STUDIES ON MICROBIAL DEGRADATION OF CHLORINATED ORGANIC COMPOUNDS

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

By

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DECLARATION

I, **Ranjani Chitrapur**, hereby declare that the Research Thesis entitled **"Studies On Microbial Degradation Of Chlorinated Organic Compounds"** which is being submitted to the **National Institute of Technology Karnataka, Surathkal** in partial fulfillment of the requirements for the award of the Degree of **Doctor of Philosophy** in **Chemical Engineering** is a bonafide report of the research work carried out by me. The material contained in this Research Thesis has not been submitted to any University or Institution for the award of any degree.

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CERTIFICATE

This is to certify that the Research Thesis entitled **"Studies On Microbial Degradation Of Chlorinated Organic Compounds"** submitted by **Ranjani Chitrapur** (Register Number: **CH05F03**) as the record of the research work carried out by her, is accepted as the Research Thesis submission in partial fulfillment of the requirements for the award of degree of **Doctor of Philosophy**.

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ABSTRACT

Chlorinated organic compounds which are toxic to higher forms of life are also recalcitrant to microbial degradation. Among the toxic chlorinated organic compounds, chlorinated phenols have been chosen for this study because of their wide spread into the environment, affecting both soil and groundwater. Chlorophenols especially mono- and dichlorophenols are formed during the chlorination of water and waste water, in the presence of some pollutants. Several techniques are available for the removal of contaminants from waste water, although not all are efficient enough to reduce to acceptable limits. Biological treatment by use of microorganisms is especially attractive because it has the potential to almost completely degrade Chlorophenol while producing innocuous end products. In addition, it has the advantage of reduced capital and operating cost because of operating at ambient conditions.

A pure culture of chlorophenol degrading bacteria used in this study was isolated from soil near waste water treatment tank of an industry manufacturing compounds using chlorophenols. Colonies of 2, 4-DCP and PCMX degrading organism were obtained from the highest dilutions on nutrient agar plates containing 10 ppm of 2, 4-DCP and PCMX. The bacteria were identified by genotype and phenotypic characterization as *Bacillus cereus*. Comparative analysis of the 16S rDNA sequence in the Gene Bank database revealed that these bacteria are related to *Bacillus cereus*. Growth of *Bacillus cereus* was optimized in concentrations ranging from 0.5 mg/L to 50mg/L of both 2, 4-DCP and PCMX.

Degradation of Chlorophenol was studied using this culture in defined salt media under various ambient conditions of pH values (pH 2,3,4,5,6,7 and 8) and different temperature (25^0C, 30^0C, 35^0C, 40^0C). The residual 2, 4-DCP estimation revealed that maximum degradation of 28 % occurred in cultures placed at 30^oC in 7pH for 264 hour with 10 mg/L of 2,4-DCP. The maximum degradation was 28.76 % at 30^oC in 7 pH for 216 hour with 50 mg/L of PCMX. In 264 hour 64.6 % 2, 4-DCP was removed at pH 3. The acidic pH influence the efficiency of degradation of 2,4-

DCP. The maximum percentage degradation of PCMX is at 7pH (28 %) when compared to other pH.

The parent strains where subjected to two types of mutation for enhancement of degradation as per the protocols reported in literature and the mutated strain have exhibited higher potential degradation for 2,4-DCP compared to PCMX, were parent strain could effectively degrade instead of mutated organism. The statistical tools to understand the interaction of the process variables by use of RSM has been carried out. The experimental results closely match the predicted values. Application studies by treating the effluent contain chlorinated organic compound was carried out by parent strain and mutated strain. Chlorinated organic compound in the effluent was effectively degraded.

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A successful man is he who receives a great deal from his fellow men, usually incomparably more than corresponds to his service to them. The value of a man, however, should be seen in what he gives, and not in what he is able to receive.
- Albert Einstein

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RANJANI CHITRAPUR

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CHAPTER 1

INTRODUCTION

"To the eyes of a god, mankind must appear as a species of **bacteria** *which multiply and become progressively virulent whenever they find themselves in a congenial culture, and whose activity diminishes until they disappear completely as soon as proper measures are taken to sterilize them."* ~ *Aleister Crowley*

Bacteria is present in diverse environments like deserts, deep sea, platues almost every where on the planet, from high up in the atmosphere to several kilometers below the Earth's crust. They have within them a very broad ability to catabolize virtually all naturally occurring compounds as their sources of carbon and energy, thus recycling the fixed, organic carbon back into harmless biomass and carbon dioxide. As humans, we depend on bacteria for our existence; for example, they colonize our skin and digestive tract as part of our immune system, certain bacteria in our gut provide us with vitamin K, and bacteria were initially responsible for the oxygenation of the Earth's atmosphere. The advances in genetics and molecular biology in the last 20-30 years has shown that bacteria are genetically extremely adaptable and, in addition to the advantage due to their rapid growth rates, have a range of mechanisms, which enable them to adapt to new environments. For example, microorganisms can be used for production of substances such as insulin in the pharmaceutical industry, for the manufacture of biodegradable plastics and as sources of novel enzymes with activities at temperature extremes.

The advent of modern chemical industry has resulted in the release of huge amounts of waste into the environment for which disposal remains an unresolved issue. Wastes contain many harmful pollutants, like pesticides, agrochemicals, novel organic compounds, etc. They are distributed widely in the environment because of their high usage, inadequate handling, accidental spills and leaching from dumpsites. Bacteria appear to beable to adapt their pre-existing catabolic breadth to enable them to attack and degrade many of these novel xenobiotic compounds. Their nutritional versatility can also be exploited for biodegradation of environmental pollutants. This has led to the possibility of using mixtures of bacteria (consortia) or even cultures of single organisms either to clean-up polluted environments or to degrade potential pollutants at source, before their release into the environment. This process is called bioremediation and is based on the capability of certain microorganisms to metabolize

toxic pollutants; obtaining energy and biomass in the process. Some compounds with complex structures are highly recalcitrant to biodegradation. Harnessing microorganisms to degrade such highly recalcitrant compounds is an attractive option for clean-up of polluted environments. However, despite the apparent simplicity of microorganisms, the different strategies for dealing with pollutants are as diverse as the organisms themselves. The process of biodegradation must therefore be investigated on several levels; biochemical, genetic and physiological.

Chlorinated organic compounds are a class of organic molecules which are implicated as one of the causes for environmental pollution. Among these, chlorophenol and chloroxylenol are two major pollutants present in soil and water which has been shown to have severe health impacts. Swoboda-Colberg et al.(1995) and Ayami Nakagawa et al.(2006) have reported that chlorophenol and chloroxylenol are produced for manufacture of pesticides, preservatives, anti-mildew agents, solvents, refrigerants, plastics etc. Chlorophenols and chloroxylenols have been used as general-purpose disinfectants and it has been found that they can also appear as degradation products of other chlorinated xenobiotic (Bollaget al.1986). Many of them are known or suspected human carcinogens (EPA, 1988). The usage of chlorinated aromatic compounds, such as chlorophenol and chloroxylenol in agricultural or industrial processes, has led to their accumulation in the environment, especially in soil and water (Keither and Tellard, 1979, Moos *et al*. 1983, Borthwick and Schimmel, 1978). Their residues and transformed products released in the environment are toxic to both terrestrial and aquatic life. Chlorophenol and chloroxylenol, which are toxic to higher forms of life, are also recalcitrant to microbial degradation (Chaudhry and Chapalamadugu, 1991). Despite the recalcitrant nature of chlorophenols, specialized strains of microorganisms have been shown to be capable of their degradation (Knackmuss and Hellwig, 1978, Balfanz and Rehm, 1991, Fava et al. 1995). According to these studies and others, the biodegradability of chlorophenols depends on the number and position of halogens in the aromatic ring. Moreover, high chlorophenol and chloroxylenol concentrations are known to be inhibitory to microbial growth.However, variation in the complexity and toxicity of chlorophenols and chloroxylenol has promoted the use of fungi, anaerobic bacteria and microbial consortia for their mineralization (Steinle et al. 1998).

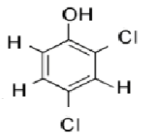
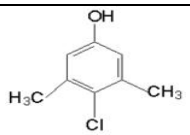
2, 4-dichlorophenol (2, 4-DCP) is a class of hazardous chlorophenol substances which are found in atleast 166 of the 1,467 National Priorities List Sites identified by the Environmental Protection Agency (EPA).They were detected first in the environment in the late 1950s and were reported globally as contaminants in almost every component of the ecosystem including air, water, soil, fish, wildlife, human blood etc.

Parachlorometaxylenol (PCMX) is a toxic chloroxylenol substance, which was first registered in US in 1959. It is used as an antimicrobial agent for control of bacteria, algae and fungi in adhesives, emulsions, paints and wash tanks. The product is a white crystalline solid and can easily be formulated into soaps such as Dettol (Ascenzi and Joseph 1996), detergents, antiseptics, disinfectants and many other products. They are used to sanitize industrial and hospital premises and pet living quarters. Its antibacterial action is due to disruption of cell membrane potentials (Aly and Malbach 1988).PCMX is registered under California register and U.S. EPA as toxic substance.

1.1. Chemical & Physical Properties

The structures and physical characteristic of chlorophenols and chloroxylenols, which have been chosen for the present study, are shown in the Table below.

Table 1.1: Chemical and physical characteristics of 2, 4-DCP and PCMX

Chemical & Physical Characteristic	2,4-DCP	PCMX
Chemical Formula	$C_6H_4Cl_2O$	$ClC_6H_2(CH_3)_2OH$
Chemical structure		
Color	White to pale Yellow solid	White to Buff
Molecular Weight	163.0	156.65
Melting Point	45 ^{0}C	112-116 ^{0}C
Boiling Point	210 ^{0}C	246^{0}C

1.2. The effects of chlorophenols and chloroxylenols on health

Phenolic compounds, especially halogenated phenolic compounds, have been found to have possible endocrine-disrupting effects, exerted by interference with the transport of thyroid hormones (Van den Berg et al. 1990). The exposure of humans to phenolic compounds becomes apparent from the results of a recent study, where some 50 brominated and chlorinated phenols were found in plasma from Swedish blood donors (Hovanderet al. 2002).

Workers who make pesticides from 2, 4-DCP and PCMX are exposed to the same through breathing or skin contact (EPA 1988). 2, 4-DCP is readily absorbed when administered by the oral, inhalation or dermal routes and causes physical hazards**.** Symptoms of exposure to concentrated forms of 2, 4-DCP includes severe irritation and burning in the eyes with corneal injury, which could result in permanent impairment of vision, even blindness. Molten or hot 2, 4-DCP is immediately absorbed through the skin in amounts that have caused death to humans. Rapid death in humans has been caused by skin exposure without immediate decontamination. Amounts of molten DCP that cover as little as 1% body surface area (e.g., palm-of-hand-sized) could result in death. Inhalation of 2, 4-DCP dust can result in severe irritation in the upper respiratory tract (nose and throat). They also accumulate mostly in the liver and kidney of laboratory test animalsand to a lesser degree in the brain, muscle and fat (World Health Organization, Draft, 1986). 2, 4-DCP is also carcinogenic (Agency for Toxic Substances and Disease Registry (ATSDR 1990).

PCMX is significantly toxic to humans and practically nontoxic to birds, highly toxic to fish, and moderately toxic to freshwater invertebrates like Molluscs, there are mitigating factors which support the conclusion that exposure to terrestrial and aquatic organisms is extremely minimal(EPA 1994). They are highly corrosive. It is a mild skin irritant and may trigger allergic reactions in some individuals. They cause eye, skin, mouth and gastrointestinal injuries. When inhaled cause nausea, vomiting, diarrhea, hypotension, myocardial failure, pulmonary edema, neurological changes, liver and renal toxicity, methemoglobinemia and hemolysis (PAN Pesticides Database - Pesticide Registration Status).

1.3. Residue level guidelines

The EPA recommends that drinking water concentrations of 2, 4-dichlorophenol should not be more than 0.02 mg/L; these are levels that can be ingested by the human body. In order for chlorophenols to be lower than levels permitted, the EPA recommends levels of 0.1 ppb for chloroxylenol, 0.3 ppb for 2,4-dichlorophenols, and 1 ppb for 2,4,5-trichlorophenol, 2,3,4,6-tetrachlorophenol(EPA 1988).

1.4. Degradation of chlorophenols and chloroxylenols

The available methods for degradation of chlorophenols and chloroxylenols are few. This includes conventional, physical and chemical methods. However, in most cases, physical and chemical methods lead to secondary pollutants, disposal being a major problem for most secondary pollutants. The commonly used methods of chlorophenols and chloroxylenols treatment are:

1.4.1. Adsorption

Chlorophenols and chloroxylenols are commonly removed from industrial effluent by adsorption using activated carbon, dried and dead fungi. The process of incineration generates many new compounds, some of which, dioxins and furans, have very severe consequences on human health.

1.4.2. Solvent Extraction

Solvent extraction process consists of three steps: extraction of the solute from water into solvent, solute removal from solvent and solvent recovery in which solvent is removed from wastewater. In wastewater treatment the third step is particularly important since there is a danger of contamination of wastewater by solvent. The solvent used for chlorophenols and chloroxylenols recovery are benzene, isopropyl ether and butyl acetate. In addition to the presence of solvent in wastewater, high cost of solvent is another factor to be considered.

1.4.3. Sonication and photochemicaland advanced oxidation method

Chlorophenols and chloroxylenols are removed from industrial effluent by sonication, photo catalysis with different types of TiO_2.Chlorophenols can also be removed by chemical treatment procedures by oxidation through reactions with ozone (O_3), hydrogen peroxide (H_2O_2) which known as Advanced oxidation processes.Advanced Oxidation Technology (AOT) has provided innovative, highly cost-effective, catalyzed chemical oxidation processes for treatment of contaminated soil, sludge, and wastewater.It also allows for the treatment of a wide variety of recalcitrant chemicals, including pesticides, chlorinated organics, MtBE, 1,4 Dioxane and many others. Additionally, the process has proven to be particularly effective for rapid treatment of sulfide related odor issues at large industrial and municipal treatment facilities.

1.4.4. Biological Treatment

Biological treatment is especially attractive because it has the potential to almost completely degrade contaminants while producing innocuous end products. In addition, it has the advantage of reduced capital and operating cost because of operating at ambient conditions. Thus biological method of treatment has turned out to be a favorable alternative for chlorophenol and chloroxylenol degradation.

1.4.4.1. Bioremediation

The use of biological systems to remove pollutants from the environment is called bioremediation. The technique may be used instead of, or as a complement to, conventional remediation techniques such as pump-and-treat or excavation and incineration. Bioremediation using bacteria takes advantage of the ability of microorganisms to degrade organic compounds and their ability to evolve degradation pathways for pollutants that are foreign (xenobiotic) in the environment. Some compounds are easily degraded by microorganisms, whereas others persist in nature – these are called recalcitrant.A number of bacteria have been identified that can degrade a variety of xenobiotic (Mishra et al. 2001). In general, xenobiotic especially chlorophenols and chloroxyenols tend to be recalcitrant.

1.4.4.1.1. Principles of Bioremediation:

The principle approaches of bioremediation are biostimulation and bio augmentation.

1.4.4.1.1.1. Biostimulation

Biostimulation is the technique of adding amendments to the soil or groundwater matrix to stimulate the native microbial population to increase in size and to degrade the hazardous chemical present in the soil. No addition of the organism is done in this method. The improvement of the conditions can be done by adding nutrients to the soil, adjusting pH, ploughing the soil to increase the oxygen and nutrient distribution. The site of pollution is located and the site is dug up for the addition of nutrients. Oxygen is provided to the lower layers of the soil by addition of hydrogen peroxide in the nutrient solution (H_2O_2). The hydrogen peroxide is highly unstable and breaks down to water and oxygen. The effects of bio augmentation with a pentachlorophenol (PCP)-adapted consortium and biostimulation with glucose as a carbon source on anaerobic bioremediation of PCP-contaminated soil were investigated by Zou and Siwei (2000) in terms of the initial PCP removal rate and the extent of PCP dechlorination and mineralization. It was found that biostimulation with glucose at 1g/kg soil or bio augmentation at 0.14 g volatile suspended solids (VSS)/kg soil could greatly improve PCP degradation.

1.4.4.1.1.2. Bio augmentation

Bio augmentation can be defined as the addition of pre grown microbial cultures to enhance microbial populations at a site to improve contaminant clean up and reduce clean up time and cost. Indigenous or native microbes are those that occur naturally at a site. They are usually present in very small quantities. They may not be able to prevent the spread of the contaminant. In some cases, native microbes do not have the ability to degrade a particular contaminant. Bio augmentation offers a way to provide specific microbes in sufficient numbers to complete the biodegradation. This can be useful if the contaminants are particularly recalcitrant to degradation or if site conditions are extreme.

1.4.4.1.2. Classification of Bioremediation

Bioremediation technologies can be generally classified as *in situ* or *ex situ*. *In situ* bioremediation involves treating the contaminated material at the site while *ex situ* involves the removal of the contaminated material to be treated elsewhere.

1.4.4.1.2.1. In Situ Bioremediation

Bioremediation technology which is used "in place", without removal of the contaminated matrix is called In-situ bioremediation. Both intrinsic and engineered bioremediation technologies can be used in-situ.

1.4.4.1.2.1.1. Intrinsic Bioremediation

Often bioremediation can be accomplished without human intervention by microorganisms that are naturally found in the contaminated matrix known as intrinsic bioremediation or natural attenuation. For this approach to be used, it is usually necessary for the rate of contaminant degradation to exceed the rate of contaminant migration. The only intervention needed is monitoring of the biodegradation processes. Knowledge of the following key site characteristics are required to evaluate the likely success of intrinsic remediation; the bioavailability of contaminants, levels of nutrients, the presence of minerals to buffer the pH of the matrix, adequate levels of electron acceptors (either oxygen, nitrate, ferric iron, or sulfate), and site specific contamination migration rates(D.H. Kampbell et al. 1994).

1.4.4.1.2.1.2. Engineered Bioremediation

In some cases, it may be desirable to construct engineered systems to supply nutrients, electron acceptors or other materials that enhance the rate or extent of contaminant degradation. This is desirable when the rate of contaminant migration, the location of environmental receptors or other liability issues dictate that steps be taken to optimize the rate of contaminant degradation in order to mitigate contaminant migration. A biological active zone (BAZ) is created in the path of pollution migration. The BAZ is created by biostimulation and bio augmentation. The key site

characteristics for engineered bioremediation are the same as for intrinsic remediation.

1.4.4.1.2.2. Ex-Situ Bioremediation

Bioremediation technologies that require removal of the contaminated matrix by excavation is called Ex- situ bioremediation. This technology uses microorganisms to degrade organic contaminants in excavated soil, sludge, and solids. The microorganisms break down contaminants by using them as a food source. The end-products typically are carbon dioxide and water. Ex-situ techniques should consider the possible removal of volatile compounds by physical processes, which must be taken into account in the biodegradation balance **(**Wolfgang Dot et al. 1995)**.** Ex- situ bioremediation is accomplished landfills and composting.

a. **Landfills:** Landfills are carried out in low value land, left over after mining activities or the barren land unsuitable for the agricultural activities. The waste are brought in trucks and weighed at the weigh bridge. Afterwards, the waste collection vehicles use the existing road network on their way to the tipping face or working front where they unload their load. After loads are deposited, compactors or dozers are used to spread and compact the waste on the working face. Landfills are carried out by area, trench and ramp variation methods.
b. **Composting:** Unlike the landfills, the prerequisite for composting is the segregation of organic and inorganic wastes. Composting is defined as the aerobic conversion of the waste into nutritionally rich humus like substance. This material can be used as fertilizers for the agricultural purposes. Composting is mainly aerobic process and is carried out in small heaps or in bioreactors unlike landfills. Composting is carried out by stationary heaps, agitated heaps or by bioreactor methods.

1.4.5. Fungal Bioremediation

A number of fungi are known to break down persistent toxins in soils, including PCP, DDT, dioxin, and PBP. *Phanerochaete chrysosporium* is the most widely studied bioremediation fungus. Degradation of phenol by *Phanerochaete chrysosporium* was

analysed by R. Rubio Peraz et al. in 1997. He investigated the influence of co-substrate concentration, mycelium acclimation and nitrogen concentration on phenol degradation and concluded that decrease in glucose and ammonium tartrate concentration decreased chlorophenol degradation.

1.4.6. Phytoremediation

Phytoremediation is the use of a plant's natural ability to contain, degrade, or remove toxic chemicals and pollutants from soil or water. It can be used to clean up metals, pesticides, solvents, explosives, crude oil, and contaminants that may leak from landfill sites. For example Hairy root cultures of Daucuscarota L., Ipomoea batatas L. and Solanumaviculare Forst were investigated for their susceptibility to the highly toxic pollutants phenol and chlorophenols and for the involvement of inherent peroxidases in the removal of phenols from liquid media by Santos De Araujo Brancilene in 1999. The term phytoremediation is a combination of two words – phyto, which means plant, and remediation, which means to remedy.

1.4.7. Unique Aspects of Biological Processes:

- The concentration of starting materials (substrates) and products in the reaction mixture are frequently low; both the substrates and the products may inhibit the process. Cell growth, the structure of intracellular enzymes and products formation depend on the nutritional needs of the cell(salts, oxygen) and on the maintenance of optimum biological conditions (temperature, concentration of reactants, and pH) within narrow limits.
- Certain substances like inhibitors effectors, precursors, and metabolic products influence the rate and the mechanism of the reactions and intracellular regulations.
- Microorganisms can metabolize unconventional or even contaminated raw materials (cellulose, molasses, mineral oil, starch, ores, wastewater, exhaust air, biogenic waste), a process which is frequently carried out in highly viscous, non-Newtonian media.
- In contrast to isolated enzymes or chemical catalysts, microorganisms adapt the structure and activity of their enzymes to the process conditions, whereby

selectivity and productivity can change. Mutations of the microorganisms can occur under sub optimal biological conditions.

- Microorganisms are frequently sensitive to strong shear stress and to thermal and chemical influences.
- Reactions generally occur in gas-liquid-solid systems, the liquid phase usually being aqueous.
- The microbial mass can increase as biochemical conversion progresses. Effects such as growth on the walls, flocculation, or autolysis of microorganisms can occur during the reaction.

Bioremediation is a viable alternative (or supplement) to conventional remediation techniques, but the success of the methodology depends on many factors, both biotic and abiotic. These factors span over a great range, from physico-chemical properties of the contaminant to genetics of the biodegrading microorganisms. Gathering of knowledge about these factors is important as it will increase the accuracy of predicting the outcome of a bioremediation strategy. Two strategies can be discerned; a "top-down" approach used by engineers, mainly relying on stimulating the intrinsic population in order to reduce contaminant levels, and a "bottom-up" approach employed by many researchers, investigating in detail the properties of a single biodegrading microorganism, or a single factor at a polluted site. When these two approaches can be fully integrated, the power of bioremediation as a treatment strategy will greatly increase.

1.5. Response Surface methodology

It has been demonstrated that treatment of small volumes of toxic compounds at the point of emission using specific microbial strains allows a higher control over the process and higher removal efficiencies (Schroder et al. 1997). Thus optimization of process variables is recognized to be an essential aspect of successful degradation (S.E.Agarry et al.2008).Hence optimisation of process variables with the application of experimental design and responsesurface methodology in degradation process can result in improved reduction and development time and overall costs (Rao et al. 2000).

Conventional and classical methods of studying a process by maintaining other factors involved at an unspecified constant level does not depict the combined effect of all the factors involved. This method is also time consuming and requires a number of experiments to determine optimum levels, which are unreliable. These limitations of a classical method can be eliminated by optimizing all the affecting parameters collectively by statistical experimental design such as response surface methodology (RSM).

Response Surface Methodology (RSM) is a collection of mathematical and statistical techniques useful for the modeling and analysis of problems in which a response of interest is influenced by several variables and the objective is to optimize this response. For example, the growth of a plant is affected by a certain amount of water x_1 and sunshine x_2. The plant can grow under any combination of treatment x_1 and x_2. Therefore, water and sunshine can vary continuously. In this case, the plant growth y is the response variable, and it is a function of water and sunshine. It can be expressed as

$$y = f(x_1, x_2) + e \text{ -----1}$$

The variables x_1 and x_2 are independent variables where the response y depends on them. The dependent variable y is a function of x_1, x_2, and the experimental error term, denoted as e.The error term e represents any measurement error on the response, as well as other type of variations not counted in f. It is a statistical error that is assumed to distribute normally with zero mean and variance s^2. In most RSM problems, the true response function f is unknown. In order to develop a proper approximation for f, the experimenter usually starts with a low-order polynomial in some small region. If the response can be defined by a linear function of independent variables, then the approximating function is a first-order model. A first-order model with 2 independent variables can be expressed as

$$y=\beta_0+\beta_1x_1+\beta_2x_2+ e\text{------2}$$

If there is a curvature in the response surface, then a higher degree polynomial should be used. The approximating function with 2 variables is called a second-order model:

$$y=\beta_0+\beta_1x_1+\beta_2x_2+ \beta_{11}x^2_{11}+\beta_{22}x^2_{22+}\beta_{12}x_1x_{2+}e\text{--------3}$$

In general all RSM problems use either one or the mixture of the both of these models. In each model, the levels of each factor are independent of the levels of other factors. In order to get the most efficient result in the approximation of polynomials the proper experimental design must be used to collect data. Once the data are collected, the Method of Least Square is used to estimate the parameters in the polynomials. The response surface analysis is performed by using the fitted surface. The response surface designs are types of designs for fitting response surface.

Therefore, the objective of studying RSM can be accomplish by

(1) Understanding the topography of the response surface (local maximum, local minimum, ridge lines), and (2) finding the region where the optimal response occurs. The goal is to move rapidly and efficiently along a path to get to a maximum or a minimum response so that the response is optimized.The response surface may be represented graphically using a counter plot or a 3-D plot (Fig.1.1).

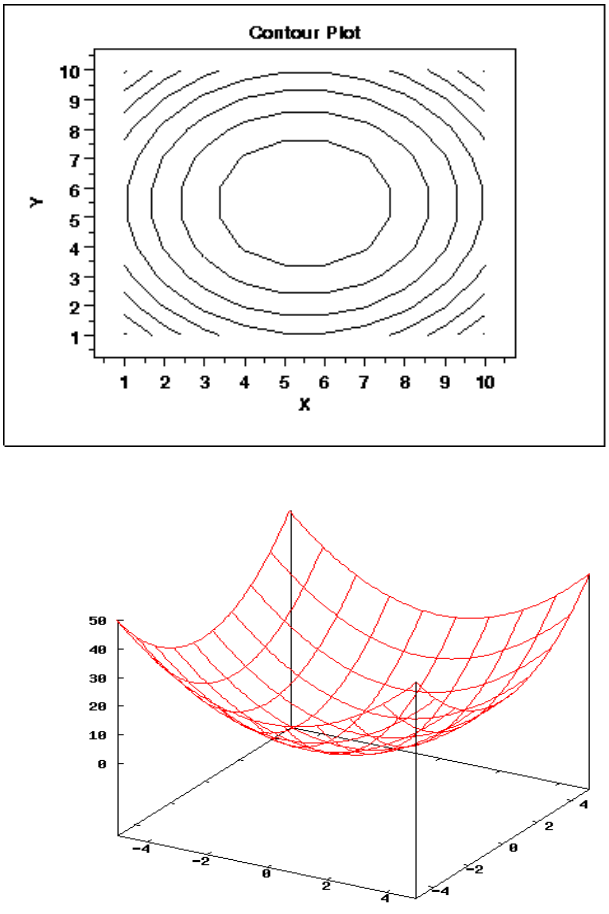


Fig. 1.1: a) The contour plot b) The surface plot

1.6. Mutations

A stress-mediated bioprocess was another designed strategy to enhance biological target productivity (Umakoshi H et al. 1998). Among stressors, mutation was a sensitive and useful stimulus for biological cells such as bacteria. In nature, the production of secondary metabolites such as antibiotics by microorganisms increases asa response to biotic and abiotic stress. In this study, we treated *Bacillus cereus* with different mutagens like ultra violet (UV) and Ethedium Bromide (EtBr) for enhancing the degradation of 2,4-DCP and PCMX.

Mutations are variations in genes that may or may not lead to changes in the phenotype of an organism. For the first time Harman Muller in 1920 reported X- rays as mutants in his Drosophila studies. During cell division specifically during DNA replication, structure of DNA can be chemically altered but this change is corrected by the cell's DNA repair machinery. The changes that remain un-repaired result in mutation. A physical or chemical substance that can change the sequence and or the structure of genomic DNA is called as a mutagen. Mutagens can be some of the radiations, mutagenic chemical substances, certain viruses and or some bacteria.

1.6.1. Radiations

Effects of radiations in the genetic material were discovered in 1920s. Radiations are first known mutagens of genomic DNA. Various types of radiation that cause mutation are electromagnetic radiation including the microwave radiations, infrared rays, visible light, ultraviolet, X rays and gamma rays, the TV and radio waves. Of these ultra violet rays is of major concern with respect to mutagenic interaction, which exerts its mutagenic effect by exciting electrons in molecules. Theexcitation of electrons in DNA molecules often results in the formation of extrabonds between adjacent pyrimidine's (specifically thymine) in DNA. When pyrimidines are bound together in this way; it is called a pyrimidine dimer (Fig. 1.2). These dimers often change the shape of the DNA in the cell and can cause problems during replication. The cell often tries to repair pyrimidine dimmers before replication, but the repair mechanism can also lead to mutations as well. In the presenceof a mutagen, however this rate can increase dramatically.

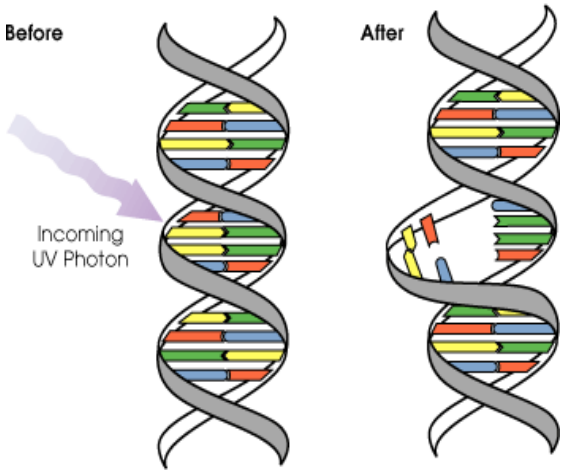


Fig. 1.2: Short wavelength UV light can cause adjacent pyrimidine bases in DNA (thymine and cytosine), to bond to one another instead of the complementary DNA strand. This disrupts DNA replication.

1.6.2. Chemical mutagens

Chemical mutagens can be DNA base analogues, intercalating agents and or chemicals like Ethedium Bromide, Acredine Orange and Proflavin that will alter DNA structure, sequence and base pairing.Cells exposed to Ethedium Bromide,a intercalating agent insert themselves between adjacent bases in the double helix of DNA, distorting the shape at the point of insertion. In that way it would behave toxic, as the cell would soon die because it cannot replicate anymore and also because no further proteins that would enable the cell to survive are synthesized (Fig. 1.3).

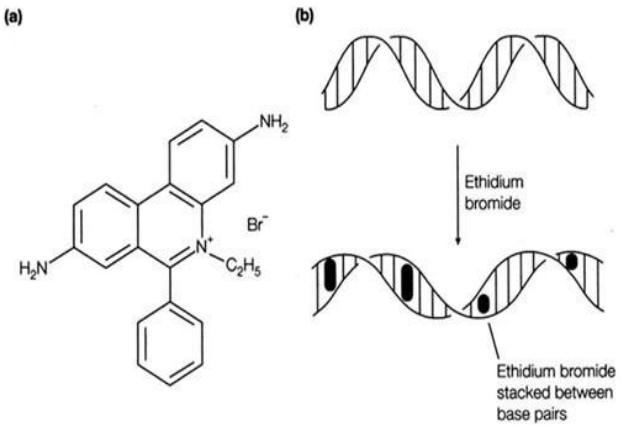


Fig. 1.3: a) Ethedium Bromide b) The process of DNA Intercalation by the Mutant Ethedium Bromide (mutagen) illustrating the lengthening and untwisting of DNA helix

1.7. Aim and Objectives of the study

The success of the chlorinated organic compound degradation is dependent on the efficient screening of microorganisms and finding the suitable conditions for the increased degradation rate. In general, biological methods are preferable methods to treat aromatic compounds because it is economical, and there is a low possibility of the production of byproducts (Marcos et al. 1997). In this concern, the extensive literature survey on isolation and screening of chlorinated organic compound degrading microorganisms like *Bacillus thuringiensis* (Roda et al. 2007), *Pseudomonas pickettii* (Hohzoh Kiyohara et al. 1992), *Azoarcus tolulyticus* (Paula et al. 1998) and *Bacillus subtilis* (James Cole et al.1994), medium formulation, studies on the effect of various physical and chemical parameters on degradation and treatment of effluent containing chlorinated organic compound was found that more number of degrading isolates need to be isolated from potential ecological sources.
Based on this the below aim and objective for the present work was outlined

The main aim of this study is to isolate a novel microorganism from soil sample around the wastewater treatment plants as to check the efficacy and efficiency of degradation of a xenobiotic compound like chlorinated organic compound.

The objectives of this study are:

- Isolation and Screening of microorganism for degrading chlorinated organic compounds.
- Identification of the microorganism.
- Studies on growth optimization of microorganism in different concentration of chlorinated organic compounds.
- Medium optimization for the enhance degradation of chlorinated organic compounds
- Increasing the degradation of chlorinated organic compound by different technique.
- Application study on untreated industrial effluent by isolated microorganism
- Analysis of intermediates of biodegradation.

1.8. Organisation of Thesis

This thesis is structured as follows. Chapter 1, a brief introduction and overview of degradation of chlorinated organic compound is presented. The steps involved in Bioremediation have also been discussed here. Additionally, Chapter 1 summarizes the objectives and presents a brief overview of this thesis.

Chapter 2 is meant to serve as a review of the degradation of chlorinated organic compound, giving a brief examination of the areas of application and the different implementations presented previously in literature.Additionally, several applications of the conventional methods to degrade chlorinated organic compound are presented. The merits and limitations of each method are discussed, and an argument is made for alternative methods that can be used for the present work.

Chapter 3 contains materials and methodology involved to isolate, identify and optimize the organism growth and media to degrade chlorinated organic compounds.Chapter 3 contains the protocol on the degradation of 2,4-DCP and PCMX in industrial effluent.

The results and conclusions of this thesis are presented in Chapter 4.

The closing chapter, Chapter 5, contains a summary of the study and suggestions for future work, while the Appendix contains the various analytical techniques used in this study.

CHAPTER 2

REVIEW OF LITERATURE

The information for this review is from journals published within the last few years that describe laboratory and field experiments of degradation techniques of chlorophenol and chloroxylenol. Reviewing this literature would be a daunting task and certainly beyond the scope for this report. Fortunately, a number of published papers and reports are readily available which were reviewed, compiled, or summarized with respect to properties, chemistry, or degradation paths of chlorinated organic compound. Citations for several of these compilations and the information summarized are listed below:

2.1 Degradation of chlorophenol and chloroxylenol by Physical and Chemical treatment

Detoxification of chlorophenol and chloroxylenol can be carried out with chemical or biological methods as shown by Atuanya et al. (2004). Conventional methods for the treatment of chlorophenol and chloroxylenol include chemical reduction using a reducing agent such as sodium sulfite and adsorption on ion exchange or chelating resins. Many researchers showed that most of these methods required high energy or large quantities of chemical reagents which is not economically feasible. However, in most cases, physical and chemical methods lead to the formation of secondary pollutants, disposal being a major problem for most secondary pollutants.

2.1.1 Adsorption

In a study by Denizli et al. (2005), the potential use of the fungus *Pleurotussajor caju* to remove phenols (*i.e.*, phenol, o-chlorophenol, p-chlorophenol and 2,4,6-trichlorophenol) from aqueous solutions was evaluated. Biosorption of phenol or chlorophenols reached equilibrium in 4 h. The maximum adsorptions of phenol and chlorophenols onto the *Pleurotussajor caju* were 0.95 mmol/g for phenol, 1.24 mmol/g for o-chlorophenol, 1.47 mmol/g for p-chlorophenol and 1.89 mmol/g for 2,4,6-trichlorophenol. The affinity order was as follows: 2,4,6-trichlorophenol> p-chlorophenol> o-chlorophenol>phenol. Phenol and chlorophenols bindings onto *Pleurotussajor caju* were clearly pH dependent. The adsorption of phenol and chlorophenols increased with increasing pH.

Chlorophenols were also removed by photolysis and adsorption technique which was studied by Pignatello et al. (1983). They examined the biodegradation, photolysis, and adsorption of pentachlorophenol (PCP) in outdoor aquatic environments with man-made channels built by the U.S. Environmental Protection Agency at a field station on the Mississippi River near Monticello, Minnesota. Four channels were used, each channel approximately 520 m long and receiving river water that flowed through the channels for about 10 hours before reentering the river. The channels were dosed continuously during the summer of 1982 with various concentrations of PCP (approximately 0, 48, 144 and 432 µg/liter) and the biotic and abiotic degradation of PCP in these channels were monitored for approximately 16 weeks. Photolysis of PCP was rapid at the water surface, but greatly attenuated with depth. Depending on sunlight conditions, photolysis accounted for a 5% to 28% decline in initial PCP concentration. Adsorption of PCP by sediment and uptake by biota accounted for <15% and probably <5% in un-acclimatized water. Microbial degradation of PCP became significant about 3 weeks after the initiation of dosing and eventually became the primary mechanism of PCP removal, accounting for a 26 to 46% (dose-dependent) decline in initial PCP.

Chlorophenols like phenol, 2-chlorophenol, 4-chlorophenol, and 2, 4-dichlorophenol from wastewater where also tried to remove using red mud a low-cost potential adsorbent from an aluminum industry waste. 2, 4-dichlorophenol and 4-chlorophenol are sorbed by the developed adsorbent up to 94-97%, while the removal of 2-chlorophenol and phenol was up to 50-81%. The order of removal was 2, 4-dichlorophenol > 4-chlorophenol > 2-chlorophenol > phenol, and the removal takes place through a particle diffusion mechanism.

2.1.2 Solvent extraction

Chin et al.(2011) extracted chlorinated aromatic hydrocarbons using supercritical fluid extraction.He concluded that supercritical fluid extraction is a better method for extracting volatile and semi volatile organic compounds.Solvent extraction of chlorinated compounds from soils and hydro-dechlorination of the extract phase was studied by Murena and Schioppa (2000).The process consists of solvent extraction followed by catalytic hydro processing (hydro-dechlorination) of

the extract phase. A mixture of ethyl acetate-acetone-water (E-A-W) was adopted as solvent in the extraction process. Tests of extraction of chlorobenzene from a model contaminated soil were carried out and the Langmuir adsorption equation was characterized. He concluded that longer times are necessary for the aromatic compounds (chlorobenzene and hexachlorobenzene) for which the CCl bond is much stronger than that in the aliphatic compound.

2.1.3 Sonication and photo chemical degradation

In a study by Herrmann et al.(1999), photo catalysis degradation of 4-chlorophenol with titanium oxide and activated carbon, on-additive adsorption capacities were observed when the two solids were mixed. Non-additive adsorption capacities were observed when the two solids were mixed and during degradation the same main intermediate products (hydroquinone and benzoquinone) were found but in much smaller quantities and during a much smaller lifetime. The synergy effect was ascribed to an extended adsorption of 4-chlorophenol on AC followed by a transfer to titania where it is photo catalytically degraded. The laboratory experiment were extrapolated to large solar set-ups and was found to be more efficient in decontaminating diluted used waters within a shorter time which could be of interest in producing drinking water in dry sunny areas.

Mineralization of 4-chlorophenol, 2, 4-dichlorophenol, 4-chloro-3, 5-dimethylphenol, 4-fluorophenol, 2, 4, 6-trinitrotoluene, 2-amino-4, 6-dinitrotoluene and 4-amino-2,6-dinitrotoluene in dilute aqueous solution using ultrasound technique was investigated by Goskondaa et al.(2002). Mineralization rates were determined as a function of substrate structure and concentration, bulk phase temperature, pH and the presence of co-solutes such as detergents and humic acids. All substrates were found to degrade sonochemically, as evidenced by the release of Cl and NO_3 respectively. Product analysis by GC–MS, HPLC and Micellar Electro kinetic Capillary Chromatography (MECC) indicated mineralization with little formation of organic byproducts, a significant advantage over other remediation methods. Chloride release from chlorophenols was approximately proportional to substrate total chlorine content, irrespective of structural differences, and reached 80% of the theoretical limit. Fluoride release from 4-fluorophenol was 10-fold lower than that of chloride

from 4-chlorophenol. Changes in the bulk phase temperature from 9.5 to 34ºC, and 12.5 to 30 ºC, respectively, were of little consequence to observed mineralization rates for nitro aromatics and chlorophenols. A significant mineralization rate increase resulted from sonication of 4-chlorophenol in acidified media. Additions of amphiphilic co-solutes resulted in modest, but statistically significant, sonolysis enhancements.

Tiehm et al. (2001) study also showed that a combination of ultrasonic pretreatment and biodegradation effectively removed the solvent chlorobenzene and the disinfectant 2, 4-dichlorophenol. In their experiments, the ultrasonic dechlorination was not influenced by the presence of other soluble organic compounds like acetate or glucose. Dechlorination of chlorobenzene by ultrasound did not lead to toxic or inhibiting reaction products. More than that, the ultrasonic pretreatment significantly reduced the toxicity of 2, 4-dichlorophenol and biological activity was initiated after sonication.

Bandara et al. (2001) studied and reported in detail the sensitized photo catalytic degradation of mono-, di- and trichlorophenol on iron oxides using aqueous suspensions of Fe_2O_3 and FeOOH. The degradation of these compounds followed pseudo first-order kinetics when -Fe_2O_3 was used as a photo catalyst. FeOOH was found to be inactive for chlorophenol degradation with the exception of 2, 4-dichlorophenol (2, 4-DCP) where a modest effect was observed. The formation of a surface complex by the chlorophenols with the iron oxide and the solubility of the particular chlorophenol in aqueous solution were observed to be the controlling parameters during the photo degradation. The results obtained with the most active catalyst Fe_2O_3 were compared with TiO_2 as total mineralization of chlorophenols was observed only on TiO_2. In either case, the intermediates produced in solution during the photo degradation were found to be significantly more biodegradable than the initial compound. For mono-, di- and trichlorophenol, the overall photo catalytic degradation was observed to increase in the order: 2,4,6-trichlorophenol (2,4,6-TCP) <2,3-dichlorophenol (2,3-DCP) <2-chlorophenol (2-CP) <2,4-DCP. The former sequence shows that the recalcitrant 2, 4-dichlorophenol degrades more rapidly than other chlorophenols tested during this study. The photo degradation of chlorophenols on - Fe_2O_3 and TiO_2 proceeds through para-hydroxylation of the initial compound as

suggested by study of the intermediates by High Pressure Liquid Chromatography (HPLC).

Gryglik et al. (2004) examined photosensitized oxidation of 2-chlorophenol using solar light where Rose Bengal, Methylene Blue and Chlorine were used as sensitizers. They were immobilized in silane gel and were used in the form of a thin film covering glass plates and xenon arc lamps were used as solar simulators. The influence of various experimental conditions (light intensity, the amount of sensitizer, oxygen concentration) on the rate of 2-chlorophenol degradation was investigated. The obtained results indicated a possibility of the application of solar radiation in the middle latitudes for wastewater treatment. The use of silane gel as a carrier for the sensitizer enables the pollutants elimination at a higher rate than in a homogenous system. The stability and durability of immobilized sensitizers were also studied.

Shchukin et al. (2004) studied the photo catalytic activity (PCA) in degradation of 2-chlorophenol in water by the Nano crystalline bicomponent TiO_2–In_2O_3 powders with various Ti/In ratio, prepared by sol–gel technique and calcined at 473 and 723 K. Their crystalline structure, surface area, surface acidity, sorption properties with respect to 2-chlorophenol, and optical absorption were determined by appropriate techniques (XRD, BET, FTIR, and UV-Vis spectroscopies). For both calcination temperatures, PCA increased with decreasing In_2O_3 contents reaching a maximum at ca. 10 wt % of In_2O_3. PCA was greatly enhanced with respect to TiO_2 for the samples pretreated at 473 K. The concentration of the main aromatic intermediate products (chlorohydroquinone and catechol) was considerably lower for TiO_2–In_2O_3 photo catalysts than for pure TiO_2. Thus the reason for PCA enhancement due to In_2O_3 included a better separation of photo generated charge carriers, an improved oxygen reduction and an increased surface acidity inducing a higher extent of adsorption of the aromatics.

Jiang et al. (2005) investigated the sonolysis of 4-chlorophenol (4-CP) in an O_2-saturated aqueous solution for a variety of operating conditions with the loss of 4-CP from solution following pseudo-first-order reaction kinetics. Hydroquinone (HQ) and 4-chlorocatechol (4-CC) were the predominant intermediates which were degraded on extended ultrasonic irradiation. The final products were identified as Cl, CO_2, CO, and HCO_2H. The rate of 4-CP degradation is dependent on the initial 4-CP

concentration with an essentially linear increase in degradation rate at low initial 4-CP concentrations but with a plateauing in the rate increase observed at high reactant concentrations. The results obtained indicate that degradation takes place in the solution bulk at low reactant concentrations while at higher concentrations degradation occurs predominantly at the gas bubble–liquid interface. The aqueous temperature has a significant effect on the reaction rate. At low frequency (20 kHz) a lower liquid temperature favors the sonochemical degradation of 4-CP while at high frequency (500 kHz) the rate of 4-CP degradation is minimally perturbed with a slight optimum at around 40ºC. The rate of 4-CP degradation is frequency dependent with maximum rate of degradation occurring (of all frequencies studied) at 200 kHz.

In conclusion, physical and chemical treatment removes the chlorophenols effectively but drawback of these techniques is that it will release some part of harmful end products which again needs further treatment. Hence, in this experiment we tried to go for biological treatment which is especially attractive because it has the potential to almost completely degrade contaminants while producing innocuous end productsand is economical, safe, and sustainable. In addition, it has the advantage of reduced capital and operating cost because of operating at ambient conditions (Zechendorf et al. 1999).

2.2 Biological treatment

Biological treatment is the ideal solution for pollution abatement, which include biostimulation (stimulating viable native microbial population), bio augmentation (artificial introduction of viable population), bioaccumulation (live cells), biosorption (dead microbial biomass), phytoremediation (plants) and rhizoremediation (plant and microbe interaction). It is the most effective innovative technology to come along that uses biological systems for treatment of contaminants. Thus, biological method of treatment has turned out to be a favorable alternative for chlorophenol and chloroxylenol degradation.

2.2.1Bioremediation

The use of biological systems to remove pollutants from the environment is called bioremediation.Bioremediation using bacteria takes advantage of the ability of

microorganisms to degrade organic compounds and their ability to evolve degradation pathways for pollutants that are foreign (xenobiotic) in the environment. The first step towards the application of bioremediation process is to select the suitable microorganism which is capable of remediation of the compounds which are of our interest. The organism of our interest has to be sought after rigorous screening procedures from a broad variety of microorganisms (bacteria, actinomycetes, fungi and yeasts).However, pure cultures of microorganisms can be obtained from public culture collections or by isolation from natural sources, e.g. from soil samples. Though the probability of success can be increased if microorganisms are selected which are already known for the desired function of bioremediation of contaminant, isolating an organism from the naturally occurring sites can prove far better than the procured cultures as they have been already acclimatized in that contaminated harsh conditions and may be growing profusely irrespective of the conditions.

Chen et al. (2005) studied the feasibility of using bioremediation technology for the remediation of chlorophenol-contaminated groundwater. In this study, a chlorophenol spill site was selected and because 2, 4-dichlorophenol was one of the major contaminants appearing at this site, it was used as the target compound in this study. Microcosm experiments were constructed to:

1. Determine the feasibility of applying different microbial consortia e.g., aquifer sediments, extracted supernatant of aquifer sediments, aerobic activated sludge collected from an industrial wastewater treatment plant, and anaerobic sludge collected from an up-flow anaerobic sludge bed for the biodegradation of 2, 4-dichlorophenol.
2. Evaluate the biodegradability of 2, 4-dichlorophenol under aerobic, anaerobic cometabolic, iron reducing, methanogenic and reductive dechlorinating conditions.
3. Determine the potential of enhancing 2, 4-dichlorophenol biodegradation using cane molasses, biological sludge cake and sodium acetate as the substitute primary substrates under aerobic cometabolic and reductive dechlorinating conditions.

The inoculum used in the microcosm study indicated that aquifer sediments, extracted supernatant of aquifer sediments and activated sludge could metabolize 2, 4-dichlorophenol directly without the addition of any extra carbon source and also indigenous microorganisms were capable of using 2, 4-dichlorophenol as their sole

carbon and energy source. Enhanced 2, 4-dichlorophenol biodegradation was not observed under co-metabolic conditions due to substrate competition. The preferential removal of added substrates caused decrease in 2, 4-dichlorophenol degradation rates. Under anaerobic conditions, the UASB sludge could biodegrade 2, 4-dichlorophenol only with the addition of sludge cake. Thus, a suitable carbon source for UASB sludge might play an important role in the removal of 2, 4-dichlorophenol. Moreover 2, 4-dichlorophenol biodegradation was not detected under any of the following anaerobic conditions such as iron reduction, reductive dechlorination and methanogenesis. This indicates that anaerobic biodegradation processes might not be the dominant biodegradation patterns at these sites and will also aid in designing a remedial system for such field application.

2.2.1.1 Influence of various factors on biodegradability of chlorophenols and chloroxylenols

The use of microbes for degradation of the chlorophenol and chloroxylenol is limited by many factors. Some of them are as follows: (1) Chemical structures of chlorophenol and chloroxylenol (2) The microbes (3) Environmentalfactors.

The less chlorinated phenols usually are less toxic and aerobic biodegradation of these chlorophenols are easy. It has been reported that mono-chlorophenols and di-chlorophenols could be easily degraded and mineralized to CO_2 and H_2O, along with the release of chloride when compared with poly-chlorophenols.

The degradability of microorganisms to chlorophenols was highly specific. Haggblom et al. (1995) used to observe the degradation of the three mono-chlorophenols under methanogenic cultures. 4-monochlorophenols was degraded the fastest, 3-monochlorophenols somewhat slower and 2-monochlorophenols the slowest. However, the reverse biodegradation rates of isomers mono-chlorophenols were observed under sulfate-reducing conditions. Generally, concentration of bacterium cells can affect degradation rate of chlorophenols. Sometimes it can change degradation pathway of substrate and final products. Deng-Yu et al. (1991) once reported resting cells of *Azotobacter sp*. strain GP1, an isolate which uses 2,4,6-trichlorophenol (TCP) as carbon source for growth, degraded 2,4,6-TCP only and transform it to 2,6-DCHQ at low cell density, however it could degrade completely or

partially mono-chlorophenols and di-chlorophenol into CO_2 and H_2O at high cell densities.Furthermore, environmental factors such as substrate concentration, medium pH, temperature and mineral salt components are important for biodegradation of chlorophenols and chloroxylenols.

2.2.1.1.1 Isolation and identification of microorganism degrading chlorophenols

The first step towards the application of bioremediation process is to select the suitable microorganism which is capable of remediation of the compounds which are of our interest. The organism of our interest has to be sought after rigorous screening procedures from a broad variety of microorganisms (Bacteria, actinomycetes, fungi and yeasts).However; pure cultures of microorganisms can be obtained from public culture collections or by isolation from natural sources like soil samples. Though the probability of success can be increased if microorganisms are selected which are already known for the desired function of remediation, isolating an organism from the naturally occurring sites can prove far better than the procured cultures as they have been already acclimatized in that contaminated harsh conditions and may be growing profusely irrespective of the conditions.The focus on the biodegradation of the chlorophenol and chloroxylenol in recent years has resulted in the isolation of a number of microorganisms that can grow on the compounds as a sole carbon and energy source.

Different types of media have been used for the isolation and enumeration of bacteria as reported by a number of researchers. Diane and Crawford (1985) isolated *Flavobacterium* strain that degrade pentachlorophenol. This bacterium was able to mineralize 100 to 200 mg/L of pentachlorophenol (PCP). Further, Schie and Young (1998) isolated and characterized phenol-degrading denitrifying bacteria. The three strains were shown to be different from each other based on physiologic and metabolic properties.Even though analysis of membrane fatty acids did not result in identification of the organisms, the fatty acid profiles were found to be similar to those of *Azoarcus species*. Sequence analysis of 16S ribosomal DNA also indicated that the phenol-degrading isolates were closely related to members of the genus *Azoarcus*. The results of this study add three new members to the genus *Azoarcus,*

which previously comprised only nitrogen-fixing species associated with plant roots and denitrifying toluene degraders.

Three strains of *Pseudomonas pickettii* that can grow with 2,4,6-trichlorophenol (2,4,6-TCP) as the sole source of carbon and energy were isolated from different mixed cultures of soil bacterial populations that had been acclimatized to 2,4,6-TCP by Kiyohara et al. (1992). These strains released 3 mol of chloride ion from 1 mol of 2, 4, 6-TCP during the complete degradation of the TCP. Of these strains, *P. pickettii* DTP0602 in high-cell-density suspension cultures dechlorinated various chlorophenols (CPs).

A pure culture of 2, 4-dichlorophenol degrading *Bacillus cereus* GN1bacteria was isolated from a natural enrichment by Matafonovaet al. (2006) that had been adapted to chlorophenols in the aeration pond of the Baikalsk pulp and paper mill (Russia). The 2, 4-dichlorophenol degradation rates by *Bacillus cereus* GN1 could be determined at concentrations up to 400 mM. However, higher concentrations of 2, 4-dichlorophenol (560 mM) were inhibitory to cell growth.

Pure cultures of 2-chlorophenol degrading bacteria were isolated from a natural enrichment that may be adapted to chlorophenols in the industrial zone at Umm-Saied city (Qatar) by Al-Thani et al. (2007). Degradation rates by isolates could be determined at concentrations up to 1.5 mM. However, higher concentrations of 2-chlorophenol (2.5 mM) were inhibitory to cell growth.

Gallizia et al. (2003) isolated nine bacterial strains capable of utilizing phenol and 2,4-dichlorophenol (DCP).One of these strains, a *Micrococcus sp*., was further investigated under aerobic conditions using phenol and DCP as sole carbon and energy sources at various pH values. Phenol degradation was enhanced under alkaline conditions, and up to 500mgdm^{-3} phenol was mineralised within 50 h at pH 10. DCP was more recalcitrant; however up to 883mgg^{-1} and 230mgg^{-1} were degraded within 10 days.

Goswami et al. (2005) studied the microbial metabolism of 2-chlorophenol, phenol and q-cresol by *Rhodococcus erythropolis* M1 in co-culture with *Pseudomonas fluorescence* P1. Rate of 2-chlorophenol degradation was higher when *Rhodococcus erythropolis* M1 was used as the pure culture as compared to the degradation rates observed with the *Pseudomonas fluorescence* P1 or with the mixed

culture. However, in case of phenol and r-cresol, degradation by the mixed culture had resulted in higher degradation rates as compared to the degradation of the substrates by both the axenic cultures.

Haggblom (1988) studied the degradation and o-methylation of chlorinated phenolic compounds by *Rhodococcus* and *Mycobacterium* strains. Three polychlorophenol-degrading *Rhodococcus* and *Mycobacterium* strains were isolated independently from soil contaminated with chlorophenol wood preservative and from sludge of a wastewater treatment facility of a Kraft pulp bleaching plant. All three strains initiated degradation of the chlorophenols by para-hydroxylation, producing chlorinated para-hydroquinones, which were then further degraded.

Decomposition of phenolic compounds and aromatic hydrocarbons by phenol-adapted bacteria was studied by Tabak et al. (1964).Bacteria from soil and related environments were selected or adapted to metabolize phenol, hydroxy phenols, nitro phenols, chlorophenols, methyl phenols, alkyl phenols, and aryl phenols when cultured in mineral salts media with the specific substrate as the sole source of carbon. Dihydric phenols were generally oxidized; trihydric phenols were not. Cresols and dimethyl phenols were oxidized; adding a chloro group increased resistance. Benzoic and hydroxybenzoic acids were oxidized; sulfonated, methoxylated, nitro, and chlorobenzoic acids were not; m-toluic acid was utilized but not the o- and p-isomers. Benzaldehyde and hydroxybenzaldehyde were oxidized. In general, nitro- and chloro-substituted compounds and the benzenes were difficult to oxidize.

Murialdo et al. (2003) investigated on degradation of phenol and chlorophenols by mixed and pure cultures. The cultures obtained were capable of degrading phenol and chlorophenols (pentachlorophenol 2,3,5,6 tetrachlorophenol and 2,4,6 trichlorophenol) but not 2,4,5 trichlorophenol.

Enrichment, isolation, and characterization of 4-chlorophenol degrading bacterium *Rhizobium sp.* 4-CP-20 was explored by Yang and Lee (2008). Strain *Rhizobium* sp. 4-CP-20 was isolated from the acclimatized mixed culture. Strain *Rhizobium sp*. 4-CP-20 could degrade 4-chlorophenol completely within 3.95 days, as the initial 4-chlorophenol concentration was 100 mg/l. If the initial 4-chlorophenol concentration was higher than 240 mg/l, the growth of bacterial cells and the activity of degrading 4-chlorophenol were both inhibited.

Kargi and Eker (2005) investigated the biological degradation of 2,4-dichlorophenol (DCP) by *Pseudomonas putida* CP1 in batch shake-flask cultures. Experiments were carried out at initial 2, 4-dichlorophenol concentrations between 50 and 750 mg/l and the rate and extent of 2, 4-dichlorophenol degradation were quantified. With increasing initial 2, 4-dichlorophenol concentrations in shake-flask cultures, percentage removal of 2, 4-dichlorophenol decreased and residual 2,4-dichlorophenol concentrations increased proportionally. The rate of DCP degradation increased with increasing initial 2, 4-dichlorophenol concentration up to 577 mg 2, 4-dichlorophenol /l. Further increase in 2, 4-dichlorophenol concentration caused decrease in the rate of degradation because of substrate inhibition. A kinetic model based on substrate inhibition was proposed. Rate and inhibition constants were determined using the experimental data**.**

Ronen et al. (2000) isolated a strain of bromophenol degrading bacteria from a contaminated desert soil. The isolate identified as *Achromobacter piechaudii* and designated as strain TBPZ was able to metabolize both 2, 4, 6-tribromophenol and chlorophenols. The degradation of halo phenols resulted in the stoichiometric release of bromide or chloride. Growth and degradation of bromophenol were enhanced in the presence of yeast extract. To follow the survival of introduced bacteria in the contaminated soil, TBPZ was transformed with a plasmid carrying a gene for kanamycin resistance and the lux CDABE operon from the luminescent bacteria Vibrio under the control of a constitutive promoter producing strain TBPZ-N61. The activity of the transformed bacteria was not affected by the insertion of the plasmid. Specific detection of the introduced isolate in the contaminated soil samples was achieved by selection on kanamycin. Survival of the introduced bacteria, TBPZ-N61, in the contaminated soil was influenced by soil moisture. Biodegradation of TBP occurred only in soil with at least 25% water content. Addition of yeast extract increased the survival and the activity of the introduced bacteria. The current study demonstrated that the limiting factors controlling pollutant degradation in a contaminated desert soil are water content, nutrient availability and the bio augmentation of an appropriate microbial population.

Gallizia et al. (2003) isolated nine bacterial strains capable of utilizing phenol and 2, 4-dichlorophenol from a mixed culture grown on substrates containing these

compounds. One of these strains, a *Micrococcus sp*., was further investigated under aerobic conditions using phenol and 2, 4-dichlorophenol as the sole carbon and energy sources at various pH values. Phenol degradation was enhanced under alkaline conditions, and up to 500mg dm^{-3} phenol was mineralised within 50 hours at pH 10. 2, 4-dichlorophenol was more recalcitrant; however up to 883mgg/1 and 230mgg /1 were degraded within 10 days, when using initial DCP concentrations of 100 and 200mgdm^{-3}, respectively. Biomass measurements showed cell growth, proving that both phenol and 2, 4-dichlorophenol are used as growth substrates for this isolate.

Valenzuela et al.(1997) studied and assessed the ability, efficiency and survival of *Alcaligenes eutrophus* JMP134(pJP4) and other indigenous microorganisms while degrading 2,4-dichlorophenoxyacetic acid, 2, 4, 6-trichlorophenol and other chlorophenols in bleached Kraft mill effluent in both short-term and long-term semi-continuous incubations. After 6 days of incubation, 2, 4-dichlorophenoxyacetate (400 mg/L) or 2, 4, 6-trichlorophenol (40 to 100 mg/L) was extensively degraded (70 to 100%). In short-term batch incubations, degradation of 2, 4, 6-trichlorophenol by strain JMP134 was significantly lowered at 200 to 400 mg/L of compound and indigenous microorganisms were unable to degrade at all thereby inhibiting the growth and survival of the JMP134 strain. This strain was also able to degrade 2, 4-dichlorophenoxyacetate, 2, 4, 6-trichlorophenol, 4-chlorophenol, and 2, 4, 5-trichlorophenol when bleached Kraft mill effluent was amended with mixtures of these compounds. In long-term (>1-month) incubations, strain JMP134 growth declined although extensive 2,4,6-trichlorophenol degradation was still observed probably due to acclimatization of the indigenous microorganisms to degrade 2,4,6-trichlorophenol. Hence, acclimatization was observed only in long-term semi-continuous incubations.

Toppl and Hanson (1990) cultured a *Flavobacterium sp*. in continuous culture limited for growth with ammonium, phosphate, sulfate, glucose, glucose + pentachlorophenol (0.065 h^{-1}) or pentachlorophenol alone. Cells were harvested, washed, and suspended to 3 x 10^7 cells /ml in shake flasks containing a complete mineral salt medium without added carbon or supplemented with 50 mg of pentachlorophenol /ml or 50 mg of pentachlorophenol / ml + 100 mg of glucose/ml. The pentachlorophenol concentration and the viable cell density were determined

periodically. Cells that were grown under phosphate, glucose, or glucose + pentachlorophenol limitation were more sensitive to pentachlorophenol and took longer to degrade 50 mg of pentachlorophenol /ml than the cells that were grown under ammonium, sulfate, or pentachlorophenol limitation. Glucose stimulated viability and pentachlorophenol degradation were found in all cases except when the cells were grown under carbon limitation with glucose and pentachlorophenol added together as the carbon source. These results indicated that there is a relationship between the nutrients.

Tikoo et al. (1997) isolated microalgae (VT-1) from pentachlorophenol treated water. Its growth and pentachlorophenol tolerance was compared with two known strains of *Chlorella sp.* and it was found to be more tolerant with an IC (24-25 mg dm^{-3}) value twice that of *C. vulgaris*. The ability of VT-1 to degrade 50e pentachlorophenol was tested using uniformly labeled pentachlorophenol and 14CO was released, indicating mineralisation. 14CO was not released in the presence of the other microalgae and only occurred in the light. Release was also stimulated by the presence of glucose in the light.

Fava et al.(1995) isolated and characterized a Gram-negative aerobic bacterium designated LD1 and identified to be a *Pseudomonas pickettii* strain, capable of using 2-chlorophenol (2-CP), 3-chlorophenol (3-CP) and chlorophenol (4-CP) as the sole carbon sources. LD1 was able to totally degrade and dechlorinate 2-CP (initial concentration: 1.51 mmol), 3-CP (initial concentration: 0.57 mmol) and 4-CP (initial concentration: 0.75 mmol) in a duration of; within 30, 30 and 40 hours of incubation respectively, under growing-cell batch conditions. LD1 was also found to be able to metabolize chlorocatechols in growing and resting-cell conditions suggesting that the bacterium degrades mono-chlorophenols through a chlorocatechol pathway. Also LD1 was found to be capable of readily metabolizing other organic compounds such as phenol, benzoic acid, hydroxybenzoic acid and hydroquinone giving it the potential for being successfully used in the bio-treatment of wastewater and in soil decontamination.

Kim and Picardal (2001) isolated bacterial strains capable of aerobic growth on ortho-substituted dichlorobiphenyls as the sole carbon and energy sources. During growth on 2, 2-dichlorobiphenyl and 2, 4-dichlorobiphenyl, strain SK-4 produced

stoichiometric amounts of 2-chlorobenzoate and 4-chlorobenzoate respectively. Chlorobenzoates were not produced when strain SK-3 was grown on 2, 4-dichlorobiphenyl.

Goswami et al. (2005) studied the interaction of *Rhodococcus erythropolis* M1 with the ability to degrade 2-chlorophenol, phenol and r-cresol (100 mg/l, each) in 18, 24 and 20 hours respectively with negligible lag with other bacteria isolated from activated sludge for water treatment like *Pseudomonas fluorescence* P1 on 2-chlorophenol, phenol and r-cresol substrates. Viable cell counts showed competitive interaction between the species on 2-chlorophenol and phenol and a higher specific growth rate for pure culture of *R. erythropolis* M1 than *P. fluorescence* P1 on 2-chlorophenol. However, in mixed culture, *P. fluorescence* P1 showed higher specific growth rate. Further studies and growth rate comparison suggested that phenol and r-cresol degradation by mixed culture resulted in higher degradation rates as compared to the degradation of the substrates by both the axenic cultures.

Haggblom et al. (1998) isolated three polychlorophenol degrading strains of *Rhodococcus sp.* and *Mycobacterium sp*. from soil contaminated with chlorophenol wood preservative and sludge from a wastewater treatment facility of a kraft pulp bleaching plant. The *Rhodococcus sp.* strain CG-1 and *Mycobacterium sp*. strain CG-2, isolated from tetrachloroguaiacol enrichment, and *Rhodococcus sp.* strain CP-2 isolated from pentachlorophenol enrichment mineralized pentachlorophenol and degraded several other polychlorinated phenols, guaiacols (2-methoxyphenols) and syringols (2,6-dimethoxyphenols) at micromolar concentrations and were sensitive to the toxic effects of pentachlorophenol. All three strains initiated degradation of the chlorophenols by para hydroxylation, producing chlorinated para-hydroquinones which were then further degraded. Parallel to degradation strains CG-1, CG-2, and CP-2 also o-methylated nearly all chlorinated phenols, guaiacols, syringols and hydroquinones.

Im et al. (2004) reclassified a 4-chlorophenol degrading bacterial strain, formerly designated as a strain of *Comamonas testosterone*; as a member of the *genus Herbaspirillum* based on its phenotypic, chemotaxonomic characteristics as well as phylogenetic analysis using 16S rDNA sequences. From this analysis, it was inferred that the strain CPW301T clusters in a phylogenetic branch that contains

Herbaspirillum sp. and the similarity was in the range 98.7-98.9% but was clearly distinguishable. Based on the study, the strain does not fix nitrogen, is not plant-associated and is an aerobic rod with one unipolar flagellum. On the basis of these characteristics, a novel *Herbaspirillum species, Herbaspirillum chlorophenolicum sp*., was proposed and the type strain was named CPW301T.

Valli and Gold (1990) reported that degradation of 2, 4-dichlorophenol by *P.chrysosporium* involves several cycles of oxidation and subsequent quinone reduction and hydroquinone methylation. These cycles led to the removal of both chlorine atoms from the substrate. The ability of *Alcaligenes eutrophus* JMP134 (pJP4) to degrade 2, 4-dichlorophenoxyacetic acid, 2, 4, 6 trichlorophenol and other chlorophenols in a bleached Kraft mill effluent was studied by Valenzuela et al. in 1997. It was observed that 2, 4-dichlorophenoxyacetate (400 mg/L) or 2, 4, 6-trichlorophenol (40 to 100 mg/L) were extensively degraded (70 to 100%).

The enrichment of mixed cultures for species capable of degrading phenol and chlorophenols, as well as the isolation of pure cultures are investigated by Murialdo et al. (2003). The cultures obtained were capable of degrading phenol and chlorophenols (pentachlorophenol, 2, 3, 5, 6-tetrachlorophenol and 2, 4, 6-trichlorophenol) but not 2, 4, 5 trichlorophenol.

Other works also reported on chlorophenol degradation by mixed cultures. Three strains of *Pseudomonas pickettii* that grew with 2,4,6-trichlorophenol (2,4,6-TCP) as the sole source of carbon and energy were isolated by Kiyohara (1991) from different mixed cultures of soil bacterial populations that had been acclimatized to 2, 4, 6-TCP. These strains released 3 mole of chloride ion from 1 mol of 2, 4, 6-TCP during the complete degradation of the TCP. Nine bacterial strains capable of utilizing phenol and 2, 4-DCP has been isolated from a mixed culture by Gallizia et al. (2003). One of these strains, a *Micrococcus* sp., was further investigated under aerobic conditions using phenol and DCP as sole carbon and energy sources at various pH values. Phenol degradation was enhanced under alkaline conditions, and up to 500mg/dm^3 phenol was mineralized within 50 h at pH 10. DCP was more recalcitrant; however up to 883mg.g^1 and 230mg.g^1 were degraded within 10 days, when using initial DCP concentrations of 100 and 200mg/dm^3, respectively.

Murialdo et al. (2003) studied the enrichment of mixed cultures for species capable of degrading phenol and chlorophenols and also the isolation of pure cultures. The cultures obtained were capable of degrading phenol and chlorophenols (pentachlorophenol 2,3,5,6 tetrachlorophenol and 2, 4, 6 trichlorophenol) but not 2, 4, 5 trichlorophenol. The results suggest the feasibility of the use of these toxic chemicals as phenols, hexadecane and other chlorophenols as co-substrates in field decontamination processes.

Chandra et al. (2008) isolated two PCP-degrading bacterial strains, *Bacillus cereus* (ITRC-S6) and *Serratia marcescens* (ITRC-S7). These two bacterial strains effectively reduced colour (45-52%), lignin (30-42%), BOD (40-70%), COD (50-60%), total phenol (32-40%) and PCP (85-90%) within 168 hour of incubation of pulp and paper mill effluent. Herrera et al. (2008) assessed the aerobic biodegradation of 2, 4-dichlorophenol (2,4-DCP) by a consortium of four *Bacillus* species that were isolated from a polluted soil by enrichment using a mixture of chlorophenols. The bacterial consortium effectively biodegraded 2-chlorophenol, 3-chlorophenol and 2, 4-dichlorophenol at degradation rates of between 1.7 and 6.7 moles/h.

2.2.1.1.2 Media composition for isolating microorganism

Different types of media have been used for the isolation and enumeration of bacteria as reported by a number of researchers. A pure culture of 2,4-DCP degrading bacteria was isolated by Matafonova et al.(2006) from a natural enrichment culture that had been adapted to chlorophenols in the aeration pond, using mineral medium (MM: g/L: K_2HPO_4-0.65; KH_2PO_4-0.19; $NaNO_3$-0.5; $MgSO_4.7H_2O$-0.1; $FeSO_4.7H_2O$-0.005; $(NH_4)_2SO_4$-0.5, supplemented with 2,4-DCP as a growth substrate. Their investigation revealed that 2,4-DCP degradation rate by isolated strain could be determined at concentration up to 400 mM. However, higher concentrations of 2,4-DCP (560 mM) were inhibitory to cell growth. Gallizia et al.(2003) reported the degradation of phenol and 2,4-dichlorophenol isolating the *Micrococcus sp.* using mineral salts medium containing (g dm^{-3}):$(NH_4)_2SO_4$-0.1; $Ca(NO_3)_2$-0.02; $FeSO_4 \cdot 7H_2O$-0.01; $MgSO_4 \cdot 7H_2O$-0.1; K_2HPO_4-0.05; KH_2PO_4-0.03; and 1cm^3 trace mineral solution per dm^3 medium containing (gdm^{-3}): $MgSO_4 \cdot H_2O$-0.5;$CoCl_2 \cdot 6H_2O$-0.1; $CaCl_2$-0.1; $ZnSO_4 \cdot 7H_2O$-0.1;$CuSO_4 \cdot 5H_2O$-0.01; H_3BO_3-0.01 at various pH values.

Phenol degradation was enhanced under alkaline conditions and up to 500 mg/dm^3 phenol was mineralised within 50 hour at pH 10. DCP was more recalcitrant; however up to 883 mg/g and 230 mg/g were degraded within 10 days, when using initial DCP concentrations of 100 and 200 mg/dm^3, respectively. Biomass measurements showed cell growth, proving that both phenol and DCP are used as growth substrates for this isolate.Minimal salt medium (MP) (2.75 g of K_2HPO_4, 2.25 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 0.2 g of $MgCl.6H_2O$, 0.1 g of NaCl,0.02 g of $FeCl_3.6H_2O$, 0.01 g of $CaCl_2$, pH 7.0 and H_2O to 1 L)was investigated by Al-Thani et al. (2007) for degradation of 2-chlorophenol.Degradation of 2-chlorophenol was studied using theses cultures in liquid medium under aerobic conditions, at initial concentrations of 0.25-2.5mM 2-chlorophenol. Degradation rates by isolates could be determined at concentrations up to 1.5mM. However, higher concentrations of 2-chlorophenol (2.5 mM) were inhibitory to cell growth.For the isolation of bacteria degrading pentachlorophenol (in grams of ingredient per liter):$NaNO_3$-0.5; K_2HPO_4-0.65; KH_2PO_4-0.17 and $MgSO_4$-0.1 were used by Saber et al. (1985). They reported that all isolated strains metabolized PCP as a sole source of carbon and energy; 73 to 83% of all carbon in the form of [U-14C] PCP was returned as $14CO_2$, with full liberation of chlorine as chloride. The isolation steps for the degradation of pentachlorophenol were performed by Stanlake and Finn (1982) in media with a mineral salts (MS) base of the following composition (in grams per liter): K_2HPO_4-1.73; KH_2PO_4-0.68; NH_3NO_3-1.0; $MgSO_4.7H_2O$-0.1; $CaCI_2.2H_20$-0.02; $MnSO_4.H_2O$-0.03 and $FeSO_4.7H_2O$-0.03. The strain was shown to degrade pentachlorophenol completely. Kiyohara and co-workers (1992) used basal mineral salts solution composed of 15 mM $(NH_4)SO_4$, 1 mM $MgSO_4.7H_20$, 10 μM $FeSO_4.7H_20$,100 μM $CaCO_3$, and8.5 mM $NaNO_3$ in phosphate buffer (10 mM Na_2HPO_4-KH_2PO_4, pH 7.0)to isolate *Pseudomonas pickettii* that can grow with 2,4,6-trichlorophenol as the sole source of carbon and energy. *Pseudomonas pickettii* released 3 mol of chloride ion from 1 mol of 2,4,6-trichlorophenol during the complete degradation of the trichlorophenol.

Six Gram-negative, oxidase- and catalase-positive, non-fermentative small rods were isolated by Puhakka et al. (1995) in tryptone-yeast extract-glucose (TGY) agar media supplemented with 10 mg/l of 2, 3, 4, 6-tetrachlorophenol (TeCP) and

pentachlorophenol (PCP). All isolates formed colonies in TGY plus 150 mg/l of PCP. The isolates degraded 2, 4, 6-trichlorophenol (TCP) and 2, 3, 4, 6-tetrachlorophenol (TeCP) but not pentachlorophenol (PCP) . But in batch-bottle bioassays, the reactor enrichment degraded PCP, TeCP and TCP both in mineral salts (MS) and tryptone-yeast extract-glucose (TGY) media.

Degradation of CP [2-CP, 3-CP, 4-CP, 2, 4-dichlorophenol (DCP) or 2, 6-DCP] under denitrifying conditions was examined in anaerobic batch culture inoculated with activated sludge by Bae et al.(2002). Although 3-CP, 4-CP, 2, 4-DCP and 2,6-DCP were not stably degraded, 2-CP was degraded and its degradation capability was sustained in a subculture. In 2-CP-degrading cultures, nitrates has consumed stoichiometrically and concomitantly during 2-CP degradation, and a dechlorination intermediate has not detected, suggesting that 2-CP degradation was coupled with nitrate reduction. A 2-CP-degrading enrichment culture degraded 2-CP in the presence of nitrate, but did not in the absence of nitrate or the presence of sulphate. This suggests that the enrichment culture strictly requires nitrate for degradation of 2-CP.

Jianlong et al. (1999) reported on the feasibility of using glucose as an added substrate for cometabolic transformation of 4-chlorophenol (4-cp). When glucose was fed as the added growth substrate, only 78% and 43% of the initial 4-cp concentrations of 100 and 200 mg / l, respectively, were transformed before the pH dropped to below 4.5 and stopped all reactions. By maintaining the medium pH, complete removal of 4-cp was achieved even at the high initial concentration of 200 mg/ l. Compared with phenol as the added growth substrate, cells grown on glucose displayed a longer acclimatization phase and, in general, a lower specific transformation rate.

The influence of high concentrations of pentachlorophenol (PCP) and readily metabolizable carbon on the activity and viability of a PCP-degrading *Flavobacterium sp.* was examined in a mineral salts medium by Topp et al. (1998). Lags preceding PCP removal by glutamate-grown *Flavobacterium* cells were greatly attenuated by the addition of glutamate, aspartate, succinate, acetate, glucose, or cellobiose. The effect of these supplementary carbon sources on the apparent lag was not mediated entirely through the stimulation of growth since PCP metabolism accompanied the

onset of growth. The specific activity of PCP-degrading cells in the absence of supplementary carbon was 1.51 x 10(-13) +/- 0.08 x 10(-13) g of PCP per cell per h and in the presence of supplementary carbon was 0.92 x 10(-13) +/- 0.09 x 10(-13) g of PCP per cell per hour.

2.2.1.1.3 *Bacillus cereus*

Chandra et al. (2008) studied on reduction of pollutants in pulp paper mill effluent treated by PCP-degrading bacterial strains. Two PCP-degrading bacterial strains, *Bacillus cereus* (ITRC-S6) and *Serratia marcescens* (ITRC-S7) were used for the treatment of pulp and paper mill effluent at conditions; 1.0% glucose and 0.5% peptone at 30^0C at 120 rpm for 168 hour of incubation. These two bacterial strains effectively reduced colour (45–52%), lignin (30–42%), BOD (40–70%), COD (50–60%), total phenol (32–40%) and PCP (85–90%) within 168 hour of incubation. However, the highest reduction in colour (62%), lignin (54%), BOD (70%), COD (90%), total phenol (90%) and PCP (100%) was recorded by mixed culture treatment.

Hu et al.(2007) investigated on the biosorption of cadmium by a Cd^{2+}hyper resistant *Bacillus cereus* strain HQ-1 newly isolated from a lead and zinc mine. The results indicated that this *Bacillus cereus* strain had excellent potential for biosorption of Cd.

Biodegradation of 2, 4-dichlorophenol by a *Bacillus* consortium was assessed by Herrera et al. (2008). The bacterial consortium effectively biodegraded 2-chlorophenol, 3-chlorophenol and 2,4-dichlorophenol at degradation rates of between 1.7 and 6.7 moles $l^{-1}h^{-1}$. In the presence of NH_4Cl or KNO_2 as nitrogen sources, 2, 4-DCP was variously degraded. Under both conditions, cell biomass attained highest values of 350 and 450 mg l^{-1} respectively, while the amounts of 2,4-DCP metabolized in 21 days reached peak values of 2.1 and 2.5 mM representing between 70 and 85% degradation, respectively.

2.2.1.2 Degradation mechanism

Mineralization of chlorophenol and chloroxylenol is a two-step process viz., cleavage of the aromatic ring and removal of the chlorine atom (Haggblom 1990).Krooneman et al. (1996) believed the presence or absence of molecular oxygen plays a crucial role

in determining the fate and biodegradation mechanisms of aromatic compounds. In general, under aerobic conditions, the 2.4-DCP and PCMX is transformed *via*.oxidative dechlorination, while in anaerobic ambience *via* reductive dechlorination.

2.2.1.2.1 Oxidative dechlorination

For the aerobic biodegradation of 2, 4-DCP and PCMX, two different mechanisms have so far been described. Degradation of mono- and dichlorophenols was shown to be initiated by oxygenation into chlorocatechols, and dechlorination occurred only after ring cleavage of the chlorocatechols (Haggblom 1990). Chlorocatechols are central metabolites in the aerobic degradation of a wide range of chlorophenol and chloroxylenols. Following transformation of the chlorophenols to chlorocatechols, ring cleavage by dioxygenases may proceed. Cleavage of the aromatic ring may occur using either the ortho- or the meta-pathway. Aromatic compounds which are found naturally in the environment, such as phenol and benzene, are typically broken down via the meta-cleavage pathway while, chlorophenol and chloroxylenol are generally broken down via the ortho-pathway. Two types of ortho-pathways exists -ortho-type I pathway, involved in the degradation of unchlorinated aromatics, *via* catechol and a modified ortho-type II pathway specific to the degradation of chlorinated aromatics, via chlorocatechol.Ortho-cleavage (Type II) of chlorophenol and chloroxylenol catalyzed by the enzyme catechol 1,2-dioxygenase is generally necessary for complete degradation with a resulting release of chloride atom. Tezukau and Iwasaki (1998) investigated the reactions of organic compounds in water induced by the plasma which was generated in a localized zone between an electrolytic solution and an anode. Each of three isomeric mono-chlorophenols, employed as models of pollutants, underwent dechlorination followed by oxidative degradation and eventually decomposed to inorganic carbon. It was also clarified that the chlorine atom could be liberated as chloride ion. On the basis of the detailed analysis of the intermediate products and kinetic consideration, a reaction pathway was proposed where the successive attack of hydroxyl radicals on the benzene rings of starting materials was presumed to be the key steps, especially in the initial stage of the overall reactions

Farrell and Quilty (1989) specifically designed a mixed microbial community with the ability to degrade mono-chlorophenols as the sole carbon source in an aerobic batch culture via the meta-cleavage pathway. During the degradation of 2-chlorophenol and 3-chlorophenol, 3-chlorocatechol was produced which led to inactivation of the catechol 2,3-dioxygenase which is toxic to cells resulting in incomplete degradation. Successful degradation of chlorinated organic compounds via 3- chlorocatechol using the meta-cleavage pathway has been demonstrated by Mars et al. (1997) but is extremely rare. Generally meta-cleavage of 3-chlorocatechol results in the inactivation of catechol 2,3-dioxygenase either by 3-chlorocatechol itself, acting as a chelating compound (Klecka and Gibson 1981) or by the production of a highly reactive acyl chloride, the product of the cleavage of 3-chlorocatechol, which binds irreversibly to the meta-cleavage enzyme (Bartels et al. 1984). The meta-cleavage of 4-chlorocatechol results in the production of a chlorinated aliphatic compound, 5-chloro-2-hydroxymuconic semi aldehyde (Weiser et al.1994) which has been widely reported as being a dead-end metabolite (Reinekeet al. 1982; Westmeier and Rehm 1987). However, recent reports have shown that further metabolism of 5-chloro-2-hydroxymuconic semi aldehyde may occur, resulting in complete degradation of 4-chlorophenol via a meta-cleavage pathway (Bae et al. 1996; Hollenderet al. 1997).*Alcaligenes eutrophus* JMP222, a derivative of *A. eutrophus* JMP134 which has lost plasmid pJP4 (encoding the tfdgenes for the ortho fission pathway), was induced for the meta-fission pathway when grown on o-cresol by Koh et al. (1997).Resting cell suspensions, grown on o-cresol, oxidized 2,4-dichlorophenol (2,4-DCP) a degradation product of 2,4-dichlorophenoxyacetic acid to 3,5-dichlorocatechol. Further degradation of 3,5-dichlorocatechol was observed by the production of a yellow ring fission product with liberation of chloride.Oxidation of 2,4-DCP (305 mM) in 47 hours resulted in 69% dehalogenation through this pathway.

A different mechanism has been described for the degradation of polychlorinated phenols. In the presence of pentachlorophenol-metabolizing *Ralstonia chlorophenolicus* andin a *Flavobacterium sp.,* degradation of PCP started by hydrolytic para-hydroxylation, yielding chlorinated para-hydroquinone. Bae et al. (1996) found that *Arthrobacter ureafaciens* and *Arthrobacter chlorophenolicus* degraded 4-chlorophenol via a new hydroquinone pathway in which the chloro-

substituent was eliminated in the first step and hydroquinone was produced as a transient intermediate. Strain *Arthrobacter chlorophenolicus* exhibited much higher substrate tolerance and degradation rate than other strains that degraded 4-chlorophenol by the hydroxylation at the second carbon position to form chlorocatechols and was also found to degrade other para-substituted phenols (4-nitro, 4-bromo-, 4-iodo- and 4-fluoro-phenol) *via* the hydroquinone pathway.Nordin et al (2005) found that *Arthrobacter chlorophenolicus* A6, a previously described 4-chlorophenol-degrading strain, was found to degrade 4-chlorophenol via hydroxyquinol, which is a novel route for aerobic microbial degradation of this compound. In addition, 10 open reading frames exhibiting sequence similarity to genes encoding enzymes involved in chlorophenol degradation were cloned and designated part of a chlorophenol degradation gene cluster (cphgenes). These open reading frames included two genes, cphA-I and cphA-II, which were shown to encode functional hydroxyquinol 1,2-dioxygenases. Disruption of the cphA-I gene yielded a mutant that exhibited negligible growth on 4-chlorophenol, there by linking the cphgene cluster to functional catabolism of 4-chlorophenol in *A. chlorophenolicus* A6. This hydroquinone pathway was also described for 2, 4, 5-trichlorophenoxyacetate-degrading *Pseudomonas cepacia.*

It may be more interesting that Li et al. (1991) have found the existence of different catabolic pathways for phenolic compounds in *Azotobacter sp*. strain GP1: the catechol pathway for phenol degrading and the hydroquinone pathway for chlorinated phenol degradation. It is deserved to be mentioned that breakdown processes of chlorocatechols occurred in the catechol pathway.

In general, after the formation of catechols, methyl-substituted aromatic compounds are degraded via the metaring cleavage pathway by catechol 2, 3-dioxygenases, whereas chloroaromatic compounds are mineralized *via* the ortho-cleavage pathway by 1, 2-dioxygenases. Fig. 2.1 illustrates the degradation pathway for the mechanism of chlorocatechol via ortho ring. However, Hollender et al. (1997) proved the pure culture in *Comamonas testosteroni* JH5, which could completely mineralize a mixture consisting of 4-CP and 4-methylphenol (4-MP), could mineralize 4-CP *via* Meta ring fission. The metabolic pathways are displayed in

Fig. 2.2. In result, it is possible to transform the chlorinated and methyl-substituted aromatic compounds simultaneously.

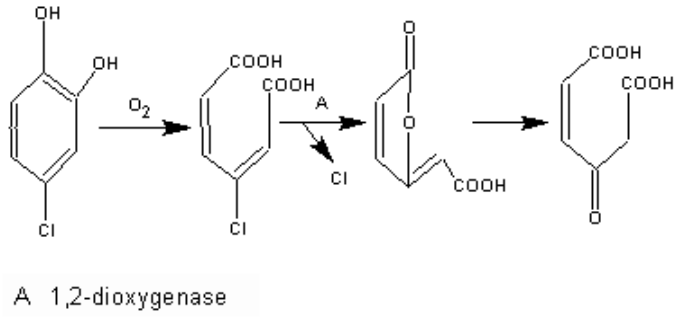


Fig. 2.1: Degradation pathway for chlorocathacol via ortho ring fission

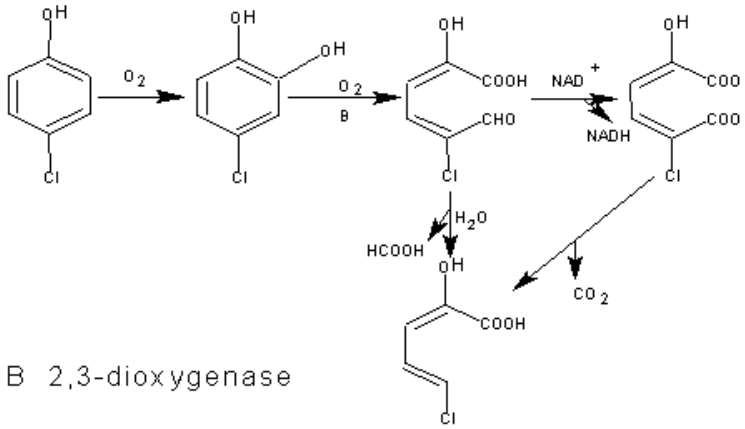


Fig. 2.2: Illustration of degradation pathway for 4-CP via Meta ring fission

2.2.1.2.2 Reductive dechlorination

Anaerobic biodegradation of chlorophenols occurs by reductive dechlorination. In this process, chlorines are replaced with hydrogen, while degrading microorganisms use chlorinated chlorophenols as terminal electron acceptors in an anaerobic respiration. Therefore reductive dechlorination is partially or completely inhibited by the presence of other electron acceptors such as sulfate, nitrate, O_2 and CO_2. Sulfate may be the important electron acceptor influencing dehalogenation and anaerobic degradation of chlorinated aromatic compounds.

Anaerobic dechlorination is very important for biodegradation of chlorophenols, especially for polychlorinated phenols. Most of the polychlorinated phenols that are resistant to aerobic microbial metabolism can be biodegraded under

anaerobic conditions. The less chlorinated metabolic products from reductive dechlorination of polychlorinated phenols are generally less toxic and degraded more easily than the parent compound. The reductive dechlorination process under the anaerobic condition is of environmental importance also because anoxic condition in soils, as well as bottom layers of aquatic sediments and freshwater and marine ecosystems are often prevailing. Studies on the distribution and fate of PCP in Japanese rice paddy fields provide some of the earliest information concerning anaerobic chlorophenol biotransformation (Nicholson et al. 1992).Cole et al. (1994) isolated a bacterium capable of anaerobic growth via reductive dehalogenation of 2-chlorophenol from a culture enriched with sediments taken from a small stream. Only the ortho position was dehalogenated and additional chlorines at other positions decreased or blocked ortho- dechlorination. Reductive dechlorination of chlorophenols has been observed for unacclimatized and acclimatized anaerobic sewage sludge, sediments, and the microbial communities, such as methanogenic consortia and sulfate-reducing bacteria. Boyd and Shelton (1984) investigated the anaerobic biodegradation of mono- and di-chlorophenol isomers by fresh (unacclimatized) sludge and also by sludge acclimatized to 2-chlorophenol, 3-chlorophenol, or 4-chlorophenol. In unacclimatized sludge, each of the monochlorophenol isomers was degraded. For the dichlorophenols in un-acclimatized sludge, reductive dechlorination of the chlorine group ortho- to phenolic OH was observed and the monochlorophenol compounds released were subsequently degraded.

Cole et al (1994) isolated a bacterium capable of anaerobic growth via reductive dehalogenation of 2-chlorophenol from a culture enriched with sediments taken from a small stream near Lansing, Michigan. Only the ortho position was dehalogenated and additional chlorines at other positions decreased or blocked ortho-dechlorination. The organism also grew with fumarate as its electron acceptor hence it was possible to induce de-chlorination. Analysis of the organism's 16S rRNA sequence revealed that it is a member of the delta proteobacteria.

In general, chlorines in positions ortho to the hydroxyl group were removed more readily than those in the Meta or para position.Nicholson et al. (1992) reported that acclimatized microbial consortia had yielded biotransformation pathways

different from those of unacclimated. They observed that the methanogenic consortium having been exposing to PCP for 10 days dechlorinated PCP principally at the ortho position. However, acclimation of the organisms to PCP over period of 6 months produced a microbial consortium with the ability to remove chlorines from the ortho, meta and para positions of PCP. The alternative processes are shown in Fig. 2.3.

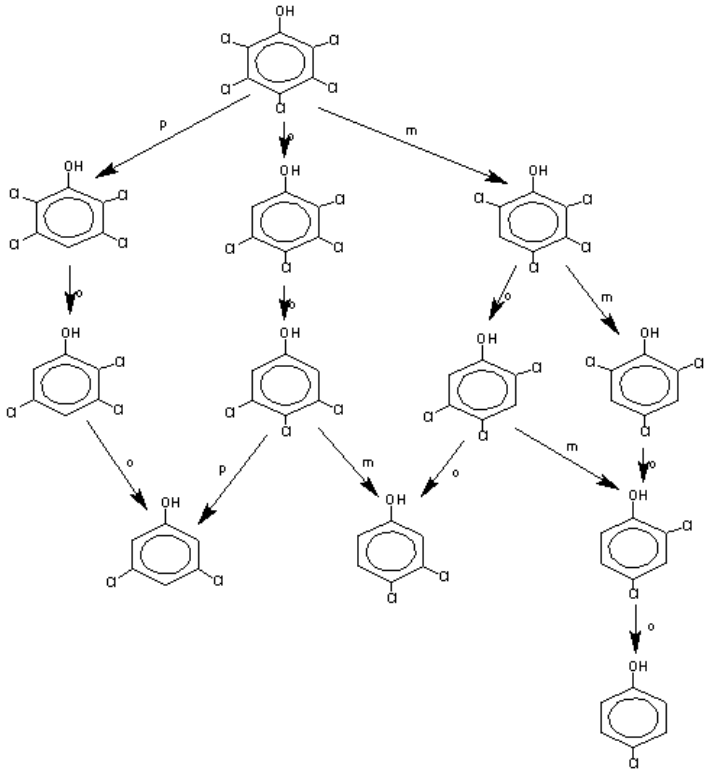


Fig. 2.3: Illustration of the reductive dechlorination pathway for PCP and its metabolites by a PCP-acclimated methanogenic consortium

Most of reductive dehalogenation studies have used with mixed cultures, and only a few stable enrichments or pure cultures of dechlorinating anaerobes exist. Ferguson et. al. (1991) believed that the anaerobic transformation of chlorinated aromatic compounds depends on a close association between different bacteria for the following understandings: Firstly, strict anaerobic bacterium needs an anaerobic or reductive circumstance which can be supplied by other bacteria; Secondly, the electron donors needed by anaerobic bacteria may be the final products of other anaerobic bacteria metabolism; Finally, the toxic products of biodegradation of

chlorophenol can be eliminated by other bacteria. Hence, reductive dechlorination of chlorophenol is liable to take place in microbial consortium.

2.2.1.3 Effect of environmental factors on degradation of chlorophenol and chloroxylenol

Various factors like pH, temperature, carbon, nitrogen etc. play important role in degradation of pollutants.The effect of these parameters on the growth and reduction of chlorophenol and chloroxylenol has been studied by many researchers.

2.2.1.3.1 Temperature

Bacteria isolated from soil often grow best at 20 to 30^0C. Low temperature may limit bioremediation by having a negative impact on enzyme activity and the ability of cells to take up the pollutant (Leung et al.1997; Nedwell 1999).Backman and Jansson (2004) investigated the cold tolerance of *Arthrobacter chlorophenolicus*, a microorganism to degrade high concentrations of 4-chlorophenol. They reported that the *Arthrobacter chlorophenolicus* strain could degrade (200–300 g/mL) 4-chlorophenol in pure cultures incubated at 5°C, although rates of degradation, growth and the metabolic status of the cells were lower at 5°C compared to 28°C.Similarly,Melin (1998) studied the effects of temperature on chlorophenol biodegradation kinetics in fluidized-bed reactors with different biomass carriers.The effect of temperature on chlorophenol degradation kinetics was studied in FBR batch tests at temperatures ranging from 4 to 16.5°C. He concluded that in the studied temperature range, a 10°C decrease in temperature generally resulted in over seven time's slower degradation rates.

Mannisto et al. (2001) studied on degradation of 2, 3, 4, 6-tetrachlorophenol at low temperature and low dioxygen concentrations by phylogenetically different groundwater and bioreactor bacteria. Effects of low temperature and low oxygen partial pressure on the occurrence and activity of 2, 3, 4, 6- tetrachlorophenol degrading bacteria in a boreal chlorophenol contaminated groundwater and a full-scale fluidized bed bioreactor were studied using four polychlorophenol degrading bacterial isolates of different phylogenetic backgrounds. These isolates with optimum growth temperatures between 23 and 25°C, degraded tetrachlorophenol faster at 8°C

than at room temperature indicating distinctly different temperature optima for chlorophenol degradation and growth on complex media. These results show efficient polychlorophenol degradation by the isolates at the boreal groundwater conditions, i.e., at low temperature and low oxygen concentrations. Differences in chlorophenol degradation and sensitivities to chlorophenols and oxygen among the isolates indicate that the phylogenetically different chlorophenol degraders have found different niches in the contaminated groundwater and thus potential for contaminant degradation under a variety of saturated subsurface conditions.

Hannaford and Kuek (1999) studied aerobic batch degradation of phenol using immobilized *Pseudomonas putida*.The degradation rate of phenol by alginate-immobilized *Pseudomonas putida* was little affected by alginate concentration between 2 and 4%. Ten degree shifts from 25^{o}C in reaction temperature resulted in approximately 30% slower degradation. Maximal degradation rates appeared to be favored at pH 5.5 - 6.0.

2.2.1.3.2 pH

Like temperature, pH also plays a role in determining the ability of bacterial growth and degradation rate in particular environments. Most commonly, bacteria grow optimally within a narrow range of pH between 6.7 and 7.5. A study of effect of pH and inoculum size on pentachlorophenol degradation by *Pseudomonas sp.* was investigated by Wolski et al.(2006).Pentachlorophenol was degraded rapidly at pH values from 6.3 to 8, but the maximum rate of pentachlorophenol degradation by *Pseudomonas sp.* was at pH 6.3. In contrast, the pentachlorophenol degradation kinetics at pH 5.5 was significantly lower, although Pentachlorophenol was totally depleted. These results show the broad range of pH for pentachlorophenol degradation for this strain. Pentachlorophenol was degraded at every inoculum size tested and pentachlorophenol degradation increased with the increasing inoculum size, but cultures inoculated with the lowest inoculum showed the highest specific consumption rate. This reveals a lower consumption of pentachlorophenol per CFU at a high population density.

Similar experiments were carried out by Gonzalez-Juarez et al. (2008) on influence of pH on the degradation 4-chlorophenol by gamma radio catalysis using

SiO_2, Al_2O_3 and TiO_2.It was found that the degradation of 4-chlorophenol is enhanced at pH between 3 and 7.

Biodegradation of phenol by native microorganisms isolated from coke processing wastewater was determined by Chakraborty et al. (2010). Phenol degrading performance of all the strains was evaluated initially. One of the strains namely ESDSPB2 was found to be highly effective for the removal of phenol, which was used as sole carbon and energy source. From an initial concentration of 200 mg l-1 it degraded to 79.84 ± 1.23 mg l^{-1}. In turn, the effect of temperature (20 to 45◦C), pH (5–10) and glucose concentration (0, 0.25 and 0.5%) on the rate of phenol degradation by that particular strain was investigated. Observations revealed that the rate of phenol biodegradation was significantly affected by pH, temperature of incubation and glucose concentration. The optimal conditions for phenol removal were found to be pH of 7 (84.63% removal), temperature, 300C (76.69% removal) and 0.25% supplemented glucose level (97.88% removal).

Gayathri and Vasudevan (2010) investigated the effect of pH, nitrogen sources and salts on the degradation of phenol by the bacterial consortium under saline conditions. The consortium contained 6 bacterial strains of *Bacillus cereus, Arthrobacter sp., Bacillus licheniformis, Halomonassalina, Bacillus subtilis and Pseudomonas aeruginosa* by 16 S rRNA sequencing. The isolated bacterial consortium showed maximum degradation in the presence of yeast extract at pH 7. Replacement of yeast extract with tryptone and urea affected the degradation efficiency of phenol. Presence of complex salts did not affect the degradation of phenol and growth of the bacterial consortium.

2.2.1.3.3 Effect of nutrient

Low biodegradation rates may be due to limited accessibility of the nutrients and growth of bacteria. The influences of mycelium acclimation, co-substrate concentration and nitrogen source on chlorophenol degradation by *Phanerochaete chrysosporium* were analyzed by Perez (1997). A decrease in glucose concentration caused a decrease in chlorophenol degradation rate. Twelve days were needed for complete degradation of O-chlorophenol with l0 g/l of glucose and 22 days when glucose concentration was decreased to 2.5g/l. The reduction of ammonium tartrate

caused a greater lag time, but not a decrease in chlorophenol degradation rate. Replacement of ammonium tartrate by ammonium chloride caused a decrease in chlorophenol degradation rate.Related work was carried out by Loh et al. (1998) by degrading phenol and 4-chlorophenol by augmenting the medium with conventional carbon sources such as sodium glutamate and glucose. Addition of 1 gl/1 sodium glutamate significantly improved the biodegradation rates of both phenol and 4-chlorophenol. On the other hand, supplementation of glucose caused a significant drop in the medium pH from 7.2 to 4.3 resulting in a reduction of degradation rate.

Tarighian et al. (2003) investigated the enhancement of 4-chlorophenol biodegradation using glucose by *Pseudomonas putida*. They verified that glucose was the superior growth co-substrate, suggesting that inexpensive sugars can be used to enhance the biodegradation of chlorophenol contaminated sites.Effect of carbon sources and shock loading on the removal of chlorophenols in sequential anaerobic-aerobic reactors was demonstrated by Majumder and Gupta (2007). Sodium formate, sodium propionate, glucose and methanol were used separately as four different carbon sources in the feed as co-substrate. Methanol was found to be the best carbon source for UASB reactors showing 95% 2-CP and 81.1% 2, 4-DCP removals. Basu et al. (2006) did a detailed study on preferential utilization of aromatic compounds over glucose by *Pseudomonas putida.* Glucose supplementation did not suppress the activity of degrading enzymes, which were induced upon addition of aromatic compounds.Topp and Hanson (1988) results indicated that there was a relationship between the nutrients. They cultured a *Flavobacterium sp*. in continuous culture limited for growth with ammonium, phosphate, sulfate, glucose, glucose+ pentachlorophenol.Cells that were grown under phosphate, glucose, or glucose + pentachlorophenol limitation were more sensitive to pentachlorophenol and took longer to degrade 50 mg of pentachlorophenol than the cells that were grown under ammonium, sulfate, or pentachlorophenol limitation. Glucose stimulated viability and pentachlorophenol degradation were found in all cases except when the cells were grown under carbon limitation with glucose and PCP added together as the carbon source.

Cho et al. (2000) studied the effect of soil moisture on bioremediation of chlorophenol-contaminated Soil.In this experiment, chlorophenol-contaminated soil

was tested for the biodegradability in a semi-pilot scale microcosm using indigenous microorganisms. More than 90% of 4-chlorophenol and 2, 4, 6-trichlorophenol, initially at 30 mg kg^{-1},were removed within 60 days and 30mg pentachlorophenol kg^{-1} was completely degraded within 140 days. The chlorophenols were degraded more effectively under aerobic condition than under anaerobic condition. Soil moisture had a significant effect with the slowest degradation rate of chlorophenols at 25% in the range of 10– 40% moisture content. At 25–40%, the rate of chlorophenol degradation was directly related to the soil moisture content, whereas at 10–25%, it was inversely related.

Loh et al. (1998) studied enhancement of biodegradation of phenol and a non-growth substrate 4-chlorophenol by medium augmentation with conventional carbon sources such as sodium glutamate and glucose.Compared with phenol as the sole carbon source, the addition of 1 gl/1 sodium glutamate increased the toxicity tolerance of cells toward 4-chlorophenol and significantly improved the biodegradation rates of both phenol and 4-chlorophenol even when the initial concentration of 4-chlorophenol was as high as 200 mgl/1. On the other hand, supplementation of glucose caused a significant drop in the medium pH from 7.2 to 4.3 resulting in a reduction of degradation rate, leaving a considerable amount of 4-chlorophenol un-degraded when the initial concentration of 4-chlorophenol was higher than 100 mgl/1. By regulating the pH of the medium, however, enhancement of degradation rates of phenol and 4-chlorophenol in the presence of glucose was achieved with a concomitant complete degradation of phenol and 4-chlorophenol.

The effect of carbon sources and shock loadings have been studied Majumder and Gupta (2007) using by two sets of sequential up flow anaerobic sludge blanket (UASB) and rotating biological contactor (RBC) reactors viz., UASB-I followed by RBC-I and UASB-II followed by RBC-II for the removal of two different priority pollutants, 2-CP and 2,4-DCP present in simulated wastewaters. Sodium formate, sodium propionate, glucose and methanol were used separately as four different carbon sources in the feed as co-substrate. Methanol was found to be the best carbon source for UASB reactors showing 95% 2-CP and 81.1% 2,4-DCP removals. The carbon sources formate and propionate were not found suitable in UASB reactors as only 22.6–46.8% 2-CP and 41.9–42.8% 2,4-DCP removals were observed. With

glucose as carbon source 93.7% 2-CP and 79.6% 2,4-DCP removals were observed in UASB reactors. For all the four carbon sources more than 97.6% 2-CP and 99.7% 2,4-DCP removals were observed in sequential reactors. Although all the four carbon sources could not serve as good carbon source for UASB reactor alone but could be successfully used by the sequential reactors for the removal of chlorophenols. The Performance of sequential reactors was also evaluated at five different chlorophenol shock loadings. During shock loading study the concentration of chlorophenols in the wastewaters was increased to 45, 60, 75, 90 and 105 mg/l as compared to the normal feed containing 30 mg/l 2-CP or 2,4-DCP. During shock loading study complete removal of 2-CP and more than 99.6% removal of 2, 4- DCP was observed in sequential reactors. Sequential reactors successfully withstood all the shock loadings and produced high quality effluents.

2.2.1.2 Studies on degradation of chlorophenol and chloroxylenol by mutated bacillus isolates

A mutation is a permanent change in the DNA sequence of a gene. Mutations in a gene's DNA sequence can alter the amino acid sequence of the protein encoded by the gene. UV-irradiation can be successfully used for the improvement of *P. Alcaligenes* for biodegradation of 2,4-dichlorophenol (Elkarmi et al.2009). Garbisu and Alkorta (1999) studied the utilization of genetically engineered microorganisms (GEMs) for bioremediation. Gentamicin production of *Micromonospora echinospora* was enhanced was treated with chemical mutagens like EtBr and MNNG and physical mutagens such as UV was investigated by Himabindu et al. (2007).Gentamicin producing strain of *Micromonospora echinospora* was treated with chemical mutagens like EtBr and MNNG and physical mutagens such as UV was carried out to obtain a mutant with enhanced production of gentamicin. After inducing mutations screening for penicillin and kanamicin resistant mutants was done. *M. echinospora* EtBr-22 strain was obtained by mutations and its gentamicin production in shake flask reaches 1354 mg/l which is 1.53-fold higher than that of the parent strain. Application of different stress conditions like heat shock, feeding high ethanol and high NaCl concentrations during fermentation has found to be effective for the increased

production of gentamicin. Production of gentamicin was increased to 1.26-fold in medium supplemented with 0.6% NaCl to 48-h-old culture.

Kim (2008) studied the structural insight into bioremediation of triphenylmethane dyes by *Citrobacter sp*. Triphenyl methane reductase (TMR) from *Citrobacter sp*. KCTC 18061P was initially isolated and biochemically characterized as an enzyme that catalyzes the reduction of triphenylmethane dyes. Information from the primary amino acid sequence suggests that TMR is a dinucleotide binding motif-containing enzyme; however, no other functional clues can be derived from sequence analysis. On the basis of simulated molecular docking using the substrate malachite green and the TMR/NADP crystal structure, together with site-directed mutagenesis, we have elucidated a potential molecular mechanism for triphenyl methane dye reduction.

2.2.1.3 Statistical optimization of process variables for the maximum degradation of chlorinated organic compound

RSM is important in designing, formulating, developing, and analyzing new scientific studying and products. It is also efficient in the improvement of existing studies and products. The most common applications of RSM are in Industrial, biological and clinical science, social science, food science, and physical and engineering sciences. Since RSM has an extensive application in the real-world, it is also important to know how and where Response Surface Methodology started in the history. According to Hill and Hunter, RSM method was introduced by G.E.P. Box and K.B. Wilson in 1951 (Wikipedia 2006). Box and Wilson suggested to use a first-degree polynomial model to approximate the response variable. They acknowledged that this model is only an approximation, not accurate, but such a model is easy to estimate and apply, even when little is known about the process (Wikipedia 2006). Moreover, Mead and Pike stated origin of RSM starts 1930s with use of Response Curves (Myers et al. 1989).

According to research conducted (Myers et al. 1989), the orthogonal design was motivated by Box and Wilson (1951) in the case of the first-order model. For the second-order models, many subject-matter scientists and engineers have a working knowledge of the central composite designs (CCDs) and three-level designs by Box

and Behnken (1960). Also, the same research states that another important contribution came from Hartley (1959), who made an effort to create a more economical or small composite design. There exist many papers in the literatures about the response surface models. In contrast, 3-level fractional design has limited works. Thus, 3-level fractional design is an open research subject. Fractional Factorial Experiment Design for Factor at 3-Levels (Connor and Zelen 1959) is a helpful resource conducting this kind of design. Many three-level fractional factorial designs and more importantly their alias tables can be found in their study. According to (Myers et al. 1989), the important development of optimal design theory in the field of experimental design emerged following Word World 4II.

One of the important facts is whether the system contains a maximum or a minimum or a saddle point, which has a wide interest in industry. Therefore, RSM is being increasingly used in the industry. Also, in recent years more emphasis has been placed by the chemical and processing field for finding regions where there is an improvement in response instead of finding the optimum response (Myers et al. 1989). In result, application and development of RSM will continue to be used in many areas in the future.

Optimization of process parameters like catalyst dose, pH and oxidant concentration for the photo catalytic degradation of 2,4-dichlorophenol in aqueous solutions was investigated by Kansal et al. (2009).The levels of various culture conditions, namely initial pH, agitation (rpm), temperature (°C), and inoculum age (h) were optimized to enhance 4-chlorophenol biodegradation and the culture specific growth rate by Sahoo et al. (2011).The enhancement in the 4-chlorophenol biodegradation efficiency was found to be 23% higher than that obtained at the un-optimized settings of the culture conditions.

Natercia Guerra Simoes et al. (2007) investigated experimental and statistical validation of SPME-GC–MS analysis of phenol and chlorophenols in raw and treated water. A procedure based on solid-phase micro extraction (SPME) and gas chromatography coupled with mass spectrometry (GC–MS) was developed and validated in order to analyse 10 phenols in water samples. The validated method is suitable for monitoring the production and distribution of potable water and was used, in field trials, for the analysis of samples from main intakes of water (surface or

underground) and from water supply system of a large area (Lisbon and neighbour municipalities).

A novel rotating brush biofilm reactor (RBBR) was used for para-chlorophenol (4-chlorophenol, 4-CP), COD and toxicity removal from synthetic wastewater containing different concentrations of 4-CP by Eker and Kargi (2006). Effects of major operating variables such as the feed 4-CP and COD concentrations and *A*/*Q* (biofilm surface area/feed flow rate) ratio on the performance of the biofilm reactor were investigated. A Box–Wilson statistical experiment design method was used by considering the feed 4-CP (0–1000 mg l^{-1}), COD (2000–6000 mg l^{-1}) and *A*/*Q* ratio (73–293m^2 $daym^{-3}$) as the independent variables while the 4-CP, COD and toxicity removals were the objective functions. The result has correlated by a response function and the coefficient has determined by regression analysis. Percentage 4-CP, COD and toxicity removals determined from the response functions were ingood agreement with the experimental results. 4-CP, COD, and toxicity removals increased with decreasing feed 4-CP and increasing *A*/*Q* ratio.Optimum conditions resulting in maximum COD, 4-CP and toxicity removals have found to be *A*/*Q* ratio of nearly 180m^2 $daym^{-3}$, feed COD ofnearly 4000 mg l^{-1} and feed 4-CP of less than 205 mg l^{-1}.

In the study conducted by Nandwani et al. (2011), Taguchi method has used to identify the several factors that may affect the percentage degradation of phenol and p-chlorophenol in wastewater in a $UV/H_2O_2/TiO_2$ system. The percentage degradation of phenol and p-chlorophenol was greatly influenced by factors suchas pH, cycle time and initial concentration of the organic pollutant. By using orthogonal experimental design and analysis technique, the $UV/H_2O_2/TiO_2$ system performance can be analysed with more objective conclusion through only a small number of simulation experiments. Analysis of variance (ANOVA) has carried out to identify the significant factors affecting the response and the best possible factor level combination was determined through. Finally, a regression model for percentage degradation of phenol and p-chlorophenol has developed, as a function of system parameters. It was found that a $UV/H_2O_2/TiO_2$ system decomposition of the organic pollutant were highly dependent on cycle time followed by pH of aqueous solution. The optimal settings of highest percentage degradation of phenol and p-chlorophenol in wastewater were also determined.

Decolourization of Vero fix Red (Reactive Red 3GL) and Lanasyam Brown Grl (Acid Brown 29) from aqueous solution was studied by adsorption technique using a hybrid adsorbent that was prepared by pyrolysing a mixture of carbon and flyash in 1:1 ratio byRavikumar et al. (2007). A 2^4 full factorial central composite design was successfully employed for experimental design and analysis of the results.The combined effect of pH, temperature, particle size and time on the dye adsorption was investigated and optimized using response surface methodology. The optimum pH, temperature, particle size and time were found to be 10.8, 59.25^{o}C, 0.0525 mm, and 395 min, respectively, for Reactive Red 3GL and those for Acid Brown 29 were 1.4, 27.5^{o}C, 0.0515 mm and 285 min, respectively. Complete removal (100%) was observed for both the dyes using the hybrid adsorbent.

Response surface models through regression on experimental data and to apply the Sequential Quadratic Programming (SQP) and Genetic Algorithms (GAs) on the models to obtain optimal processing conditions for dairy tofu was studied by Chen et al.(2005).The two-stage effort of obtaining a surface model using response surface methodology (RSM), and optimizing this model using GAs or SQP techniques was demonstrated to be an effective approach. Both SQP and GAs techniques were able to determine the optimal conditions for manufacturing the probiotic dairy tofu. The conditions were 1% of glucono-delta-lactone (GDL), 0% of peptides level, 3% of isomalto-oligosaccharides (IMO) and 18% of milk concentrations, and they were confirmed by verification experiments. Among the SQP and two GAs employed, the SQP, modified with the multi-start capability, is the most efficient one.

Fu et al. (2009) studied the multivariate-parameter optimization of acid blue-7 wastewater treatment by Ti/TiO_2 photo-electrocatalysis *via* the Box–Behnken design.On the basis of a three-variable Box-Behnken design (BBD), RSM was used to determine the effect of pH values (range 3.2–6), light intensity (range 10–20×10^2 μW/cm^2) and bias potential (range 0.1–1.1 V) on the levels of response, *i.e.* decolourization efficiency. By applying the quadratic regression analysis, the equations describing the behaviors of the response as simultaneous functions of the selected independent variables were developed. Accordingly, the optimal conditions were determined as pH of 3.41, light intensity of 16.02×10^2 μW/cm^2 and bias potential of 0.68 V. Decolourization efficiency of 90.13%, obtained experimentally

under such optimal conditions, agreed was highly with that of 90.44%, estimated by the

The optimization of nutrient levels for the production of pristinamycins by *Streptomyces pristinaespiralis* CGMCC 0957 in submerged fermentation was carried out by Jia et al. (2008) using the statistical methodologies based on the Placket–Burman design, the steepest ascent method, and the central composite design (CCD). First, the Plackett–Burman design was applied to evaluate the influence of related nutrients in the medium. Soluble starch and MgSO4·7H2O were then identified as the most significant nutrients with a confidence level of 99%. Subsequently, the concentrations of the two nutrients were further optimized using response surface methodology of CCD, together with the steepest ascent method. Accordingly, a second-order polynomial regression model was finally fitted to the experimental data. By solving the regression equation from the model and analyzing the response surface, the optimal levels for soluble starch and MgSO4·7H2O were determined as 20.95 and 5.67g/L, respectively. Under the optimized medium, the yield of pristinamycins in the shake flask and 5-L bioreactor could reach 1.30 and 1.01g/L, respectively, which is the highest yield reported in literature to date.

Response surface methodology was employed to optimize culture medium for production of lipase with *Rhodotorula sp*. MTCC 8737 by Chennupati et al. (2009). In the first step, a Plackett–Burman design was used to evaluate the effects of different inducers qualitatively. Of all the seven inducers tested, soybean oil showed significant influence on the lipase production. Further, response surface studies were conducted to quantitatively optimize by considering linear, interactive, and quadratic effects of test variables. A novel approach was proposed to optimize the lipase production system by optimizing the responses in terms of yield kinetics rather than optimizing the direct responses like lipase titer and biomass growth. The coefficient of determination (R^2) calculated for $Y_{P/S}$ (0.769), $Y_{P/X}$ (0.799), and $Y_{X/S}$ (0.847) indicated that the statistical model could explain 76.9%, 79.99%, and 84.7% of variability in the response.

Optimization of the medium for biosurfactants production by probiotic bacteria (*Lactococcus lactis* 53 and *Streptococcus thermophilus* A) was carried out by Rodrigues et al.(2006) using response surface methodology. Both biosurfactants were

proved to be growth-associated, thus the desired response selected for the optimization was the biomass concentration. With the optimization procedure, a biosurfactant mass recovery 2.1 times higher was achieved. The application of response surface methodology resulted in an enhancement in biosurfactants production.

Optimization of chloroxylenol degradation by *Aspergillus Niger* using Plackett-Burman design and response surface methodology was explored by Ghanemet al. (2012). It was concluded that *Aspergillus Niger*, local isolate, was an efficient fungus that degraded 99.72% of 2 mg/L of chloroxylenol after 7 days of fermentation. It also had a high capacity to degrade 91.83% of higher chloroxylenol concentration of 20 mg/L after 6 days of incubation on mineral medium amended with 2 g/L of glucose. Statistical experimental designs have used to optimize the process of chloroxylenol degradation by the fungus. The most important factors influencing chloroxylenol degradation, as identified by a two-level Plackett-Burman design with 11 variables, were NaCl, $(NH4)_2SO_4$, and inoculum size. Response surface analysis was adopted to further investigate the mutual interactions between these variables and to identify their optimal values that would generate maximum chloroxylenol degradation. Under the optimized medium compositions and culture conditions, *Aspergillus Niger* was able to degrade completely (100%) chloroxylenol (20 mg/L) after 134.6 h of fermentation. The predicted values of Plackett-Burman conditions and response surface methodology has further verified by validation experiments. The excellent correlation between predicted and experimental values confirmed the validity and practicability of this statistical optimum strategy.

2.2.1.4 Fungal bioremediation

A number of fungi are known to break down persistent toxins in soils, including PCP, DDT, dioxin, and PBP. *Phanerochaete chrysosporium* is the most widely studied bioremediation fungus. Degradation of phenol by *Phanerochaete chrysosporium* was analysed by Peraz in 1997. He investigated the influence of co-substrate concentration, mycelium acclimatization and nitrogen concentration on phenol degradation and concluded that decrease in glucose and ammonium tartrate concentration decreased chlorophenol degradation. Denizli et al. (2005) evaluated the

use of the fungus *Pleurotus sajorcaju* to remove phenols (i.e., phenol, o-chlorophenol, p-chlorophenoland 2, 4, 6-trichlorophenol) from aqueous solutions. The maximum adsorptions of phenol and chlorophenols onto the *Pleurotussajor caju* were 0.95 mmol/g for phenol, 1.24 mmol/g for o-chlorophenol, 1.47 mmol/g for p-chlorophenol and 1.89 mmol/g for 2,4,6-trichlorophenol.

Zouari et al. (2002) investigated 4-Chlorophenol (4-CP) degradation by suspending and immobilizing *Phanerochaete chrysosporium* in static and agitated cultures. The best results were achieved when experiment was carried out in a rotating biological contactor instead of an Erlenmeyer flask, for both batch degradation and repeated batch degradation. The relative contribution of lignin peroxidase (LiP) versus manganese peroxidase (MnP) to the 4-CP degradation by *P. chrysosporium* was investigated. 4-CP degradation slightly increased and a high level of MnP (38 nKat) was produced when *Phanerochaete chrysosporium* was grown at high concentration. High LiP production in the medium had no significant effect on 4-CP degradation; rather 4-CP degradation occurred when *Phanerochaete chrysosporium* was grown in a medium that repressed LiP and MnP production. This result indicated that LiP and MnP are not directly involved in 4-CP degradation by *Phanerochaete chrysosporium.*

Sedarati et al.(2003) compared free-cell cultures of *Trametes versicolor* with cultures immobilized on a nylon mesh in a 2-litre bioreactor for transformation of pentachlorophenol (PCP) and 2,4-dichlorophenol (2,4-DCP) added at intervals to the liquid culture medium over a period of 816 hrs. Increasing amounts of PCP from 200 mg/L to 2000 mg/L added batchwise to cultures permitted acclimatization of the fungus to these toxic pollutants. A total addition of 2000 mg/L of 2,4-DCP and 3400 mg/L PCP were removed from the immobilized cultures with 85% of 2,4-DCP and 70% of PCP transformed by enzymes (laccase and Mn-peroxidase), 5% 2,4-DCP and 28% PCP adsorbed by the biomass and 10% 2,4-DCP and 2% PCP retained in the medium at the termination of the fermentation after 1020 hrs. In contrast, free-cell cultures in the same medium with the same addition regime of PCP and 2,4-DCP, transformed 20% 2,4-DCP and 12% PCP by enzyme action, adsorbed 58% 2,4- DCP and 80% PCP by the biomass, and retained 22% 2,4-DCP and 8% PCP in the medium. The use of nylon mesh as an immobilization matrix for removal of PCP and

2,4-DCP facilitates more efficient removal of chlorophenols and can be adapted to scale-up application for removal in large volumes of chlorophenol-containing aqueous effluents.

2.2.1.5 Phytoremediation

Phytoremediation is the use of a plant's natural ability to contain, degrade, or remove toxic chemicals and pollutants from soil or water. It can be used to clean up metals, pesticides, solvents, explosives, crude oil, and contaminants that may *Daucuscarota L., Ipomoea batatas L. and Solanumaviculare Forst* leak from landfill sites. For example hairy root cultures of were investigated for their susceptibility to the highly toxic pollutants phenol and chlorophenols and for the involvement of inherent peroxidases in the removal of phenols from liquid media by Brancileneet al. in 2006.

2.2.1.6 Cometabolism Bioremediation

Cometabolism is the process by which a contaminant is fortuitously degraded by an Enzyme or cofactor produced during microbial metabolism of another compound. Typically, there is no apparent benefit to the microorganism involved. Matsumura and Boush (19967) reported the degradation of dieldrin by 12 soil isolates growing in mannitol-yeast extract medium. The lack of growth of these microorganisms at the expenseof dieldrin, in the absence of the additional carbon and energy source, mannitol, indicates that co-metabolism was responsible for breakdown of this pesticide by the soil isolates.

SUMMING UP

The degradation of chlorophenol and chloroxylenol in environment is a major environmental challenge. Currently, various chemical, physical and biological treatment methods are used to degrade chlorophenol and chloroxylenol in environment. Because of the high cost and disposal problems, many of these methods for degrading have not been widely applied in the field. A literature survey shows that research has been and continues to beconducted in the areas of chemical and combined chemical-biological treatments in order to improve the biodegradation of chlorophenol and chloroxylenol. Previous studies have shown that many of the chlorophenol and chloroxylenol are carcinogenic, mutagenic, and detrimental to the

environment. As toxicity standards become more common and stringent, the development of new techniques for minimizing the concentration of chlorophenol and chloroxylenol and their breakdown products in the wastewater becomes necessary. Chemical degradation mechanism for chlorophenol and chloroxylenol, which are reported in the literature, include adsorption by activated carbon and dried cell, electrochemical treatment and sonication and photochemical precipitation. The high cost and disposal problems have opened the door for further investigation of new techniques. Application of conventional biological processes in the treatment of chlorophenol and chloroxylenol wastewater had been extensively reported in the literature.

The results from the literature review support the conclusion that wastewaters containing chlorophenol and chloroxylenol are generally not degraded effectively by the meta cleavage pathway, due to the production of dead-end or suicide-metabolites. In general, chlorines in positions ortho to the hydroxyl group were removed more readily than those in the metaor para position.Microorganisms like *Azotobacter sp., Flavobacterium sp., Pseudomonas pickettii, Bacillus cereus, Micrococcus sp., Rhodococcus erythropolis, Pseudomonas fluorescence, Rhizobium sp.*etc. were used to treat the different types of chlorophenols and concluded that there was upto 75% of degradation. According to literature review many investigators have examined a wide range of physical and chemical factors for degradation of chlorophenol and chloroxylenol, from which it can be concluded that degradation of chlorophenol and chloroxylenol was maximum at temperature above 20^0C and pH 5-8. Nutrients like glucose, sodium glutamate, sodium formate, sodium propionate and methanol were used for studying degradation profile of chlorophenol. The carbon sources formate and propionate were not found suitable for degradation as there was only negligible percentage of degradation of chlorophenol. With glucose as carbon source it was observed that there was maximum percentage of chlorophenol and chloroxylenol. Further literature review discloses that optimization of physical and biological parameters using statistical methods have improved the degradation capacity of microorganism.

CHAPTER 3

MATERIALS & METHODS

This chapter describes the experimental details of various methodologies adopted in the present study. The detailed procedure of enrichment and isolation of the surfactant producing microorganisms; selection of the surfactant producers using various screening methods and identification of the candidate surfactant producing bacteria has been presented. The chapter presents the details on the extraction of the extracellular surfactant from the cell-free broth. The procedures employed for the studies on the various factors affecting the production of surfactant, optimization of process variables using statistical method and kinetics of surfactant production have been presented. The experimental procedures followed in partial purification and characterization of surfactant, studies on the effect of various environmental factors affecting surfactant stability and efficiency of surfactant in the biodegradation of crude oil have been presented. In the present study, all the experiments were conducted in triplicates.

The following experiments have been carried out to meet the status of justice listed in chapter.Isolation screening and identification through biochemical and molecular technique of microorganism; optimization of growth and degradation parameter for the selected strain; design of experiment using RSM method was carried out with efficiency to degrade 2,4-DCP and PCMX. Mutation studies of the selected strain to check the efficiency for degradation and degradation studies with industrial effluent were also performed. The methodology used for the above experiment is as per standard procedures reported in literature and the analysis of the required parameters to generate the required information is carried out using standard protocols. The experiment where carried out in duplicates for all the runs and the average of the observation where used for further interpretation. The methodology used for the experiment and the different protocols used in the study are listed as follows.

3.1. Isolation of bacteria

Soils used for isolating microorganism in this study were collected by simple soil sampling method (Galina Matafonova et al. 2006, Roda F. Al-Thani et al. 2007) from various geographic locations (at a depth of 5 to 7 inches) that had been exposed to chloroxylenol and chlorophenol near a wastewater treatment plant from Rickitt

Benckiser Dettol manufacturing company located in Mysore, Karnataka. They were transported intact at ambient temperature in sealed polyethylene bags. The soil samples were sieved through a 2mm screen and stored at 4°C until further use.

3.2. Sample collection point

Sample 1: Near untreated sludge drying tank

Sample 2: Near chlorine treatment tank

Sample 3: Near treated effluent tank

Sample 4: Near untreated effluent tank

Sample 5: Control from place where there is no trace of effluent

3.3. Chemicals and reagents

Pure and analytical grade chemicals were used throughout the experiments including media preparation for growth of microorganisms. Nutrient agar used for isolation was supplied by Merck chemicals, India. Chemicals used for analysis of 2, 4-DCP and PCMX in HPLC was procured from Merck, Chemical, India. 2,4-DCP was purchased from Sigma and PCMX (98%) was obtained from Alfa Aesar. The chemicals contained in the minimal salt media used for the study (KH_2PO_4, Na_2HPO_4, Peptone, and Glucose) were purchased from Merck India Ltd. Deionized water was obtained using Millipore deionization system.HCl and NaOH for adjusting pH were purchased from Merck chemicals, India and NICE Chemicals.

3.4. Glassware and apparatus

All glass wares (Conical flasks, Measuring cylinders, Beakers, Petriplates and Test tubes, burette, pipettes etc.) were of Borosil grade. The instruments and apparatus used throughout the experiment are listed in Table 3.1

Table 3.1: List of Instruments used during the whole experiment their make and function

Equipment	Make and Model	Use
Vertical Autoclave	Tomy	Sterilization
Analytical Balance	Sartorius	Weight Measurement
Laminar airflow	Clean Air	Aseptic Environment
pH	Elico Instruments	Measurement of pH
Temperature controlled incubator shaker	Scigenics Biotech Orbitek	Incubation and shaking of cultures
Ultra Low Temperature freezer	Vest frost	Preservation of cultures
Ultra-pure water system	Millipore Elix	Preparation of solutions and stocks.
Spectrophotometer(UV/Vis)	Hitachi	Estimation studies
Centrifuge	R-8C REMI	Collection of pellet
Colony counter	Vijaya Enterprises	Cell count
Vortex Mixture	Heidolph	Serial Dilution
SEM-EDX	JEOL JSM-6330F, Japan	Elemental analysis of sample
HPLC	Dionex	Estimation of 2,4-DCP and PCMX

3.5. Enrichment and isolation of 2,4-DCP and PCMX degrading microorganism

A defined mineral salt medium was used for isolation and cultivation of the 2, 4-DCP and PCMX degrading strains (Madhumathi et al. 2010).Mineral salts medium (MSM) used in this study consisted of (in g/L): Solution A- KH_2PO_4, 3g; Na_2HPO_4, 6g; Peptone, 1g;and Solution B -Glucose, 10 g. Isolation of bacteria was carried out from soil samples enriched with 2, 4-DCP and PCMX with a starting concentration of 10mg/L. The enriched soil samples were incubated on a rotary shaker at 30°C and 120 rpm in dark. After fifteen days of incubation period, 1ml of sample was drawn and 10^{-10} fold serial dilutions was prepared and streaked on both nutrient agar and mineral media. Standard plate count method was used for the colony count at different serial dilution ranging from 10^{-1} to 10^{-10}.Colonies of 2, 4-DCP and PCMX degrading organisms were obtained from the highest dilutions on nutrient agar plates containing 10 mg/L of 2, 4-DCP and PCMX. Six strains were isolated from soil samples and isolates were tentatively named as 01-C-My-NITK, 03-M-My-NITK, 03-G-My-NITK, 02-Y-My-NITK, 04-O-My-NITK and 03-P-My-NITK.

3.6. Maintenance of stock cultures

The strains were then maintained on both mineral media and nutrient agar slants at 4°C (Appendix I and II).For optimization study organism were subjected to different concentration of 2,4-DCP and PCMX. For the study of morphological characteristics, the strains were grown on agar plates and observed. For microscopic observation, the strains were mounted on a slide and examined with oil immersion microscope.

3.7. Cell density and pH analysis

2ml sample from each flask was taken for determination of biomass. Cell growth was monitored spectrophotometrically (Spectrophotometer Labomed, Inc., USA) by measuring the absorbance at 610 nm (Appendix IV). pH of the minimal media was measured using a pH meter (M/s Equiptronics Instruments). In the experiments in which pH regulation was required, a few drops of a sterilized stock solution of HCl (2M) and NaOH (2M) were manually addedto achieve the desired pH.

3.8. Analysis of 2, 4-DCP and PCMX using HPLC

2, 4-DCP and PCMX degradation was established by measuring the residual concentration of 2, 4-DCP and PCMX using HPLC system (Dionex make with UVD 170U), Pump P680A HPG-2, Thermostat column compacter TCC-100 and Chromelon software 6.60 version (Appendix V). As the stationary phase, a Eurospher-100 C18 reversed-phase column with a 4-mm internal diameter and 125-mm length was used. The mobile phase was an aqueous solution of methanol water and Acetic acid (100:100:2) (Vol/Vol). The progress of the chromatography was monitored simultaneously at 280 nm with 32°C as the annealing temperature (Hee Sung Bae et al. 1996).

3.9. Screening of isolates using different concentration of 2, 4-DCP and PCMX

Six isolated strains were screened by subjecting them for growth at concentrations ranging from 0.5 mg/L to 50mg/L of both 2, 4-DCP and PCMX. Strains were grown in 100 ml of defined mineral salt medium. The flask was placed on shaker at 120 rpm at 35°C. Growth was monitored by measuring the biomass concentration at 610nm using Hitachi Spectrophotometer. Cells were harvested at 20 hours during mid-log

phase. Cultures (5 ml) were centrifuged at 8000 rpm for 5 min, the pellet was washed with 0.85% NaCl and sterile distilled water. This was used immediately as inoculums for the screening experiment. The inoculum size of 0.1 ml was spread on mineral media in duplicate containing different concentration of 2, 4-DCP and PCMX. It was then incubated for 24 hours at 30°C. Colonies were counted after incubation.To confirm the result 0.1 OD size of both the isolated organism were inoculated in an Erlenmeyer flask containing 2, 4-DCP and PCMX. It was incubated for 24 hours at 30°C and sample were drawn at different time intervals (0, 24, 48, 72, 192 hours.) and observed growth pattern through spectrophotometer at 610 nm.

The analysis results revealed that the isolated strain 01-C-My-NITK and 03-P-My-NITK recorded maximum growth in 2, 4-DCP and PCMX when compared to the other isolated strains.

3.10. Identification of the isolate

In the present study, phenotypic characteristics, biochemical and phylogenetic analysis on the basis of 16S rDNA sequences were examined to determine the precise taxonomic position ofthis strain.

3.10.1. Morphological characteristics

The young cultures (18 hour.) were inoculated in nutrient agar plates and were used to identify colonial characteristics like shape, size, and elevation, margin type, opacity, colour and chromo genesis (pigmentation).

3.10.2. Biochemical characteristics

Various biochemical testes were done for the isolated cultures.Gram-staining was performed according to procedures outlined by Smibert and Krieg 1981. Biochemical tests included Amylase activity , hydrolysis of starch, Catalase, Methyl red, Vogues – Proskauer, Indole, Citrate utilization, Glucose ,Sucrose, Lactose, Urease, H_2S fermentation, Hydrolysis of gelatin and Oxidase.

3.10.2.1. Simple staining

Thin smear was prepared on slide and heat fixed. Few drops of Methylene blue have added. Slide was stained off and washed gently with distilled water and blot dried. Shape of the cell was observed under microscope.

3.10.2.2. Gram staining

Thin smear of the isolate was prepared and heat fixed aseptically in laminar air flow chamber. Crystal violet was used on the smear and left for 30 seconds. Slide was washed with distilled water and the slide was flooded with Gram's iodine for 60 seconds. The iodine was washed with 95% ethanol drop by drop. Slide was drained off with distilled water. Safranine was applied for 30 seconds and blot dried before observing under microscope.

Results: Purple color - **Gram positive**

Pink color **- Gram negative**

3.10.2.3. Capsule staining

The primary stain applied was crystal violet, which stains both the bacterial cell and the surrounding capsule. A 20% copper sulfate solution was then applied, which serves a dual function both to decolorize and counter stain. It removes and replaces the crystal violet in the capsule only. At the end of the staining procedure, the capsule appears as a faint blue or white halo around a purple cell.

3.10.2.4. Endospore staining

Using aseptic technique, bacterial smear was prepared on a clean slide, air dried and gently heat fixed. Slide was placed on a staining rack over the water bath and was stained with Malachite green (primary stain). Then slide was steamed for five minutes. The slide was removed from the water bath and allowed to cool, and then rinsed with deionized water until the water ran clear. Excess water was drained and Safranine (counter stain) was applied and kept for two minutes. Excess Safranine was washed off with deionized water, and the slide was blot dried. The slide was observed under a light microscope with oil immersion.

Result: The vegetative cells are pink/red and the endospore are green.

3.10.2.5. Motility test

A small drop of liquid bacterial culture was placed in the center of a cover slip. The slide was inverted with a central depression over the cover slip such that the cover slip will stick to the slide and when the slide is inverted the drop of bacterial culture will be suspended in the well. The slide was examined microscopically for motile organisms.

Positive result: A darting, zigzag, tumbling or other organized movement

Negative result: No movement or Brownian motion only

3.10.2.6. Amylase production test

Sterile starchplate (Appendix III 2.1) was inoculated with the isolate and incubated at optimum temperature for at least 48 hour. Plate was flooded with iodine, and results were observed.

Result: Blue color indicates no hydrolysis, while a clear zone indicates hydrolysis.

3.10.2.7. Catalase test

3% H_2O_2 was added to the slide containing the isolate. The tube was observed for bubble indicating a positive reaction.

3.10.2.8. Methyl red test

Buffered glucose broth (Appendix III 2.2) was inoculated and incubated at 37°C for 48 hour few drops of methyl red solution was added to the culture. The colour was observed immediately.

Result: Red color indicates positive and yellow indicates negative.

3.10.2.9. Vogues- Proskauer test

Buffered glucose broth (Appendix III 2.2) was inoculated with the organism and incubated at 37°C for 3 days. Few drops of alpha Napthol was added, followed by 1 ml of 40% KOH. It was mixed well and kept 30 minutes.

Result: Pink color indicates positive, no color change is negative.

3.10.2.10. Indole production test

Tryptone broth (Appendix III 2.3) was inoculated and incubated at 37°C for 48 hour. 1mL Kovacs reagent was added and observation was done immediately.

Result: A bright pink color in the top layer indicates the presence of Indole.

3.10.2.11. Citrate utilization test

Simmon's Citrate agar plate (Appendix III 2.4), sterilized before, was inoculated with the organism and incubated at 37°C for 48 hour.

Result: Growth on the medium is accompanied by a rise in pH to change the medium from its initial green color to deep blue.

3.10.2.12. Fermentation of carbohydrates

In order to test for these fermentation products, the tubes containing a single carbohydrate (Appendix III 2.5) (such as glucose, lactose and sucrose with phenol red and Durham tube, a small inverted tube to detect gas production) was inoculated and incubated for 48 hour at 37°C.

Result: If the particular carbohydrate is fermented by the bacterium, acid end products will be produced which lowers the pH, causing the pH indicator to change color of the phenol red to yellow. If gas is produced along with the acid, it collects in the Durham tube as a gas bubble. If the carbohydrate is not fermented, no acid or gas will be produced and the phenol red will remain red.

3.10.2.13. Urease test

The surface of a urea agar slant (Appendix III 2.6) was streaked with the isolate to be tested. The tube was incubated for 8-24 hour at 35°C with the loose cap.

Result: **Positive result:** When organisms utilize urea, ammonia is formed which makes the reaction of the media alkaline, producing a pink-red color (due to the change in the phenol red indicator). *Proteus* is rapidly positive; usually within 6 h.

Negative result: Agar slant and butt of the tube remain light orange.

3.10.2.14. Oxidase test

A small suspension of the pure culture was prepared and oxidation disc was dipped into the suspension. The disc was removed immediately and observed for the color change.

Result: Color change to purple within

- 10 seconds = **Positive**
- 10 - 60 seconds = **Delayed positive**
- >60 seconds = **Negative**

3.10.2.15. Hydrogen Sulphide production test

Hydrogen Sulphide producing Media (Appendix III 2.7) was prepared and was inoculated with the pure culture. The plates were incubated at 37°C for 24-48 hour.

Result: **Positive:** H_2S production = Black

Negative: No H_2S production = No blackening of medium

3.10.2.16. Hydrolysis of gelatin

Gelatin medium was prepared according to composition given in Appendix III 2.8. The medium was poured into sterile test tubes and were inoculated with the culture. The tubes were incubated at 37^{o}C for 24 - 48 hour. At the end of the incubation period, tubes were placed into a 4^{o}C refrigerator for 30 minutes to 1 hour.

Result: **Positive**: liquefied form.

Negative: gel form.

3.10.3. Molecular analysis of the isolates

Genetic identification of microorganisms is basically a comparison study. The genetic identification of microorganisms utilizes molecular technologies to evaluate specific regions of the genome and uniquely determines the genus, species, or strain of a microorganism (Liisa J. Nohynek et al. 1996, Chu-Fang Yang et al. 2008). To identify an unknown organism, appropriate sequences from the unknown are compared to documented sequences from known organisms. Homology between the sequences results in a positive test. An exact match will occur when the two organisms are the same. Related individuals have genetic material that is identical for some regions and

dissimilar for others. Unrelated individuals will have significant differences in the sequences being evaluated. Developing a database of key sequences that are unique to and characteristic of a series of known organisms facilitates this type of analysis. The sequences utilized fall into two different categories, 1) fragments derived from the transcriptional active, coding regions of the genome, and, 2) fragments present in inactive, noncoding regions. Of the two, the noncoding genomic material is more susceptible to mutation and will therefore show a higher degree of variability.Depending on the level of specificity required, an assay can provide information on the genus, species, and/or strain of a microorganism.

01-C-My-NITK showing maximum growth in different concentration of 2,4-DCP and PCMX was selected for genotypic identification and was sent to Anshul Biotech, Hyderabad.

In conclusion six different bacterial strains were isolated from soil near waste water treatment tank of an industry, manufacturing compounds using PCMX. Growth of six different isolated strainswasstudied in different concentration of 2, 4-DCP and PCMX. One of the bacterial cultures which had higher tolerant capacity for 2, 4-DCP and PCMX was used for further studies. This bacterium was further identified by morphological, physiological and biochemical characterization as *Bacillus* sp. For confirmation of size and shape, 5 Days old culture was viewed under scanning electron microscopy. Comparative analysis of the 16SrRNA sequence in the Gene Bank database revealed that this bacterium is related to *Bacillus cereus*.

3.10.4. Effects of 2, 4-DCP and PCMX on morphology of *Bacillus cereus* observed under Scanning electronic microscopic

Scanning Electron Microscopy analysis is done to understand the morphological and topological changes that occurred in the microbial cells before and after degradation (Appendix VII). The culture was grown overnight in media containing 10 mg/L 0f 2,4-DCP and 50 mg/L of PCMX.1 ml media was centrifuged at 10000 rpm to separate the cells .The cell pellet was mixed with 2.5% glutaraldehyde solution and incubated at 4 $^{\circ}$C overnight for fixation. It was then centrifuged to get the pellet. Then cells were dehydrated with a serial concentration (10 to 95%) of ethanol every 15 min and then 100% ethanol for 20 min and then air-dried. The dried cells were coated with gold

under vacuum for 20-30 min using a sputter coater (IB-3, Giko Engineering Co., Japan) and finally photographed by a scanning electron microscope(S-2500C, Hitachi Co., Japan)

3.11.1 Effect of inoculum density on the growth of *Bacillus cereus*

Young cultures of *Bacillus cereus* were inoculated in 500 ml of mineral media in 1000 ml Erlenmeyer flask and incubated for 24 hours at 30°C at 120 rpm. One ml of the sample was drawn, centrifuged and diluted using 0.85% of saline solution to achieve 0.01, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 OD by measuring the optical density (OD) at 610 nmusing UV spectrophotometer. Different OD (0.01, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5) was further inoculated to the 100ml mineral media in 250 ml Erlenmeyer flask. One ml of the sample were drawn from flask at different time intervals and observed the growth patterns by measuring the optical density at 610 nm in spectrophotometer.

3.11.2. Effect of concentration of glucose on the growth of *Bacillus cereus*

In the present study of bacterial growth curve, the number of bacteria present in defined mineral salt medium was measured using a direct method (counting the number of cells in a culture of bacteria (viable count)) and also by an indirect method (counting the number of cells in a culture of bacteria (optical density)) during the course of 2 hours. Concentration of glucose was varied.

Bacillus cereus was grown in 250 ml Erlenmeyer flask in minimal media. The cultures were placed on shaker at 120 rpm at 30°C. Growth was monitored by measuring the optical density at 610nm using Hitachi Spectrophotometer. Cells were harvested at 18 hour during mid-log phase. Cultures (5 ml) were centrifuged at 8000 rpm for 5 min, the pellet was washed with 0.85% NaCl and sterile distilled water. This was used immediately as inoculum for the optimization experiment. The inoculum size of 0.2 OD was transferred into 250ml Erlenmeyer flask and was placed on shaker at 120 rpm at concentration of glucose (0.1, 0.5, 0.8, 10g/L).

3.11.3. Effect of concentration of peptone on the growth of *Bacillus cereus*

Bacillus cereus were inoculated into 250ml Erlenmeyer flask containing 100ml minimal media and incubated at 30°C at 120rpm. 0.2 OD Cell growth was transferred to a fresh minimal salt media containing different concentration of peptone (0.1, 0.5 and 1 g/L) and the growth of *Bacillus cereus* was monitored by measuring the absorbance at 610 nm.

3.11.4. Effect of different temperature and pH on the growth of *Bacillus cereus*

Growth proceeds most rapidly at the optimum growth temperature and pH for a particular bacterium. For any bacteria, there is a minimum and maximum temperature and pH beyond which growth is not supported.

Experiments were conducted to find the optimum growth temperature and pH of *Bacillus cereus. Bacillus cereus* was grown in 100ml defined mineral salt medium in a 250ml Erlenmeyer flask. The cultures were placed on shaker at 120 rpm at 35°C. Growth was monito. Cells were harvested at 18 hour during mid-log phase. Cultures (5 ml) were centrifuged by measuring the optical density at 610nm using Hitachi Spectrophotometer at 8000 rpm for 5 min, the pellet was washed with 0.85% NaCl and sterile distill water. This was used immediately as inoculum for the optimization experiment. The inoculum size of 0.2 OD were transferred into 100ml of defined mineral salt medium and were placed on shaker at 120 rpm at different temperature(25°C, 30°C, 35°C, 40°C, 45°C) and different pH(5,6,7,8).

3.12. Optimization of chemical and physical parameters to increase the percentage degradation of 2, 4-DCP and PCMX by *Bacillus cereus*

Chemical and physical parameters also play a role in determining the ability of bacterial growth and degradation of pollutant in particular environments. Generally parameters optimization is done with one parameter at various levels while the other parameters are kept at one constant level. This was done for various physical parameters like pH and temperature as well as chemical parameters like media components. The present investigation describes optimization studies to increase the percentage degradation of 2, 4-DCP and PCMX by *Bacillus cereus.* The optimum pH and temperature for growth of *Bacillus cereus* and degradation of 2, 4-DCP and

PCMX was determined by monitoring the residual 2, 4-DCP and PCMX concentrations in cultures inoculated into medium. The medium optimization was carried with different pH values (pH 2,3,4,5,6,7and 8),different temperature (25°C, 30°C, 35°C, 40°C), glucose(5g/L, 7.5g/L, 10g/L, 12.5g/L, 15 g/L), peptone (0.5 g/L, 0.75g/L, 1g/L,1.25g/L,1.5g/L), phosphate(4.5 g/L, 6.75g/L, 9g/L, 11.25g/L, 13.5g/L) and inoculum size (0.1 OD, 0.15 OD, 0.2 OD, 0.25 OD, 0.3 OD).

The difference in response between the levels of one factor is not the same at all levels of the other factor. This is described as the interactive between the two factors statistical experimental designs allows simultaneous systems and efficient variation of all components. The effect of various parameters on bacterial growth, compound degradation and change in pH was studied in such a way that the interaction of various impact parameters was studied as a factorial design. In the factorial experiment, each complete trail and replication of the experiment of all possible combination of the levels of the factors are increased and the effects of factors is defined to be the change in response produced by a change in the level of factor. This is called a main effect.

3.12.1. Optimization of the parameters to increase the percentage degradation of 2, 4-DCP by *Bacillus cereus*

3.12.1.1. Degradation studies of 2, 4-DCP by *Bacillus cereus* at different temperatures

Bacillus cereus cells were grown in varying concentrations of 2, 4-DCP (10, 30 and 75 mg/L) in separate 250ml Erlenmeyer flasks containing 100 ml of defined mineral salt medium. The flask was placed on shaker at 120 rpm at 30°C. Growth was monitored by measuring the optical density at 610nm using Hitachi UV Spectrophotometer. Cells were harvested at 20 hours during mid-log phase. Cultures(5 ml) were centrifuged at 8000 rpm for 5 min, the pellet was washed with 0.85% NaCl and sterile distill water. These were used immediately as inoculum for the optimization experiment. The inoculum size of 0.2 OD was transferred into each 100ml of defined mineral salt medium containing different concentration and (10,30 and 75 mg/L) of 2, 4-DCP and was placed on shaker at 120 rpm at different

temperature (25°C, 30°C, 35°C, 40°C). Control with 2, 4-DCP in media without cells was also placed on shaker at 120 rpm at different temperature (25°C, 30°C, 35°C, 40°C).

During the bioremediation experiment 5 ml of sample was taken for every 24 hour and the amount of 2, 4-DCP, pH of medium and growth of *Bacillus cereus* was analyzed using Dionex HPLC system, Elico pH meter and Hitachi UV visible spectrophotometer.The same experiment was repeated for 2, 4-DCP (10 mg/L) in different pH (2, 3, 4, 5, 6, 7and 8)

3.12.2 Optimization of the parameters to increase the percentage degradations of PCMX by *Bacillus cereus*

3.12.2.1 Degradation studies of PCMX (50 and 350 mg/L) by *Bacillus cereus* at different temperatures

Bacillus cereus cells were grown in 100 ml of defined mineral salt medium containing different concentration of PCMX (50 and100 mg/L). The flask was placed on shaker at 120 rpm at 30^0C. Growth was monitored by measuring the optical density at 610nm using Hitachi UV Spectrophotometer. Cells were harvested at 20 hour during mid-log phase. Cultures (5 ml) were centrifuged at 8000 rpm for 5 min, the pellet was washed with 0.85% NaCl and sterile distill water. This was used immediately as inoculum for the optimization experiment. The inoculum size of0.2 OD was transferred into 100ml of defined mineral salt medium containing different concentration of PCMX (50 and 350 mg/L) and was placed on shaker at 120 rpm at different temperature (25°C, 30°C, 35°C, 40°C).

5 ml of sample was harvested for every 24 hours and the amount of PCMX, pH of medium and growth of *Bacillus cereus* were analyzed using Dionex HPLC system, Elico pH meter and Hitachi UV visible spectrophotometer. Control with PCMX in media without cells was also analyzed.

Similar procedures were repeated for PCMX (50mg/L) in different pH (5, 6, 7 and 8)

3.13. Studies on degradation of 2, 4-DCP and PCMX by parent and mutated *Bacillus* isolates

The aim of this study was to obtain *Bacillus cereus* mutant with enhanced capability to degrade 2, 4-DCP. For achieve this aim, chemical mutagenesis, Ethedium Bromide (EtBr) and physical mutagenesis UV irradiation were employed to increase degradation capacity of *Bacillus cereus*.

3.13.1. Chemical mutagen and UV Irradiation of *Bacillus cereus*

A 10^8 CFU/ml of bacterial suspension taken in the aseptic plate without cover was exposed to UV light for time duration of 10, 20, 30, 40 and 60 min at a distance of 30 cm from UV lamp with a wavelength of 2537 A° and a power of 30,000 micro-watts. A 10^8 CFU/ml of bacterial suspension from a slant culture of *Bacillus cereus* was treated with 80 µg/ml of EtBr for duration of 30 min. (Appendix VI).

The petriplates was examined after 24 and 48 hours to count the number of bacteria colonies that have mutated as the result of the UV light and chemical mutagen. EtBr and UV irradiation of parent-type *Bacillus cereus* gave rise to 42 colonies.The cell suspension of the parent strain after inducing mutations was spread on the medium containing 2, 4-DCP and the resistant mutants were screened. A total of 6 colonies were screened and 2, 4-DCP concentration was measured and compared. *Bacillus cereus* UV-10, UV-20, UV-30, UV-40, UV-60 and EtBr-12 strain were selected and shake flask experiments were conducted to compare their degradation capabilities with that of the parent strain.

3.14. Degradation studies of 2, 4-DCP by mutated *Bacillus cereus*

Parent strain of *Bacillus cereus* and UV and Ethedium bromide mutated *Bacillus cereus* cells were grown in 100 ml of defined mineral salt medium. The cultures were placed on shaker at 120 rpm at 30°C in the dark. Growth was monitored by measuring the optical density at 610nm using Hitachi spectrophotometer. Cells were harvested at 20 hr. during mid-log phase. Cultures (5 ml) were centrifuged at 8000 rpm for 5 min, the pellet was washed with 0.85% NaCl and sterile distilled water. This was used immediately as inoculum for the optimization experiment. The inoculum size of 0.2 OD of *Bacillus cereus*, UV and Ethedium mutated *Bacillus cereus* cells were

transferred into different flasks containing 100ml of defined mineral salt medium along with 10, 30 and 75 mg/L of 2, 4-DCP concentration. The flasks were placed on shaker at 120 rpm at 30°C. 5 ml of sample was harvested every 24 hours and the cells were pelleted by centrifugation at 10,000 rpm for 5 min. 2, 4-DCP degradation was assessed in supernatant by standard HPLC method. Control sample with 2, 4-DCP in media without the microbial cells was also analysed. The same mutation experiment was carried out for 50,100 and 150mg/L concentration of PCMX.

3.15. Experimental design and optimization

Physical conditions such as pH, temperature and inoculum size and inoculum age play a vital role in a degradation process.A wide range of optimum and operational temperatures have been disclosed by many investigators for degradation work. In addition to physical parameters, medium composition is also very strongly influenced the degradation of chlorinated organic compound. Therefore, the degradation conditions and medium optimization studies are very important.

Response surface is an optimization methodology, mainly based on statistical techniques. Response surface methodology has been successfully used to modal and optimizes biochemical and biotechnological processes. The application of response surface methodology in degradation process can result in improved degradation of compound, reduced process variability, development time and overall costs. The objectives of a statistically designed optimization study are to (i) confirm previous effects and interactions, (ii) estimate specific curvature or quadratic effects, and (iii) determine optimal settings of the critical factors. Screening should be done when the investigator is faced with a large number of factors. Once a list of variables has been made, the settings for each variable must be determined. In a screening experiment, two settings are chosen for each variable. When the critical variables have been identified via screening, the investigator can proceed to the optimization stage of the experimental design.In general, optimization by traditional one variable at a time technique was used.This technique is not only laborious and time consuming but also often leads to an incomplete understanding of the systems behavior, resulting in confusion and a lack of predictive ability. Response surface methodology (RSM) is a powerful and efficient mathematical approach applied in the optimization of different

processes. It gave information necessary for design and process optimization and also helpful in the analysis of multiple responses at the same time.

In this study, the optimum conditions for maximum degradation of 2, 4, DCP and PCMX by *Bacillus cereus* were determined by means of RSM. According to this design, the total number of treatment combinations was 2^k + 2k + n0 where k is the number of independent variables and n0 is the number of repetitions of the experiments at the center point. Based on the best results of one at a time approach, four critical components of the growth medium were selected and further evaluated for their interactive behaviors by using a statistical approach like response Surface Method. The experimental design and statistical analysis were performedusing Minitab 15.0. A five-level four-factor central composite design(CCD) was chosen to evaluate the combined effect of four independent variables Peptone,Glucose, Phosphate and inoculum coded as X1, X2, X3 and X4 respectively. The minimum and maximum values for peptone were set at 0.5 -1.5 g/l and, glucose between 5-15 g/l and, phosphate between 4.5-13.5g/l and Inoculum 0.1-0.3 ml/l(Table 3.2).The response was percentage degradation. The complete design consisted of 30 combinations including five replicates of the center point (Myers & Montgomery 2002).Each of the variable were coded at five levels –2, -1, 0, 1, and 2 by using equation 1.

The range and levels of the variables in coded units for RSM studies are given in Table 2.

$$Xi=X_i-X_0/\Delta X \text{ ---------- (1)}$$

Where x_i is the dimensionless coded value of the variable X_i, X_0 the value of the X_i at the center point, and ΔX the step change.

The behavior of the system was explained by the following quadratic model 2.

$$Y= \beta_0 + \sum\beta_i x_{ia} + \sum\beta_{ii} x_i^2 + \sum\beta_{lj} X_l X_e \text{----------(2)}$$

Where Y is the predicted response, β_0 the intercept term, β_i the linear effect, β_{ii} the squared effect, and β_{ij} the interaction effect.

$$Y = \beta_0 + \beta_1 x1 + \beta_2 x2 + \beta_3 x3 + \beta_4 x4 + \beta_{11} x1*x1 + \beta_{12} x1*x\ 2+$$
$$\beta_{13} x1*x3 + \beta_{14} x1*x\ 4 + \beta_{22} x2*x2+ \beta_{23} x2*x3 + \beta_{24} x2*x\ 4 +$$
$$\beta_{33} x3*x3 + \beta_{34} x3*x4 + \beta_{44} x4*x4 \text{ ---------- } (3)$$

Several experimental designs have been considered for studying such models, and central composite design was selected. For this study, a 2^4 factorial design with eight star points and six replicates at the central points were employed to fit the second order polynomial model, which indicated that 30 experiments were required for this procedure. STATISTICA 6.0 (Stat Soft, Inc, Tulsa, OK, USA) software was used for regression and graphical analysis of the data obtained.In order to search for the optimum combination of major components of the production medium, experiments were performed according to the CCD experimental plan. The results of CCD experiments for studying the effect of four independent variables are presented along with the mean predicted and observed responses in Table 3.3.

Table 3.2: Independent Variables and their coded and actual values used for optimization studies

Independent Variables	Symbols	Code Levels				
		-2	**-1**	**0**	**+1**	**+2**
Glucose g/l	X1	5	7.5	10.00	12.5	15
Peptone g/l	X2	0.5	0.75	1.00	1.25	1.5
Inoculum Size	X3	0.1	0.15	0.2	0.25	0.3
Phosphate g/l	X4	4.50	6.75	9.0	11.25	13.5

Table 3.3: Design of experiments by Central composite design (CCD) for RSM studies

Run number	x1	x2	x3	x4	Coefficients assessed by
1	-1	-1	-1	-1	Full factorial 2^4 design (16 experiments)
2	1	-1	-1	-1	
3	-1	1	-1	-1	
4	1	1	-1	-1	
5	-1	-1	1	-1	
6	1	-1	1	-1	
7	-1	1	1	-1	
8	1	1	1	-1	
9	-1	-1	-1	1	
10	1	-1	-1	1	
11	-1	1	-1	1	
12	1	1	-1	1	
13	-1	-1	1	1	
14	1	-1	1	1	
15	-1	1	1	1	
16	1	1	1	1	
17	-2	0	0	0	
18	2	0	0	0	Star points
19	0	-2	0	0	(8 experiments)
20	0	2	0	0	
21	0	0	-2	0	
22	0	0	2	0	
23	0	0	0	-2	
24	0	0	0	2	
25	0	0	0	0	
26	0	0	0	0	Central points
27	0	0	0	0	(6 experiments)
28	0	0	0	0	
29	0	0	0	0	
30	0	0	0	0	

3.16. Degradation studies on industrial effluent containing 2,4-DCP and PCMX by parent and UV treated bacillus isolates

The present investigation is an attempt to study the effect of UV-treated and parent strain *Bacillus cereus* on bioremediation of industrial waste effluents.

3.16.1. Effluent sample collection from industry

Industrial effluent samples containing 2,4-DCP and PCMX were collected from M/s Sequent Scientific and Rickitt Benckiser Dettol manufacturing company which is located in Karnataka, India. The effluent was collected in sterilized plastic can from the inlet of Rickitt Benckiser containing untreated effluent with significant amount of PCMX and from inlet of M/s Sequent Scientific Ltd. containing untreated effluent with significant amount of 2,4-DCP. In laboratory the effluent was stored in a refrigerator at 4°C and further used for biodegradation studies.

3.16.2. Measurement of PhysicoChemical parameters of the effluent samples

The physicochemical parameters like pH and COD of collected effluent were measured by digital pH meter and by titration method.

3.16.3. Determination of 2,4-DCP and PCMX concentration in industrial effluent

The estimation of 2,4-DCP and PCMX was done by Dionex make High Performance Liquid Chromatography (HPLC).

3.17. Degradation studies of 2,4-DCP and PCMX by mutated and parent strain *Bacillus cereus* in industrial effluent

Batch culture experiments for the degradation of 2, 4-DCP and PCMX using *Bacillus cereus*, were carried out in Erlenmeyer flasks (250 ml).One set containing 100ml of effluent with initial concentration of 0.2 mg/L of 2,4-DCP with initial pH 5 and 3000 mg/L of PCMX and initial pH of 7 was incubated in shaker at 30°C at 120 rpm. It also contained nutrients ((in $g \cdot \ell^{-1}$) KH_2PO_4, 3g; Na_2HPO_4, 6g; Peptone, 1g;and Glucose, 10g) and 0.2 OD *Bacillus cereus*.One more set of Erlenmeyer flasks containing 100 ml of effluent and 0.2 OD *Bacillus cereus* without nutrient was placed on a shaker at 120 rpm at 30°Cin dark, to avoid photodecomposition of 2,4-DCP and

PCMX. All experiments were carried out in duplicates. 5 ml of sample are collected at regular intervals over a period of 20 days and analyzed for the amount of 2,4-DCP and PCMX, pH of medium and growth of *Bacillus cereus* using Dionex HPLC system, Elico pH meter and Hitachi UV visible spectrophotometer respectively.

CHAPTER 4

RESULTS & DISCUSSION

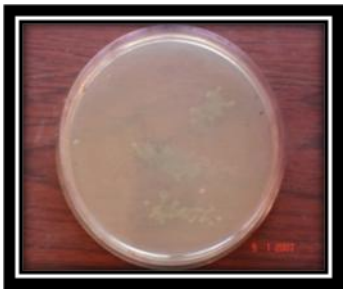
The present chapter deals with the experimental results obtained using the methodologies presented in Chapter 3 to meet the stated objectives presented in Chapter 1. The observation of experiments carried out namely enrichment and isolation of microorganisms and screening based on the degradation are presented. The results from identification of the potential degrading microorganism using various tests, namely, microscopic observations, biochemical tests as well as partial 16S ribosomal DNA sequencing are presented. Further experiments on the chosen microorganism in terms of its efficacy and efficiency for the degradation of 2,4-DCP and PCMX are presented in this chapter. This chapter also presents the experimental results with respect to mutated strains and degradation studies on industrial effluent.The results of various experiments in the present study arepresented in the form of Tables and Figures; the data obtained has been compared with relevant literature. The experimental observations presented in this chapter are the average of the experimental results in duplicate.

4.1. Selection of high-efficiency strains

This study intended to isolate a bacterium which is effective in the degradation of 2, 4-DCP and PCMX. Soil samples for isolation were collected from an area around the wastewater treatment plant from Rickitt Benckiser Dettol manufacturing company located in Mysore, Karnataka. A defined mineral salt medium was used for isolation and six strains were isolated by the spread plate cultivation and streak cultivation method. They were tentatively named as 01-C-My-NITK, 03-M-My-NITK, 03-G-My-NITK, 02-Y-My-NITK, 04-O-My-NITK and 03-P-My-NITK respectively which are projected in Figure 4.1.

04-O-My-NITK
(Microroganism isolated near untreated effluent storage tank)

03-P-My-NITK
(Microroganism isolated near treatment plant)



03-G-My-NITK (Microroganism isolated near treatment plant)

03-M-My-NITK (Microroganism isolated near treatment plant)

01-C-My-NITK (Microroganism isolated near sludge drying tank)

02-Y-My-NITK (Microroganism isolated near chlorination tank)

Fig 4.1: Six different bacterial strains isolated from soil

4.2. Screening studies

Screening was carried out by counting the colonies using colony counter to identify the microorganism which was most suitable for the degradation of different concentration of 2,4-DCP and PCMX. The six isolated strains were inoculated on defined mineral salt medium with different concentrations of 2, 4-DCP and PCMX (0.5mg/L, 1mg/L, 2.5mg/L, 5mg/L, 10mg/L, 25mg/L, 50mg/L) and incubated at 30°**C**. The results of these studies are presented in Table 4.1 and 4.2 for 2,4 DCP and PCMX respectively.All the six isolates were resistant to lower concentrations of 2, 4-DCP and PCMX. However, as the concentration of 2, 4-DCP and PCMX increased, their resistivity also got reduced. From Tables it is evident that out of six isolated strain 03-M-My-NITK, 04-O-My-NITK, 02-Y-My-NITK and 03-G-My-NITK showed low resistance up to 10mg/L of 2, 4-DCP and PCMX concentration.

Remaining two isolates 03-P-My-NITK and01-C-My-NITK could effectively utilize both 2, 4-DCP and PCMX up to 25 mg/L.

Hence for further confirmation, screening studies based on biomass concentration was carried out for only two isolates 03-P-My-NITK and 01-C-My-NITK.The results of biomass concentration of 03-P-My-NITK and 01-C-My-NITK with respect to growth (OD) are presented in Tables4.3, 4.4, 4.5 and 4.6 and Figures 4.2,4.3,4.4 and 4.5 respectively.From the Tables and Figures it is evident that 03-P-My-NITK has shown good tolerance to 2, 4-DCP up to 25 mg/L and PCMX up to 5 mg/L. High growth was observed in control without 2,4-DCP and PCMX. Further from the Figures and Tables it is evident that 01-C-My-NITK has shown good tolerance to 2, 4-DCP up to 50 mg/L and PCMX up to 25 mg/L. Low growth was observed in control without the presence of 2, 4-DCP and PCMX.

Hence it can be concluded that among the six isolated strains, 01-C-My-NITK and 03-P-My-NITK, can be considered to be the best for the reduction of 2, 4-DCP and PCMX. Thus, further studies on 2, 4-DCP and PCMX reduction were carried out by these strains.

Table 4.1: Screening of six isolates using different concentration of 2, 4-DCP

Organism	Hours	0.5 mg/L	1 mg/L	2.5 mg/L	5 mg/L	10 mg/L	25 mg/L	50 mg/L	75 mg/L	100 mg/L	150 mg/L	350 mg/L
03-M-My-NITK	24	++	++	++	++	+	+	-	-	-	-	-
	48	++	++	++	++	+	+	-	-	-	-	-
	72	++	++	++	++	+	+	-	-	-	-	-
	192	++	++	++	++	+	+	-	-	-	-	-
01-C-My-NITK	24	++	++	++	++	++	+	+	-	-	-	-
	48	++	++	++	++	++	+	+	-	-	-	_
	72	++	++	++	++	++	+	+	-	-	-	-
	192	++	++	++	++	++	+	+	-	+	+	+
03-G-My-NITK	24	+++	++	++	++	+	-	+	-	-	-	-
	48	++	++	++	++	+	-	+	-	-	-	-
	72	++	++	++	++	+	-	+	-	-	-	-
	192	++	++	++	++	+	+	+	-	-	-	-
04-O-My-NITK	24	++	++	++	++	++	+	-	-	-	-	-
	48	++	++	++	++	++	+	-	-	-	-	-
	72	++	++	++	++	++	+	-	-	-	-	-
	192	++	++	++	++	++	+	-	-	-	-	-
03-P-My-NITK	24	+++	+++	+++	+++	+++	+++	-	-	-	-	-
	48	+++	+++	+++	+++	+++	+++	+	+	+	-	-
	72	+++	+++	+++	+++	+++	+++	+	+	+	-	-
	192	+++	+++	+++	+++	+++	+++	-	-	-	-	-
02-Y-My-NITK	24	+++	+++	+++	+++	+++	+++	-	-	-	-	-
	48	++	++	++	++	++	++	-	-	-	-	-
	72	++	++	++	++	++	++	-	-	-	-	-
	192	++	++	++	++	++	++	-	-	-	-	-

+++ --- Colony Counts above 1000
++ --- Colony Counts above 600
+ --- Colony Counts above 300

Table 4.2: Screening of six isolates using different concentration of PCMX

Organism	Hours	0.5 mg/L	1 mg/L	2.5 mg/L	5 mg/L	10 mg/L	25 mg/L	50 mg/L	75 mg/L	100 mg/L	150 mg/L	350 mg/L
03-M-My-NITK	24	+++	+++	+++	++	++	+	-	-	-	-	-
	48	++	++	++	++	++	+	+	-	-	-	-
	72	+++	++	++	++	++	+	+	-	-	-	-
	192	+++	++	++	++	++	+	+	-	-	-	-
01-C-My-NITK	24	+++	+++	+++	+++	+++	+++	+++	-	-	-	-
	48	++	++	++	++	++	++	++	-	+	+	_
	72	++	++	++	++	++	++	++	-	+	+	+
	192	++	++	++	++	++	++	+	-	+	+	+
03-G-My-NITK	24	+++	++	++	++	+	-	-	-	-	-	-
	48	++	++	++	++	+	-	-	-	-	-	-
	72	++	++	++	++	++	-	-	-	-	-	-
	192	++	++	++	++	+	+	+	-	-	-	-
04-O-My-NITK	24	+++	+++	+++	+++	+++	-	-	-	-	-	-
	48	++	++	++	++	++	-	-	-	-	-	-
	72	++	++	++	++	++	-	-	-	-	-	-
	192	++	++	++	++	++	+	+	-	-	-	-
03-P-My-NITK	24	+++	+++	+++	+++	+++	+	+	-	-	-	-
	48	+++	++	++	++	++	+	+	+	+	-	-
	72	+++	++	++	++	++	+	+	+	+	-	-
	192	+++	++	++	-	-	-	-	-	-	-	-
02-Y-My-NITK	24	+++	+++	+++	+++	++	-	-	-	-	-	-
	48	++	++	++	++	++	-	-	-	-	-	-
	72	++	++	++	++	++	-	-	-	-	-	-
	192	++	++	++	++	++	-	-	-	-	-	-

+++ --- Colony Counts above 1000
++ --- Colony Counts above 600
+ --- Colony Counts above 300

Table 4.3: Growth (OD) of 03-P-My-NITK in different concentration of 2, 4-DCP with time

Time(hr.)	0.5 mg/L	1 mg/L	2.5 mg/L	5 mg/L	10 mg/L	25 mg/L	50 mg/L	Control
0	0.002	0.003	0.003	0.002	0.001	0.002	0.002	0.003
24	0.324	0.31	0.309	0.267	0.283	0.218	0.086	0.264
48	0.341	0.332	0.379	0.309	0.342	0.265	0.149	0.333
72	0.186	0.146	0.234	0.414	0.193	0.217	0.097	0.467
192	0.133	0.141	0.44	0.415	0.135	0.185	0.065	0.291

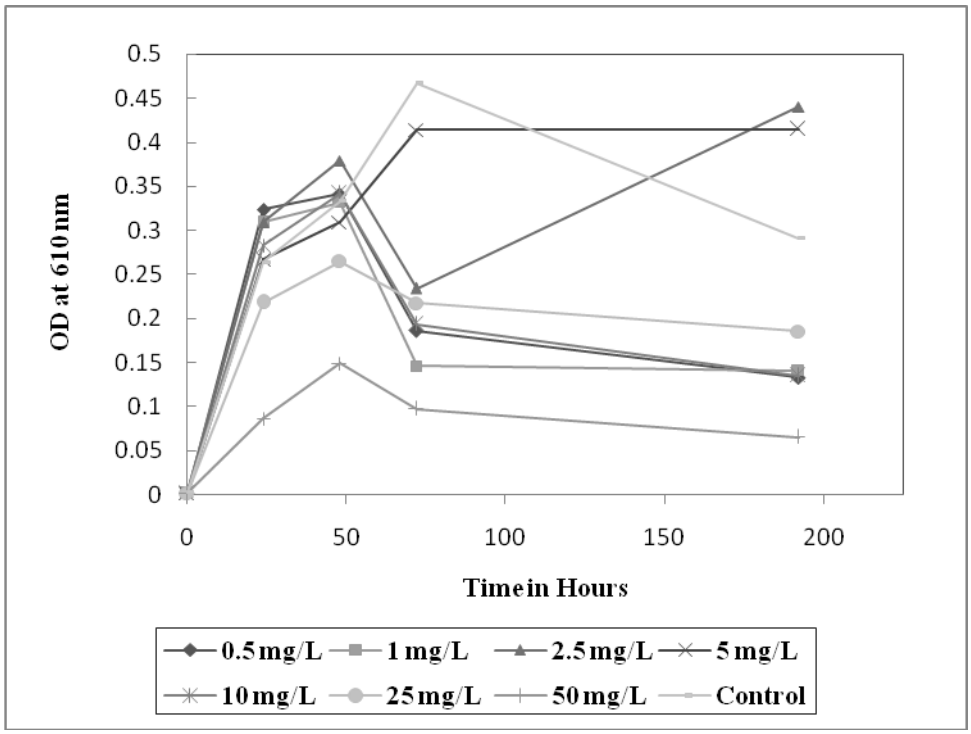


Fig. 4.2: Growth (OD) of 03-P-My-NIK in different concentration of 2, 4-DCP with time

Table 4.4: Growth (OD) of 03-P-My-NITK in different concentration of PCMX with time

Time(hr.)	0.5 mg/L	1 mg/L	2.5 mg/L	5 mg/L	10 mg/L	25 mg/L	50 mg/L	Control
0	0.002	0.003	0.003	0.002	0.001	0.002	0.002	0.003
24	0.229	0.211	0.037	0.002	0.002	0.001	0.002	0.264
48	0.268	0.233	0.198	0.003	0.003	0.004	0.002	0.333
72	0.268	0.241	0.164	0.003	0.003	0.004	0.006	0.467
192	0.476	0.398	0.073	0.035	0.004	0.005	0.007	0.291

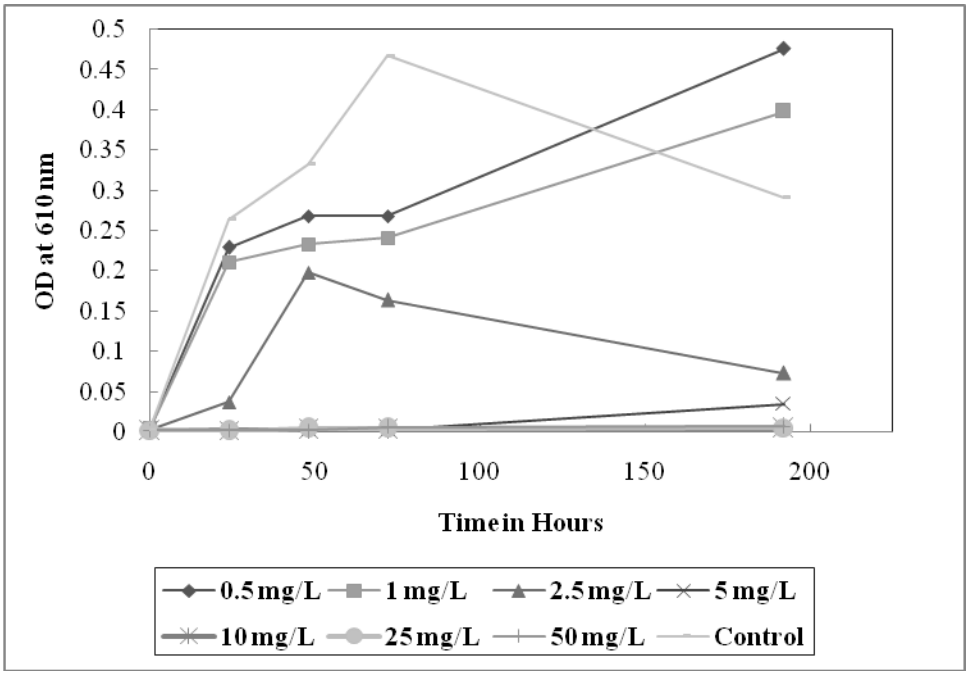


Fig.4.3: Growth (OD) of 03-P-My-NITK in different concentration of PCMX with time

Table 4.5: Growth (OD) of 01-C-My-NITK in different concentration of 2, 4-DCP with time

Time(hr.)	0.5 mg/L	1 mg/L	2.5 mg/L	5 mg/L	10 mg/L	25 mg/L	50 mg/L	Control
0	0.002	0.003	0.003	0.002	0.002	0.004	0.002	0.003
24	1.176	1.101	1.356	1.245	0.898	1.138	0.987	0.216
48	0.94	0.91	1.233	1.045	1.753	1.234	0.913	0.266
72	0.608	0.342	0.363	0.537	0.481	1.631	0.843	0.316
192	1.326	1.041	0.507	0.48	0.525	0.42	0.342	0.613

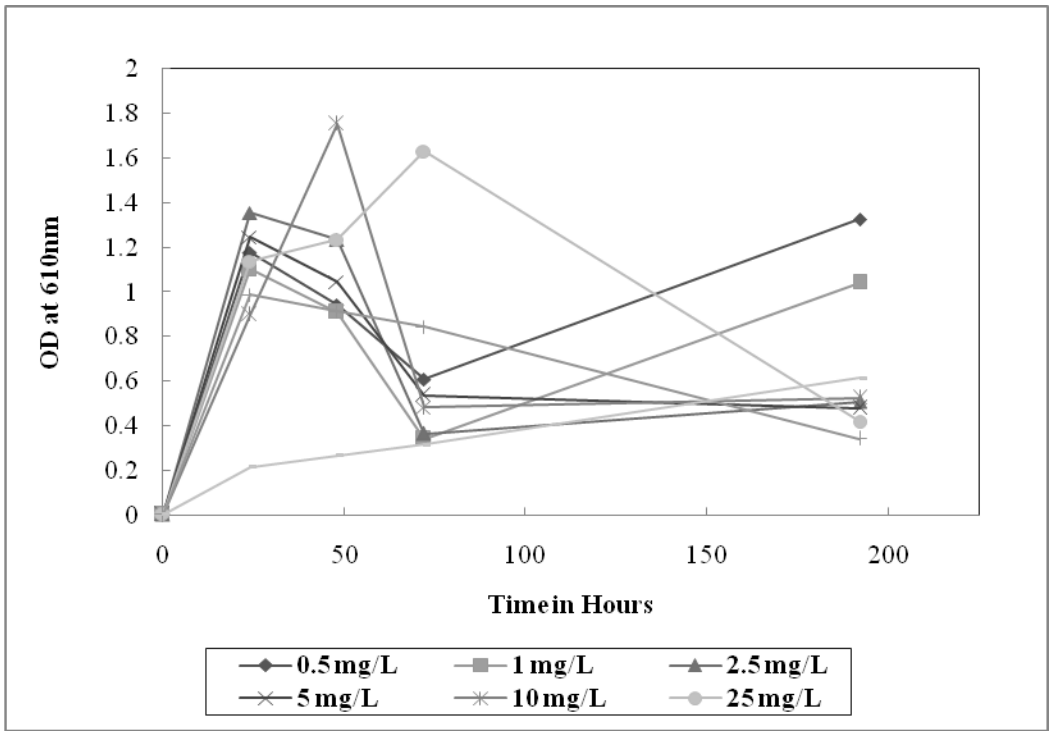


Fig. 4.4: Growth (OD) of 01-C-My-NITK in different concentration of 2, 4-DCP with time

Table 4.6: Growth (OD) of 01-C-My-NITK in different concentration of PCMX with time

Time(hr.)	0.5 mg/L	1 mg/L	2.5 mg/L	5 mg/L	10 mg/L	25 mg/L	50 mg/L	Control
0	0.004	0.003	0.002	0.003	0.003	0.002	0.001	0.002
24	1.153	1.11	0.327	0.064	0.001	0.009	0.007	0.216
48	1.199	1.102	0.358	0.023	0.002	0.005	0.006	0.266
72	1.192	0.998	0.402	0.003	0.001	0.004	0.005	0.316
192	0.482	0.401	0.131	0.07	0.033	0.047	0.032	0.613

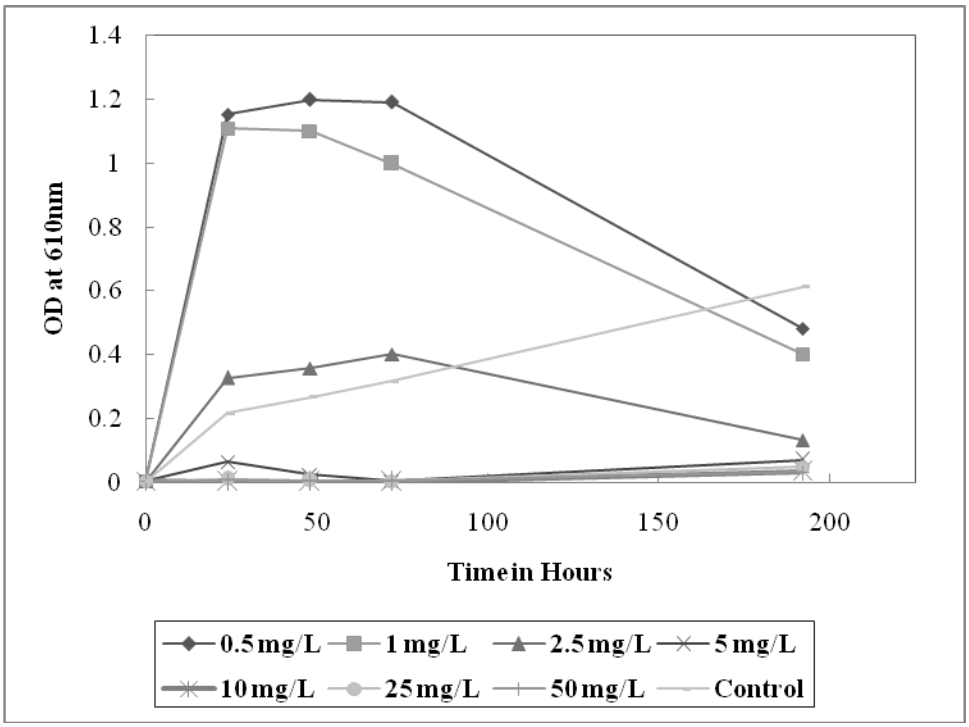


Fig. 4.5: Growth (OD) of 01-C-My-NITK in different concentration of PCMX with time

4.3. Identification of the isolate

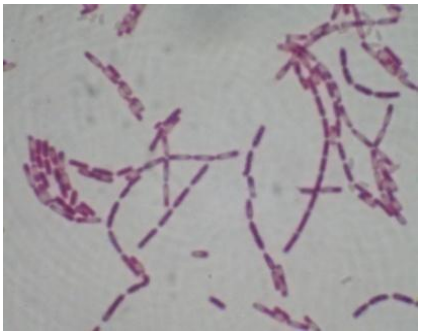
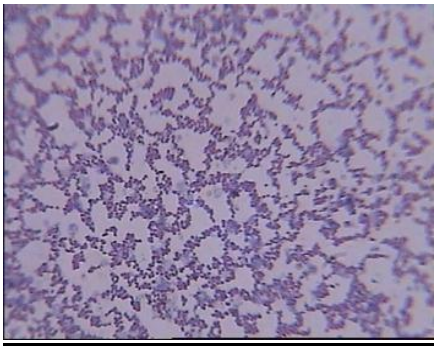
4.3.1. Biochemical analysis

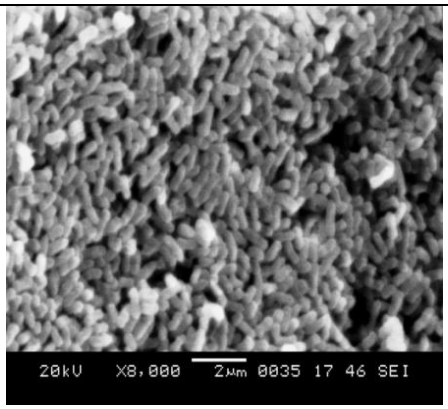
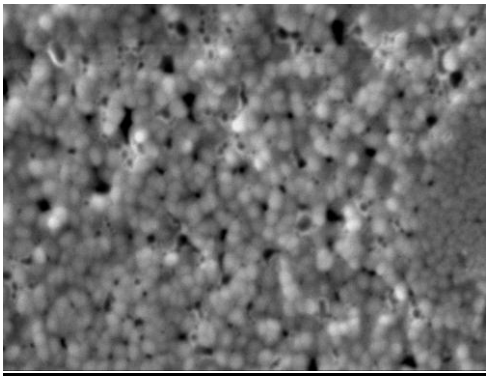
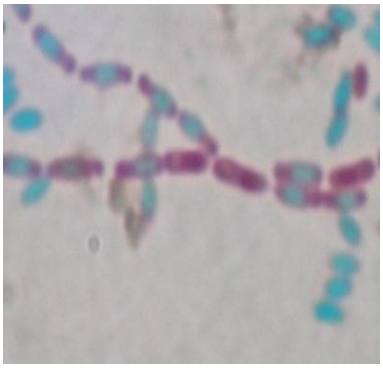
The morphological, physiological, biochemical and sequence analysis of selected bacterial isolate are shown in the Table 4.7 (*Bergey's Manual of Systemic Bacteriology*). Strain 01-C-My-NITK is an aerobic, Gram-positive, motile, endospore forming rod-shaped bacteria and they produced no fluorescent pigment. Colonies that formed on nutrient agar plates were flat, translucent, white, irregular, undulate and convex colonies. The strain gave positive results for Amylase, Catalase, Methyl red, Glucose and Oxidase. In contrast, the tested isolate recorded negative results with theVoges–Proskauer, Indole, Starch hydrolysis, Citrate utilization, Sucrose, Lactose, Urease, H_2S production and Hydrolysis of gelatin. The strain grew well in a simple defined medium without supplemental micronutrients. It also grewv well at 30°C and pH 6–8, but did not grown wellat 45°C. On the basis of the results obtained in this study, it is likely that the strain 01-C-My-NITK could belong to the genus *Bacillus*.

Strain 03-P-My-NITK is an aerobic, Gram-negative, short rod bacterium with approximate size (μm) 0.8-1.5. Colonies that formed on nutrient agar plates produced red pigment. The strain gave positive results for Starch hydrolysis, Hydrolysis of gelatin, Catalase, Hydrolysis of gelatin and Oxidase.In contrast, the tested isolate recorded negative results with the Vogues–Proskauer, Indole, Citrate utilization, methyl red, Sucrose, Lactose, Urease and H_2S production.The strain grew well in a simple defined medium without supplemental micronutrients. It also grew well at 30°C and pH 6–8, but did not grow well at 45°C. Based on the results obtained in this study, it is likely that the strain 03-P-My-NITK could belong to the genus *Serratia.*

Table 4.7: Characteristics of the bacterial isolate 01-C-My-NITK and 03-P-My-NITK

Morphological tests:

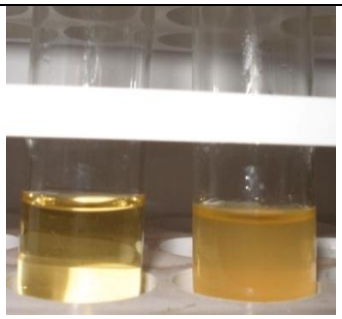
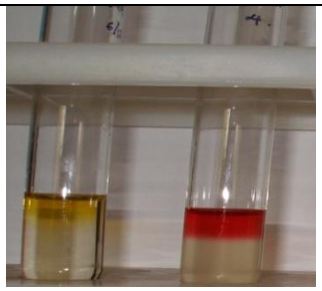
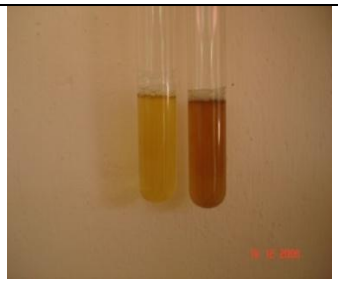
Test	01-C-My-NITK	03-P-My-NITK
Colony morphology		
Configuration	Circular	Circular
Margin	Irregular	Entire
Elevation	Flat	Convex
Surface	Rough	Smooth
Pigment	Cream	Red
Opacity	Opaque	Opaque
Cell shape	Rod	Short rod
Size (μm)	1.5-3.0	0.8-1.5
Arrangement	Chains	Irregular
Spore(s)	+	-
Position	Central	-
Shape	Ellipsoidal	-
Sporangia bulging	Non-bulged	-
Motility	-	+
Gram's reaction	Gram +ve, bacilli 	Gram -ve, cocci 

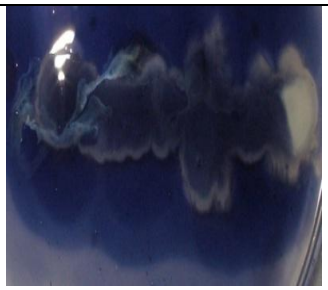
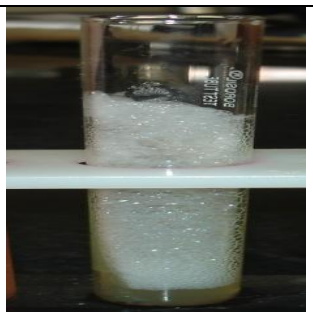
Scanning electron microscope Image		
Endospore staining		
Cell shape	Rod	Short rod
Size (µm)	1.5-3.0	0.8-1.5
Arrangement	Chains	Irregular
Spore(s)	+	-
Position	Central	-
Shape	Ellipsoidal	-
Sporangia bulging	Non-bulged	-
Motility	-	+

Physiological tests:

Tests	01-C-My-NITK	03-P-My-NITK
Growth at temp		
10^{o}C	+	+
15^{o}C	+	+
20^{o}C	+	+
25^{o}C	+	+
30^{o}C	+	+
37^{o}C	+	+
42^{o}C	+	+
45^{o}C	+	+
Growth at pH		
pH 6.0	+	+
pH 8.0	+	+
pH 9.0	+	+
pH 10.5	-	-
Growth at NaCl (%)		
2.0	+	+
4.0	+	+
6.0	+	+
Anaerobic growth	+	+

Biochemical tests:

Tests	01-C-My-NITK	03-P-My-NITK	Photos
Indole test	-	-	
Methyl red test	+	-	
Voges Proskauer test	-	-	
Citrate utilization	-	-	
Gelatin hydrolysis	-	+	

Starch hydrolysis	-	+	
Amylase Test	+	-	
Catalase test	+	+	
Oxidase test	+	-	
Fermentation of carbohydrates:			
Glucose	+	+	

Sucrose	-	+	
Lactose	-	+	
Urease test	-	-	

4.3.2. Molecular analysis

After studying the morphological and physiological characteristic of the 01-C-My-NITK and 03-P-My-NITK it was concluded that the strains fall under *Bacillus and Serratia* species respectively. As per the literature survey *Serratia* species are human opportunistic pathogen.Hence further 16srDNA and phylogenetic studies were carried out with only *Bacillus* species and wassent to Anshul Biotech, Hyderabad.Figure 4.6 presents the phylogenetic tree and it is observed from that this isolate is closely related to *Bacillus cereus*.

GATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGA
AGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAA
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ACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGC
GTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAG
GCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAA
GGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTG
ACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCA
CGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTC
CATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCT
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TGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACC
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GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGG
CACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCC
TTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAG
CTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGC
TAGTAATCGCGGATCAGCATGCCGCGGGTGAATACGNTCCCGGGCCTTGTACACACCG

Fig 4.6: 16S ribosomal DNA sequence of the isolate *Bacillus cereus*

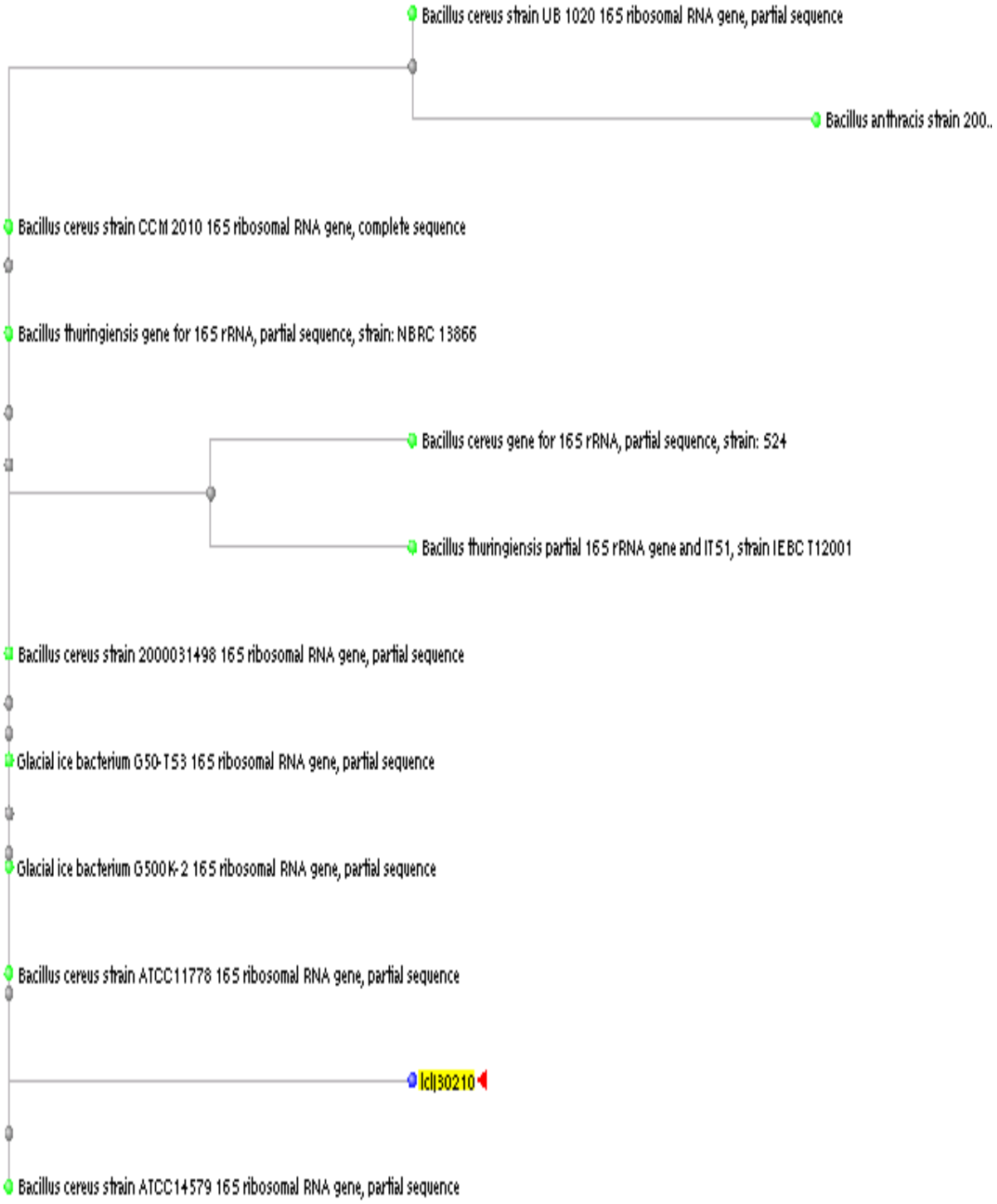


Fig.4.7: Phylogenetic cladogram based on comparison of related partial 16srDNA sequences for 01-C-My-NITKby CLUSTALW (Accelerys GCG software).

SUMMING UP

In conclusion the present study was undertaken to isolate and characterize the 2, 4-DCP and PCMX resistant bacteria from soil near a waste water treatment plant of an industry handling/ manufacturing these chemicals. Six bacterial isolates capable of degrading 2, 4-DCP and PCMX were obtained on agar plates using culture enrichment technique. Growth rate of these six bacteria were assessed in different concentrations of 2, 4-DCP and PCMX (5–50mg/L). Two of these strains 03-P-My-NITK and 01-C-My-NITK were found resistant up to 50 mg/L and 5mg/L of 2, 4-DCP and PCMX respectively. Biochemical characterization was carried out in laboratory and with this investigation it has been demonstrated that 01-C-My-NITK and 03-P-My-NITK belong to *Bacillus* and *Serratia species* respectively. *Serratia species* was first thought to be harmless (non-pathogenic). Due to its ability to produce red pigmentation, it was first used in 1906 as a marker in order to trace bacterial activity or transmission (Cappuccino et al.2005). It was not until the 1950's that the US government experimented with the *Serratia species* and the harmful effects that the bacteria cause were revealed. Further study using *Serratia species* was carried out to determine the possibility of biological weapons being transmitted by wind current by Lazachek et al. (1971) and Leopold Kurz et al. (2003).He concluded that *Serratia species* is a human opportunistic pathogen. Hence, studies in the present work were continued only with *Bacillus species* rather than *Serratia species*.For further confirmation the organism 01-C-My-NITK was sent to Anshul Biotech, Hyderabad for 16s rDNA and phylogenetic studies. Based on the results the isolate belonged to *Bacillus cereus*.

4.4. Effect of inoculum density on the growth of *Bacillus cereus*

The effects of quantum of inoculum taken initially on the growth of *Bacillus cereus* were studied for different time (0-49 hours).The growth (OD) of *Bacillus cereus* for different concentrations (0.01, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5) of inoculum density with respect to optical density are presented in Table 4.8 and Figure 4.8. From the Figures and Tables it is observed that maximum growth of *Bacillus cereus* was at 0.2 OD of inoculum density and observed that growth inhibition increased with higher inoculum density. This work can be substantiated with the investigation done by Rita Grosch et al. (2003) who reported the plant growth of infected plants was also influenced by inoculum density of the pathogen and growth inhibition increased with higher inoculum density.

Table 4.8: Growth of *Bacillus cereus* in different concentration of inoculum density

Time (hr.)	0.01	0.05	0.1	0.2	0.3	0.4	0.5
0	0.001	0.001	0.001	0.001	0.001	0.001	0.001
2	0.001	0.001	0.001	0.001	0.001	0.001	0.001
4	0.001	0.001	0.001	0.001	0.001	0.001	0.001
6	0.001	0.001	0.001	0.001	0.001	0.001	0.001
8	0.001	0.001	0.001	0.001	0.001	0.001	0.001
10	0.001	0.001	0.001	0.001	0.001	0.001	0.001
12	0.001	0.001	0.001	0.001	0.001	0.001	0.001
14	0.001	0.001	0.001	0.001	0.001	0.001	0.001
16	0.001	0.001	0.001	0.001	0.001	0.001	0.001
24	0.001	0.001	0.001	0.001	0.001	0.001	0.001
27	0.001	0.001	0.002	0.001	0.001	0.001	0.002
30	0.001	0.002	0.002	0.006	0.001	0.002	0.002
33	0.002	0.002	0.002	0.008	0.002	0.002	0.002
36	0.002	0.003	0.006	0.009	0.002	0.003	0.006
39	0.001	0.004	0.006	0.009	0.001	0.004	0.006
49	0.002	0.004	0.007	0.01	0.002	0.004	0.007

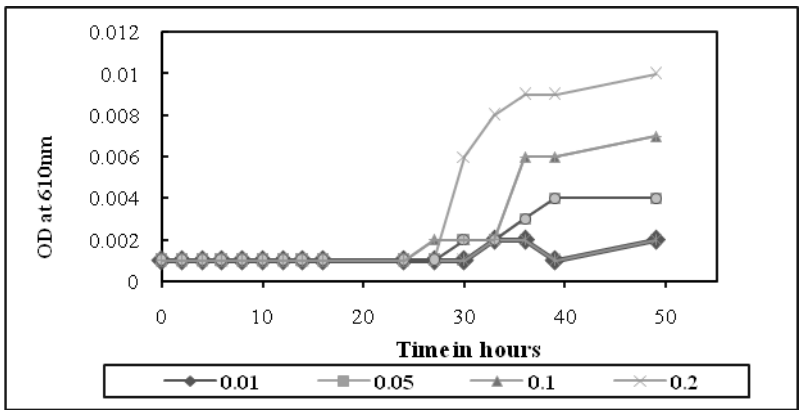


Fig. 4.8: Growth (OD) of *Bacillus cereus* in different concentration of inoculum density

4.5. Effect of concentration of glucose on the growth of *Bacillus cereus*

The biomass growth studies were carried out using various glucose concentrations (0.1, 0.5, 0.8 and 10g/l) for different time duration (0 – 49 hour). Table 4.9 and Figure 4.9 shows growth (OD) of *Bacillus cereus* for different concentrations of glucose. It is observed that maximum growth of *Bacillus cereus* was observed at 10 g/L of Glucose.Hence it can be concluded that glucose is acting as the primary growth substrate.As per reports reported by different authors glucose was found to be the superior growth co substrate, suggesting that inexpensive sugars can be used to enhance the biodegradation of chlorophenol.

Table 4.9: Growth of *Bacillus cereus* in different concentration of glucose

Time (hr.)	0.1 g/L Glucose	0.5 g/L Glucose	0.8 g/L Glucose	10 g/L Glucose
0	0.001	0.001	0.001	0.001
2	0.001	0.001	0.001	0.001
4	0.001	0.001	0.001	0.001
6	0.001	0.001	0.001	0.001
8	0.001	0.001	0.001	0.001
10	0.001	0.001	0.001	0.001
12	0.001	0.001	0.001	0.001
14	0.001	0.001	0.001	0.001
16	0.001	0.001	0.001	0.001
24	0.001	0.001	0.001	0.001
27	0.001	0.001	0.002	0.001
30	0.001	0.002	0.002	0.006
33	0.002	0.002	0.002	0.008
36	0.002	0.003	0.006	0.009
39	0.001	0.004	0.006	0.009
49	0.002	0.004	0.007	0.01

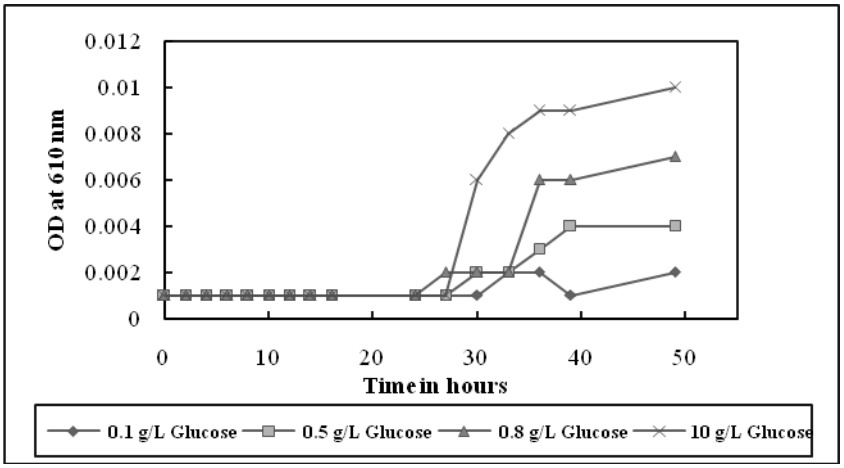


Fig.4.9: Growth of *Bacillus cereus* in different concentration of glucose

4.6. Effect of concentration of peptone on the growth of *Bacillus cereus*

The growth of *Bacillus cereus* in different concentrations of peptone (0.1, 0.5 and 1g/L) at different time interval was studied. Table 4.10 and Figure 4.10 show growth (OD) of *Bacillus cereus* for different concentration of Peptone. It is observed that maximum growth of *Bacillus cereus* was observed at 1 g/L of Peptone and exponential growth commenced immediately after inoculation with peptone and with no noticeable lag phase and peptone above certain limit was inhibitory on growth.This result coincides with Chandana Lakshmi et al, (2011) who reported that phenol degradation by a bacterial population was also enhanced by adding nitrogen sources like ammonium salts

Table 4.10: Growth of *Bacillus cereus* in different concentration of peptone

Time(hr.)	0.1g/L peptone	0.5g/L peptone	1g/L peptone
0	0.001	0.002	0.002
2	0.001	0.002	0.003
4	0.001	0.005	0.001
6	0.001	0.008	0.008
8	0.008	0.032	0.062
10	0.027	0.077	0.237
12	0.034	0.19	0.344
14	0.036	0.249	0.39
16	0.056	0.283	0.382
24	0.061	0.297	0.267
27	0.142	0.294	0.262
30	0.167	0.255	0.225
33	0.152	0.243	0.218
36	0.134	0.227	0.228
39	0.123	0.213	0.226
49	0.111	0.223	0.23

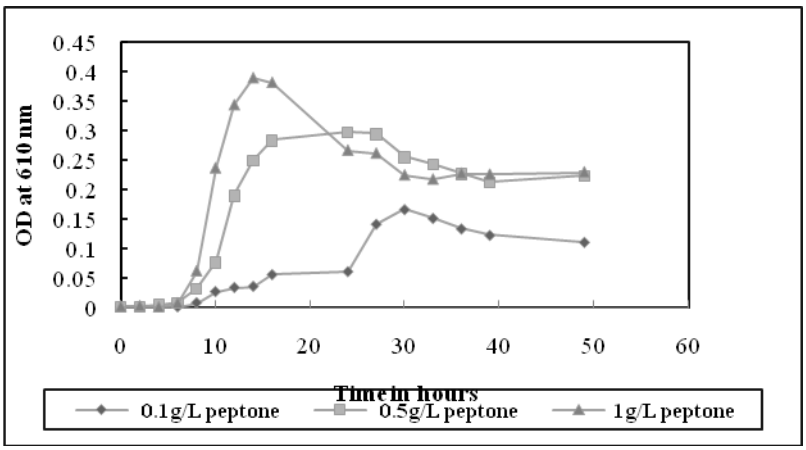


Fig.4.10: Growth of *Bacillus cereus* in different concentration of peptone

4.7. Effect of different temperature and pH on the growth of *Bacillus cereus*

The study was carried out for the growth of *Bacillus cereus* at varying temperatures 25, 30, 35, 40 and 45°C and pH 5, 6, 7 and 8. Table 4.11 and Figure 4.11 shows growth (OD) of *Bacillus cereus* at different temperatures (25°C, 30°C, 35°C, 40°C, 45°C). Table 4.12 and Figure 4.12 shows growth (OD) of *Bacillus cereus* in different pH (5, 6, 7 and 8). It is observed from the Figures and Tables that optimum growth temperature for *Bacillus cereus* is 25°C. This work can be validated with the work carried by Johnson and Snygg et al., (1974) wherein it has been stated that the optimum temperature for *Bacillus cereus* is between 30 and 37°C but some strains can grow at temperature as low as 4.5°C and up to 55°C. It is also observed that optimum growth pH for *Bacillus cereus* is pH 5.This finding is in agreement with the work of Goepfert et al. (1972) who reported that pH 4.9 - 9.3 permitted growth of *Bacillus cereus* in laboratory media.

Table 4.11: Growth (OD) of *Bacillus cereus* at different temperatures

Time(hr.)	25°C	30°C	35°C	40°C	45°C
0	0.003	0.003	0.003	0.004	0.004
24	0.208	0.036	0.086	0.128	0.078
48	0.048	0.046	0.064	0.049	0.097
72	0.058	0.046	0.056	0.054	0.078
96	0.056	0.047	0.052	0.048	0.078

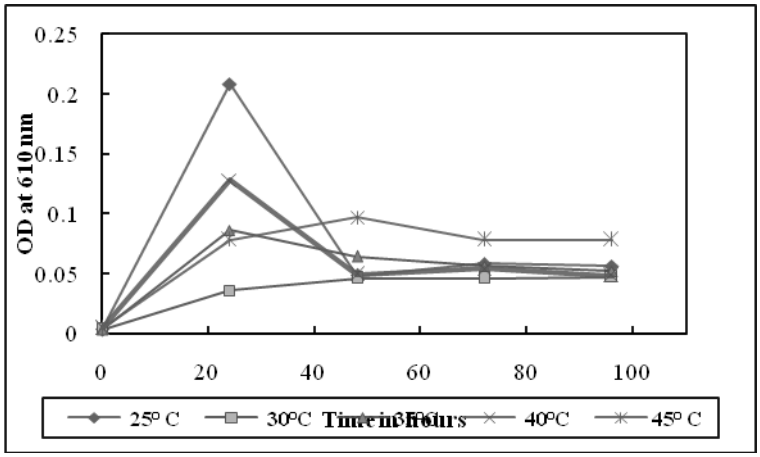


Fig. 4.11: Growth (OD) of *Bacillus cereus* at different temperatures

Table 4.12: Growth (OD) of *Bacillus cereus* at different pH

Time(hr.)	pH 5	pH 6	pH 7	pH 8
0	0.003	0.002	0.003	0.002
24	0.094	0.067	0.071	0.04
48	0.104	0.056	0.065	0.065
72	0.112	0.056	0.068	0.059
96	0.113	0.042	0.062	0.059

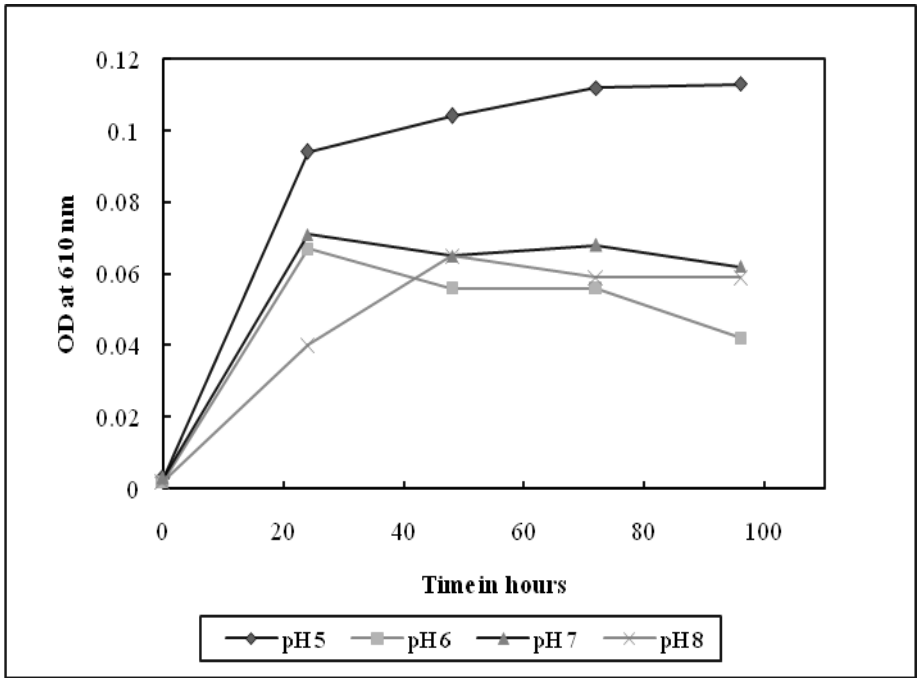


Fig. 4.12: Growth (OD) of *Bacillus cereus* at different pH

4.8. Optimization of temperature and pH to increase the percentage degradation of 2, 4-DCP and PCMX by *Bacillus cereus*

From the earlier studies it has been established that temperature and pH play a vital role in determining the ability of bacterial growth of *Bacillus cereus*. Hence further studies were carried out to find the effect of the different temperatures (25°C, 30°C, 35°C, 40°C) and different pH (2, 3, 4, 5, 6, 7, 8 and 10) in the presence of 2,4-DCP and PCMX on the bacterial growth and degradation varying concentration of 2,4-DCP (10, 30, 75 mg/L) and PCMX (50, 100, 150, 350 mg/L).

For the determination of optimum temperature on 2,4-DCP and PCMX removal by the bacterium,an inoculum size of 0.2 OD was added to the minimal medium with an initial pH 7 and incubated at different temperatures (25°C, 30°C, 35°C, 40°C) in different concentrations 10 mg/L ,30 mg/L and 75 mg/L of 2, 4-DCP and 50 mg/L, 100 mg/L,150 mg/L and 350 mg/L of PCMX and incubated under shaking at 120 rpm in an orbital shaker. Similarly for the determination of pH dependency on 2,4-DCP and PCMX removal by the bacterium, the pH of the minimal medium was adjusted to various pH values (2,3,4,5,6,7,8,10) in different concentrations 10 mg/L ,30 mg/L and 75 mg/L of 2, 4-DCP and 50 mg/L, 100 mg/L, 150 mg/L and 350 mg/L of PCMX and incubated at 30^{o}C at 120rpm in an orbital shaker for desired period. After incubation, cells were separated from culture medium by centrifugation. Clear supernatant was used for the assay of 2,4-DCP and PCMX using the HPLC. pH and biomass level were also determined at different intervals.

4.8.1. Degradation studies of 2, 4-DCP (10mg/L) by *Bacillus cereus* at different temperature

Effect of incubation temperature on 2, 4-DCP removal by *Bacillus cereus* was carried out at different temperatures. *Bacillus cereus* was able to grow and degrade 2,4-DCP at temperature ranging from 25^{o}C to 40^{o}C (Table 4.13 and Figure 4.13).However, the highest growth of *Bacillus cereus* and 2,4-DCP degradation was observed at 30^{o}C, where 28 % of 2,4-DCP was degraded after 264 hr. of cultivation.Although 2,4-DCP (10mg/L) was degraded at 25^{o}C (4%), 35^{o}C (25%) and 40^{o}C (3.7%), the time taken was significantly longer thanat 30^{o}C.This observation can be substantiated with the work carried out by Nor Suhaila et al., (2010) who reported that the highest cell

growth and the amount of phenol degraded (0.5g/L) were observed inoptimized cultivation conditions (30^oC and initial pH 7.5) after 21 hour.

Results presented in the Table 4.14 and Figure 4.14 indicated that the degradation of 2,4-DCP was not associated with growth of *Bacillus cereus*.High degradation rate was related to low growth rate obtained in cultivation at temperature ranging from 30^oC to 35^oC. High growth of *Bacillus cereus* at low temperature (25^oC) caused reduction in the degradation of 2, 4-DCP. This indicates that lower growth rate of cell will be contributed to higher rate of degradation. G.C.Okpokwasili and C.O. Nweke (2005) studied on microbial growth and substrate utilization kinetics and explained that there are also situations in which the organism concentration remains essentially constant even as the substrate is degraded (i.e. no growth situation). It is also observed from the Table 4.15 and Figure 4.15 that there is no significant change in pH of the medium.

Table 4.13: Concentration of 2, 4-DCP (10mg/L) of *Bacillus cereus* versus time at different temperatures

Time(hr.)	25°C	30°C	35° C	40°C
0	10	10	10	10
12	10	9.72	9.42	9.85
24	10	9.9	9.03	9.83
36	10.14	9.48	8.17	9.82
48	9.45	9.47	7.81	9.86
60	10.17	8.4	7.78	9.77
72	9.53	8.2	7.73	9.75
96	10.42	8	7.65	9.72
156	9.6	7.2	7.5	9.63
264	9.7	7	7.45	9.63
%	**4**	**28**	**25**	**3.7**

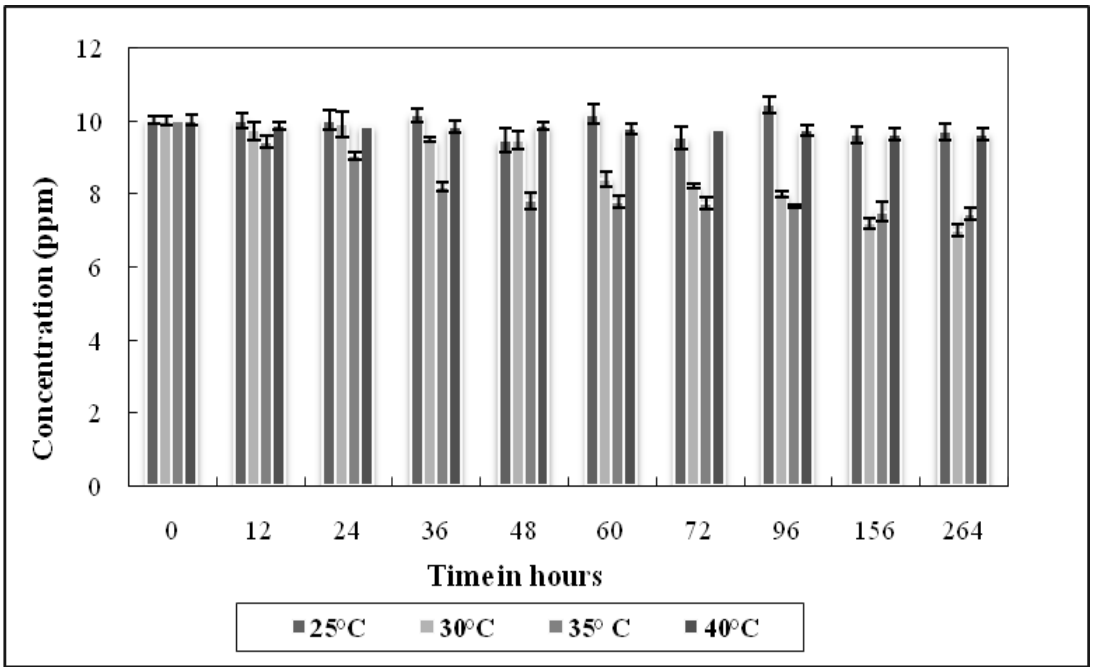


Fig.4.13: Concentration of 2, 4-DCP (10mg/L) of *Bacillus cereus* versus time at different temperatures

Table 4.14: Growth (OD) of *Bacillus cereus* with 2, 4-DCP (10mg/L) versus time at different temperatures

Time(hr.)	25° C	30°C	35°C	40°C
0	0.03	0.02	0.04	0.036
12	0.112	0.063	0.08	0.093
24	0.095	0.073	0.106	0.061
36	0.103	0.052	0.103	0.063
48	0.099	0.056	0.108	0.067
60	0.099	0.055	0.104	0.068
72	0.092	0.05	0.1	0.069
96	0.098	0.052	0.095	0.069
156	0.094	0.054	0.096	0.069
264	0.094	0.053	0.092	0.067

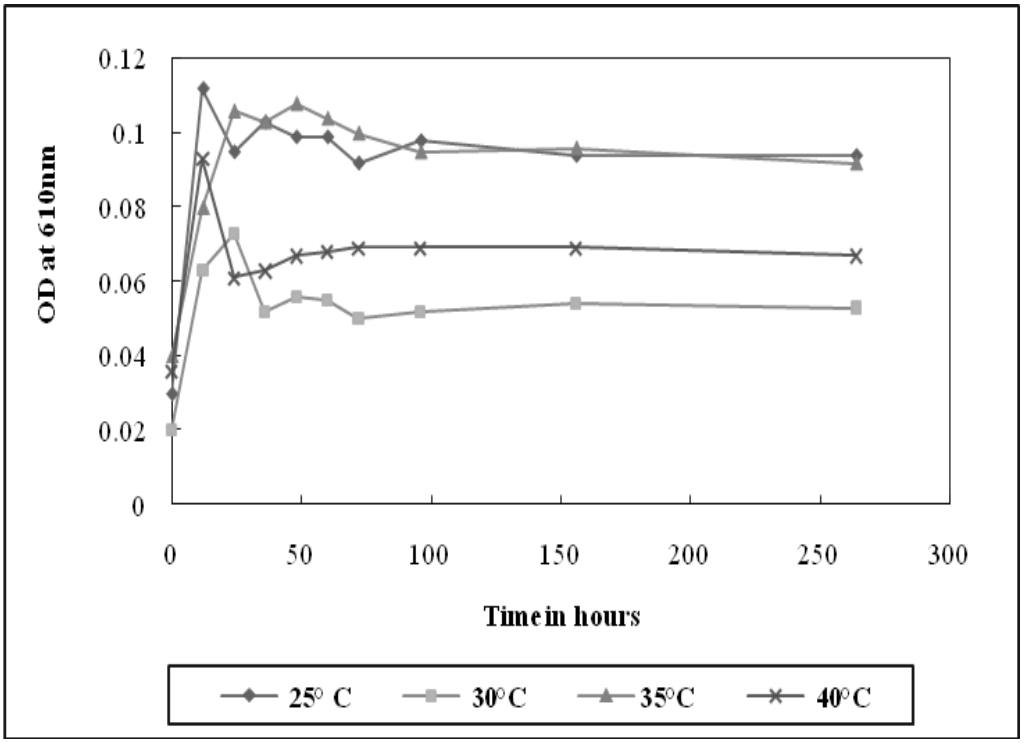


Fig.4.14: Growth (OD) of *Bacillus cereus* with 2, 4-DCP (10mg/L) versus time at different temperatures

Table 4.15: pH of minimal media with 2, 4-DCP (10mg/L) versus time at different temperatures

Time(hr.)	25° C	30°C	35°C	40°C
0	7.03	7.14	7.24	7.08
12	7.26	7.26	7.22	7.24
24	7.28	7.27	7.21	7.29
36	7.27	7.26	7.24	7.29
48	7.23	7.24	7.2	7.23
60	7.33	7.30	7.21	7.23
72	7.30	7.25	7.20	7.30
96	7.25	7.24	7.22	7.29
156	7.30	7.24	7.20	7.29
264	7.29	7.23	7.19	7.28

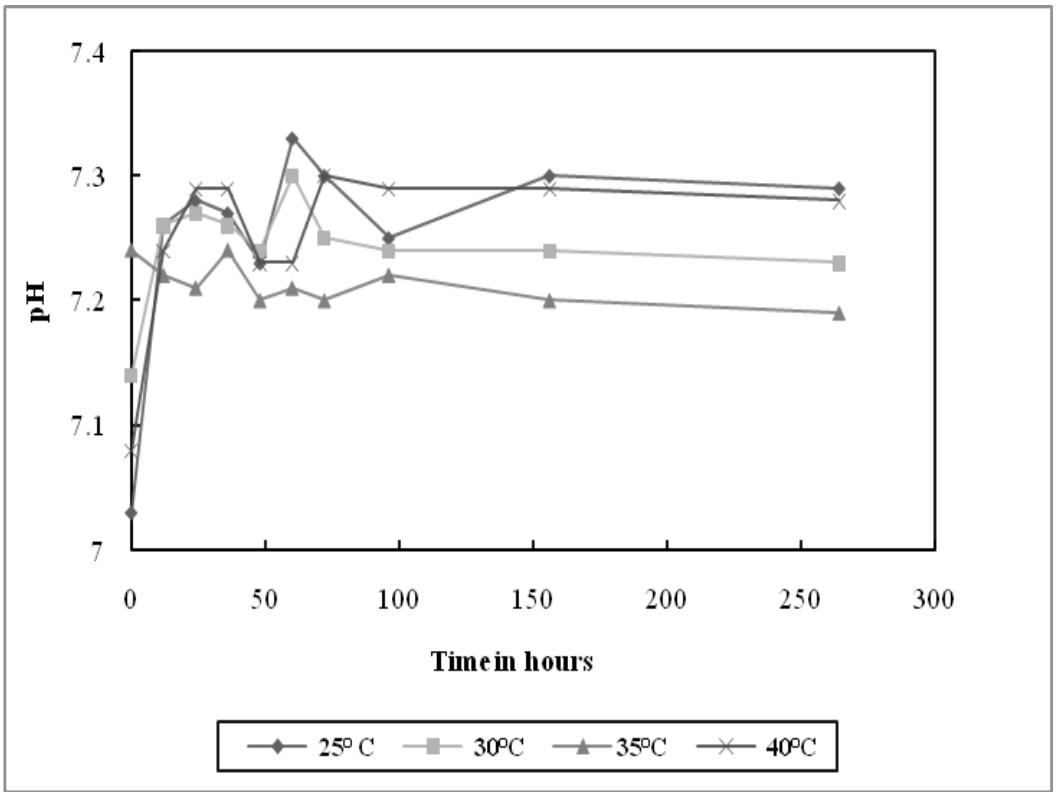


Fig.4.15: pH of minimal media with 2, 4-DCP (10mg/L) versus time at different temperatures

4.8.2. Degradation studies of 2, 4-DCP (30mg/L) by *Bacillus cereus* at different temperature

The average concentration of the duplicate analysis of 2, 4-DCP versus time is presented in Table 4.16 and Figure 4.16. For a higher initial concentration of 30 mg/L of 2,4-DCP Growth (OD) of *Bacillus cereus* versus time is presented in Table 4.17 and Figure 4.17. pH of media with 2, 4-DCP versus time is presented in Table 4.18 and Figure 4.18. In 30mg/L of 2, 4-DCP it was observed that degradation was slightly lower when compared with 10mg/L of 2,4-DCP. At the end of 264 hour the percentage degradation of 2,4-DCP by *Bacillus cereus* was 21.23% at 30^{o}C and 20.07% at 35^{o}C in 7pH.While at temperatures of 25^{o}C and 40^{o}C degradation of 2,4-DCP was only 3.66% and 0.69% respectively. V.M. Monsalvo et al., (2009) who stated that only at the highest temperature tested (35°C) it was possible to increase the maximum assimilative 4-CP concentration by the biological sludge up to 2100 mg/L recorded similar findings. From the Tables and Figures it is apparent that maximum growth is at 35°C and can be concluded that degradation of 2, 4-DCP is not growth related as the high degradation was observed at 35°C and it is also observed that there is no significant change in pH of the medium.

Table 4.16: Concentration of 2, 4-DCP (30mg/L) of *Bacillus cereus* versus time at different temperatures

Time(hr.)	25°C	30°C	35°C	40°C
0	30	29.99	30.03	30
12	29.99	28.74	28.8	29.36
24	30.07	28.8	26.87	29.82
36	29.65	28.82	25.13	29.82
48	28.97	27.8	25.22	29.8
60	28.78	27.68	25.24	29.76
72	28.89	26.01	24.73	29.74
96	28.93	25.99	24.98	29.74
156	28.88	23.63	23.95	29.78
264	28.89	23.5	23.9	29.8
%	**3.69**	**21.64**	**20.31**	**0.666**

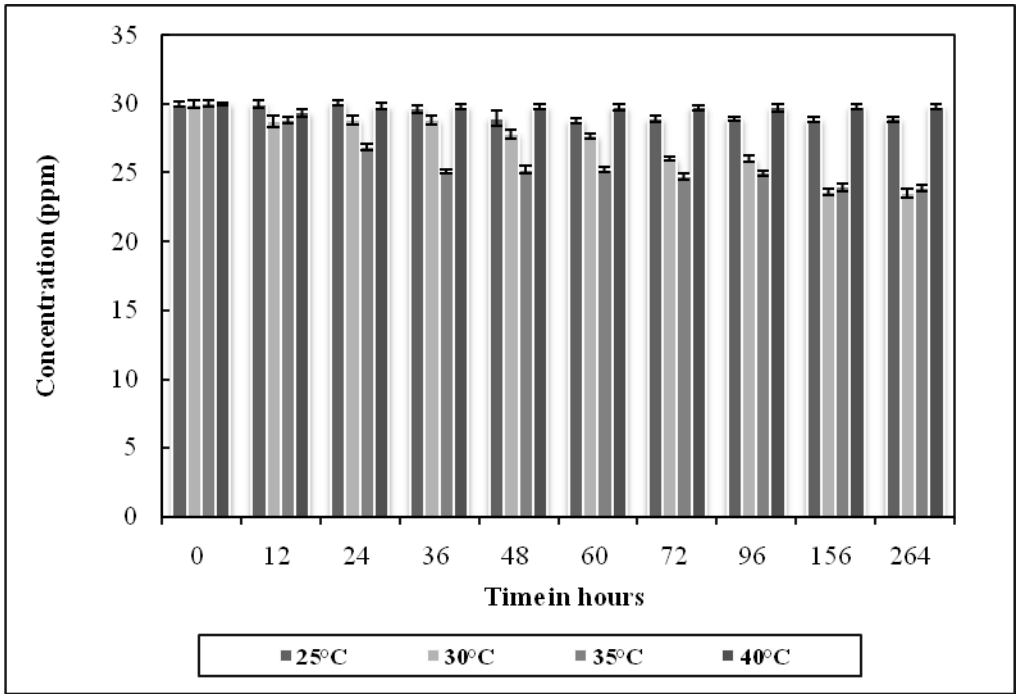


Fig.4.16: Concentration of 2, 4-DCP (30mg/L) of *Bacillus cereus* versus time at different temperatures

Table 4.17: Growth (OD) of *Bacillus cereus* with 2, 4-DCP (30mg/L) versus time at different temperatures

Time(hr.)	25° C	30°C	35° C	40° C
0	0.002	0.002	0.004	0.002
12	0.076	0.029	0.047	0.031
24	0.076	0.039	0.101	0.039
36	0.079	0.043	0.096	0.039
48	0.079	0.038	0.092	0.055
60	0.073	0.028	0.087	0.046
72	0.055	0.03	0.086	0.045
96	0.068	0.029	0.08	0.044
156	0.061	0.029	0.081	0.046
264	0.066	0.036	0.079	0.044

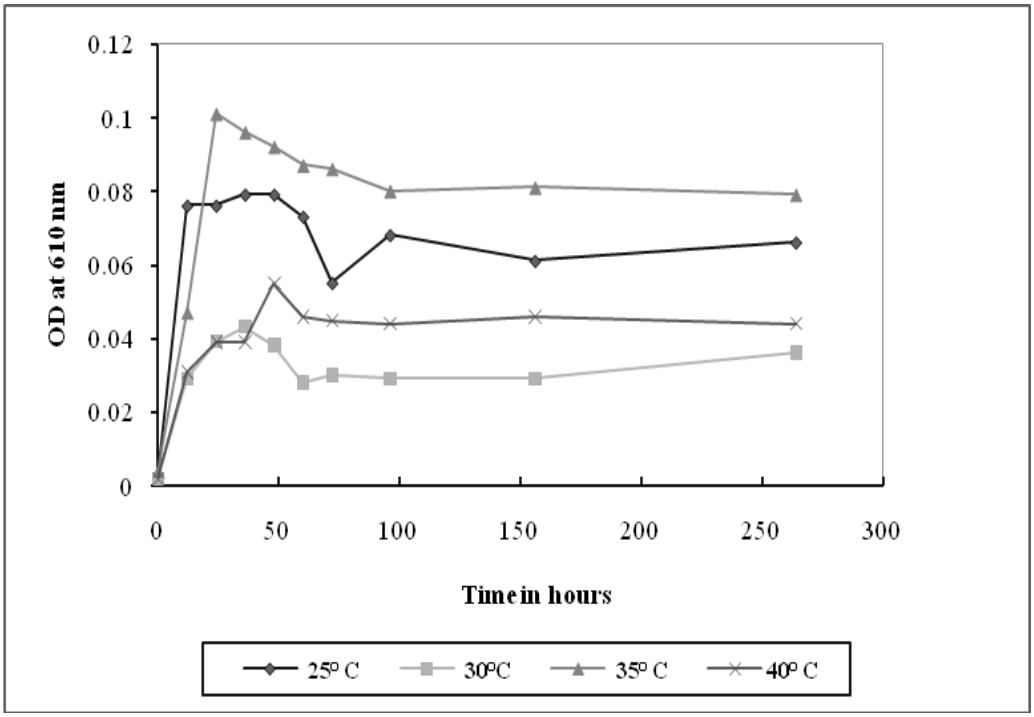


Fig.4.17: Growth (OD) of *Bacillus cereus* with 2, 4-DCP (30mg/L) versus time at different temperatures

Table 4.18: pH of minimal media with 2, 4-DCP (30mg/L) versus time at different temperatures

Time(hr.)	25° C	30°C	35° C	40° C
0	7.07	7.14	7.22	7.1
12	7.23	7.07	7.24	7.14
24	7.28	7.15	7.22	7.24
36	7.27	7.16	7.29	7.25
48	7.35	7.11	7.27	7.22
60	7.37	7.3	7.26	7.27
72	7.32	7.22	7.29	7.28
96	7.32	7.21	7.26	7.27
156	7.33	7.22	7.27	7.26
264	7.31	7.2	7.24	7.28

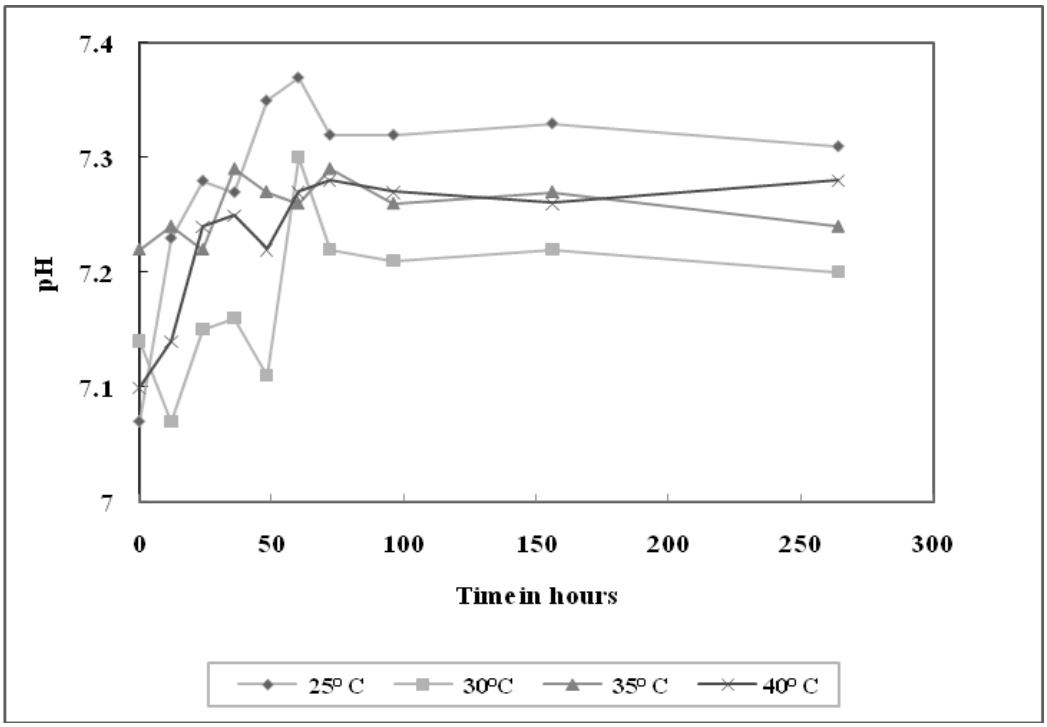


Fig.4.18: pH of minimal media with 2, 4-DCP (30mg/L) versus time at different temperatures

4.8.3. Degradation studies of 2, 4-DCP (75mg/L) by *Bacillus cereus* at different temperatures

The effect of temperature on the degradation 2,4-DCP with the initial concentration of 75 mg/L versus time by *Bacillus cereus* is shown in Table 4.19 and Figure 4.19. From the Tables and Figures it is evident that percentage degradation of 75 mg/L of 2, 4-DCP at the end of 264hour was 9.17% at 30^{o}C ,8.11% at 35^{o}C,0.053% at 25^{o}C and 2.34% at 40^{o}C in 7pH.The degradation rate was high between 30^{o}C to 35^{o}C and difference within this range was not significant. Below 30^{o}C and above 35^{o}C the degradation rate decreased significantly.This work can be justified with the similar work carried by Young –Gyun Cho et al., (2000) on the influence of environmental parameters on bioremediation of chlorophenols. From the Table 4.20 and Figure 4.20 it is evident that the high growth of *Bacillus cereus* with 75 mg/L of 2, 4-DCP is between 30^{o}C to 35^{o}C.pH of media with 2, 4-DCP versus time is presented in Table 4.21 and Figure 4.21.There is no significant change in pH of the medium also.

Table 4.19: Concentration of 2, 4-DCP (75mg/L) of *Bacillus cereus* versus time at different temperatures

Time(hr.)	25°C	30°C	35° C	40°C
0	75	75	75	75
12	74.99	74.69	72.14	74.89
24	74.98	73.09	71.33	74.79
36	74.96	72.74	70.34	74.78
48	74.98	69.59	69.32	74.76
60	74.95	69.49	69.54	74.68
72	74.97	69.17	69.31	74.69
96	74.95	68.22	68.99	73.69
156	74.96	68.1	68.9	73.24
264	74.92	68.15	68.89	73.14
%	**0.1064**	**9.110**	**8.126**	**2.473**

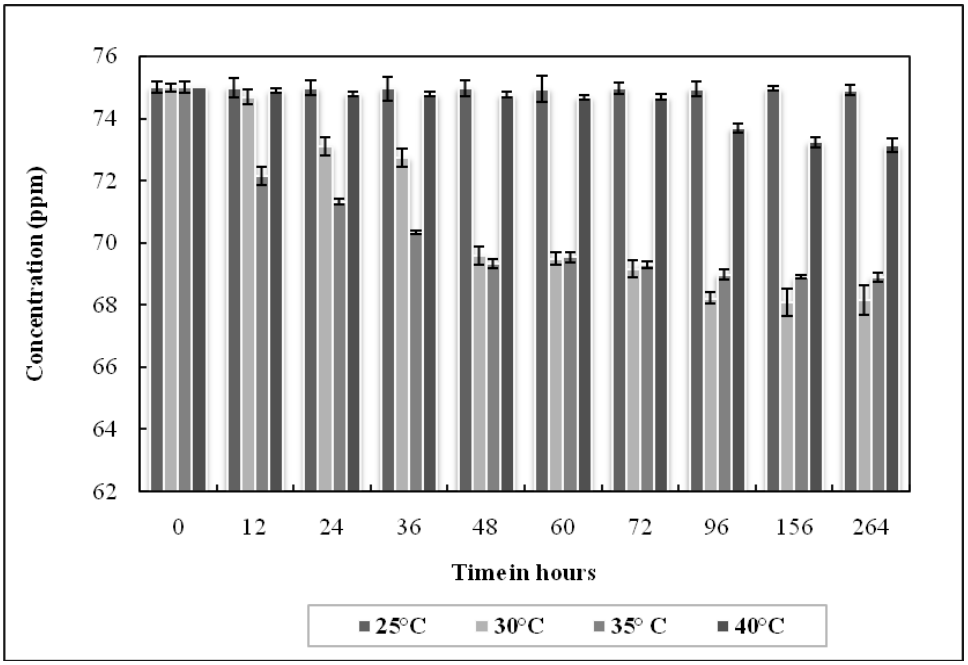


Fig.4.19: Concentration of 2, 4-DCP (75mg/L) of *Bacillus cereus* versus time in different temperatures

Table 4.20: Growth (OD) of *Bacillus cereus* with 2, 4-DCP (75mg/L) versus time at different temperatures

Time(hr.)	25° C	30° C	35° C	40° C
0	0.003	0.004	0.004	0.01
12	0.03	0.05	0.01	0.02
24	0.012	0.049	0.02	0.022
36	0.014	0.03	0.023	0.017
48	0.012	0.017	0.017	0.019
60	0.01	0.012	0.017	0.02
72	0.012	0.012	0.015	0.02
96	0.01	0.013	0.019	0.022
156	0.011	0.014	0.017	0.023
264	0.011	0.013	0.014	0.024

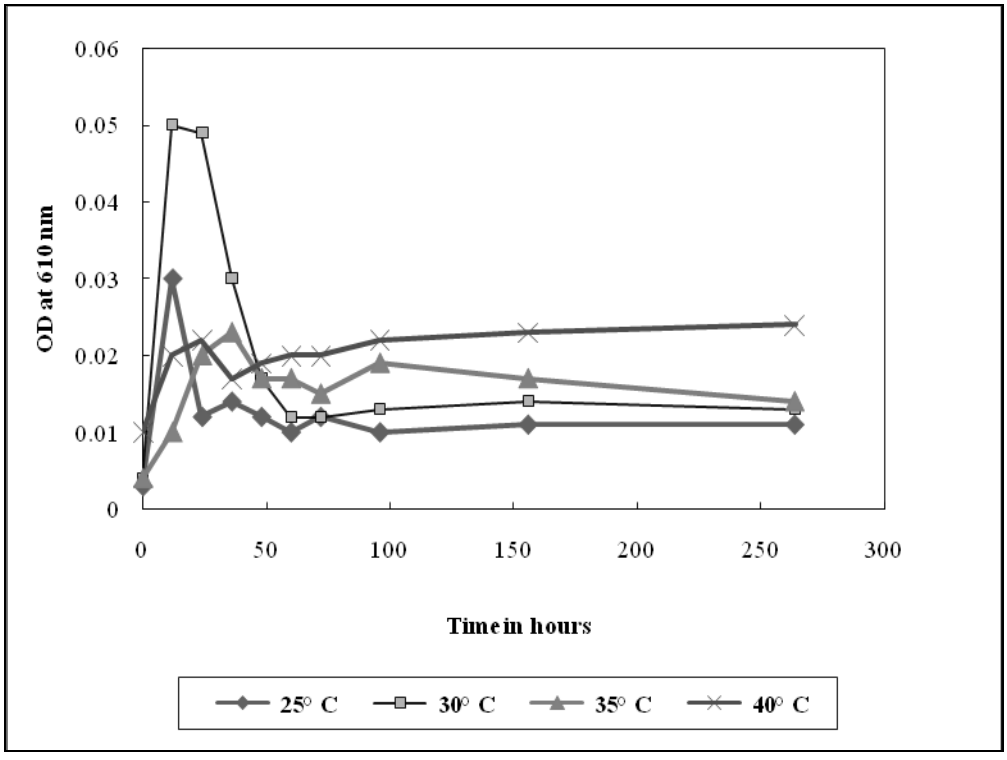


Fig.4.20: Growth (OD) of minimal media with 2, 4-DCP (75mg/L) versus time at different temperatures

Table 4.21: pH of minimal media with 2, 4-DCP (75mg/L) versus time at different temperatures

Time(hr.)	25° C	30° C	35° C	40° C
0	7.16	7.03	7.27	7.08
12	7.06	6.9	7.37	7.11
24	7.07	7.14	7.33	7.18
36	7.08	7.17	7.32	7.19
48	7.11	7.21	7.39	7.16
60	7.1	7.2	7.38	7.16
72	7.11	7.19	7.46	7.17
96	7.09	7.21	7.34	7.18
156	7.13	7.2	7.33	7.19
264	7.14	7.19	7.32	7.2

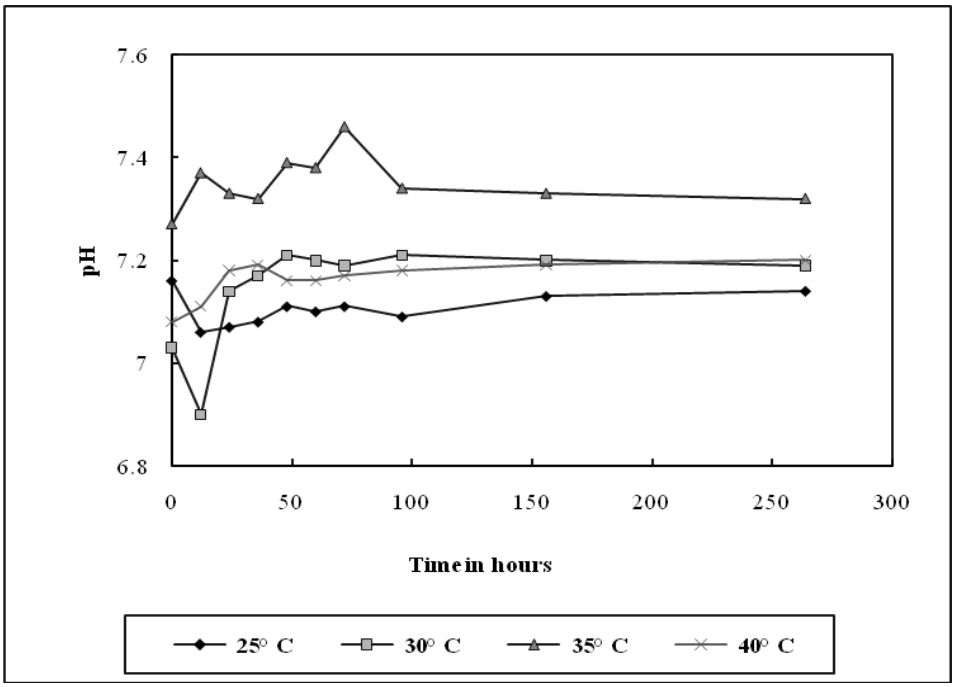


Fig.4.21: pH of minimal media with 2, 4-DCP (75mg/L) versus time at different temperatures

4.8.4. Percentage degradation of different concentration of 2.4-DCP

The impact of temperature on the different concentration of 2, 4-DCP by *Bacillus cereus* is presented in Table 4.22 and Figure 4.22. It is observed that as the concentration of 2, 4-DCP was increased from 10 mg/L to 75 mg/L at 30°C the percentage degradation by *Bacillus cereus* decreased drastically. Further from the previous experiment carried it is observed that the growth of *Bacillus cereus* declined as the concentration of 2, 4-DCP was increased. This could reflect that toxicity of 2, 4-DCP increased as the concentration increased thereby decreasing the growth of *Bacillus cereus.*Similar work was carried out by Muftah H. El-Naas et al., (2009) on biodegradation of phenol by *Pseudomonas putida* immobilized in polyvinyl alcohol (PVA) gel.He reported that increase in the initial phenol concentration beyond 75 mg/L reduces the removal rate of phenol. He attributed to the inhibitory effect of phenol.

Table 4.22: Percentage degradation of different concentration of 2, 4-DCP at 30°C

Concentration of 2,4-DCP	**10 mg/L**	**30 mg/L**	**75 mg/L**
Percentage Degradation	28	21.64	9.11

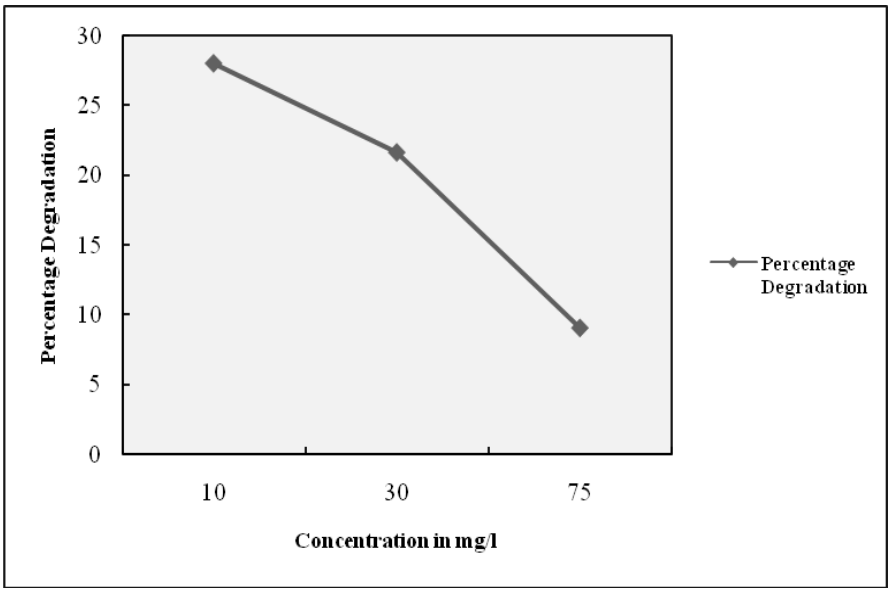


Fig. 4.22: Percentage degradation of different concentration of 2, 4-DCP at 30°C

4.8.5. Degradation studies of PCMX (50mg/L) by *Bacillus cereus* at different temperatures

The average concentration of the duplicate analysis of PCMX versus time is presented in Table 4.23 and Figure 4.23. pH of media with PCMX versus time is presented in. From the Tables and Figures the residual PCMX estimation revealed that optimum temperature was between 30^{o}C to 35°C. The percentage degradation was 28.76 % and 25.08% respectively at pH 7 at the end of 216 hour with initial concentration of 50 mg/L of PCMX. Degradation was hampered both at low as well as high temperatures. Enrichment cultures at 25^{o}C and 40^{o}C temperatures were unable to degrade PCMX and the percentage degradation recorded was only 7.24% and 5.68% respectively. Our data was similar to work carried by David R. Boone and Luying Xun (1987) on the effects of temperature on propionate degradation by methanogenic cultures.

Results presented in Table 4.24 and Figure 4.24 indicated that the degradation of PCMX was associated with growth of *Bacillus cereus*. High degradation rate was related to high growth rate obtained in cultivation at temperature ranging from 30^{o}C to 35^{o}C. Reduced growth of *Bacillus cereus* at high and low temperature (25^{o}C and 40^{o}C) caused reduction in the degradation of PCMX at 10 mg/L. This indicates that higher growth rate of cell will contribute to higher rate of degradation.It is also observed that there is no significant change in pH of medium as presented in Table 4.25 and Figure 4.25.

Table 4.23: Concentration of PCMX (50mg/L) versus time by *Bacillus cereus* at different temperatures

Time(hr.)	25° C	30° C	35° C	40° C
0	50	50	50	50
24	49.9	36.55	48.53	45.5
48	47.38	37.08	48.97	42.91
72	47.1	37	48.62	41.54
96	43.9	38.03	48.49	42.91
120	45.46	38.79	43.82	43.53
144	47.86	35.99	43.67	47.36
168	45.82	36.17	44.26	42.79
192	44.08	36.78	40.9	43.76
216	46.38	35.62	37.46	47.16
%	**7.24**	**28.76**	**25.08**	**5.68**

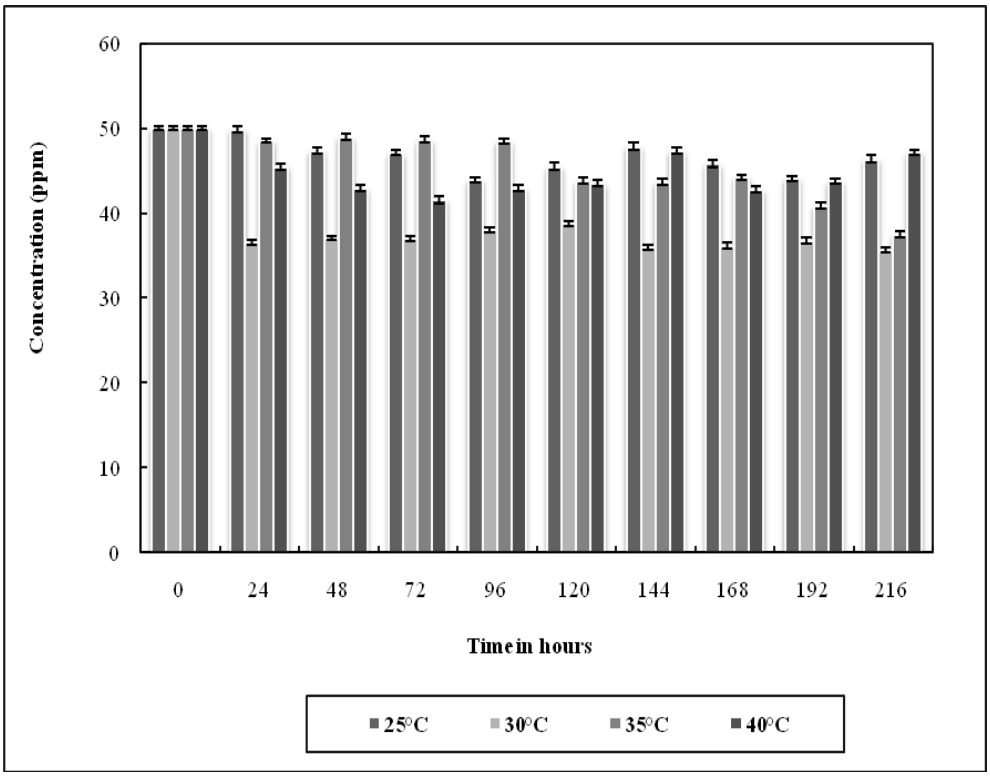


Fig.4.23: Concentration of PCMX (50 mg/L) versus time by *Bacillus cereus* at different temperatures

Table 4.24: Growth (OD) of *Bacillus cereus* with PCMX (50 mg/L) versus time at different temperatures

Time(hr.)	25° C	30° C	35° C	40° C
0	0.003	0.003	0.003	0.004
24	0.158	0.1	0.145	0.047
48	0.196	0.188	0.223	0.074
72	0.284	0.177	0.276	0.181
96	0.344	0.363	0.217	0.278
120	0.383	0.411	0.303	0.305
144	0.404	0.407	0.328	0.35
168	0.389	0.272	0.284	0.324
192	0.348	0.249	0.317	0.299
216	0.342	0.227	0.296	0.266

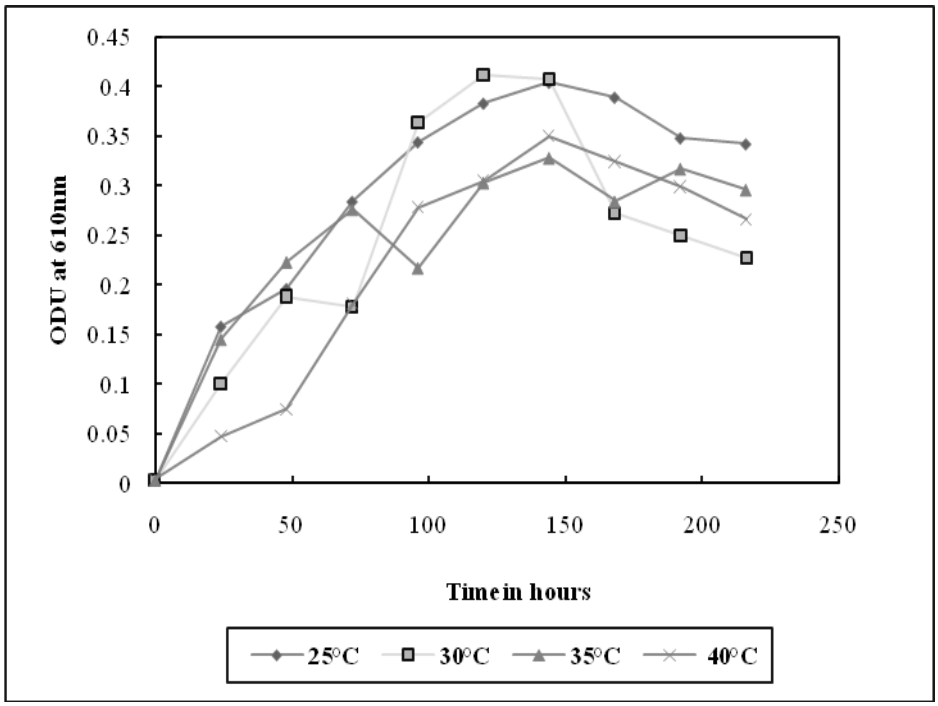


Fig. 4.24: Growth (OD) of *Bacillus cereus* with PCMX (50 mg/L) versus time at different temperatures

Table 4.25: pH of minimal media with PCMX (50mg/L) versus time at different temperatures

Time(hr.)	25° C	30° C	35° C	40° C
0	7.11	7.13	7.15	7.12
24	7.30	7.18	7.27	7.24
48	7.29	7.21	7.15	7.18
72	7.21	7.15	7.06	7.16
96	7.04	7.01	7.04	7.20
120	6.93	6.94	7.02	7.19
144	7.16	7.08	7.02	7.18
168	7.18	7.11	7.11	7.16
192	7.14	7.15	7.16	7.15
216	7.17	7.12	7.17	7.14

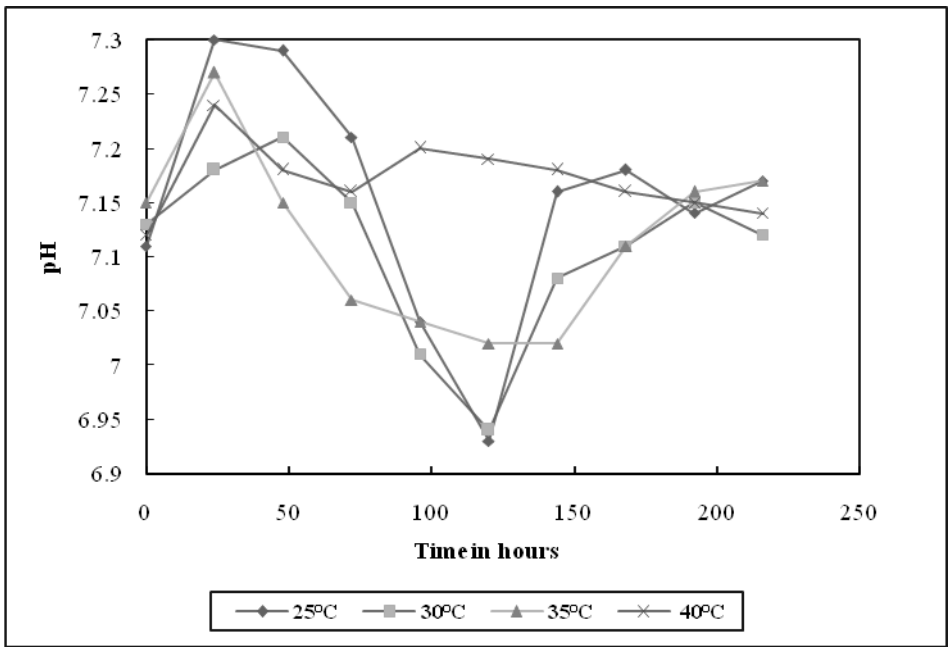


Fig.4.25: pH of minimal media with PCMX (50mg/L) versus time at different temperatures

4.8.6. Degradation studies of PCMX (100 mg/L) by *Bacillus cereus* at different temperatures

The average concentration of the duplicate analysis of PCMX versus time is presented in Table 4.26 and Figure 4.26 for a higher concentration of 100 mg/L of PCMX. Growth (OD) of *Bacillus cereus* in PCMX versus time is presented in Table 4.27 and Figure 4.27. pH of media with PCMX versus time is presented in Table 4.28 and Figure 4.28. It was observed that degradation was slightly lower when compared to 50mg/l of PCMX and percentage degradation in 100 mg/L of PCMX at the end of 216 hour was 8.57% at 30^{o}C and 3.44% at 35^{o}C in 7pH. While at temperatures of 25^{o}C and 40^{o}C it was only 4.2% and 3.5% respectively. It is evident that maximum percentage degradation of PCMX is at 30^{o}C (8.57 %) when compared to other concentration and growth rate of *Bacillus cereus* is maximum at 30^{o}C, as presented in the Tables 4.26 and Figure 4.29. It is also observed from the data presented in Table 4.27 and Figure 4.30 that there is no significant change in pH of medium.

Table 4.26: Concentration of PCMX (100 mg/L) versus time by *Bacillus cereus* at different temperatures

Time(hr.)	25°C	30°C	35°C	40°C
0	100	100	100	100
24	99.4	98.5	99.5	99.7
48	99.5	96.5	99.72	99
72	98.5	98.75	99.65	99.54
96	98.7	99.1	98.3	99.4
120	97.8	95.6	98.5	98.3
144	96.4	93.4	97.5	98
168	96.3	93.43	96.8	97.3
192	96.7	91.7	97.6	96.6
216	96.8	91.43	96.56	96.5
%	**3.2**	**8.57**	**3.44**	**3.5**

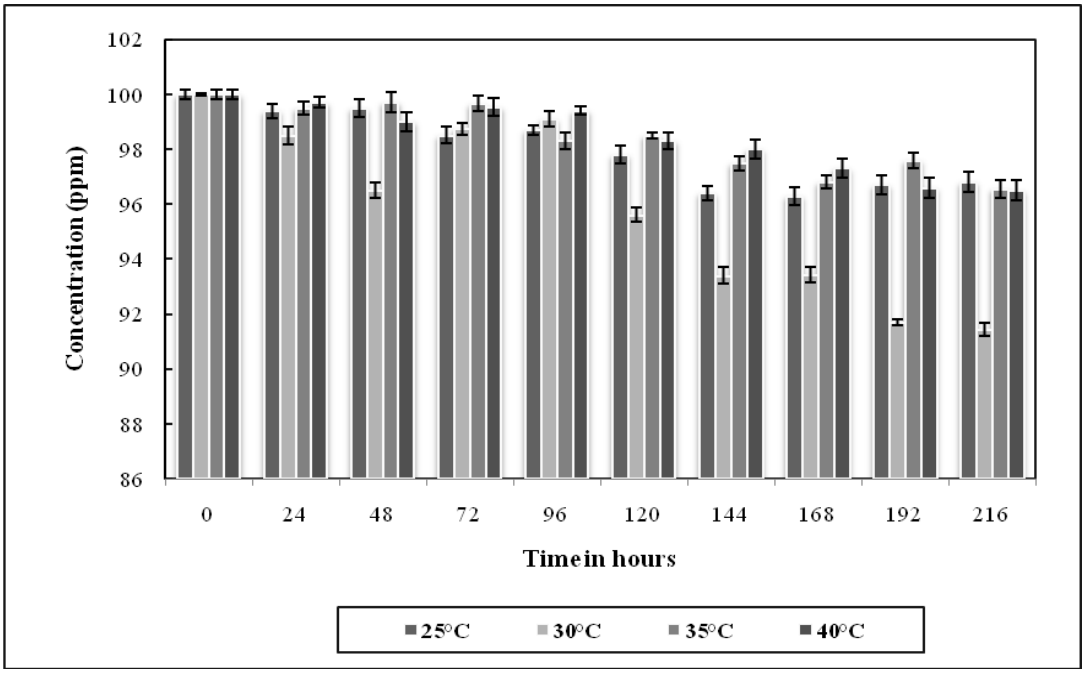


Fig.4.26: Concentration of PCMX (100 mg/L) versus time by *Bacillus cereus at* different temperatures

Table4.27: Growth (OD) of *Bacillus cereus* with PCMX (100 mg/L) versus time at different temperatures

Time(hr.)	25°C	30°C	35°C	40°C
0	0.003	0.003	0.003	0.004
24	0.154	0.145	0.146	0.168
48	0.179	0.181	0.184	0.178
72	0.278	0.301	0.314	0.298
96	0.243	0.311	0.264	0.276
120	0.213	0.289	0.236	0.253
144	0.224	0.282	0.233	0.249
168	0.221	0.279	0.222	0.246
192	0.213	0.265	0.225	0.232
216	0.219	0.264	0.223	0.231

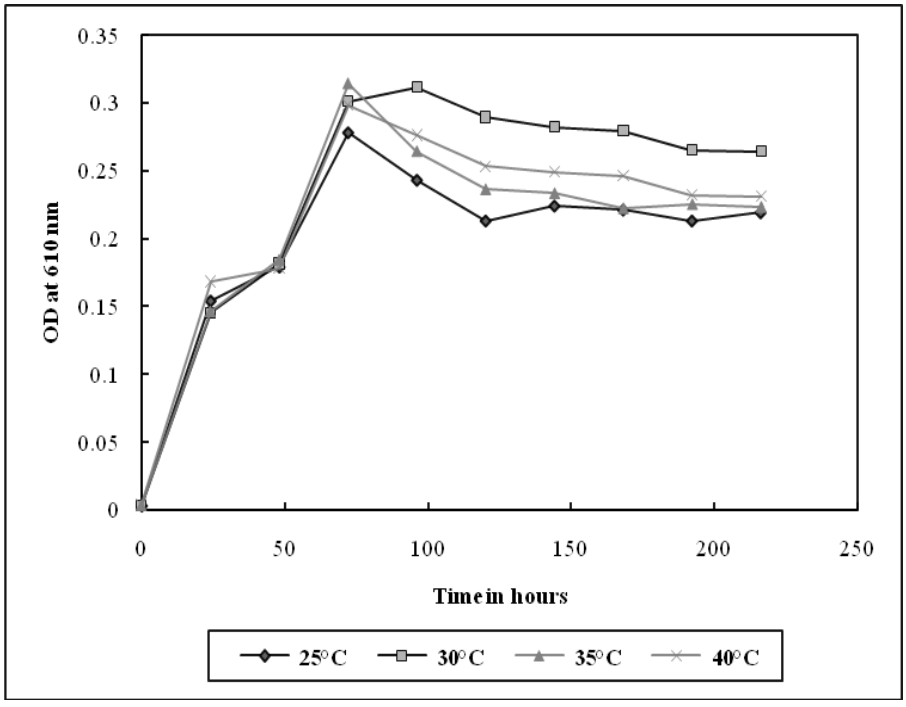


Fig. 4.27: Growth (OD) of *Bacillus cereus* with PCMX (100 mg/L) versus time at different temperatures

Table 4.28: pH of minimal media with PCMX (100 mg/L) versus time at different temperatures

Time(hr.)	25^0C	30^0C	35^0C	40^0C
0	7.00	6.99	6.99	7.01
12	7.01	7.07	7.01	7.00
24	6.99	6.99	7.00	7.02
36	7.02	6.96	6.99	7.04
48	7.00	6.95	7.02	7.01
60	7.09	6.96	7.09	6.99
72	7.03	6.98	7.04	7.09
96	7.06	7.14	7.10	6.90
120	7.10	7.10	7.11	7.04
156	7.11	7.12	7.09	7.11
216	7.08	7.11	7.13	7.06

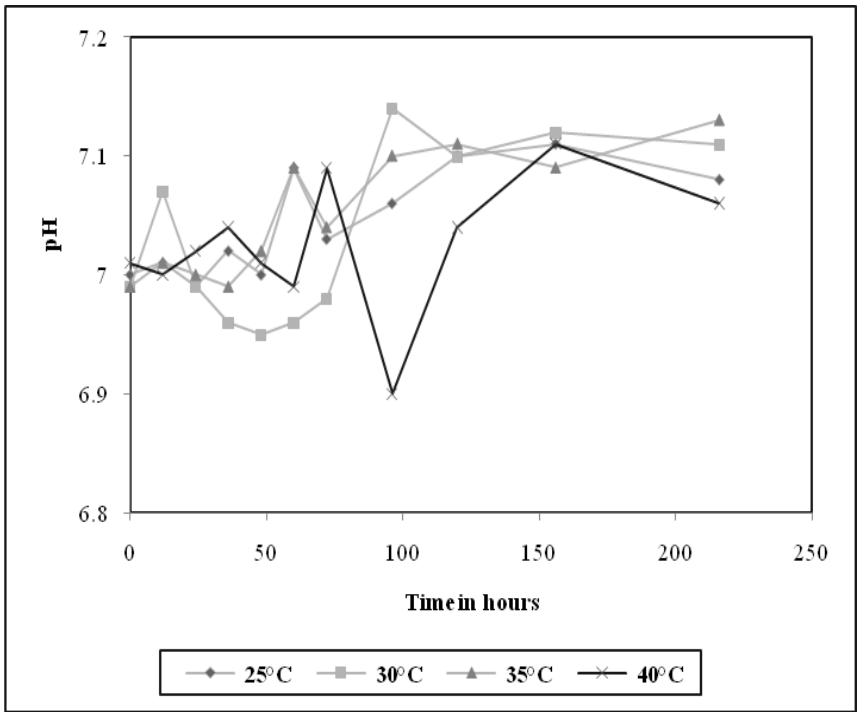


Fig.4.28: pH of minimal media with PCMX (100 mg/L) versus time at different temperatures

4.8.7. Degradation studies of PCMX (150 mg/L) by *Bacillus cereus* at different temperatures

Table 4.29 and Figure 4.29 contain the presentation of the results of the degradation of PCMX at 150 mg/L at average concentration versus time. From the presented Tables and Figures it is evident that maximum percentage degradation of 150 mg/L of PCMX at the end of 216 hour was 2.64% at 30^{o}C and 1.69% at 35^{o}C in 7pH. While at temperatures of 25^{o}C and 40^{o}C it was only 2.64% and 1.32% respectively. Maximum percentage degradationof PCMX is at 30^{o}C (4%) when compared to other temperature.It is observed that maximum growth is at 25^{o}C and there is no significant change in pH of medium as can be observed from Tables 4.30 and 4.31and Figures 4.30 and 4.31.

Table 4.29: Concentration of PCMX (150 mg/L) versus time by *Bacillus cereus* at different temperatures

Time(hr.)	25°C	30°C	35°C	40°C
0	150	150	150	150
24	144	148.78	148.5	149
48	147	145.66	146.5	148
72	145	146.34	148.75	150
96	147	147.2	149.1	147
120	149	148.4	145.6	149
144	148	147.67	143.4	143
168	148	146.08	146.43	146
192	147.1	147.78	147.7	146.1
216	147.09	146	147.43	148.09
%	**1.98**	**2.66**	**1.69**	**1.32**

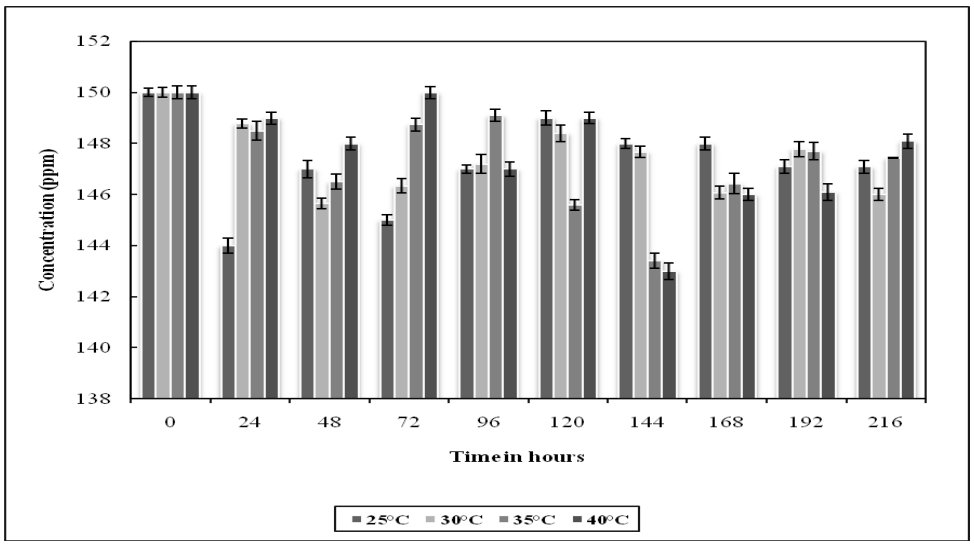


Fig. 4.29: Concentration of PCMX (150 mg/L) versus time by *Bacillus cereus* at different temperatures

Table 4.30: Growth (OD) of *Bacillus cereus* with PCMX (150 mg/L) versus time at different temperatures

Time(hr.)	25°C	30°C	35°C	40°C
0	0.012	0.006	0.003	0.003
24	0.122	0.134	0.146	0.021
48	0.176	0.292	0.184	0.066
72	0.323	0.301	0.314	0.124
96	0.379	0.343	0.264	0.136
120	0.294	0.278	0.236	0.143
144	0.286	0.251	0.233	0.146
168	0.25	0.305	0.222	0.163
192	0.23	0.304	0.225	0.164
216	0.224	0.313	0.223	0.174

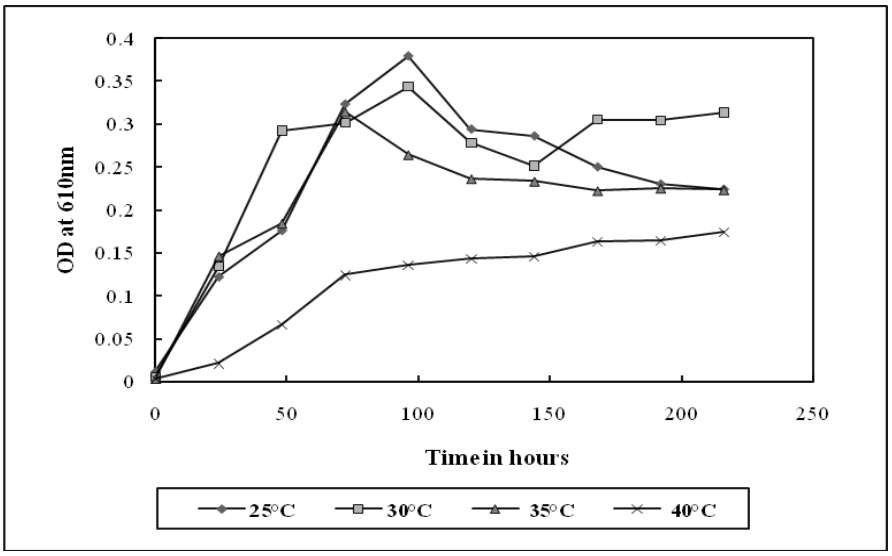


Fig. 4.30: Growth (OD) of *Bacillus cereus* with PCMX (150 mg/L) versus time at different temperatures

Table 4.31: pH of minimal media with PCMX (150 mg/L) versus time at different temperatures

Time(hr.)	25^0C	30^0C	35^0C	40^0C
0	6.82	6.90	6.99	7.15
24	6.86	6.95	7.07	7.08
48	6.9	6.87	6.99	7.11
72	6.96	6.82	6.96	6.79
96	6.98	6.80	6.95	6.99
120	7.00	6.76	6.96	6.98
144	7.14	6.77	6.98	6.91
168	7.07	6.67	7.14	6.89
192	7.11	6.60	7.10	6.88
216	7.10	6.67	7.12	6.89

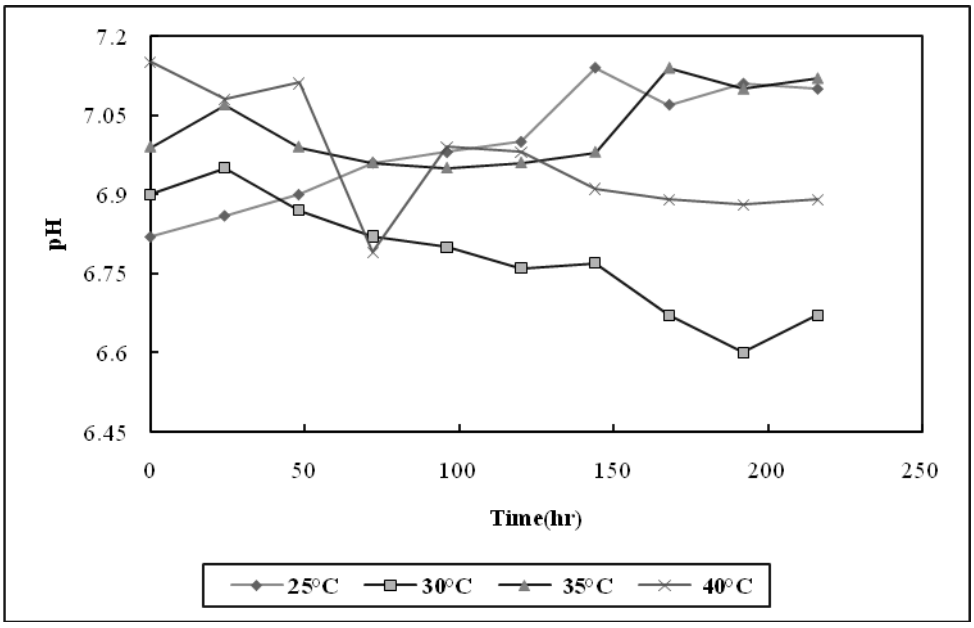


Fig.4.31: pH of minimal media with PCMX (150 mg/L) versus time at different temperatures

4.8.8. Degradation studies of PCMX (350mg/L) by *Bacillus cereus* in at different temperature

The duplicate analysis of higher concentration of 350 mg/L of PCMX versus time is presented in Table 4.32 and Figure 4.32. Growth (OD) of *Bacillus cereus* in PCMX versus time is presented in Table 4.33 and Figure 4.33. pH of media with PCMX versus time is presented in Table 4.34 and Figure 4.34. From the Tables and Figures it is evident percentage degradation of 350 mg/L of PCMX at the end of 216 hour was 6.85% at 30^{o}C and 2.00% at 35^{o}C in7pH. While at temperatures of 25^{o}C and 40^{o}C it was only 0% and 1.14% respectively that maximum percentage degradation of PCMX is, at 30^{o}C (24 %) when compared to other temperature.It is observed that maximum growth is at 25^{o}C and there is no significant change in pH of the medium as presented in Tables and Figures.

Table 4.32: Concentration of PCMX (350mg/L) versus time by *Bacillus cereus* at different temperatures

Time(hr.)	25°C	30°C	35°C	40°C
0	350	350	350	350
24	349	349	349	350
48	349	348	345	349
72	351	350	346	349
96	350	340	346	350
120	349	349	345	345
144	350	348	345	346
168	349	330	343	345
192	350	328	343	345
216	350	326	343	346
%	**0**	**6.84**	**1.99**	**1.14**

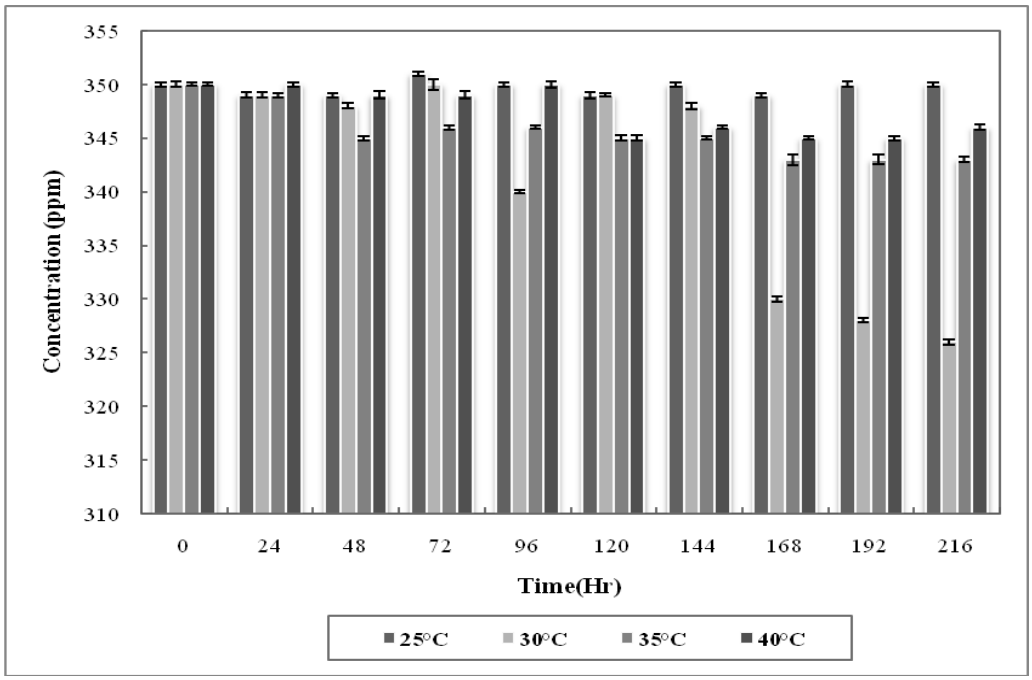


Fig. 4.32: Concentration of PCMX (350 mg/L) versus time by *Bacillus cereus* at different temperatures

Table 4.33: Growth (OD) of *Bacillus cereus* with PCMX (350 mg/L) versus time at different temperature

Time(hr.)	25°C	30°C	35°C	40°C
0	0.003	0.003	0.004	0.032
24	0.009	0.021	0.012	0.015
48	0.015	0.066	0.067	0.012
72	0.021	0.124	0.055	0.011
96	0.02	0.136	0.064	0.015
120	0.045	0.143	0.062	0.018
144	0.097	0.146	0.059	0.017
168	0.144	0.163	0.038	0.047
192	0.145	0.166	0.036	0.048
216	0.156	0.177	0.037	0.055

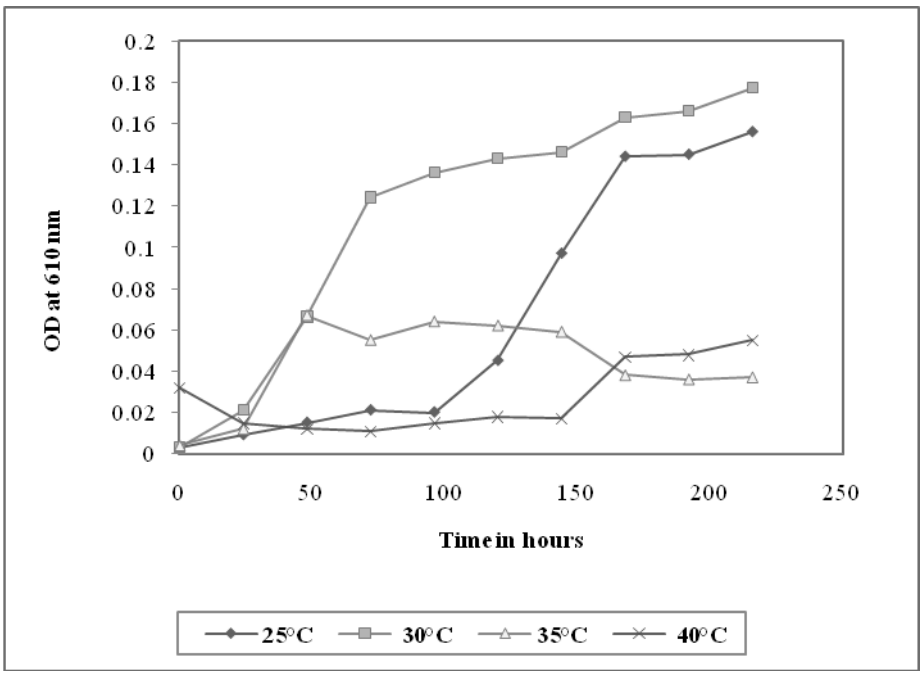


Fig. 4.33: Growth (OD) of *Bacillus cereus* with PCMX (350 mg/L) versus time at different temperature

Table 4.34: pH of minimal media with PCMX (350mg/L) versus time at different temperatures

Time(hr.)	25^0C	30^0C	35^0C	40^0C
0	7.20	7.15	7.38	7.09
24	7.15	7.08	7.30	7.10
48	7.11	7.11	7.33	7.09
72	7.14	6.79	7.30	7.08
96	7.13	6.99	7.42	7.02
120	7.21	6.98	7.49	7.03
144	7.21	6.91	7.43	7.11
168	7.20	6.89	7.45	7.17
192	7.24	6.88	7.46	7.14
216	7.21	6.87	7.45	7.16

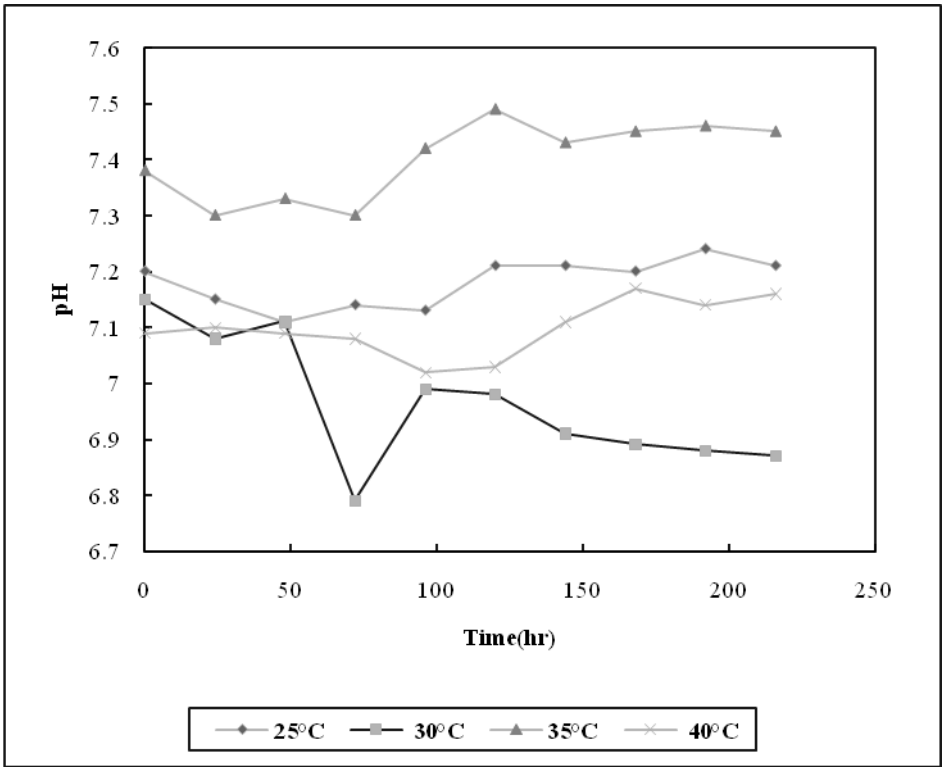


Fig.4.34: pH of minimal media with PCMX (350mg/L) versus time at different temperatures

4.8.9. Percentage degradation of different concentration of PCMX

Experiments were carried out to assess the effect of temperature ranging from 25^{o}C to 40^{o}C on the biodegradation of PCMX. Table 4.35 and Figure 4.35 indicate the effect of temperature on degradation of PCMX at concentration ranging from 50 mg/L to 350 mg/L by *Bacillus cereus* at 30^{o}C. From the Figure, 28.76 percentage of biodegradation rate was obtained at 50mg/L concentration of PCMX and a slight increase in degradation rate of 8.57 was obtained at 100mg/L concentration of PCMX temperature of 30^{o}C. Higher concentration of PCMX seen to negatively affect the activity of the *Bacillus cereus* culture and hence hindered its biodegradation capabilities. It is believed that exposure of the *Bacillus cereus to* high concentration of PCMX may have detrimental effect on the Bacterial enzymes hence decreasing the percentage degradation.

Table 4.35: Percentage degradation of different concentration of PCMX at 30^{0}C

Concentration of PCMX	**50 mg/L**	**100 mg/L**	**150 mg/L**	**350 mg/L**
Percentage Degradation	28.76	8.57	2.66	6.84

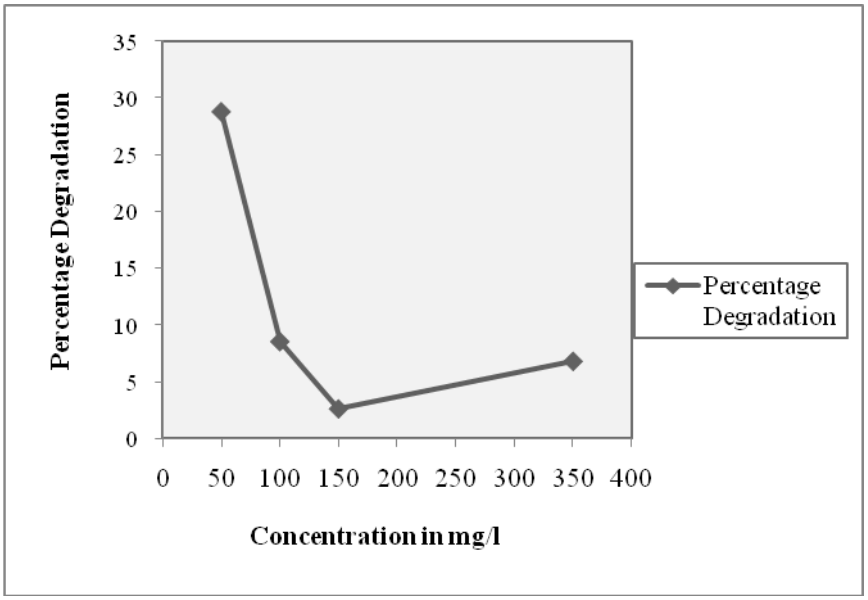


Fig. 4.35: Percentage degradation of different concentration of PCMX at 30^{0}C

SUMMING UP

In conclusion, degradation was hampered both at low as well at high temperatures. It was not possible to degrade 2,4-DCP and PCMX at varying concentration from10mg/L to350mg/L at temperatures above and below 30°C. This corroborates with previous studies by Leung et al. (1997) and Nedwell et al. (1999), on bioremediation of chlorophenols. They recorded that low and high temperature may limit bioremediation by having a negative impact on enzyme activity and the ability of cells to take up the pollutant. Backman and Jansson (2004) have reported similar results on the *Arthrobacter chlorophenolicus* at 16.5^{o}C.They reported that the *Arthrobacter chlorophenolicus* strain could degrade (200–300 mg/L) 4-chlorophenol in pure cultures incubated at 5^{o}C, although rates of degradation, growth and the metabolic status of the cells were lower at 5^{o}C compared to 28^{o}C.

Further, Melin E.S. et al. (1998) studied the effect of temperature on chlorophenol degradation kinetics in FBR batch tests at temperatures ranging from 4 to 16.5°C. They concluded that a 10°C decrease in temperature generally resulted in over seven time's slower degradation rates. To conclude many researchers recorded maximum degradation rates for chlorophenol to be at 30^{o}C.Temperature of 35^{o}C also showed considerable degradation but level of degradation was much lower than 30^{o}C.

4.8.10. Degradation studies of 2, 4-DCP (10mg/L) by *Bacillus cereus* at different pH

The effect of pH values in the range of 2 to 10in the degradation of 2,4-DCP were investigated.The average concentration of the duplicated experiments of 2, 4-DCP versus time is presented in Table 4.36 and Figure 4.36. In 264 hour 64.6 % 2, 4-DCP was removed at pH 3, while the rest of the pH conditions could not degrade 2, 4-DCP more efficiently. Both neutral and alkaline pH had a marked inhibition on 2, 4-DCP removal efficiency. At pH 4 and 5, 2, 4-DCP removal was up to 57.1% and 54.6%. In acidic pH 3 the degradation of 2, 4-DCP was higher as compared to the other pH at 30^{o}C. It can be inferred from the results that low pH ranging from 2 to 7 degradation was fairly good but at pH 8 and 10 no degradation was observed. However it can be further seen that as the pH increases from 2 to 7 percentage degradation decreases from 64.2% to 22% which clearly establishes that acidic pH influence the efficiency

of degradation of 2,4-DCP.From the Tables 4.37 and 4.38 and Figures 4.37 and 4.38 it is observed that maximum growth is at pH 8 and there is no change in pH of the medium.

Table 4.36: Concentration of 2, 4-DCP (10mg/L) versus time by *Bacillus cereus* at different pH

Time(hr.)	2pH	3pH	4 pH	5pH	6 pH	7 pH	8 pH	10 pH
0	10	10	10	10	10	10	10	10
12	9.9	9.97	7.19	7.99	9.59	9.76	9.23	10
24	9.98	8.67	6.25	7.56	9.012	8.47	9.47	10.11
36	9.97	7.89	6.03	6.98	8.49	8.54	9.54	10.02
48	9.87	5.99	5.28	7.48	9.54	8.61	10.11	10.01
60	9.89	5.89	5.65	7.23	9.43	8.55	10.05	9.99
72	9.87	5.79	5.87	7.13	9.33	8.52	10.22	9.89
96	9.9	5.76	4.54	7.16	8.47	8.42	10.06	9.96
156	8.11	5.41	4.38	5.9	8.25	8.46	10.02	10
264	8.3	3.54	4.29	4.54	8.18	7.8	10	10.09
%	**17**	**64.6**	**57.1**	**54.6**	**18.2**	**22**	**0**	**0**

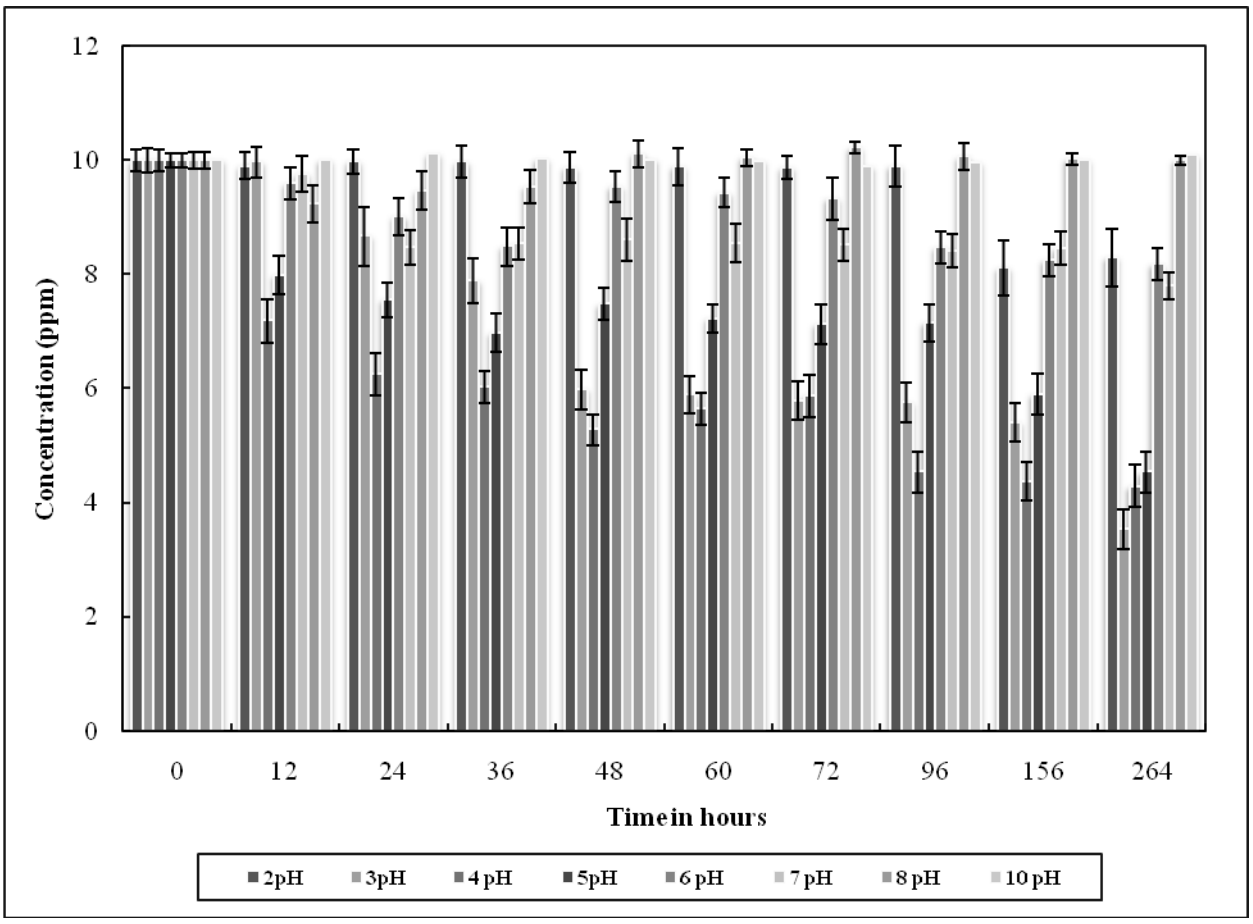


Fig 4.36: Concentration of 2, 4-DCP (10mg/L) versus time by *Bacillus cereus* at different pH

Table 4.37: Growth (OD) of *Bacillus cereus* with 2, 4-DCP (10mg/L) versus time at different pH

Time(hr.)	2pH	3pH	4 pH	5pH	6 pH	7 pH	8 pH	10 pH
0	0.003	0.004	0.003	0.003	0.003	0.003	0.004	0.004
12	0.036	0.069	0.076	0.177	0.114	0.185	0.101	0.006
24	0.041	0.079	0.097	0.2	0.125	0.172	0.139	0.019
36	0.046	0.08	0.083	0.124	0.114	0.147	0.136	0.034
48	0.054	0.081	0.086	0.108	0.1	0.13	0.118	0.078
60	0.052	0.078	0.067	0.116	0.101	0.125	0.118	0.109
72	0.049	0.066	0.063	0.116	0.101	0.112	0.11	0.111
96	0.043	0.058	0.051	0.11	0.095	0.111	0.105	0.098
156	0.044	0.045	0.05	0.112	0.096	0.115	0.105	0.094
264	0.043	0.049	0.052	0.111	0.101	0.114	0.116	0.083

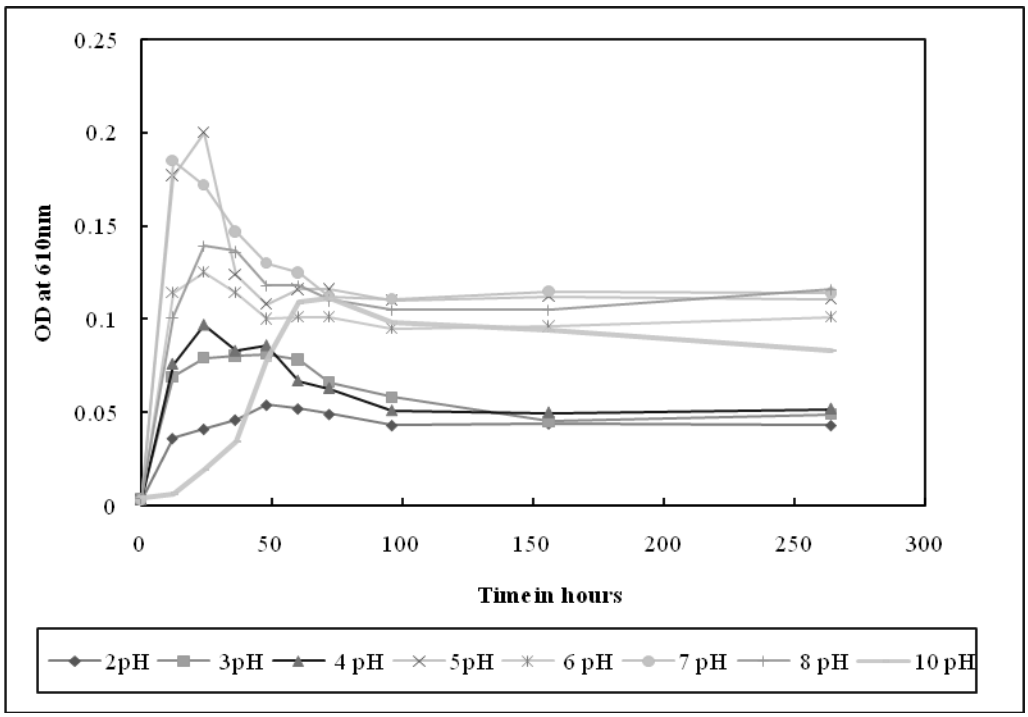


Fig. 4.37: Growth (OD) of *Bacillus cereus* with 2, 4-DCP versus time at different pH

Table 4.38: pH of minimal media with 2, 4-DCP (10mg/L) versus time at different pH

Time(hr.)	2pH	3pH	4 pH	5 pH	6 pH	7 pH	8 pH	10 pH
0	2.24	3.01	4	5.1	5.97	7.02	8.02	10
12	2.23	3.01	4.89	5.84	6.08	7.07	7.79	10.01
24	2.24	3.02	5.04	5.98	6.22	7.13	7.99	10
36	2.14	3.04	5.1	5.93	6.3	7.22	8.31	10.01
48	2.01	3	5.23	5.92	6.37	7.23	8.45	9.97
60	2.09	3.01	5.3	5.94	6.34	7.27	8.53	9.98
72	2.03	3.02	5.37	5.98	6.36	7.16	8.58	9.94
96	2.04	3	5.43	6.02	6.31	7.18	8.6	9.96
156	2.04	3	5.56	6.02	6.35	7.22	8.63	9.63
264	2.02	3.03	5.54	6.01	6.33	7.25	8.64	9.64

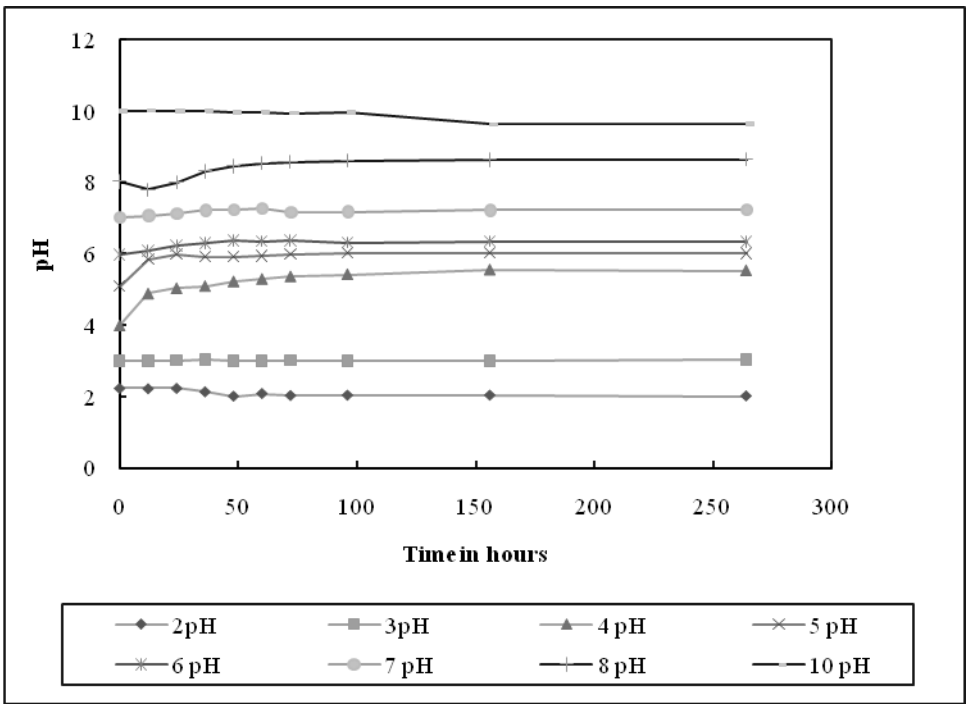


Fig. 4.38: pH of minimal media with 2, 4-DCP versus time at different pH

4.8.11. Degradation studies of PCMX (50mg/L) by *Bacillus cereus* in different pH

Four pH values from 5 to 8 were studied on the degradation of PCMX.The average concentration of the duplicate analysis of PCMX versus time is presented in Table 4.39 and Figure 4.39. PCMX degraded rapidly at pH 6 and 7. However, the PCMX degradation at pH 6 was slower and the PCMX concentration decreased rapidly after 216 hour post-inoculation. *Bacillus cereus* cells grew better at pH values within a range of 5 to 8 in minimal salts medium. From the result,it is evident that maximum percentage degradation of PCMX is at 7pH (28 %) when compared to other pH. These behaviors are consistent with result reported by Brandt et al. (1997) on the effect of pH on the biosorptive uptake of Pentachlorophenol by *Mycobacterium chlorophenolicum*. At very low or high pH values, acids or bases can penetrate into cells more easily, because they tend to exist in undissociated form under these conditions and electrostatic force cannot prevent them from entering cells.From the Tables 4.40 and 4.41 and Figures 4.40 and 4.41 it is observed that maximum growth is at 7pH and there is no significant change in pH of the medium.

Table 4.39: Concentration of PCMX (50mg/L) versus time by *Bacillus cereus* at different pH

Time(hr.)	5 pH	6 pH	7 pH	8 pH
0	50	50	50	50
24	48.91	36.19	36.55	42.61
48	41	38.27	37.08	43.95
72	41.28	37.51	37.4	43.56
96	43.5	37.6	38.04	43.67
120	42.34	37.86	38.03	43.78
144	40.27	38.09	38.02	44.26
168	41.2	37.89	37.6	44.35
192	40.32	37.9	35.99	44.56
216	40.21	38.04	35.62	44.23
%	**19.76**	**23.92**	**28.76**	**11.54**

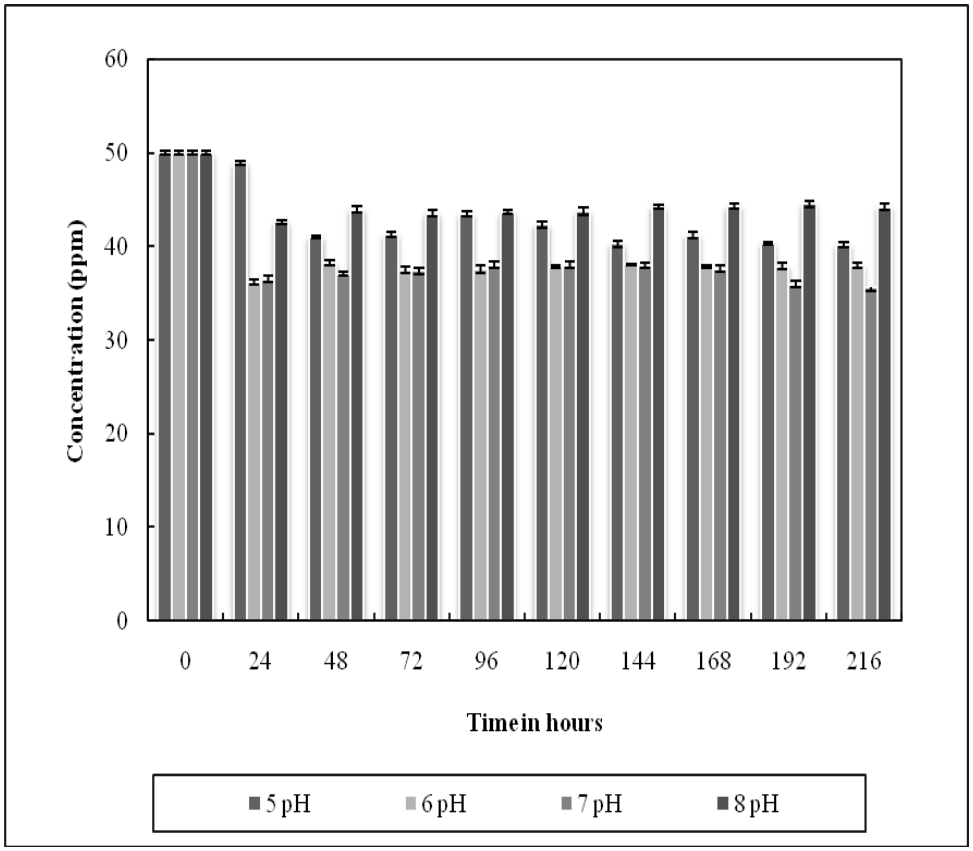


Fig.4.39: Concentration of PCMX (50mg/L) versus time by *Bacillus cereus* at different pH

Table 4.40: Growth (OD) of *Bacillus cereus* with PCMX (50 mg/L) versus time at different pH

Time(hr.)	5 pH	6 pH	7 pH	8 pH
0	0.003	0.003	0.004	0.003
24	0.085	0.025	0.208	0.206
48	0.072	0.131	0.409	0.419
72	0.049	0.061	0.491	0.675
96	0.056	0.068	0.394	0.408
120	0.054	0.063	0.342	0.31
144	0.043	0.063	0.347	0.312
168	0.042	0.064	0.332	0.341
192	0.044	0.062	0.34	0.32
216	0.041	0.061	0.341	0.301

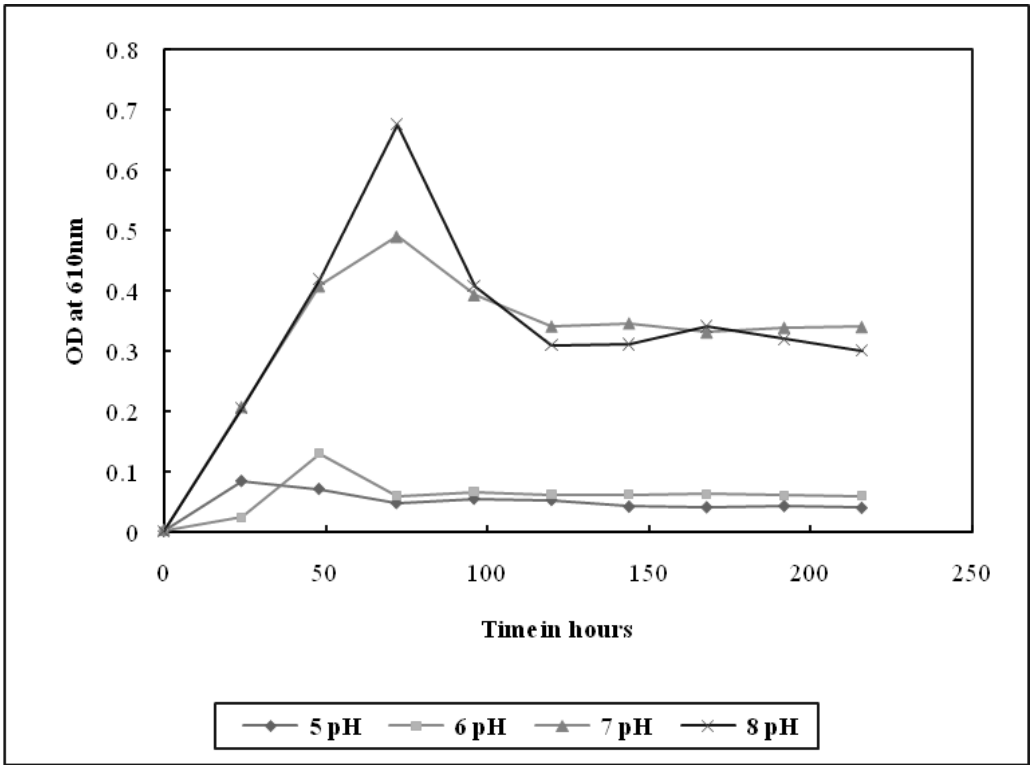


Fig. 4.40: Growth (OD) of *Bacillus cereus* with PCMX (50 mg/L) versus time at different pH

Table 4.41: pH of minimal media with PCMX (50mg/L) versus time at different pH

Time(hr.)	5 pH	6 pH	7 pH	8 pH
0	5.02	6	7.2	8.04
24	5.02	5.97	7.26	7.7
48	4.23	5.27	6.91	7.29
72	4.25	4.92	7.01	7.84
96	4.25	4.91	7.24	7.96
120	4.25	4.91	7.23	7.98
144	4.26	4.89	7.25	8.22
168	4.29	4.56	7.24	8.32
192	4.03	4.57	7.2	8.24
216	4.01	4.74	7.19	8.23

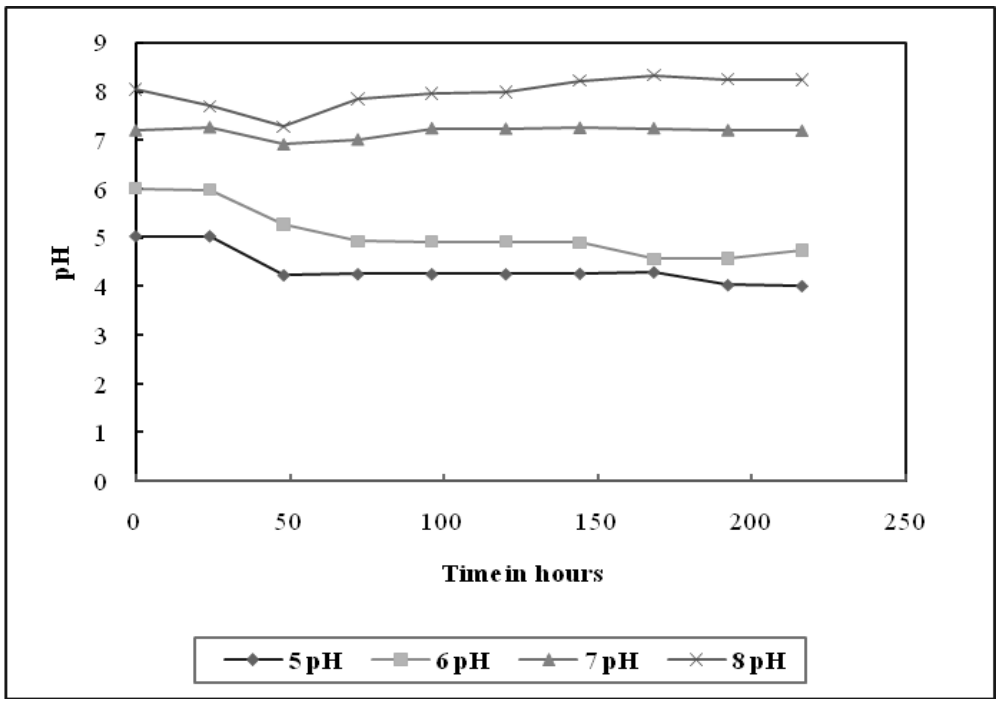


Fig. 4.41: pH of minimal media with PCMX (50mg/L) versus time at different pH

SUMMING UP

The experimental results for the effect of initial pH on the growth, concentration and the biodegradation rate of 2,4-DCP and PCMX are shown in the form of Tables and Figures. From the Tables and Figures, highest percentage degradation of 64.2 is observed at 3 pH for 2, 4-DCP at 10 mg/L and 28.76 percentage degradation at 7 pH was observed for PCMX at 50 mg/L.These results are similar to some researchers describing the biosorptive uptake of chlorophenol on suspended biomass, which is being primarily influenced by pH. Antizar-Ladislao and Galil (2004) who reported that an increase in pH value decreased the equilibrium sorption carried similar work. This observation may be because as the pH increased, the overall surface charge on the cells became negative and this led to a lower electrostatic attraction between negatively charged chlorophenol and binding sites of the biomass surface. Similarly, Daughney and Fein (1998) showed that an increase in pH from 5 to 9 showed a decrease in the percentage of 2, 4, 6 -TCP sorbed by *Bacillus subtilis*. It is known that there is some relationship between biosorption and biodegradation. Our results are in agreement with reports mentioned above and are correlated with the fact that there would be an increase in biosorption and in the specific rate of 2,4-DCP and PCMX degradation at pH 3 and 7 in *Bacillus cereus.* On the other hand, *Bacillus cereus* cells grew better at pH values within a range of 6 to 8 in mineral salt medium containing 2, 4-DCP than at pH 3. The same behavior was described by Tyler and Finn (1974) and Rutgers et al. (1998) who recorded that the dependence of pH on growth rate; at a given pH there is a certain amount of substrate present in dissociated and undissociated form. The undissociated form is the species that is believed to penetrate the cell membrane and to be responsible for both metabolic and inhibitory activity. This could explain the lower growth rate of *Bacillus cereus* at pH 3 than at higher pH in our work.

Within the work done by Karigar (2006) on *Arthrobacter citreus* they have reported that due to the effect of pH on the ionization and therefore binding and interaction of a myriad of molecular processes, which in turn affect the metabolic pathway. It could even cause denaturing of proteins, which might result in lethal

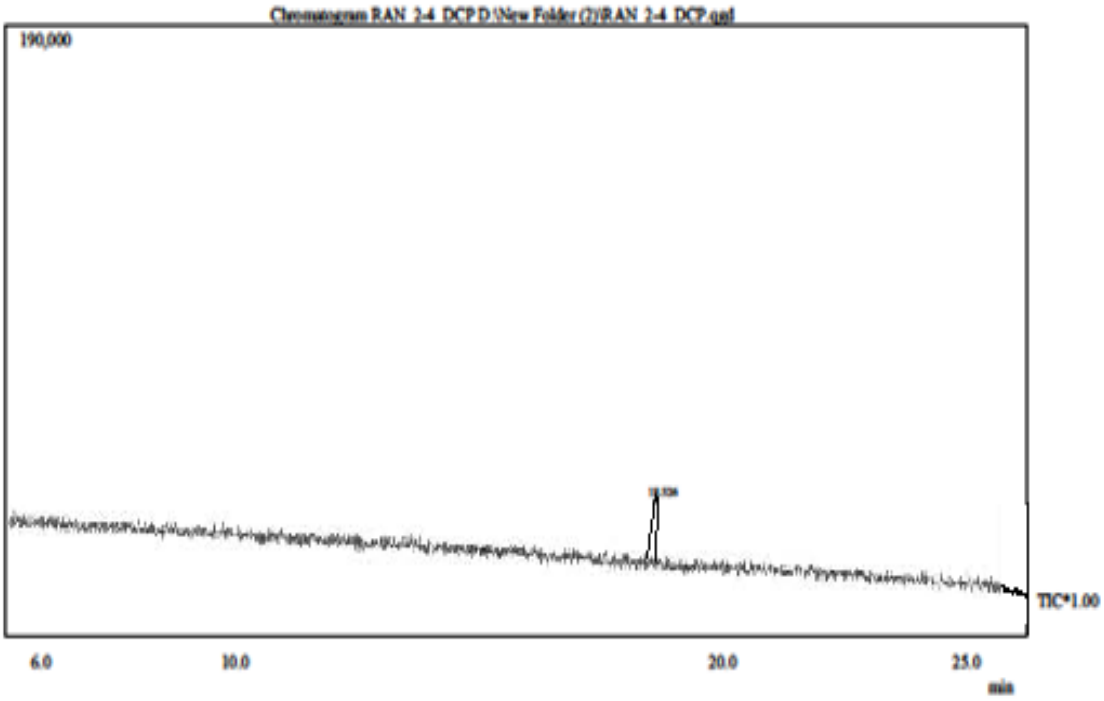
toxicity. At a room temperature and under normal pressure degradation of Atrazine is maximum at pH 6 for an optimum concentration of 5mg/L.

In conclusion, the results showed that changes in the temperature of the culture medium, and changes in the pH values affected the degradation of 2, 4-DCP and PCMX by *Bacillus cereus.* A temperature value between 30°C and 35°C were found to be optimal to obtain the maximum degradation of 2, 4-DCP and PCMX in this batch experimental system. The optimal pH for the reductive stage of remediation of 2, 4-DCP and PCMX showed that degradation was most rapid at pH value of 3 and 7 for 2, 4-DCP and PCMX respectively.

4.9. Qualitative Experiments to verify the degradation of 2, 4-DCP and PCMX

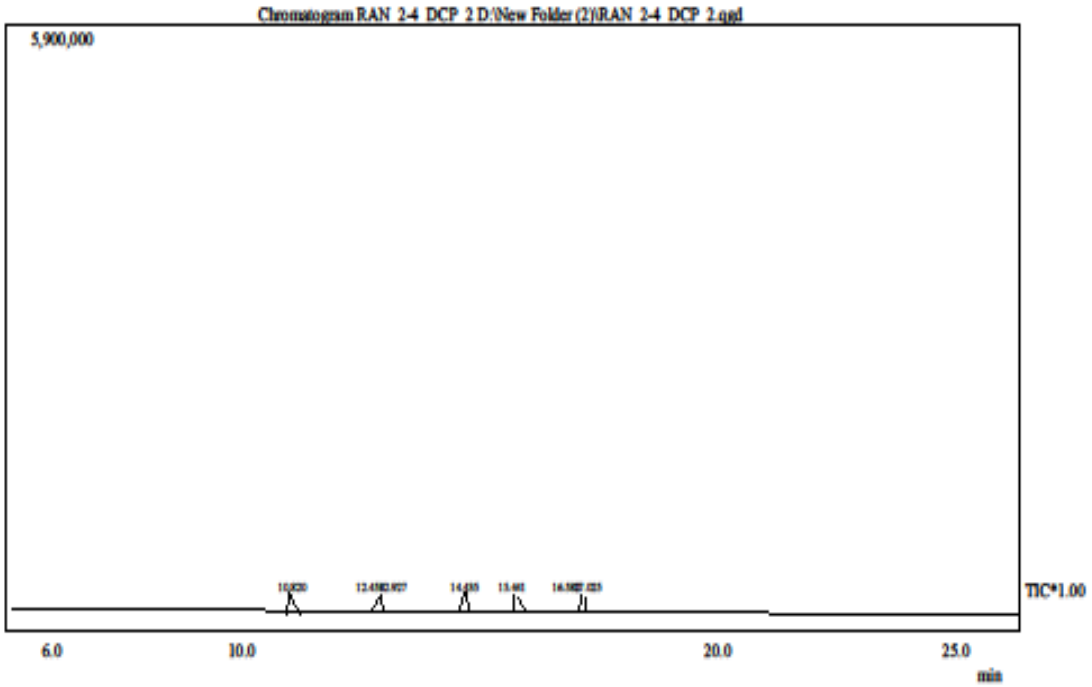
4.9.1. GCMS analysisof 2,4-DCP and PCMX

To verify the degradation process it was further studied using the analysis of intermediate products released through GCMS. Figures 4.41 to 4.46 presents the observation of the studies carried out through GCMS. It can be inferred from the chromatogram that the quantity of 2,4-DCP and PCMX has considerably reduced and new peaks have emerged.Fig. 4.42, 4.43 and 4.44 presents the results of GCMS analysis with respect to the progressive reduction of 2,4-DCP.Fig. 4.42 refers to the 2,4-DCP at 0 hour and from Fig. 4.43 new intermediate compounds are seen in the chromatogram for 2,4-DCP at 144 hour after the degradation was started. The results at the end of 336 hour are presented in Fig.4.44 where 2,4-DCP has been reduced completely beyond the detectable limit. These chromatograms indicate qualitatively the progressive removal of 2,4-DCP which is in conformity with the experimental data presented earlier.Fig.4.45, 4.46 and 4.47 presents the results of GCMS analysis at different time intervals for PCMX.Fig.4.45 presents the chromatogram initially and Fig. 4.46 indicates the presence of intermediate compounds in the biodegradation pathway of PCMX at the end of 144 hour and the Fig.4.47 presents the chromatogram at the end 336 hour. It can be observed from the chromatogram since the degradation was less as per the experimental results reported earlier the intermediate compound was still seen at the end of 336 hour.



Peak#	R.Time	I.Time	F.Time	Peak Report TIC Area% Name
1	18.526	18.408	18.692	65701100.00 65701100.0

Fig.4.42: Chromatogram of GCMS analysis of intermediate of 2, 4-DCP at 0th hr.



Peak#	R.Time	I. Time	F.Time	Peak Report TIC Area% Name
1	10.820	10.792	10.850	2015519.56
2	12.454	12.400	12.475	2504624.31
3	12.927	12.883	12.950	1872118.17
4	14.435	14.425	14.450	81827.94
5	15.441	15.425	15.475	1287512.50
6	16.582	16.567	16.600	62896.10
7	17.025	17.000	17.058	1177011.42

Fig.4.43 : Chromatogram of GCMS analysis of intermediate of 2, 4-DCP at 144th hr.

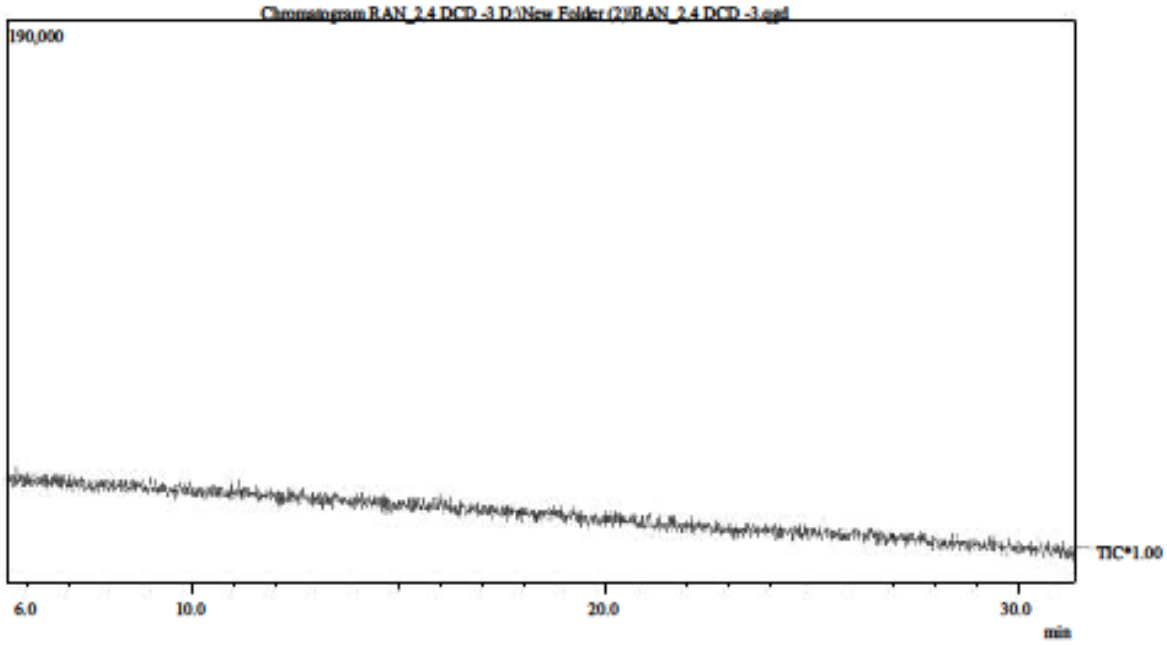
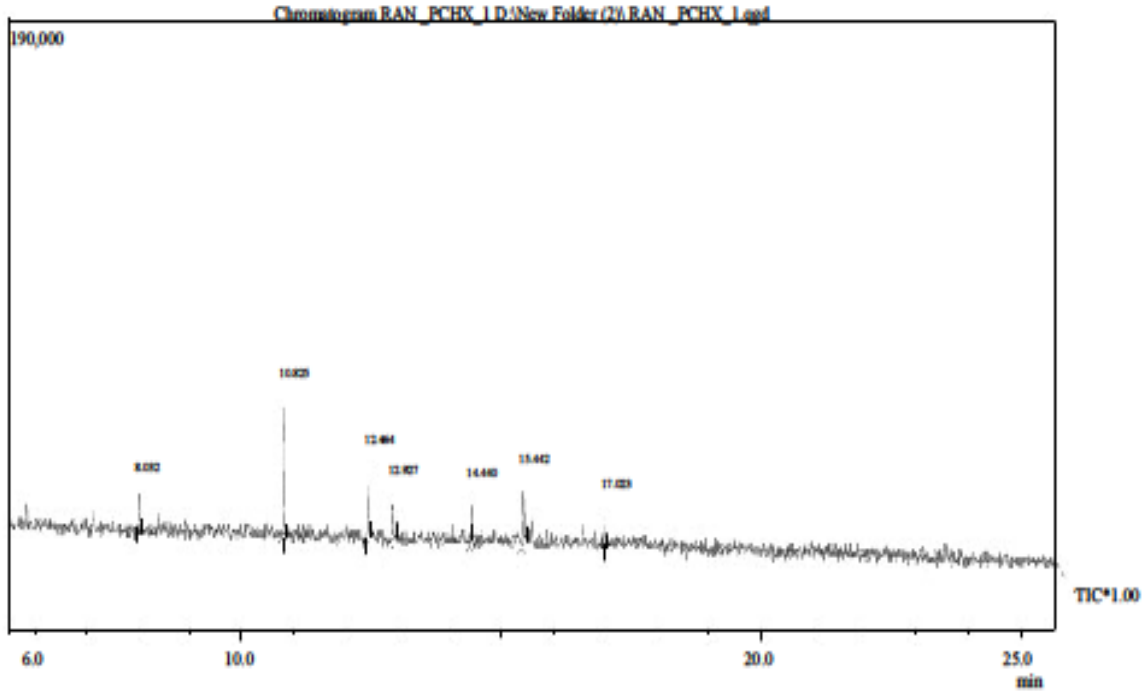


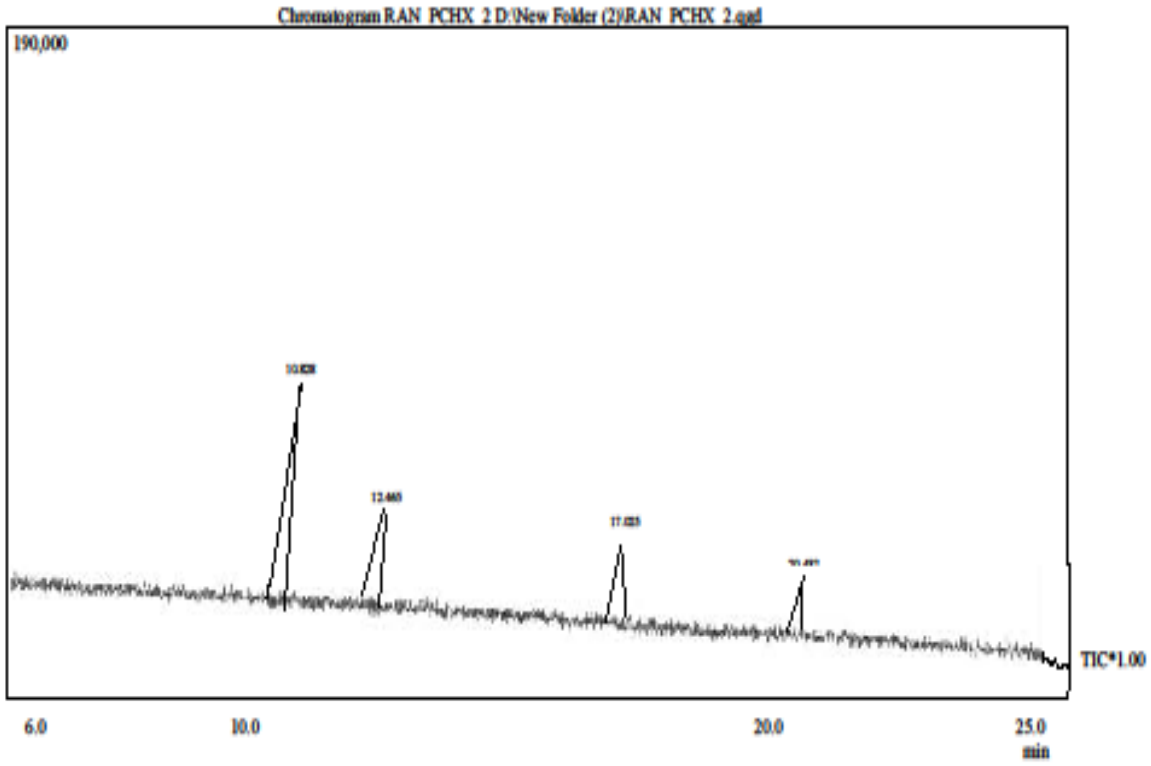
Fig.4.44 : Chromatogram of GCMS analysis of intermediate of 2,4-DCP at 336th hr.

PCMX



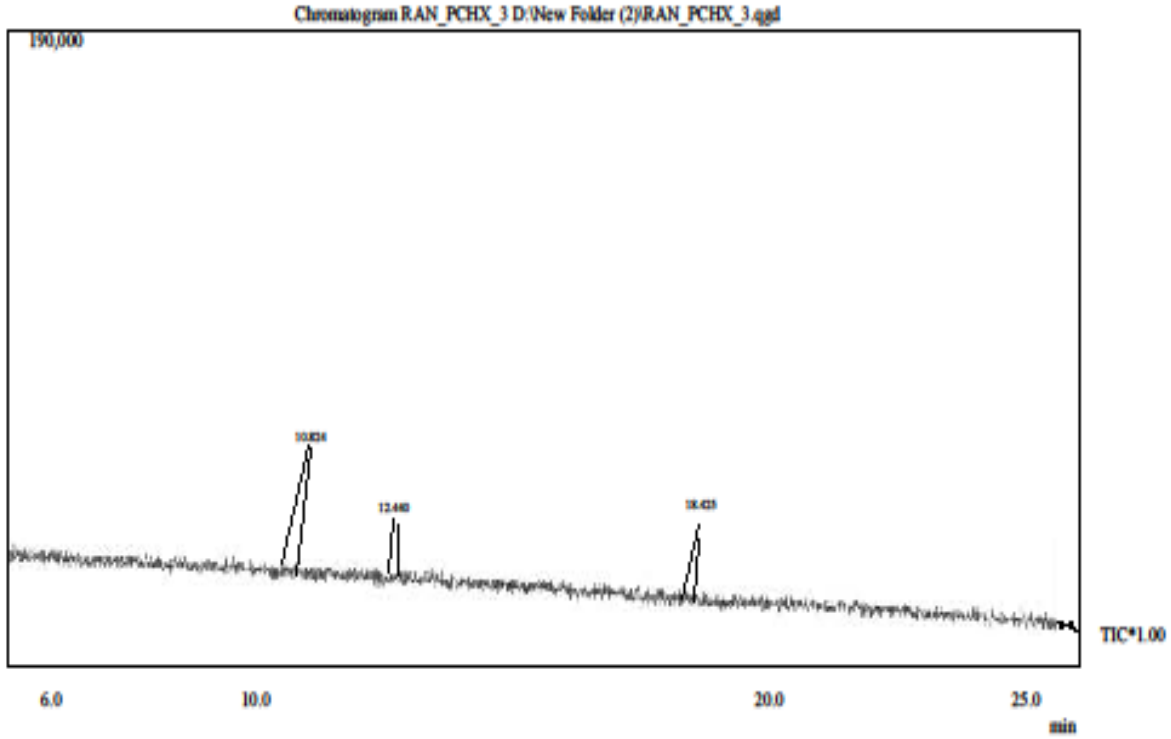
Peak#	R.Time	I. Time	F.Time	Peak Report TIC Area% Name
1	8.032	7.975	8.058	188248.58
2	10.825	10.783	10.867	7346533.48
3	12.464	12.400	12.500	4563620.79
4	12.927	12.867	12.967	206919.43
5	14.440	14.408	14.450	139776.37
6	15.442	15.400	15.500	2848612.98
7	17.023	16.983	17.058	183798.37

Fig.4.45 : Chromatogram of GCMS analysis of intermediate of PCMX at 0^{th} hr.



Peak#	R.Time	I. Time	F. Time	Peak Report TIC Area% Name
1	10.828	10.792	10.858	10252648.87
2	12.465	12.408	12.508	6269929.89
3	17.025	17.000	17.058	2234710.65
4	20.432	20.342	20.517	2221110.59

Fig.4.46: Chromatogram of GCMS analysis of intermediate of PCMX at 144th hr.



Peak#	R.Time	I. Time	F. Time	Peak Report TIC Area% Name
1	10.824	10.792	10.858	5269435.03
2	12.440	12.400	12.492	2504816.65
3	18.425	18.300	18.508	7269648.32

Fig.4.47: Chromatogram of GCMS analysis of intermediate of PCMX at 336th hr.

4.9.2. Degradation studies of 2, 4-DCP and PCMX imparting yellow colour to media

During degradation process of 2, 4-DCP and PCMX by *Bacillus cereus* yellow colour was observed in the media at the end of 264 hour and 216 hour respectively (Figure 4.48). According to the report published by many researchers chloride released is imparting yellow colour to the media indicating that degradation of chlorinated organic compound have occurred. Jae Jun Jeong et al. (2003) reported that during growth with p-cresol and 4-ethylphenol, *Pseudomonas sp.* KL28 produced a yellow-coloured diffusible product, presumably a meta-ring cleavage product. Similar work was carried out by H. Movahedyan et al. (2008) reported that metabolization of 4CP results in production of yellowish intermediate.

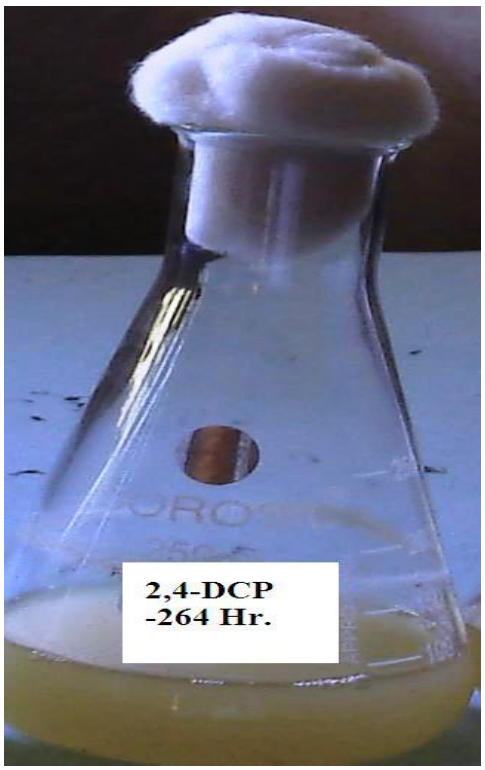


Fig.4.48.Degradation studies of 2, 4-DCP imparting yellow colour to media at 264 Hr.

4.9.3. Effects of 2, 4-DCP and PCMX on morphology of *Bacillus cereus* observed under Scanning electronic microscope

Experiments were carried out to understand the structural changes of the selected strain due to exposure of 2, 4-DCP and PCMX. The structural changes in terms of morphology was analysed through Scanning Electron Microscope. The results of the present study are presented in the form of Figures 4.49 to 4.51. It can be observed from the Figures that morphology changes after exposure to selected chemicals and the survival rate reduces which can be observed due to wrinkle surface and opening in cell wall.

After the organism was exposed to 2, 4-DCP, the images of scanning electron microscope in Fig. 4.50 of *Bacillus cereus* showed that the cells were swollen and the cell size decreased with irregular rod shapes and wrinkled surfaces. This decrease in the cell size might be due to the cellular oxidative stress of 2,4- DCP on the non-adapted cells of *Bacillus Cereus*. Ackerley et al., (2006), carried out similar work on the structural morphology of Escherichia coli K-12 before and after chromate stress. It was clearly observed in the E. coli cell that when the cell was exposed to chromate it becomes elongated compared to the normal cells due cellular oxidative stress of chromium on the non-adapted cells of E. coli.

From the Figures, it is clearly observed that when cells were exposed to PCMX they underwent cell lysis with destructive openings in their cell envelopes. The cell wall disruption can be clearly seen in images shown in Fig. 4.51. Sang-Ho Park and C.K. Kim(2001)reported the same type of work that the biphenyl, 4-chlorobiphenyl, 4-hydroxybenzoate and 4-chloro benzoate at high concentrations conferred a toxic effect on the cells due to the disruption of membrane components, and led to cell death.

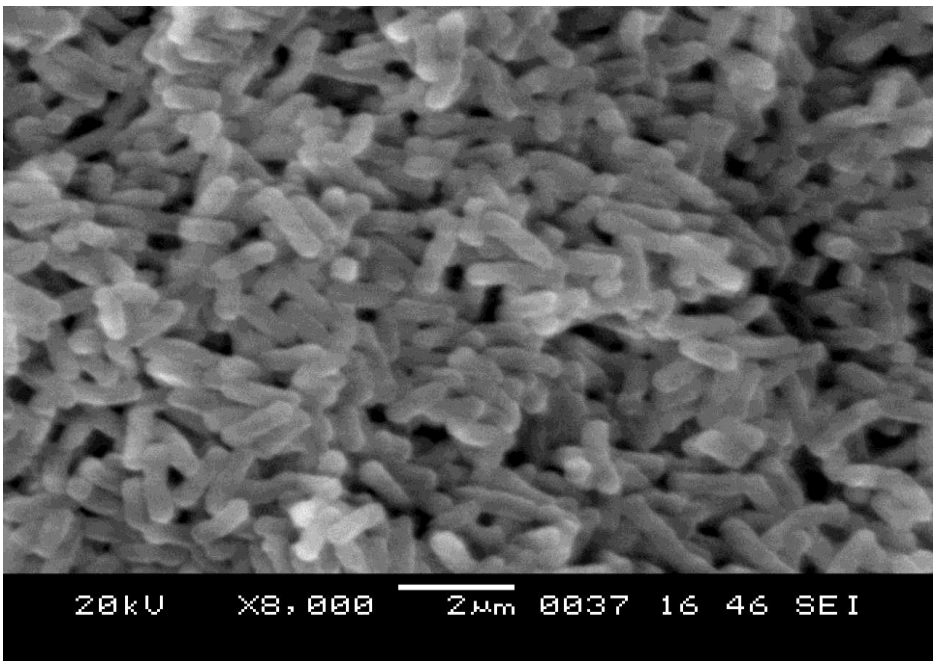


Fig. 4.49: Scanning electron micrographs of *Bacillus cereus*

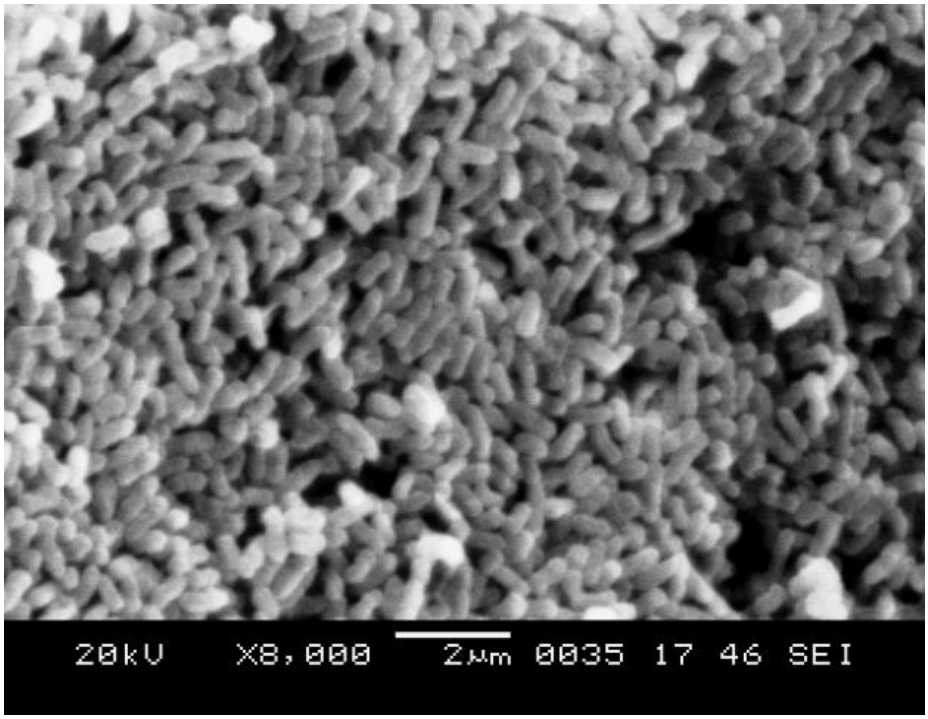


Fig. 4.50: Scanning electron micrographs of *Bacillus cereus* treated with 2,4-DCP for 264 hour.

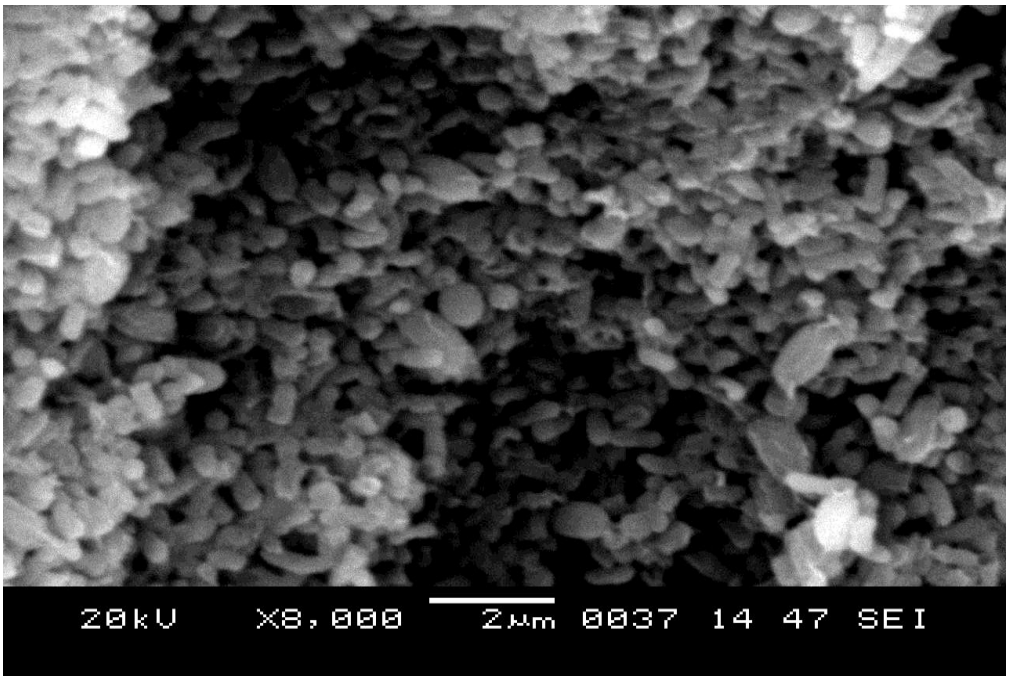


Fig. 4.51: Scanning electron micrographs of *Bacillus cereus* treated with PCMX for 264 hour.

4.10 Degradation studies of different concentration of 2,4-DCP and PCMX by mutated *Bacillus cereus*

The earlier experiment using parent strain at optimized conditions could degrade 2,4-DCP and PCMX 64.6% and 54.6% respectively. It has been reported through many studies in literature that mutation of parent strain has yielded better degradation of the selected chemicals.Hence it was proposed to conduct the mutation of parent strain through different methodology and standard protocols used by various researchers.

The present investigation was to increase the degradation rate of 2,4-DCP and PCMX by mutating the isolated strain using UV and Ethedium bromide. UV Irradiation (10, 20, 30, 40 and 60min) and chemical mutagen, Ethedium bromide were used in the mutation studies and then screened. A total of 42 mutants, 8 mutants after EtBr treatment and 34 mutants after UV treatment were screened. Among them one mutant from EtBr treatment and five mutants from UV treatment were selected for further studies based on their 2,4-DCP degradation profile. Further degradation studies were carried out with different concentrations of 2,4-DCP (10, 30 and 75mg/L) and PCMX (50, 100 and 150 mg/L) at 30^0C.

4.10.1 Degradation studies of 2, 4-DCP (10mg/L) by mutated *Bacillus cereus* and parent strain

The percentage removal efficiency by mutated and parent strain of *Bacillus cereus* (BC) at 10 mg/L of 2, 4-DCP was determined at a neutral pH of 7 and 30^0C temperature and are presented in Table 4.42 and Figure 4.52. From the Tables and Figures it is evident that percentage degradation of 2, 4-DCP (10mg/L) by mutated *Bacillus cereus* wasUV-10(29.6%), UV-20(39.1%), UV-30(34.6%), UV-40(31.8%), UV-60(36.4%), EtBr-12(31.7%) and parent type *Bacillus cereus* (BC-24.4%). The maximum percentage degradation was found with UV-20 (39.1%). It is observed from the results presented in Table 4.44 and Figure 4.53 that there is no change in pH of the medium and from the Table 4.43 and Figure 4.54 it is observed that maximum growth was obtained in the case of the parent strain *Bacillus cereus*.

Table 4.42: Concentration of 2, 4-DCP (10mg/L) versus time by mutated *Bacillus cereus* and parent strain

Time (hr.)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12	BC
0	10	10	10	10	10	10	10
24	8.78	7.94	8.57	8.93	8.44	9.96	9.98
48	8.65	7.95	8.53	8.65	8.12	8.85	8.86
72	9.04	8.01	8.42	8.47	8.29	9.46	8.45
96	8.76	7.99	7.08	7.17	8.07	9.02	8.67
120	6.77	6.38	6.68	6.34	6.74	8.76	8.52
144	7.04	6.71	6.7	6.21	7.16	6.43	8.32
168	7.23	6.58	6.65	6.5	7.52	7.57	8.24
192	6.77	6.83	6.45	6.49	6.01	8.16	8.12
216	7.41	6.04	6.4	6.75	6.51	8.18	7.98
264	7.04	6.09	6.54	6.82	6.36	6.83	7.56
%	**29.6**	**39.1**	**34.6**	**31.8**	**36.4**	**31.7**	**24.4**

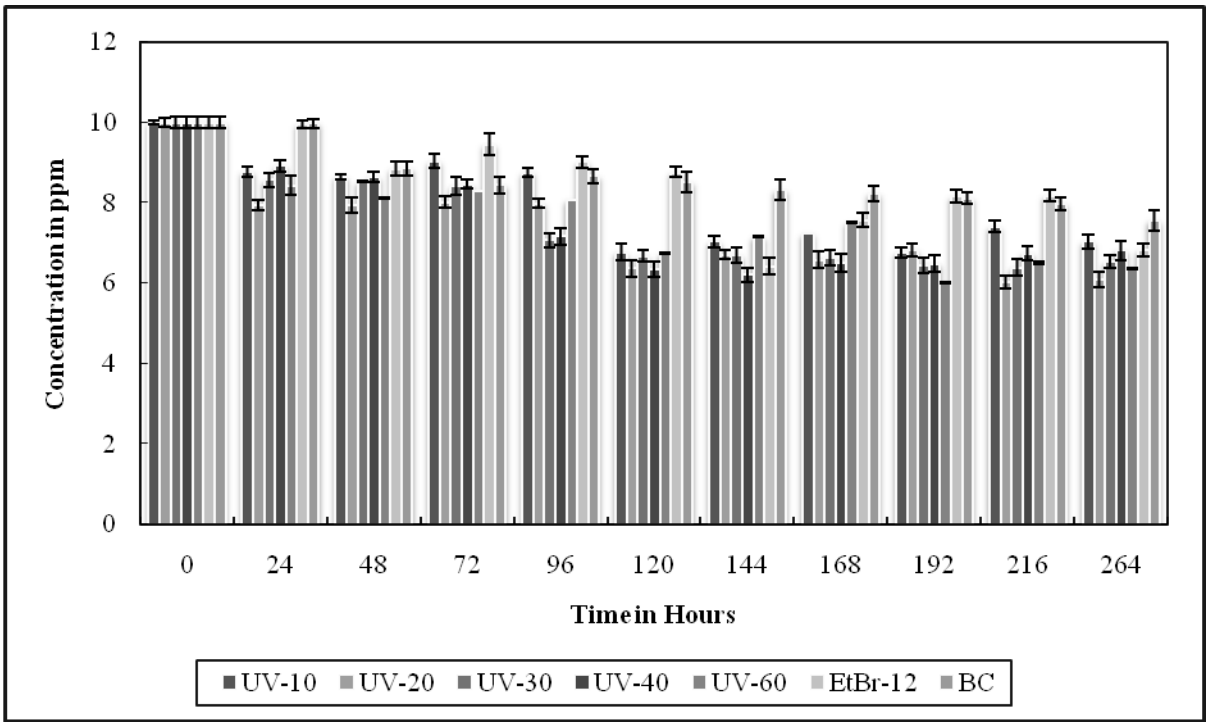


Fig.4.52: Concentration and growth (OD) of 2, 4-DCP (10mg/L) versus time by mutated Bacillus *cereus* and parent strain

Table 4.43: Growth (OD) of *Bacillus cereus* with 2, 4-DCP (10mg/L) versus time by mutated *Bacillus cereus* and parent strain

Time(hr.)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12	BC
0	0.003	0.002	0.002	0.002	0.004	0.006	0.003
24	0.078	0.089	0.079	0.081	0.117	0.098	0.195
48	0.077	0.086	0.078	0.083	0.115	0.069	0.186
72	0.065	0.08	0.07	0.072	0.114	0.074	0.157
96	0.066	0.071	0.07	0.065	0.107	0.065	0.145
120	0.057	0.07	0.065	0.062	0.099	0.06	0.132
144	0.052	0.064	0.066	0.069	0.099	0.065	0.123
168	0.051	0.05	0.062	0.063	0.087	0.057	0.112
192	0.048	0.054	0.058	0.057	0.076	0.057	0.114
216	0.047	0.057	0.06	0.06	0.083	0.056	0.114
264	0.047	0.055	0.061	0.06	0.072	0.042	0.113

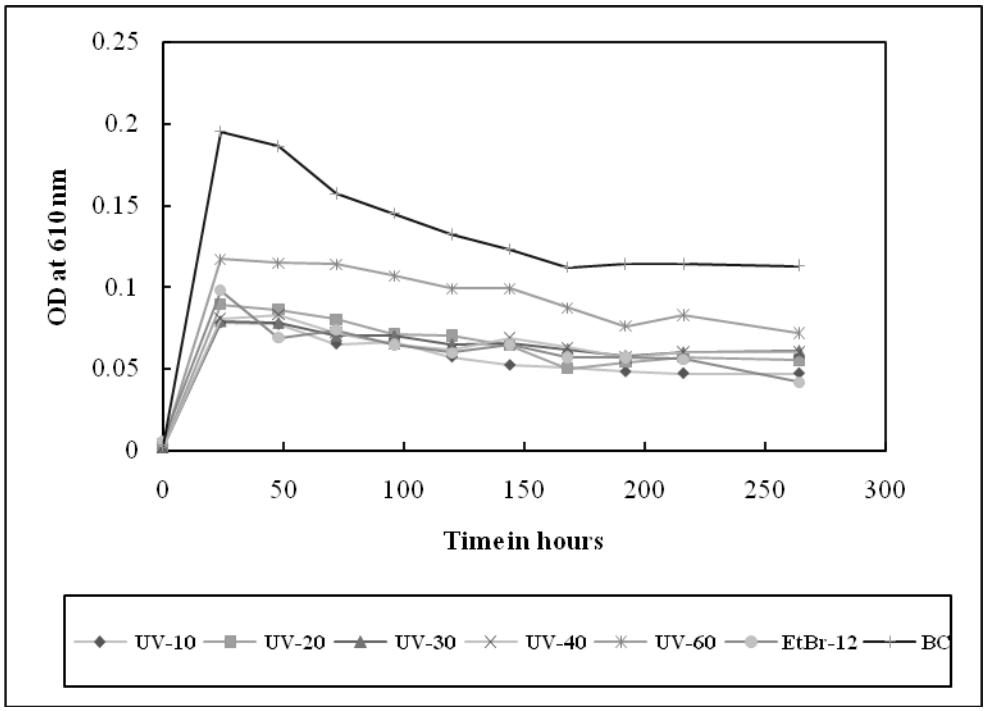


Fig. 4.53: Growth (OD) of *Bacillus cereus* with 2, 4-DCP (10mg/L) versus time by mutated *Bacillus cereus* and parent strain

Table 4.44: pH of minimal media with 2, 4-DCP (10mg/L) versus time by mutated *Bacillus cereus* and parent strain

Time(hr.)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12	BC
0	7.12	7.10	7.16	7.11	7.25	7.09	7.20
24	7.14	7.18	7.19	7.14	7.29	7.20	7.21
48	7.18	7.24	7.26	7.18	7.39	7.33	7.11
72	7.27	7.30	7.30	7.27	7.41	7.28	7.10
96	7.21	7.27	7.33	7.26	7.49	7.24	7.04
120	7.32	7.27	7.29	7.26	7.43	7.24	7.13
144	7.22	7.28	7.26	7.28	7.38	7.25	7.21
168	7.28	7.31	7.31	7.31	7.44	7.27	7.20
192	7.32	7.30	7.31	7.32	7.47	7.10	7.12
216	7.27	7.26	7.27	7.28	7.44	7.10	7.20
264	7.31	7.30	7.31	7.31	7.47	7.19	7.26

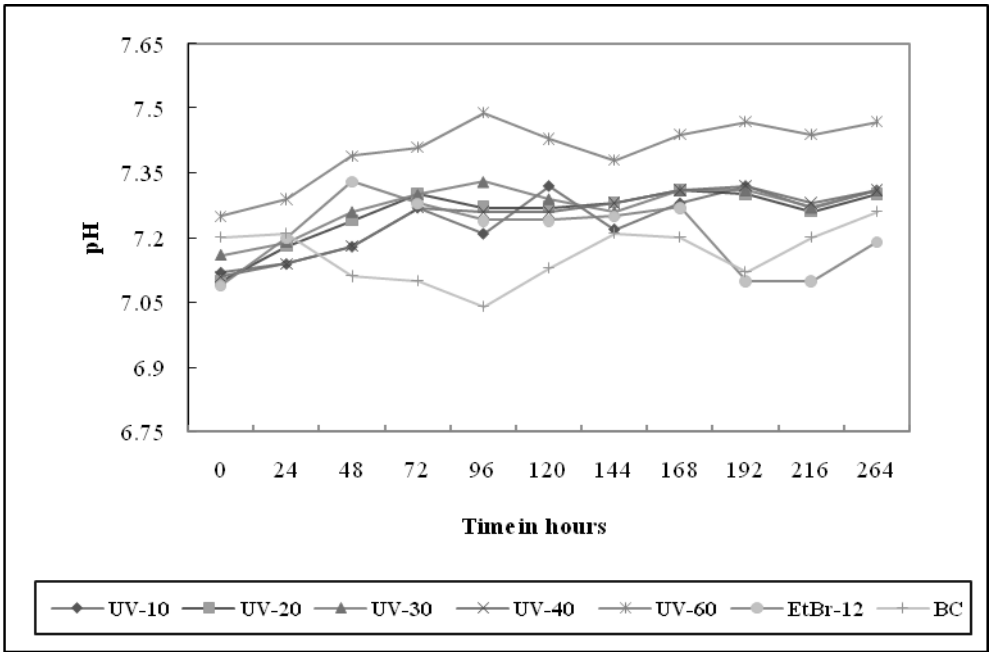


Fig. 4.54: pH of minimal media with 2, 4-DCP (10mg/L) versus time

4.10.2. Degradation studies of 2, 4-DCP (30mg/L) by mutated *Bacillus cereus* and parent strain

The average concentration and growth (OD) of the duplicate analysis of 2, 4-DCP (30mg/L)by mutated and parent strain of *Bacillus cereus* versus time is presented in Table 4.45 and Figure 4.55.Growth (OD) of *Bacillus cereus* in PCMX versus time is presented in Table 4.46 and Figure 4.56. pH of media with 2, 4-DCP versus time is presented in Table 4.47 and Figure 4.57. From the result it is seen that the percentage degradation of 2,4-DCP(30mg/L) by mutated *Bacillus cereus* was UV-10(47.46%), UV-20(39.70%),UV-30(32.10%), UV-40(31.56%), UV-60(31.20%), EtBr-12(29.23%) and parent type *Bacillus cereus* BC(21.21%).The maximum percentage degradation was found to be by mutated *Bacillus cereus* UV-10(47.46%). It is evident that there is no change in pH of the mediumand maximum growth is by UV-40.

Table 4.45: Concentration of 2, 4-DCP (30mg/L) versus time by mutated *Bacillus cereus* by mutated *Bacillus cereus* and parent strain

Time(hr.)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12	BC
0	30.00	30.00	30.00	30.00	30.00	30.00	30.00
24	22.34	25.93	25.67	26.34	26.26	29.87	28.84
48	21.10	25.83	25.68	26.03	25.24	29.21	28.80
72	21.22	25.84	26.65	25.08	24.13	27.70	29.89
96	21.64	25.87	26.05	24.36	24.05	28.45	27.80
120	21.10	25.70	23.48	23.01	23.88	26.65	27.67
144	17.22	23.87	22.00	23.51	23.43	26.92	26.03
168	16.64	19.31	21.35	22.56	22.56	25.54	26.03
192	16.08	19.03	20.25	21.39	20.28	26.27	25.68
216	16.92	18.83	20.39	20.52	20.20	22.45	25.45
264	15.76	18.09	20.36	20.52	20.63	21.22	23.63
%	**47.46**	**39.70**	**32.10**	**31.56**	**31.20**	**29.23**	**21.21**

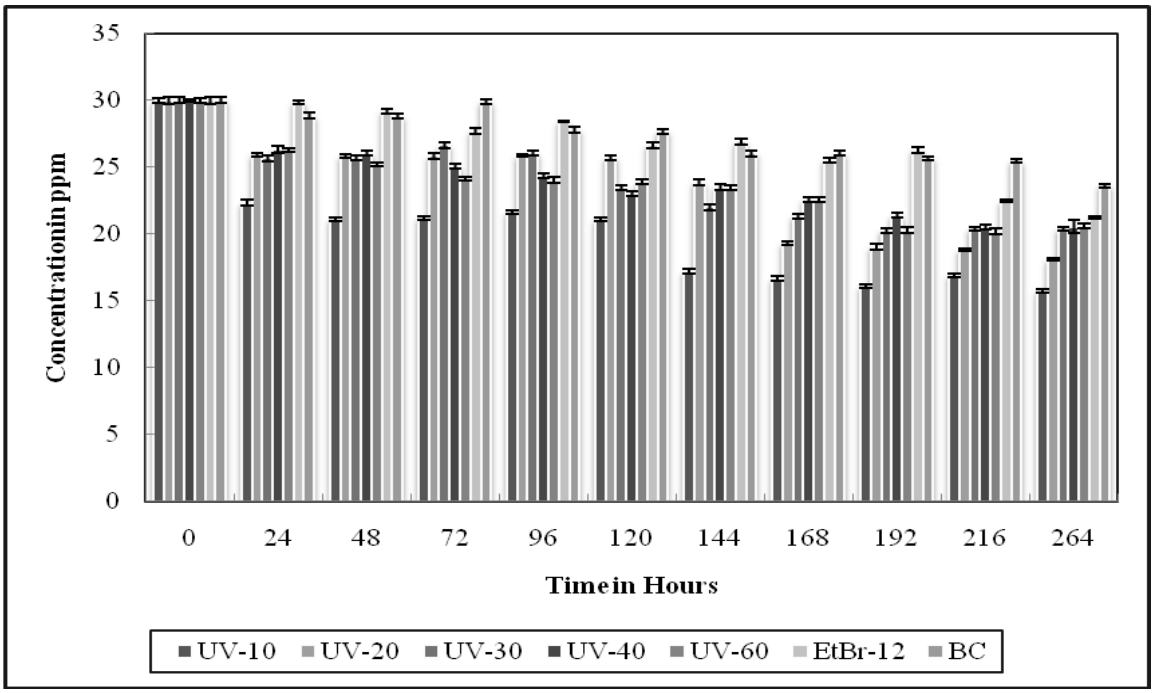


Fig. 4.55: Concentration of 2, 4-DCP (30mg/L) versus time by mutated *Bacillus cereus* by mutated *Bacillus cereus* and parent strain

Table 4.46: Growth OD of mutated *Bacillus cereus* and parent strain with 2, 4-DCP (30mg/L) versus time

Time(hr.)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12
0	0.003	0.003	0.002	0.003	0.003	0.006
24	0.076	0.069	0.079	0.078	0.058	0.088
48	0.065	0.056	0.07	0.08	0.052	0.048
72	0.065	0.052	0.07	0.07	0.045	0.047
96	0.066	0.045	0.07	0.067	0.037	0.039
120	0.057	0.048	0.062	0.044	0.037	0.036
144	0.048	0.038	0.056	0.047	0.029	0.035
168	0.044	0.027	0.047	0.043	0.027	0.028
192	0.03	0.026	0.035	0.042	0.025	0.025
216	0.029	0.026	0.034	0.046	0.026	0.023
264	0.029	0.028	0.036	0.043	0.025	0.02

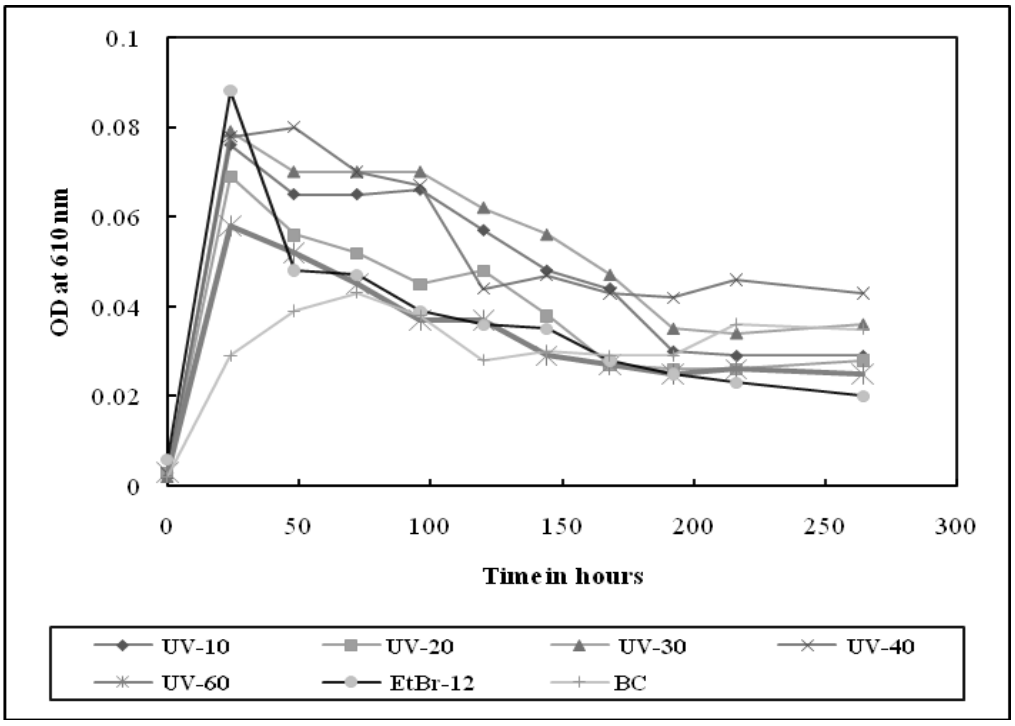


Fig. 4.56: Growth OD of mutated *Bacillus cereus* and parent strain with 2, 4-DCP (30mg/L) versus time

Table 4.47: pH of minimal media with 2, 4-DCP (30mg/L) versus time by mutated *Bacillus cereus* and parent strain

Time(hr.)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12	BC
0	7.13	7.11	7.17	7.13	7.12	7.06	7.13
24	7.24	7.28	7.19	7.31	7.23	7.20	7.08
48	7.28	7.24	7.27	7.31	7.28	7.30	7.09
72	7.27	7.30	7.31	7.31	7.32	7.24	7.06
96	7.21	7.27	7.33	7.35	7.37	7.26	7.13
120	7.26	7.29	7.30	7.35	7.36	7.28	7.32
144	7.27	7.28	7.36	7.35	7.38	7.30	7.36
168	7.28	7.31	7.32	7.34	7.38	7.27	7.24
192	7.32	7.32	7.31	7.35	7.36	7.11	7.19
216	7.37	7.36	7.32	7.36	7.38	7.08	7.22
264	7.36	7.34	7.32	7.36	7.38	7.10	7.24

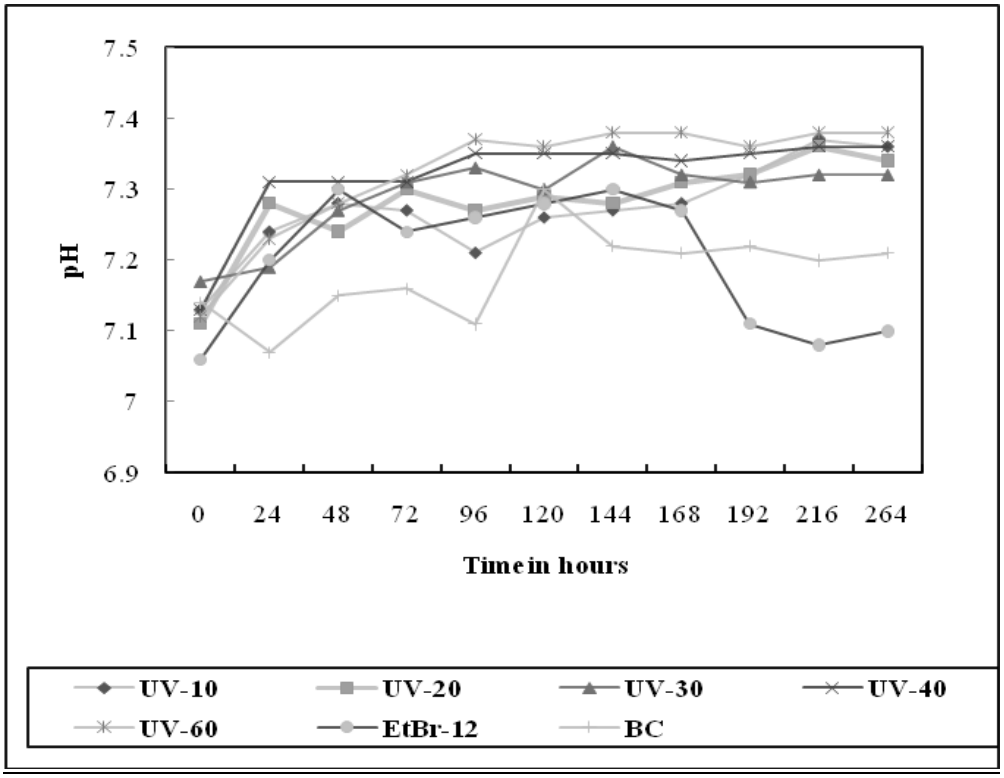


Fig. 4.57: pH of minimal media with 2, 4-DCP (30mg/L) versus time by mutated *Bacillus cereus* and parent strain

4.10.3 Degradation studies of 2, 4-DCP (75 mg/L) by mutated *Bacillus cereus* and parent strain

In Table 4.48 and Figure 4.58 are presented the residual 2,4-DCP estimation data by mutated *Bacillus cereus* and parent strain at 30^{o}C at pH 7.Results show that percentage degradation of 2,4-DCP(75mg/L) were as follows: UV-10(53.28%), UV-20(47.12%), UV-30(44.59%), UV-40(42.62%), UV-60(43.73%), EtBr-12(7.50%) and parent type *Bacillus cereus* BC(9.47%). Maximum degradation was with UV-10 (53.28%). Growth (OD) of *Bacillus cereus* in PCMX versus time is presented in Table 4.49 and Figure 4.59. pH of media with 2,4-DCP versus time is presented in Table 4.50 and Figure 4.60. From the Tables and Figures it is evident that there is no change in pH of the medium and maximum growth was observed in case of mutated organism EtBr-12.

Table 4.48: Concentration of 2, 4-DCP (75 mg/L) versus time by mutated *Bacillus cereus* and parent strain

Time(hr.)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12	BC
0	75.00	75.00	75.00	75.00	75.00	75.00	75.00
24	55.45	60.80	64.81	67.40	58.23	75.28	74.80
48	55.48	60.56	60.43	65.28	58.63	74.39	73.69
72	55.57	60.07	58.95	64.32	58.83	75.78	72.54
96	45.62	56.72	54.06	60.16	54.10	75.76	69.89
120	43.68	54.72	53.62	59.07	49.10	72.01	69.67
144	43.11	53.83	46.75	44.65	47.43	69.02	69.34
168	43.65	52.64	46.17	44.58	46.54	68.39	68.56
192	43.08	46.50	47.62	43.65	47.89	70.29	68.34
216	35.25	48.17	45.57	43.73	43.72	69.64	68.14
264	35.04	39.65	41.47	42.95	42.12	69.36	67.89
%	**53.28**	**47.12**	**44.59**	**42.62**	**43.73**	**7.50**	**9.47**

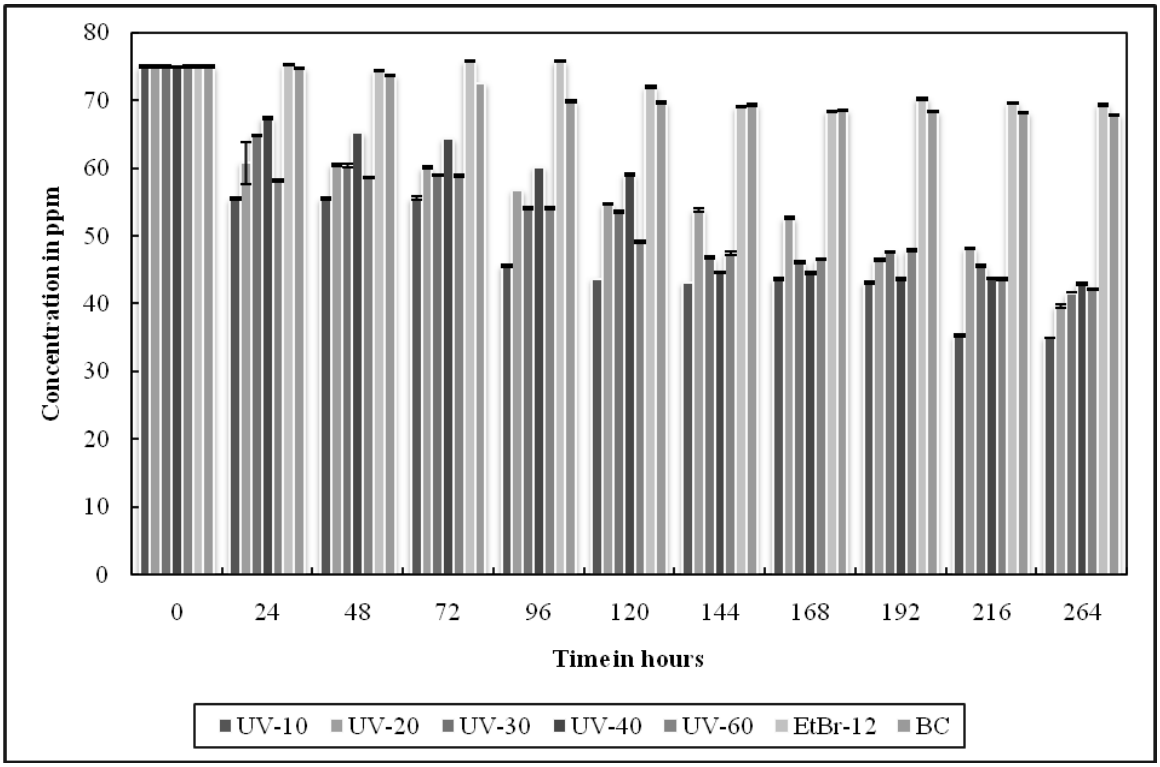


Fig.4.58: Concentration of 2, 4-DCP (75 mg/L) versus time by mutated *Bacillus cereus* and parent strain

Table 4.49: Growth (OD) of mutated *Bacillus cereus* and parent strain with 2, 4-DCP (75mg/L) versus time

Time(hr.)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12	BC
0	0.003	0.003	0.004	0.002	0.002	0.003	0.005
24	0.041	0.011	0.009	0.006	0.004	0.014	0.06
48	0.045	0.009	0.011	0.025	0.003	0.034	0.09
72	0.037	0.006	0.011	0.026	0.003	0.061	0.059
96	0.032	0.008	0.022	0.034	0.003	0.053	0.043
120	0.029	0.006	0.024	0.035	0.003	0.044	0.027
144	0.026	0.006	0.05	0.047	0.008	0.042	0.018
168	0.025	0.011	0.019	0.038	0.011	0.049	0.019
192	0.029	0.013	0.016	0.036	0.018	0.05	0.023
216	0.026	0.015	0.016	0.032	0.018	0.054	0.017
264	0.025	0.019	0.017	0.024	0.016	0.055	0.014

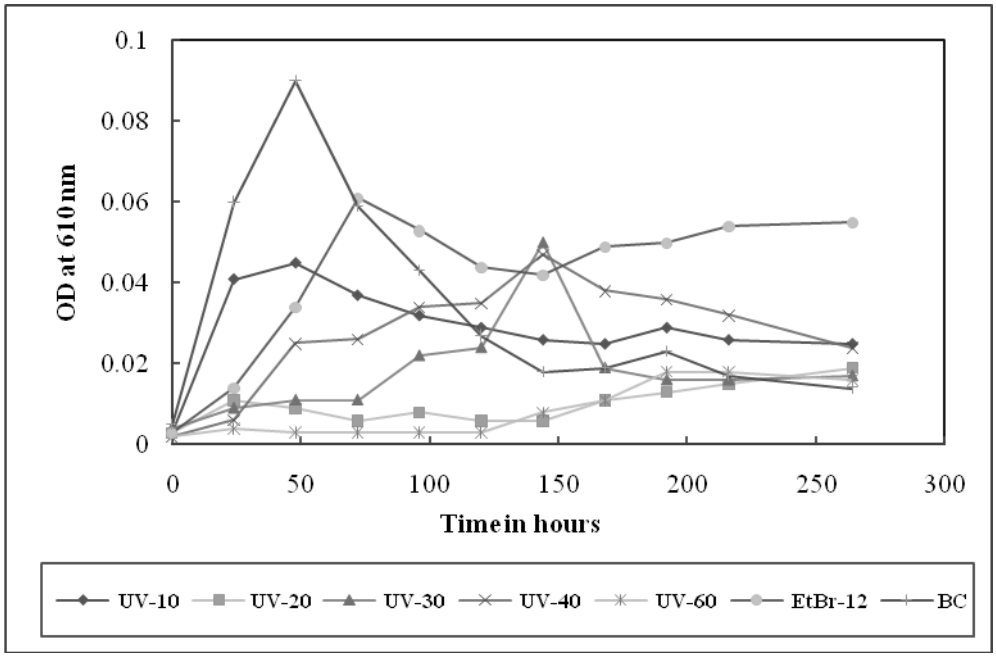


Fig. 4.59: Growth (OD) of mutated *Bacillus cereus* and parent strainwith 2, 4-DCP (75mg/L) versus time

Table 4.50: pH of minimal media with by mutated *Bacillus cereus* and parent strainand 2, 4-DCP (75 mg/L) versus time

Time(hr.)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12	BC
0	7.16	7.01	7.16	7.18	7.10	7.07	7.04
24	7.25	7.27	7.19	7.21	7.23	7.13	6.98
48	7.26	7.24	7.27	7.25	7.28	7.22	7.15
72	7.27	7.30	7.33	7.29	7.32	7.24	7.16
96	7.29	7.27	7.31	7.32	7.37	7.20	7.23
120	7.36	7.29	7.32	7.38	7.36	7.21	7.19
144	7.37	7.28	7.36	7.39	7.38	7.18	7.20
168	7.58	7.31	7.35	7.40	7.38	7.19	7.22
192	7.52	7.32	7.37	7.42	7.36	7.20	7.16
216	7.67	7.36	7.38	7.52	7.38	7.20	7.18
264	7.68	7.63	7.40	7.62	7.59	7.20	7.19

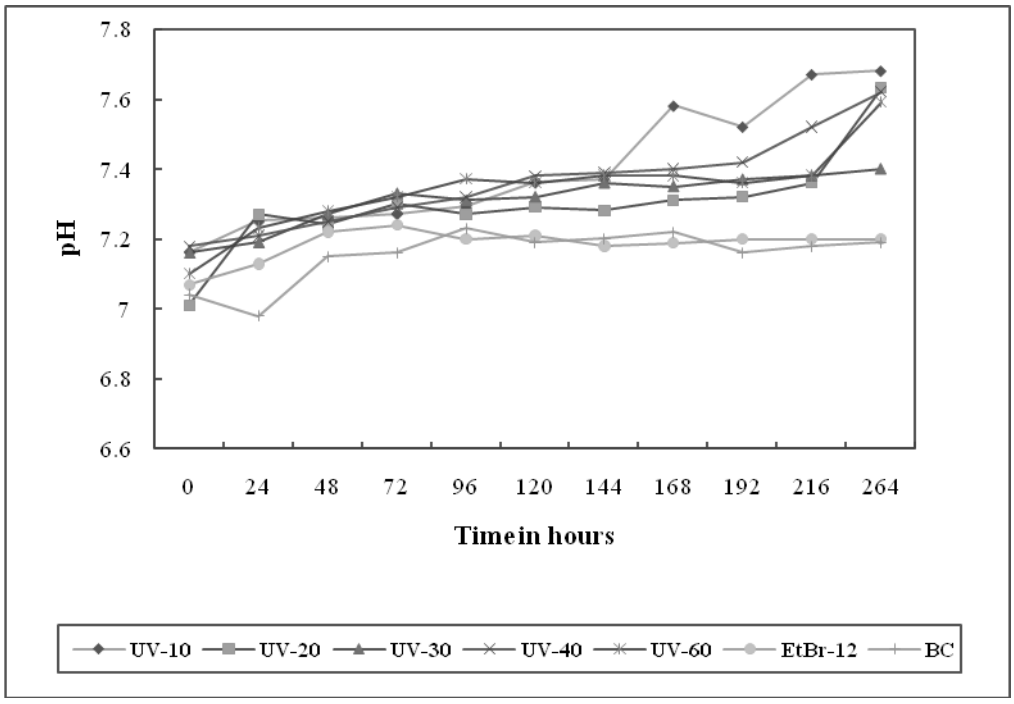


Fig.4.60: pH of minimal media with 2, 4-DCP and mutated *Bacillus cereus* and parent strain (75 mg/L) versus time

4.10.4. Percentage degradationby UV-10, EtBR-12 and BC at different concentration of 2,4-DCP

Results of the degradation experiments by mutated strain (UV-10 and EtBr-12) and parent strain(BC) at different concentration of 2,4-DCP at 30^{o}C at 264 hour are presented in the Table 4.51 and Figure 4.61.It is found that as the concentration of 2,4-DCP is increased from 10 to 75 mg/L the percentage degradation obtained by UV mutated strain(UV- 10)was more when compared to parent type(BC) and chemically mutated strain (EtBr-12).

Table 4.51: Percentage degradation by UV-10, EtBr-12 and BC at different concentration of 2,4-DCP at 264 hr.

Concentration of 2,4-DCP	10 mg/L	30 mg/L	75 mg/L
UV-10	29.6	47.46	53.28
EtBr-12	31.7	29.23	7.5
BC	24.4	21.21	9.47

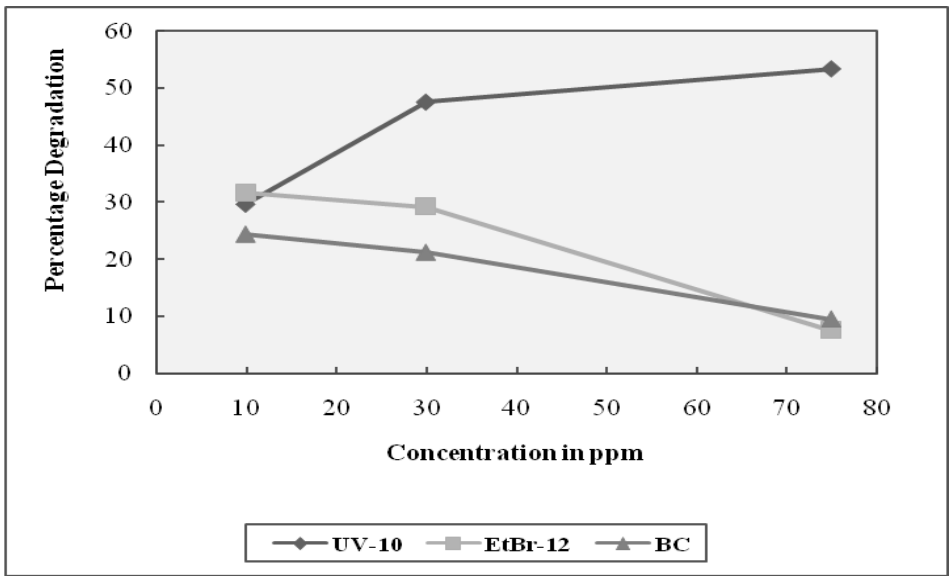


Fig.4.61: Percentage degradation by UV-10, EtBR-12 and BC at different concentration of 2, 4-DCP at 264 hr.

SUMMING UP

In conclusion degradation of 2, 4-DCP by mutant strain (UV-10) was 2 fold higher than the parent strain (BC).Further it was found that the growth of parent type *Bacillus cereus* is more when compared to mutated strain. This indicates that the enhancement of degradation by mutant strain was not because decrease in growth but may be due to the enhancement in production of enzyme degrading 2, 4-DCP. Comparing with other studies *Pseudomonas alcaligenes* which could degrade 2,4-dichlorophenol before UV-irradiation was 220 and 380 mg/L after UV-irradiation(Elkarmi et al., 2009).Mutating the microorganism for enhancing the degradation and production of enzymes has been reported by many authors.Yan Jiang et al. (2007) mutated *Candida tropicalis* by Irradiation with a He-Ne Laser to increase its ability to degrade phenol.He cloned the phenol hydroxylase gene in parent and mutant strains, which was responsible for degradation of phenol. From his findings, it was clear that the activity of phenol hydroxylase was promoted after irradiation with a He-Ne laser. In addition, he described the cell growth and intrinsic phenol biodegradation kinetics of mutant strain CTM 2 in batch cultures as explained by Haldane's kinetic equation with a wide range of initial phenol concentrations from 0 to 2,600 mgliter. He concluded that specific growth and degradation rates the CTM 2 mutant strain possessed a higher capacity to resist phenol toxicity than parent *C. tropicalis*.Keratinase production and keratin degradation by a mutant strain of *Bacillus subtilis* was studied by Cheng-gang Cai, in 2008 concluding that elevated keratinolytic activity using *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine mutagenesis resulted in a mutant strain KD-N2 producing keratinolytic activity about 2.5 times that of the parent-type strain. Similar type of work was carried out by M. Himabindu et al.(2007) on enhancement of gentamicin production by *Micromonospora echinospora.* He reported that production of gentamicin in shake flask by *Micromonospora echinospora* EtBr-22 strain reaches 1354 mg /L which is 1.53-fold higher than that of the parent strain.Further studies in this area are needed to elucidate the genetic alterations and the new biodegradation pathway that occurred due to chemical and physical mutagens.

4.11 Degradation studies of different concentration of PCMX by mutated *Bacillus cereus*

4.11.1 Degradation studies of PCMX (50mg/L) by mutated *Bacillus cereus*

The average concentration and growth (OD) of the duplicate analysis of PCMX by mutated and parent strain *Bacillus cereus* versus time is presented in Table 4.52 and Figure 4.62. Growth (OD) of *Bacillus cereus* in PCMX versus time is presented in Table 4.53 and Figure 4.63. pH of media with PCMX versus time is presented in Table 4.54 and Figure 4.64. The percentage degradation of PCMX (50mg/L) by mutated *Bacillus cereus* was UV-10(10.26%), UV-20(14.24%), UV-30(21.88%), UV-40(5.6%), UV-60(3.16%), EtBr-12(4.86%) and parent type *Bacillus cereus* BC (29.14%) as shown in Tables and Figures.The maximum percentage degradation was observed by BC (parent type) (29.14%).It is evident that there isno change in pH of the medium and maximum growth was observed in case of EtBr-12.

Table 4.52: Concentration of PCMX (50mg/L) versus time by mutated *Bacillus cereus* and parent strain

Time (hr.)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12	BC
0	50.00	50.00	50.00	50.00	50.00	50.00	50
24	49.71	29.93	31.11	49.87	48.04	32.12	43.55
48	48.88	36.95	32.68	45.98	48.10	39.95	37.75
72	49.42	45.35	41.08	46.51	48.78	34.22	37.48
96	49.05	44.21	40.56	45.62	46.54	31.92	37.23
120	48.15	48.56	40.80	44.90	44.51	46.79	37.05
144	45.54	40.30	38.27	47.13	44.28	49.49	37.19
168	45.26	40.92	40.94	44.67	39.88	45.89	36.78
192	45.18	42.73	38.12	44.58	35.45	33.83	35.97
216	46.81	43.54	36.98	45.61	46.34	34.87	35.78
264	44.87	42.88	39.06	47.20	48.42	47.57	35.43
%	**10.26**	**14.24**	**21.88**	**5.6**	**3.16**	**4.86**	**29.14**

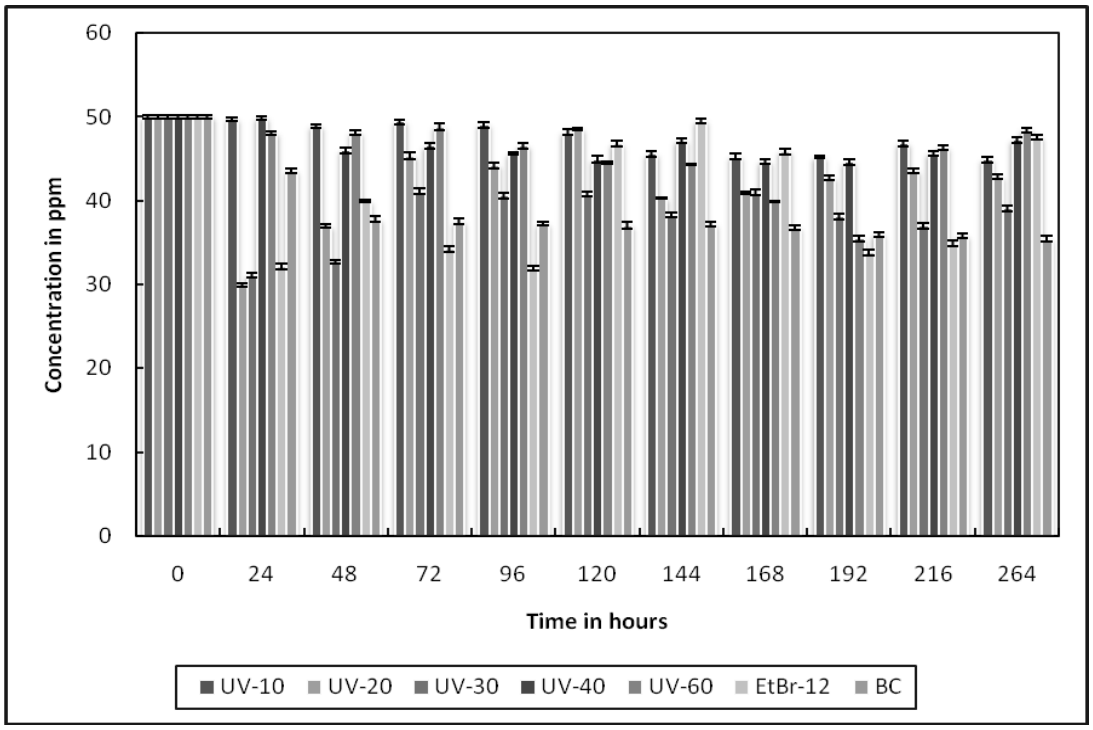


Fig.4.62: Concentration of PCMX (50mg/L) versus time by mutated *Bacillus cereus* and parent strain

Table 4.53: Growth (OD) of *Bacillus cereus* with PCMX (50mg/L) versus time by mutated *Bacillus cereus* and parent strain

Time (hr.)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12	BC
0	0.027	0.014	0.005	0.013	0.003	0.002	0.003
24	0.184	0.12	0.169	0.163	0.133	0.272	0.098
48	0.227	0.342	0.188	0.226	0.191	0.398	0.108
72	0.268	0.276	0.314	0.268	0.143	0.45	0.176
96	0.302	0.203	0.345	0.245	0.214	0.405	0.19
120	0.34	0.201	0.365	0.25	0.276	0.316	0.299
144	0.303	0.225	0.28	0.22	0.273	0.324	0.398
168	0.264	0.282	0.253	0.218	0.291	0.32	0.457
192	0.225	0.187	0.194	0.171	0.28	0.344	0.367
216	0.209	0.219	0.248	0.19	0.244	0.355	0.345
264	0.215	0.205	0.208	0.188	0.181	0.34	0.308

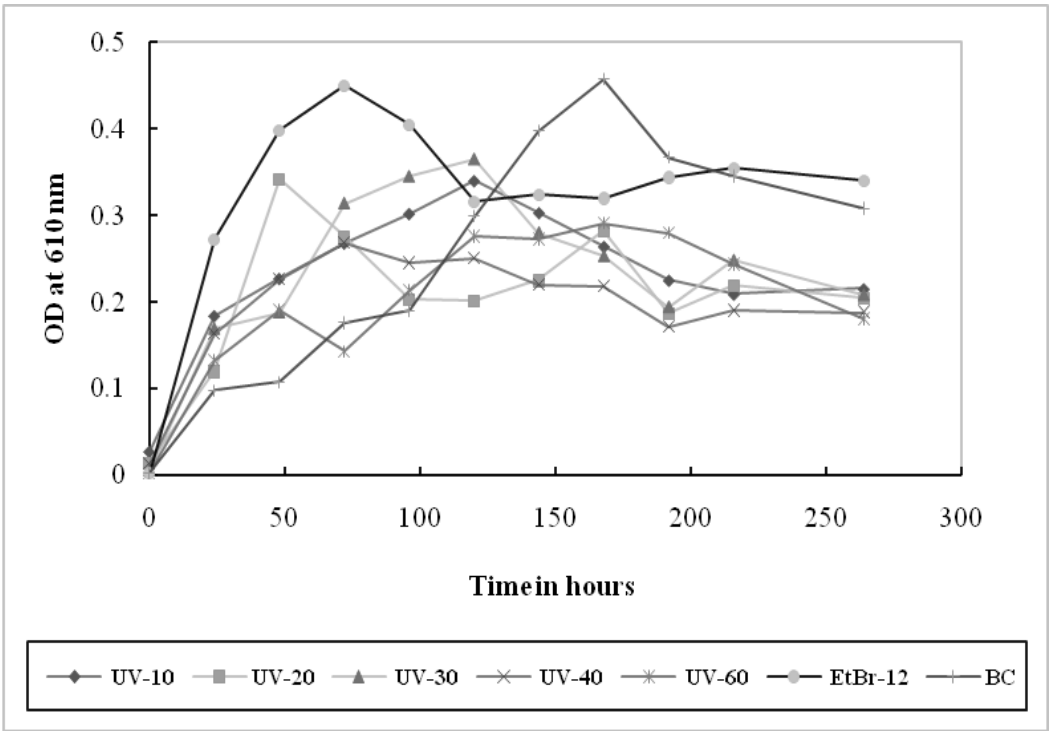


Fig. 4.63: Growth (OD) of *Bacillus cereus* with PCMX (50mg/L) versus time by mutated *Bacillus cereus* and parent strain

Table 4.54: pH of minimal media with by mutated *Bacillus cereus* and parent strainand PCMX (50mg/L) versus time

Time(hr)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12	BC
0	7.12	7.11	7.12	7.10	7.11	7.08	7.13
24	6.97	7.15	6.99	6.95	7.17	6.90	7.18
48	6.94	7.11	7.04	6.90	7.20	6.84	7.21
72	6.95	7.13	6.87	6.81	7.19	6.87	7.15
96	6.74	7.12	6.85	6.82	7.18	7.01	7.01
120	6.83	7.12	6.95	6.99	7.15	7.02	6.94
144	7.03	7.08	7.07	7.09	7.09	7.05	7.08
168	7.08	7.08	7.08	7.11	7.06	7.11	7.11
192	7.11	7.03	7.01	7.14	7.04	6.50	7.15
216	7.14	7.08	7.15	7.19	7.10	6.98	7.12
264	7.15	7.12	7.15	7.20	7.12	7.12	7.12

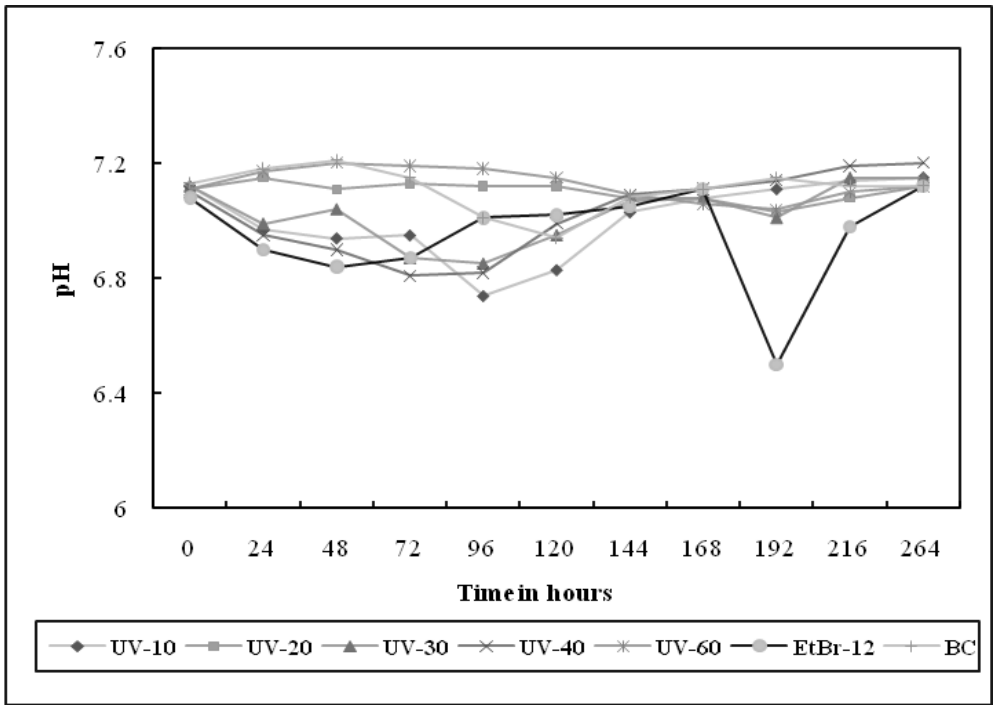


Fig.4.64: pH of minimal media with PCMX (50mg/L) and by mutated *Bacillus cereus* and parent strainversus time

4.11.2 Degradation studies of PCMX (100 mg/L) by mutated *Bacillus cereus*

The average concentration and growth (OD) of the duplicate analysis of PCMX by mutated and parent strain *Bacillus cereus* versus time is presented in Table 4.55 and Figure 4.65. Growth (OD) of *Bacillus cereus* in PCMX versus time is presented in Table 4.56 and Figure 4.66. pH of media with PCMX versus time is presented in Table 4.57 and Figure 4.67. The percentage degradation of PCMX (100mg/L) by mutated *Bacillus cereus* was UV-10(3.19%), UV-20(3.32%), UV-30(3.4%), UV-40(3.43%), UV-60(2.58%), EtBr-12(2.53%) and parent type *Bacillus cereus* BC (8.09%). The maximum percentage degradation was observed by BC (parent type) (8.09%) and maximum growth was observed in case of EtBr-12.It is evident that there is no change in pH of the medium.

Table 4.55: Concentration of PCMX (100mg/L) versus time by mutated *Bacillus cereus* and parent strain

Time (hr.)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12	BC
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00
24	99.81	99.93	99.11	99.96	99.04	99.12	99.25
48	99.86	99.65	98.80	99.85	99.70	99.95	98.57
72	99.72	99.15	98.58	98.71	99.68	98.82	98.65
96	99.75	98.81	98.36	98.61	98.84	98.92	98.31
120	98.54	98.79	98.08	97.87	98.51	98.79	96.63
144	97.65	98.30	98.27	97.54	98.28	98.49	94.74
168	97.34	97.92	97.64	97.17	97.98	97.89	93.76
192	97.18	97.83	97.42	96.78	97.54	97.73	92.87
216	96.98	96.74	97.68	96.65	97.34	97.88	92.03
264	96.81	96.68	96.60	96.57	97.42	97.57	91.91
%	**3.19**	**3.32**	**3.4**	**3.43**	**2.58**	**2.53**	**8.09**

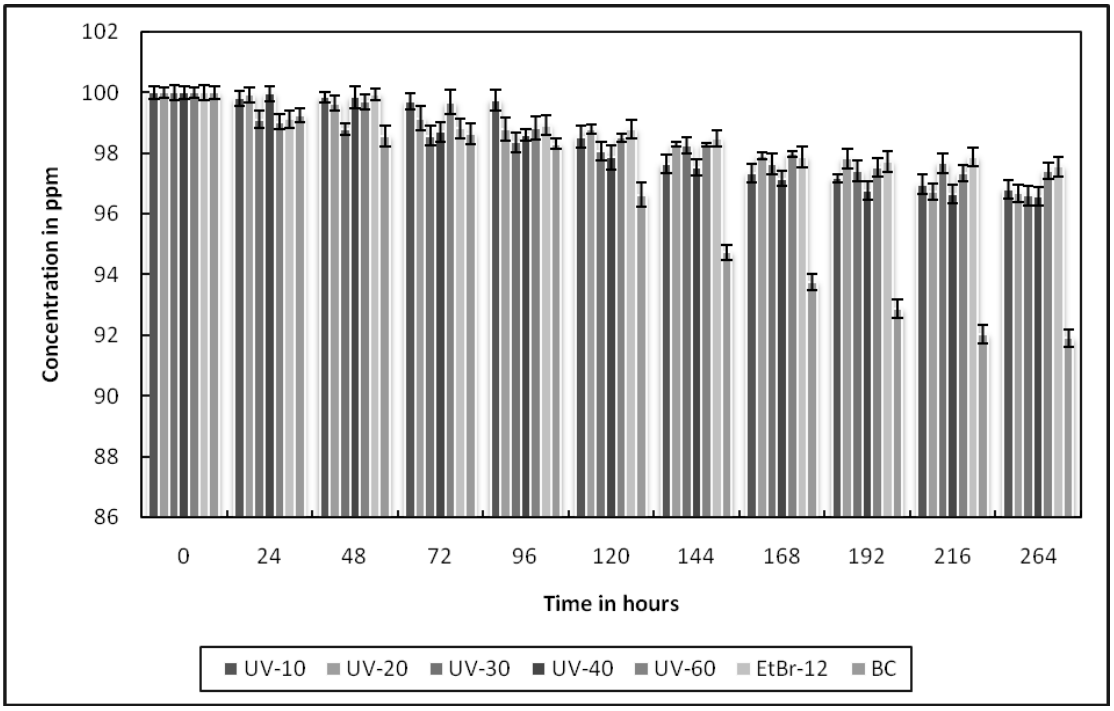


Fig.4.65: Concentration of PCMX (100mg/L) versus time by mutated *Bacillus cereus* and parent strain

Table 4.56: Growth (OD) by mutated *Bacillus cereus* and parent strain with PCMX (100mg/L) versus time

Time (hr.)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12	BC
0	0.002	0.004	0.005	0.005	0.005	0.004	0.006
24	0.174	0.167	0.159	0.185	0.199	0.237	0.087
48	0.207	0.234	0.178	0.28	0.277	0.278	0.108
72	0.213	0.257	0.223	0.31	0.326	0.336	0.178
96	0.254	0.27	0.264	0.333	0.34	0.355	0.22
120	0.263	0.293	0.278	0.242	0.309	0.328	0.373
144	0.29	0.302	0.28	0.267	0.322	0.325	0.426
168	0.303	0.334	0.263	0.254	0.292	0.292	0.467
192	0.276	0.298	0.256	0.25	0.279	0.285	0.372
216	0.267	0.276	0.246	0.252	0.275	0.293	0.349
264	0.215	0.205	0.208	0.268	0.285	0.335	0.297

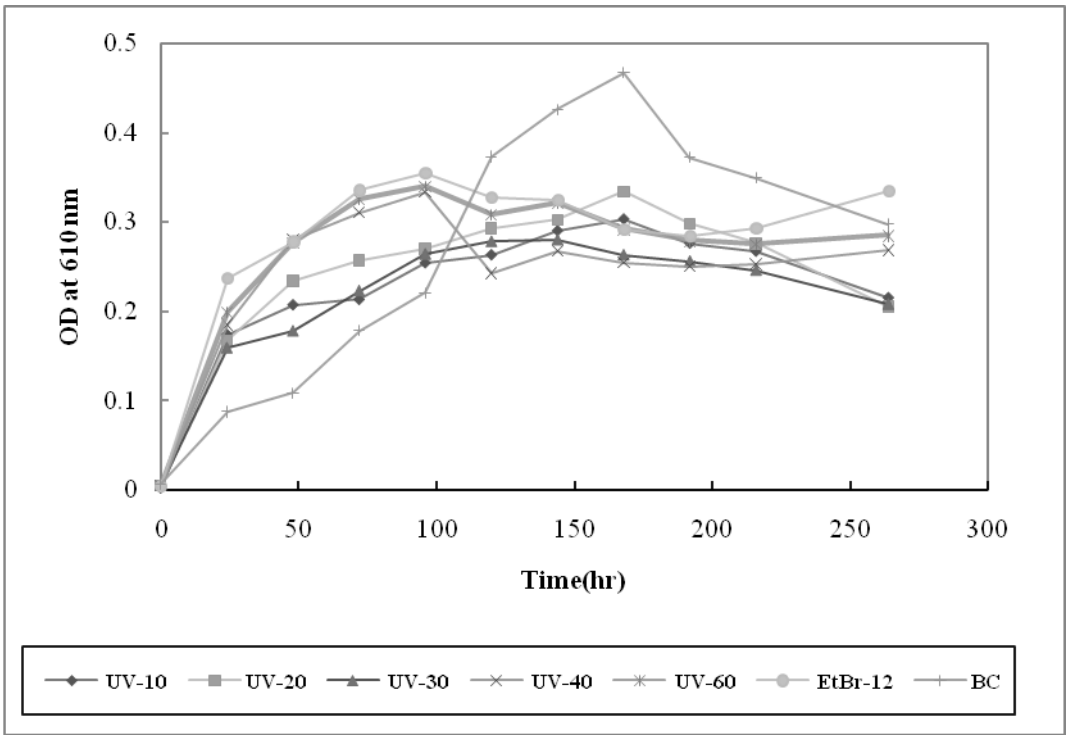


Fig. 4.66: Growth (OD) of *Bacillus cereus* with PCMX (100mg/L) versus time by mutated *Bacillus cereus* and parent strain

Table 4.57: pH of minimal media by mutated *Bacillus cereus* and parent strain and PCMX (100mg/L) versus time

Time(hr)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12	BC
0	7.02	7.12	7.09	7.11	7.11	7.10	7.11
24	6.87	7.09	7.07	6.87	6.89	6.81	7.16
48	6.94	7.11	7.03	6.83	6.81	6.79	7.19
72	6.96	7.08	6.97	6.77	6.81	6.81	7.15
96	6.85	7.13	6.95	6.71	6.73	6.80	7.09
120	6.73	7.16	6.85	6.72	6.78	6.79	7.04
144	6.83	7.06	7.06	6.65	6.75	6.73	6.98
168	7.06	7.08	7.05	6.58	6.63	6.59	7.01
192	7.21	7.05	7.04	6.27	6.45	6.38	7.16
216	7.14	7.09	7.12	6.29	6.54	6.38	7.13
264	7.18	7.11	7.16	6.50	6.95	6.86	7.11

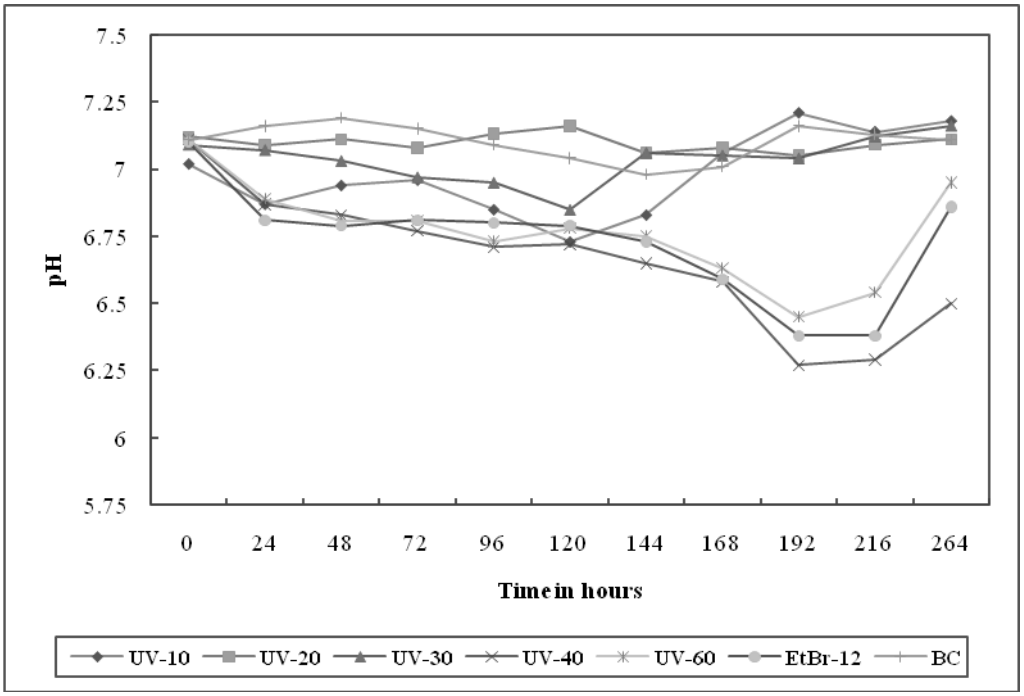


Fig.4.67: pH of minimal media with PCMX (100mg/L) versus time by mutated *Bacillus cereus* and parent strain

4.11.3 Degradation studies of PCMX (150mg/L) by mutated *Bacillus cereus*

The duplicate analysis of higher concentration of 150 mg/L of PCMX versus time is presented in Table 4.58 and Figure 4.68. Growth (OD) of *Bacillus cereus* in PCMX versus time is presented in Table 4.59 and Figure 4.69. pH of media with PCMX versus time is presented in Table 4.60 and Figure 4.70. From the Tables and Figures it is evident that the percentage degradation of PCMX (150mg/L) by mutated *Bacillus cereus* was UV-10(2.08%), UV-20(1.88%), UV-30(1.56%), UV-40(1.66%), UV-60(1.52%), EtBr-12(1.64%) and parent type *Bacillus cereus* BC (2.67%).The maximum percentage degradation was observed by BC (parent type) (2.67%)and maximum growth was observed in case of UV-60.It is evident that there is no change in pH of the medium.

Table 4.58: Concentration of PCMX (150mg/L) versus time by mutated *Bacillus cereus* and parent strain

Time (hr.)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12	BC
0	150.00	150.00	150.00	150.00	150.00	150.00	150
24	149.91	149.93	149.91	149.87	149.84	149.92	149.89
48	149.88	149.95	149.68	149.98	149.90	149.95	147.76
72	149.52	149.35	149.78	149.51	149.78	148.66	146.48
96	149.35	149.21	149.69	149.62	149.84	148.79	147.62
120	148.75	148.67	149.80	148.90	148.85	148.89	147.94
144	146.54	148.50	148.72	148.63	148.68	149.69	148.97
168	146.66	147.72	148.94	148.67	148.58	148.69	146.78
192	146.78	147.73	148.12	148.58	148.45	148.33	146.48
216	146.81	147.54	147.98	147.61	147.98	147.87	146.54
264	146.87	147.18	147.66	147.50	147.72	147.53	145.99
%	**2.08**	**1.88**	**1.56**	**1.66**	**1.52**	**1.64**	**2.67**

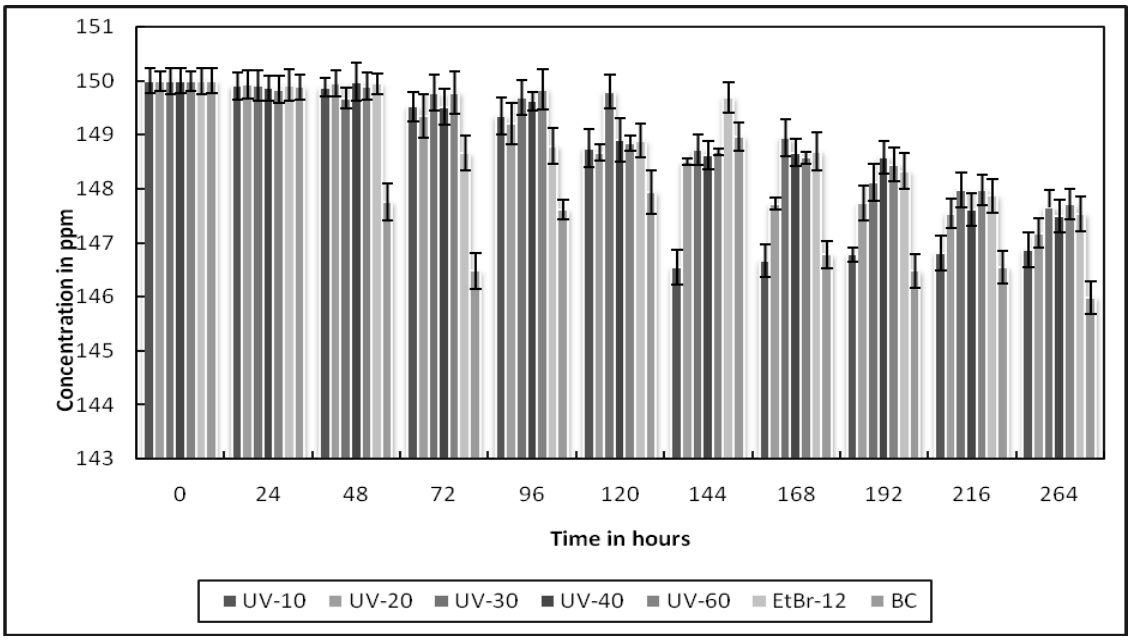


Fig.4.68: Concentration of PCMX (150mg/L) versus time by mutated *Bacillus cereus* and parent strain

Table 4.59: Growth OD of mutated *Bacillus cereus* and parent strain with PCMX (150mg/L) versus time

Time (hr.)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12	BC
0	0.007	0.011	0.006	0.004	0.004	0.003	0.003
24	0.198	0.188	0.153	0.184	0.27	0.204	0.084
48	0.208	0.245	0.178	0.239	0.311	0.249	0.176
72	0.279	0.262	0.274	0.283	0.367	0.29	0.182
96	0.298	0.283	0.283	0.308	0.391	0.287	0.271
120	0.309	0.299	0.265	0.281	0.35	0.268	0.298
144	0.343	0.309	0.278	0.244	0.387	0.278	0.378
168	0.307	0.301	0.251	0.231	0.357	0.253	0.417
192	0.298	0.318	0.204	0.227	0.332	0.254	0.392
216	0.269	0.298	0.238	0.231	0.34	0.267	0.299
264	0.226	0.275	0.203	0.233	0.364	0.282	0.263

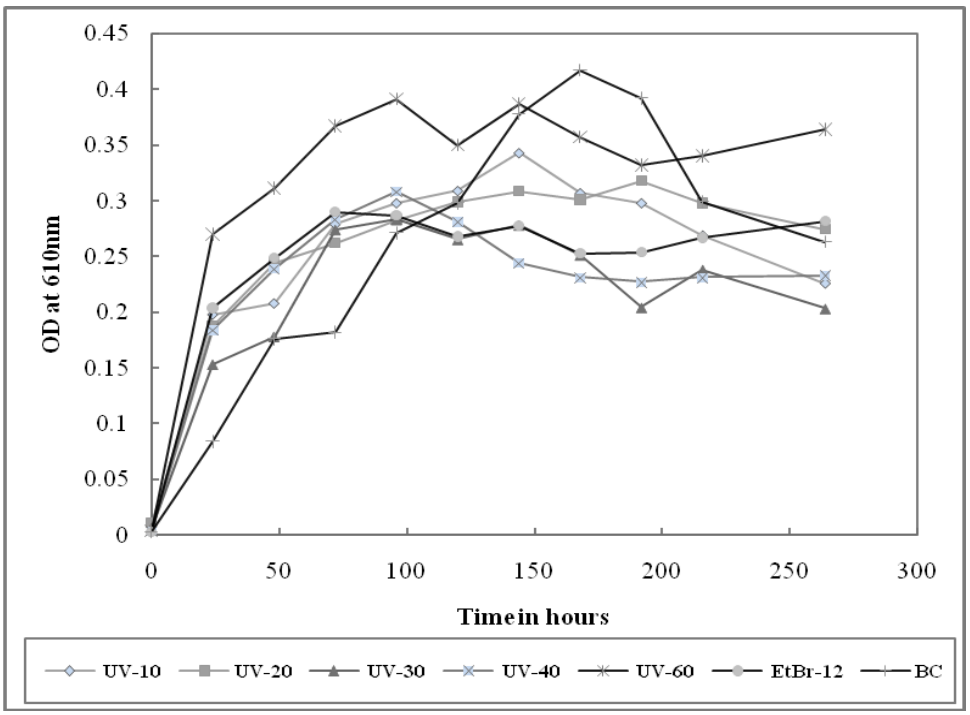


Fig. 4.69: Growth OD by mutated *Bacillus cereus* and parent strain with PCMX (150mg/L) versus time

Table 4.60: pH of minimal media with by mutated *Bacillus cereus* and parent strain and PCMX versus time

Time(hr.)	UV-10	UV-20	UV-30	UV-40	UV-60	EtBr-12	BC
0	7.08	7.06	7.1	7.08	7.12	7.09	7.13
24	7.07	7.13	7.09	6.89	6.83	6.85	7.15
48	7.01	7.12	7.05	6.83	6.66	6.82	7.2
72	6.96	7.09	6.98	6.52	6.76	6.79	7.18
96	6.84	7.11	6.99	6.69	6.79	6.78	7.15
120	6.71	6.98	6.94	6.62	6.76	6.67	6.98
144	6.93	6.87	7.03	6.53	6.72	6.48	7.08
168	7.02	7.07	7.03	6.48	6.74	6.72	7.16
192	7.07	7.06	7.04	6.3	6.78	6.81	7.11
216	7.11	7.08	7.12	6.29	6.89	6.86	7.1
264	7.12	7.1	7.16	6.98	6.96	6.59	7.14

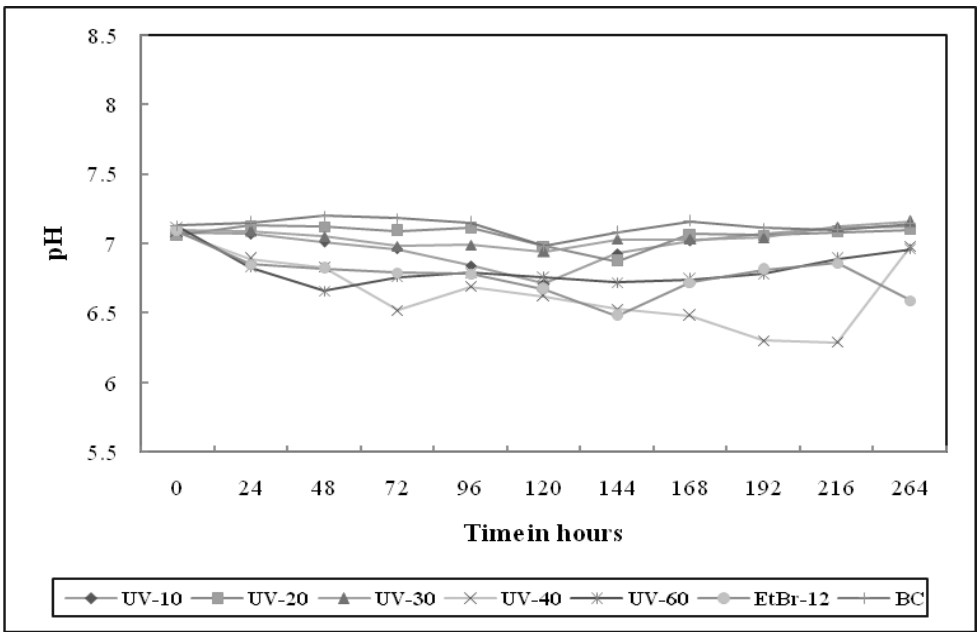


Fig.4.70: pH of minimal media with PCMX (150mg/L) and mutated *Bacillus cereus* and parent strain versus time

4.11.4. Percentage degradation by UV-30, EtBR-12 and BC at different concentration of PCMX

Results of percentage degradation by UV-30, EtBR-12 and BC at different concentration of PCMX are presented in Table 4.61 and Figure 4.71. From the results, it is found that as the concentration of PCMX was found to increase from 50 to 150 mg/L the percentage degradation of parent type (BC) increased when compared to UV mutated strain (UV- 10) and chemically mutated strain (EtBr-12).

Table 4.61: Percentage degradation by UV-10, EtBr-12 and BC at different concentration of PCMX at 264 hour.

Concentration of PCMX	50mg/L	100 mg/ L	150 mg/L
UV-30	21.88	3.4	1.56
EtBr-12	4.86	2.53	1.64
BC	29.14	8.09	2.67

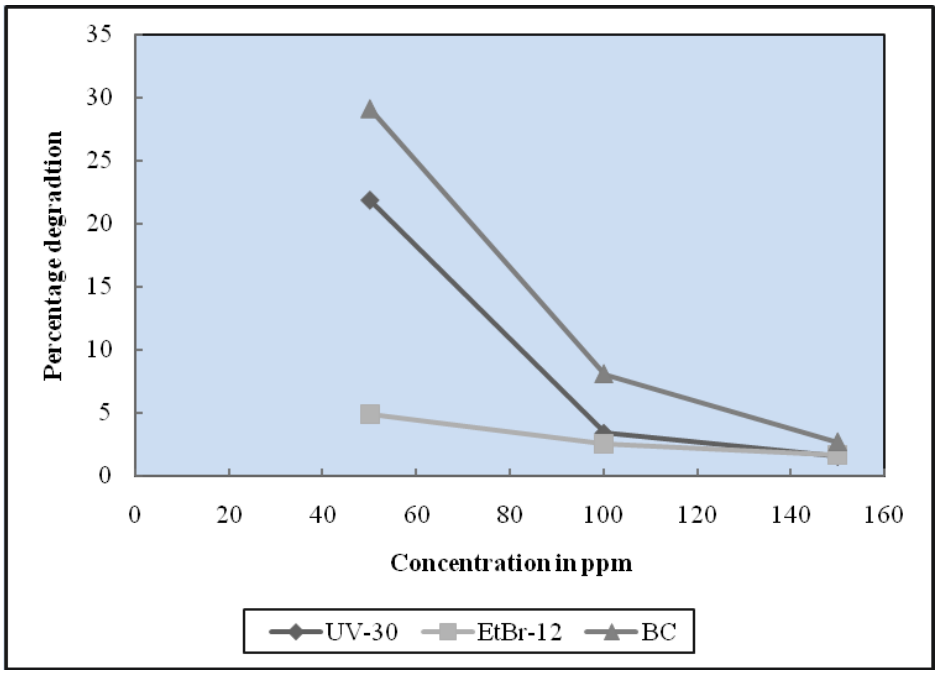


Fig. 4.71: Percentage degradation by UV-10, EtBR-12 and BC at different concentration of PCMX at 264 hour.

SUMMING UP

Degradation of PCMX by parent strain was higher than mutant strain. Degradation by parent strain BC was 2 fold higher than the mutant strain. The reason for this trend might be that the activity of enzyme, which is responsible for degradation of PCMX, might not be promoted by UV irradiation. Further, it was found that the growth of *Bacillus cereus* was more when compared to mutated strain. Hence, the degradation of PCMX is related to growth of the *Bacillus cereus.*Concluding that mutated strain did not yield significant enhancement of selected chemicals hence it was not proceeded for further experiments.

4.12. Statistical optimization studies

4.12.1 Optimization of inoculum size, glucose, phosphate and peptone using Response Surface Methodology to get maximum degradation of 2,4-DCP.

The Response Surface Methodology (RSM) is an effective sequential and stepwise procedure that could be applied for optimization of process parameters. The quantitative description of the process variables like inoculum size, glucose, phosphate and peptone effects on 2,4-DCP degradation was performed. The results of CCD experiments for studying the effects of four independent variables on degradation of 2, 4-DCP by *Bacillus cereus* were carried. By applying multiple regression analysis on the experimental data, the experimental results of the CCD design (Table 4.62 and 4.63) were fitted with the second order full polynomial equation. The empirical relationship for four factors and degradation of 2,4 DCP in coded units obtained by the application of RSM is given by equation 1.

Y= -447.43 +39.28x X1 +59.94x X2 +1978.93x X3+22.53x X4 -1.55x X1 x X1-63.98xX2 xX2 -3341.42x X3xX3 -0.48xX4 xX4+2.44xX2 xX2 -25.68x X1xX3 -0.38xX1xX4+8.70xX2xX3+2.20xX2xX4-45.94xX3xX4 ---------- (1)

Table 4.62: The CCD design and experiment data used for optimizing the % degradation of 2,4-DCP

Run Order	Glucose X1	Peptone X2	Inoculum X3	Phosphate X4	Degradation of 2, 4-DCP (%)	Predicted Values
1	7.5	0.75	0.15	6.75	42.05	52.0129
2	12.5	0.75	0.15	6.75	50.29	70.3113
3	7.5	1.25	0.15	6.75	21.46	35.2429
4	12.5	1.25	0.15	6.75	60.59	59.6463
5	7.5	0.75	0.25	6.75	71.63	66.6296
6	12.5	0.75	0.25	6.75	51.42	72.0879
7	7.5	1.25	0.25	6.75	26.55	50.2946
8	12.5	1.25	0.25	6.75	52.33	61.8579
9	7.5	0.75	0.15	11.25	72.08	78.1729
10	12.5	0.75	0.15	11.25	99.62	88.0263
11	7.5	1.25	0.15	11.25	74.87	66.3529
12	12.5	1.25	0.15	11.25	61.69	82.3113
13	7.5	0.75	0.25	11.25	59.02	72.1146
14	12.5	0.75	0.25	11.25	67.29	69.1279
15	7.5	1.25	0.25	11.25	65.13	60.7296
16	12.5	1.25	0.25	11.25	61.66	63.8479
17	5	1	0.2	9	51	40.5058
18	15	1	0.2	9	79.2	61.9225
19	10	0.5	0.2	9	98.73	85.0742
20	10	1.5	0.2	9	77.14	63.0242
21	10	1	0.1	9	69.38	58.5525
22	10	1	0.3	9	71.65	54.7058
23	10	1	0.2	4.5	98.22	66.2242
24	10	1	0.2	13.5	90.15	94.3742
25	10	1	0.2	9	85.64	90.0433
26	10	1	0.2	9	88.61	90.0433
27	10	1	0.2	9	87.42	90.0433
28	10	1	0.2	9	92.38	90.0433
29	10	1	0.2	9	91.32	90.0433
30	10	1	0.2	9	94.89	90.0433

Table 4.63: Coefficient of the model.

Term	Coefficient Value	Standard Error	t-value	p-value
Constant	-447.43	220.46	-2.030	0.061
Glucose	39.28	16.87	2.328	0.034
Peptone	59.94	168.72	0.355	0.727
Inoculum	1978.93	843.61	2.346	0.033
Phosphate	22.53	18.75	1.202	0.248
Glucose*Glucose	-1.55	0.56	-2.794	0.014
Peptone*Peptone	-63.98	55.58	-1.151	0.268
Inoculum*Inoculum	-3341.42	1389.58	-2.405	0.030
Phosphate*Phosphate	-0.48	0.69	-0.701	0.494
Glucose*Peptone	2.44	7.28	0.336	0.742
Glucose*Inoculum	-25.68	36.39	-0.706	0.491
Glucose*Phosphate	-0.38	0.81	-0.464	0.649
Peptone*Inoculum	8.70	363.88	0.024	0.981
Peptone*Phosphate	2.20	8.09	0.272	0.789
Inoculum*Phosphate	-45.94	40.43	-1.136	0.274

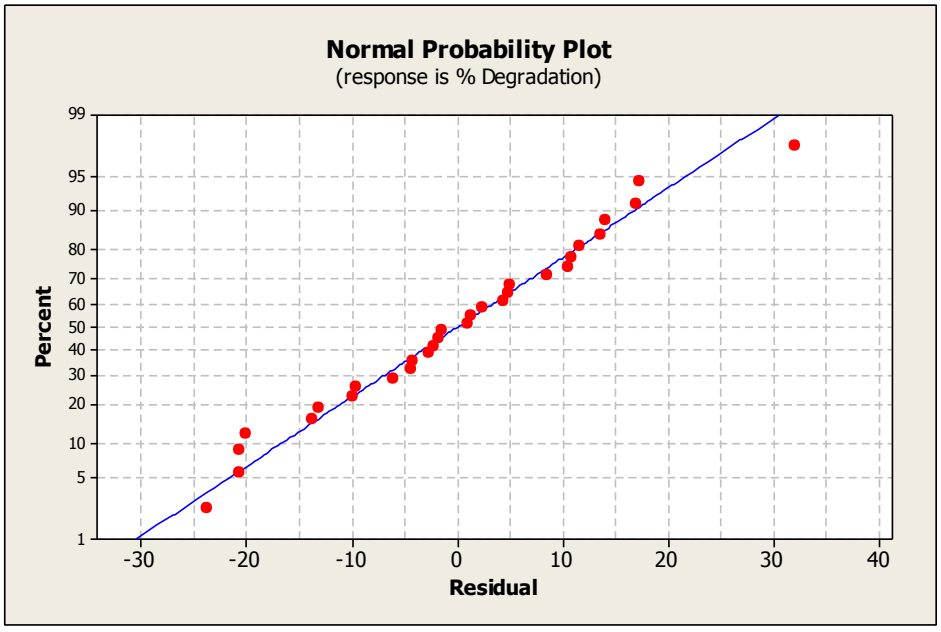


Fig. 4.72(a): 2, 4 – Dichlorophenol Normal Probability Plot

The degradation of 2,4-DCP due to different concentrations of variables can be predicted from the contour and surface plots as shown in Figures 4.72 to 4.77 .The response surfaces and contour plot shown in Figures were based on the regression model holding other variable constant at their zero level, while varying the other two within their experimental range.These plots demonstrate that the degradation of 2,4-DCP is dependent on linear, interactive effects of glucose, peptone, phosphate and inoculum size.

From Figure 4.72, it can be seen that 2,4-DCP degradation is highest when Peptone and Phosphate concentration in medium is in the range of 0.75 – 1.0 g/L and 11 – 13 g/L respectively.

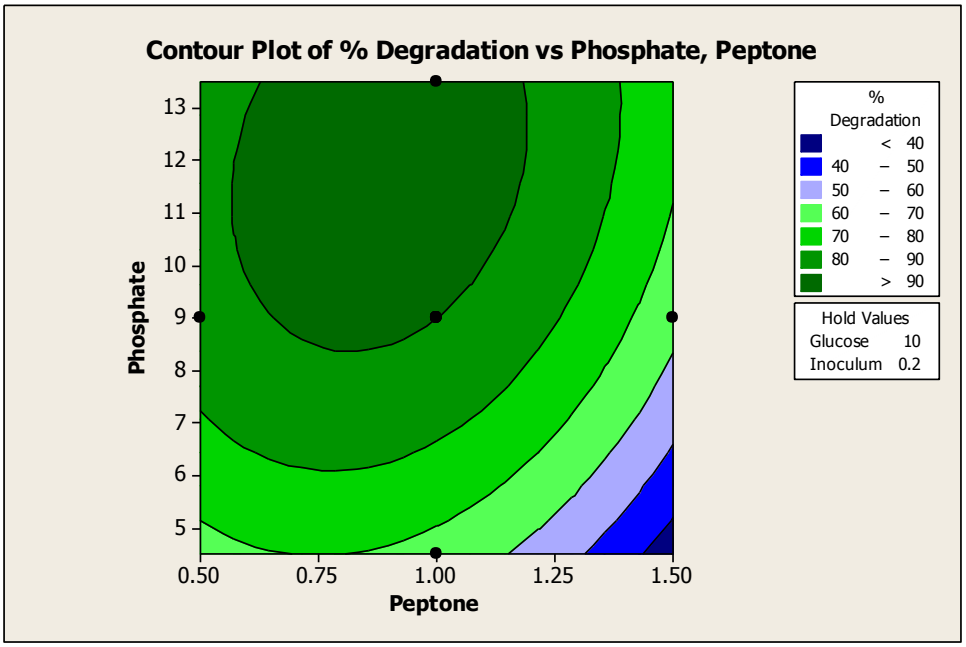
From Figure 4.73, it can be seen that 2,4-DCP degradation is highest when Peptone and Glucose concentration in medium is in the range of 0.75 – 1.0 g/L and 9-11 g/L respectively.

From Figure 4.74, it can be seen that 2,4-DCP degradation is highest when Inoculum and Peptone concentration in medium is in the range of 0.2 O.D and 0.8 – 0.9 g/L respectively.

From Figure 4.75, it can be seen that 2, 4-DCP degradation is highest when Phosphate and Inoculum concentration in medium is in the range of 11 - 13 g/L and 0.18 – 0.2O.D respectively.

From Figure 4.76, it can be seen that 2, 4-DCP degradation is highest when Inoculum and Glucose concentration in medium is in the range of 0.2 O.D and 10 – 11 g/L respectively.

From Figure 4.77, it can be seen that 2, 4-DCP degradation is highest when Phosphate and Glucose concentration in medium is in the range of 11 - 13 g/L and 10 g/L respectively.



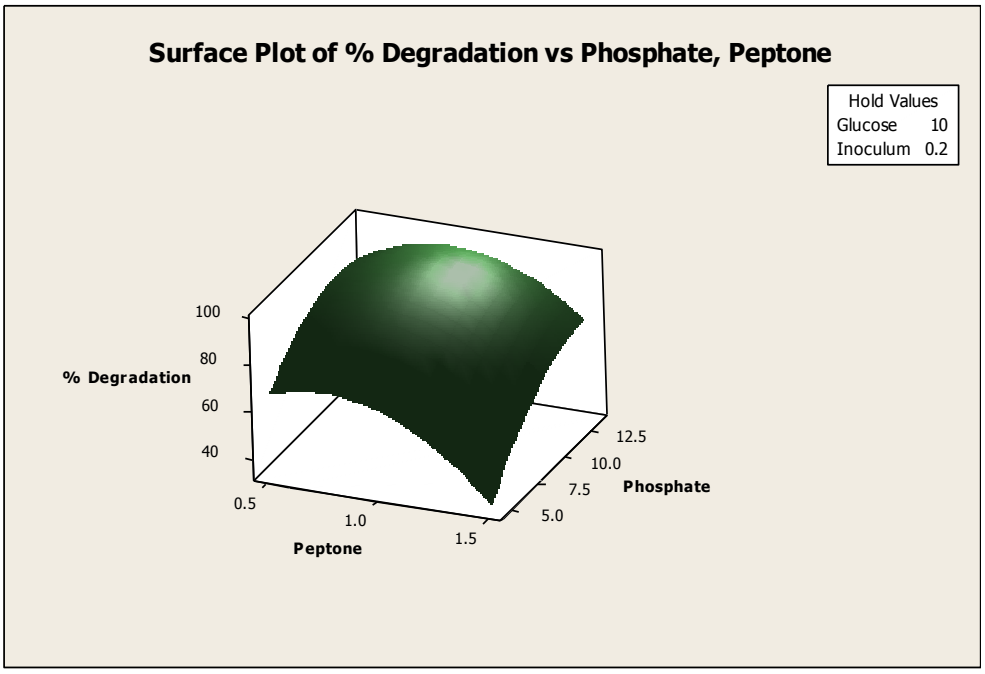


Fig.4.72: Contour and Surface plots for 2, 4-D degradation v/s Phosphate, Peptone;Glucose=10 g/l, Inoculum=0.2 O.D.

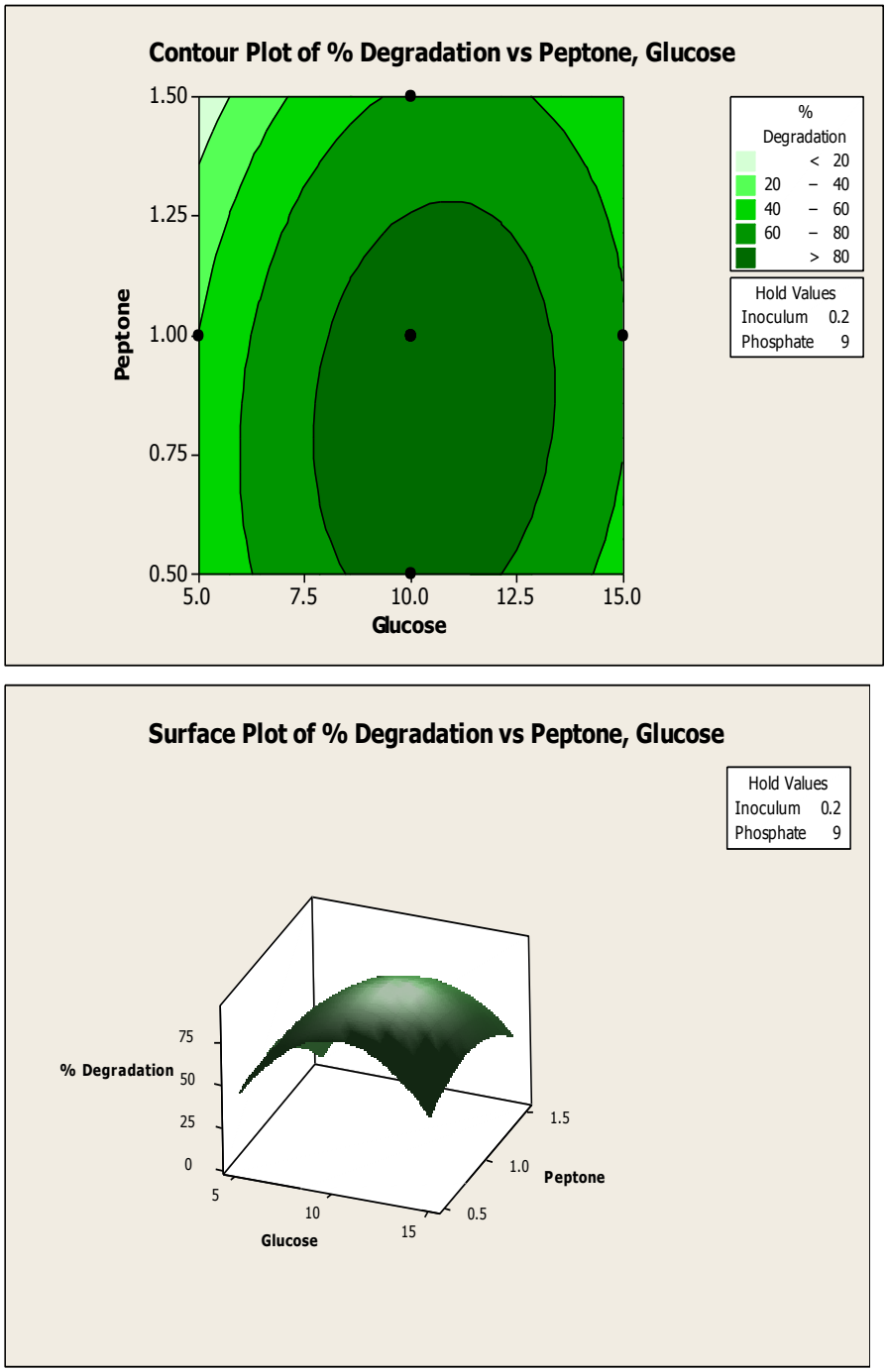


Fig. 4.73: Contour and Surface plots for 2, 4-D degradation v/s Peptone, Glucose; Phosphate=9.0 g/l, Inoculum=0.2 O.D.

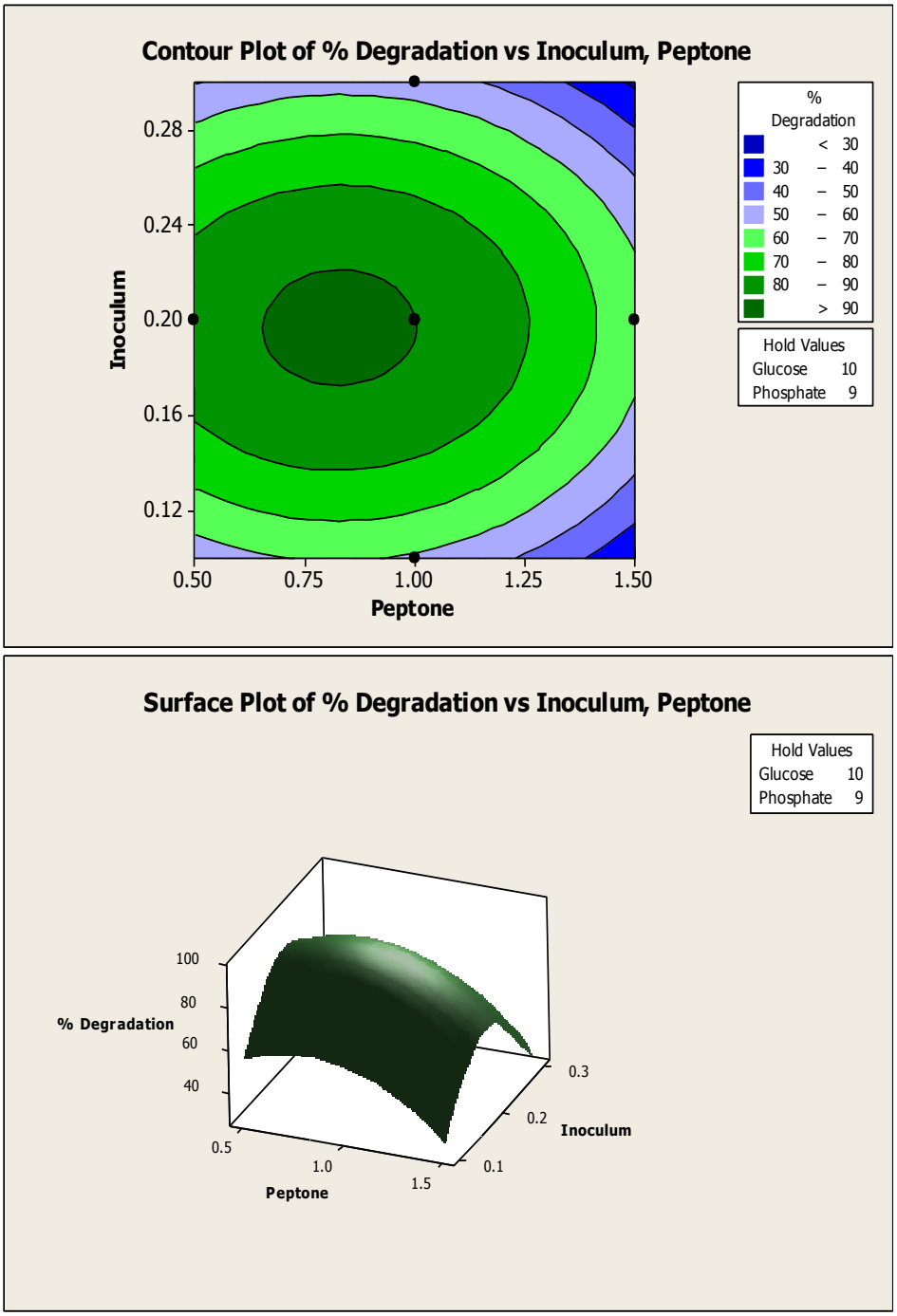
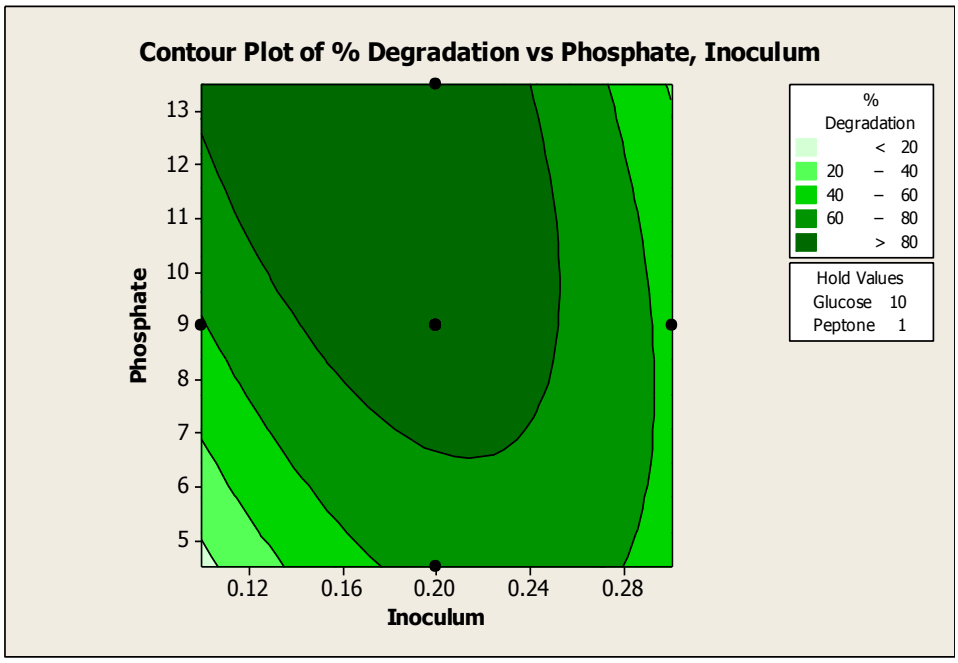


Fig. 4.74: Contour and Surface plots for 2, 4-DCP degradation v/s Inoculum, Peptone; Phosphate=9.0 g/L, Glucose=10.0 g/L.



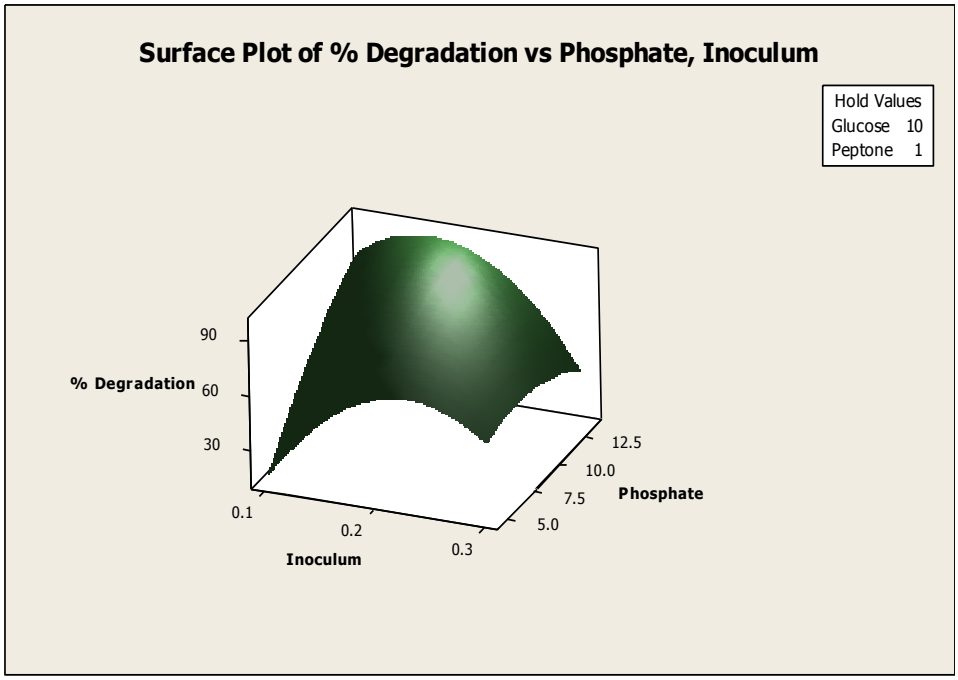
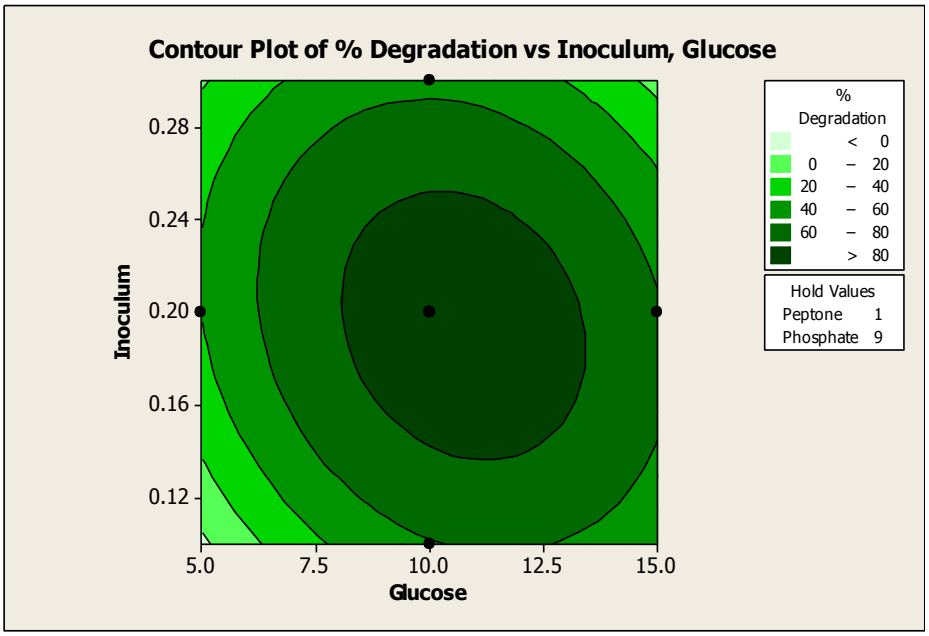


Fig.4.75: Contour and Surface plots for 2, 4-D degradation v/s Phosphate, Inoculum; Peptone=1.0 g/L, Glucose=10.0 g/L.



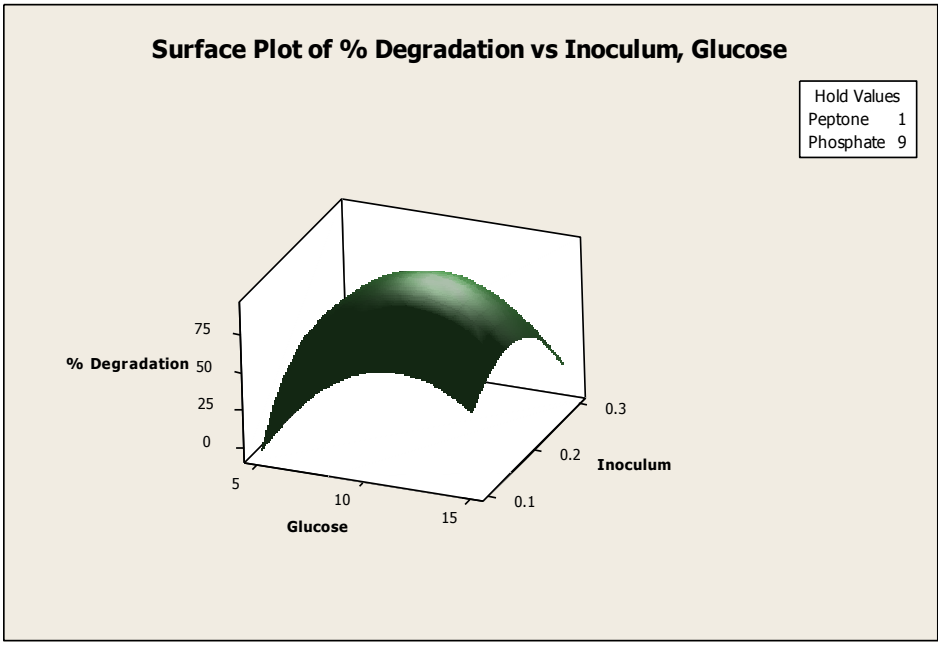
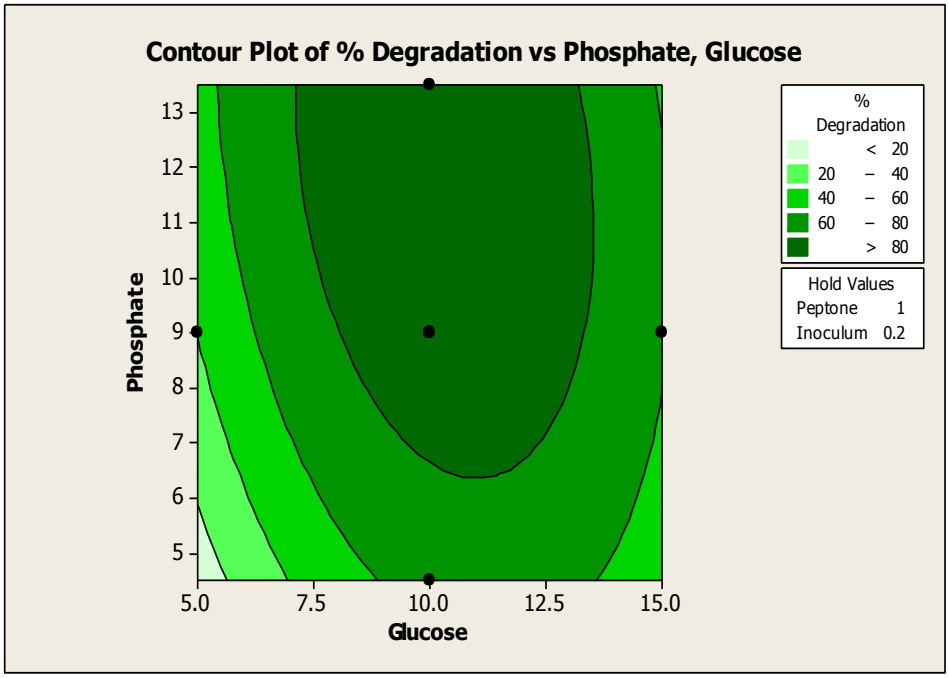


Fig. 4.76: Contour and Surface plots for 2, 4-D degradation v/s Inoculum, Glucose; Phosphate=9.0 g/L, Peptone=1.0 g/L.



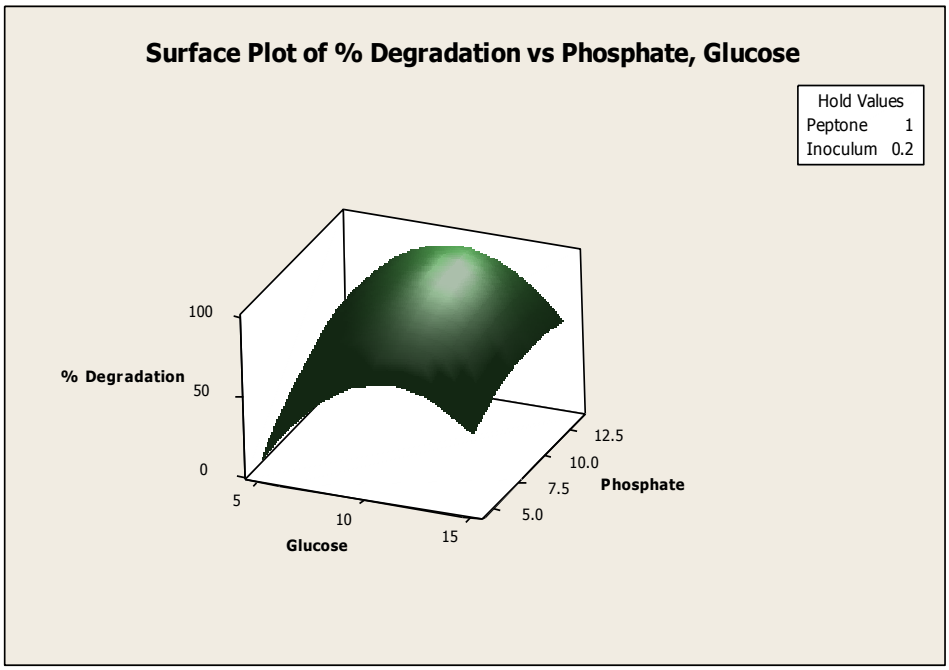


Fig. 4.77: Contour and Surface plots for 2, 4-D degradation v/s Phosphate, Glucose; Peptone=1.0 g/L Inoculum=0.2 O.D.

SUMMING UP

The present study shows the potential of the isolated indigenous *Bacillus cereus* for 2,4-DCP degradation. The performance of the indigenous strain in biodegradation of 2,4-DCP in the minimal medium is excellent. The response surface methodology using 30 full-factorial composite design was adopted to optimize the process variables like inoculum size, glucose, phosphate and peptone for the microbial degradation of 2,4-DCP.In order to determine the optimum levels of each variable for maximum percentage degradation, three-dimensional response surface plots were constructed by plotting the response (percentage degradation) on the Z-axis against any two independent variables, while maintaining other variables at their optimum levels. The response at the centre point corresponds to a maximum degree of achievable degradation process for that factor. An increase in percentage degradation was observed when the concentrations of inoculum and phosphate were increased till a certain level 11.25g/L of phosphate and 0.2 OD inoculum size; percentage degradation decreased after a certain concentration of 0.25 OD inoculum and 12g/L of Phosphate. A similar convex shape trend was observed for variation in concentrations of inoculum and glucose; for variation in concentrations of inoculum and peptone, for variation in concentrations of phosphate and glucose, for variation in concentrations of peptone and phosphate and for variation in concentrations of glucose and peptone, suggesting that these were well-defined process conditions for degradation process. The shapes of contour plots indicate the nature and extent of the interactions. Negligible interaction is shown by circular nature of contour plots. Furthermore, the studies of the contour plot also reveal that optimal values of variables lie within the range: phosphate 11.25g/L, inoculum 0.15 OD, peptone at 0.75g/L and glucose 12.5 g/L. The contour plots show a rather broad plateau region in which the percentage degradation changes relatively little when the nutrient concentrations were varied. This indicated that the optimal solution can accommodate small errors or variability in the experimental factors. These plots are helpful in studying the effects of the factors variation in the studied field and consequently, in determining the optimal experimental conditions.

The highest 2, 4-DCP degradation that can be achieved according to the model prediction under the optimal experimental conditions is 94.37%. The experimental results indicated a maximum degradation of about 99.62% under the optimal process conditions. This confirms the closeness of the model to the experimental results.

4.12.2. Optimization of inoculum size, glucose, phosphate, and peptone using response surface methodology maximum degradation of PCMX

The results of CCD experiments for studying the effects of four independent variables on degradation of PCMX by *Bacillus cereus* were carried.The coefficient of determination (R^2) was calculated as 38.27% for the degradation of PCMX. By applying multiple regression analysis on the experimental data, the experimental results of the CCD design (Table 4.64 and 4.65) were fitted with the second order full polynomial equation. The empirical relationship of four factors and degradation of PCMX in coded units obtained by the application of RSM is given by equation 2.

PCMX % degradation = 42.0408+1.4279*Glucose+3.8388*Peptone+1.8938*Inoculum-1.9746*Phosphate+1.4078*Glucose *Glucose-2.8034 *Peptone *Peptone 2.8034*Inoculum*Inoculum-3.5909*Phosphate*Phosphate-1.4881*Glucose*Peptone+2.5706*Glucose*Inoculum+5.4494*Glucose*Phosphate+0.1131*Peptone*Inoculum+4.1094*Peptone*Phosphate+1.1906*Inoculum*Phosphate ---------- (2)

Table 4.64: The CCD design and experiment data used for optimizing the % degradation of PCMX

Glucose	Peptone	Inoculum	Phosphate	% Degradation	Predicted value
10	1	0.3	9	37.01	35.28
10	0.5	0.2	9	25.27	23.14
5	1	0.2	9	44.88	44.81
10	1.5	0.2	9	40.39	38.49
10	1	0.2	4.5	23.33	31.62
10	1	0.2	13.5	36.03	23.72
10	1	0.2	9	33.96	42.03
10	1	0.1	9	30	27.70
10	1	0.2	9	34.42	42.03
15	1	0.2	9	54.47	50.52
12.5	0.75	0.15	11.25	24.39	27.31
7.5	0.75	0.25	11.25	7.2	16.52
7.5	1.25	0.25	6.75	30.97	39.87
12.5	1.25	0.25	11.25	22.89	51.54
7.5	1.25	0.15	6.75	44.08	43.38
10	1	0.2	9	36.33	42.03
10	1	0.2	9	58.4	42.03
12.5	0.75	0.15	6.75	21.91	30.96
12.5	0.75	0.25	11.25	45.48	38.39
12.5	0.75	0.25	6.75	36.08	37.28
7.5	0.75	0.25	6.75	36.78	37.21
12.5	1.25	0.15	6.75	24.72	27.22
7.5	0.75	0.15	6.75	58	41.17
7.5	1.25	0.25	11.25	52.46	35.62
7.5	0.75	0.15	11.25	6.43	15.72
7.5	1.25	0.15	11.25	23.75	34.37
12.5	1.25	0.15	11.25	48.22	40.00
10	1	0.2	9	63.09	42.03
10	1	0.2	9	25.98	42.03
12.5	1.25	0.25	6.75	51.07	33.99

Table 4.65: Coefficient of the model.

Term	Coefficient Value	Standard Error	t-value	p-value
Constant	90.47	188.71	0.479	0.639
Glucose	-14.38	14.44	-0.996	0.335
Peptone	61.31	144.42	0.425	0.677
Inoculum	149.47	722.11	0.207	0.839
Phosphate	-64.98	144.42	-0.45	0.659
Glucose*Glucose	0.23	0.48	0.473	0.643
Peptone*Peptone	-44.85	47.58	-0.943	0.361
Inoculum*Inoculum	-1053.88	1189.43	-0.886	0.39
Phosphate*Phosphate	-57.45	47.58	-1.208	0.246
Glucose*Peptone	-2.38	6.23	-0.382	0.708
Glucose*Inoculum	20.57	31.15	0.66	0.519
Glucose*Phosphate	8.72	6.23	1.4	0.182
Peptone*Inoculum	9.05	311.47	0.029	0.977
Peptone*Phosphate	65.75	62.29	1.055	0.308
Inoculum*Phosphate	95.25	311.47	0.306	0.764

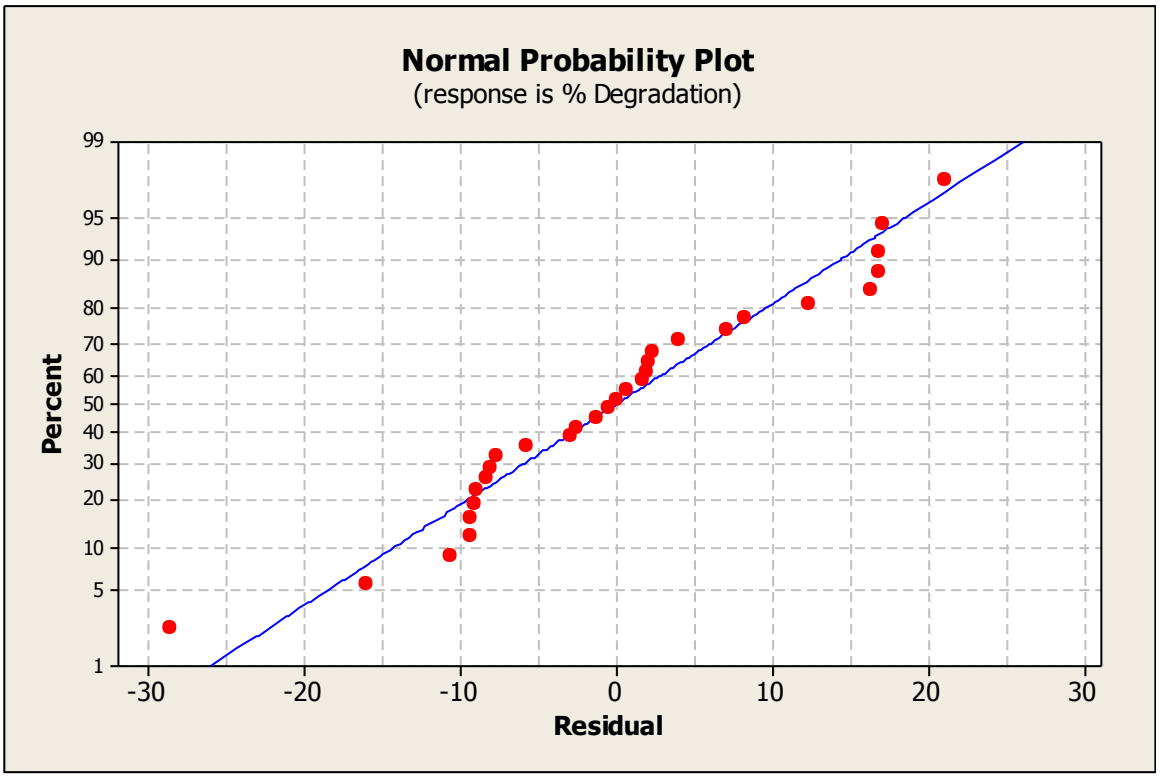


Fig. 4.78(a) : PCMX Normal Probability Plot

The degradation of PCMX due to different concentrations of variables can be predicted from the contour and surface plots as shown in Figures 4.78 to 4.83. The response surfaces and contour plot shown in Figures were based on the regression model holding other variable constant at their zero level, while varying the other two within their experimental range.These plots demonstrate that the degradation of PCMX is dependent on linear, interactive effects of glucose, peptone, phosphate and inoculum size.

From Figure 4.78, it can be seen that PCMX degradation is highest when Peptone and Inoculum concentration in medium is in the range of 1.0 – 1.25 g/L and 0.2-0.25 OD respectively.

From Figure 4.79, it can be seen that PCMX degradation is highest when Phosphate and Glucose concentration in medium is in the range of 8.0-9.0 g/L and 10.0 – 12.5 g/L respectively.

From Figure 4.80, it can be seen that PCMX degradation is highest when Phosphateand Inoculum concentration in medium is in the range of 7.5-9.0g/L and 0.2 – 0.25 OD respectively.

From Figure 4.81, it can be seen that PCMX degradation is highest when Peptone and Phosphate concentration in medium is in the range of 1.0 – 1.25 g/L and 9-10 g/L respectively.

From Figure 4.82, it can be seen that PCMX degradation is highest when Peptone and Glucose concentration in medium is at 1.0g/L and 10.0 g/L respectively.

From Figure 4.83, it can be seen that PCMX degradation is highest when Inoculum and Glucose concentration in medium is at 0.2O.D and 10.0 g/L respectively.

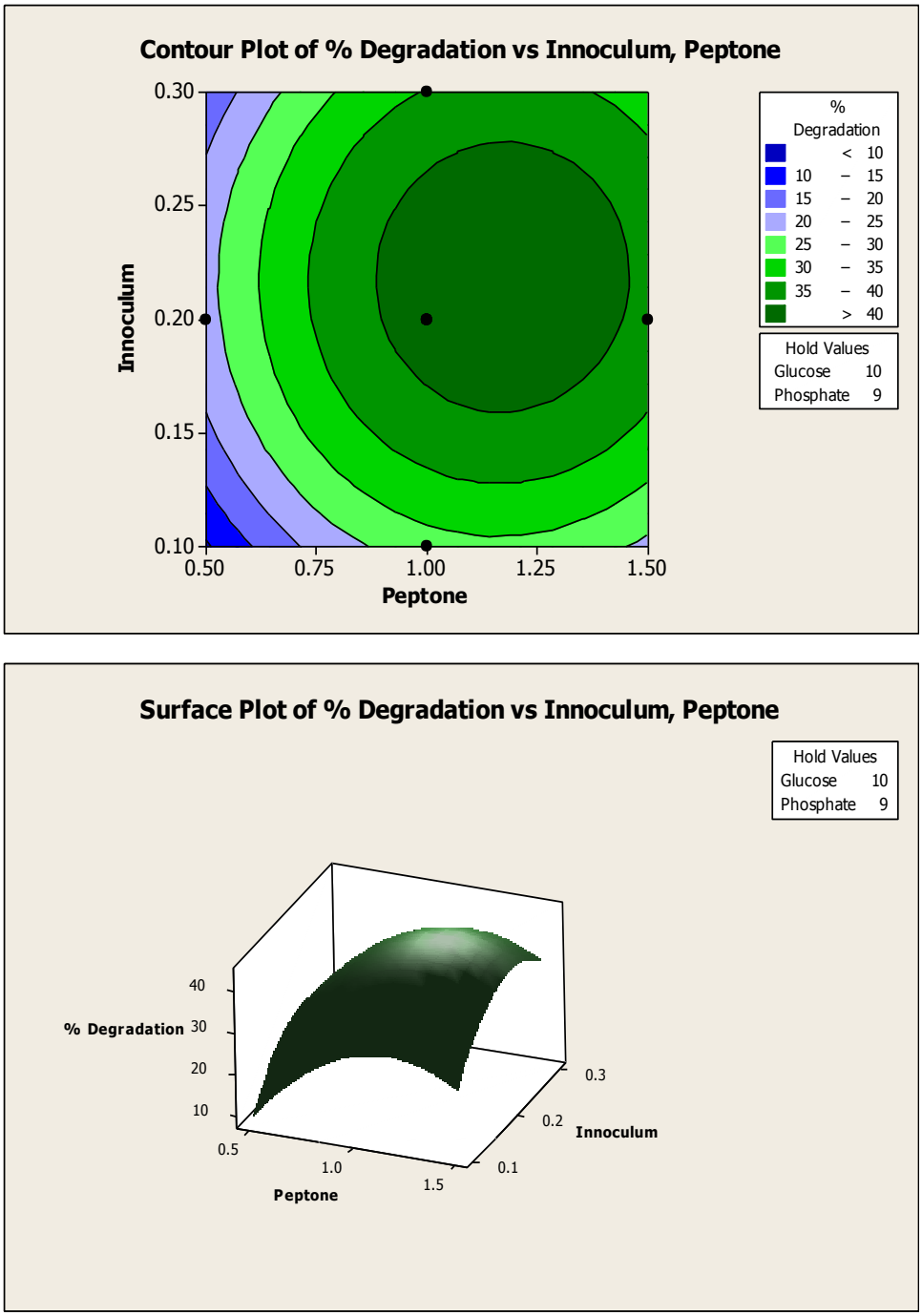
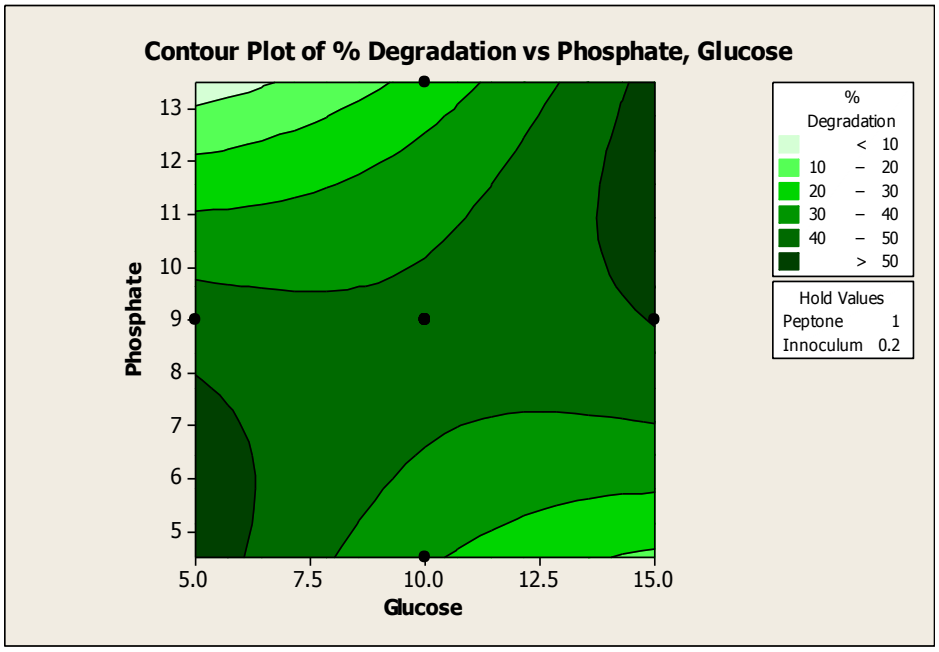


Fig.4.78: Contour and Surface plots for PCMX degradation v/s Inoculum, Peptone;Phosphate=9.0 g/l,Glucose=10 g/l.



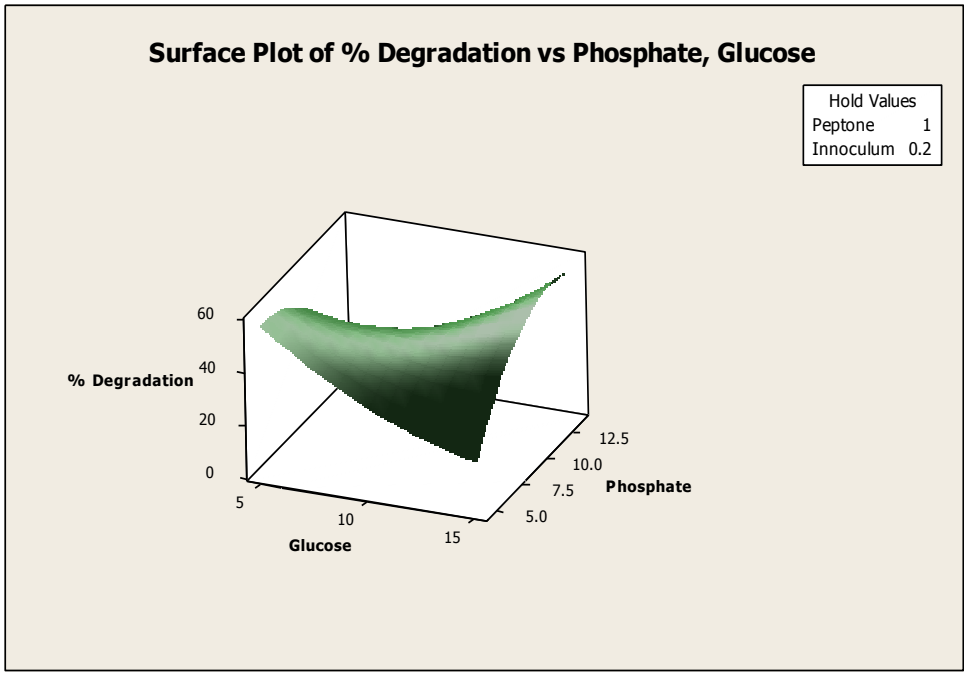
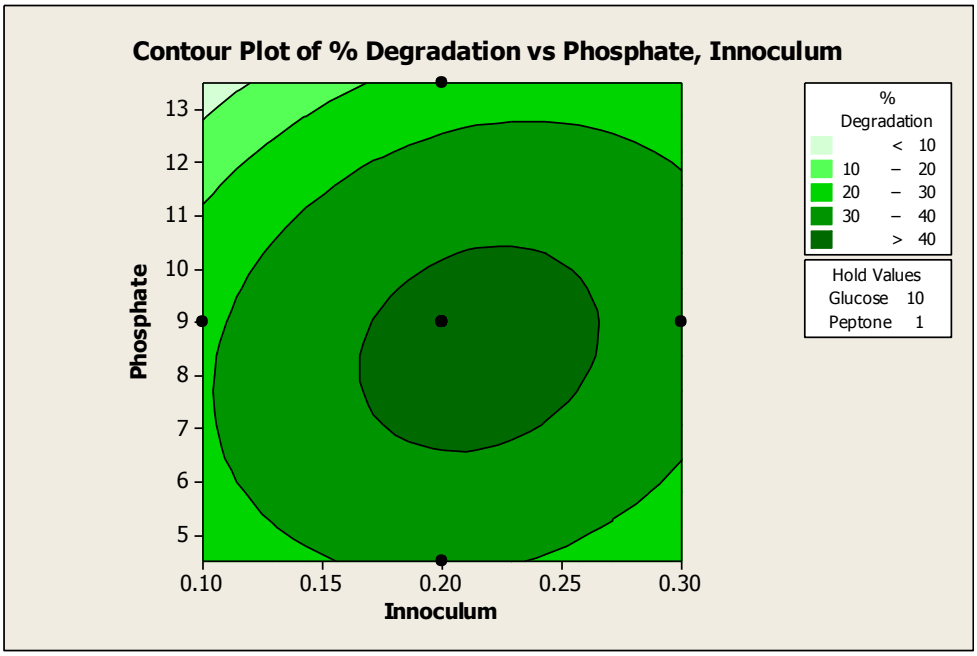


Fig. 4.79: Contour and Surface plots for PCMX degradation v/s Phosphate, Glucose; Peptone =1.0 g/l, Inoculum=0.2 O.D.



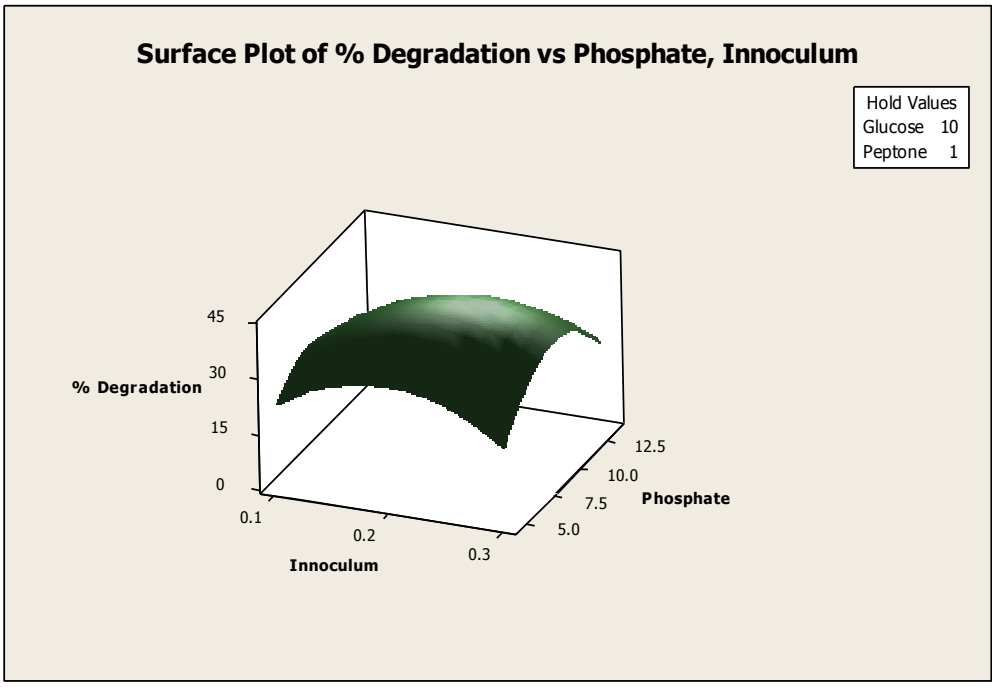
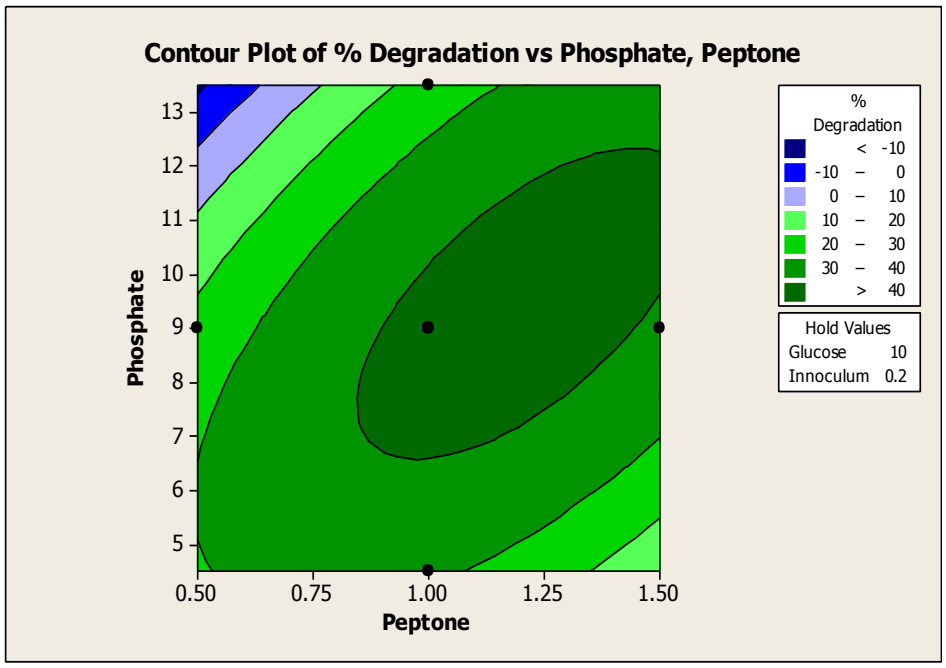


Fig.4.80: Contour and Surface plots for PCMX degradation v/s Phosphate, Inoculum; Peptone =1.0 g/l, Glucose=10 g/l.



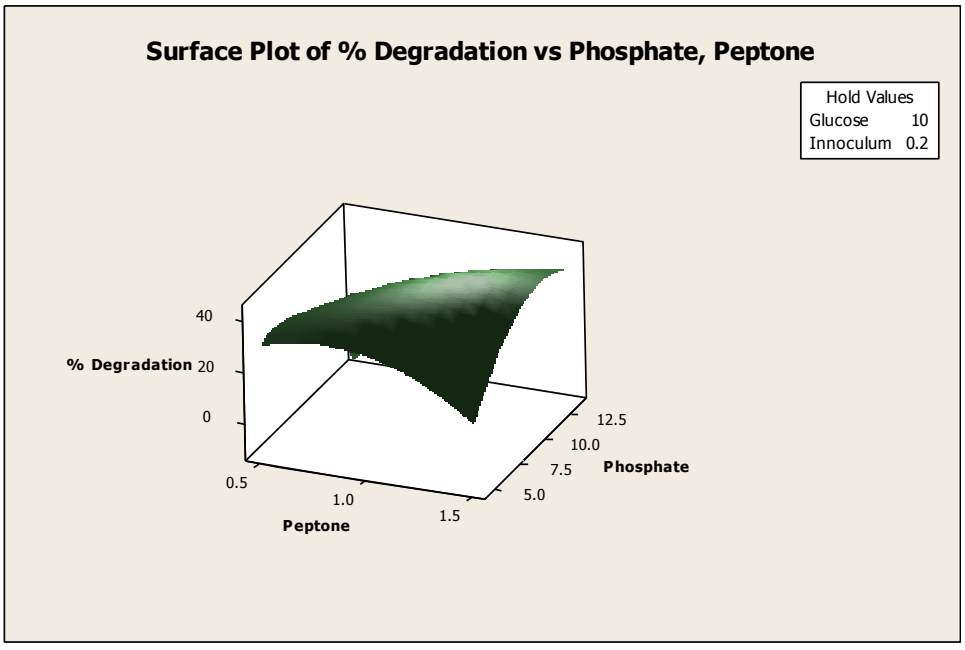
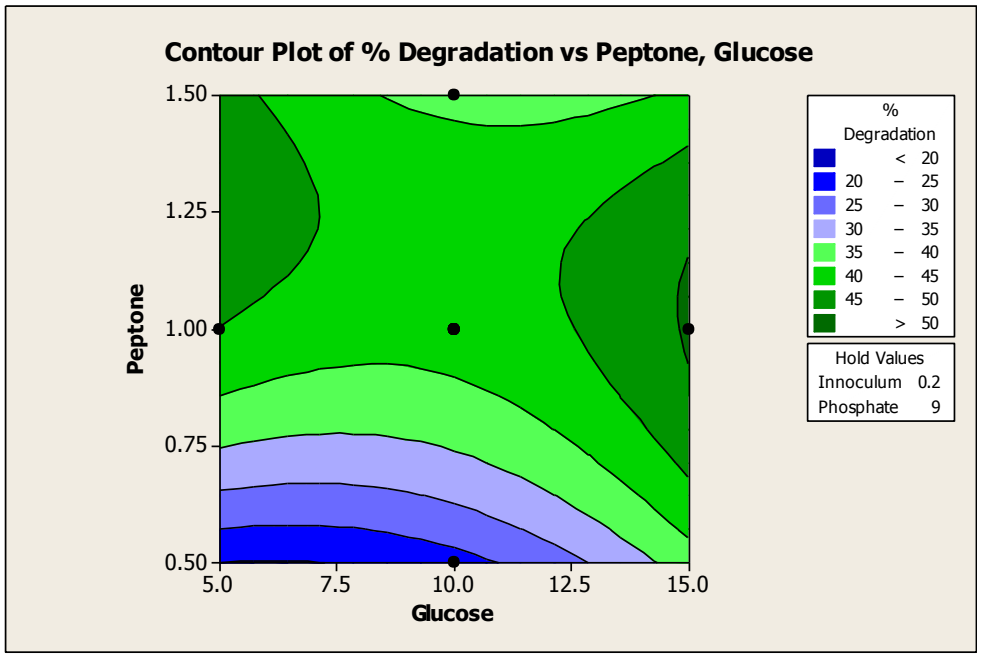


Fig. 4.81: Contour and Surface plots for PCMX degradation v/s Phosphate, Peptone; Glucose=10 g/l, Inoculum=0.2 O.D.



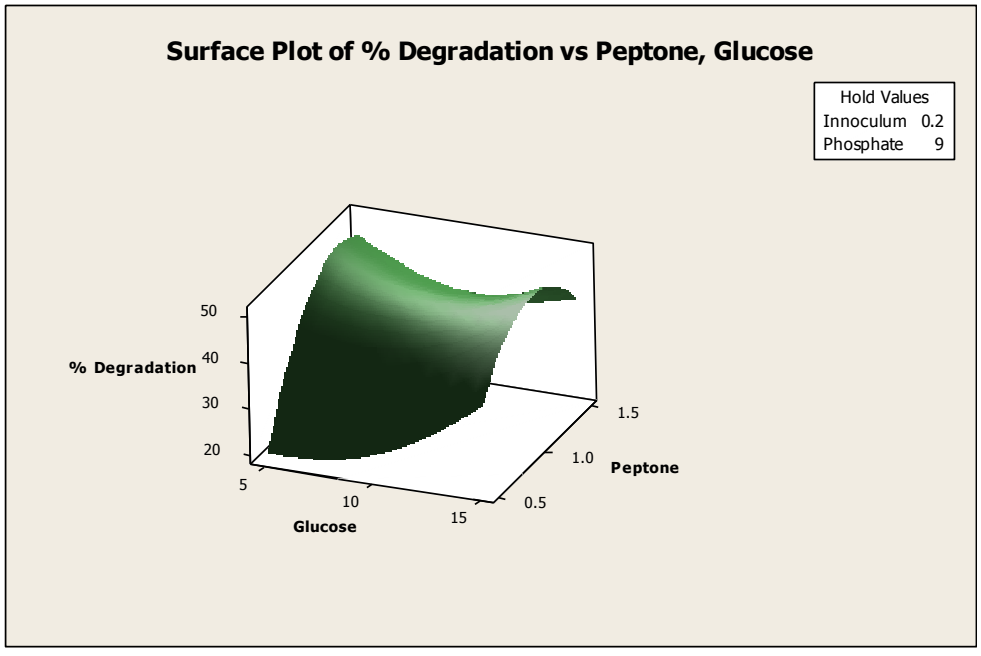
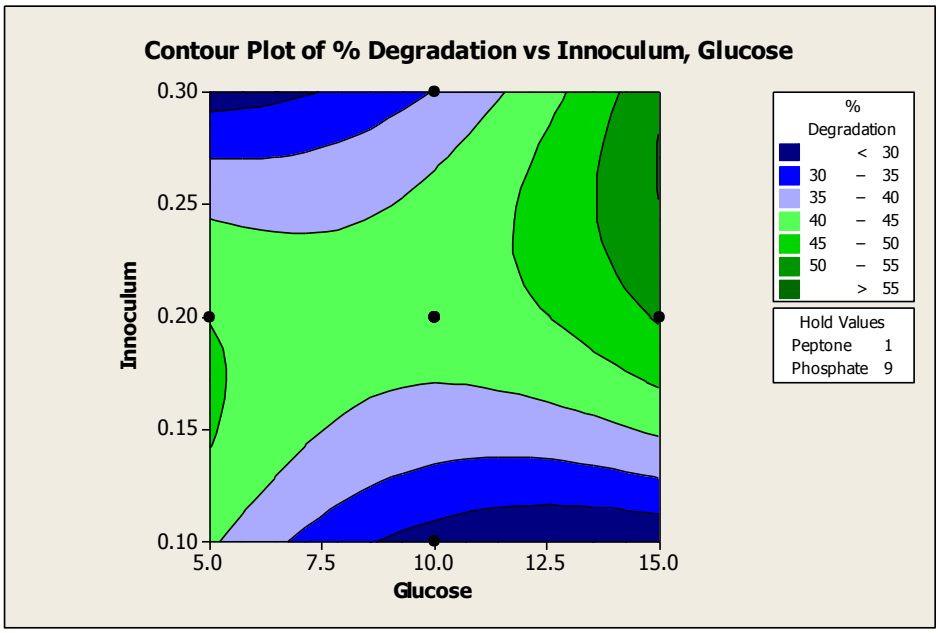


Fig. 4.82: Contour and Surface plots for PCMX degradation v/s Peptone, Glucose; Inoculum=0.2 O.D, Phosphate=9.0 g/l.



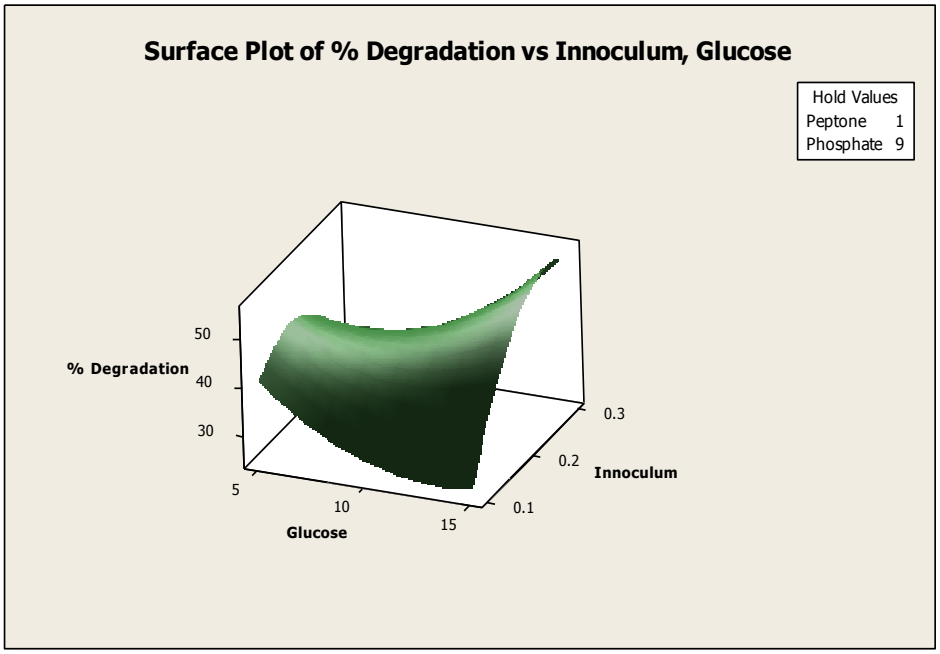


Fig. 4.83: Contour and Surface plots for PCMX degradation v/s Inoculum, Glucose; Peptone =1.0 g/l, Phosphate =9.0 g/l.

SUMMING UP

Similar optimization work was carried out for PCMX.The minimum, maximum values for peptone were set at 0.5 -1.5 g/l and, glucose between 5-15 g/l and, phosphate between 4.5-17 g/l, and Inoculum 0.1-0.3 OD.The present study shows the potential of the isolated indigenous *Bacillus cereus* for PCMX degradation. The performance of the indigenous strain in biodegradation of PCMX in the minimal medium is excellent. The response surface methodology using 30 full-factorial composite designs was adopted to optimize the process variables like Inoculum Size, Glucose, Phosphate and Peptone for the microbial degradation of PCMX.In order to determine the optimum levels of each variable for maximum percentage degradation, three-dimensional response surface plots was constructed by plotting the response (percentage degradation) on the Z-axis against any two independent variables, while maintaining other variables at their optimum levels. The response at the centre point corresponds to a maximum degree of achievable degradation process for that factor. An increase in percentage degradation was observed when the concentrations of inoculum and peptone were increased till a certain level 1.00g/L of peptone and 0.2 OD inoculums size.A similar convex shape trend was observed for variation in concentrations of phosphate and glucose; for variation in concentrations of inoculum and phosphate, for variation in concentrations of phosphate and peptone, for variation in concentrations of peptone and glucose and for variation in concentrations of inoculum and glucose, suggesting that these were well-defined process conditions for degradation process. The shapes of contour plots indicate the nature and extent of the interactions. Negligible interaction is shown by circular nature of contour plots. Furthermore, the studies of the contour plot also reveal that optimal values of variables lie within the range: phosphate 17 g/L, inoculum 0.3 OD, peptone at 1.5g/L and glucose 15.0 g/L. The contour plots show a rather broad plateau region in which the percentage degradation changes relatively little when the nutrient concentrations were varied. This indicated that the optimal solution could accommodate small errors or variability in the experimental factors. These plots are helpful in studying the effects of the factors variation in the studied field and consequently, in determining the optimal experimental conditions.

The highest PCMX degradation that can be achieved according to the model prediction under the optimal experimental conditions is 51.54%. The experimental results indicated a maximum degradation of about 63.09 % under the optimal process conditions. This confirms the closeness of the model to the experimental results.

4.13. Effluent treatment by *Bacillus cereus*

From the previous experiments it can be concluded that 99.62% of 2,4-DCP and 63.09% of PCMX degradation was achieved after medium optimization. So an attempt was made to treat the effluent from industry discharging the same. The initial concentration of 2,4-DCP and PCMX of the untreated effluent from the industry were 0.2mg/L and 3000 mg/L respectively. And it was observed that the initial pH of effluent was 5.26 and 9.14 for effluent containing 2,4-DCP and PCMX respectively.

Experiments were conducted by inoculating mutated and parent strain *Bacillus cereus* with nutrient and without nutrient to untreated effluent from different industries and incubated for 15 days at 30^0C. Concentration of 2,4-DCP and PCMX was analyzed for every consecutive days.The results of the experiments are presented in Table 4.66 to 4.71 and Figures 4.84 to 4.88.From the Figure 4.84 it is apparent that percentage degradation of 2, 4-DCP with nutrient in effluent by parent strains *Bacillus cereus* BC (98.51 %) and mutated *Bacillus cereus* was UV-10(65.66%), UV-20(66.67%), UV-30(45.60%), UV-40(84.86%), UV-60(82.84%). The maximum percentage degradation was found by parent strain *Bacillus cereus* BC(98.51 %).Further from the Figure 4.87 it is evident that percentage degradation of 2, 4-DCP without nutrient in effluent by parent strains *Bacillus cereus* BC(87.64%) and mutated *Bacillus cereus*was UV-10(56.06%), UV-20(49.89%), UV-30(11.96%),UV-40(57.24%), UV-60(6.02%). The maximum percentage degradation was found by parent strain *Bacillus cereus* BC (87.64 %).

The percentage degradation of PCMX with nutrient by mutated *Bacillus cereus* was UV-10(12.23%), UV-20(48.81%), UV-30(17.31%), UV-40(6.68%), UV-60(7.86%) and parent strain *Bacillus cereus* BC (53.18%) as shown in Figure 4.84. The maximum percentage degradation was observed by BC (parent strain)(53.18%).The percentage degradation of PCMX without nutrient by mutated *Bacillus cereus* which was UV-10(9.05%), UV-20(34.36%), UV-30(17.56%), UV-

40(6.73%), UV-60(6.60%) and parent strain *Bacillus cereus* BC (41.57%) and these results are presented in Figure 4.87. The maximum percentage degradation was observed by BC (parent strain) (41.57%).

Under optimized conditions *Bacillus cereus* could degrade 94.89% of 10 mg/L of 2,4-DCP and 54% of 50 mg/L of PCMX with 264 hour and 216 hour of incubation respectively.Whereas when the industry effluent was subjected to degradation by *Bacillus cereus* percentage degradation was 98.51% for 2,4-DCP and 53.18 % for PCMX at 456 hour. The reason behind the prolonged time required for degradation is the presence of other pollutants in the industrial effluent which extends the time required to degrade the 2,4-DCP and PCMX. Debadatta Das (2010) explained similar type of work on phenol degradation in effluent that took longer time compared to the phenol degradation in aqueous solution.

Table 4.66: Concentration of 2, 4-DCP versus time by mutated and parent strain *Bacillus cereus* in effluent.

Time(hr.)	BC (With nutrient)	BC (Without nutrient)	UV-10 (With nutrient)	UV-10 (Without nutrient)	UV-20 (With nutrient)	UV-20 (Without nutrient)	UV-30 (With nutrient)	UV-30 (Without nutrient)	UV-40 (With nutrient)	UV-40 (Without nutrient)	UV-60 (With nutrient)	UV-60 (Without nutrient)
0	0.1345	0.1335	0.1302	0.1361	0.1302	0.1361	0.1364	0.1362	0.1302	0.1361	0.136	0.1362
96	0.0678	0.0869	0.0863	0.0899	0.0832	0.0981	0.0896	0.1332	0.0803	0.0781	0.071	0.1378
144	0.068	0.0834	0.0845	0.0889	0.0879	0.0989	0.0878	0.1312	0.0799	0.0789	0.07	0.1302
192	0.0546	0.0549	0.0876	0.0798	0.0812	0.0999	0.0867	0.1289	0.0791	0.0799	0.057	0.1389
216	0.0436	0.0622	0.0778	0.0745	0.0754	0.0805	0.0843	0.131	0.0698	0.0505	0.044	0.1333
264	0.0423	0.0629	0.0687	0.065	0.0689	0.0867	0.0776	0.1299	0.0677	0.0567	0.043	0.1308
336	0.012	0.0626	0.0572	0.0618	0.0599	0.0798	0.0756	0.1223	0.0572	0.0598	0.027	0.1323
456	0.002	0.0165	0.0447	0.0598	0.0434	0.0682	0.0742	0.1199	0.0197	0.0582	0.023	0.128
%	**98.51**	**87.64**	**65.66**	**56.06**	**66.67**	**49.89**	**45.60**	**11.96**	**84.86**	**57.24**	**82.84**	**6.02**

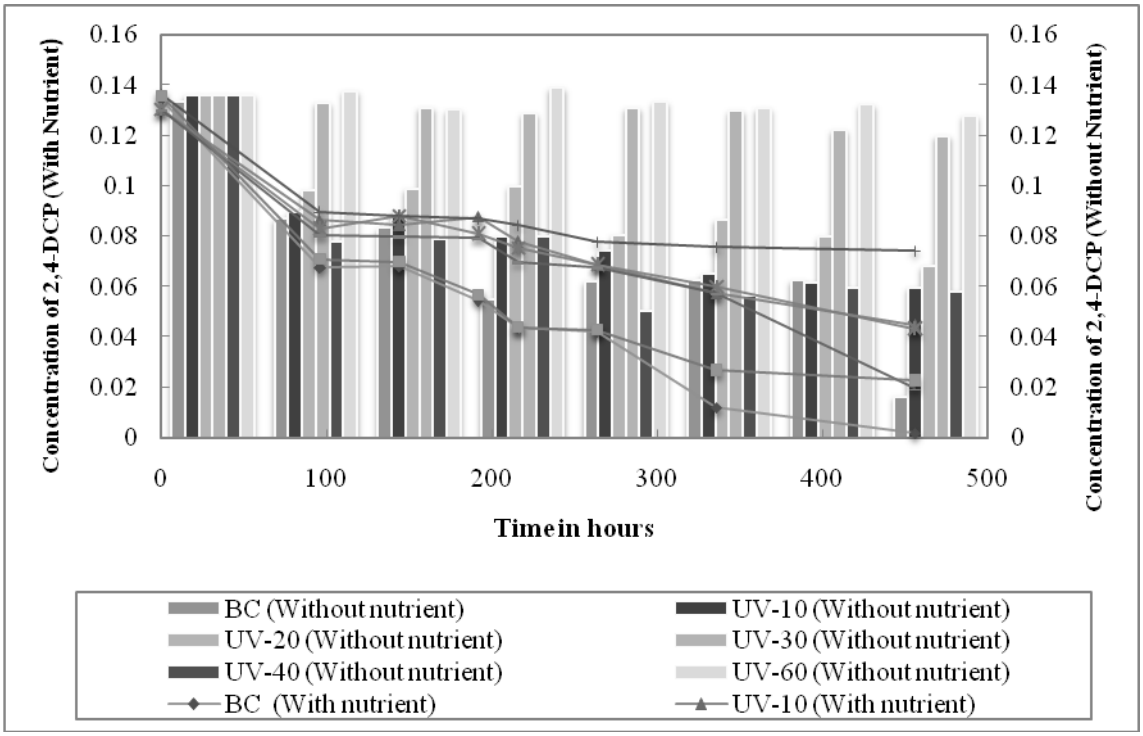


Fig.4.84: Concentration of 2, 4-DCP versus time by mutated and parent strain *Bacillus cereus* in effluent.

Table 4.67: Growth (OD) of mutated *Bacillus cereus* and parent strain versus time with 2, 4-DCP in effluent.

Time(hr.)	BC (With nutrient)	BC (Without nutrient)	UV-10 (With nutrient)	UV-10 (Without nutrient)	UV-20 (With nutrient)	UV-20 (Without nutrient)	UV-30 (With nutrient)	UV-30 (Without nutrient)	UV-40 (With nutrient)	UV-40 (Without nutrient)	UV-60 (With nutrient)	UV-60 (Without nutrient)
0	0.003	0.002	0.003	0.002	0.003	0.003	0.002	0.003	0.003	0.004	0.002	0.003
96	0.897	0.74	0.832	0.612	0.878	0.679	0.805	0.614	0.725	0.634	0.832	0.623
144	0.967	0.732	0.908	0.626	0.898	0.706	0.879	0.72	0.863	0.734	0.908	0.736
192	1.032	0.667	0.974	0.745	0.962	0.768	0.943	0.701	1.206	0.772	0.962	0.767
216	1.24	0.479	1.058	0.889	1.008	0.847	1.041	0.591	1.38	0.89	0.999	0.808
264	1.234	0.466	1.223	0.898	1.16	0.637	1.132	0.469	1.081	0.713	1.068	0.599
336	1.25	0.451	1.097	0.585	0.844	0.447	1.18	0.408	0.888	0.527	1.167	0.489
456	1.081	0.439	0.999	0.372	0.817	0.228	1.056	0.334	0.855	0.323	0.974	0.375

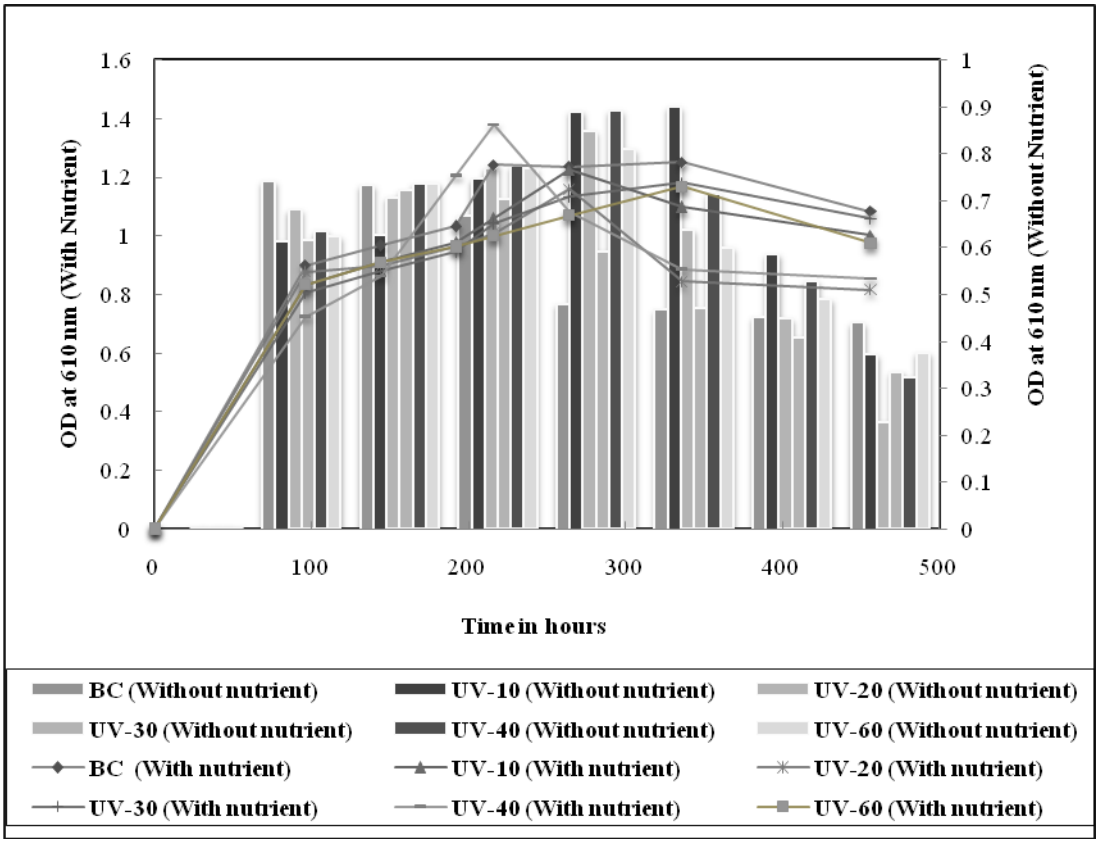


Fig. 4.85: Growth (OD) of mutated *Bacillus cereus* and parent strain versus time with 2, 4-DCP in effluent.

Table 4.68:pH of effluent with mutated *Bacillus cereus* and parent strain and 2, 4-DCP versus time.

Timr(hr.)	BC (With nutrient)	BC (Without nutrient)	UV-10 (With nutrient)	UV-10 (Without nutrient)	UV-20 (With nutrient)	UV-20 (Without nutrient)	UV-30 (With nutrient)	UV-30 (Without nutrient)	UV-40 (With nutrient)	UV-40 (Without nutrient)	UV-60 (With nutrient)	UV-60 (Without nutrient)
0	5.6	5.6	5.6	5.5	5.7	5.5	5.6	5.5	5.6	5.4	5.6	5.5
96	5.5	5.5	5.3	5.6	5.7	5.4	5.6	5.5	5.6	5.4	5.5	5.5
144	5.4	5.4	5.4	5.5	5.6	5.3	5.5	5.4	5.6	5.5	5.5	5.5
192	5.4	5.3	5.4	5.5	5.6	5.4	5.4	5.4	5.6	5.4	5.4	5.6
216	5.5	5.4	5.4	5.4	5.5	5.4	5.5	5.4	5.6	5.3	5.4	5.6
264	5.4	5.4	5.5	5.4	5.5	5.5	5.5	5.5	5.5	5.3	5.3	5.5
336	5.3	5.3	5.6	5.5	5.5	5.3	5.4	5.5	5.4	5.3	5.3	5.5
456	5.3	5.3	5.6	5.5	5.4	5.3	5.4	5.4	5.4	5.2	5.3	5.3

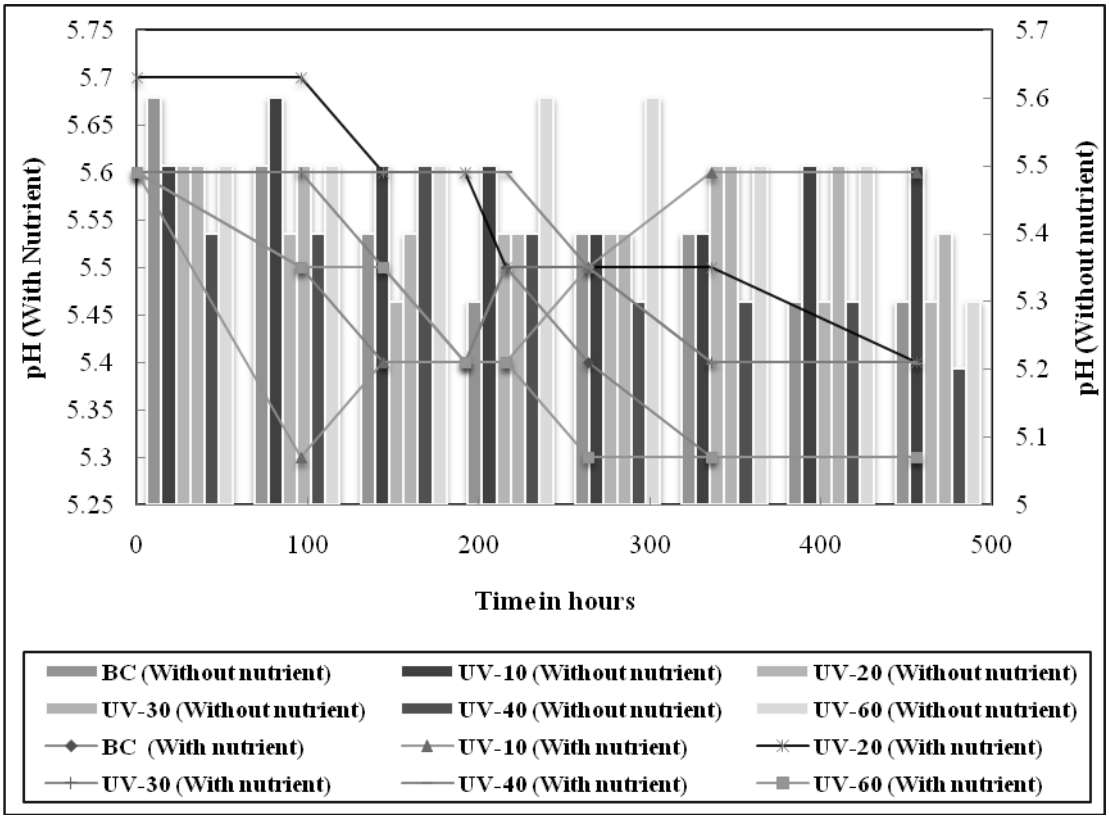


Fig. 4.86: pH of effluent with mutated *Bacillus cereus* and parent strain and 2, 4-DCP versus time.

Table 4.69: Concentration of PCMX versus time by mutated and parent strain *Bacillus cereus* in effluent.

Time (hr.)	BC (With nutrient)	BC (Without nutrient)	UV-10 (With nutrient)	UV-10 (Without nutrient)	UV-20 (With nutrient)	UV-20 (Without nutrient)	UV-30 (With nutrient)	UV-30 (Without nutrient)	UV-40 (With nutrient)	UV-40 (Without nutrient)	UV-60 (With nutrient)	UV-60 (Without nutrient)
0	58.17	43.94	56.9	55.2	53.27	40.1	47	47.95	58.8	57.57	57.58	57.96
96	48.3	33.72	54.8	56.64	43.09	38.75	46.51	47.1	58.15	57.02	57.6	57.16
144	44.68	33.68	54.09	54.79	40.87	36.89	44.56	46.76	57.77	57.99	56.98	56.78
192	37.89	32.77	53.89	54.41	38.79	34.44	43.43	46.23	57.09	56.9	56.08	56.07
216	31.78	31.89	52.9	53.99	36.98	32.91	42.98	45.61	56.48	54.73	55.12	55.56
264	29.98	28.65	51.59	51.84	30.13	29.34	42.45	45.99	55.49	54.87	55.08	55.98
336	27.3	25.84	50.89	51.53	27.14	26.53	41.18	46.29	55.31	53.72	54.18	55.45
456	27.23	25.67	49.94	50.2	27.27	26.32	38.86	39.53	54.87	53.69	53.05	54.13
%	**53.18**	**41.57**	**12.23**	**9.05**	**48.81**	**34.36**	**17.31**	**17.56**	**6.68**	**6.73**	**7.86**	**6.6**

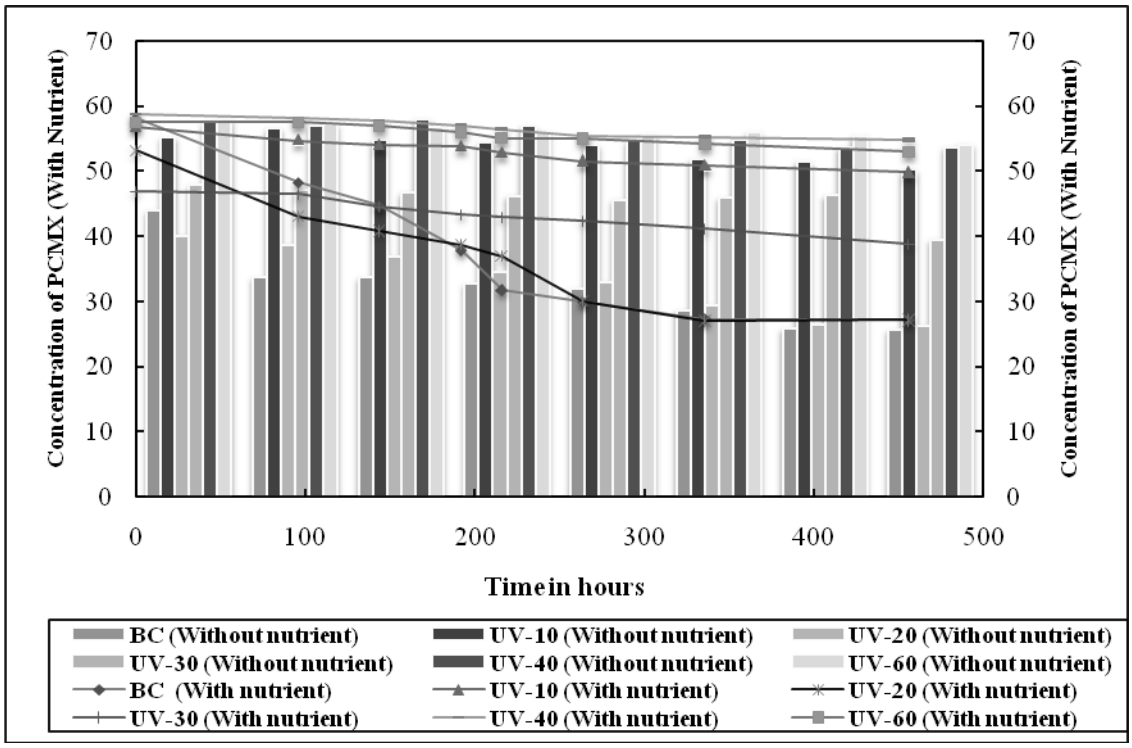


Fig. 4.87: Concentration of PCMX versus time by mutated and parent strain *Bacillus cereus* in effluent.

Table 4.70: Growth OD of mutated *Bacillus cereus* and parent strain versus time with PCMX in effluent.

Time (hr.)	BC (With nutrient)	BC (Without nutrient)	UV-10 (With nutrient)	UV-10 (Without nutrient)	UV-20 (With nutrient)	UV-20 (Without nutrient)	UV-30 (With nutrient)	UV-30 (Without nutrient)	UV-40 (With nutrient)	UV-40 (Without nutrient)	UV-60 (With nutrient)	UV-60 (Without nutrient)
0	0.003	0.002	0.003	0.002	0.003	0.003	0.002	0.003	0.003	0.004	0.002	0.003
96	0.58	0.019	0.432	0.012	0.608	0.023	0.475	0.014	0.452	0.017	0.532	0.023
144	0.61	0.134	0.478	0.021	0.698	0.056	0.489	0.024	0.563	0.034	0.578	0.036
192	0.63	0.167	0.564	0.045	0.752	0.129	0.543	0.067	0.664	0.072	0.612	0.067
216	0.637	0.08	0.598	0.076	0.688	0.074	0.576	0.086	0.657	0.098	0.654	0.078
264	0.645	0.074	0.623	0.098	0.643	0.037	0.612	0.089	0.502	0.109	0.678	0.099
336	0.656	0.055	0.567	0.085	0.539	0.017	0.578	0.078	0.488	0.028	0.567	0.089
456	0.579	0.056	0.532	0.072	0.307	0.013	0.56	0.054	0.455	0.03	0.534	0.075

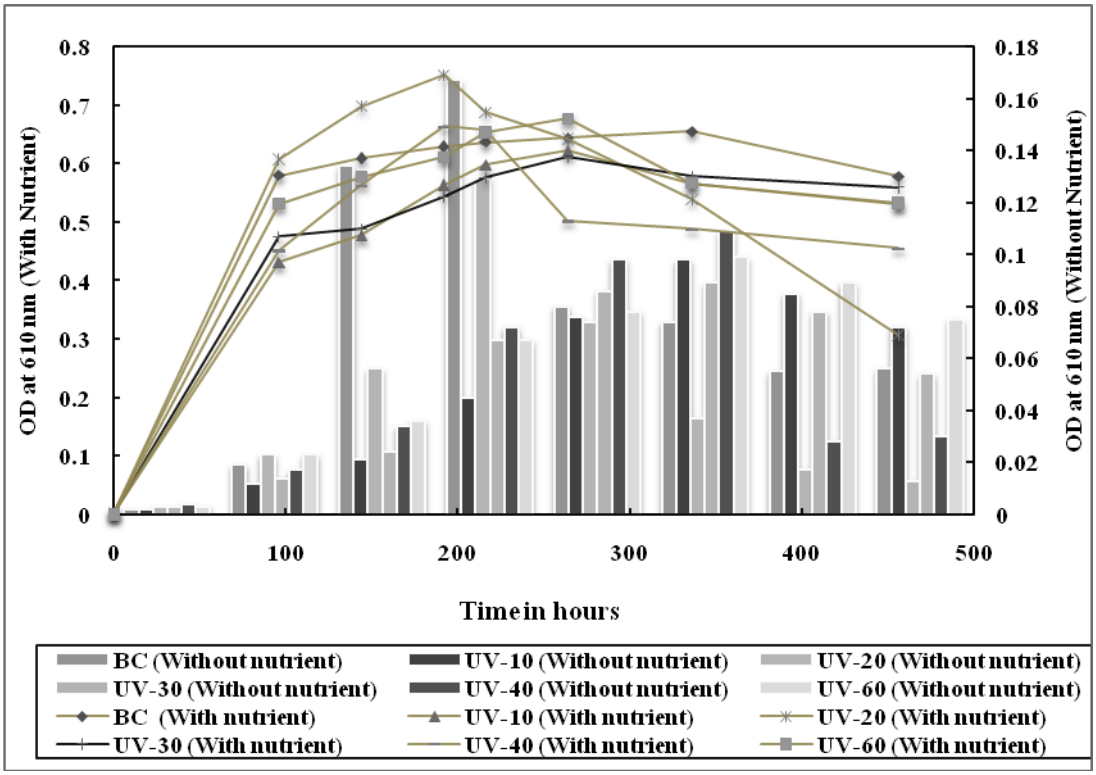


Fig. 4.88: Growth OD of mutated *Bacillus cereus* and parent strain versus time with PCMX in effluent.

Table 4.71:pH of effluent with mutated *Bacillus cereus* and parent strainand PCMX versus time.

Time(hr.)	BC (With nutrient)	BC (Without nutrient)	UV-10 (With nutrient)	UV-10 (Without nutrient)	UV-20 (With nutrient)	UV-20 (Without nutrient)	UV-30 (With nutrient)	UV-30 (Without nutrient)	UV-40 (With nutrient)	UV-40 (Without nutrient)	UV-60 (With nutrient)	UV-60 (Without nutrient)
0	7.4	7.3	7.4	7.4	7.1	7.2	7.3	7.6	7.6	7.4	7.5	7.5
96	7.3	7.4	7.3	7.5	7.2	7.3	7.4	7.5	7.6	7.4	7.5	7.6
144	7.2	7.4	7.4	7.5	7.4	7.3	7.3	7.6	7.6	7.6	7.5	7.5
192	7.3	7.3	7.4	7.5	7.3	7.2	7.4	7.4	7.5	7.4	7.4	7.5
216	7.3	7.4	7.4	7.4	7.4	7.1	7.5	7.4	7.5	7.3	7.4	7.5
264	7.4	7.2	7.5	7.4	7.5	7.2	7.3	7.3	7.5	7.3	7.3	7.5
336	7.3	7.3	7.6	7.3	7.3	7.2	7.2	7.5	7.4	7.3	7.3	7.4
456	7	7.1	7.6	7.2	7.2	7.1	7.1	7.3	7.4	7.2	7.3	7.3

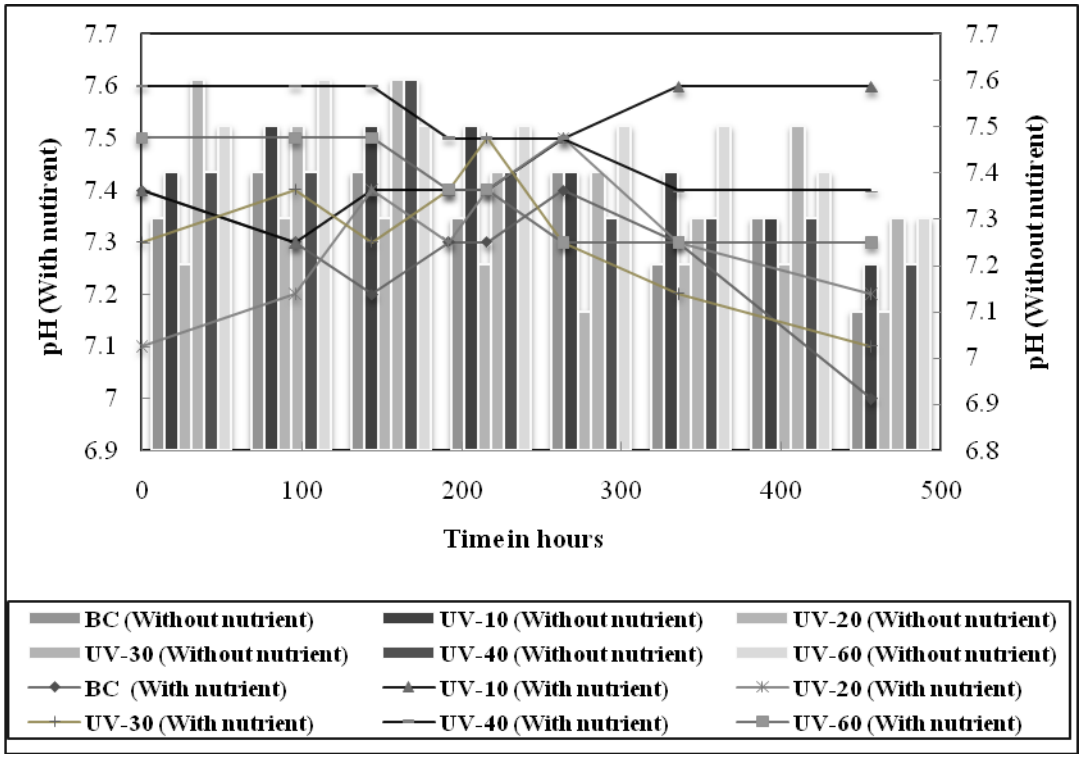


Fig. 4.89:pH of effluent with mutated *Bacillus cereus* and parent strain and PCMX versus time.

SUMMING UP

From this study it can be concluded that, after 15 days of incubation, the 2,4-DCP and PCMX concentration was reduced to the maximum by parent strain *Bacillus cereus* than by the mutated strains up to 98 and 53% for 2,4-DCP and PCMX respectively.The reason for this trend might be that the mutagen might not have increased the secretion of degrading enzymes.Effluents without nutrient, indigenous microorganisms were unable to degrade 2,4-DCP and PCMX effectively.

2,4-DCP degradation in industrial effluent is growth related when compared to the degradation of 2,4-CP in synthetic effluent which was not growth related. Probably this type of growth related degradation of 2,4-DCP might be due to the presence of other organic and inorganic pollutants acting as a nutrient for the growth of *Bacillus cereus*.Few researcher have studied the removal of heavy metals from industry effluent Halimoon and Yin, (2010) studied the removal of heavy metals from textile wastewater using zeolite, the amount of heavy metals like Cr, Cu, Pb present in textile industry effluent. Similarly, Benazir et al., (2009) also estimated total chromium to know the total chromium present in tannery effluent after degradation by the microbial consortia.

CHAPTER 5

SUMMARY

&

CONCLUSION

The contamination of biosphere by chlorinated organic compounds during the last several decades has given rise to major concern about the Eco toxicological effects and ultimate fate of these compounds on humans. The recalcitrant pollutants that are considered to be most hazardous because of their intrinsic toxicity, high exposure level, or recalcitrant behaviour in the environment have been placed on blacklists and other policy priority lists and has been reported by many researchers. The authors Alexandersson and Hedenstierna (1982) concluded that inhalation exposure to trichlorophenol may cause pulmonary dysfunction and possibly fibrosis following chronic duration exposure. Porphyria cutaneatarda has been reported in workers employed in the manufacture of 2,4-DCP and 2,4,5-TCP (Bleiberg et al. 1964). Exposure to chlorophenols and intermediates was probably through inhalation and dermal contact. A number of investigators have studied the potential association between chlorophenol-based pesticide production and carcinogenicity (Eriksson et al. 1981, 1990; Hardell et al. 1981; Hoar et al. 1986; Honchar and Halperin 1981; Kogevinas et al. 1992; Lynge 1985; Smith et al. 1984; Woods et al. 1987). Reports from Sweden indicate significantly increased relative risk ratios for soft tissue sarcomas (STS) and/or non-Hodgkin's lymphomas (NHLs) in exposed workers.

The fate of chlorinated organic compounds, particularly chlorophenols that enter the environment ismainly determined by their rate of biodegradation, which therefore also has a major effect on the degree of bioaccumulation and risk of Eco toxicological effects. The degree and rate of biodegradation are also of criticalimportance for the feasibility of biological techniques to clean up contaminated sites and waste streams. The biodegradation of the chlorinated organic compounds has thus been the subject of numerous studies. These studies led to a deeper understanding of the diversity of biodegradation processes. Dechlorination is the first critical step in the bacterial degradation of many chlorinated pollutants. The mechanism of biodegradation depends on the conditions under which the studies are carried out. Under the aerobic condition, degradation of mono- and dichlorophenols was shown to be initiated by oxygenation into chlorocatechols, and dechlorination occurred only after ring cleavage of the chlorocatechols, while degradation of polychlorinated phenols started with hydrolytic para-hydroxylation, yielding chlorinated para-hydroquinone. Anaerobic biodegradation of chlorophenols occurs by reductive

dechlorination, a process by which the chlorines were replaced with hydrogen. Focus on literature revealed that *Pseudomonas spp*., *Clostridium spp., Bacillus spp. etc*. could dechlorinate various chlorophenols. Out of these *Pseudomonas spp*., can mineralize a variety of hazardous chlorophenols.For instance, of 206 isolates from a petroleum waste lagoon, 46% were able to degrade chlorophenols as a sole source of carbon after acclimation to the particular chlorophenol (Tabak et al. 1964).More recently, aerobic microorganisms in clay loam soils were able to degrade most of the 2-MCP, 4-MCP, 2,4-DCP, 2,6-DCP, or 2,4,6-T_3CP present (100 mg/kg) within a few days without a lag phase (Baker & Mayfield, 1980).

The results of many other studies have confirmed that most chlorophenols can be metabolized by certain microorganisms in water (Aly& Faust, 1964; Baker et al. 1980; Hwang et al. 1986), sediment, soil (Walker, 1954; Loos et al. 1967; Spokes & Walker, 1974), and activated sludge (Baird et al. 1974).

Most of the previously reported chlorophenols and chloroxylenols degradation was around neutral pH in the range of 7–8. Only few reports have revealed that chlorophenols and chloroxylenols can be degraded in acidic condition. It can be inferred from published reports that optimum temperature for the reduction of chlorophenols and chloroxylenols is 28°C. Very few records demonstrate that chlorophenols and chloroxylenols degrade in extreme low temperature at 5°C (A. Backman et al.2003). Degradation of chlorophenol and chloroxylenol with different types of conventional carbon sources such as sodium glutamate, sodium formate, sodium propionate, glucose and methanol are reported. Glucose as carbon source was most suitable source.

Although extensive research work has been carried out on the isolation and screening of chlorinated phenol degrading microorganisms, medium formulation and application study on industrialeffluent to reduce the pollutant, it was found that more number of bacterial strains with high antimicrobial agent degrading capability need to be isolated from potential ecological sources. It was found that there is a need to optimize medium at various conditions in order to increase the percentage degradation by studying the effect of various parameters. In addition, in the literature,reports on the degradation of strong antimicrobial agent areless. Hence, there is a need for the isolation, screening, and identification of effective bacteria degrading chlorinated

organic compound. The effect of various process parameters leading to the maximum degradation requires future studiesto bring significant result in degrading chlorinated organic compounds. Chlorinated organic compounds are highly toxic and are used widely as antimicrobial agent. Therefore, the present study was taken to meet the stated objectives, which included isolation, screening, identification, and optimisation studies of potential degrading microorganism. The effect of various process variables influencing degradation has also studied. Studies have been carried out to degrade the chlorinated organic compound present in Industrial effluent. In addition, studies were initiated to increase the biodegradation rate by mutating the isolated microorganism.

In order to meet the stated objectives, soil and water samples were collected from various sampling points from an area around the wastewater treatment plant from Rickitt Benckiser Dettol manufacturing company located in Mysore, Karnataka,India.The microorganisms from various samples collected were isolated by enrichment technique. The soil samples exposed to chlorophenol showed greater number of microorganisms compared to the control samples. The six isolated microorganisms were screened using different concentration of 2,4-DCP and PCMX. Among the six isolates, a bacterial isolate 01-C-My-NITK, showed optimum growth in higher concentration of both 2, 4-DCP and PCMX. Hence, this organism was subjected to biochemical and 16srDNA sequencing and identified as a *Bacillus cereus*. Further degradation studies were carried out using different concentrations of 2, 4-DCP and PCMX in defined salt media under various ambient conditions of pH and temperatures.*Bacillus cereus was* grown overnight in a 100ml defined mineral salt medium containing 10g/L of glucose. Maximum CFU/ml with 10g/L Glucose was 11.76 at 30 hour. From the literature, it was understood that the low level of glucose ensured that cultures were carbon limited rather than nitrogen limited and prevented lags in growth of *Bacillus cereus.* Further Peptone concentration was varied. It was observed that maximum growth OD and CFU/ml with 1g/L peptone of *Bacillus cereus* was 0.382 and 29.22 at 24 hour respectively.

The optimum temperature and pH range for degradation of 2, 4-DCP and PCMX was obtained by conducting experiments using cultures inoculated into medium with *Bacillus cereus* having different initial temperature (25^0C, 30^0C, 35^0C and 40^0C) and pH values (pH 2 to 8). A 0.2 OD inoculum and a starting 2, 4-DCP and

PCMX concentration of 50 and 10 mg/L were used in these experiments. The growth for each strain and final pH was measured as well.

The effect of temperature on 2,4DCP degradation was studied and the2, 4-DCP estimation data revealed that maximum degradation of 28 % occurred in cultures placed at 30^{o}C in 7pH for 264hour with 10 mg/L of 2,4-DCP. At 35^{o}C also degradation occurred significantly up to 25%, but less than at 30^{o}C. At extreme temperatures of 25^{o}C and 40^{o}C it was only 4.00 and 3.7% respectively. Very less degradation was observed in all the other concentration of 2,4-DCP. In 30mg/L and 75mg/L of 2, 4-DCP it was observed that degradation was slightly lower. Percentage degradation by *Bacillus cereus* in 30 mg/Lof 2, 4-DCP at the end of 264 hour was 21.23% at 30^{o}C and 20.07% at 35^{o}C in 7pH. While at low and high temperatures of 25^{o}C and 40^{o}C it was only 3.66% and 0.69% respectively. Similarly percentage degradation of *Bacillus cereus* in 75mg/L of 2, 4-DCP at the end of 264 hour was 9.17% at 30^{o}C and 8.11% at 35^{o}C in 7pH. While at extreme low and high temperatures of 25^{o}C and 40^{o}C it was only 0.053% and 2.34% respectively.

The effect of temperature on degradation of PCMX by *Bacillus cereus* revealed that maximum degradation of 28.76 % occurred in cultures placed at 30^{o}C in 7pH for 216hour with 10 mg/L of PCMX. At 35^{o}C also degradation occurred significantly up to 25.08%, but less than at 30^{o}C. Degradation was hampered both at low as well as high temperatures. At temperatures of 25^{o}C and 40^{o}C it was only 7.24% and 5.68% respectively.

Very less degradation was observed in all the other concentration of PCMX. In 100mg/L, 150mg/Land 350 mg/L of PCMX it was observed that degradation was slightly lower. Percentage degradation by *Bacillus cereus* in 100 mg/L of PCMX at the end of 216 hour was 8.57% at 30^{o}C and 3.44% at 35^{o}C in 7pH. While at low and high temperatures of 25^{o}C and 40^{o}C it was only 4.2% and 3.5% respectively. Similarly percentage degradation of *Bacillus cereus* in 150mg/L of 2, 4-DCP at the end of 216hour was 2.64% at 30^{o}C and 1.69% at 35^{o}C in 7pH. While at low and high temperatures of 25^{o}C and 40^{o}C it was only 2.64% and 1.32% respectively. Similarly percentage degradation of *Bacillus cereus* in 350 mg/L of 2, 4-DCP at the end of 216 hour was 6.85% at 30^{o}C and 2.00% at 35^{o}C in 7pH. While at low and high temperatures of 25^{o}C and 40^{o}C it was only 0% and 1.14% respectively.

Decreasing the pH of the media from 7 to 2 at 30^{o}C increased the rate of 2, 4-DCP degradation. On increasing the pH further it had a reserved effect on 2, 4-DCP removal potentiality. In 264 hour 64.6 % 2, 4-DCP was removed at pH 3, while the rest of the pH conditions it could not degrade 2, 4-DCP more efficiently. Both neutral and alkaline pH had a marked inhibition on 2, 4-DCP removal efficiency. At pH 4 and 5, 2, 4-DCP removal was upto 57.1% and 54.6% respectively. Acidic pH (pH-3) could degrade 2, 4-DCP at higher rates as compared to the other pH at 30^{o}C. This shows that pH influences the degradation process. Because the pH decreases (acidic) the growth of organism is also affected when compared with neutral pH. The results corroboratewith work done by Karigar (2006) on *Arthrobacter citreus*. He has reported that at room temperature and under normal pressure degradation of atrazine is maximum at pH 6 for an optimum concentration of 5mg/L. This may be due to the effect of pH on the ionization and therefore binding and interaction of a myriad of molecular processes, which in turn affect the metabolic pathway. It could even cause denaturing of proteins which might result in lethal toxicity.

Effects of pH values from 5 to 8 on degradation of PCMX were analysed.PCMX degraded rapidly at pH 6 and 7. However, the PCMX degradation at pH 6 was slower and the PCMX concentration decreased rapidly after 216 hour post-inoculation. In control cultures no degradation occurred. *Bacillus cereus*cells grew better at pH values within a range of 5 to 8 in minimal salts medium. It is evident that maximum percentage degradation of PCMX is at 7pH (28 %) when compared to other pH.This corroborates with previous studies by Erika Alejandra Wolski et al., (2005), on pentachlorophenol degradation by soil *Pseudomonas.* They recorded maximum degradation rates for pentachlorophenol at pH values from 6.3 to 8.

In conclusion, the results showed that changes in the temperature of the culture medium, and changes in the pH values affected the degradation of 2,4-DCP and PCMX by *Bacillus cereus.* A temperature range between 30°C and 35°C were optimal to obtain the maximum degradation of 2,4-DCP and PCMX in this batch experimental system. There was no significant difference in the rates of removal at these temperatures.

The *Bacillus cereus* studied in thiswork was able to degrade 2,4-DCP and PCMX over a wide range of pH also, particularly at pH as low as 3. Experiments

performed to determine the optimal pH for the reductive stage of remediation of 2,4-DCP and PCMX showed that degradation was most rapid at pH value of 3 and 7 for 2,4-DCP and PCMX respectively. This is an important characteristic for the bioremediation of changeable environments. The temperature and pH is another tool to achieve a good rate of 2,4-DCP and PCMX degradation, since usually, when remediation of contaminated soils is done, temperature and pH of soil plays a main role.

It was observed from the experiments that maximum percentage degradation of 2, 4-DCP was 64.6 % at pH 3 and PCMX is at 7pH (28 %).Biological treatment of wastewater and contaminated soils always involves degradation of some incompatible mixtures, rather than degradation of single compounds. The observed degradation capability of certain microbe is specific for certain or similar compounds. Therefore, isolating such microbes with the ability to degrade incompatible substrate mixtures is vital. Up to date, there are several ways to get such organisms: one of which is mutagenesis. Recently some reporters described that *Pseudomonas* strain JS150, which was obtained by mutagenesis of a 1, 4-dichlorobenzene-degrading organism, degraded simultaneously chloro- and methyl phenols in the presence of phenol. Hence to increase the percentage degradation of 2, 4-DCP and PCMX the *Bacillus cereus* was subjected to mutation using Ethedium Bromide and UV rays. The degradation of 2,4-DCP and PCMX was promoted when *Bacillus cereus* was subjected to mutagenesis. The degradation of different concentration of 2, 4-DCP (50,100,150 mg/L) by mutated *Bacillus cereus* was found to be higher when compared to parent strain.Further chemical and physical mutation on *Bacillus cereus* did not affect degradation of PCMX.

Response surface methodology was used to find the effect of process variables affecting 2,4-DCP and PCMX degradation by *Bacillus cereus*.*Bacillus cereus* showed maximum 2,4-DCP percentage degradation at optimal values of variables within the range: phosphate 11.25g/L, inoculum 0.15 OD, peptone at 0.75g/L and glucose 12.5 g/L. The highest 2,4-DCP degradation that could be achieved according to the model prediction under the optimal experimental conditions was 94.37%. The experimental results indicated a maximum degradation of about 99.62% under the optimal process conditions.

The contour plot from response surface method for PCMX degradation reveal that optimal values of variables to be: phosphate 17 g/L, inoculum 0.3 OD, peptone at 1.5g/L and glucose 15.0 g/L. The highest PCMX degradation that could be achieved according to the model prediction under the optimal experimental conditions was 51.54%. The experimental results indicated a maximum degradation of about 63.09 % under the optimal process conditions.

During last several years, researchers have primarily focused their studies on the isolation and activities of the chlorophenol-degrading bacteria in shaken liquid media, and reported data mainly are limited to laboratory experiments. However, the results observed in the laboratory often differ from those observed in the field or in treatment processes in practice. For instance, bioavailability of organic chemicals in soil or waste water can be limited or controlled by physicochemical processes including diffusion, sorption/desorption and dissolution. Hence an attempt was made to degrade the 2,4-DCP and PCMX in industrial effluent.The results of this study indicates that percentage degradation by *Bacillus cereus* for 2,4-DCP was 98.51% and for PCMX percentage degradation ineffluent was 53.18 % at 456hr. Crawford R L et al have reported similar work using industrial effluents. They introduced the laboratory strains, *Flavobacterium sp.*, *Arthrobacter sp.*, and *Rhodococcus chlorophenolicus* to bio remediate PCP contaminated soil, and in each case removal of PCP was accelerated.

In conclusion, our results suggest that the bacterial community in the waste water treatment area of industry contains strains such as *Bacillus cereus*, which exhibit a high potential for effective degradation of 2, 4-DCP and PCMX under aerobic conditions. In spite of these chemicals being potent antimicrobial agents *Bacillus cereus* has been found to be efficient in growing in their presence and also bring about their degradation. It is clear that the use of isolated bacteria can lead to considerable decrease of treatment time as well as promotion of 2, 4-DCP and PCMX removal rate.

Major findings in this thesis are:

- *Bacillus cereus* microorganism was isolated from the area exposed with PCMX in spite of it being a potent antimicrobial agent.

- *Bacillus cereus* can grow on unusually high concentrations of 2,4-DCP and PCMX without the need for any cofactors or vitamins.

- It is observed that pH and temperature highly influence the degradation of 2,4-DCP (64.6%)and PCMX (28%).

- The time course study revealed that the 2,4-DCP degradation was not related with the growth of *Bacillus cereus.* Probably this might be because of the co-oxidation process in which microorganism oxidised a substance without being able to utilize the energy derived from oxidation to support growth.This type of mechanism has been reported by many researchers wherein they have explained the co metabolism nature of microorganism in degrading the organic compounds in nature (Raymond S. Horvath 1972)

- During the degradation of PCMX by *Bacillus cereus*a good growth was observed. This might be due to "primary metabolite" as the degradation of PCMX coincided with the exponential growth phase of the bacterial strains.

- The mutated *Bacillus cereus* strain can remove 29.6 % of 2,4-DCP and 21.88% PCMX.

- By optimising the media composition degradation of both 2,4-DCP and PCMX increased exponentially up to 99.62% and 63.09% respectively.

- *Bacillus cereus* can grow on a effluent sample containing 2,4-DCP and PCMX and degrade upto 98 and 53% respectively.

Conclusions

The present work on the degradation of 2,4-DCP and PCMX using the isolated strains from waste water treatment plant of industries having effluent containing these chemicals have shown important information with respect to the enhanced degradation of these chemicals. Although 6 strains were initially isolated, based on the screening studies two were chosen for further degradation studies.The isolated organism has been identified through biochemical and 16srDNA studies.It can be seen from the present study that inoculum density,glucose as carbon source and peptone as nitrogen source have shown important effect on growth and degradation. Similarly both the effect for temperature and pH have been studied and at 30^0C and at pH 3 and pH 7 maximum degradation has occurred .The present work has shown that the degradation efficiency of 2,4-DCP and PCMX were around 98% and 59% which is fairly higher than what is reported in the literature at optimum condition. An attempt was also made to qualitatively check through GCMS and variation in shape of *Bacillus cereus* through Scanning electron microscope to verify the actual degradation at different time interval. The data reported in the present work through batch study at optimum condition will provide the necessary inputs to column studies to develop a bioreactor for the removal of PCMX and 2, 4-DCP in the case of industrial waste water treatment.

SCOPE FOR FUTURE WORK

1. Further understanding of the mechanism of chlorophenols and chloroxylenols transformation during the bacterial degradation can be studied. Knowledge of biotransformation pathways is essential.
2. Enzymes instead of the microbes can be studied to degrade chlorophenols and chloroxylenols. Compared to physical and chemical methods, biodegradation is cost-effective and safe. Furthermore enzymes may prove more economical in treatment processes, because they are biochemically stable and can be used repeatedly to detoxify xenobiotic compounds.
3. Biological treatment of wastewater using Bio reactors can be studied.

APPENDIX I

NUTRIENT BROTH MEDIUM

Nutrient broth of the following composition was used in the study:

Components	g/L
Peptone	5.0
Sodium Chloride	5.0
Yeast Extract	2.0
Beef Extract	1.0

The components of the nutrient broth medium were weighed and dissolved in 1 liter of water. The initial pH of the medium was set to 7.4±0.2. The medium was sterilized at 15 psi pressure at 121°C for 15 minutes.

APPENDIX II

MINERAL MEDIA COMPOSITION

Solution A	**Solution B**
KH_2PO_4 -3g Na_2HPO_4 -6g Peptone -1g Distilled water -800ml	Glucose -10g Distilled water-200ml

To prevent caramalisation two solutions “A “ and“B” where sterilized separately.one liter of media “ A” was prepared in distilled water ,with components in concentrations shown in table 100ml of media “A “ was prepared in distilled water, as shown in Table above.1 ml of media “B” was added

APPENDIX III

MEDIA FOR BIOCHEMICAL TESTS

2.1 AMYLASE HYDROLYSIS TEST

Starch agar Medium	
Beef extract	3.0 g
Soluble starch	10.0 g
Agar	12.0 g
Distilled water	1 L

The components of the medium were dissolved in 1 litre of distilled water and the medium was sterilized by autoclaving at 15 psi pressure for 20 minutes at 121°C.

2.2 METHYL RED AND VOGES- PROSKAUER TEST

Buffered Glucose Broth (pH 6.9)	
Peptone	7.0 g
D-glucose	5.0 g
Na_2HPO_4	5.0 g
Distilled water	1 L

The components of the medium were dissolved in 1 litre of distilled water and the pH of the broth was set to 6.9. The medium was autoclaved at 15 psi pressure for 20 minutes at 121°C.

2.3 INDOLE TEST

10.0 grams of Tryptone was dissolved in 1 litre of distilled water. The broth was poured into test tubes. The test tubes containing the medium were autoclaved at 15 psi pressure for 20 minutes at 121°C.

2.4 CITRATE UTILIZATION TEST

Simmons Citrate Agar (pH 6.9)	
$MgSO_4.7H_2O$	0.2 g
$(NH_4)H_2PO_4$	1.0 g
K_2HPO_4	1.0 g
Sodium Citrate	2.0 g
NaCl	5.0 g
Bromothymol blue	0.8 g
Agar	15.0 g
Distilled water	1 L

The components of the medium were dissolved in 1 liter of distilled water and the pH of the broth was set to 6.9. The medium was autoclaved at 15 psi pressure for 20 minutes at 121°C.

2.5 FERMENTATION OF CARBOHYDRATES (pH 7.3)

Carbohydrate Fermentation Medium	
Peptone	10 g
Carbohydrate (glucose, lactose and sucrose)	5.0 g
NaCl	15 g
Phenol red	0.018 g
Distilled water	1 L

The components of the medium were dissolved in 1 litre of distilled water; the pH of the medium was set to 7.3. The medium was poured into test tubes and they were autoclaved at 15 psi pressure for 20 minutes at 121°C.

2.6 UREASE TEST

Urea Agar (pH 6.8)	
Peptone	1 g
K_2HPO_4	2.0 g
NaCl	5.0 g
Agar	20.0 g
Distilled water	1 L

Autoclave this and cool for 50°C. Add glucose- 1 g and phenol red- 6 ml to the molten base and steam for 1 hr. and cool to 50°C. Urea of 20% aqueous solution is filter sterilized and added to the basal media.

2.7 HYDROGEN SULPHIDE PRODUCTION TEST

Triple Sugar Iron Agar Medium	
Peptone	30 g
Beef extract	3.0 g
Ferrous Ammonium Sulphate	0.2 g
Sodium thiosulfate	0.025 g
Agar	3.0 g
Distilled water	1 L

The components of the medium were dissolved in 1 litre of distilled water. The initial pH of the medium was adjusted to 7.4. The medium was autoclaved at 15 psi pressure for 20 minutes at 121°C.

2.8 GELATIN HYDROLYSIS TEST

Gelatin agar medium (pH 7.3)	
Peptone	10 g
Meat extract	10.0 g
NaCl	5.0 g
Gelatin	10.0 g
Agar	15 g
Distilled water	1 L

The components of the medium were dissolved in 1 litre of distilled water; the initial pH of the medium was set to 7.3. The medium was poured into test tubes and the tubes were autoclaved at 15 psi pressure for 20 minutes at 121°C.

APPENDIX 1V

OPTICAL DENSITY MEASUREMENT

The determination of optical density is a method of enumeration of bacterial count (Cappuccino and Natalie 1999). Bacterial growth was determined by measuring the culture optical densities at 600nm (OD600) using spectrophotometer (GBC Scientific Equipment, Australia). 3 ml of the culture broth was added to a glass cuvette and the optical density was measured. The optical densities of the samples removed from cultures were read against uninoculated broth, which was used as blank throughout the study for growth determination.

APPENDIX V

CALIBRATION PROCEDURE FOR ESTIMATING 2, 4-DCP AND PCMX USING HPLC SYSTEM

Chlorinated organic compound degradation was established by measuring the aromatic ring cleavage using HPLC system (model UVD 170U), Pump P680A HPG-2, Thermostat column compacter TCC-100 and Chromelon software 6.60 version. As the stationary phase, a Eurospher-100 C18 reversed-phase column with a 4-mm internal diameter and 125-mm length was used. The mobile phase was an aqueous solution of methanol water and Acetic acid (100:100:2) (Vol/Vol). The progress of the chromatography was monitored simultaneously at between 275 and 280 nm. with 32°C as the annealing temperature.

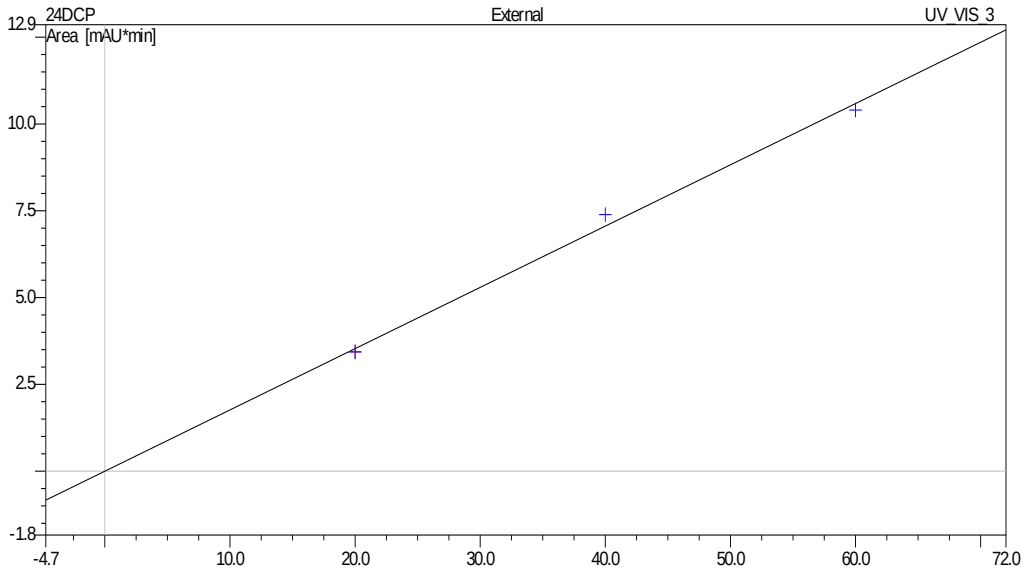


Fig. 1: Calibration curve for 2,4-DCP estimation at 280 nm

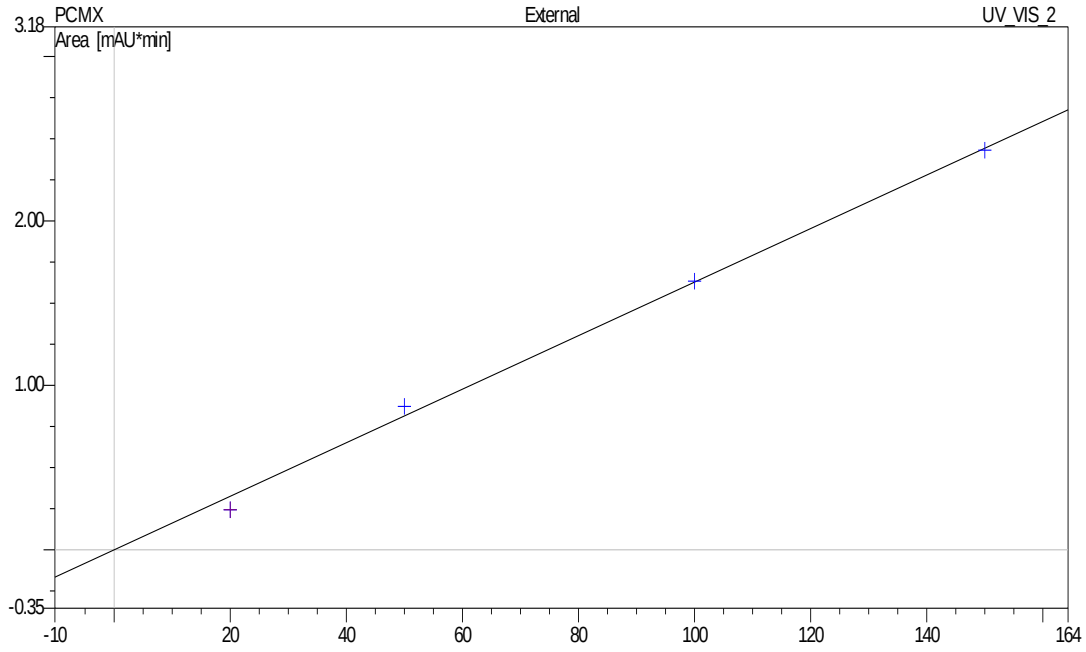


Fig. 2: Calibration curve for PCMX estimation at 280 nm

APPENDIX VI

UV INDUCED AUXOTROPHIC MUTANTS PRODUCTION AND ISOLATION OF MUTANTS BY REPLICA PLATING TECHNIQUE

An auxotroph is a bacterial mutant that requires one or more growth factors that the parent-type, or phototrophs can synthesize. A lysine auxotroph, for example, will grow on lysine supplemented media but not on a medium lacking an adequate supply of lysine because it cannot synthesize this amino acid. Auxotrophs are produced in the laboratory by treating phototrophs with mutagenic agents such as ultra violet radiation generated by a 15 or 25-W germicidal lamp or nitrosoguanidine or mitomycin C.

In 1952, Joshua Lederberg and E.M. Lederberg devised a technique called replica plating for detecting and isolating mutant strains of microorganisms. This technique is used to detect auxotrophic mutants (An auxotroph is a mutated phototroph that lacks the ability to synthesize an essential nutrient and therefore must obtain it or a precursor from its surroundings). In other words, it distinguishes between mutants and the parent strainstrain based on their ability to grow in the absence of a particular biosynthetic end product. The principal application of this technique is that it permits the simultaneous transfer of a large number of colonies from one plating medium to another in a single operation by use of a cotton velveteen cloth and one can readily examine thousands of colonies in short time, without having to pick and transfer each one separately.

This exercise deals with the production of auxotrophic mutants by UV-irradiations and isolation of lysine auxotroph of E.coli that will grow on lysine supplemented media but not on a media lacking an adequate supply of this amino acid because it does not have the ability to synthesize lysine, by the use of replica plating technique. By appropriate supplementation of the replica plate, auxotrophs of a specific type (e.g. a single amino acid, vitamin or nucleic acid precursor can be identified when used together with a highly specific mutagen such as nitrosoguanidine, this technique can yield upto 20% total auxotrophs or 1 to 2% of a particular mutant type.

In this technique, the mutants are produced by treating a culture with a mutagen. The culture containing both parent-type and auxotrophs (i.e. the mutant survivors) is plated on complete medium. After the colonies have developed, the bacteria are transferred by replica plating technique in which velveteen-covered colony transfer device is used. The sterile velveteen carrier is pressed on the plate surface to pick up bacteria from each colony. Then the velveteen carrier is pressed on the surface of other plates (i.e. plate with complete medium and other plate with minimal medium, i.e. medium minus lysine) and organisms are transferred to the same position as on the master plate. The plates are incubated for the growth of colonies and both the replica plates compared to identify the Lys $^-$auxotrophic mutant colonies.

REQUIREMENTS

- 24 hour nutrient broth culture of Escherichia coli
- Trypticase soy agar (TSA) plates
- LB broth tube
- Minimal agar plate
- Cotton velveteen cloth
- Beaker of 95% alcohol
- Bent glass rod
- Sterile 1-ml pipettes
- UV germicidal lamp (15W)
- Phosphate buffered saline (PBS)

PREPARATION OF MATERIALS

1. Preparation of LB broth and agar medium (pH 7.0):

Tryptone	10g
Yeast extracts	5g
NaCl	5g
Distilled water	1000ml

Weigh and dissolve the above constituents in distilled water. Add IM NaOH to adjust pH to 7. Pour into 250ml Erlenmeyer flasks and culture tubes. Autoclave the media at

121°C for 15 minutes. LB broth is a complex medium for rapid growth of E.coli and other members of Enterobacteriaceae.

LB agar medium:

Add 15g of agar per litre of LB broth. Pour 20 to 25ml into sterile plates.

2. Preparation of sterile velveteen carrier:

(i) Cut ordinary cotton velveteen cloth into 15cm square pieces.

(ii) Wrap them in packets of 10-20 in aluminium foil.

(iii) Autoclave them at 121°C for 15 minutes.

(iv) Secure the sterile velveteen cloth on the bottoms of sterile 400ml beakers (beaker acting as a replica block) with rubber bands or metal rings.

ASSAY PROCEDURE

A. Irradiation of E.coliculture with UV radiation:

1. Inoculate LB broth tube with E.coli and incubate it for 16 hours at 37°C.
2. Dilute the overnight culture 100 fold into fresh LB broth and grow to a cell density of 2×10^6 cells/ ml.
3. Wash the bacteria by filtration or centrifugation and suspend them in PBS to have a cell density of 2×10^6 cells/ ml.
4. Plate 0.1ml aliquots of 10^{-5} dilution in duplicate on solid LB agar medium.
5. Incubate the plates at 37°C for 24 to 48 hours.
6. Observe the plates for the development of E.coli colonies. Count the number of viable cells per ml prior to irradiation.
7. Irradiate the culture of E.coli with UV to get UV induced mutants of the bacterium as follow :-

   Pipette 5 to 6ml aliquots of bacteria suspended in PBS (Phosphate Buffer Saline) as prepared in step 3 above, at 2×10^6 cells/ ml to sterile 100mm glass petri dishes. Remove the petri dishes (as glass and plastic are UV opaque) and place these on a shaker underneath the UV light source, at a distance of 30 cm, and expose the plates for 15-20 minutes.
8. Transfer 1ml of the UV treated culture in 10 ml nutrient broth and incubate overnight to stationary phase at 37°C.

9. Pipette 0.1ml of undiluted as well as 10^1 or 10^2 fold dilutions of cultures on to nutrient agar plates i.e. complete medium.
10. Spread the organisms over the plate with a sterile bent glass rod.
11. Incubate these plates in an inverted position at 37°C for 24 hours.

OBSERVATION

Observe the plates for the appearance of colonies (parent-type and mutant survivors) which should be in the range of 100-200 colonies per plate and allow the colonies to attain 3 mm in diameter.

APPENDIX VII

SAMPLE PREPARATION FOR SCANNING ELECTRON MICROSCOPE

The culture was grown overnight in media.1 ml media was centrifuged at 10000 rpm to separate the cells .The cell pellet was mixed with 2.5% glutaraldehyde solution and incubated at 4 $^{\circ C}$ Overnight for fixation. It was then centrifuged to get the pellet. Then cells were mixed 10% alcohol and incubated for 15 min at room temperature.It was then centrifuged to separate the cells and fixed with 20% alcohol. The procedure was repeated till 100% alcohol was added .One drop of this suspension was placed on glass cover slip and allowed to dry .the cover slip was placed on the holder and gold coating was done under vacuum for 20-30 min using sputter coater

APPENDIX VIII

SAMPLE PREPARATION FOR GAS CHROMATOGRAPHY MASS SPECTROPHOTOMETER

The culture was grown overnight in media.1 ml media was centrifuged at 10000 rpm to separate the cells .The cell pellet was mixed with 2.5% glutaraldehyde solution and incubated at 4 ^{0} C Overnight for fixation. It was then centrifuged to get the pellet. Then cells were mixed 10% alcohol and incubated for 15 min at room temperature.It was then centrifuged to separate the cells and fixed with 20% alcohol. The procedure was repeated till 100% alcohol was added.One drop of this suspension was placed on glass cover slip and allowed to dry .the cover slip was placed on the holder and gold coating was done under vacuum for 20-30 min using sputter coater

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POSTER PRESENTED AT CONFERENCE

- "Studies on microbial degradation of chlorinated organic compounds by *Paenibacillusmacerans*" **Proceedings of 2nd International Conference on Biotechnology for Sustainable Development Biotechnology (green chemicals and materials, bioenergy, bio refinery and clean water)**, during 7th -9th January 2008, National Chemical Laboratory, Pune, p.53 2008.
- "Toxic effects of chlorinated organic compound and stresses on gram positive and gram negative bacteria "**AP Science Congress 2008**, during 14-16 November 2008, Osmania University, Hyderabad.

PAPER PRESENTED AT CONFERENCE

- "Studies on microbial degradation of chlorinated organic compounds by Bacillus cereus" **Proceedings of 2nd National Conference on Biotechnology for Rural and Industrial Development (NCBIRD-2008)** during 17th-19th January 2008, GulbargaUniversity, Gulbargap.60-61 2008.
- "Isolation, biochemical and molecular characterization of Chloroxylenol degrading Bacillus isolates" Proceedings of **2nd International Conference on Environment 2008 (ICENV 2008),** during 15-17 December 2008, University of Sains, Penang, Malaysia.

AWARD

- Awarded first place for poster presentation titled "Toxic effects of chlorinated organic compound and stresses on gram positive and gram negative bacteria "**AP Science Congress 2008**, during 14-16 November 2008,Osmania University, Hyderabad.

PAPER PUBLISHED

- Ranjani C., Saidutta M. B. and Srinikethan G. (2012), Application studies on untreated industrial effluent containing chlorophenol and chloroxylenol by parent and UV treated bacillus isolates, accepted in **International Journal of Industrial Biotechnology**, ISSN 2277-6109, Vol.1 No.2 2011, Pages: 105-109.

CURRICULUM VITAE

RANJANI CHITRAPUR

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EDUCATION

- **Doctor of Philosophy (Ph.D)*** in Industrial Biotechnology from National Institute of Technology Karnataka, Surathkal, Mangalore India **(CGPA = 8.5/10)** 2005.
- **Master of Science** (**M.Sc.)** in Ecology and Environmental Science Sikkim Manipal University of Health, Medical and Technological Sciences India (**First Class**), 2003-2004, **61.1%.**
- Bachelor of Science (B.Sc.) in **University College, Hampankatta, Mangalore,Karnataka India** (First class), 1994-1999, **69%.**

WORK EXPERIENCE

Total experience: 7 years

Research

- **Worked as** Water Analyst **for a period of six months at Suhas Enterprises, Water analysis, Mangalore India, 1st June 1999 to 30thNovember 1999.**
- Worked as **Lab Assistant** for a period of four monthsat Department of Chemistry, NITK, Surathkal, India, 3rd December 1999 to 10th April 2000.
- Worked as **Scientific Assistant** for a period of four yearsat Department of Chemical Engineering, NITK, Surathkal India, 20th July 2000 to 30th April 2005.

Industry

- Worked as **Chemist** for a period of eight months in M/s Primacy Industries Private Limited, Baikampady Industrial Area, Panambur, Mangalore, 25 th May 2005 to 31st December 2005.
- Working as **EHS-officer** in M/s Sequent Scientific Company *(USFDA approved API Manufacturing unit),* Baikampady Industrial Area, Panambur, Mangalore, 16th May 2010.

JOB RESPONSIBILITY

- Have experience of working in Effluent Treatment Plant.
- Have experience in Handling of ETP and STP operations.
- Have experience in Hazardous & Solid Waste Management and Disposal options.
- Have experience in monitoring water consumption and waste water recycling.
- Have experience in conducting EHS training sessions.
- Performance evaluation and trouble shooting of Pollution control facilities.

- Analysing the report of waste Water Chemistry, COD, BOD, TDS, TSS, MLSS, MLVSS.
- Have experience in Monitoring & control of Air, Water Pollution.
- Experience in interacting with Local monitoring bodies like KSPCB.
- Preparation of Annual and monthly reports.
- Have experience in assisting the reporting officer in day to day activities.

TRAINING

- Short Term Course on **"Advanced Instrumentation"** (HPLC, GCMS, IC, AAS, SEM) conducted by TIFAC-CORE in Industrial Biotechnology, NITK, Surathkal between June 22nd and 27th 2006.
- Short Term Course on **"Molecular Biology Techniques in Microbiology**" from 04-06-07 to 08-06-07 in CFTRI, Mysore, Karnataka
- Short Term Course on **"INDUSTRIAL BIOTECHNOLOGY"**conducted by AICTE/MHRD at NITK, Surathkal between 21st July -2nd August, 2008

RESEARH AREAS OF INTEREST

- Optimization of bioprocesses for the Effluent treatment operation
- Working in the interface of the microbiology and chemical engineering
- Up-scaling and Down scaling of Bioprocesses

PROJECTS

1. Studies on the coastal area environmental project funded by **Karnataka Urban Infrastructure Development and Finance Corporation (KUIDFC)**
2. Environmental Assessment studies for water, soil, noise, air of **New Mangalore Port Trust (NMPT)**
3. Phytoremediation of heavy metals using aquatic weeds.
4. Bioremediation of municipal waste.
5. Studies on microbial degradation of Chlorinated Organic Compound

ANALYTICAL AND OTHER SKILLS

- Strong skills in biochemical engineering and Microbiology
- Isolation, enrichment and maintenance of microbes from different habitats, all microbiological procedures etc.
- Knowledge in handling of instruments like GC-MS, HPLC, UV/VIS Spectrophotometer, TOC, AAS etc.
- Estimation of all environmental parameters like COD, BOD, TOC, Sulfates, Sulfides, Sulfur, Heavy metals etc.
- Administered many students (M. Tech, B.E) for their dissertation works with the help of professors
- Strong knowledge in computers required for research work.
- Planning of research work and its execution in stipulated time and preparation of manuscripts, technical and project reports.

PAPERS/POSTER PRESENTED AT CONFERENCES/WORKSHOPS

- "Studies on Ground Water Contamination due to Leachate from Municipal Solid Waste Dump Yard" in **State level symposium on current trends in Biotechnology** during 15 - 16 September, 2005 at Nitte, Karkala.
- "Studies on microbial degradation of chlorinated organic compounds by Bacillus cereus" **Proceedings of 2nd National Conference on Biotechnology for Rural and Industrial Development (NCBIRD-2008)** during 17th -19th January 2008, Gulbarga University, Gulbarga.
- "Studies on microbial degradation of chlorinated organic compounds by Bacillus cereus" Proceedings of **2nd International Conference on Biotechnology for Sustainable Development Biotechnology (green chemicals and materials, bioenergy, biorefinery and clean water)**, during 7th -9th January 2008, National Chemical Laboratory, Pune.
- "Toxic effects of chlorinated organic compound and stresses on gram positive and gram negative bacteria "**AP Science Congress 2008**, during 14-16 November 2008, Osmania University, Hyderabad.
- "Isolation, biochemical and molecular characterization of Chloroxylenol degrading Bacillus isolates" **2nd International Conference On Environment 2008 (ICENV 2008),** during 15-17 December 2008, University of Sains, Penang, Malaysia.
- "Studies on degradation of chlorinated organic compound by mutated Bacillus isolates "**Socio-Economic development Challenges before Women Scientists, Technologist and Engineers**, during 13-15 February 2009, National Institute of Technology, Rourkela, Orissa.

PROFESSIONAL PARTICIPATION

National

- **Coastal Environmental issues and problems in West Coast of Karnataka** by Environmental Management & Policy Research Institute
- **National Workshop on "**Bioseparation-Theory & Practice by Centre of Bioseparation & Technology", **Vellore Institute of Technology, Tamilnadu**
- National conference on **"Synergetic solutions for sustainable development"** December 28-30th 2005 National Institute of Technology Karnataka, Surathkal, Mangalore India
- National Workshop on "**Trends in Aerosol research climate and health"** on November 25to 26, 2005 at NITK, Surathkal.
- State level symposium on **"Current trends in Biotechnology"** on September 15 to 16, 2005 at Nitte, Karkala.
- National Symposium Biotechnology**" Bridging the Industry-Academic Gap"** on January 20-21, 2006 at St. Aloysius, Mangalore.
- National workshop on **"Recent Developments in Industrial Biotechnology"** June 16th 2006 National Institute of Technology Karnataka, Surathkal, Mangalore India
- National workshop on **"Emerging Trends in Environmental Biotechnology" (TW-ETEB-2009)** January 12th 2009 National Institute of Technology Karnataka, Surathkal, Mangalore India

RESEARCH/TECHNICAL PUBLICATIONS

International proceedings

- **Ranjani C**, M.B.Saidutta, G.Srinikethan "Studies on degradation of chlorinated organic compound by mutated Bacillus isolates "ISBN 978-93-80043-03-6, Excel India publishers.
- **Ranjani C**, M.B.Saidutta, G.Srinikethan "Studies on microbial degradation of chlorinated organic compounds by Bacillus isolates" Proceedings of 2nd International Conference On Biotechnology for Sustainable Development Biotechnology (green chemicals and materials, bioenergy, biorefinery and clean water), National Chemical Laboratory, Pune, p.53 2008.
- **Ranjani C**, M.B.Saidutta, G.Srinikethan "Isolation, biochemical and molecular characterization of Chloroxylenol degrading Bacillus isolates" Proceedings of 2ndInternational Conference On Environment 2008 (ICENV 2008)**,**during 15-17 December 2008 , University of Sains, Penang, Malaysia.

National proceedings

- **Ranjani C**, M.B.Saidutta, G.Srinikethan "Studies on microbial degradation of chlorinated organic compounds by Bacillus cereus" Proceedings of 2nd National Conference on Biotechnology for Rural and Industrial Development (NCBIRD-2008) Gulbarga University, Gulbarga, p.60-61 2008.
- **Ranjani C**, M.B.Saidutta, G.Srinikethan "Toxic effects of chlorinated organic compound and stresses on gram positive and gram negative bacteria " Proceedings of **AP Science Congress 2008** ,during 14-16 November 2008,Osmania University, Hyderabad, p.

AWARDS/ACHIEVEMENTS/HONORS

- Awarded **best poster presentation** during AP Science Congress 2008 November 14-16 2008,Osmania University, Hyderabad for paper titled "Toxic effects of chlorinated organic compound and stresses on gram positive and gram negative bacteria "
- Awarded **International travel grant** to attend an International conference on Environment 2008 (ICENV 2008)**,** during 15-17 December 2008, University of Sains, Penang, Malaysia by Department of Biotechnology, Govt. of India. New Delhi India.
- **Organizing committee member** for one day national workshop on "Recent Developments in Industrial Biotechnology"16th June 2006, Department of Chemical Engineering, NITK, Surathkal, Mangalore, India.
- **Organizing committee member** for three day national workshop on "Emerging Trends in Environmental Biotechnology" (TW-ETEB-2009) January 12th 2009 National Institute of Technology Karnataka, Surathkal, Mangalore India.
- Faced**USFDA** Audit.

COMPUTER KNOWLEDGE

- Operating Systems: All Versions of WINDOWS
- **Languages Known: C.**

PERSONNEL INFORMATION

Father's Name	:	Jagadeesh C.
Mother's Name	:	Shakunthala C.
Date of Birth	:	6th April 1978
Nationality	:	Indian
Sex	:	Female
Languages Known	:	English, Hindi, Kannada and Tulu (To Read, Write and Speak)
Address	:	"SVAJAN NIVAS", Behind Fisheries School, Chitrapur, P.O.KULAI, Mangalore, Karnataka, India-575019. Phone: +91-0824-2408073

EXTRA CURRICULAR ACTIVITIES

- Active participant of various events held at Department of Chemical Engineering, NITK, Surathkal
- Organizing event management for chemical engineering department symposia at NITK , Surathkal
- Reporter for one technical session in National Conference on Socio-Economic development Challenges before Women Scientists, Technologist and Engineers, during 13-15 February 2009, National Institute of Technology, Rourkela, Orissa.
- Basic course in Pranic Healing.

REFERENCES

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I hereby declare that the above-furnished information is true to the fullest of my knowledge.

Thanking You

(**Ranjani C.)**