP was present in industrial effluents. WW was stored at 4˚C.

Table 4.5: Physiochemical characterization of WW collected from industry located in Multan, Pakistan

S.No	Parameters	Unit	Range
1	Temperature	Degree Celsius	32.5 ±2
2	pH	-	2.92±0.05
3	Electrical conductivity	mS/cm	2.86 ±0.08
4	Chemical Oxygen Demand (COD)	mg/L	8000±23
5	Phosphate (PO_4^{+3}—)	mg/L	825±6
6	Nitrate (NO_3^-—N)	mg/L	40±3
7	Nitrite (NO_2^-—N)	mg/L	60±5.2
8	Chloride (Cl^-)	mg/L	1053±12.4
9	TSS	mg/L	140±6.3
10	Optical Density	600 nm	0.33±0.001
11	Metals (XRF)	--	K, Ti, Cr, Fe, Co, Zn, Ni, Mn, Ru, Hg, Cl, Ca, As, Hg
12	Chlorpyrifos	mg/L	545±10.7

4.3.2. Biodegradation of CP in Real Industrial Wastewater

As a seeding inoculum bacterial consortium isolated in laboratory consisting of five CP degrading strains was used. During startup period, batch mode of operation with a variable dilution of WW was adopted; this provided biomass with acclimation period sufficient to start biodegradation activity and develop tolerance for varying concentration of pollutants present in

WW and to avoid accumulation of pollutants in reactor. When CP concentration was low enough new batch was started. Gradual reduction of HRT indicated adaptation of microbial biomass to recalcitrant compounds present in WW.

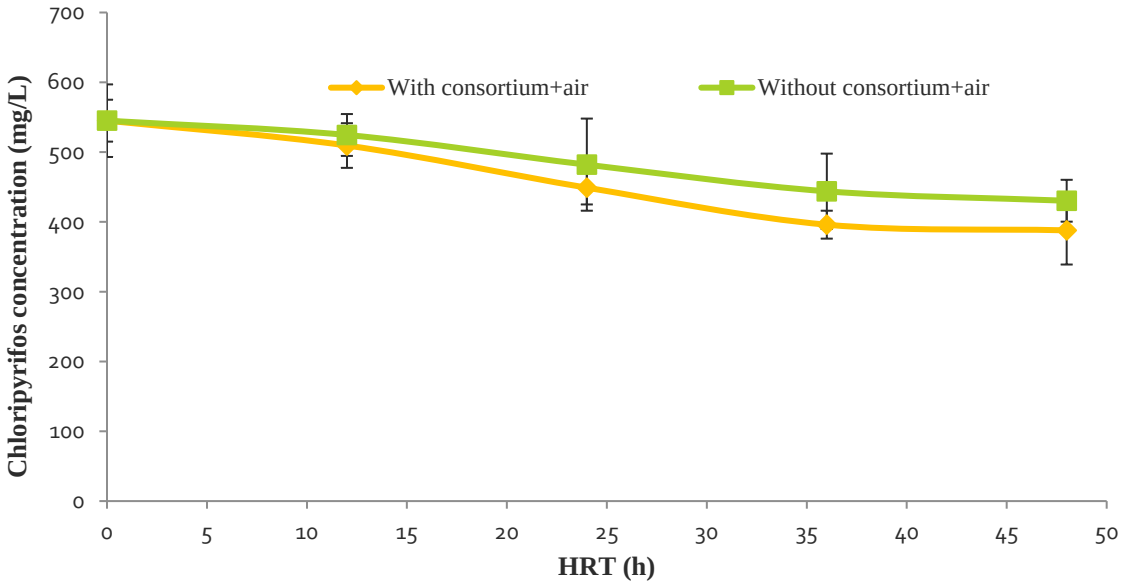


Figure 4.13: Biodegradation of CP in WW when bacterial consortium was used as seeding inoculum initial CP concentration 545 mg/L; hydraulic retention time; 48 h.

In order to enhance the biodegradation of CP, WW was bioaugmented with bacterial consortium. Bacterial consortium consisted of five members *Pseudomonas kilonensis* SRK1, *Serratia marcescens* SRK2, *Bacillus pumilus* SRK4, *Acromobacter xylosoxidans* SRK5 and *Klebsiella sp.* T13. CP removal efficiency in the batch seeded with microbial consortium was 29% after 48h of incubation. In control where indigenous biomass was present 21% CP removal was observed (Figure 4.13). Bacterial consortium performed efficient biodegradation of CP in synthetic medium but CP removal in industrial WW was low. Possible reason for this could be extremely low pH of WW because CP degrading microbes work best in pH range of 7-8 (Yang *et al*. 2005; Lu *et al*., 2013; Jabeen *et al* ., 2014). Although there was a slight increase in CP degradation with consortium addition but desired results were not achieved. Possible reason for

this could be inhospitable environment. According to Kauffmann and Mandelbaum (1998), extreme pH, predators and large quantity of solvents are limiting factor for bacterial acclimation in WW. Bacterial consortium could not perform considerable degradation activity. Further experiments were designed for optimization of the process to achieve significant CP removal.

4.3.3. Effect of Environmental Conditions on Biodegradation of CP in Real Industrial Wastewater

Environmental factors play important role in acclimation of exogenous microbes in new environment. According to Van der Gast *et al*. (2004) bioaugmentation may take advantage of microbial consortium designed for specific physiochemical properties of bioprocess since this approach was shown to be more efficient than using undefined inocula. To optimize the performance efficiency of bioreactor, experiments were conducted under different sets of conditions by varying one and keeping all other constant. Optimum value of one factor was adopted for succeeding experiments.

Effect of pH on CP biodegradation in real industrial wastewater

Experiments were conducted by adjusting pH of WW from an acidic to basic pH range of 5 to 8. At pH 5 and 6 CP degradation was 27 and 88% respectively after HRT 48 h. Maximum biodegradation was observed at pH 7 i.e. 92.2% (Figure 4.14). However at pH 8, a slight decline in CP degradation was observed. Biodegradation at altered pH followed the order 5< 6 <7>8. At pH 9 and 10, a severe decrease in CP biodegradation was detected. As shown in Figure 4.14 when pH was increased from 5 to 7 biodegradation of CP was increased. pH 7 was selected as optimum for performance of microbial community. Successful biodegradation of chlorpyrifos was reported at pH 8 by Yang *et al*. (2005) and Jabeen *et al*. (2014). Lu *et al*., 2013 reported efficient degradation of CP at pH 7 by *Cupriavidus* sp. optimal biodegradation activity at pH 7

might be result of optimum enzyme activity. Biodegradation of CP requires production of Organophosphate hydrolase (OPH) enzyme (Gao *et al*., 2012). OPH is highly pH specific and works well at pH 7. Substrate affinity towards enzyme depends upon charge at active site which is affected by pH changes. Other possible effect with varying pH could be degree of ionization, dissociated and undissociated form of substrate, acidification of environment at low pH (Olson *et al*., 2003; Reddy *et al*., 2014). Glucose breakdown results in formation of organic acids, this may leads to a pH drop (Cycon *et a*., 2009).

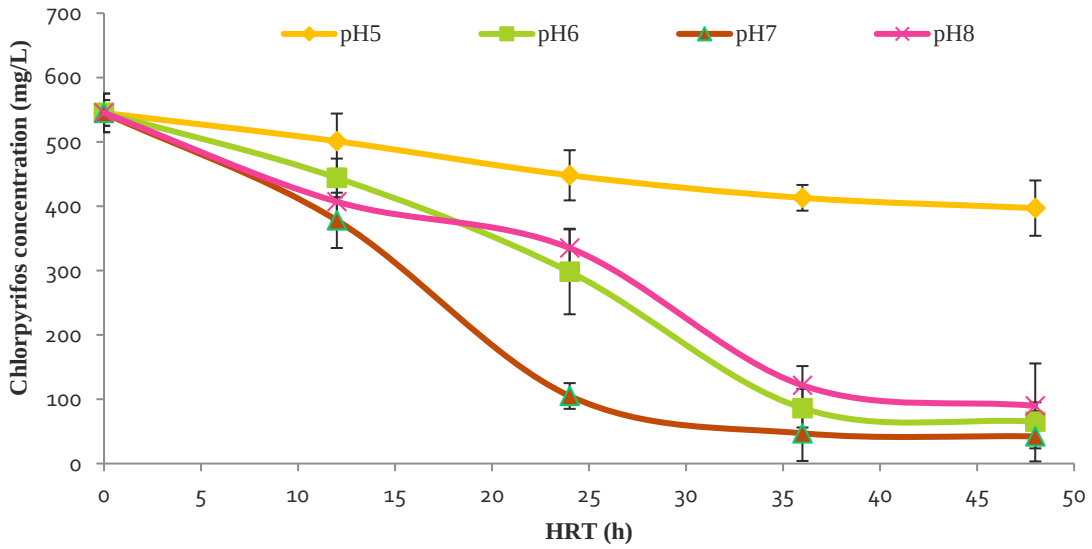


Figure 4.14: Biodegradation of CP in WW at different pH (5, 6, 7, 8) using bacterial consortium as seeding inoculum (CP; 545 mg/L, HRT; 48h)

Effect of ACS addition or biostimulation on CP biodegradation in real industrial wastewater

Influents of wastewater treatment plants usually have large concentration of ACS than pesticides (Koeck-Schulmeyer *et al*., 2013). Presence of ACS may affect biodegradation process. Easily biodegradable ACS were added in the medium to investigate their effect in large concentration on CP biodegradation. For this experiment glucose, sucrose and yeast extract were used at the rate of 2 g/L. With glucose addition reduction in lag phase was observed which accelerated over

all degradation process and 96% CP removal was achieved at HRT 24 h. Glucose addition has accelerated biodegradation of CP. In contrast medium without glucose addition has achieved only 92% degradation in double time (48 h). With sucrose and yeast extract 84 and 78.3% removal was observed after 24 h (Figure 4.15). Possible reason for this may be enhanced bacterial growth with availability of easily degradable carbon substrate, reduction in lag phase and large population. Multiple carbon substrates in medium may help in survival of microbial population and relatively sustainable population could be achieved.

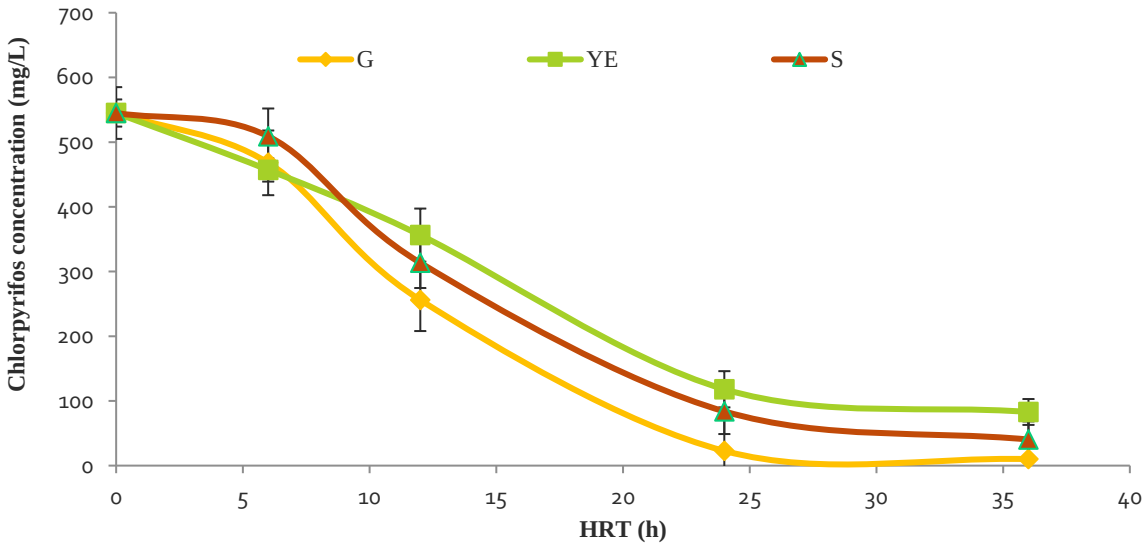


Figure 4.15: Biodegradation of CP in WW with addition of assimilable carbon substrates i.e. glucose, yeast extract, sucrose (each 2000 mg/L) using bacterial consortium as seeding inoculum. (CP 545 mg/L; pH 7; HRT 36 h)

CP degradation in the presence of glucose, sucrose and yeast extract revealed that CP degrading enzymes were expressed even in the presence of other carbon substrates. Pino and Penuela, (2011) reported enhanced biodegradation of CP and methyl parathion in the presence of glucose. This is because glucose presence increased microbial population (Qiu *et al*., 2007). Higher concentration of cosubstrates may produce negative impact by enhanced dependence of microbes

on cosubstrate which may result in accumulation of toxic metabolites (Pino and Penuela, 2011). Therefore utilization of optimized concentration of cosubstrates to enhance biodegradation of CP in contaminated environment is suggested. Addition of yeast extract and peptone resulted in complete degradation of DDT as compared to control (Bidlan and Manonmani *et al.,* 2002). Similar results were reported elsewhere where yeast extract and glucose served as supportive cosubstrates in synthetic medium (Anwar *et al*., 2009).

4.3.4. Batch Operation for CP Biodegradation, Mineralization and Dechlorination at Optimized Conditions

In order to investigate the kinetics of CP degradation, generation of metabolites, water soluble products and fluctuation in pH experiment was performed.

CP Biodegradation and Mineralization

Initial concentration of CP was reduced to 19.6 mg/L after 24 h HRT which means almost 97% removal (Figure 4.16a). In order to investigate that biodegradation is because of adsorption or assimilation; sludge samples were collected and analyzed. In start 24 mg/L adsorption was observed on sludge sample. With the passage of time it was released again into the medium and at the end of cycle 2 mg/L CP was found adsorbed on sludge. Efficient biodegradation was observed using microbial consortium in WW because consortia degrade contaminant more efficiently because of competition between microbes for substrate and reduction in growth inhibition of specific microbial population (Pino and Penuela, 2011). These consortia are usually obtained from contaminated sites where microbes are exposed to harsh environmental conditions. Exposure to harsh environmental conditions for longer period makes them more tolerant towards toxic compounds (Krishna and Ligy, 2008).

Initial pH was 7 but slight fluctuation was observed from low pH of 6.94 to high 7.12 (Figure 4.16 a). As observed in pH experiments, consortium was able to perform degradation activity in this range. Glucose breakdown results in formation of organic acids; this may leads to a pH drop (Cycon *et al*., 2009).

CP contains aromatic ring in its structure. Catalytic cleavage of aromatic ring involves various types of enzymes (Vaillancourt *et al*., 2006). OPH and/or OPAA enzymes are involved in biodegradation of CP (Supreeth and Raju, 2017). Metabolites like TCP, TMP and DETP were detected. TCP and DETP are produced when microbes follow different pathway for degradation (Figure 4.16b). Concentration of TCP at 3 h was 14.3 mg/L and increased to maximum 72.6 mg/L at 12 h however at the end of cycle 9.3 mg/L was detected. TCP contains halogen in its structure; this is responsible for its toxicity. Halogen group is either cleaved or transformed to obtain metabolites for further processing in central metabolism (Zhang *et al*., 2013, Vesela *et al*., 2012). After catalytic cleavage of TCP, a secondary metabolite TMP is formed. Concentration of TMP at 6 h was 3.8 mg/L and increased to maximum 22 mg/L at 12 h at the end of cycle 4 mg/L was detected. Presence of 44.5 mg/L DETP at 9 h confirms the microbe present in the medium follow different degradation pathways. At the end of cycle 5.98 mg/L DETP was detected. Some microorganisms can use CP and its primary metabolite TCP at the same time, which revealed their potential for reclamation of environment polluted with CP and TCP (Kim and Ahn, 2009; Briceno *et al*., 2012). In current study an increase in concentration of all metabolites was observed followed by a drop in their concentration; this indicates metabolic capability of bacterial community toward these metabolites.

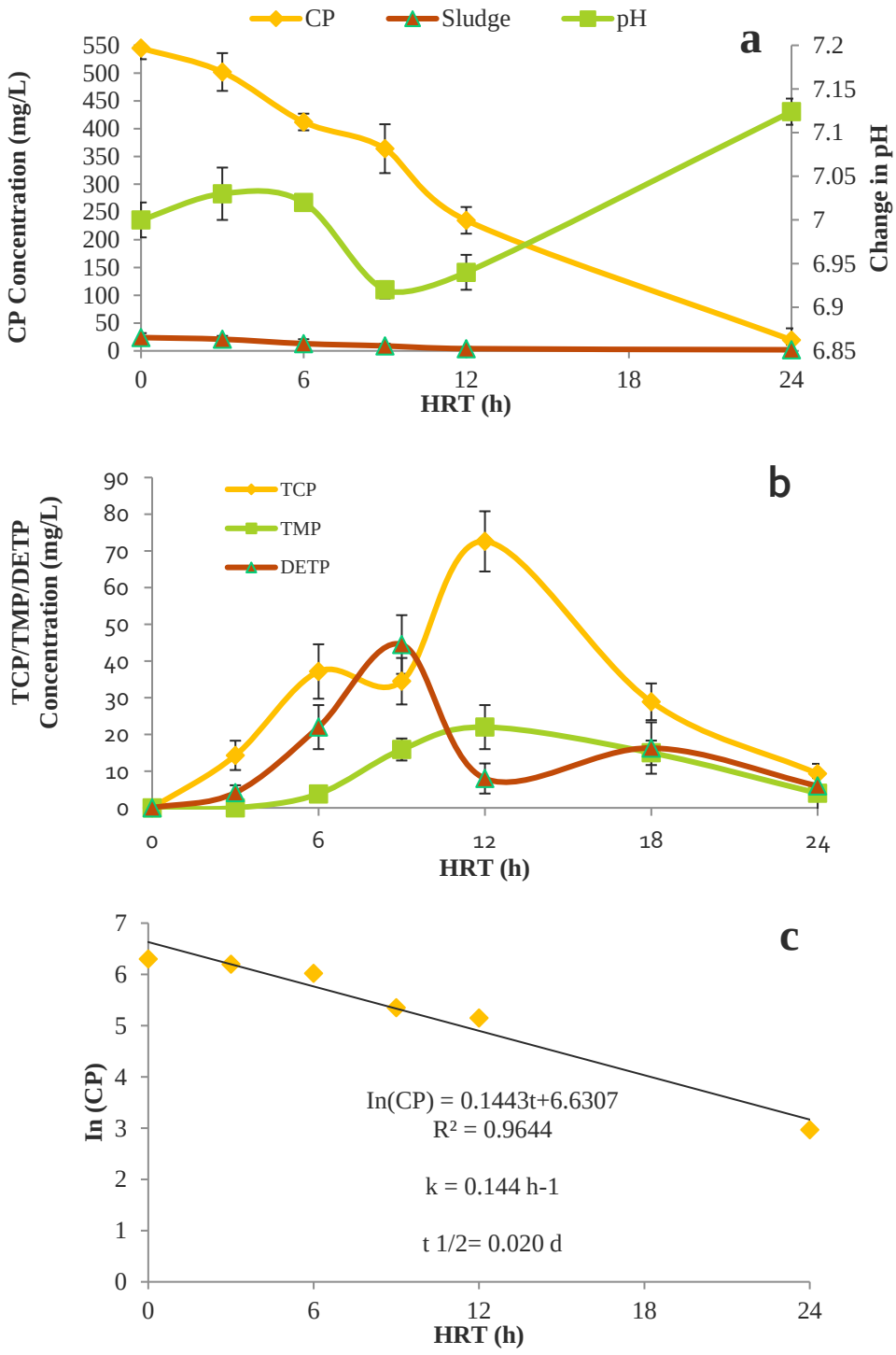


Figure 4.16: Biodegradation of CP in WW (a) CP concentration and pH (b) concentration of metabolites (c) change in In(CP) with time (CP; 545 mg/L, Ph; 7, HRT; 24 h, Glucose; 2 g/L)

Data from these observations was used for kinetic calculations.

Using hyperbolic saturation function degradation may be expressed as equation 4

$$\boldsymbol{R = \frac{R_m .C}{k+C}} \qquad (10)$$

First order and zero order rates constant may be expressed as (equation 4 and 5) (Yang *et al*., 2014)

$$\boldsymbol{k1=Rm/k} \qquad (11)$$

$$\boldsymbol{k_o = Rm} \qquad (12)$$

Figure 4 (e) presents regression coefficient for CP biodegradation is 0.96 this confirms first order model fit to experimental data. From slope of straight line regression equation first order rate constant was calculated. First order rate constant k(h^{-1}) for CP biodegradation was 0.144. This indicates fast degradation. CP is recalcitrant to biodegradation and has long half-life. Another study reported rate constant for CP degradation 1.364 d^{-1} (Silambarasan and Abraham, 2013). Moscoso *et al*., (2013) observed depletion rate of 0.054 h^{-1} for CP using *Pseudomonas stutzeri.*

With addition of CP degrading bacterial consortium at pH 7, HRT 24 h and glucose as cosubstrate half-life of CP was reduced to 0.02 days (Figure 4.16c). Obtained results have demonstrated faster degradation of CP. CP was efficiently degraded in short period of time in WW inoculated with indigenous microbial community and isolate bacterial consortium.

COD, Phosphate and Nitrate removal

As WW was used in this experiment without any alteration number of pesticides, their residues, intermediate compounds and organic solvents could be present. Chemical oxygen demand could be used as an indicator of overall degradation process. Initial COD 8000 mg/L was reduced to 74% of its initial concentration at the end of cycle (Figure 4.17). This indicated that microbes have ability to mineralize most of organic carbon compounds present in WW. Further concentration of nitrate nitrate (NO_3^-—N) and phosphorous (PO_4^{+3}—P) was also evaluated

initially and after completion of process. Results revealed 68.2% and 65% removal was achieved for phosphates and nitrates respectively (Figure 4.17). Decrease in concentration of phosphate and nitrate is an indicator of ring breakage (Tiwari and Guha 2014).

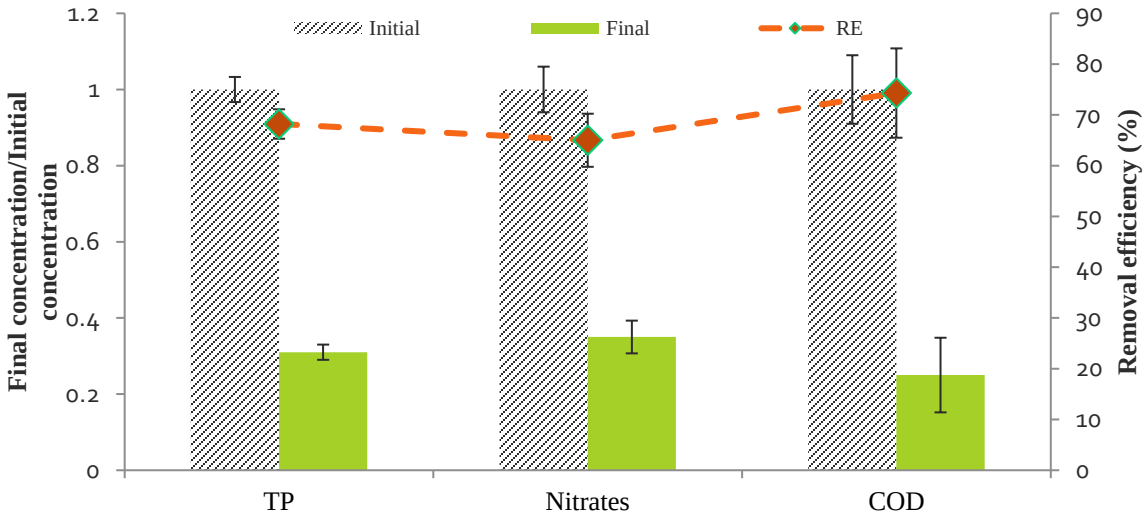


Figure 4.17: Initial/final concentration and removal efficiency of nitrate (NO_3^--N) and phosphate (PO_4^{+3}-P) and chemical oxygen demand during biodegradation of CP in WW (CP; 545 mg/L, pH; 7, HRT; 24 h, Glucose; 2 g/L)

Dechlorination

Concentration of chloride ions was monitored throughout the batch process. As it was WW, initial chloride ion concentration was 1053 mg/L (Figure 4.18). With gradual increase in concentration a maximum of 1252 mg/L was observed at 12 h. A drop in concentration was observed after 18 h. A gradual increase in chloride ion concentration means dechlorination of CP. Primary metabolite of CP has three chlorine atoms in its ring (Supreeth and Raju, 2017). Toxic nature of halogenated compounds is mainly because of halogen group present and dehalogenase is responsible for release of chloride ions. These chlorine atoms are toxic for microorganisms and they inhibit further degradation (Singh and Walker 2006; Supreeth *et al*., 2016). In case of CP, chlorine is responsible for its toxic nature. Release of chloride ion is an

indicator of dechlorination step (ring breakage), this means decrease in toxicity (Camacho-Perez *et al*., 2012, Saez *et al*., 2015).

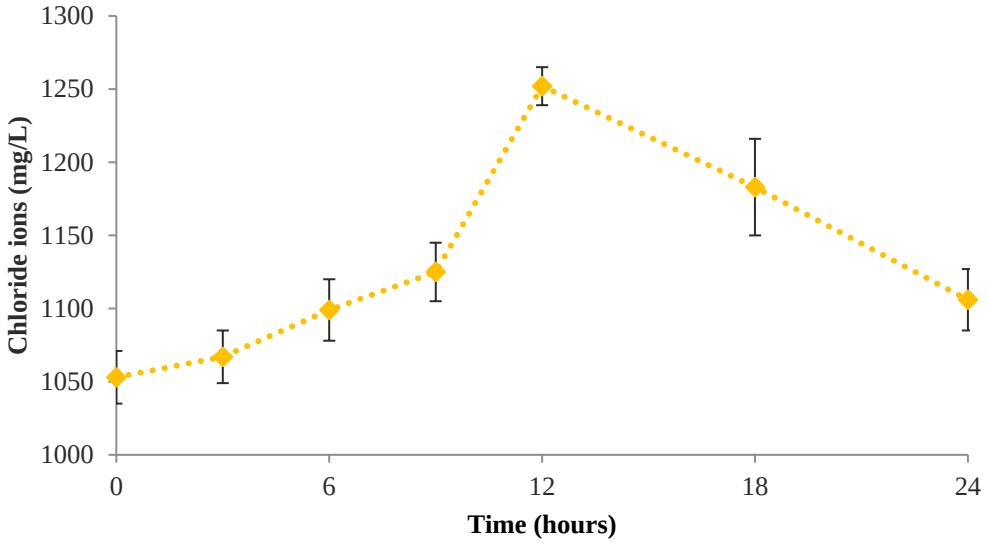


Figure 4.18: Change in concentration of chloride ions during CP biodegradation in WW (CP 545 mg/L; pH 7; HRT 24 h; Glucose 2 g/L)

Phytotoxicity assay

Phytotoxicity assay is a promising tool to investigate toxicity level of bioremediated effluents. For evaluation of phytotoxic effects seeds of *Triricum aestivum* were selected. Germination % of seeds irrigated with tap water (TI) bioremediated effluents (TII), abiotic control (TIII) was 95, 80 and 65% respectively. Maximum root and shoot length was observed for tap water i.e. 8.12 and 11.2 cm respectively. Root and shoot length with treatment II were 6.26 and 9.73 cm respectively (Figure 4.19). With treatment III root and shoot length were 2.2 and 4.21 cm. Seed vigour is indicator of overall plant health. Vigour index of treated effluents (127.9) was higher than untreated effluents (41.7) (Figure 4.19). Bergero and Lucchesi, (2015) reported 43% and 75% germination of *Lactuca sativa* seeds in untreated and biologically treated effluents respectively. With untreated effluents germination percentage and root elongation of *L. sative* was adversely affected. Saez *et al*. (2014) observed that seeds of *L. sativa* were adversely

affected by untreated effluents in term of germination, root and hypocotyl length in slurry contaminated with lindane as compared to uncontaminated slurry. Lower germination could be result of imbalance in biochemical process because of poisonous pesticides (Bidlan *et al*., 2004; Fuentes *et al*., 2013). Growth of *T. aestivum* was inhibited when irrigated with untreated effluent; this is an indicator of CP toxicity towards *T. aestivum.* It is evident from data that toxicity of bioremediated effluents was significantly reduced. Results suggested suitability of bacterial consortium for bioremediation of industrial effluents.

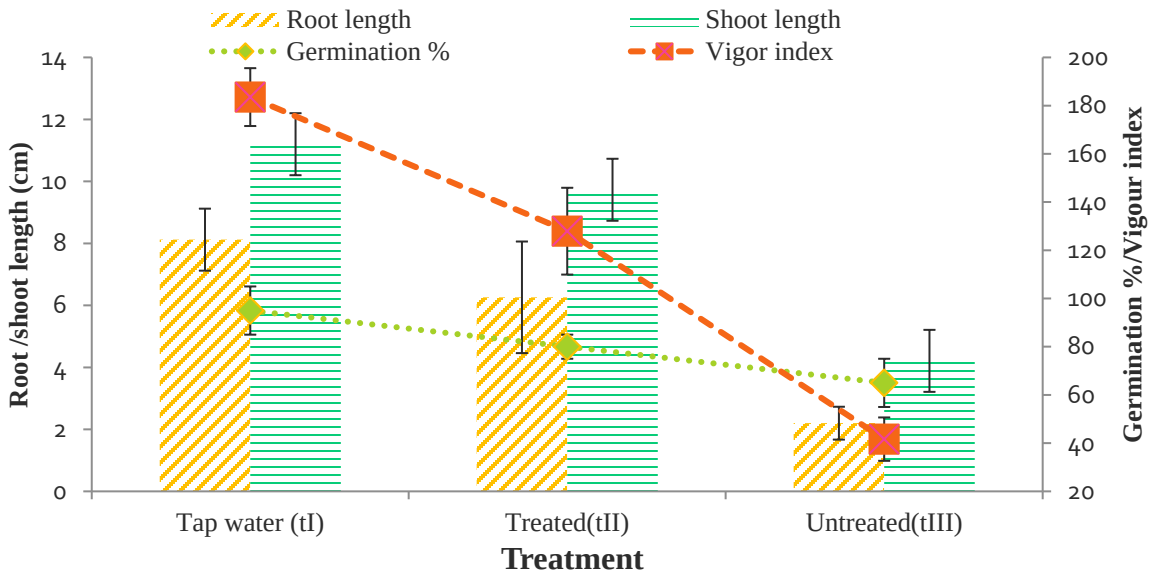


Figure 4.19: Growth of triticum aestivum seeds after treatment with tap water (TI), treated effluents (TII), abiotic control (TIII), root and shoot length (primary y-axis), germination % and vigor index (secondary y-axis).

PHASE III

4.4. BIODEGRADATION OF CHLORPYRIFOS USING IMMOBILIZED BACTERIA

Separation of cells for reuse has been a major problem associated with the free cells. Freely suspended cells used in the biodegradation studies have some disadvantages such as cell washout, stability loss and non-reusability making biotreatment costly (Kourkoutas *et al*., 2004; Chen *et al*., 2013). Thus there is a need to investigate methods that governs bacterial application more cost-effective and durable.

4.4.1. Effect of Different Factors on Residual Activity of Calcium Alginate Microsphere

Effect of sodium alginate concentration

Experiments were performed to estimate the effect of sodium alginate concentration (1- 4 g/100 ml) on residual activity of MCs in terms of CP biodegradation in MSM. Figure 4.20 (a) represents residual activity of MCs with different concentration of sodium alginate after 12 h of incubation at 35˚C. MCs formed with 1 g/100ml of sodium alginate has shown least residual activity and initial CP concentration decreased to 70 % after 12 h of incubation. It was followed by 1.5 and 2 g/100ml where residual activity in terms of CP biodegradation was 84.5 and 92% respectively. Highest residual activity (99.8%) was observed at 2.5 g/100 ml sodium alginate concentration. With further increase in concentration of sodium alginate to 4 % residual activity decreased to 53%. Sodium alginate concentration of 2.5 g/100ml was selected as optimum for MCs formation. At low concentration bacteria have enough space to get diffused nutrients. In contrast at high concentration nutrient diffusion become slow and difficult, so bacteria may not get enough substrate to degrade (Idris and Suzana, 2006). This may be the reason for decrease in

CP degradation efficiency. Lin *et al*. (2013) reported similar results where higher concentration of sodium alginate resulted in decreased biodegradation of TNT by *Bacillus mycoides.* Higher concentration result in lower pore size leading to lower immobilization efficiency. Increase in concentration results in increased viscosity which makes MCs formation difficult. At very low concentration crosslinking will be loosely bound and this results in formation of fragile cell beads (Daassi *et al*., 2014).

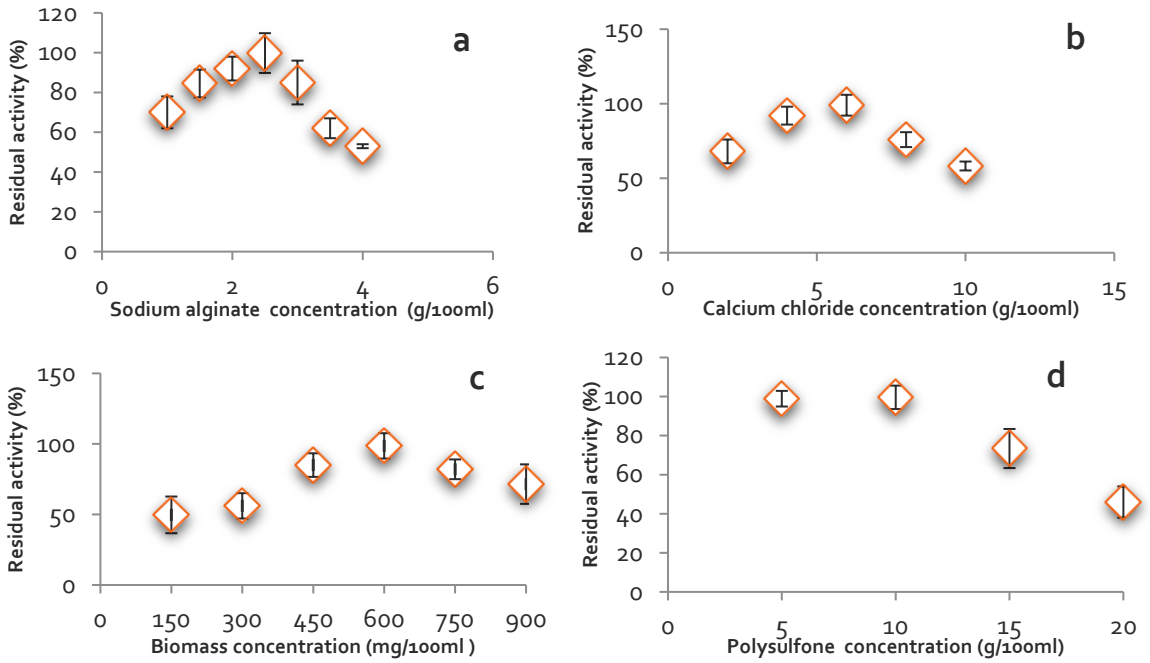


Figure 4.20: Effect of different concentrations of (a) sodium alginate (b) calcium chloride (c) biomass concentration (d) polysulfone on residual activity of MCs

Effect of calcium chloride concentration

$CaCl_2$ is used as cross-linking agent to start gel formation for MCs. Experiments were performed to estimate the effect of cross linking agent $CaCl_2$ concentration (2-10% w/v) on residual activity of MCs in terms of CP biodegradation and results are presented in Figure 4.20 (b). With 2 g/100ml calcium chloride residual activity was 68 %. With the increase in concentration an increase in residual activity was observed upto 6 g/100ml of $CaCl_2$. Maximum (99 %) CP

removal potential of bacterial consortium was observed at 6 % of $CaCl_2$. With further increase in concentration (10 %) a gradual decrease in residual activity to 58 % was noticed. Above and below this concentration residual activity in terms of CP biodegradation was significantly decreased. Criteria for selection suitable cell immobilization method include mechanical strength (Sarma and Pakshirajan, 2011). Type and concentration of cross linking agent effect stability and activity of immobilized cells. However concentration of sodium alginate plays more important role than $CaCl_2$ concentration. Gelling process starts from outer towards inner core of microspheres. At low concentration enough cations were not present to diffuse inside core and initiate cross linking in inner core, which results in fragile structure. Diffusion of substrate was slightly affected by $CaCl_2$ concentration upto 6 g/100ml. *Pseudomonas oleovorans* entrapped in calcium alginate matrix has shown higher removal percentage for xenobiotic than PVA and polyacrylamide (Chen *et al*., 2013), this could be attributed to toxicity level and increased tightness (Chen *et al*., 2008; Ha *et al*., 2009).

Effect of biomass concentration

The effect of biomass concentration (150 to 900 mg/100ml) on residual activity of MCs in terms of CP removal potential was evaluated. MSM broth supplemented with CP was inoculated with MCs. Figure 4.20(c) represents residual activity by different MCs when incubated at 35˚C for 12 h. MCs formed with 150, 300 and 450 mg/100ml of sodium alginate has shown 49.7, 56 and 85 % residual activity (Figure 4.20c). MCs formed with 600 mg/100ml of biomass concentration has obtained 98.6 % removal of initial CP concentration (Figure 4.20c). Above this concentration a gradual decrease in residual activity was noticed. Least residual activity (71%) was observed with MCs formed at 900 mg/100ml of biomass concentration. A sharp decrease in biodegradation was observed with further increase in biomass concentration after 600 mg/100

ml. Siripattanakul *et al*., (2009) reported less atrazine removal at biomass loading of 300mg and higher removal at 600 mg, but there was no significant difference with further increase in biomass to 900 mg. Lin *et al*. (2013) observed similar trend in biodegradation of TNT with increase in biomass concentration beyond 10% (v/v). Possible reason for this could be decrease in porosity and surface area because cells occupy pore spaces (Lin *et al*., 2013; Tsai *et al*., 2013). Therefore less biomass (600 mg/100 ml) provided more porosity and greater surface area to carry out biodegradation. With high biomass concentration mechanical strength also decreased (Rodger and Bruce, 2001).

Effect of polysulfone concentration

Concentration ranging from 5, 10 and 15g/100ml (w/v%) polysulfone solution in N-methyl-2-pyrrolidone (NMP) was used for coating of CAMs, to study their effect on CP removal CAMs were inoculated into MSM broth supplemented with CP and incubated for 12 h at 35˚C. At 5% Polysulfone (PSf) concentration 98% residual activity in terms of CP removal was observed after 12 h. With the increase in concentration from 5 to 10% change in residual activity was negligible (Figure 4.20d). With further increase in concentration of PSf to 15% a sharp decline was observed in residual activity to 73% (Figure 4.20d). PSf concentration of 10% was selected as optimal for coating of MC to form macrocapsules. Hence our result demonstrated 10% PSf concentration was suitable for polymeric coating. Criteria for selection suitable cell immobilization method include mechanical strength and biological activity (Sarma and Pakshirajan, 2011). Kim *et al*., (2015) reported mechanical strength of calcium alginate bead with polysulfone coating was higher (1.195 mJ) than without coating (0.73 mJ). 10% PSf improves mechanical stability to the level that it allows sufficient biodegradation as well. Higher concentration of polymers may result in more compact structure which hinders diffusion of

substrate and other essential nutrients into matrix (Muhammad and Bustard, 2008; Chen *et al*., 2008). Thickness and stability of pore walls increased when PSf concentration increased but number and size of pores decreased. Other reason could be less cell viability at high polymer concentration (Cheetham *et al*., 1979, Ha *et al*., 2009).

4.4.2. Characterization of Calcium Alginate Microsphere and Macrocapsules

Physical stability

Figure 4.21 (a) depicts residual activity in terms of CP biodegradation and cell leakage (absorbance at 600 nm) at different temperatures (-70 to 65 ˚C) for 24 h. At -70 and -20 ˚C CAMs alone and inside the macrocaspules were disintegrated. Cell leakage for MCs and CAMs was negligible at 4, 15, 25 and 35˚C. MCs have maintained their structure even at high temperature like 65˚C and negligible cell leakage was observed. In contrast 0.59 OD_{600nm} was observed with CAMs showing that most of cells were leaked from CAMs. From 4 to 35˚C the residual activity for both MCs and CAMs was >98 %. At 45 ˚C >76 and 91.2 % CP degradation activity was observed with CAMs and MCs respectively (Figure 4.21 a). No significant difference in residual activity was observed at 35˚C and their initial activity was >98 %. It is interesting to note that 43% biodegradation activity was observed with MCs even after incubation at high temperature (65˚C). In contrast CAMs have shown only 6% residual activity (Figure 4.21 a). It may be concluded that MCs have higher thermal stability as compared to CAMs.

Figure 4.21 (b) show stability of MCs and CAMs at acidic to basic pH range of 3 -12. MCs have maintained physical structure and cell leakage was negligible at all pH range. However, CAMs have retained most of cells inside at pH 7-9 above and below this pH considerable cell loss was

observed. At acidic pH (3, 4) and basic pH (10, 11, 12) at high cell leakage was observed with CAMs (Figure 4.21 b).

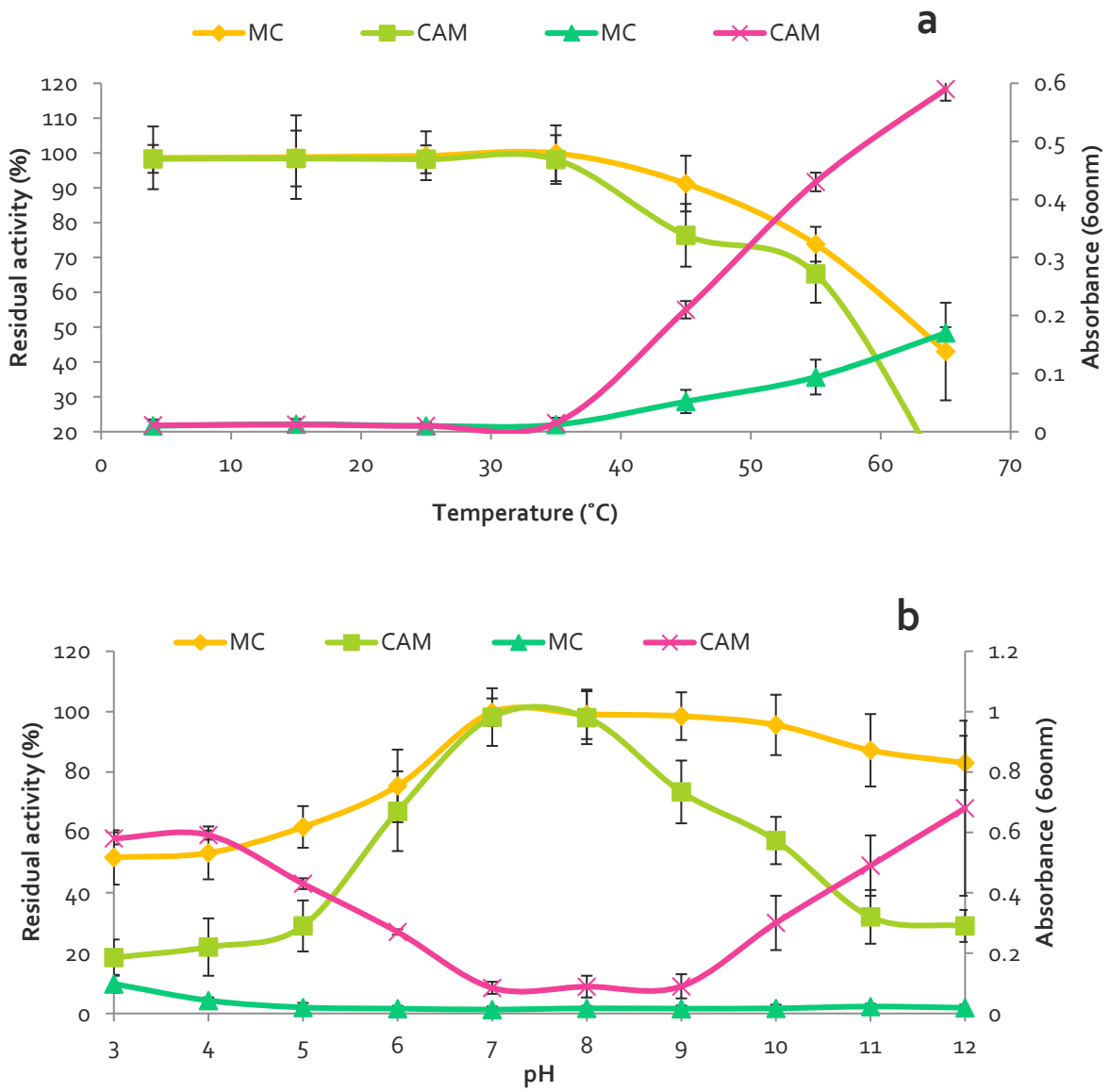


Figure 4.21: Residual activity in terms of CP biodegradation and cell leakage (absorbance at 600 nm) of MC and CAM (a) at different temperatures (-70 to 65˚C) for 24 h (b) at acidic to basic pH range of 3 -12.

MCs have maintained more than 50 % of residual activity at pH 3-5 and more than 95% of residual activity at pH 7-10. In contrast CAMs have maintained >50% degradation in pH range of 6-10(Figure 4.21 b). Above pH 10 and below pH 6 CP biodegradation activity was not much

appreciable with CAMs. Results infer that MCs were more stable at wide pH range than CAMs. According to Covarrubias et al. (2011), entrapment in alginate matrix provides sufficient physical protection to microbes against harsh environmental conditions of wastewater. Our results are in agreement with above description; however polymeric coating with polysulfone (MCs) is a step further in protection against physical harms.

Chemical stability

Initially both formulation (MCs and CAMs) produced equal amount of biomass and have similar biodegradation potential. Chemical stability of MCs and CAMs was investigated by incubating them in harsh conditions. A gradual increase in absorbance (OD_{600}) was observed in suspension with CAMs throughout incubation period.

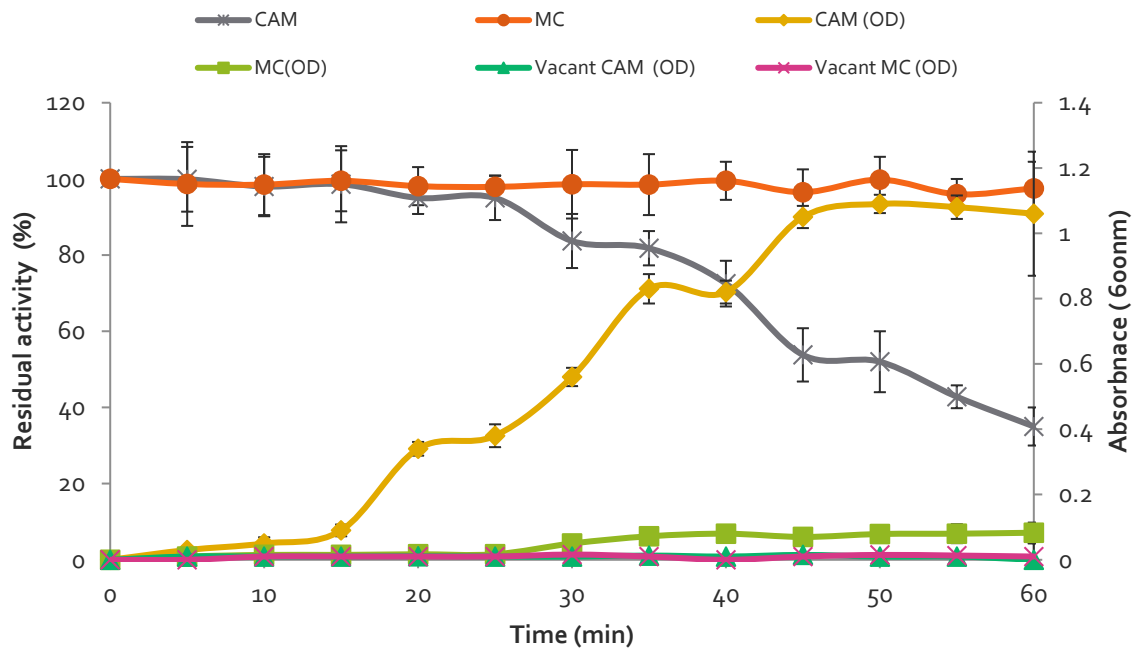


Figure 4.22: Residual activity in terms of CP biodegradation and cell leakage (absorbance at 600nm) of MCs, CAMs with bacterial consortium and vacant MCs and CAMs in chemically harsh environment upto 60 min

Sharp decrease was observed after 30 min when CAMs were used for CP removal. Initially absorbance was 0.03 and after completion of period absorbance was 1.06 after 60 min of incubation. Suspension with MCs has not shown any considerable absorbance upto 60 min. Results of current study are in agreement with Kim *et al*. (2015). Possible cell leakage from CAMs could be results of disruption of alginate matrix in harsh environment. Contrary to this membrane layer has saved alginate matrix and cell leakage from MCs (Kim *et al*., 2015). Residual activity after chemical treatment was also observed. MCs have maintained more than 97% of their initial activity even after incubation for 60 min. In contrast CAMs have lost their residual activity because of cell leakage and only 35% degradation was observed after 60 min of incubation.

Reusability

One of the merits associated with use of immobilized bacteria is separation from medium at the end of process this facilitates reuse and reduces cost of process. In order to evaluate/ compare reusability of MCs versus CAMs were used repeatedly for biodegradation experiments in MSM and results are presented in Figure 4.23. Freshly prepared MCs and CAMs obtained 98% CP removal in first cycle. An increase in CP removal upto 5 cycles using MCs. Results showed MCs could be reused for 9 cycles without any change in degradation efficiency (~99.5%) . Contrary to this, CAMs maintained 92% degradation efficiency upto 6 cycles after this a gradual decrease in degradation efficiency was observed. 91.7 % and 48 % CP removal efficiency with MCs and CAMs was obtained at 15^{th} cycle. Results indicate polysulfone layer has provided sufficient protection against all these factors. With the passage of time reduction in biodegradation ability was observed possibly because of mechanical strength, loss of viability (viable cells), enzyme inactivation, pore blocking with substrate (Parsad and Suresh, 2015; Daassi *et al*., 2014).

Patil (2006) reported loss of biodegradation capability after repeated use of sodium alginate CAMs. Parasad and Suresh (2015), reported >97% biodegradation of DMP with freshly entrapped cells it was reduced to 75 and 63% by 9th and 12th cycle. Tallur *et al*., (2009) also presented similar results. In current study 48% CP removal was observed after 15th cycle with CAMs, contrary to this MCs maintained much higher removal percentage (~ 92%). Results strongly inferred suitability of MCs for long term use for biodegradation of CP as they are mechanically strong, retains cells, saves time and decreases cost of operation.

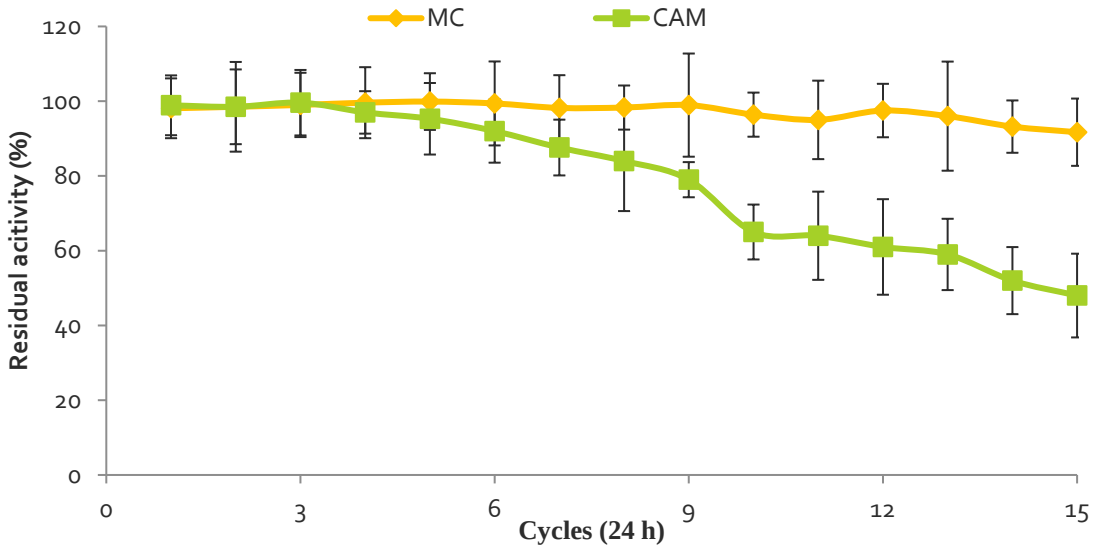


Figure 4.23: Residual activity in terms of CP biodegradation with MCs and CAMs for repeated cycles (initial CP 600 mg/L; Incubation time 24 h; pH: 7; cycles 15)

Storage Stability and Shelf life evaluation

Storage stability is factor that plays most important role in determination of suitability of material for immobilization of bacteria for practical use (Wu *et al*., 2009). The data on shelf life evaluation of MCs and CAMs is presented in Figure 4.24. MCs exhibited most consistent performance in terms of biodegradation of CP. Efficiency of CAMs and MCs was 13 and 87.5 % in terms of CP biodegradation after 20 weeks of storage (Figure 4.24). CAMs maintained more than74% efficiency upto 12 weeks, after this a sharp decline was observed and 13% CP

biodegradation activity was observed after 20 weeks. Contrary to this MCs removed more than 88 % CP even after 20 weeks of storage at 4˚C. In contrast CAMs have lost biodegradation activity completely. Ability of MCs to maintain residual activity after storage for longer period of time makes it suitable and cost effective for industrial use. Lin *et al*. (2013) compared storage stability of free cells and entrapped cells and observed complete loss of biodegradation activity after 14 days, contrary to these entrapped cells have maintained their activity at 91.3% even after 42 days. Above results indicate that nature of material plays an important role in determining the suitability of material for immobilization of bacteria. Further to facilitate commercial use of these products effect of temperature, pH, possible harsh environment was also evaluated. MCs have shown more potential for application in terms of thermal stability, chemical stability, storage and reusability.

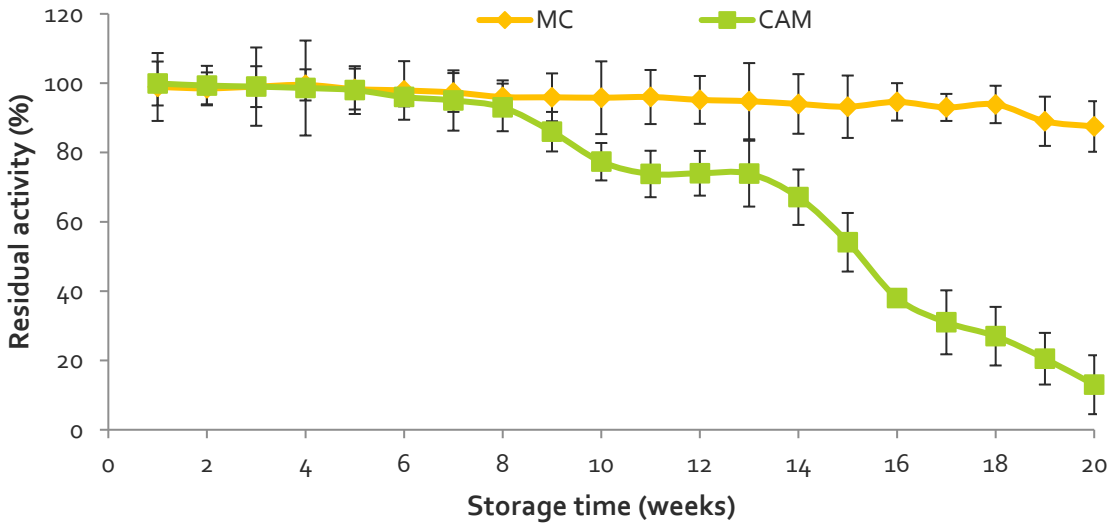


Figure 4.24: Residual activity in terms of CP biodegradation of MCs and CAMs after several weeks of storage (initial CP 600 mg/L; incubation time 24 h; pH 7; cycles 15)

Scanning electron microscopy

Photographs, microscopic images and scanning electron micrograph were used to study morphology of CAMs and MCs.

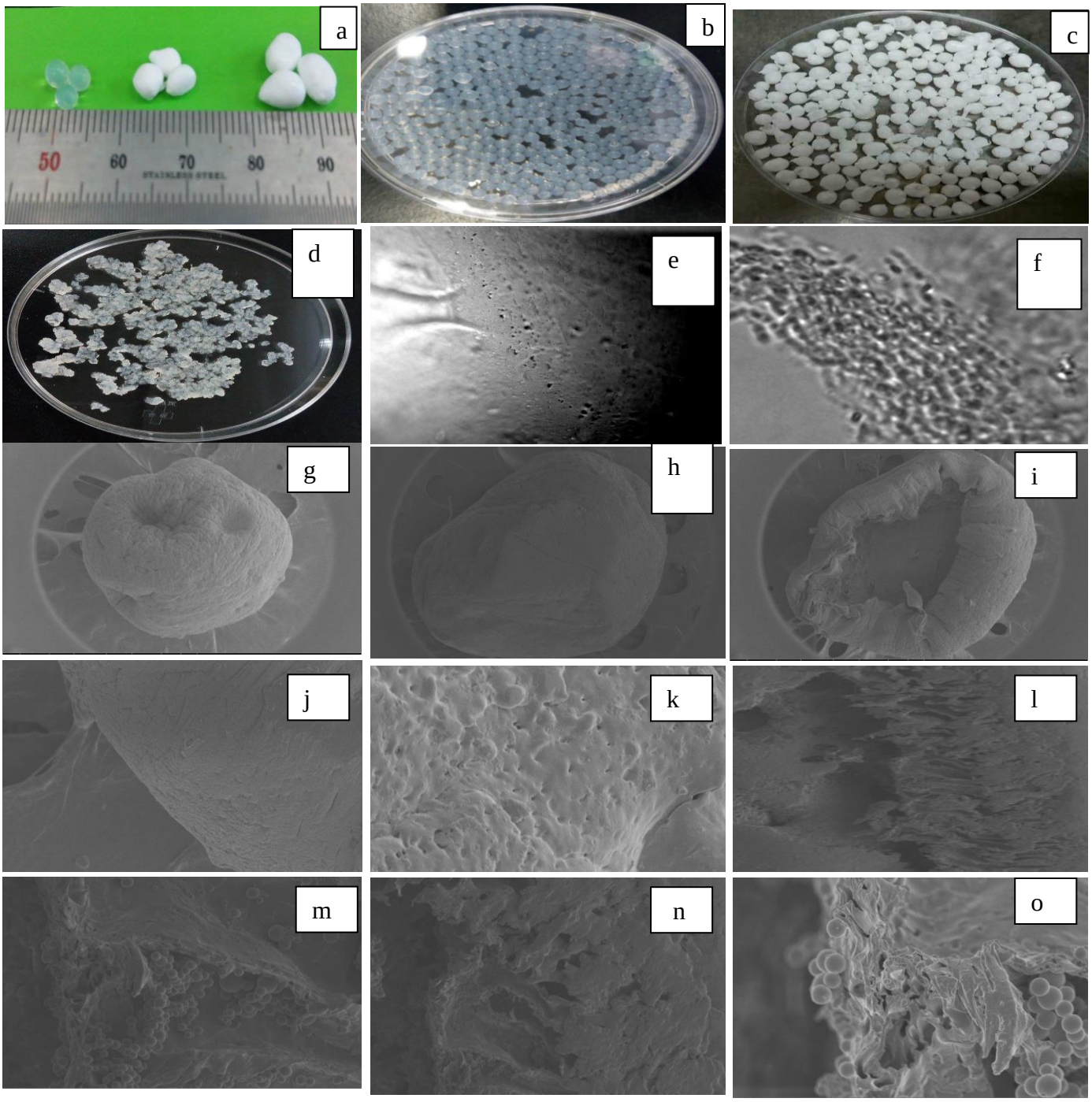


Figure 4.25: (a, b, c) present CAMs, MCs with single coating, MCs with double membrane coating, photographs of calcium alginate microspheres and macrocapsules (d) disintegrated CAMs (e) microscopic image of MCs (f) microscopic image of bacterial cells

Figure 4.26 shows distribution of bacterial consortium inside immobilized in gel matrix. SEM images clearly showed inner and cross section of MCs and CAMs. Figure 4.26 (e, g, h, j) shows rough and microporous outer surface of MCs and CAMs. Porous on outer and inner surface are

also clearly visible. Pores are convenient for transport of nutrient substances into gel matrix. Porous membrane layer and cross-sectional area is clearly visible as shown in Figure 4.26 (i, k, l), this surface is suitable for immobilization of bacteria. Bacterial cells inside CAMs and macrocapsules are apparent in SEM image (Figure 4.26 m, n, o). Microscopic images and SEM image showed presence of rod shaped and round bacterial cells were immobilized inside gel matrix. Cross sectional area is showing finger-like structure that is asymmetric which could be attributed to spherical shape instead of flat sheet. These mages confirmed immobilization of bacteria in CAMs and MCs.

4.4.3. BIODEGRADATION OF CP USING MACROCAPSULES

Biodegradation of CP

CP concentration (100-600 mg/L) was used to investigate biodegradation potential of bacterial consortium (SRK1+SRK2+SRK4+SRK5+T13) after encapsulation/immobilization in macrocapsules (calcium alginate microspheres coated with polysulfone). MSM amended with CP was inoculated with MCs. Figure 4.27 shows CP removal efficiency and time required for its complete degradation. When initial CP concentration was low (300 mg/L) complete removal was achieved within 12 h. However, with further increase in concentration time required for complete CP removal was prolonged. From 400 to 600 mg/L more than 98 % removal was achieved within 18 h. With further increase in concentration biodegradation slowed down and 38% CP removal was observed after 24 h. Prasad and Suresh, (2015) compared performance of free cells and entrapped cells for biodegradation of dimethyl phalate ester (300 mg/L) and observed complete biodegradation in 8 h and 30 h using entrapped cells and free cells respectively. Lin *et al*. (2013) observed increase in biodegradation rate of TNT upto 60mg/L using immobilized cells, with further increase in concentration inhibited biodegradation rate.

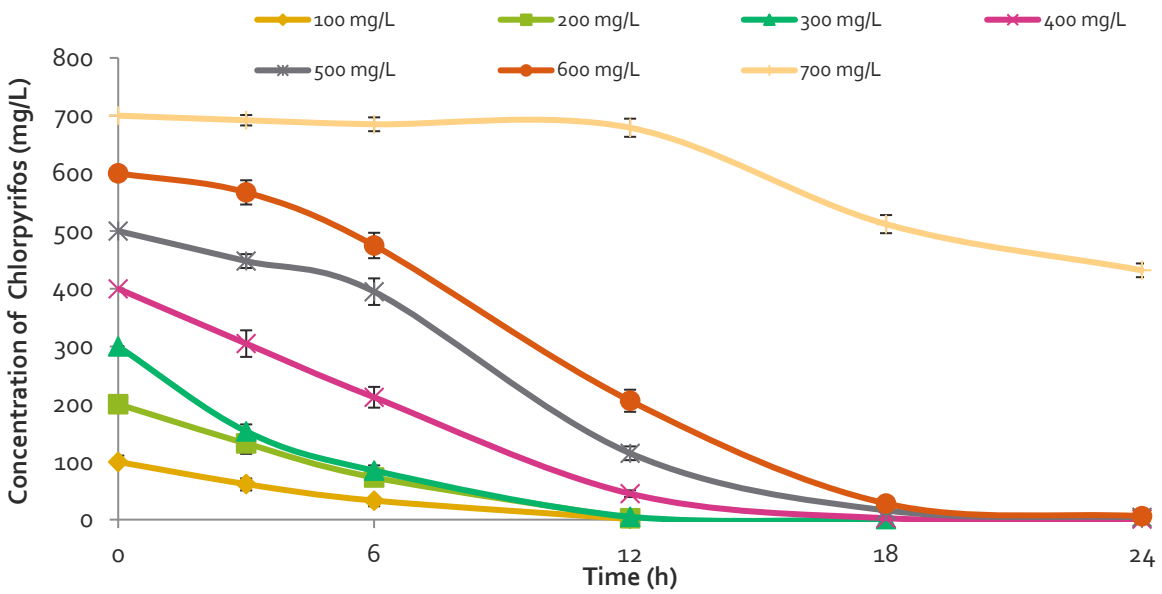


Figure 4.26: Biodegradation of CP using macrocapsules (encapsulation bacterial consortium) at varying initial CP concentration from 100 to 700 mg/L in MSM at pH 7 and incubation time 24 h.

These results showed that initial concentration of CP (contaminant) plays a vital role on overall biodegradation process this may be attributed to substrate inhibition at higher concentration (Singh *et al*.,2008). In addition to that mass transfer resistance of substrate by polymer did play a significant role (Chen *et al*., 2013). Longer lag phase of 6-7 hours was observed using free cells for biodegradation whereas lag phase was absent when entrapped cells were used (Prasad and Suresh, 2015). This phenomenon exhibits that cell immobilization is a viable method to treat high concentrations of xenobiotic, as it protects cells from shock concentrations.

GCMS and GC-ECD Analysis of metabolites

Figure 4.28 (a) represents chromatogram obtained through GCMS analysis of biodegradation products. GCMS analysis revealed presence of four peaks at 2.33, 2.67, 3.48 and 9.88 min. Molecular ion fragment pattern of these intermediate may be observed in Figure 4.28 (b, c, d, f). Molecular fragment pattern of peak observed at 9.88 min retention time was identified as CP. Peak eluted at 3.48 and 2.67 min were identified as 3, 5, 6-trichloro-2-pyridinol (TCP) and 3, 5,

6-trichloro-2-methoxypyridine (TMP). Peak detected at 2.33 min retention time was identified as diethyl-thiophosphate (DETP). Based on analysis of GCMS chromatogram a metabolic pathway could be postulated. CP was metabolized resulted in formation of TCP and DETP, then TCP was further degraded to TMP. TMP and DETP were degraded to water soluble products. Bacterial consortium utilized CP as a source of carbon and released TCP and DETP as metabolites. Similar CP metabolites using microbial consortium under aerobic conditions were reported by Tiwari and Guha (2014). CP degradation results in the formation of TCP and DETP (Yadav *et al*., 2015). In anaerobic conditions Metabolites of CP degradation reaction are TCP and DETP (Tiwari and Guha, 2014). CP mineralization results in the formation of primary metabolites TCP that form secondary metabolite TMP. Metabolites formed after CP degradation produces ethanol after hydrolysis that serve as a source of sulphur, inorganic phosphate and carbon (Singh and Walker, 2006). No other metabolites were observed in GCMS analysis which indicates complete mineralization of CP.

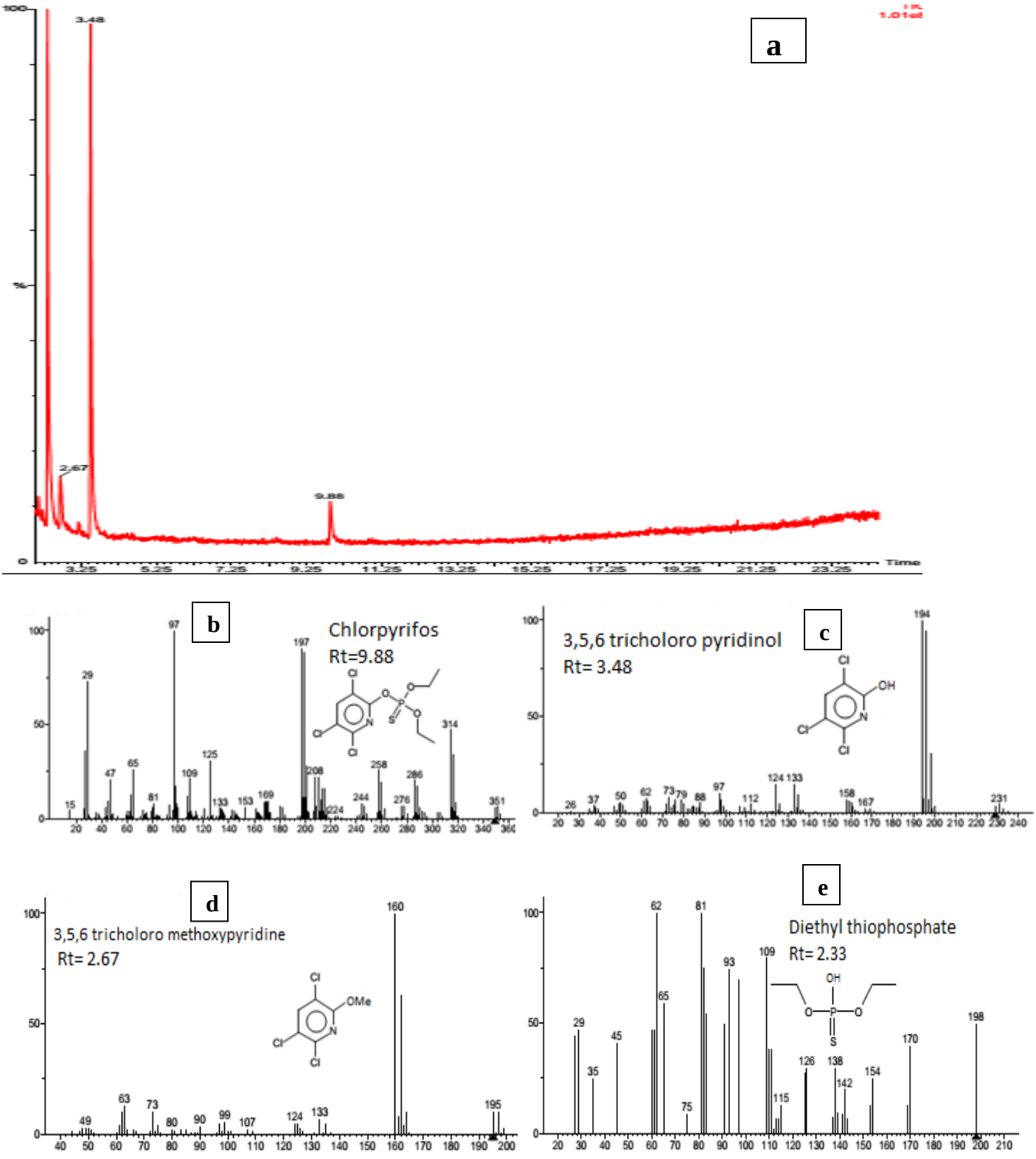


Figure 4.27: (a) GCMS chromatogram showing peaks for different metabolites and molecular ion fragmentation pattern of CP (b) and metabolites TCP (c), TMP (d), DETP (e).

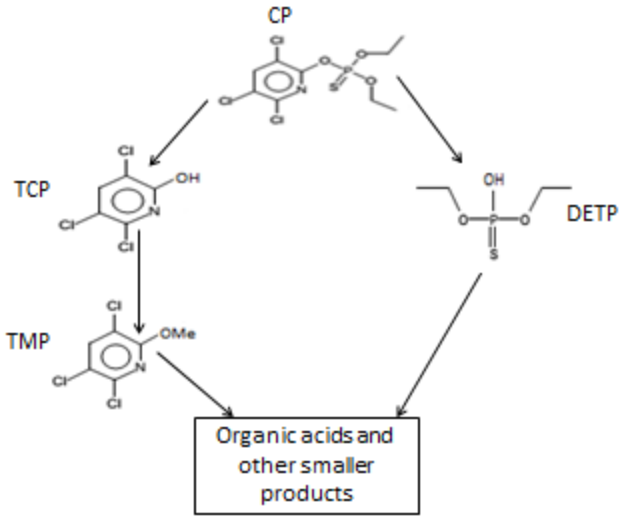


Figure 4.28: Metabolic pathway of chlorpyrifos constructed from GCMS results

After identification of metabolites using GCMS, their concentration was determined using GC-ECD. Figure 4.29 represents that initially no metabolite was observed. With the rapid decrease in concentration of CP a metabolite TCP was detected in the medium after 3 h (Figure 4.30). The concentration of TCP increased from 12.3 to 63.5 mg/L after 12 h of incubation (Figure 4.30).. Biodegradation of CP results information of toxic intermediate TCP has been reported in numerous studies (Xu *et al*., 2007; Abraham *et al*., 2014; Tiwari and Guha, 2014). Accumulation of TCP in medium may hinder growth of CP degrading microbes (Liu *et al*., 2012). In current study a gradual decrease in concentration of TCP was observed this may be attributed the fact that bacterial consortium used TCP as a source of carbon and energy to carry out cellular functions. 2.76 mg/L of DETP was detected in medium after 3 h of incubation. GC analysis revealed appearance of TMP after 6 h of incubation (Figure 4.30). After maximum concentration (29.86 mg/L) at 12 h a decline in concentration of TMP was observed. Concentrations of metabolites was increased upto certain level than a gradual decrease in their concentration was observed, this indicates capability of microbial consortium to utilize intermediate compounds formed after degradation of parent compound as a source of carbon and energy. Absence of any

peak in abiotic control indicates that degradation of CP occurred because of biological process. Catalytic cleavage of aromatic ring involves various types of enzymes (Vaillancourt *et al*., 2006). OPH and/or OPAA enzymes are involved in biodegradation of CP (Supreeth and Raju, 2017). However no other organic metabolite was detected. This indicated products of inorganic acids or other water-soluble products. Some microorganisms can use CP and its primary metabolite TCP at the same time, which revealed their potential for reclamation of environment polluted with CP and TCP (Kim and Ahn, 2009; Briceno *et al*., 2012).

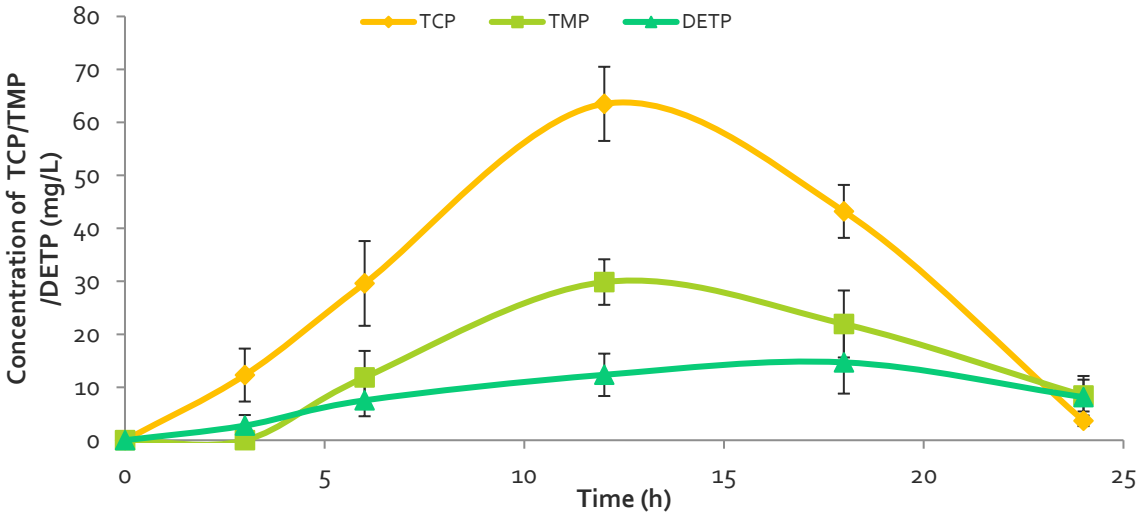


Figure 4.29: Quantification of metabolites formed after CP biodegradation using MCs through GC-ECD analysis

Chemical Oxygen Demand (COD) removal

Estimation of chemical oxygen demand (COD) is a measure of overall extent of mineralization. In order to estimate COD removal efficiency of bacterial consortium immobilized in macrocapsules experiments were performed and results are presented in Figure 4.30.

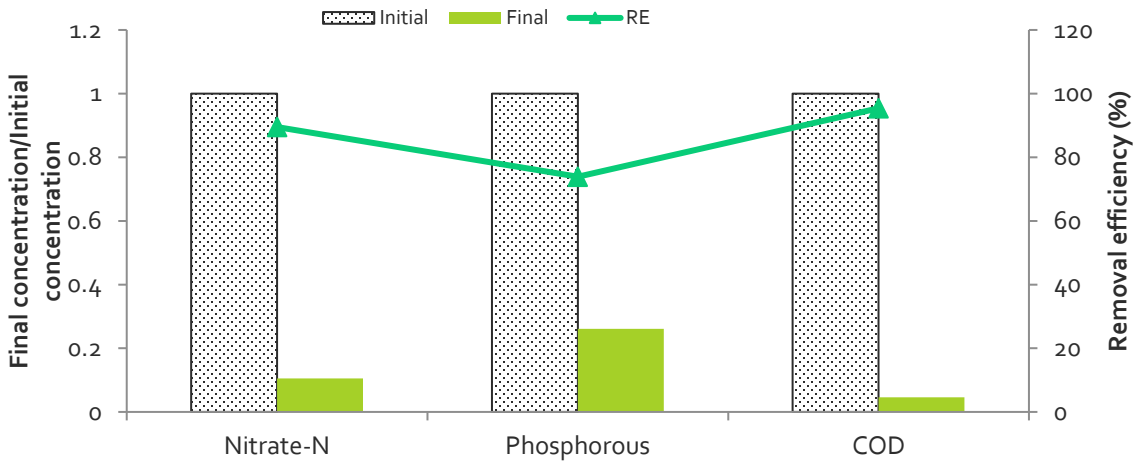


Figure 4.30: Initial/final concentration and removal efficiency of nitrate-nitrogen (NO_3^-—N), phosphate-phosphorous (PO_4^{+3}—P) and chemical oxygen demand during biodegradation of CP in MSM inoculated with MCs.

Initially COD of medium was decreased as a function of time. After 24 h of incubation COD was reduced and 95% removal efficiency was obtained. First CP and then its intermediates like TCP and TMP constitute COD. No accumulation of organic metabolites or end products was observed. Reduction in COD concentration after 24 h of treatment suggested biodegradation of CP via mineralization or conversion to bacterial biomass. These results were complementary to those obtained from GCMS and GCECD analysis where CP was degraded to its products and then a decrease in their concentration was observed with time. Results indicate that bacterial consortium has effectively degraded all organic intermediate compounds formed after the biodegradation of parent compound thereby decreasing organic load. Concentration of nitrates and phosphates was determined initially and at the end of 24 h cycle. Figure 4.30 indicates removal of nitrate and phosphate to be 89 and 73 %. Decrease in concentration of nitrate is an indicator of ring breakage. Phosphorous atom is present in side chain of chlorpyrifos. Decrease in concentration of (PO_4^{+3}—P) exhibits detachment of side chain and further degradation of diethyl thiophosphate (DETP) to water soluble products (Tiwari and Guha, 2014)

Chloride ion release after CP degradation

Primary metabolite of CP has three chlorine atoms in its ring (Supreeth and Raju, 2017). Chloride ions remain attached with ring in metabolites TCP and TMP. These halogen atoms are main cause of toxicity of chlorinated pesticides.

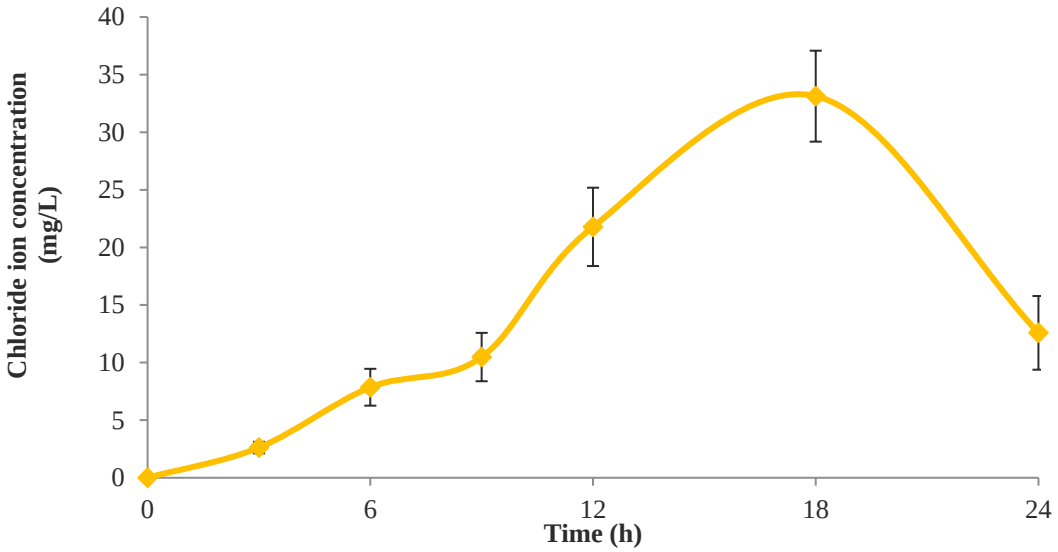


Figure 4.31: Change in concentration of chloride ions during CP biodegradation in mineral salt medium at pH7 and inoculated with MCs.

In order to evaluate extent of biodegradation and toxicity reduction release of chloride ions was determined throughout degradation period. Initially free chloride ions were not present. After 3 h of incubation 2.62 mg/L of chloride ions were detected in system. With a gradual increase in chloride ions concentration 33.12 mg/L was observed after 18 h (Figure 4.32). These chlorine atoms are toxic for microorganisms and they inhibit further degradation (Singh and Walker 2006; Supreeth *et al*., 2016). Release of free chloride ions exhibits ring breakage (Tiwari and Guha, 2014), this results in complete detoxification. Release of chloride ions occur when TMP is further degraded into water soluble products. This dehalogenation requires enzymes called dehalogenase (Camacho-Perez *et al*., 2012, Saez *et al*., 2015). These results indicated presence

of dehalogenase enzyme in bacterial consortium. Further this confirms that metabolites and other water-soluble products were not confined within immobilized matrix and were released back to the medium.

4.4.4. Biodegradation of CP in Real Wastewater Inoculated with MCs and CAMs

Most of the microbes a having capability of complete biodegradation in synthetic media, don't perform biodegradation activity when introduced into real wastewater. As real wastewater (WW) has complex composition and chemicals present other than targeted compound effect performance of microbes. In current studies microbes were protected against these environmental harms through immobilization in CAMs or MCs.

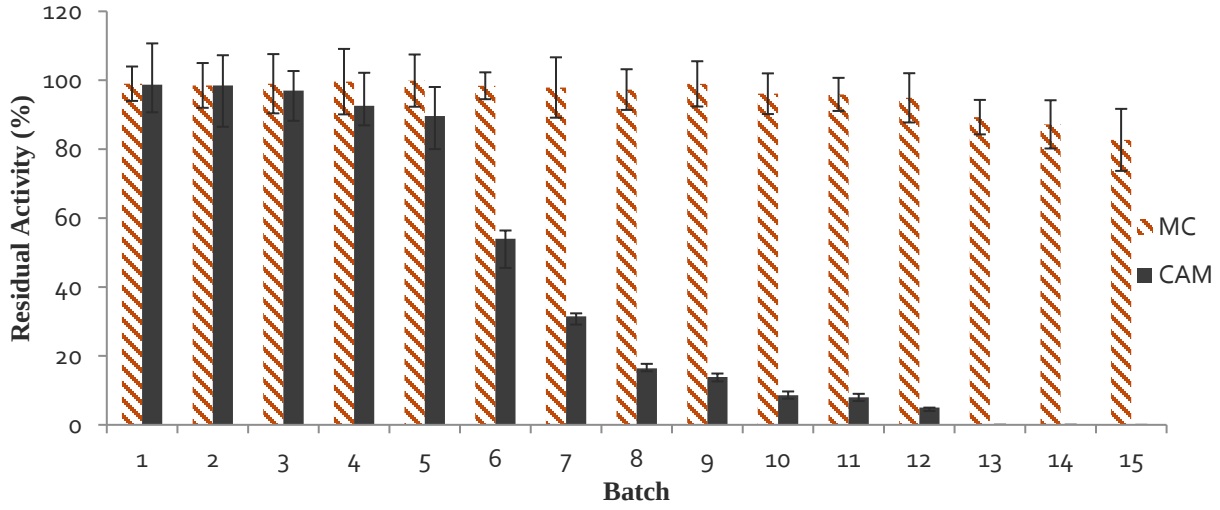


Figure 4.32: Biodegradation of chlorpyrifos (CP) in real wastewater inoculated with MCs or CAMs for repeated cycles

Performance of CAMs and MCs in terms of CP biodegradation was compared in real wastewater for repeated cycles. At the end of each cycle MCs and CAMs were washed and reused for new batch. Initially both MCs and CAMs take little longer than in synthetic medium i.e. 32 h for 3 batches after wards time decreased gradually and complete degradation was achieved in 24h both with MCs and CAMs. MCs maintained >90 residual activity upto 11batches. More than 80% residual activity was observed even after 15 batches with MCs.

Contrary to this CAMs performed well upto 5cycles and achieved >90% residual activity. CAMs suddenly lost their residual activity by almost 50% in 6 cycles. CAMs started losing their residual activity and almost completely lost their activity by 10cycle. Therefore, it can be concluded that polysulfone has provided sufficient strength to calcium alginate matrix in MCs and maintained residual activity for longer period. In the phase II of study free cells were used for biodegradation of CP in real wastewater (section 4.8) and 97% removal of initial CP concentration (545mg/L) was achieved in 24h with addition of glucose as cosubstrate. However, with MCs complete removal of higher CP concentration (583 mg/L) could be treated within 24 h without addition of any cosubstrate. In addition to complete removal, MCs have shown other considerable benefits over free cells and CAMs i.e. reusability, stability, protecting bacterial cells against nontargeted compounds present in wastewater. MCs have shown more potential for field application for biotreatment of pesticide contaminated wastewater.

Chapter 5

5. CONCLUSION AND RECOMMENDATIONS

5.1. CONCLUSIONS

PHASE I

Stable bacterial consortium comprised of *Pseudomonas kilonensis* SRK1 *(*KT013088)*, Serratia marcescens* SRK2 (KT013089), *Bacillus pumilus* SRK4 (KT013091), Acromobacter *xylosoxidans* SRK5 (KT013092) and *Klebsiella sp.* T13 (KT013093) was developed (Figure 4.1, Table 4.3). CP biodegradation and kinetic study demonstrated that bacterial consortium has broad tolerance to wide range of environmental conditions (inoculum intensity, pH, CP concentration) (Figure 4.4, 4.5, 4.6). Bacterial consortium was able to remove 99% of initial CP concentration within 36 h and data has shown excellent fit to first order rate model (R^2 >0.96). Half-life of CP is of several weeks, this was reduced to 0.284 days only (Figure 4.8). Bacterial consortium has ability to degrade CP in SWW having organic (xylene, toluene and benzene) and inorganic ($CuSO_4$, $ZnSO_4$ and $HgSO_4$) pollutants, a task that may not be accomplished by single strain. However removal efficiency decreases significantly above 300mg/L (Figure 4.2). Results suggests low substrate concentration (<300mg/L) and extended HRT is suitable for treatment of WW contaminated with CP. Results of phytotoxicity assay demonstrated higher vigor index of *T. aestivum* after treatment with bioremediated effluents (4.11), these results were in agreement with higher CP removal, suggesting that CP and its toxic metabolites (TCP, TMP, DETP) were effectively removed by bacterial consortium. Results demonstrated that bacterial consortium performed efficient biodegradation activity this indicated possibility for field application.

PHASE II

Feasibility of experimental setup and bacterial consortium for biotreatment of pesticide industry wastewater was investigated under laboratory conditions. Physiochemical characterization of WW collected from industry "A" has shown presence of higher value for COD (8000 mg/L), phosphate (825 mg/L), chlorides (1053 mg/L), chlorpyrifos (545 mg/L) and numerous trace metals. For efficient biodegradation of CP in WW bioaugmentation with specific microbes and cosubstrates addition for sustained operation was required. pH of WW collected from industry was extremely low (2.92± 0.05). pH of WW plays vital role in biodegradation of CP therefore it was altered, and results indicated that pH in the range of 7-8 was best for successful operation (Figure 4.14). Addition of cosubstrates enhanced biodegradation rate of CP in WW. However higher concentration of cosubstrates may produce negative impact by enhanced dependence of microbes on cosubstrate which may result in accumulation of toxic metabolites. Therefore, utilization of optimized concentration of cosubstrates to enhance biodegradation of CP in contaminated environment is suggested. The wide and low cost availability of ACS, along with its confirmed efficacy in CP biodegradation, makes it feasible for environmental remediation of CP. 97% of initial CP concentration (550 mg/L) was removed from WW after bioaugmentation with bacterial consortium. An increase in concentration of toxic intermediated (TCP, TMP, DETP) followed by gradual decrease suggests successful degradation of toxic metabolites (Figure 4.16). Release of chloride ion indicated ring breakage and toxicity reduction (Figure 4.18). Bacterial consortium was able to reduce 74.3 % of overall organic load (Figure 4.17); this indicates possibility for degradation of other organic compounds. On the basis of obtained results it could be concluded that in harsh environment such as extreme pH, exotic microbes lose their activity and do not perform degradation activity. In addition, harsh conditions such as pH, effect

performance of microbes to great extents. Adjustment of pH level optimum for microbial performance is essential. Repeated cycles could be performed but free cells washout and predation may occur. If invasion is unlikely immobilization or encapsulation of exotic microbe into safe structures such as sodium alginate beads is recommended for future studies. This may protect cells from predators, prevent washout and consequently extend the lifetime of biodegradation activity.

PHASE III

Cell separation, washout, stability loss and non-reusability have been major problems associated with the use of free cells. Thus, there is a need to investigate methods that governs bacterial application more cost-effective and durable. Application of cell immobilization technique for biodegradation of CP was investigated. There are number of problems associated with use of calcium alginate matrix. Therefore, a new method of polymeric coating of calcium alginate microspheres with polysulfone was investigated. Effect of different concentrations of sodium alginate, $CaCl_2$, biomass and polysulfone was investigated. Highest residual activity among all concentration was observed at 2.5 g/100 ml of sodium alginate, 6 g/100ml of $CaCl_2$, 600 mg/100ml of biomass and 10% Polysulfone (PSf). Storage stability is factor that plays most important role in determination of suitability of material for immobilization of bacteria for practical use. MC may remove more than 88 % CP even after 20 weeks of storage at 4˚C. Contrary to this CAM has lost biodegradation activity completely. Macrocapsules are reusable and have longer shelf life as compared to calcium alginate matrix. This would provide economic advantage when used in large scale application. With MC from 400 to 600 mg/L CP more than 98 % removal was achieved within 18 h, with free cells 36 h was required for complete removal possibly because of longer lag phase or substrate inhibition. It may be concluded that membrane

layer protected bacteria from leaking out thereby protecting it from physically (thermal, pH) and chemically harsh environment. Entrapment in alginate matrix provides sufficient physical protection to microbes against harsh environmental conditions of WW. However polymeric coating with polysulfone (MC) is a step further in protection against physical harm. GC-MS results demonstrate that bacterial consortium may effectively transform CP to low molecular weight products (TCP, TMP and DETP). It also opens a novel perspective for relevant agroindustry wastewater treatment. Study is one step forward to application of immobilized bacteria for more stable option.

5.2. RECOMMENDATIONS

1. Capability of bacterial consortium for biodegradation of other organophosphate pesticides should be also be investigated.
2. To bring utility of immobilization matrix closer to a practical solution it should be studied with multiple pesticides and other polymers.
3. Enzymatic studies should be carried to explore enzyme capable for biodegradation for wide range of xenobiotic.

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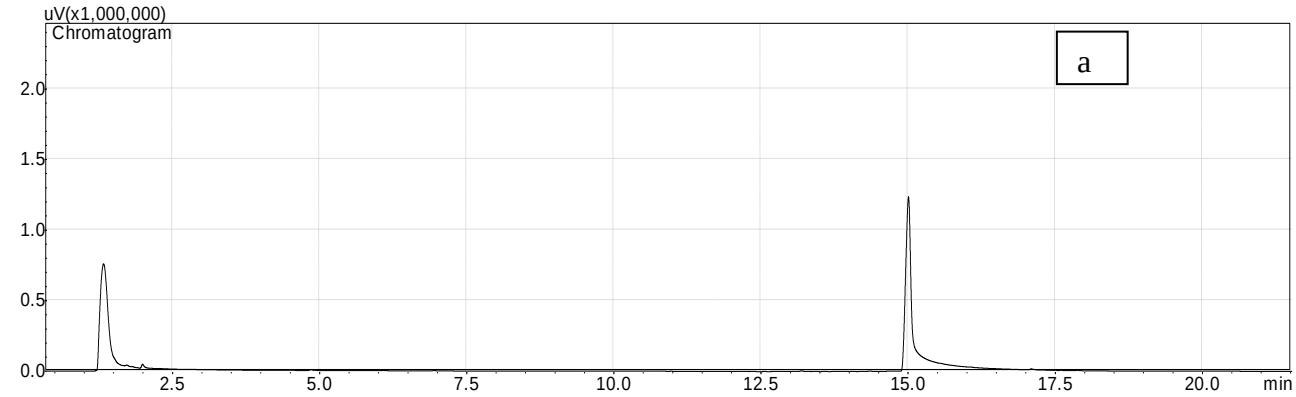
ANNEX I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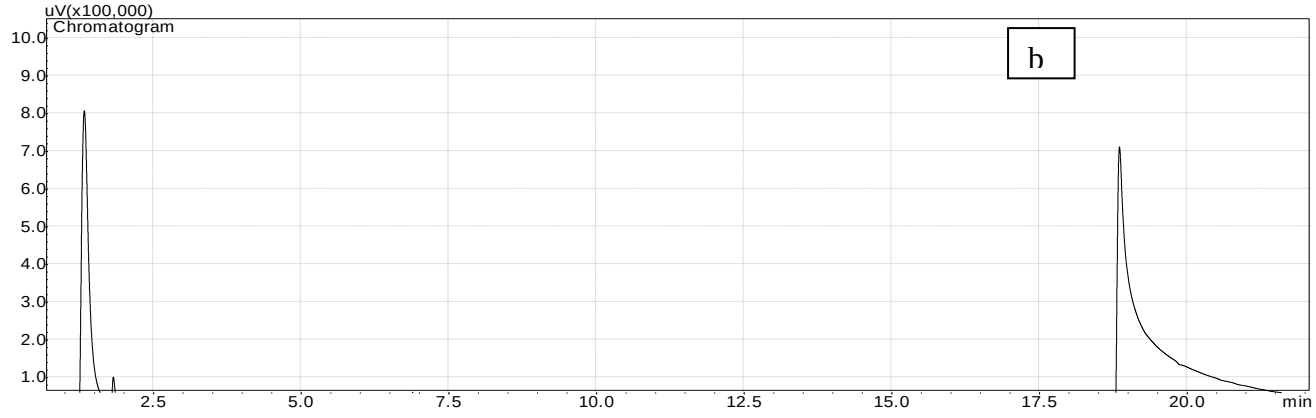
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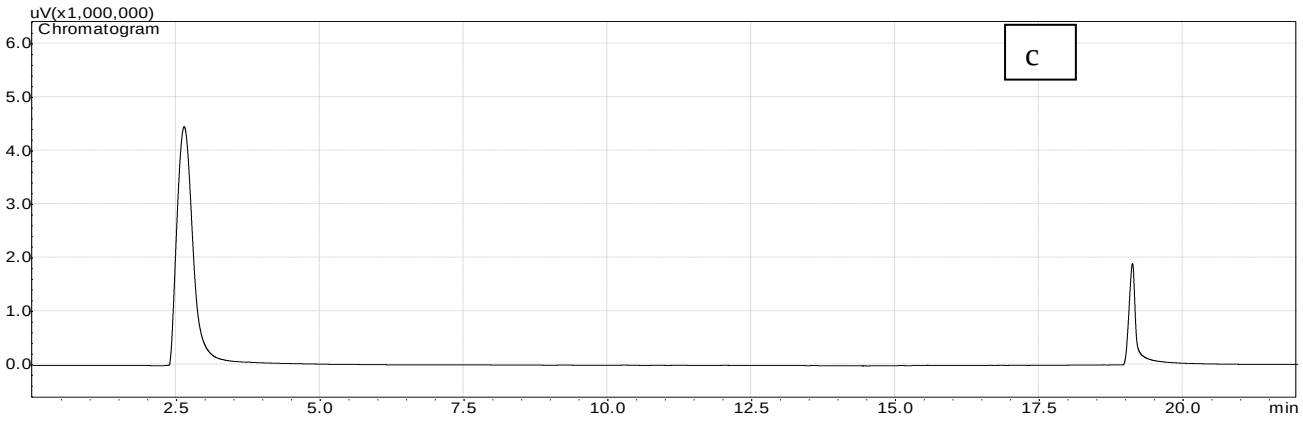

Figure I: Typical GC chromatogram (a) Chlorpyrifos CP (retention time; 15.00min) (b) TCP (retention time; 18.85min) (c) TMP (retention time; 19.12 min).

ANNEX II

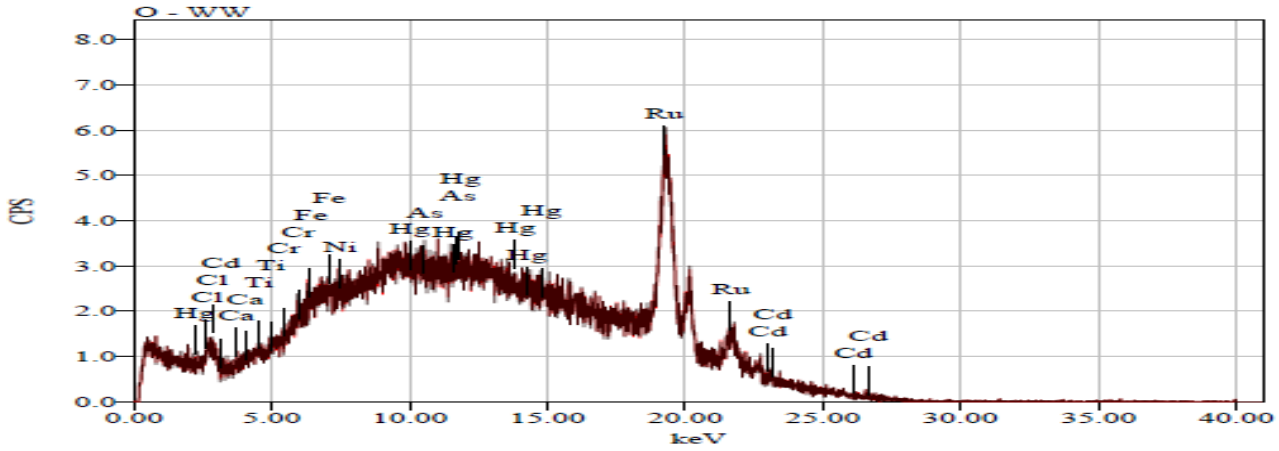

Figure II a: XRF pattern of two wastewater samples (1) collected from agrochemical industry for various metals (voltage; 30Kv, Current; 1mA, Energy range; 0 - 41KeV, Counting rate; 10565counts/sec).

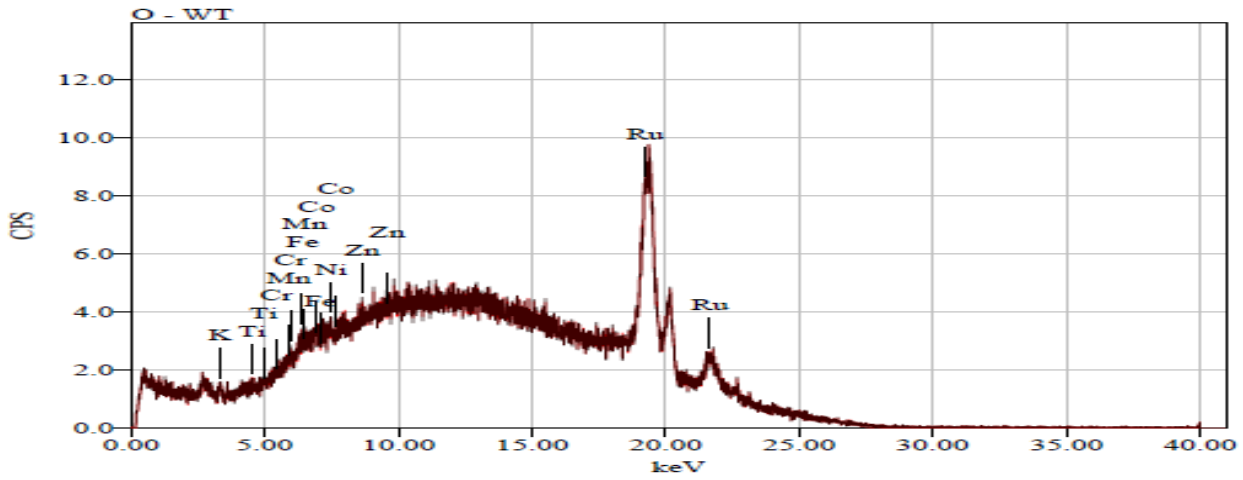

Figure II b: XRF pattern of wastewater samples (2) collected from agrochemical industry for various metals (voltage; 30Kv, Current; 1mA, Energy range; 0 - 41KeV, Counting rate; 10565counts/sec).

ANNEX III

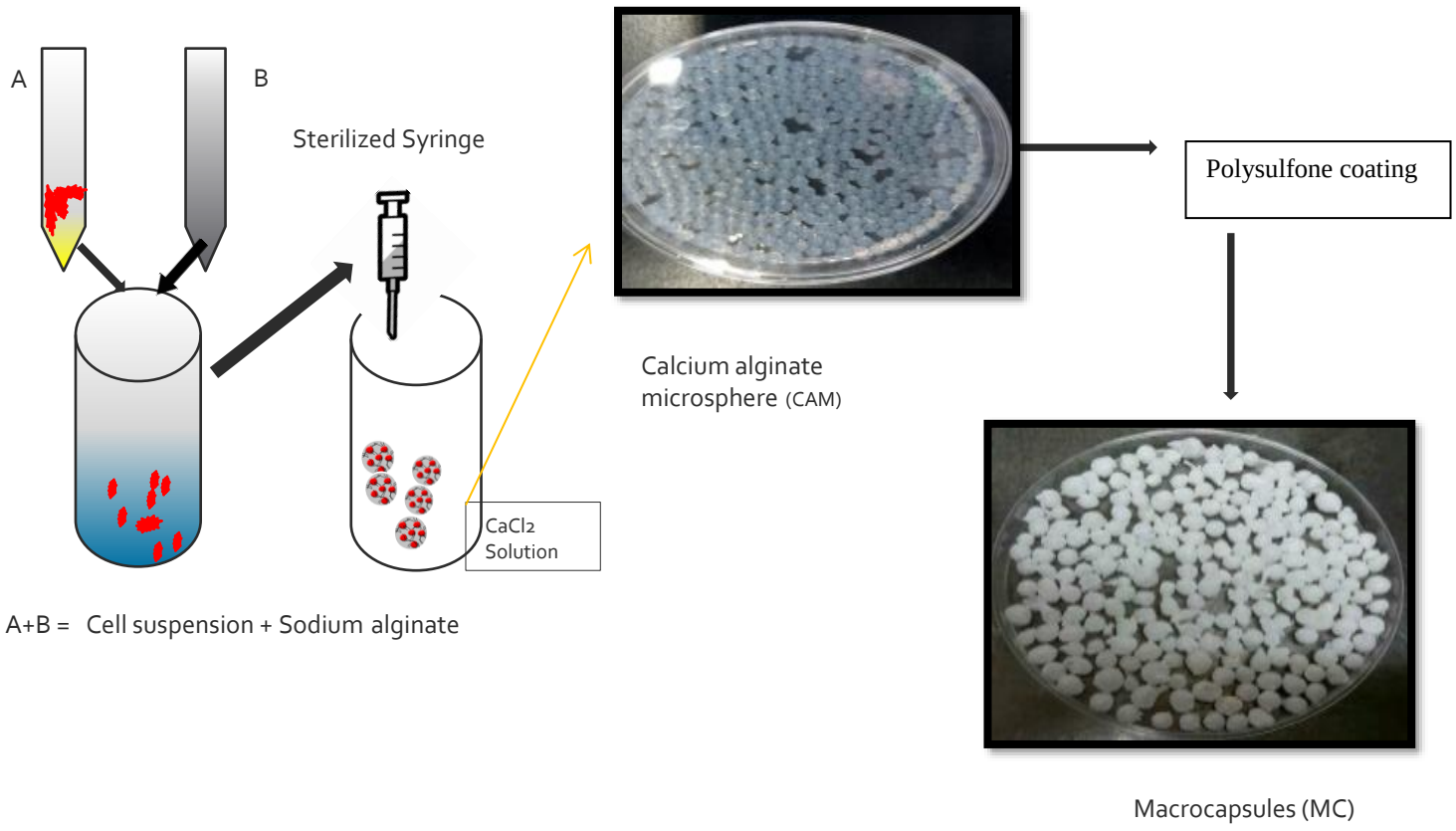

Figure III a: Schematic of calcium alginate microspheres (CAMs) and macrocapsules (MCs) preparation process.

ANNEX IV

RESEARCH PUBLICATIONS

List of ISI indexed Journal Publications

1. **Saira Khalid,** Imran Hashmi, (2016) "**Biotreatment of Chlorpyrifos in a Bench Scale Bioreactor using Psychrobacter alimentarius T-14**" **published** in ISI indexed journal "Environmental Technology" Vol 37, 316-326pp. DOI:10.1080/09593330.2015.1069406 (Impact factor: 1.76)
2. **Saira Khalid**, Imran Hashmi, Sher Jamal Khan (2016) "**Bacterial Assisted Degradation of Chlorpyrifos: The Key Role of Environmental Conditions, Trace Metals and Organic Solvents**" **published** in ISI indexed journal "Journal of Environmental

Management" Vol 168, 1-9pp. DOI: 10.1016/j.jenvman.2015.11.030. (Impact factor: 4.005)

3. **Saira Khalid**, Imran Hashmi, Sher Jamal Khan, Ishtaiq A. Qazi, Habib Nasir, (2016) "**Effect of Metal Ions and Petrochemicals on Bioremediation of Chlorpyrifos in Aerobic Sequencing Batch Bioreactor (ASBR)**" **published** in ISI indexed journal "Environmental Science and Pollution Research" Vol 23 pp.20646–20660. DOI 10.1007/s11356-016-7153-8 (Impact factor: 2.8)
4. Muhammad Ajaz Ahmed, Muhammd Saif Ur Rehman, Ruly Terán-Hilares, **Saira Khalid**, Jong-In Han (2017) "**Optimization of twin gear-based pretreatment of rice straw for bioethanol production" published** in ISI indexed Journal "Energy Conversion and Management, Vol 141,120-125. DOI org/10.1016/j.enconman.2016.06.022 (Impact factor: 6.377)
5. **Saira Khalid**, Jong-In Han, Imran Hashmi, Ghalib Hasnain, Muhammad Ejaz Ahmed, Sher Jamal Khan , Muhammad Arshad (2018) **"Strengthening Calcium Alginate Microspheres Using Polysulfone and it's Performance Evaluation: Preparation, Characterization and Application for Enhanced Biodegradation of Chlorpyrifos" published** in ISI indexed journal "Science of the Total Environment" Vol 631-632, 1046-1058pp. (Impact factor: 4.9)
6. **Saira Khalid**, Imran Hashmi, Jong-In Han (2018) "**Bioaugmentation and Biostimulation Plays Important Role in biodegradation of Chlorpyrifos Present in Agrochemical Industry Wastewater" submitted** in ISI indexed journal "Journal of Environmental Management"

List of Conference Publications

7. **Saira Khalid**, Imran Hashmi, (2014) "**Biotreatment of chlorpyrifos in a bench scale activated sludge bioreactor (ASBR) using *Psychrobacter alimentarius* T14**" In proceedings of "7th International Conference on Challenges in Environmental Sciences and Engineering " held on 12-16 September, 2014 at Johar Bahru, Malaysia.
8. **Saira Khalid**, Imran Hashmi, Jong-In Han (2015) "**Effect of immobilization on biotransformation of Chlorpyrifos using *Bacillus Pumilus* SRK4**" In proceedings of

International Environmental Engineering conference IEEC-2015 held from 28-30 October, 2015 at Busan, South Korea.

9. **Saira Khalid**, Imran Hashmi, (2015) "**Role of *Bacillus sp.* SRK3 in biotransformation of Chlorpyrifos using simulated pesticide wastewater** " In proceedings of International Environmental Engineering conference IEEC-2015, held from October 28-30 October, 2015 at Busan, South Korea.
10. **Saira Khalid**, Imran Hashmi, Jong-In Han, (2015) "**Biodegradation of Chlorpyrifos in Sequencing Batch Reactor at Different Hydraulic Retention Times**" In proceedings of 6th International Conference on "Environmentally Sustainable Development" held in 2016 at Abbottabad, Pakistan.
11. **Saira Khalid**, Imran Hashmi (2016) "**Riboflavin mediated Degradation of Chlorpyrifos by Alcaligens sp. T11**" **Published** in proceedings of 5th International Conference on Biological, Chemical and Environmental Sciences" pp. 6-9, DOI:http://dx.doi.org/10.15242/IICBE.C0316014 held on 24-25 March, 2016 at London, United Kingdom

ANNEX IV

GenBank KT013087-KT013093

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admin@ncbi.nlm.nih.gov>To: saira@iese.nust.edu.pk, sayraraja@yahoo.com

Dear GenBank Submitter:

Thank you for your direct submission of sequence data to GenBank. We have provided GenBank accession numbers for your nucleotide sequences:

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BankIt1829823 Seq1 KT013088

BankIt1829823 Seq2 KT013089
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BankIt1829823 Seq4 KT013091
BankIt1829823 Seq5 KT013092
BankIt1829823 Seq6 KT013093

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