CHAPTER-VI

DEGRADATION OF 2, 4, 6-TRICHLOROPHENOL (2, 4,6TCP) WITH POLYPHENOL OXIDASE (PPO) PRODUCED BY KOCURIA ROSEA AND BACILLUS CEREUS

This chapter describes about degradation of 2, 4,6TCP with KPPO, BPPO. And also discussed how process parameters like reaction time, pH, substrate concentration, enzyme concentrations will effect the degradation of 2, 4,6TCP in aqueous phase.

6.1 Review of Literature

6.1.1 Chlorophenols

Chlorophenols are used as biocides, chlorinated phenols are known to be present in drinking water, resulting either from contamination or raw water sources or from chlorination of water containing phenolic compounds. Tri chlorophenol have been detected in raw water at levels of 1-1.5µg/l and occasionally higher. Chlorophenols are well known for their taste and odour thresholds. Accepted levels up to 100µg/l in drinking water. It should be borne in mind that same chlorophenols exert toxic effects at some what higher concentration. Chemical and physical information of 2,4,6-trichlorophenol are reported in Table 6.1.1 & 2.

Table: 6.1.1 Chemical identity

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Table 6.1.2 Physical and chemical properties

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<td>Flashpoint</td>
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6.1.2 Application and disposal of trichlorophenol

6.1.2.1 Application of trichlorophenol

The trichlorophenol have been used as bactericides, algicides, molluscicides, acaricides, fungicide, and mould inhibitors, and for less specific uses, such as general antiseptics and disinfectants. Chlorophenols are also used as intermediates in the production of certain herbicides, dyes, and drugs. For example, in Sweden and Finland, chlorophenols use is severely restricted in the wood preservation or pulp and paper industries (Lindoos et al. 1987). Most chlorophenols are applied in the form of a chlorophenol-oil mixture, but some are dissolved in a "clean" carrier that can be recovered, such as methylene chloride (Jones, 1981). In contrast, the sodium salts of higher chlorophenols (particularly T₃CP, T₄CP, and PCP) are readily soluble in water.

Wood treatment: Large quantities of higher chlorophenols are used in wood preservation. In Canada in 1981, most chlorophenol-treated wood was preserved by pressure treatment with chlorophenols. These compounds have been evaluated previously (WHO, 1987b). The preservative is usually added to the glue.
**Agriculture:** Chlorophenol-treatment was widely used in agriculture, to prevent wood decay in buildings, food containers, and horticultural timbers. Recently, such chlorophenol applications have been considerably restricted in some countries (Jones, 1981). 2,4,6-trichlorophenol Precursor for higher CPs; germicide, particularly for preservation of wood, leather, glue, and textiles; intermediate in preparation of insecticides and soap germicides (From: US EPA (1979); From: US EPA (1980c)). Sodium salts of T₃CP and T₄CP have been used to inhibit microbial growth in a diverse array of products. These applications make up only a small fraction of the total consumption of chlorophenols (Jones, 1981).

**Intermediates in industrial syntheses:** chlorophenols also serve as intermediates in the production of pesticides, manufacture of some dyes and drugs.

**6.1.2.2 Disposal of trichlorophenol**

Available treatment methods for such waste should prove satisfactory, if they are carefully applied. Gravity separation is the primary treatment method most often used to recover oil and the associated chlorophenol for recycling and treatment. Organisms during secondary treatment degrade roughly 90% of most chlorophenol waste, provided that they are acclimated to the waste, and precautions are taken against shock loadings. Adsorption on activated carbon as a final clean-up step removes almost 100% of remaining waste chlorophenols in waste-streams. Incineration appears to be an effective means of disposal, if the temperatures are high enough and residence times long enough to ensure complete combustion and prevent the formation of PCDDs and PCDFs in the incinerator.
6.1.3 Environmental transport, distribution and transformation

6.1.3.1 Movement in atmospheric

While chlorophenols are considered to be primarily water and soil contaminants, atmospheric movement also occurs. Measurable quantities of chlorophenols have been detected in air, rainfall, and snow, sometimes far from obvious point sources (Paasivirta et al. 1985). Furthermore, considerable quantities of chlorophenols are released as part of incinerator emissions.

6.1.3.2 Movement in soil

Adsorption: Environmental transport of chlorophenols, particularly in soils, can be affected by adsorption on particulates. Such deposition is quite variable. Acidic soils bind chlorophenols strongly, while adsorption is minimal under alkaline conditions. Chlorophenols also adsorb on organic matter, with the result that adsorption is strong in organic soils, but low in mineral soils. (Seip et al. 1986) compared the migration rates of tritiated water and of dilute solutions (12.5-25 µg/litre) of 2,4-DCP, 2,4,6-T₃CP, 2,3,4,6-T₄CP, and PCP through packed soil columns. All of the chlorophenols migrated more slowly than water. Adsorption was moderate in a weakly acid inorganic soil and a basic soil with a higher organic content, while no chlorophenols were detected in the elute from a soil with both a low pH and a high content of organic matter. Miller & Faust (1973) confirmed that sorption of a number of phenolic compounds on organo-clay was pH-dependent.

Leaching: In instances when adsorption is minimal, leaching will be an important means of chlorophenol transport in the soil.
6.1.3.3 Movement in aquatic environments

While a large fraction of the chlorophenols entering waters is probably degraded in situ, they are nonetheless moderately soluble and fairly persistent, and so can be transported considerable distances by water (Fox & Joshi 1984). Chlorophenols that are not degraded are concentrated in the sediments, perhaps through adsorption on sediment particulates. Adsorption was quite strong on non-mineral sediments (Xie et al. 1986).

6.1.3.4 Biodegradation

Although chlorophenols are quite toxic for microorganisms in general, they are nonetheless readily metabolized by a large number that occur in soils, natural waters, sediments, and sewage sludges. 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). 2,4,6-T3CP (initially 300 mg/litre) disappeared in 7-10 days. In batch cultures enriched with 50 mg chlorophenol/litre and inoculated with soil, 2-MCP, 4-MCP, 2,4-DCP, and 2,4,6-T3CP were readily biodegraded and were often removed completely in less than 10 days. Using an acclimated, activated sludge derived from soil, Ingols et al. (1966) observed complete ring degradation of 2,4,6-T3CP in 3 days at 100 mg/litre. Aerobic microorganisms in clay loam soils were able to degrade 2, 4, 6-T3CP 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 (Baker et al. 1980; Blades-Fillmore et al. 1982; Hwang et al. 1986), sediment (Baker et al. 1980), soil (Baker et al. 1980; Pal et al. 1980), and activated sludge (Pal et al. 1980; Boyd & Shelton, 1984).
The relative rate of degradation of chlorophenols generally decreases as the number of chlorine atoms on the phenolic ring increases (Ingols et. al. 1966; Baker & Mayfield, 1980). Rates of biodegradation are further affected by the relative position of the chlorine atoms on the phenolic ring. Compounds with chlorine in the meta position are generally more stable than those without (Baker & Mayfield, 1980).

Microorganisms that have been previously exposed to a compound are usually able to metabolize it immediately when re-exposed, and at a faster rate than unexposed organisms (Pal et. al. 1980; Blades-Fillmore et. al. 1982), presumably because exposure induces the enzymes necessary to metabolize the chlorophenol. Microorganisms not previously acclimated often exhibit a lag time of as much as several days before they begin to degrade the compound (de Kreuk & Hantsveit, 1981). Similarly, prior exposure to a structurally related compound can facilitate the metabolism of chlorophenols, indicating that the enzymes induced by the original compound are somewhat nonspecific. PCP-adapted microorganisms utilize T₃CPs and T₄CPs readily (Chu & Kirsch, 1973), while bacteria raised on phenol, lower chlorophenols, or phenoxyacetic acids are able to metabolize various other lower chlorophenols (Walker, 1973; Boyd & Shelton, 1984).

Research workers have found little or no anaerobic biodegradation of chlorophenols (Baker & Mayfield, 1980; Horowitz et. al. 1982; Pignatello et. al. 1986). Sterile sediments or several inert substances enhanced the degradation of 50 μg 2,4,6-T₃CP/litre in river water (Blades-Fillmore et. al. 1982). In other reports, chlorophenol degradation in water has proceeded more rapidly (eliminated in 1-3 weeks) (Blades-Fillmore et. al. 1982; Hwang et. al. 1986). It is possible that chlorophenols are generally
degraded faster in soils and aerobic sediments than in water but, wherever a suitable combination of microflora and physical and chemical factors occurs, these general differences can be overridden.

In summary, a number of microorganisms from a variety of habitats can readily degrade chlorophenols, especially if previous exposure to these compounds has induced the enzymes necessary for their metabolism. This process is slowest with exposure to the higher chlorophenols, particularly those that are meta-substituted. The results of incubation studies in the laboratory suggest that biodegradation is most rapid in aerobic soils and sediments, and is reduced in anaerobic or nutrient-poor habitats.

6.1.4 Potential for human exposure

Humans and wildlife are exposed to aromatic pollutants in the environment, as has been shown by the detection of these compounds in samples from many different organisms. Furthermore, pollutants may be metabolized in the body, giving rise to potentially more harmful compounds. For example, PCPs and their metabolites have been found both in human breast milk and blood plasma (Huvander et al. 2002; Norén and Meironytė, 2000).

6.1.5 General population exposure

The general population is exposed to chlorinated phenols through diverse sources and routes, which have been summarized by the NRCC (1982). Chlorophenols can be ingested as contaminants in food including produce sprayed with phenolic pesticides, flesh of livestock given feed contaminated with these pesticides, and general food items, usually at mg/kg levels. In addition, sub-µg/litre quantities of chlorophenol congeners have been detected in drinking-water. These 2 routes of exposure are generally considered to be the major sources of exposure of the general population to
chlorophenols (US EPA, 1980c). On the basis of preliminary estimates from the literature of total chlorinated phenol residues in food, water, air, and miscellaneous sources, the Canadian Department of National Health and Welfare (NIHW, 1988) estimated typical non-occupational exposure to all chlorophenols to be: 6.0 μg/person per day in food; 2.8 μg/person per day in water; 1.9 μg/person per day in air; 2.0 μg/person per day from other sources; 12.7 μg/person per day in total (= 0.18 μg/kg body weight per day for 70-kg adult).

6.1.6 Occupational Exposure

The potential for both acute and long-term exposure to chlorophenols may be heavy for workers from industries using these compounds. The routes of exposure for Canadian workers have been summarized by NRCC (1982). In-service treatment of wood by painters, wood preservation workers, or telephone linemen could result in similar dermal and inhalation exposure. Employees in the chemical industry, who are involved in the manufacture of chlorophenols or their derivatives, may also be exposed to high levels. The same is true of employees in manufacturing industries that use chlorophenols as preservatives, such as the photographic, paint, textile, rubber, construction, electrical, pharmaceutical, and disinfectant industries. Finally, employees working with products containing chlorophenols may be exposed. For such occupational exposures, inhalation and dermal absorption are the major routes of uptake.

Ott et al. (1987) examined worker exposure to T₄CP at a manufacturing plant in the USA. The time-weighted average concentrations of T₄CP in the air at work locations adjacent to the reactor, salt wheel, acid wheel, and dryer, were 2.1, 2.1, 9.7, and 1.6 mg/m³ respectively. Kauppinen & Lindroos (1985) showed much lower average
atmospheric chlorophenol levels in 10 Finnish sawmills. Levels of 2,4,6-T,C,CP were measured; particularly high concentrations were noted at the machine stacking site (58 µg/m³) and the outdoor dipping site (44 µg/m³), while it could not be detected at the preparation site. 230 sawmill workers in Finland were examined for urinary levels of chlorophenols (Lindroos et al., 1987). In occupations where dermal exposure was greatest, workers (n = 112) had a median urinary chlorophenol level of approximately 1.8 mg/litre (range, 0.02-49 mg/litre) where as employees (n = 34) exposed mainly via the respiratory route had a median urinary level of 0.2 mg chlorophenols/litre (range, 0.02-3.1 mg/litre).

6.1.7 Health effects on man

As a result of the diverse range of applications of chlorophenols, there is considerable potential for human exposure to these compounds and their associated contaminants. Knowledge concerning the toxic effects of chlorophenols on people is based primarily on studies on persons employed in the chemical-manufacturing industry, wood-preservacion/protection industries, where T₃CP, T₄CP, and PCP are the major forms used (Kozak et al. 1979, and Ahlborg & Thunberg, 1980).

6.1.7.1 Acute exposure

Accidental and suicidal poisonings with commercial chlorinated phenols have been reported (WHO, 1987b), and a number of the most heavy acute exposures have resulted in death. With the support of animal studies, the signs and symptoms of acute exposure to chlorinated phenols include: convulsions (especially with less-chlorinated phenols), ataxia, mental and physical fatigue, headache, dizziness, disorientation, tachycardia, body temperature change and increased sweating. Cyanosis and asphyxia spasms shortly
precede death. Death is apparently due to cardiac arrest and is followed, at least in animals, by rapid rigor mortis, especially with T₃CP and T₄CP poisoning.

In man, the only published estimate of a minimum lethal oral dose (LD₅₀) for an: chlorophenol is for PCP (29 mg/kg body weight, approximately 2 g for an average person) (WHO, 1987b). To date, acute exposure of the general population to lower chlorinated chlorophenols has been documented only from the ICMESA plant accident in Seveso, Italy. An over-heated chemical reactor discharged a cloud containing sodium hydroxide, Na-T₃CP, and TCDD into the atmosphere, contaminating an area south of the factory containing 37,000 people (Hay, 1976; Del Como et al.1982). Within 2 weeks of the accident, toxic effects were being treated in some 500 people (Hay, 1976). The most prevalent signs of exposure were skin burns and chloracne (a persistent form of acne with keratotic follicles associated with exposure to chlorinated compounds), which was evident in 193 of the inhabitants.

6.1.7.2 Long-term exposure

Effects on skin and mucous membranes: Workers may display a variety of symptoms of chlorophenol exposure. Persons often complain of irritations of the skin, mucous membranes and respiratory tract as a result of direct airborne contact. In addition, chronic skin ailments, particularly chloracne, but also other skin lesions, ulcerations, and porphyria cutanea tarda have been reported, mainly from plants manufacturing chlorophenols for phenoxy-acetic acid herbicides. Clinical indications of liver damage and haematological and neurological effects have also been reported, particularly in association with high exposures.
A detailed study of chlorophenol exposure in sawmill in the same geographical area was carried out by Embree et al. (1984). From health histories, the only symptoms that occurred significantly more frequently in exposed workers were a productive cough and a reduced rate of forced exhalation in the "airborne" group. These symptoms could not be attributed to chlorophenol exposure, as the "dermal-plus-airborne" group were exposed to similar atmospheric chlorophenol levels and had higher levels of overall exposure, yet recorded a significantly lower incidence of productive coughing.

Alexandersson & Hedenstierna (1982) examined the effects of long-term exposure to T3CP vapour in workers at a gas-mask factory. Trichlorophenol vapour, because of its characteristic smell, was used at the factory for checking leaks in gas masks. Complaints of eye, nose, and airway irritation were voiced by 7 individuals who had been employed in testing masks for from 2 to 10 or more years. Pulmonary function tests revealed that exposed workers displayed reduced forced expiratory flow and increased closing volume in the lungs compared with controls.

**Systemic effects:** Effects on liver and kidney function and haematological parameters have also been investigated in workers exposed to chlorophenols. The findings have been generally negative. In studies on Canadian sawmill workers (Enarson et al. 1986), serum levels of creatinine, bilirubin, glutamic oxaloacetic transaminase, and alkaline phosphatase, and patient histories of jaundice, liver, kidney, and heart disease did not differ from those of the controls. Blood-leukocyte counts and haematocrit decreased, and urine-erythrocyte levels increased following chlorophenate exposure. These effects were significant only for the haematocrit and haematuria, and only for workers handling
treated lumber. Sterling et al. (1982) reported that chlorophenol-exposed sawmill workers filling out self-administered questionnaires reported significantly increased incidences of gastrointestinal, musculoskeletal, acute systemic, liver, kidney, and neurological symptoms.

Psychological and neurological effects: A range of psychological and neurological symptoms have also been associated with exposure to chlorophenols, often in association with other chemicals. Similarly, Kleu & Goltz (1971) reported that 10 persons suffering from chloracne as a result of 15 years' exposure to a T3CP formulation complained of "decreased sexual activity, easy fatigability, alcohol intolerance, and loss of interest, reduced vital psychic and intellectual capacities combined with neurasthenia and mental depression". The actual occupations of these individuals were not stated. Gilioli et al. (1983) conducted electroencephalographic analyses of workers exposed to T3CP and TCDD at the Seveso plant in Italy, site of an accident in 1976. Exposed workers generally exhibited an increased incidence of abnormal EEG tracings.

Carcinogenicity: A large number of epidemiological studies have been published concerning human cancer outcomes following occupational exposure to chlorophenols, phenoxy herbicides, and chlorinated dibenzo-\( p \)-dioxins and dibenzofurans (microcontaminants found in some chlorophenols and phenoxy herbicides). Most of these studies have been described and reviewed in several publications by the International Agency for Research on Cancer (IARC, 1979, 1986, 1987). Data from occupational mortality statistics in which exposure data are inferred only from job titles are also not included (Milham, 1985).
6.1.8 Methods for reducing toxic effects

6.1.8.1 Reducing peak absorption following exposure

Work-place exposure to chlorophenols should be minimized, and absorption of these compounds through the dermal and inhalation routes prevented, by: enclosure and automation of industrial processes that use chlorophenols, adequate ventilation of the work area, provision of appropriate protective clothing for employees working with chlorophenols, instruction of workers in the safe use and handling of chlorophenols, the importance of personal hygiene (washing before eating or smoking, showering before leaving work, and daily laundering of clothing).

The availability and use of consumer products containing chlorophenols should be reduced wherever practicable. Products containing chlorophenols should be clearly labelled by the manufacturer to alert the consumer to their toxicity and to instruct consumers in the safe use and handling of these products (WHO, 1989).

6.1.8.2 Reducing body burden

Phenol is excreted in the breath, urine, and feces. Mitigation strategies to increase urinary output and dilute the chemical once it is in the bloodstream may be useful. One method for this may be increased hydration of the individual in order to stimulate diuresis.

6.1.9 Levels monitored or estimated in the environment

Work-place air concentrations of chlorophenols are much higher. Facilities in which chlorophenols are used, such as sawmills, often have air levels of several tens of μg/m³, while in manufacturing facilities, concentrations may be in the mg/m³ range.

Residues of all chlorophenol isomers have been detected in aquatic systems. Generally, residues are present at measurable concentrations in discharges from
such sources as manufacturing plants, wood-treatment facilities, municipal waste discharges, and in the receiving waters adjacent to these sources Fox & Joshi (1984).

Similarly, in a Great Lakes survey conducted by the Ontario Ministry of the Environment (Jones, 1981), chlorophenol congeners were detected in receiving waters Folke (1984) analysed effluent from a Danish sewage-treatment plant (8 µg 2,4,6-T3CP/litre). Concentrations of chlorophenols in the effluent from a Swedish sawmill on two separate dates were, respectively: 40 and 22 µg 2,4,6-T3CP/litre (Xie et al. 1986).

Chlorophenol concentrations in sediments are for the most part much greater than those in the overlying water. This may reflect adsorption of the chlorophenols on suspended particulates in the water column, with subsequent sedimentation. For instance, Eder & Weber (1980) reported higher levels of chlorophenols. High concentrations of DCP and T2CP were present in sediments adjacent to hazardous waste dumps near the Niagara River, at maximum levels of approximately 2000 and 500 µg/kg, respectively (Elder et al. 1981). In one lake, contaminated only from sawmills upstream, concentrations of T3CP was 4.68 µg/kg of sediment, while in another lake downstream from a pulp-mill, the corresponding values was 27.7µg/kg. The third lake, further downstream from pulp and paper inputs, contained intermediate level of T3CP. Similar levels were found in Baltic Sea sediments from a site 2 km distant from a sulfate pulp-mill, these contained 0.4 µg/kg dry sediment of 2,4,6-T3CP/kg, higher levels of the same congeners were found at this location (Xie et al. 1986).

Garrett (1980) reported that soil samples from the former site of a pesticide plant in Richmond, British Columbia contained 0.18 mg T3CP/kg dry soil. Kitunen et al. (1987) determined the concentrations of chlorophenols and their contaminants in soil
near the preserving facilities at 4 different sawmills. Concentrations of chlorophenols in soil ranged from 500 to 3500 mg/kg.

6.1.10 Analytical methods

6.1.10.1 Sample collection and storage

Proper sampling and sample storage are essential prerequisites for residue determinations, particularly as picogram or nanogram quantities are often encountered in environmental samples. It is, therefore, important to minimize contamination, and to collect representative samples. Chlorophenols in the air have been collected by drawing air through an absorbent liquid at a given rate for a given period, using absorbents such as potassium carbonate (Dahms & Metzher, 1979). If a significant proportion of the chlorophenols present is likely to bind to container walls, as occurs with water samples, glass containers are preferable to plastic ones (Kozak et al. 1979). The American Public Health Association (Greenberg et al. 1985) recommends preserving waste-water samples containing phenolic compounds by acidification with phosphoric acid and treatment with copper sulfate, prior to refrigeration.

6.1.10.2 Sample preparation and analysis

The early procedures used to analyse for chlorophenols were reviewed by Bevenue & Beckman (1967). Most were colorimetric techniques, the most popular being the 4-aminoantipyrine method; none of the methods was either very specific or sensitive. They are no longer widely used. Instead, more sophisticated analytical techniques are being increasingly used, including thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC), ion exchange chromatography, infrared (IR) and ultraviolet (uv) spectroscopy, mass spectrometry (MS).
6.1.11 Regulations and advisories

EPA listed TCP as a hazardous pollutant. A guideline value of 10 µg/litre was recommended by WHO (WHO, 1984) for 2,4,6-trichlorophenol in drinking-water, based on animal carcinogenicity data using a conservative mathematical model. In the supporting documentation for this guideline value (WHO, 1985), it was noted that the taste threshold level for 2, 4, 6-T₃CP was 1.0 µg/litre and that, on the basis of aesthetic qualities, the level would be 0.1 µg/litre. The National Toxicology Program has classified 2,4,6-T₃CP chemical is reasonably anticipated to be a human carcinogen (NTP 2005). The International Agency for Research on Cancer has classified 2,4,6-T₃CP as Group 2B carcinogen (IARC 2005)
6.2 Materials and Methods

2,4,6TCP was purchased from sigma chemicals (99.9% purity). The aqueous solution of the 2,4,6TCP was prepared prior to the experiments by dissolving the requisite amount of 2,4,6 TCP in double distilled water.

6.2.1 Enzyme production:

0.5 ml of 24 hr culture of *Bacillus cereus* and *Kocuria rosea* in basal medium was used to inoculate 500 ml Erlenmeyer flasks containing 100 ml of phenol containing mineral media. (Mineral media A: 0.2 g MgSO₄.7H₂O, 0.05 g CaCl₂.2H₂O, 0.5 g KCl and 1 g NH₄Cl per liter. Mineral Media B: 2.5 g FeSO₄.7H₂O, 1.5 g EDTA, 1.0 g CuSO₄.5H₂O, 2.5 g ZnSO₄.4H₂O, 2.5 g MnSO₄.4H₂O, 0.5 g CoCl₂.6H₂O and 1.0 g H₃BO₃ [pH 6.8-7.4]). 1 ml of Mineral media B was added to 999 ml of mineral media A and made up to 1 liter to this 1g phenol was added, mineral media pH maintained at neutral conditions (pH 6.8 – 7.4). And incubated for 48 hours at 25°C, 150 rpm.

6.2.2 Extraction of enzyme

The culture was centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant obtained was lyophilized and used as crude enzyme preparation for degradation studies.

6.2.3 Analytical methods

6.2.3.1 Quantitative estimation of the 2,4,6TCP

Quantitative estimation of the 2,4,6TCP in the aqueous phase was carried out by UV-visible spectrophotometer. A solution of 2 mg/l. concentration was scanned over a range of 190-800nm by using UV-visible spectrophotometer and λₘₐₓ (wave length) was determined as 292nm. The spectral curve was prepared at maximum wave length, λₘₐₓ was used for the estimation of the 2,4,6TCP concentration in aqueous phase. After the
treatment, the sample was centrifuged and the supernatant was assayed for the residual 2, 4,6TCP concentrations.

High performance liquid chromatography (HPLC) was employed to understand the 2, 4,6TCP removal during the enzyme catalysed treatment. HPLC (Agilent 1100 series) instrument with binary pump system, multiwave length UV-Visible detector; manual injection port with fixed loop system; reverse phase column (ZORBAX SB-C18, 4.6X250 mm length column packed with 5 \(\mu\)m particle size) was used for the 2, 4,6TCP estimation. 2, 4,6TCP and degradation products were analysed at 292 nm. Acetonitrile and water in the ratio of 80:20 respectively was used as a mobile phase with a flow rate of 1 ml/min.

6.2.3.2 Estimation of protein content by lowery method (Lowry et al. 1951)

Bovine serum albumin (1\(\mu\)g/1\(\mu\)l) was used as a standard. Aliquots of 0.2 ml, 0.4 ml, 1.0 ml of BSA is taken in test tubes and make up to 1.0 ml with distilled water. A test tube with 1 ml of the sample (culture filtrate) was taken. 5 ml of solution C [100 ml of Solution A: 0.4 g of NaOH and 2 g of Na\(_2\)CO\(_3\) in 100 ml of distilled water + 1 ml of Solution B: 0.05 g of CuSO\(_4\) and 0.1 g Sodium potassium tartarate dissolved in 1% (w/v) aqueous solution (to be prepared fresh)] was added to all the test tubes and vortexed. These are incubated for 15 minutes at room temperature. 0.5 ml of 1N Folins reagent was then added to all the test tubes. After vortexing they were incubated for 30 minutes at room temperature. A test tube of 1.0 ml of water with the solution C and 1N Folins reagent was kept as control. The absorbance was then observed at 750 nm taking the control as blank. A standard graph was plotted and the concentration of protein in culture filtrate was estimated from the graph.
6.2.3.3 Qualitative check for PPO in crude extract

Nutrient agar plates contained colour indicator remazol brilliant blue(0.25 mg remazol brilliant blue(C₄₅H₄₄N₃NaO₇S₂) dissolved in 50 ml of methanol+ 40 ml of water+10 ml of glacial acetic acid) were used for screening of polyphenol oxidase. In that agar plates Bacillus cereus, Kocuria rosea was inoculated. Inhibition zone was formed around the inoculum indicates polyphenol oxidase positive (Laura-Leena Kiiskinen et.al.2004)

6.2.3.4 Quantitative check for PPO in crude extract

Polyphenol oxidase activity was determined by Christiane Galhaup et.al.(2002) method with using 2, 3-azino-bis (3-ethylbenzthiazoline-6-sulfonate) [ABTS] as the substrate. The assay mixture contained 1mM ABTS, 20mM sodium acetate buffer (pH3.5), and 10μl aliquots of enzyme extract. Oxidation of ABTS was monitored by following the increase in absorbency at 436 nm. One unit of laccase activity was defined as amount of enzyme required to oxidize 1μmol of ABTS per min at 25°C.

6.2.4 Experimental methodology

6.2.4.1 Removal of 2, 4,6TCP in aqueous phase by crude enzyme

Experiments were conducted at a constant temperature by varying processes parameters such as pH, 2, 4,6TCP concentration, enzyme concentration and reaction time.

6.2.4.2 Optimum contact time

Initially kinetics were carried out in a series of vials containing 20 mg/l 2,4,6TCP ,by maintaining the aqueous phase pH at 7.0,enzyme concentration at 1 ml .The reaction mixtures in the vials were kept for agitation on a horizontal shaker at 100 rpm for the requisite concentration .In aqueous phase after centrifugation at 5000 rpm for 5 min ,At
room temperature. Each vial was removed at a predetermined time and residual 2,4,6TCP concentration in aqueous phase was estimated to determine the optimum contact time.

6.2.4.3 Optimum pH

pH optimization studies were carried out at what pH optimum removal of 2,4,6TCP was observed. By varying the aqueous phase pH of the reaction mixture between 2 and 10 by keeping the all other reaction conditions constant i.e. reaction temperature, 2, 4,6TCP concentration, enzyme concentration and reaction time.

6.2.4.4 Optimum substrate concentration

To assess out the optimum substrate concentration the amount of enzyme concentration was kept constant and the concentration of substrate was gradually increased till the reaction reaches its maximum and attains the equilibrium state. Reaction temperature, pH, enzyme concentration, contact times were kept constant.

6.2.4.5 Optimum enzyme concentration

Experiments were also carried out to find out the optimum enzyme concentration required to bring out the degradation of 2, 4,6TCP. By varying enzyme concentration between 0.25 to 1.5ml and keeping all the other experimental conditions like reaction temperature, pH, 2, 4,6TCP concentration and contact time were constant.
6.3 Results and Discussion

This section describes about degradation of 2,4,6TCP in aqueous phase by crude KPPO, BPPO. And also discussed about effect of various operation parameters like contact time, pH, substrate concentration, enzyme concentrations on degradation of 2,4,6TCP in aqueous phase.

6.3.1 Removal of phenol in aqueous phase by crude enzyme

6.3.1.1 Optimum contact time

Initially experiments were conducted to assess the optimum contact time required for 2,4,6TCP removal. To a series of vials containing 5 ml of 2,4,6TCP solution (20mg/L) and 1ml of crude enzyme at pH 7.0, the mixture was kept for agitation in horizontal shaker at room temperature.

From figure 6.3.1 it is evident that 105-120 minutes contact time was sufficient to achieve 100% removal of 2,4,6TCP by crude enzymes of *Kocuria rosea* (KPPO) and *Bacillus cereus* (BPPO). From 0-120 minutes there is a gradual increase in the percentage removal of 2,4,6TCP by these enzymes. But in different periods the percentage removal rate varies differently.

For the first 15 minutes the removal rate of 2, 4,6TCP by two crude enzymes *Kocuria rosea* and *Bacillus cereus* was observed to be 31% and 28% respectively. For next 15-30 minutes there is an increase of 18 and 14% in the rate of removal of 2,4,6TCP by *Kocuria rosea* and *Bacillus cereus* respectively. From 30-45 minutes there is an increase of 17 and 13% in the rate of removal of 2,4,6TCP by both enzymes respectively. From 45-90 minutes duration there is an increase of rate of removal by 36%, after that 90-105 minutes only 9% increase by *Bacillus cereus* crude enzyme
was observed. From 45-75 minutes duration there is an increase of 18% in the rate of removal, after that 75-90 minutes duration 14% rate of removal and 90-105 minutes only 2% increase in rate of removal of 2,4,6TCP by *Kocuria rosea* crude enzyme was achieved.

![Graph showing the influence of reaction time on removal of 2,4,6-trichlorophenol in the presence of crude enzymes of *Kocuria rosea* and *Bacillus cereus*](image)

**Fig 6.3.1:** Influence of reaction time on removal of 2, 4, 6-trichlorophenol in the presence of crude enzymes of *Kocuria rosea* and *Bacillus cereus*.

### 6.3.1.2 Optimum pH

The enzyme has an optimum pH range at which their activity is maximum. The studies were carried out on the 2,4,6TCP by varying the aqueous phase pH of the reaction mixture between 2-10 at 20 mg/L 2,4,6TCP concentration and 1 ml of crude enzyme at the contact time of 120 minutes. Variation of rate of removal of 2, 4,6TCP at various pH values is depicted in figure 6.3.2.
From figure 6.3.2 it is evident that pH 7.0 is sufficient for achieving optimum (100%) removal of 2, 4,6TCP. Good removal of 2, 4,6TCP was also achieved at pH 5-8 (i.e. 90% rate of removal) by *Kocuria rosea* crude enzyme. Optimum (100%) removal of 2, 4,6TCP was achieved at pH 6. 90% rate of removal of 2,4,6TCP was also achieved at pH 4-7 by *Bacillus cereus* crude enzyme. The enzymes polyphenol oxidases from bacteria, *B. licheniformis*, *B. natto*, and *B. sphaericus*, are useful for oxidizing and bleaching a variety of substrates including phenolic compounds at an optimum pH of 7.0 (United States Patent 6184014).

Results indicate that below pH 4.0 and above pH 8.0 a sudden fall in the rate of removal of 2, 4,6TCP was observed.

![Graph](image)

**Fig 6.3.2:** Influence of pH (2-10) on removal of 2, 4, 6-trichlorophenol in the presence of crude enzymes of *Kocuria rosea* and *Bacillus cereus*.
6.3.1.3 Optimum substrate concentration

The concentration of substrate present in the aqueous phase has significant influence on any enzyme mediated reaction. If the amount of enzyme concentration is kept constant and the substrate concentration is gradually increased, the reaction will increase until it reaches maximum. After attaining the equilibrium state, any further addition of the substrate will not change the rate of reaction. Studies were carried out at different concentration of the 2, 4,6TCP (20-120mg/L) keeping the other parameters constant.

From figure 6.3.3 it is evident that at 20 mg/L of 2,4,6TCP was the optimum substrate concentration for 1ml Kocuria rosea and Bacillus cereus crude enzyme. From 40-120 mg/L concentration there is gradual decrease in the percentage removal rate of 2,4,6TCP by both these enzymes. At 40 mg/L concentration there has been a rate of removal of 86% and 81%. At 60 mg/L concentration there has been a rate of removal of 71% and 65% by crude enzymes of Kocuria rosea, Bacillus cereus respectively. At 80 mg/L concentration of 2,4,6TCP concentration there has been 59% and 52% removal by both the enzymes. Percentage reduction in the removal rate is approximately 20% in both cases. At 100mg/L and 120mg/L there is a 37% and 25% removal was observed in case of Kocuria rosea crude enzyme. Kocuria varians strain having the highest resistance to chlorine (Leriche et.al.2003). 32% and 18% removal was observed in case of Bacillus cereus crude enzyme. Degradation of 2, 4-DCP (2, 4-dichlorophenol) was studied using this culture in liquid medium under aerobic conditions, at initial concentrations of 20-560 µM concentration of 2, 4-DCP. The 2, 4-DCP degradation rates by B. cereus GN1 could be determined at concentrations up to 400 µM. However, higher concentrations of 2, 4-DCP (560 µM) were inhibitory to cell growth (Galina Matafonova et. al. 2006)
6.3.3 Influence of substrate concentration on removal of 2, 4, 6-trichlorophenol in the presence of crude enzymes of \textit{Kocuria rosea} and \textit{Bacillus cereus}.

### 6.3.1.4 Optimum enzyme concentration

Generally as the concentration of enzyme increases the rate of removal of substrate also increases. At optimized experimental conditions by achieving the 100% removal of substrate at certain enzyme concentration, after that any increase in enzyme concentration there was decrease in reaction time.

From figure 6.3.4 it is evident that at 1.25 ml enzyme concentration is sufficient for optimum (100%) removal of 2,4,6TCP, at 1.5ml of enzyme concentration also 100% removal was observed by \textit{Kocuria rosea}, \textit{Bacillus cereus} crude enzymes respectively. At 0.25 ml 58% and 42% and at 0.5 ml crude enzyme concentration 74% and 69% removal was achieved by both \textit{Kocuria rosea} and \textit{Bacillus cereus} crude enzymes respectively. At 0.75 ml and 1 ml crude enzyme concentration 86 and 92% rate of removal was achieved.
with *Kocuria rosea* crude enzyme and 78%, 89% rate of removal was achieved with *Bacillus cereus* crude enzyme.

**Fig 6.3.4:** Influence of crude enzymes of *Kocuria rosea* and *Bacillus cereus* concentration on removal of 2, 4, 6-trichlorophenol

### 6.3.2 HPLC analysis

HPLC profile of the control sample (2, 4, 6 TCP) (a) shows a peak at a 2.93 retention time at 292 nm (Fig 6.3.5). After crude enzymes treatment, the HPLC profile of the 2, 4,6TCP shows no peak at 2.93 retention time. But seven peaks were observed at the retention time of 3.71, 3.88, 4.14, 4.92, 5.64, 7.02, 8.27 in case of *Kocuria rosea* and four peaks were observed at the retention time of 1.99, 3.23, 3.43, 3.91 at 292nm in case of *Bacillus cereus* (Fig 6.3.6;6.3.7). These results indicate the possible breakdown of the parent molecule. Comparison of HPLC chromatogram of enzyme treated samples with control showed enhanced 2, 4,6TCP degradation due to *Kocuria rosea* and *Bacillus cereus* crude enzymes catalyzed treatment processes.
Proposed pathway for the degradation of 2,4,6-trichlorophenol (Tai Man Louie et al.):

Fig 6.3.5: HPLC profile of 2,4,6TCP at 292 nm

Fig 6.3.6: HPLC profile of *Kocuria rosea* crude enzyme treated 2,4,6TCP sample.
6.3.3 Conclusions

1. Degradation of 2, 4,6TCP occurs effectively with KPPO and BPPO.

2. KPPO was more promising enzyme to degrade 2, 4,6TCP in aqueous phase than BPPO.

3. Optimized process parameters for removal of 2,4,6TCP were 105 minutes contact time, 20 mg/L substrate concentration, 1.25 ml of crude enzyme and pH 7 for KPPO and pH 6 for BPPO

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**Table 6.3.7:** HPLC profile of *Bacillus cereus* crude enzyme treated 2, 4,6TCP sample at 292 nm

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6.4 References


