BACTERIAL DEGRADATION OF 2,4-DICHLOROPHENOL: CATABOLIC GENES DETECTION AND ENZYME CHARACTERIZATION

by

BOITUMELO SETLHARE

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School of Life Sciences

College of Agriculture, Engineering and Science

University of KwaZulu-Natal (Westville Campus)

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PREFACE

The research contained in this thesis was completed by the candidate while based in the Discipline of Microbiology, School of Life Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville Campus, South Africa. The research was financially supported by National Research Foundation (NRF).

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

Supervisor: Dr M. P. Mokoena

_____

Signed:

Date: 16 August 2017

Co-Supervisor: Prof A. O. Olaniran

_____

Signed:

Date: 16 August 2017

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

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ABSTRACT

The widespread use of chemicals and their frequent release into the environment is the major cause of pollution, worldwide. Chlorophenolic compounds, such as 2,4-dichlorophenol (2,4-DCP), are classified as priority pollutants due to their recalcitrance, persistence and toxicity. They are also of environmental and human health concern due to their carcinogenic and mutagenic properties. The aims of this study were: to isolate 2,4-DCP degrading microorganisms indigenous to contaminated sites in Durban, South Africa, and establish their degradation potential, to detect the presence of 2,4-DCP catabolic genes in selected isolates, and to characterize the enzymes involved in the degradation process. Following enrichment in mineral salt medium (MSM) supplemented with 2,4-DCP (40 ppm) as the sole carbon and energy source, three 2,4-DCP degrading bacteria were isolated and identified as *Pseudomonas chlororaphis* strain UFB2, *Klebsiella pneumoniae* strain KPNIH39 and *Klebsiella pneumoniae* strain DHQP1002001 based on the 16S rRNA gene sequence analysis. These isolates were able to degrade between 49.01% and 75.11% of 2,4-DCP within 10 days, with the degradation rate constant ranging between 0.07 and 0.14 mg/L/d. The PCR amplification of the catabolic genes involved in 2,4-DCP degradation revealed the presence of the phenol hydroxylase (600 and 715 bp), catechol 1,2-dioxygenase (467 and 507 bp), muconate isomerase (651 and 494 bp), *cis*-dienelactone hydrolase (491 and 567 bp), and *trans*-dienelactone hydrolase (491 and 567 bp) in *Pseudomonas chlororaphis* and *Klebsiella pneumoniae* respectively. The absence of catechol 2,3-dioxygenase gene in these isolates suggests that the organisms most likely follow *ortho*-pathway for 2,4-DCP degradation. This was further confirmed by enzyme assays from the crude extracts of the isolates. The extracts showed specific activities (in mU/mg protein) of 21840, 15630, 1480, 2340 and 1490 for phenol hydroxylase, catechol 1,2-dioxygenase, muconate isomerase, *cis*-dienelactone hydrolase and *trans*-dienelactone hydrolase, respectively. The crude extracts did not show any catechol 2,3-dioxygenase activity. Phenol

hydroxylase (61.6 kDa) and catechol 1,2-dioxygenase (35 kDa) enzymes were purified from the crude extracts of *Pseudomonas chlororaphis* UFB2 by anion exchange (1mL HiTrap ANX column) and gel filtration (Sephacryl HR100) chromatography to a purification fold of 1.6 and 1.5, and a yield of 57.69% and 13.02%, respectively. Phenol hydroxylase was observed to be optimum at pH 8 and temperature of 30°C while catechol 1,2-dioxygenase was optimum pH 7.5 and optimum temperature of 30°C. The structural predictions for phenol hydroxylase and catechol 1,2-dioxygenase was conducted by tryptic digestion and mass spectrometry and showed two models for each enzyme. Models for phenol hydroxylase were 2-hydoxybinyl-3-monooxygenase (19.18%) and 3-hydroxybenzoate hydroxylase (19.53%). Models for catechol 1,2-dioxygenase were homogentisate 1,2-dioxygenase (81.06%) and 3 oxalate decarboxylase oxide (14.62%). The biophysical properties of phenol hydroxylase from *Pseudomonas chlororaphis* UFB2 showed that it is not stable and it is highly hydrophilic in water while catechol 1,2-dioxygenase was stable and highly hydrophilic.

This study will therefore assist in contaminated sites and alleviate pollution of chlorophenolic compounds in the environment.

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CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

An increasing population growth results in modern technologies being developed to sustain the evolving human needs. In industries, chemicals are produced daily to improve agricultural productivity and for domestic uses, but once they are released, some of them become toxic to the environment (Igbinosa *et al*., 2013; Arora and Bae, 2014; El-Naas *et al*., 2017). Chlorophenolic compounds are some of the leading chemical pollutants that are found at high levels in the environment (Michałowicz and Duda, 2007; Igbinosa *et al*., 2013; Arora and Bae, 2014; Hubbe *et al*., 2016). The United States Protection Agency (US EPA) has classified chlorophenols as priority pollutants due to their carcinogenic, mutagenic and teratogenic properties (Olaniran and Igbinosa, 2011; Igbinosa *et al*., 2013). The release of these compounds in the environment is due to accidental spillage, anthropogenic activities and negligence from petrochemical companies (Jain *et al*., 2011; Varsha *et al*., 2011; Sihag *et al*., 2014).

Due to their high levels in the environment and their toxicity, it is imperative to develop methods for the degradation of chlorophenols compounds (Olaniran and Igbinosa, 2011; Igbinosa *et al*., 2013; Hubbe *et al*, 2016). Physical and chemical methods have been used, however, biological degradation has been the most attractive method due to its effectiveness, cost effectiveness and the fact that it is environmentally friendly (Olaniran and Igbinosa, 2011; Arora and Bae, 2014; Herrera *et al*., 2015). There are 19 different chlorophenols that have been discovered in the environment, and they differ in the amount and position of the chlorine atom. The mostly commercialized ones are 2-chlorophenol (2-CP), 4-chlorophenol (4-CP), 2,4-dichlorophenol (2,4-DCP), 2,4,6-trichlorophenol (2,4,6-TCP) and pentachlorophenol (PCP) (Gaofeng *et al*., 2004; Olaniran and Igbinosa, 2011; Igbinosa *et al*., 2013). This study focused on 2,4-DCP, a derivative of the mostly used herbicide, 2,4-Dichlorophenoxyacetic acid (Arora

and Bae, 2014; Kumar *et al*., 2014). This compound has been classified as a leading priority pollutant and has been detected in water, soil and air at high concentrations (Igbinosa *et al*., 2013; Lamastra *et al*., 2016). Bioremediation has been employed to degrade this compound and different microorganism have been used such as *Pseudomonas* spp., *Rhodococcus* spp., *Candidas tropicalis*, *Klebsiella* spp. and *Bacillus* spp. (Vidali, 2001; Watanabe *et al*., 2002; Gallizia *et al*., 2003; Quan *et al*., 2004; Elkarmi *et al*., 2009; Herrera *et al*., 2015). These microorganisms produce enzymes that facilitate the degradation of these compounds via different metabolic pathways (Arora and Bae, 2014; Hou *et al*., 2016).

There are three different metabolic pathways involved in the degradation of 2,4-DCP; i.e. *ortho-*, *meta-* and *hydroquinone*-pathways. However, most 2,4-DCP degrading organisms have been reported to follow *ortho-* and *meta*-pathways (Gaofeng *et al*., 2004; Seo *et al*., 2009*;* Sridevi *et al*., 2012). The *ortho*-pathway is facilitated by the enzyme catechol 1,2-dioxygenase encoded by gene *C120* and the *meta*-pathway is facilitated by catechol 2,3-dioxygenase encoded by gene *C230* (Lillis *et al*., 2010; Silva *et al*., 2012). An increasing 2,4-DCP pollution in developing countries such as South Africa makes it a priority to remove pollutants from the environment. Thus, the aims of the study were: to isolate 2,4-DCP degrading microorganisms and analyze their degradation potential, phylogenetic relationships, to determine the metabolic pathway(s) followed by microorganism during the degradation; and detection of the catabolic genes and enzymes.

1.1 Chlorophenols in the environment

1.1.1 Production of chlorophenols and their release into the environment

The continuous increase of the world population has been linked to pollution in the environment. Due to urbanization, increased industrial, agricultural and domestic utilization, industrially produced chemical substances have become essential in the daily lives of humans and are widely used for various economic activities (García-Peña *et al*., 2012; Arora and Bae, 2014; Sihag *et al*., 2014). The chemicals are widely used and their accumulation in the environment is due to anthropogenic activities, toxic waste disposal, accidental spillage, storage tanks and municipal landfills (Sharma *et al*., 2013). The most dominant chemical pollutants widespread in the environment are phenolic compounds and their intermediates. They are recalcitrant and persistent in nature. These properties have made them to be classified as priority pollutants (Gallizia *et al.*, 2003; Lin *et al*., 2008; Cao *et al*., 2009; Liu *et al*., 2012; Mahiudddin *et al*., 2012; Olaniran and Igbinosa, 2011; Nweke *et al*., 2014).

Chlorophenols have many applications in industries, agriculture, also with wide domestic and forestry utilization. Previously, many products have been produced that have chlorophenol compounds, such as herbicides, insecticides, fungicides, detergents, dyes, resins, pharmaceutical drugs and wood preservatives (Lin *et al*., 2008; Olaniran and Igbinosa, 2011). Anthropogenic activities and many mega-corporations such as petroleum processing entities, coke conversion and steel manufacturing disposal have also led to the introduction of chlorophenols in the environment resulting in high levels of pollution (Raung, 1984; Lin *et al*., 2008; Olaniran and Igbinosa, 2011; Arora and Bae, 2014).

A study conducted by Jain *et al*. (2011) reported that although there is widespread pollution of the compounds in the environment, in wastewater there are multiple processes and operations

that eliminate the pollutants resulting in the water that is free of the compounds. However, this is contrary to the study that was conducted by Kargi and Cikla (2006), where it was found that some of the treated effluents from wastewater had pollutants. Chlorophenols are ubiquitous in nature and they are commonly found in many areas this includes water, soil and sediments (Michałowicz and Duda, 2007; Igbinosa *et al*., 2013; Patel and Kumar, 2016). This results in severe environmental problems due to the recalcitrant nature of chlorophenols (Kumar *et al*., 2011; Igbinosa *et al*., 2013). It has been reported by Ivanciuc *et al*. (2006) that the broad use of many compounds in industry, agriculture, health care, and household is the main source of soil and water contamination. El-Naas *et al*. (2017) reported that the major sources of chemical pollution are chlorophenols, which are weak acids that differ due to their chemical and physical properties. They are all solids except 2-chlorophenol which is liquid, and the number of chlorine atoms attached to the molecule determines their toxicity and solubility. Their structures and the number of chlorine atoms play a vital role on their dissociation constants (K_a). The dissociation constant (K_a) and partition coefficient ($K_{o/w}$) of chlorophenols are important factors that are responsible for distribution of the compounds in the environment by determining their absorption and solubility shown in Table 1.1.

Table 1.1: Physiochemical properties of chlorophenols (El-Naas *et al*., 2017)

Number	Compounds	Formula	Molecular weight	Boiling point (°C)	Melting point (°C)	Solubility at 20°C (gl^{-1})	p*Ka*	Log*Ko/w*
1	2-Chlorophenol	C_6H_5ClO	128.56	174.9	9.3	28	8.3–8.6	2.12–2.17
2	3-Chlorophenol	C_6H_5ClO	128.56	214	33–34	26	8.8–9.1	2.48–2.50
3	4-Chlorophenol	C_6H_5ClO	128.56	217–219	42–44	27	9.1–9.4	2.35–2.44
4	2,3-Dichlorophenol	$C_6H_4Cl_2O$	163.00	206	57–58	NA	6.4–7.8	3.15–3.19
5	2,4-Dichlorophenol	$C_6H_4Cl_2O$	163.00	210	45	4.50	7.5–8.1	2.75–3.30
6	2,5-Dichlorophenol	$C_6H_4Cl_2O$	163.00	211	58–59	NA	6.4–7.5	3.20–3.24
7	2,6-Dichlorophenol	$C_6H_4Cl_2O$	163.00	219	68	NA	6.7–7.8	2.57–2.86
8	3,4-Dichlorophenol	$C_6H_4Cl_2O$	163.00	253–254	65–68	NA	7.4–8.7	3.13–3.44
9	3,5-Dichlorophenol	$C_6H_4Cl_2O$	163.00	233	68	NA	6.9–8.3	2.57–3.56
10	2,3,4-Trichlorophenol	$C_6H_3Cl_3O$	197.45	Sublimes	77-84	0.22	6.5–7.7	3.49–4.07
11	2,3,5-Trichlorophenol	$C_6H_3Cl_3O$	197.45	248–255	57–62	0.22	6.8–7.4	3.84–4.56
12	2,3,6-Trichlorophenol	$C_6H_3Cl_3O$	197.45	246	58	NA	3.49–4.07	3.88
13	2,4,5-Trichlorophenol	$C_6H_3Cl_3O$	197.45	Sublimes	67-70	0.948	7.0–7.7	3.72–4.10
14	2,4,6-Trichlorophenol	$C_6H_3Cl_3O$	197.45	243-249	69	0.434	6.0–7.4	3.60–4.05
15	3,4,5-Trichlorophenol	$C_6H_3Cl_3O$	197.45	271–277	101	NA	7.7–7.8	4.01–4.39
16	2,3,4,5-Tetrachlorophenol	$C_6H_2Cl_4O$	231.89	Sublimes	116–117	0.166	6.2–7.0	4.21–5.16
17	2,3,4,5-Tetrachlorophenol	$C_6H_2Cl_4O$	231.89	150	70	0.183	5.3–6.6	4.10–4.81
18	2,3,5,6-Tetrachlorophenol	$C_6H_2Cl_4O$	231.89	188	114–116	0.100	5.2–5.5	3.88–4.92
19	Pentachlorophenol	C_6Cl_5OH	266.34	300	190	0.014	4.7–4.9	5.01–5.86

1.1.2 Chlorophenols in water, air and soil

1.1.2.1 Water

The World Health Organization (WHO) reports show that the scarcity of water affects about 40% of the world population and this is partly due to economic, political factors and the climate of the particular environments (Koop and van Leeuwen, 2017). Developing countries tend to be victims of water shortage and unhygienic water due to the pollutants in the water (Koop and van Leeuwen, 2017). Chlorophenols have been introduced to the environment as effluents from different industrial processes, anthropogenic activities and industrial process, such as water disinfection and pulp bleaching with chlorine (Olaniran and Igbinosa, 2011; Sartori, 2012; Mohammadi and Sabbaghi, 2014; Stepanowa *et al*., 2014). Their high solubility, persistence and toxicity have led to the pollution of water bodies (Anju *et al*., 2010; Sartori, 2012). They are found in natural water, ground water, coastal seawater, ocean water and wastewater. Due to their high sorption and lower desorption properties, they persist in the environment (Li *et al*., 2013; Hassine *et al*., 2015; Duran and Cravo-Laurea, 2016). As a result, there are different quantities and qualities of chlorophenols that have been discovered in lakes and rivers (Diez, 2010; Al-Janabi *et al*., 2011; El-Naas *et al*, 2017). This has been shown in the experiments that were conducted in Canadian surface waters (Czaplicka, 2004; Sartori, 2012). Studies have shown that even at low concentrations, chlorophenols that are residues in water consumed by humans impart a peculiar taste and odour when formed due to the chlorination cleaning process of drinking water (Alam *et al*., 2007; Santana *et al*., 2009; Al-Janabi *et al*., 2011; Mohammadi and Sabbaghi, 2014). The concentration of phenolic compounds that are allowed in the water bodies has been reported to be 1 mg/L (Mohite *et al*., 2010). Singh and Srivastava, (2002) reported that there are different concentrations of various chlorophenols in drinking water. However, Santana *et al*. (2009) reported that the standard maximum concentration of 0.5 µg/L chlorophenols is acceptable for drinking water and this standard was set by the European Union

Council (EU) Directive 2455/2001/EC (Sarafraz-Yazdi *et al*., 2012). There have been many international standards and regulations regarding the acceptable concentrations of the chlorophenols that can be allowed in clean water and this differs in each country (Al-Janabi *et al*., 2011; Olaniran and Igbinosa, 2011). The concentration of the chlorophenols also differs in reservoirs where they are found (Czaplicka, 2004; Seo *et al*., 2009; Durruty *et al*., 2011; Olaniran and Igbinosa, 2011). The presence of chlorophenol compounds such as 4-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol was detected in surface water in the other rivers (Michałowicz and Duda *et al*., 2007; Igbinosa *et al*., 2013). Most studies have reported the presence of pentachlorophenol in drinking water, although detected at low concentrations (Czaplicka, 2004; Olaniran and Igbinosa, 2011).

1.1.2.2 Air

The release of manufactured chemicals, gunpowder, combustion and fireworks leads to pollution of air. Chlorophenols are present in the atmospheric air as vapour or smoke (Czaplicka, 2004; Michałowicz and Duda *et al*., 2007). The US-EPA has reported the total emission of the compounds in air and the most common chlorophenol was pentachlorophenol (Czaplicka, 2004; Durruty *et al*., 2011; Igbinosa *et al*., 2013). Monochlorophenols and dichlorophenols such as 2-chlorophenol and 2,4-dichlorophenol are highly volatile. They react with the photochemically produced free radicals and this results in high pollution of atmospheric air (Olaniran and Igbinosa, 2011; Trivedi *et al*., 2015; El-Naas *et al*., 2017).

1.1.2.3 Soil

The use of herbicides, pesticides and other chemicals has led to the pollution of the soil and they pose a challenge for farmers, particularly for crop production. Chlorophenols which are highly toxic aromatic compounds are found in the soil sediments and surface soil (Gaofeng *et*

al., 2004; Seo *et al*., 2009; Mohammadi and Sabbaghi, 2014). Studies have reported high levels of 2,4-dichlorophenol, pentachlorophenol and 2,3,4,6-tetrachlorophenol in the soil (Igbinosa *et al*., 2013; Yadid *et al*., 2013; Mohammadi and Sabbaghi, 2014; Karn *et al*., 2015). The pH, temperature, moisture and particles of the soil have an impact on the chlorophenol contamination since soil serves as a medium for transportation and facilitates absorbance of the compounds (Diez, 2010, Olaniran and Igbinosa, 2011; Kumar *et al*., 2015). An increase in the pH results in an increased ionization of the compounds and therefore also facilitates their absorption in the soil (Ivanciuc *et al*., 2006; Olaniran and Igbinosa, 2011). The soil has a high surface area and activity which makes it highly absorbent to the compounds (Stearman *et al*., 2014). Soil can result in pollution due to the formation of toxic metabolites after degradation of the compounds and leaching (Shayler *et al*., 2009; Chibuike and Obiora, 2014; Stearman *et al*., 2014). This is a challenge globally, especially in countries that have high crop production where fungicides, insecticides, and herbicides are frequently used (Czaplicka, 2004; Diez, 2010).

1.2 Toxicity and health risks of chlorophenol

1.2.1 Toxicity

The toxicity and acidity of chlorophenols increases in proportion to the number of chlorine atoms (Olaniran and Igbinosa, 2011; Liu *et al*., 2012). The chlorophenols are ubiquitous in the environment and due to their widespread applications, they pose a health risk to all life forms (Olaniran and Igbinosa, 2011).

1.2.2 Health Risks

The release of chemicals in the environment results in increased pollution. This affects the quality of air, water, soil and living organisms which results in interference with the biodiversity (Arora and Bae, 2014; Kadmi *et al*., 2015). It is imperative to identify the pollutants so that environmental policies can be established (Xing *et al*., 2012; Bazrafshan *et al*., 2013). European Union (EU) categorized 132 hazardous pollutants in terms of their toxicity, stability, bioaccumulation and this is used in regulations of water (European Environment Agency (CSI 015), 2007; Olaniran and Igbinosa, 2011; Sarafraz-Yazdi *et al*., 2012; Igbinosa *et al*., 2013, Kadmi *et al*., 2015).

The structures of chlorophenols are an indication of how toxic the compound is and an illustration of its persistence in the environment when degraded by either chemical, physical or biological methods (Seo *et al*., 2009; Diez, 2010). In other countries, regulatory measures have been employed in the use of pesticides due to their high levels of toxicity and production of more hazardous metabolites consistent with the Environmental Protection Agency (EPA) in the US and the World Health Organization (WHO) (Michałowicz and Duda, 2007; Lillis *et al* 2010; Igbinosa *et al*., 2013; Hou *et al*., 2016). This is vital as the pesticides that are highly toxic enter the food chain and cause severe diseases (Igbinosa *et al*., 2013). The Food and Agriculture Organization (FAO) and WHO stipulated toxicity levels of pesticides that can be allowed to be residues in food and the environment (Damalas *et al*., 2011).

The effects of hazardous chemicals on human beings depend on how long the person has been exposed to the compound, the concentration of the compound, the dose, personal traits and whether the person is immune compromised (Czaplicka, 2004). Humans can be exposed to the toxic chlorophenols via ingestion, inhalation and occupational exposure (Stearman *et al*., 2014). There are several diseases that are associated with the exposure to chlorophenols; and

most people are exposed to chlorophenols in their work environment (Igbinosa *et al*., 2013; Stearman *et al*., 2014). Employees that were exposed to chlorophenols in the working environments were mostly treated for hematopoietic tumors (Cline *et al*., 1989; Stearman *et al*., 2014). Most of these employees included farmers and agricultural workers, people who manufacture herbicides, pesticides, people who work in textile and petrochemical industries (Michałowicz and Duda, 2007; Igbinosa *et al*., 2013). Diseases such as cancer of the respiratory system, lymphoma and myocardial ischemia have been diagnosed from employees who work with the herbicides 2,4-dichlorophenoxyacetic acid, due to its high toxicity (Czaplicka, 2004; Michałowicz and Duda, 2007; Igbinosa *et al*., 2013). In the vinyl chloride production factories, there have been thousands of people who have been suffering from the lung and liver diseases (Becker *et al*., 2003; Michałowicz and Duda, 2007; Igbinosa *et al*., 2013). Due to their detrimental properties, methods have been employed for the degradation of chlorinated compounds in other studies. Some of the methods have been used simultaneously to achieve degradation (García-Peña *et al*., 2012; Hossain and Ismail, 2015).

1.3 Different methods used for the degradation of chlorophenols

Pollution has been regulated by different approaches in the world. In some countries, the chemicals that are discharged are checked for any hazards or toxicity potential. If the pollutants are harmful they are stored in containers to prevent their spread in the environment via spillages (Lin, 2002; Igbinosa *et al*., 2013).

These approaches have been used in countries such as the USA, however, there are still challenges in monitoring the release of harmful chemicals into the environment. Therefore, organizations such as the European Union (EU) have restricted the use of highly toxic compounds (Lin, 2002; Igbinosa *et al*., 2013).

1.3.1 Photocatalysis

Advanced oxidation methods such as photocatalysis have been used for the degradation of the highly toxic compounds that are detected as pollutants during wastewater treatment. Photocatalysis is facilitated by a combination of TiO_2 and UV light (Uysal and Türkman, 2007; Liu *et al*., 2012). A photocatalyst depends on several factors to be effective. These include the size of the particle, the light intensity, the surface area, the porosity, nature of the compound to be degraded, the alkalinity and acidity of the compound (Liu *et al*., 2012). Though this method has been effective for treatment of other compounds, the disadvantages of using it are its cost and the production of highly toxic secondary pollutants such as dibenzo-p-dioxins and polychlorinated dibenzofurans (Liu *et al*., 2012).

1.3.2 Activated carbon adsorption

The activated carbon adsorption is mostly used in the treatment of wastewater and has been used widely for phenolic compounds (Laidislao and Galil, 2004; Kumar *et al*., 2007). Some of the absorbents that have been studied in the removal of chlorophenol compounds are palm seed coat carbon, clay, rice husks, bituminous coals, petroleum coke and wood pulp XAD-4 resin (Kumar *et al*., 2007; Uysal and Türkman, 2007).

1.3.3 Low-temperature plasma

This is a new method that has been used for the degradation of the chlorophenol compounds. Plasma is a group of positive and negative particles which include ions that are positively or negatively charged, including the active and the free radicals (Zhang *et al*., 2009; Aggelopolous *et al*., 2013). The process includes the high-energy electron irradiation, radiation particles, oxidation and O_3 ultraviolet radiations. The method employs the use of low-temperature plasma to degrade the compound and this results in physical and chemical processes such as the production of high energy electrons and ultraviolet radiation. Active particles which are formed

from the latter are used for the degradation of the compounds (Zhang *et al*., 2009; Aggelopoulos *et al*., 2013).

Although physical and chemical methods such photocatalysis, activated carbon adsorption, low-temperature plasma have been proven to effectively reduce the broad spectrum of contaminants, they have major disadvantages because they are uneconomical and not environmentally friendly due to the production of more toxic secondary metabolites (Karigar and Rao, 2011).

1.3.4 Biological degradation

Biological methods have been recommended for the removal of phenolic compounds from environment due to their efficiency, cost-effectiveness and the production of nontoxic secondary metabolites (Wang *et al*., 2000; Wei *et al*., 2010). These include biodegradation of aromatic compounds (Elkarmi *et al*., 2008). Bioremediation has been used over the years for the degradation of contaminants in the environment (Mailin and Firdausi, 2006; Arora and Bae, 2014). This process is facilitated by organisms such as plants and microorganisms that form the contaminants into secondary metabolites that are not hazardous. A wide spectrum of microorganisms capable of degrading phenolic compounds includes *Acinetobacter* spp., *Pseudomonas* spp., *Bacillus* spp., *Rhodococcus* spp. and *Ralstonia* spp. Some microorganisms show the ability to mineralize the compound completely while others were incapable of degrading the compound (Elkarmi *et al*., 2009; Sridevi *et al*., 2012). Some microorganisms are highly adaptable and can thrive in extreme conditions. They can utilize hazardous compounds as their carbon and energy sources for their adaptation in waste streams (Diaz, 2010). Biological treatment is generally preferable for the degradation of chlorophenols among other methods due to its numerous advantages such as its relatively low cost, effectiveness, non-toxic end products and improved biodiversity. Hence, bioremediation is a superior method (Đokić *et*

al., 2011; Kumar and Gopal, 2015). The main challenge in the degradation of chlorophenols using the microorganisms is substrate inhibition. High concentrations of substrates tend to negatively affect the degradation potential of some microorganisms as the substrates become too toxic for them, resulting in lesser degradation (Nweke and Okpokwasilia, 2014). Microorganisms have enzymes that are involved in the degradation of the compounds. These enzymes are specific for each reaction that takes place in the degradation of these xenobiotics (Karigar and Rao, 2011). The process of bioremediation is facilitated by microorganisms that are able to degrade the compounds and affected by the environment in which the microorganisms are cultivated (Karigar and Rao, 2011; Lin *et al*., 2008). Gallizia *et al*. (2003) have reported that bioremediation has been accepted due to its cost-effectiveness, environmental friendliness and the non-toxic formation of secondary metabolites. Microorganisms are involved in processes such as quorum sensing and other interactive processes and this leads to structural changes and sometimes complete degradation of the products (Michałowicz and Duda, 2007).

1.3.4.1 Plants

Plants such as *Spirodela polyrrhiza* have shown the ability to degrade several pollutants together with bacteria and this process is known as phytoremediation (Michałowicz and Duda, 2007; Hoang *et al*., 2013; Weyens *et al*., 2015). Mechanisms in phytoremediation have been employed for an enhanced remediation such as *in planta* process and *ex situ* degradation. *In planta* process is sequestrated through the plant where there is an uptake of the organic pollutants through the different structures of the plants such as the roots, stem or leaves. *Ex situ* degradation occurs at the rhizosphere of the plant when there is an enhanced activity of microorganisms or due to nonspecific activity from the protein and cofactors (James and Strand, 2009; Weyens *et al*., 2015). Genetically modified plants such as *Arabidopsis thaliana*

which expresses two bacteria genes have been used for the degradation of chlorophenols (Karigar and Rao, 2011).

1.3.4.2 Fungi

Fungi have been used over the years for the degradation of chlorophenols and this process is known as mycoremediation (Haggblorn, 1990; Rao *et al*., 2010). Studies conducted by Gianfreda and Rao (2014), showed that pesticides and other aromatic pollutants are degraded by fungi and this results in minor structural changes in the molecule, and ultimately in the formation of nontoxic compounds. Fungi such as white-rot fungi have been used in several experiments for the degradation of chlorophenols and other toxic xenobiotics (Michałowicz and Duda, 2007). Fungi are known to produce a lot of enzymes that facilitate the degradation of chlorophenols. White-rot fungi have extracellular peroxidase enzymes which are non-specific and have the ability to degrade chlorophenols and other toxic pollutants (Diez, 2010; Rao *et al*., 2010). Some white-rot fungi such as *Trametes versicolor, Pleurotus ostreatus, Bjerkandera adusta, Irpex lacteus, Agaricus bisporus* and *Gloeophyllum striatum* have ligninolytic enzymes such as laccase that are involved in the degradation of chlorophenolic compounds. However, *Phanerochaete chrysosporium* has been proven to be more efficient for degradation of the compounds as compared to the other fungi (Kasai *et al*., 2010; Rhodes, 2014). Several yeast strains have also been reported to be capable of degrading toxic phenolic compounds; examples are *Candida lipolytic*, *Candida tropicalis*, *Rhodotorula mucilaginosa* and *Trichosporon mucoides* (Jiang *et al*., 2007; Das and Chandran, 2011; Karimi and Hassanshahian, 2016).

1.3.4.3 Algae

Algae discovered in polluted water have been used as indicators of pollutants in water. Some algae are capable of forming pollutants such as phenolic compounds and other aromatic hydrocarbons into non-toxic forms (Lima *et al*., 2005; Rao *et al*., 2010; El-Sheekh *et al*., 2012; Lakshmi and Sridevi, 2015). *Ochromonas danica* has been extensively studied due to its ability to degrade a range of pollutants that are found in the environment. Other examples are *Chlorella fusca and Skeletonema costatum* that have been shown to degrade 2,4-dichlorophenol effectively (Lima *et al*., 2005; Rao *et al*., 2010).

1.3.4.5 Bacteria

Several studies have been conducted using bacteria for the degradation of pollutants over the years. The strains that have been studied are *Pseudomonas*, *Arthrobacter, Mycobacterium*, *Sphingomonas,* and *Rhodococcus* (Gallizia *et al*., 2003; Quan *et al*., 2004; Das and Chandran, 2010). *Pseudomonads* and *Alcaligenes eutrophus* have been widely studied due to their ability to use a range of aromatic compounds as their substrates (Wanga *et al*., 2012; Kumar *et al*., 2014; Igbinosa *et al*., 2013; El-Naas *et al*., 2017). During the degradation of the compounds, there are metabolic pathways that are followed by the organism (Solyanikova and Golovleva, 2004; Mahiudddin *et al*., 2012; Sridevi *et al*., 2012; Arora and Bae, 2014; El-Naas *et al*., 2017).

Majority of the enzymes are induced during the mineralization of chlorophenols. Pseudomonads are non-specific, thus allowing them to use broad substrate range of chlorophenol compounds and the potential to simultaneously mineralize the different compounds. However, they follow different metabolic pathways for the mineralization of the compound (Hutchinson and Robinson, 1988; Gurusamy *et al*., 2002; Rao *et al*., 2010; Mahiudddin *et al*., 2011; El-Naas *et al*., 2017).

The microorganisms and their ability to degrade different concentrations of phenolic compounds are illustrated in Table 1.2**,** where it is shown that most microorganisms have the ability to completely degrade high concentrations of phenol at a very short period. The chlorophenols have chlorine atom attached and this influences the degradation rate of the compounds. This suggests that the microorganisms find these chlorophenols to be highly toxic and their concentrations can affect the degradation rate of the microorganisms (El-Naas *et al*., 2017). More studies must be conducted in order to improve the degradation of the compounds by microorganisms.

Table 1.2: Microorganisms capable of degrading phenolic compounds

Microorganisms	**Compounds**	**Concentration (ppm)**	**Time (h)**	**Percentage degradation (%)**	**References**
Alcaligenes (AG21)	2-chlorophenol	100	27	99.5	Gallego *et al*., 2001
Alcaligenes (AG23)	Phenol	50	20	99.5	Gallego *et al*., 2001
Streptococcus epidermis OCS-B	Phenol	200	84	100	Mohite *et al*., 2010
Rhodococcus sp. RSP8	Phenol	150	24	100	Sinha *et al*., 2011
Streptomyces rochei	Pentachlorophenol	400	144	100	Elkarmi *et al*., 2009
Candida tropicalis	4-chlorophenol	150	30	100	Wanga *et al*., 2012
Lentinula edodes	2,4-dichlorophenol	1620	672	92	Tsujiyama *et al*., 2013

1.4 2,4-dichorophenol

2,4-Dichlorophenol (2,4-DCP) is used in the production of toxic chlorinated compounds such as pentachlorophenol (Elkarmi *et al*., 2008; Padhye and Chakrabarti, 2015). It is also the first intermediate formed during degradation of the herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D) (Fukumori and Hausinger, 1993; Boivin *et al*., 2005; Igbinosa *et al*., 2013; Arora and Bae, 2014, Trivedi *et al*., 2015). Despite its widespread use, it has been reported to be carcinogenic, mutagenic and teratogenic (Arora and Bae, 2014). Hence, the compound is a priority pollutant and efforts have been made to remove it from the environment (Igbinosa *et al*., 2013; Arora and Bae, 2014). 2,4-DCP has been restrained for usage due to its toxicity. However, studies have shown that there is still the presence of the compound in potable water, wastewater, surface water and industrial effluents (Igbinosa *et al*., 2013; Arora and Bae, 2014). The combustion emissions of solid waste from municipal incinerators also cause pollution. Therefore, an effective method is needed to degrade the compound. Biological treatment is an effective method of degrading 2,4-DCP to yield non-toxic end products (Igbinosa *et al*., 2013; Arora and Bae, 2014). The challenging part of using biological treatment is that it takes a long time to remove the compounds by microbial cells. The compound concentration can also be inhibitory to the growth of the microorganisms (Arora and Bae, 2014).

During the degradation of 2,4-DCP by microorganisms, the enzyme 2,4-dichlorophenol-hydroxylase catabolizes the compound through the 3,5-dichlorocatechol pathway (Arora and Bae, 2014; Igbinosa *et al*., 2015). 3,5-Dichlorocatechol is *ortho*-cleaved to 2,4-dichloromuconic acid by 3,5-dichlorocatechol dioxygenase. The compound is further mineralised by 2,4-dichloromuconic acid isomerase that catalyses the conversion of 2,4-dichloromuconic acid to *cis*-2-chlorodienelactone via the removal of one chloro-group, the lactone is further converted to *cis*-2-chlorodienelactone by an isomerase that is subsequently

degraded via formation of chloromaleylacetate by a hydroxylase (Sridevi *et al*., 2012; Igbinosa *et al*., 2015). The chloromaleylacetate is further degraded to maleylacetate by removal of the chloro-group and then to 3-oxodipic acid by maleylacetate reductase via a *meta*-cleavage pathway (Pieper *et al*., 2010; Ebert *et al*., 2011). In that process, 2,4-DCP can also be alternatively mineralized to 3,5-dichlorocatechol via a *meta*-cleavage pathway through the formation of 2-hydroxy-3,5-dichloro-6-oxohexa-2,4-dienoic acid. This pathway normally leads to dead-end products or inactivation as illustrated in Figure 1.1 (Sridevi *et al*., 2012; Arora and Bae 2014).

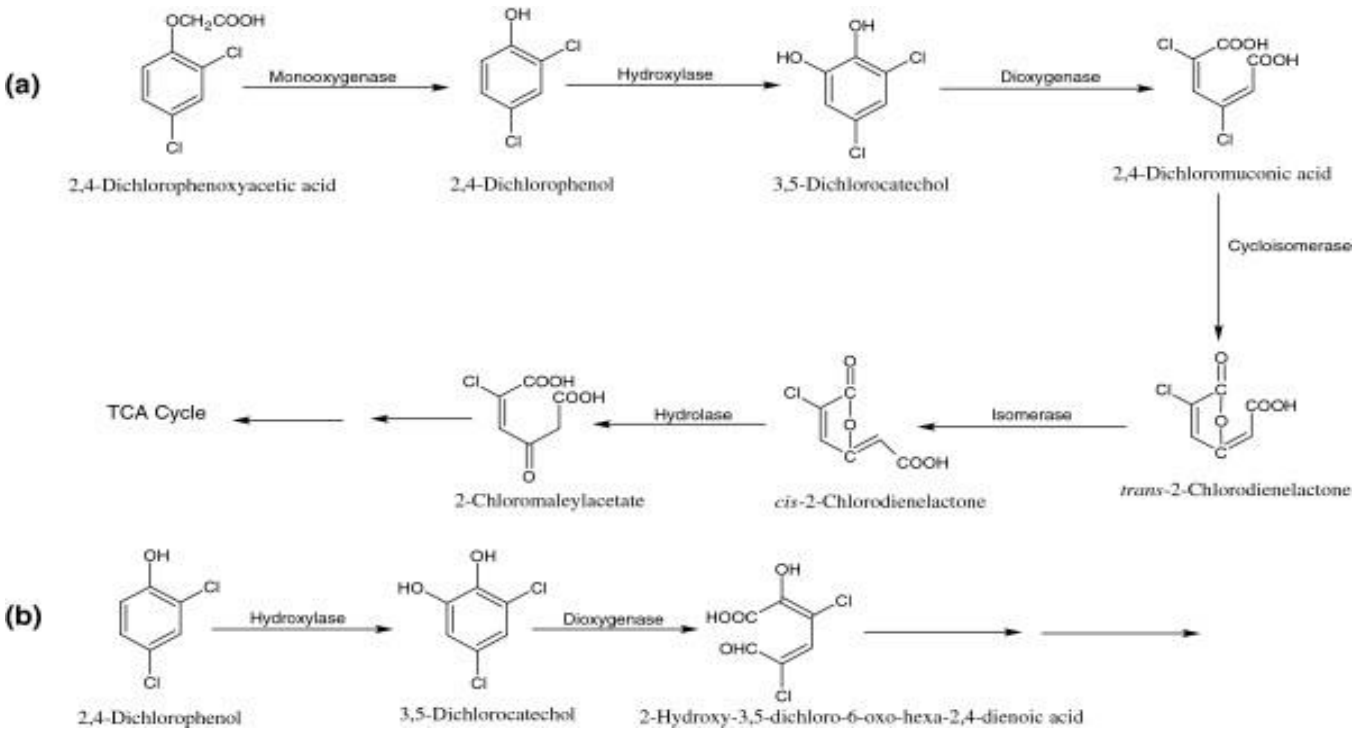

Figure 1.1: The degradation of 2,4-dichlorophenoxyacetic acid and its derivative 2,4-dichlorophenol via **(a)** *ortho*-pathway and **(b)** *meta*-pathway (Arora and Bae, 2014).

1.4.1 Metabolic pathways and enzymes involved in the degradation of chlorophenolic compounds

During the biodegradation of chlorophenols such as 2,4-DCP, there are different metabolic pathways that microorganisms follow to mineralize the compound. Gaofeng *et al*. (2004) showed that there are three different metabolic pathways that are followed by microorganisms during the degradation of chlorophenols *hydroquinone, ortho*- and *meta*-pathway. However, the mostly studied degradation pathways are the *ortho*- and *meta*-pathway. The *hydroquinone* -pathway is not extensively studied since it is only detected in few microorganisms and during the degradation of complex chlorophenols (Gaofeng *et al*., 2004; El-Naas *et al*., 2017). Catechol is the central metabolite for the degradation of chlorophenols compounds under aerobic conditions. There are challenges in the degradation of the chlorophenol compounds via the *meta*-pathway due to the inactivation of the enzyme catechol 2,3-dioxygenase which is a result of the accumulation of the toxic metabolites. It has been reported that the *ortho*-pathway is required for the complete mineralization of chlorophenol compounds (Patel and Kumar, 2016; El-Naas *et al*., 2017).

1.4.1.1 *Ortho*-pathway

The *ortho*-pathway for the chlorophenol compounds is facilitated by the enzyme catechol 1,2-dioxygenase, illustrated in Figure 1.2 (Lillis *et al*., 2010; Mahiudddin *et al*., 2012). During the biodegradation of chlorophenols such as 2-chlorophenol and 2,4-dichlorophenol via the *ortho*-pathway there is a cleavage of 3-chlorocatechol (3CC) into 2-chloro-*cis*-*cis*-muconate. This is further broken down to dienelactone by the enzyme chloro-muconate cycloisomerase. This enzyme conducts this by using *cis/trans*-dienelactone substrates. The degradation products then enter the Krebs cycle through the formation of maleylacetate by the enzyme maleyacetate reductase (Solyanikova and Golovlela, 2004; Arora and Bae, 2014; El-Naas *et al*., 2016).

Depending on the organisms that are used to degrade the chlorophenols, the latter pathway can be modified. In other cases, some microorganisms have less degradation efficiency of the compound (Farhana and New, 1997; Gaofeng *et al*., 2004). *Trichosporon cutaneum* operates through *ortho*-pathway for the mineralization of phenolic compounds (El-Naas *et al*., 2017).

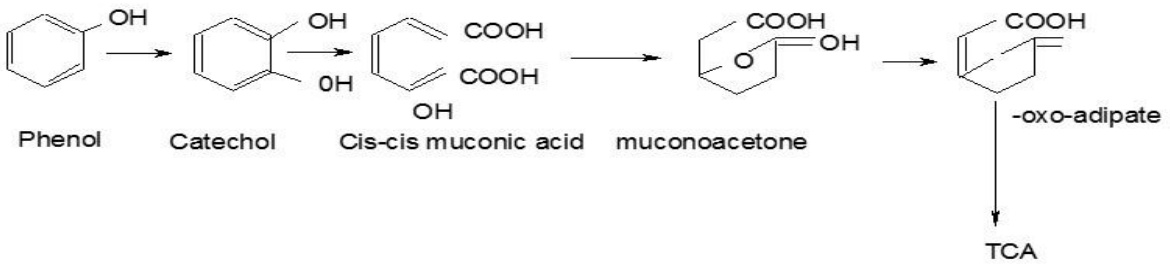

Figure 1.2: *Ortho*-pathway degradation of phenol (Mahiudddin *et al*., 2012).

1.4.1.2 *Meta*-pathway

The *meta*-pathway is catalyzed by the enzyme catechol 2,3-dioxygenase, illustrated in Figure 1.3 (Lillis *et al*., 2010; Mahiudddin *et al*., 2011). Mineralization of phenol via the *meta*-pathway is initialized by phenol hydroxylase for the hydroxylation of phenol to catechol, the catechol is then cleaved into 2-hydroxymuconic semialdehyde by catechol 2,3-dioxygenase. The compound is further broken down into the products of the tricarboxylic acid cycle (Krastanov *et al*., 2013). *Pseudomonas* spp. and *Comamonas testosterone* JH5, *Alcaligenes eutrophus*, *Sphingomonas* spp. BN6 *Azotobacter* sp. GP1 have been reported to degrade the phenolic compounds through the *meta*-pathway (Hollender *et al*., 1997; Lillis *et al*., 2010; Arora and Bae, 2014; El-Naas *et al*., 2017).

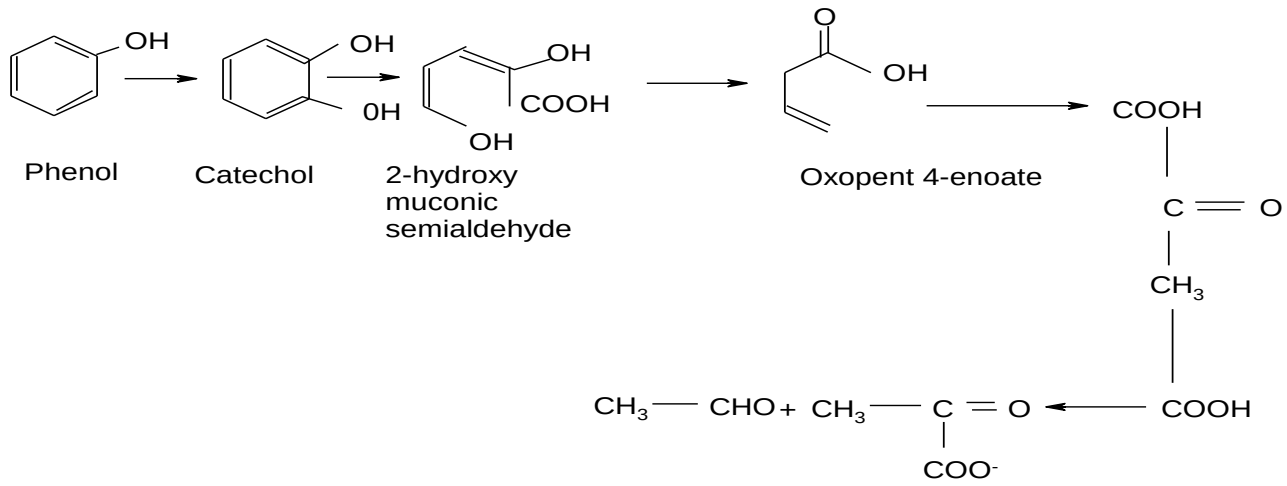

Figure 1.3: *Meta*-pathway degradation of phenol (Mahiudddin *et al*., 2012).

1.4.2 Microbial enzymes from the metabolic pathway that facilitate biodegradation of 2,4-dichlorophenol

There are key enzymes that are involved in the different metabolic pathways during the biodegradation of 2,4-dichlorophenol and they facilitate the mineralization of the compound. Each enzyme is responsible for a step in the pathway.

1.4.2.1 Phenol hydroxylase

The enzyme phenol hydroxylase is the first enzyme that is involved in the cleavage of phenol and chlorophenols to catechol (Powlowski *et al*., 1994; Ahuatzi-Chacon *et al*., 2004; van Schie *et al*., 2000; Páca *et al*., 2007; Paisioa *et al*., 2013; Hasan *et al*., 2015). Phenol hydroxylase is the monooxygenase enzyme that has been shown to have many components, and it is found in most microorganisms that can utilize phenol and phenol derivatives as substrates (Vilímková *et al*., 2008; Qu *et al*., 2012). Microorganisms such as *Pseudomonas* spp., *Sphingomonas* spp., *Ralstonia eutropha, Candidas tropicalis, Bacillus* spp., *Rhodococcus* spp.), have been shown to be the best producers of the enzyme when they utilize phenol and chlorophenols as the substrate and the only carbon and energy source (Dokic *et al*., 2011; Long *et al*., 2014).

1.4.2.2 Catechol 1,2-dioxygenase

Catechol 1,2-dioxygenase is the enzyme that is involved in the degradation of catechol into *cis*-*cis*-muconate, and this is the enzyme of the *ortho*-pathway (Saxena and Thakur, 2005; Nair *et al*., 2008; Mahiudddin *et al*., 2012; Hupert-Kocurek *et al*., 2014). Most catechol 1,2-dioxygenase enzymes are studied in different organisms, especially Gram-negative bacteria, but there is less information about these enzymes in Gram-positive bacteria (Shumkova *et al*., 2009; Sridevi *et al*., 2012). New research has shown that there are microbial enzymes that are capable of degrading aliphatic, mono and poly-aromatic hydrocarbons in culture medium and in the soil. Some of the isolates are further studied to improve their rate of degradation so that they can be used in the bioremediation of wastewater (Silva *et al*., 2012). Catechol 1,2-dioxygenase contains Iron(III) oxide as a prosthetic group and it is part of the enzymes that cleave catechol via the *ortho*-cleavage, resulting in the formation of *cis*-*cis*-muconic acid (Tsai and Li, 2007). The environmental conditions for a successful usage of catechol 1,2-dioxygenase are very important and need to be considered since they affect the activity (Silva *et al*., 2012; Sihag *et al*., 2014). Catechol dioxygenases add two oxygen atoms to the aromatic ring of catechol, disrupting chemical bonds and allowing opening in this ring (Silva *et al*., 2012; Knoot *et al*., 2015).

1.4.2.3 Catechol 2.3-dioxygenase

Catechol 2,3-dioxygenase is involved in the degradation of catechol through the *meta*-cleavage pathway (Nair *et al*., 2008; Lillis *et al*., 2010; Hupert-Kocurek; Guo *et al*., 2015). The enzyme has been shown to cleave a larger array of substrates and has been found to be involved in numerous pathways for the degradation of aromatic compounds (Park *et al*., 2003; Weiss *et al*., 2013). This enzyme has been studied throughout the years in different microorganisms. It belongs to the extradiol dioxygenases family of monoaromatic compounds. The enzyme uses

non-heme ferrous ion, magnesium and sometimes manganese to cleave the aromatic ring (Weiss *et al*., 2013; Guo *et al*., 2015). Catechol 2,3-dioxygenase has been studied in the Gram-positive organisms such as *Planococcus* spp. strain S5 and *Pseudomonas fluorescens*. These enzymes are substrate-specific and they have received the attention over the years due to their distinct qualities (Mahiudddin *et al*., 2012; Hupert-Kocurek *et al*., 2014; Guo *et al*., 2015). However, there are drawbacks using these enzymes. Most extradiol dioxygenases are found to be inactivated by the strong chelating ability of 3-chlorocatechol (3CC), which happens during the degradation of chlorophenols via the *meta*-pathway (Weiss *et al*., 2013). Inactivation of the enzyme catechol 2,3-dioxygenase results in a low turnover capacity of the 3CC that results in the insufficient growth of the microorganisms and the inability to utilize the substrate (Weiss *et al*., 2013).

Catechol 2,3-dioxygenases (*C*23*O*s) belong to the extradiol dioxygenases family. These enzymes have been found in numerous microorganisms such as Gram-negative (*Pseudomonas, Sphingomonas, Acinetobacter, Ralstonia, Burkholderia, Stenotrophomonas*) and Gram-positive strains (*Nocardia, Rhodococcus* and *Bacillus*) (Jiang *et al.,* 2004; Junca *et al.,* 2004; Viggiani *et al.,* 2004; Ma *et al.,* 2010; Wei *et al.,* 2010; Tancsics *et al.,* 2010; Wojcieszyńska *et al.,* 2011). All known catechol 2,3-dioxygenases are homotetramers and most of them contain non-heme Fe (II) in their active site but some are also active with Mn (II) (Viggiani *et al.,* 2004; Vaillancourt *et al.,* 2006). The catechol 2,3-dioxygenases of the *meta*-pathway can convert catechol, both isomeric methylcatechols and 4-chlorocatechol at respectable rates (Kaschabek *et al*., 1997).

1.4.2.4 Muconate Isomerase

During the biodegradation of phenol and chlorophenol, catechol is converted to *cis*-*cis*-muconate by the enzyme catechol 1,2-dioxygenase (Murakami *et al*., 2003; Matsumura *et al*., 2006; Camara *et al*., 2008; Sridevi *et al*., 2012; Arora and Bae, 2014). *Cis*-*cis*-Muconate

cycloisomerase catalyses the cycloisomerization of *cis*-*cis*-muconate in the second step of the pathway. This enzyme has been purified and characterized in various bacteria, such as *Arthrobacter, Pseudomonas, Cupriavidus necator* JMP134 and *Bacillus* spp. (Pollmann *et al*., 2002; Murakami *et al*., 2003; Matsumura *et al*., 2006; Pérez-Pantoja *et al*., 2008; Arora and Bae, 2014).

1.4.2.5 Dienelactone hydrolase

Dienelactone hydrolase is the enzyme that is involved in the hydrolysis of dienelactone to maleylacetate (Camara *et al*., 2008; Marín and Pieper, 2009; Park *et al*., 2010; Arora and Bae, 2014; Kumar *et al*., 2014). This enzyme takes part in the aerobic microbial degradation of natural or synthetic aromatic compounds that are recalcitrant and persistent in the environment (Park *et al*., 2010; Igbinosa *et al*., 2015). Dienelactone hydrolases have been classified into diverse groups and this is dependent on the target substrate. The different substrates are *trans*- and *cis*-dienelactone hydrolase (Camara *et al*., 2008; Park *et al*., 2010; Kumar *et al*., 2014). Microorganisms that have these enzymes have been studied throughout the years. Some of the common microbes used are *Pseudomonas putida* RW10 and *Pseudomonas reinekei* MT1 that are able to hydrolyze *trans*-dienelactone (Camara *et al*., 2008; Park *et al*., 2010; Kumar *et al*., 2014; Arora and Bae., 2014). Other microorganisms can hydrolyze the *cis*-dienelactone substrate such as *Pseudomonas*, *Burkhoderia cepacia* possess the same ability (Park *et al*., 2010). Some microorganisms can hydrolyze both the *cis*- and *trans*-dienelactone substrate. *Alcaligenes eutrophus* JMP134 and *Pseudomonas* spp. strain B13 (*Pseudomonas knackmussii*) are examples of the microorganisms that have this ability (Camara *et al*., 2008; Park *et al*., 2010). Studies have shown that most dienelactone hydrolases belong to the α/β-hydrolase fold enzymes which contain a Nucleophile-His-Acid catalytic triad and the most conserved sequence, Sm-X-Nu-X-Sm-Sm (Sm=small residue), around the nucleophile (Park *et al*., 2010).

In *Pseudomonas knackmussii*, it was observed that the dienelactone hydrolase has an active site and the sequence around the cysteine is Gly-Tyr-Cys-Leu-Gly-Gly (Park *et al*., 2010; Kumar *et al*., 2014).

1.4.2.6 Maleylacetate reductase

Maleylacetate reductase is one of the key enzymes that result the complete mineralization of phenolic compounds and their derivatives due to its degradation of ring-fission products (Sridevi *et al*., 2012; Fujii *et al*., 2016). In microorganisms, such as fungi the enzyme maleylacetate reductase is induced during the degradation of different substrates and this is through pathways of halo-catechols, hydroxyquinol, chloro-hydroxyquinol, hydroquinone and chloro-hydroquinone, as ring cleavage intermediates of a broad array of metabolized compounds (Perez-Pantoja *et al*., 2009; Arora and Bae., 2014). The enzyme catalyzes maleylacetate to 3-oxoadipate through a NADPH-dependent reduction, 2 moles of NAD(P)H per mole of the substrate are consumed during the reaction and this results into 3-oxoadipate. Once the reaction has taken place and the product is formed, 3-oxoadipate is channeled into the Krebs cycle (Perez-Pantoja *et al*., 2009; Marín *et al*., 2010). *Ralstonia eutropha* (*Cupriavidus necator*), *Rhodococcus* spp., *Actinobacteria*, *Proteobacteria*, *Pseudomonas* spp. are amongst microorganisms that are have induction of the enzyme maleylacetate reductase during catabolism of chlorophenolic compounds, via the *ortho*-pathway into the Krebs cycle (Trefault *et al*., 2004; Perez-Pantoja *et al*., 2009; Arora and Bae., 2014).

1.5 Genetics of bacterial degradation of chlorophenols

During the biodegradation of chlorophenols by microorganisms, microbial enzymes are induced in the metabolic pathways which are specific to each reaction, these enzymes are encoded by the different genes. It is therefore imperative to understand and to evaluate which

genes encode the enzymes to understand the genetics of the microbes. The genes that are involved in the degradation of chlorophenols are found in the plasmid. A study that was conducted by Solyanikova and Golovleva. (2004) showed a modified *ortho*-pathway for the degradation of 3-chlorocatechol degradation by *Rhodococcus opacus* 1CP, the pathway showed a cluster of genes that encode the enzymes of the metabolic pathway during the degradation of 3-chlorocatechol degradation, the genes *clcA2, clcB2; clcD2; clcF* encodes for the enzymes chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, dienelactone hydrolase (ClcD2) and muconolatone isomerase, respectively (Gobel *et al*., 2002). In Figure 1.4 the pathways for 3-chlorocatechol and 4-chlorocatechol are illustrated to understand the newly modified 3-chlorocatechol pathway that was analyzed (Solyanikova and Golovleva, 2004). The latter modified pathway was different from the other studies that showed a formation of maleyacetate which leads to the Krebs cycle (Solyanikova and Golovleva, 2004; Arora and Bae, 2014). *Cupriavidus necator* JMP134 has been shown to express the genes that are different to the modified pathway 4-chlorocatechol *tfdC, tfdD, tfdE and tfdF* are the different genes that encode the enzymes chlorocatechol-1,2-dioxygenase, chloromuconate cycloisomerase, dienelactone hydrolase, and maleylacetate reductase, respectively (Laemmli *et al*., 2002; Laemmli *et al*., 2000; Lillis *et al*., 2010; Arora and Bae, 2014; Dobslaw and Karl-Heinrich, 2015; Kumar *et al*., 2014).

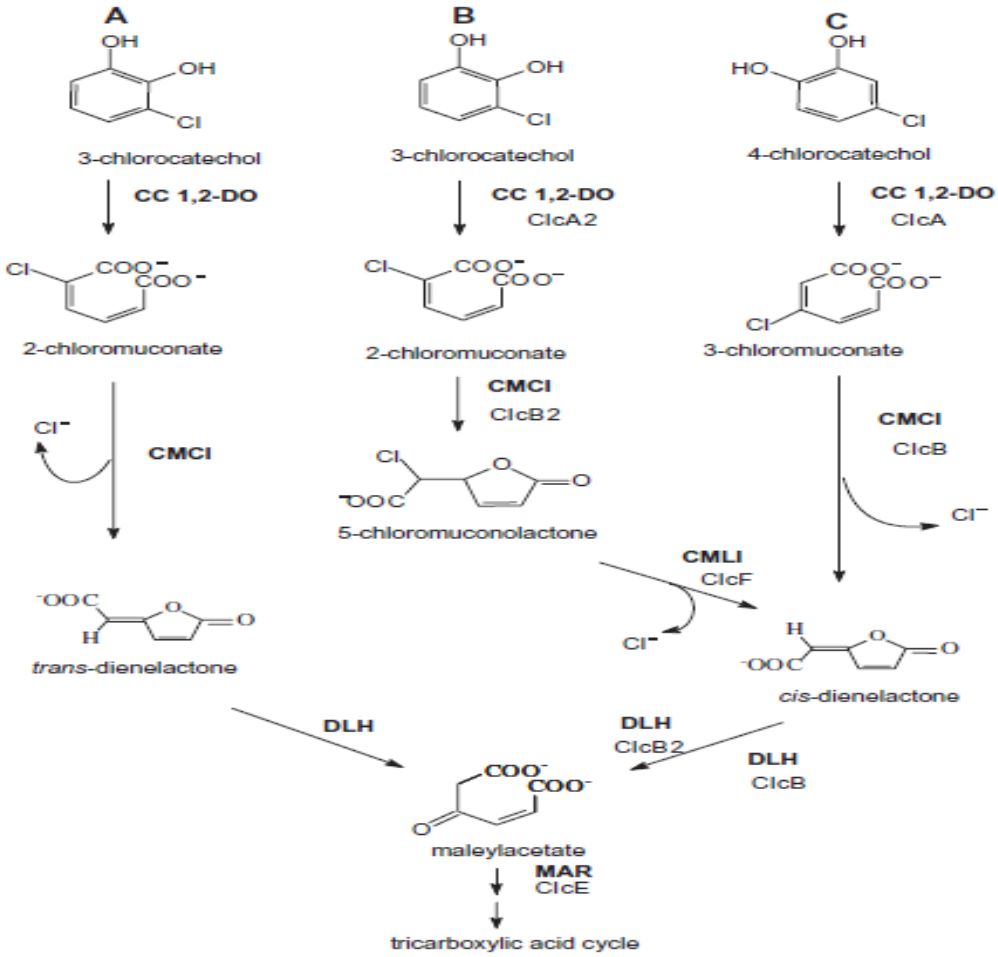

Figure 1.4: Modified pathways for the mineralization of (chloro)catechols by bacteria (A) 3-chlorocatechol mineralization by Gram-negative bacteria, (B) Mineralization of 4-chlorocatechol by *R. opocus* CP1. (C) Mineralization of 3-chlorocatechol by *R. opocus* CP1 (Solyanikova and Golovleva, 2004).

Gram-positive bacteria have been studied extensively for the complete operons of 3-chlorocatechol and 2-dichlorocatechol, there was a significant similarity of the structure of the operon and the amino acid sequence of the enzymes from the isolates *Pseudomonas putida* pAC27, *Cupriavidus necator* JMP134, *Pseudomonas* spp. P51, and *Burkholderia* spp. NK8 (Figure 1.5 and 1.6) (Solyanikova and Golovleva, 2004).

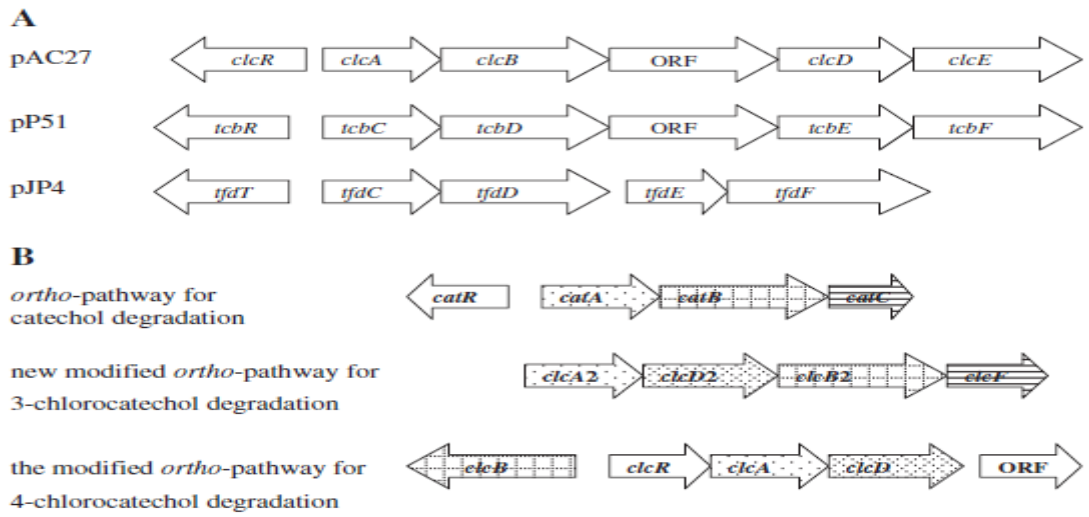

Figure 1.5: Gene clusters for chlorophenol degradation: (A) Similarity in the genes that encode for the enzymes of the chlorocatechol pathway in Gram-negative bacteria. Chlorocatecho 1,2-dioxygenase (*clcA, tcbC, tfdC*), Chloromuconate cycloisomerase (*clcB, tcbD, tfdD*) Dienelactone hydrolase (*clcD, tcbE, tfdE*) and Maleylacetate reductase (*clcE, tcbF, tfdF*); (B) for *Rhodococcus opacus* 1CP, the genes are the same as in Figure 5 (Solyanikova and Golovleva, 2004).

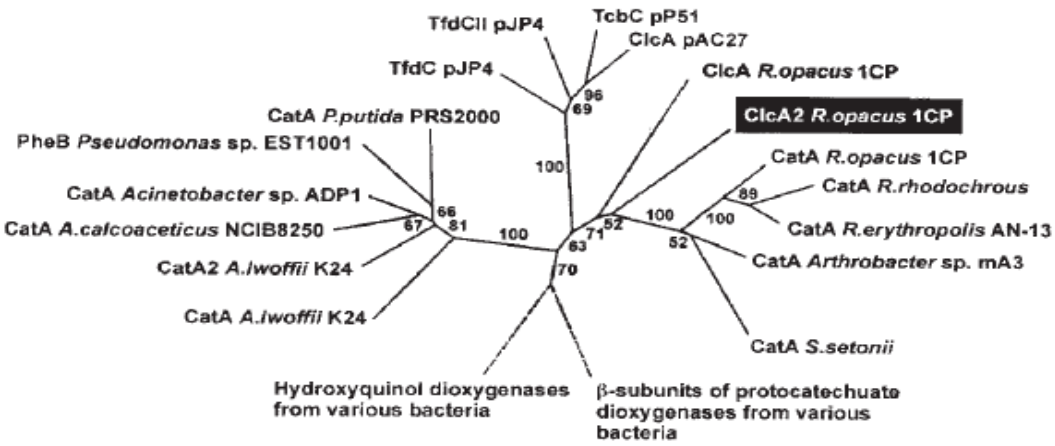

Figure 1.6: The relationship of the dioxygenases enzyme from the different Gram-negative bacteria (Solyanikova and Golovleva, 2004).

1.6 Rationale

Pollution is a global problem and is increasing in South Africa due to the use and inappropriate disposal of chemicals in agriculture and industries. Microorganisms that are found in contaminated sites possess enzymes capable of degrading chlorophenol compounds. It is therefore imperative to optimize the conditions for the enzymes to facilitate an improved degradation of the compounds by microorganisms. This study aimed at determining the efficiency of indigenous bacterial isolates for 2,4-DCP degradation and characterizing the enzymes involved in the biodegradation process, with the view of establishing the metabolic pathways in these organisms. Such microorganisms and/or their enzymes may be used as solutions to treat chlorophenol-contaminated sites. Another objective of this study was to improve enzyme activity and optimise the conditions for proper degradation of aromatic compounds.

1.7 Hypothesis

It is hypothesized that microorganisms indigenous to contaminated sites possess the enzymes capable of mineralizing 2,4-dichlorophenols via the *ortho*-pathway and/or *meta*-pathway. It is further hypothesized that establishing the biodegradation potential of these organisms and characterization of the enzymes involved will allow for the possible application of the organisms and/or their enzymes for treating chlorophenol-polluted sites.

1.8 Aims

1.8.1 To Isolate and identify microorganisms capable of degrading 2,4-dichlorophenol.

1.8.2 To characterize the organisms for 2,4-DCP biodegradation.

1.8.3 To determine the pathway (s) followed by the microorganisms during the degradation of 2,4-DCP.

1.8.4 To purify phenol hydroxylase and catechol 1,2-dioxygenase from isolated strains.

1.8.5 To design primers for the isolates.

1.8.6 To characterize the enzyme phenol hydroxylase and catechol 1,2-dioxygenase.

1.8.7 To determine the kinetic parameters of the purified enzymes.

1.8.8 To perform tryptic digestion and amino acid sequencing of the purified enzyme sliced from Sodium Dodecyl Sulfate- Polyacrylamide gel electrophoresis (SDS-PAGE).

1.9 Objectives

1.9.1 Isolation of the microorganisms by culture enrichment technique and identification via 16S rRNA gene sequence amplification and analysis.

1.9.2 To determine growth profile and biodegradation kinetics.

1.9.3 To characterize various enzymes involved in 2,4-DCP and phenol degradation pathways in the crude extract of the bacterial isolates.

1.9.4 To conduct gel filtration and anion exchange chromatography for purification of the enzymes.

1.9.5 To determine the molecular weight of the proteins using SDS-PAGE.

1.9.6 To detect genes that encode for phenol hydroxylase, catechol 1,2-dioxygenase, muconate isomerase and dienelactone hydrolase.

1.9.7 The kinetics parameters such as K_m and v_{max} of the partially purified enzymes will be determined by plotting Lineweaver-Burk plot and applying Michaelis-Menten equation.

1.9.8 To deduce the nucleotide sequence from amino acid sequence and reverse translation.

1.9.9 Prediction of the three-dimensional structure of phenol hydroxylase and catechol 1,2-dioxygenase.

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CHAPTER TWO: ISOLATION AND CHARACTERIZATION OF BACTERIA INDIGENOUS TO CONTAMINATED SITES IN DURBAN FOR 2,4-DICHLOROPHENOL DEGRADATION

Abstract

Chlorophenolic compounds are of environmental and human health concern due to their mutagenic and carcinogenic properties. 2,4-Dichlorophenol (2,4-DCP), in particular, is classified as a priority pollutant due to its recalcitrance, persistence and toxicity. The aims of this study were to isolate 2,4-DCP degrading bacteria from contaminated sites in Durban and establish their degradation potential during growth in 2,4-DCP as the sole carbon and energy source, and to detect the presence of 2,4-DCP catabolic genes in selected isolates. Following enrichment in mineral salt medium (MSM) supplemented with 40 ppm of 2,4-DCP as the sole carbon and energy source, three 2,4-DCP degrading bacteria were isolated and identified as *Pseudomonas chlororaphis* strain UFB2, *Klebsiella pneumoniae* strain KPNIH39 and *Klebsiella pneumoniae* strain DHQP1002001 via PCR amplification and analysis of their 16S rRNA genes. These isolates could degrade between 49.01% and 75.11% of 2,4-DCP within 10 days with the degradation rate constant ranging between 0.07 and 0.14 mg/L/d. PCR amplification of the catabolic genes involved in 2,4-DCP degradation from the genomic DNA of two of the isolates (*Pseudomonas chlororaphis* and *Klebsiella pneumoniae*) revealed the presence of phenol hydroxylase, catechol 1,2-dioxygenase, muconate isomerase, *cis*-dienelactone hydrolase and *trans*-dienelactone hydrolase genes, this was confirmed by the specific activities of the enzymes (in mU/mg protein) of 21840, 15630, 1480, 2340 and 1490 for phenol hydroxylase, catechol 1,2-dioxygenase, muconate isomerase, *cis*- dienelactone hydrolase and *trans*-dienelactone hydrolase, respectively when phenol is used as a carbon source. The ability of the microorganisms to degrade 2,4-DCP may assist in the treatment of environments that are contaminated with other aromatic compounds.

2.1 Introduction

Widespread use of chemicals and their release into the environment by anthropogenic activities leads to the deteriorating quality of life (Tornero and Hanke, 2016; Arora and Bae, 2014). One of the leading chemical pollutants is 2,4-dichlorophenol (2,4-DCP), a chlorinated aromatic compound mainly used in the synthesis of pentachlorophenol and 2,4- dichlorophenoxyacetic acid (2,4-D), the widely-used herbicide (Elkarmi *et al*., 2009; Abdullah *et al*., 2010; Arora and Bae, 2014; Trivedi *et al*., 2015). It has also been used in the manufacturing of plastics, pesticides, fungicides used widely as a wood preserver, anticorrosive rust product and as an additive in dyes and drugs (Liu *et al*., 2015; Trivedi *et al*., 2015; Tornero and Hanke, 2016). 2,4-DCP, has been listed as a priority pollutant due to the carcinogenic, mutagenic and teratogenic properties as reported by the US Clean Water Act as well as the European Union (EU) Directive 2455/2001/EC (Abdullah *et al*., 2010; Al-Khalid and El-Naas, 2013; Igbinosa *et al*., 2013; Liu *et al*., 2015).

Anthropogenic activities and accidental spillages are the main causes of the release of 2,4-DCP in the environment, but there are certain factors that contribute to the recalcitrance of the compound in the environment. The compound is distributed through the air, water, soil and living organisms (Trivedi *et al*., 2015; El-Naas *et al*., 2017). The distribution and degradation of the compound are dependent on its physicochemical properties. 2,4-Dichlorophenol is volatile and hydrophobic, especially at low temperatures but soluble in inorganic solvents such as ethanol and methanol (El-Naas *et al*., 2017).

Several studies showed that the concentration limit of the 2,4-DCP set for drinking water is 0.5 mg/L. This regulation is important since 2,4-DCP is found in water, due to the use of chlorine for disinfection (Hassine *et al*., 2015; Kadmi *et al*., 2015; Zhu *et al*., 2016). Liu *et al*. (2015) reported that though there are regulations concerning drinking water, the elevated levels of 2,4-

DCP in wastewater cause pollution in the environment when its effluent is discharged into different water bodies (Abdullah *et al*., 2010; Jin *et al*., 2012), where it affects living organisms, especially animals and subsequently human beings as they consume meat from the animals (Hader 2013; Gryglik *et al*., 2016). Also, 2,4-DCP has been found to be an endocrine disruptor and has cytotoxic properties (Jin *et al*., 2012; Gryglik *et al*., 2016; Lurquin, 2016). Therefore, it is imperative to eliminate this compound from the environment.

Several methods have been employed to degrade 2,4-DCP over the years, such as carbon adsorption, ion exchange, reverse osmosis, solvent extraction and flocculation, and these have shown the ability to remove 2,4-DCP (Stoilova *et al*., 2006; Elkarmi *et al*., 2009; Li *et al* 2011; Albadarin *et al* 2012; Liu *et al*., 2015), albeit at varying level. However, there were drawbacks to the methods as they are expensive and produce toxic secondary metabolites. Biological formation of the compound was therefore conducted using microorganisms such as yeast, bacteria, algae and fungi, especially those that can mineralize the compound into metabolites that are non-toxic to the environment and living organisms. Bioremediation therefore serves as an attractive method for the degradation of 2,4-DCP. Bacteria such as *Pseudomonas* spp. have been used to degrade the compound and other chlorophenols (Elkarmi *et al*., 2009; Al-Khalid and El-Naas, 2013; Arora and Bae, 2014; Chen *et al*., 2015). Also, fungi such as *Candidas tropicalis* have been shown to have the ability to degrade 2,4-DCP (Al-Khalid and El-Naas, 2013).

There are several microorganisms that are involved in the biodegradation of 2,4-DCP. However, microorganisms have been reported to only be able to degrade a certain concentration of the compound since high concentration is detrimental to the microorganism and may inhibit enzyme activity (Elkarmi *et al*., 2009). Stoilova *et al*. (2006) reported that concentrations that are above 300 ppm have inhibitory effects on the growth of the microorganism. Fungi such as *Aspergillus awamori* have high degradation potential because

they possess enzymes that can mineralize the compounds. The genes and the enzymes of microorganisms are easy to manipulate to optimize the degradation rates. Literature and studies show that there are only a few microorganisms that can utilize 2,4-DCP as the sole carbon and energy source. The bacteria, *Cupriavidus necator* JMP134 has been used as a model organism for the degradation of chloroaromatic compounds (Perez *et al*., 2008). Most of the studies used chemical or physical methods for the degradation of the compound, however they result in toxic secondary metabolites and are very expensive. Thus, the aims of the study were to isolate indigenous bacteria that can degrade 2,4-DCP, to determine the phylogenetic relationship of the isolated bacteria to those previously reported in literature, to determine growth and degradation kinetics, and to identify the genes involved in the catabolism of 2,4-DCP.

2.2 Materials and Methods

2.2.1 Sample collection

Groundwater sample with known history of contamination with chlorinated organic compounds, was collected from an environmental site, while activated sludge sample was collected from New Germany wastewater treatment plant, both located in Durban, South Africa. Samples were collected in 500 mL bottle and immediately stored at 4°C until used for enrichment set-up.

2.2.2 Enrichment and isolation

The mineral salt medium (MSM) used for the culture enrichment comprise of (in mg/L): KH_2PO_4, 800; Na_2HPO_4, 800; $MgSO_4 \cdot 7H_2O$, 200; NH_4SO_2, 500. The pH was adjusted to 7.5 using 2M NaOH prior to autoclaving at 121°C for 15 minutes. One mL of trace metal which comprised of (mg/L): $FeSO_4 \cdot 7H_2O$, 5; $ZnSO_4 \cdot 7H_2O$, 4; $MnSO_4 \cdot 4H_2O$, 0.2; $NiCl \cdot 6H_2O$, 0.1;

H_3BO_3, 0.15; $CoCl_2 \cdot 6H_2O$, 0.5; $ZnCl_2$, 0.25; EDTA, 2.5; was added by syringe filter (0.2 µm pore) into 1 litre MSM. Ten percent of the groundwater/sludge sample was inoculated into MSM that was supplemented with 40 ppm of 2,4-DCP in a 250 mL Erlenmeyer flask and incubated at 30°C shaking at 150 rpm for a week (Stoilova *et al*., 2006; Karn *et al*., 2015). Sub-culturing in a fresh media was carried out until a stable and consistent culture was obtained. Aliquots from each culture were spread on MSM agar plates supplemented with 40 ppm of 2,4-DCP and incubated at 30°C until the growth of the microorganisms were visible. Pure cultures were obtained by streaking individual morphologically different colonies on nutrient agar plates. The pure colonies were stored at -70°C as 20% (v/v) glycerol stocks.

2.2.3 Growth and degradation studies

Six bacteria were isolated from the contaminated groundwater and activated sludge samples. However, only three isolates showed the ability to use 2,4-DCP as the sole carbon and energy source during screening step and therefore used for further studies.

2.2.3.1 Standardization of inoculum and experimental set-up

The inoculum was prepared by streaking bacterial cultures on nutrient agar plates and incubating them at 30°C overnight. A pure colony of the culture was transferred into 100 mL nutrient broth and incubated at 30°C, at 150 rpm. The cell pellets were collected by centrifugation at 8000 rpm and suspended in MSM, washed twice and adjusted to an OD of 1.0 at 600 nm. Ten milliliters of the standardized culture were added to 250 mL Erlenmeyer flask containing 90 mL MSM supplemented with 40 ppm of 2,4-DCP. The flasks were incubated at 30°C at 150 rpm and monitored for growth, chloride release and 2,4-DCP degradation over a period of time.

2.2.3.2 Growth determination

One milliliter sample was withdrawn periodically from the culture flask and used for growth measurement at 600 nm. All experiments were performed in triplicate. Haldane growth inhibition model (Eq. 2.1) was used to calculate the growth kinetic parameters (Kargi and Eker, 2005)

$$\mu = \mu_m S / (S + K_s + S^2/Ki) \quad \text{(Eq. 2.1)}$$

Where, μ is the specific growth rate (h^{-1}), μ_m is the maximum specific growth rate (h^{-1}), K_s is the half saturation constant (mg/L), K_i is the substrate inhibition constant (mg/L) and S is the substrate concentration (mg/L).

2.2.3.3 2,4-DCP degradation kinetics

2,4-dichlorophenol concentration was determined spectrophotometrically at 284 nm. A 5000-ppm stock solution of 2,4-DCP was prepared by dissolving analytical grade 2,4-DCP in MSM and sterilized by syringe filter (0.22μm) and stored in a dark colored bottle and closed cabinet to avoid light oxidation of the compound. The standard curve was plotted by diluting 2,4-DCP in MSM to the concentration of 0-50 ppm and measuring the OD at 284 nm (Gaya, 2010; Leszczynska *et al*., 2010; Gryglik *et al*., 2016). The data were plotted and fitted in the linear equation y= mx+c to determine the 2,4-DCP concentration in unknown samples. All experiments were performed in triplicate. The Haldane/Andrew`s substrate inhibition model (Eq. 2.2) was used to calculate the biodegradation kinetics constants during chlorophenols degradation (Kargi and Eker, 2005).

$$R_s = (R_m S/K_s + S)(K_i / K_i + S) = R_m (1 + Ks/S)(1 + SK_i) \quad \text{(Eq. 2.2)}$$

Where R_s and R_m are the actual and maximum rate of chlorophenol degradation (mg 2,4-DCP/L/h). S is the initial [2,4-DCP] (mg/L); K_s is the saturation constant (mg/L) and K_i is the 2,4-DCP inhibition constant (mg/L).

At lower substrate concentration, the inhibition constant can be neglected. Hence, the above equation can be derived as:

$$R_s = R_m S / (K_s + S) \quad \text{(Eq. 2.3)}$$

The reciprocal of equation can be plotted linearly,

$$1/R_s = 1/R_m + (K_s/R_m)(1/S) \quad \text{(Eq. 2.4)}$$

2.2.3.4 Chloride release measurement

Chloride release during 2,4-DCP degradation was measured spectrophotometrically at 460 nm (Bergmann and Sanik, 1957). One hundred microliters of Reagent A were added to 1 mL of the sample to be analyzed. Consequently, the addition of 200 µL of Reagent B develops a color and absorbance was measured at the wavelength 460 nm (UV-1800 Shimadzu UV spectrophotometer). The Cl^{-1} standard curve was derived by mixing 100 µL of reagent A to 1mL sample (in 1.5 mL tubes constituting of 0-50 µg Cl^{-1}) followed by 200 µL reagent B and measuring the developed color at 460 nm. 14.1 mM of NaCl equivalent to stock of 500 µg/mL Cl^{-1} was prepared by dissolving 824 µg of NaCl in 1mL of autoclaved double distilled water. The curve was fitted to the linear equation $y = mx + c$ and Cl^{-1} released determined in unknown samples. The composition of reagent A and B are shown in Appendix A (7.2).

2.2.4 Identification and phylogenetic analysis of the bacterial isolates

2.2.4.1 DNA extraction

The bacterial cells were grown on the nutrient agar for 24 h at 30°C. Thereafter, a single colony was inoculated into 10 mL nutrient broth and incubated at 30°C, shaking at 150 rpm for 16 h. The cells were collected by centrifuging at 8000 rpm and used for DNA extraction using the Genomic DNA Purification Kit (Thermo Scientific, USA). The purity and concentration of DNA were analyzed by NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE) and the DNA integrity was checked by loading 200 ng DNA on 1.0% (v/v) agarose gel, electrophoresis (Powerpac^{TM}, BIORAD) run at 85 volts, 60 minutes and viewed by Syngene G: Box. The DNA was stored at -20°C and used for further experiments.

2.2.4.2 Polymerase Chain Reaction (PCR) amplification of 16S rDNA

For the identification of microorganisms, PCR experiments were performed in T100^{TM} Thermal Cycler (BIO-RAD) using universal primers for 16S rRNA amplification using the purified DNA as a template. The primers: forward primer 63F-5'-CAGGCCTAACACATGCAAGTC-3' and reverse primer 1387R-5'-GGGCGGTGTGTACAAGGC-3' were synthesized at Inqaba Biotech (Pretoria, South Africa). Ten microliter PCR reaction mixture contained: 1 µL buffer (10x), 0.6 µL $MgCl_2$ of (25 mM), 0.2 µL of 200 µM dNTP, 0.2 µL of each primer (10 µm) and 0.05 µl of AmpliTaq polymerase, ~20 ng DNA template and 7.3 µL autoclaved ddH_2O. The final PCR reactions were scaled up to the volume of 50 µL. The PCR conditions were as follows: 95°C for 5 min, (1 cycle) 95°C for 30 s, 55°C for 1 min and 72°C for 1 min (35 cycles) and a final elongation at 72°C for 10 min (1 cycles). The amplified PCR product was checked through 1.0% gel electrophoresis (Powerpac^{TM}, BIORAD) run at 85 volts, 60 minutes and viewed by Syngene G: Box.

2.2.4.3 Sequencing and analysis of 16S rRNA genes

The amplified 16S rRNA gene was sequenced by Inqaba Biotechnical Industries (Pty) Ltd (Pretoria, South Africa). The gene sequences were retrieved using SnapGene Viewer (3.3.4) and submitted to the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/blast/ using the blastn algorithm) for the identification of organisms.

2.2.4.4 Phylogenetic analysis

The 16S rRNA sequences were retrieved from NCBI and the phylogenetic tree was constructed by rooted neighbour-joining method using DNAMAN (version 7), Lynnon Corporation, CA, USA (Demo version). The numbers on branching points are bootstrap values with 1000 replicates (values <95% were not included).

2.2.5 Detection of catabolic genes involved in 2,4-DCP degradation via PCR

The very nearly related strains to the 2,4-DCP degrading bacteria isolated in this study, based of 16S rDNA homology, were searched for whole genome sequences in NCBI database. The whole genome database of the individual strain was searched for the presence of genes involved in 2,4-DCP degradation. The gene sequences were retrieved and aligned in DNAMAN (version 7), Lynnon Corporation, CA, USA (Demo version) and used to manually design degenerate primers for PCR amplification of these genes. The primer sequences were checked for self-annealing, complementarity and hair loop formation before sending for synthesis. The PCR assays were conducted at 95°C for 5 min, (1 cycle) 95°C for 30 s (denaturation), annealing was at different annealing temperatures indicated in Table 2.1 and and a final elongation at 72°C for 10 min (1 cycles).

Table 2.1: Primers designed in this study for the PCR detection of catabolic genes involved in 2,4-DCP degradation by the bacterial isolates, the annealing temperature and expected product size

Pseudomonas chlororaphis UFB2

Enzyme	Nucleotide sequence (5′ → 3′)	Annealing Temperature (ºC)	Expected Size (bp)
Phenol Hydroxylase	**pPhe-F**-ATG AAG CTG CTC GCC GTC CGC	60	600 bp
	pPhe-R-CAG GTC GAG TTC GTC GTA GAT GG		
Catechol 1,2-dioxygenase	**pC120-F**- AAT CTC GAG ATG TCT ATC CGA ATT TCC CAG	62	467 bp
	pC120-R- AAT GCT TAG CTT AGT CTT CGA GGG CGC G		
Muconate Isomerase	**pMuc-F**- ATT CAG CTC AGG CAG TCA AAG CAA A	60	651 bp
	pMuc-R- CGA GCA ATC AAC TGT TCG ACG CTT		
Dienelactone Hydrolase	**pDh-F**-CTG GCC ATC GAC ATG TAC GGC GA	60	491 bp
	pDh-R-TGT TGT AGC CAA TGT CCG GCC C		

Klebsiella pneumoniae

Enzyme	Nucleotide sequence (5′→ 3′)	Annealing Temperature (ºC)	Expected Size (bp)
Phenol Hydroxylase 1	**kPhe1-F**-TGC TGT TCT GGA TGC CAA ACC	55	715 bp
	kphe1-R-CGG AAC ACC TGG GTG AAG AA		
Phenol Hydroxylase 2	**kPhe2-F**-TGG GCC AGG TGG TTG AAC TC	57	304 bp
	kphe-2-R-CG GTA AAG CGC TCG AAG GC		
Catechol 1,2-dioxygenase	**kCat-F**-ATG GCT AAC ATT CTC GGC GG	55	507 bp
	kCat-R-TGG CCG AGT TTG TAA CAA CGG		
Muconate Isomerase	**kMuc2-F=** ATT CTC GAG ATG CCG CAT TTT ATC GCC	62	494 bp
	kMuc2-R= ATT GCT CAG CTT ATT TAA ATA ACG CGT GC		
Dienelactone Hydrolase	**kDh-F**-ATG ACG CGA TTA ACG GCC AAA G	60	567 bp
	kDh-R-CCG TAG CAA AAA CCG GTG ATA C		

2.2.6 Enzymes induction and activities

Cells of *Pseudomonas chlororaphis* strain UFB2, *Klebsiella pneumoniae* strain KPNIH39 and *Klebsiella pneumoniae* strain DHQP1002001 were grown in nutrient broth overnight and the culture was standardized to OD=1 at 600 nm. A 10% inoculum of the standardized culture was inoculated into Mineral Salt Medium (MSM) supplemented with 600 ppm of phenol as a sole carbon and energy source. The MSM comprised of (in g/L): K_2HPO_4, 2.75; KH_2PO_4, 0.1; NH_4Cl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.01; $CaCl_2 \cdot 2H_2O$, 1.0; NH_4Cl, 0.5, and Yeast extract, 1.0. The pH was adjusted to 7 with 2M NaOH prior to autoclaving at 121°C for 15 minutes. 1 mL of trace metal solution composed of (mg/L): $FeSO_4 \cdot 7H_2O$, 5; $ZnSO_4 \cdot 7H_2O$, 4; $MnSO_4 \cdot 4H_2O$, 0.2; $NiCl \cdot 6H_2O$, 0.1; H_3BO_3, 0.15; $CoCl_2 \cdot 6H_2O$, 0.5; $ZnCl_2$, 0.25; EDTA, 2, was added separately by syringe filter (0.22 µm) into the media. The inoculated media were incubated at 30°C for 36 h with shaking at 150 rpm. The cells were harvested in the late exponential phase of growth by centrifugation at 10000 ×*g* for 15 min at 4°C. The cells were washed twice with 50 mM sodium phosphate buffer, pH 7.5 (1 mM EDTA and 1mM β-Mercaptoethanol was added to deactivate proteases and avoid hydrolysis of proteins by the proteases). A total of 24 g of the cell pellet was collected and suspended in 100 mL of the same buffer. Cell-free extracts were prepared by lysing the pellet by sonication with 400 Ultrasonicator (OMNI International) 8 cycles each with a pulse of the 30s on/off for 4 min). The lysed cell extract was centrifuged at 20000× *g* for 30 min at 4 °C. The clear supernatant was kept on ice to prevent inactivation of the enzymes and used as a crude extract for enzyme assays, while the remaining extract was kept in -20°C for further studies (Mahiudddin *et al*., 2011). The substrate used, product formed and wavelength used to measure product during different enzyme assays are summarized in Table 2.2.

Table 2.2: Details of substrates and wavelengths used for the different enzyme assays and the products measured.

Enzymes	Substrates	Wavelengths	Products	References
Phenol hydroxylase	2, 4-Dichlorophenol Phenol (NADH)	340 nm (Decrease in Absorbance)	Catechol NAD^+	(Kotresha and Vidyasagar, 2012).
Catechol 1,2-dioxygenase	Catechol	260 nm (Increase in Absorbance)	*Cis*-*cis*-muconic acid	(Mahiudddin *et al*., 2012).
Catechol 2,3- dioxygenase	Catechol	375 nm (Increase in Absorbance)	2-Hydroxymuconic semialdehyde	(Mahiudddin *et al*., 2012).
Muconate isomerase	*Cis*-*cis*-muconate	260 nm (Increase in Absorbance)	Dienelactone Hydrolase	(Matsumura *et al*., 2006).
Dienelactone hydrolase	*Cis*-dienelactone and *trans*-dienelactone	280 nm (decrease in Absorbance)	Maleylacetate	(Kumar *et al*., 2014).
Maleylactetate reductase	Maleylacetate	340 nm (Decrease in Absorbance)	3-oxoadipate	(Matsumura *et al*., 2006)

2.2.6.1 Phenol hydroxylase assay

Phenol hydroxylase activity was measured by the modified method of Kotresha and Vidyasagar (2012). One milliliter reaction mixture contained 2.5 µM phenol, 1.0 mM NADH in 50 mM Sodium phosphate buffer (pH 8.0). The reaction was initiated by adding 100 µL of enzyme extract to the reaction mixture and incubated for 30 min at 30°C. The initial and final absorbance was measured at 340 nm using Shimadzu UV-1800 UV-Vis Spectrophotometer fitted with temperature controller CPS-240A unit set at 30°C. The proper controls for each reaction were conducted along with the assay. One unit of enzyme activity was defined as the amount of the enzyme reducing 1 µm of NADH per minute under standard assay conditions.

The OD at 340 nm representing the reduction of NADH was converted in the micromole of the product by using extinction coefficient of NADH i.e. 6225 µM/min/cm. The unit of enzyme activity was calculated by the equation below:

Enzyme activity = (Δ Abs/ E × L)/ time (min)

Where Abs=Optical density at the different wavelengths; E= molar extinction coefficient and L =path length (Mahiudddin *et al*., 2011).

2.2.6.2 Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activity assay

Catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activity were measured by the method described Kotresha and Vidyasagar (2012). One milliliter reaction mixture contained 10 mM of catechol (substrate) in 50 mM Sodium phosphate buffer (pH 8.0). The reaction was initiated by adding 100 µL of enzyme extract to the reaction mixture and incubated for 30 min at 30°C. The initial and final absorbance at 260 nm and 375 nm were measured using Shimadzu UV-1800 UV-Vis Spectrophotometer fitted with temperature controller CPS-240A unit set at 30°C. The proper controls for each reaction were conducted along with the assay. One unit of enzyme activity was defined as the amount of the enzyme forming 1 µM of either *cis*-*cis*-muconic acid at 260 nm (Catechol 1,2-dioxygenase) or 2-hydroxymuconic semialdehyde at 375 nm (Catechol 2,3-dioxygenase) under standard assay conditions. The enzyme activity was calculated by using the following equation:

Enzyme activity (µm of product formed/min) = [(E × L/V) (ΔOD /min)

Where ΔOD=Optical density at the different wavelengths; E= molar extinction coefficient, V= volume, and L = path length. Molar extinction coefficient of 16 800 µM/min/cm (muconic acid) and 14 700 µM/min/cm (2-hydroxysemialdehyde) were used for catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activity, respectively (Mahiudddin *et al*., 2011).

2.2.6.3 Dienelactone hydrolase activity assay

The dienelactone hydrolase activity was measured using 0.5 µM of *cis*- and *trans*-dienelactones as the substrates. The enzyme activity was determined spectrophotometrically by measuring the decrease in OD at 280 nm. In 900 µL of 50 mM of Tris-Cl buffer (pH 7) in a quartz cuvette containing 0.5 µM of the substrate, 100 µL enzyme solution was added and incubated for 30 min. Absorbance at 280 nm was recorded using Shimadzu UV-1800 UV-Vis Spectrophotometer fitted with temperature controller CPS-240A unit set at 30°C. The OD at 280 nm represents the reduction of *cis*-dienelactone or *trans*-dienelactone under standard assay conditions. The molar extinction coefficient of ε_{280}=17000 $M^{-1}cm^{-1}$ and ε_{280}=15625 $M^{-1}cm^{-1}$ for *cis*-dienelactone or *trans*-dienelactone, respectively, was used to calculate the units of dienelactone hydrolase enzyme (Kumar *et al*., 2014).

2.2.6.4 Muconate cycloisomerase activity assay

The *cis*-*cis*-muconate cycloisomerase enzyme activity was measured in a reaction mixture containing 30 mM Tris-HCl (pH 8.0), 1 mM $MnSO_4$, 100 µL enzyme extract, and 0.1 mM *cis*-*cis*-muconate as a substrate. The activity of muconate cycloisomerase was measured spectrophotometrically at 260 nm using Shimadzu UV-1800 UV-Vis Spectrophotometer fitted with temperature controller CPS-240A unit set at 30°C (Murakami *et al*., 2003; Matsumura *et al*., 2006). One unit of the enzyme activity is defined as the amount of enzyme that liberates 1.0 µL of dienelactone hydrolase per minute under standard assay conditions.

2.2.6.5 Maleylacetate reductase activity assay

The crude enzyme extract (100 µL) was added into a quartz cuvette containing 0.2 mM NADH in 50 mM Tris -HCL (pH 7.5). After nonspecific NADH oxidation at 340 nm had been recorded for 2 min, maleylacetate was added to a final concentration of 0.1 mM as a substrate and NADH

oxidation after 30 min incubation was recorded. The activity of maleylacetate reductase was calculated from the difference of the two oxidation rates at 340 nm (Kasberg *et al*., 1995).

2.3 Results

2.3.1 Enrichment and Isolation of 2,4-DCP degrading bacteria

The 2,4-DCP degrading microorganisms were enriched in MSM supplemented with 40 ppm of 2,4-DCP and a plate count technique was used to culture and enumerate bacteria that were able to grow on MSM plates supplemented with 40 ppm of 2,4-DCP. The Colony Forming Units (CFUs) were found to be 1.3×10^6 and 1.1×10^7 for the groundwater and activated sludge sample, respectively. Axenic culture of the individual bacterial isolate was obtained by streaking on nutrient agar plates (Figure 2.1 and 2.2). Six microorganisms showed the ability to grow and utilize 2,4-DCP for growth. Of the isolates cultured from both samples, six dominant organisms (designated as a, b, c, d, e and f) were purified on nutrient agar plates and screened for growth and ability to degrade 2,4-DCP in MSM medium at an initial concentration of 40 ppm.

2.3.2 Screening and selection of isolates.

Preliminary screening of the six isolates when grown in MSM containing 40 ppm of 2,4-DCP as sole carbon and energy source revealed a prolonged lag phase and very short exponential phases for some of the organisms, indicating that they were unable to effectively utilize 2,4-DCP as a substrate (Figure 2.3). Compared to *Cupriavidus necator* JMP134 (a well characterized chlorophenol degrading bacteria) used as a positive control, two isolates (YR and MC) from activated sludge sample and one isolate (GW) from groundwater sample could grow effectively in 40 ppm of 2,4-DCP. A peak OD ranging from 0.16-0.26 was obtained for the three isolates after 5 days of growth (Figure 2.3).

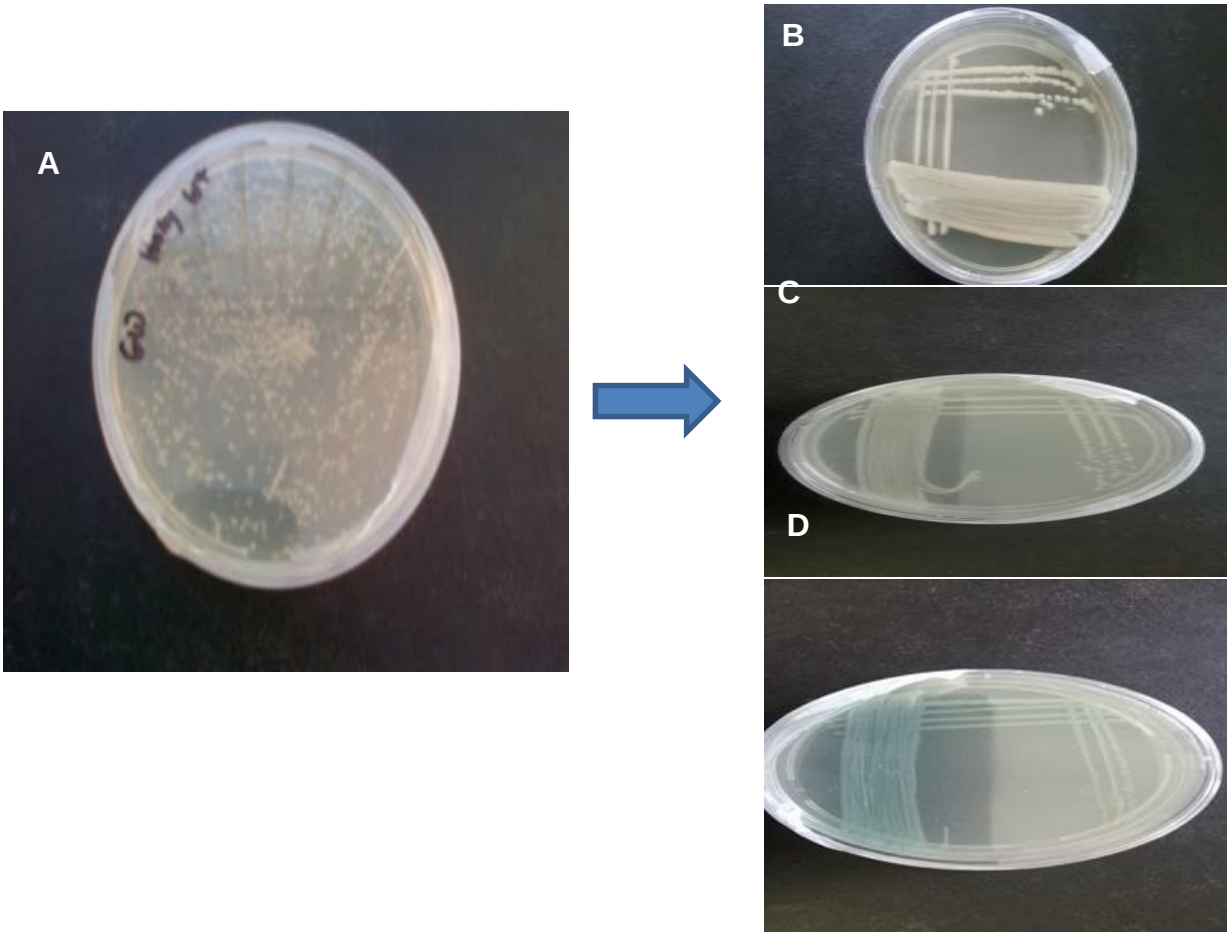


Figure 2.1: Culture plates showing growth of bacteria capable of growth on 2,4-DCP, following enrichment of MSM with groundwater sample (A) and subsequent isolation of pure cultures (B, C and D).

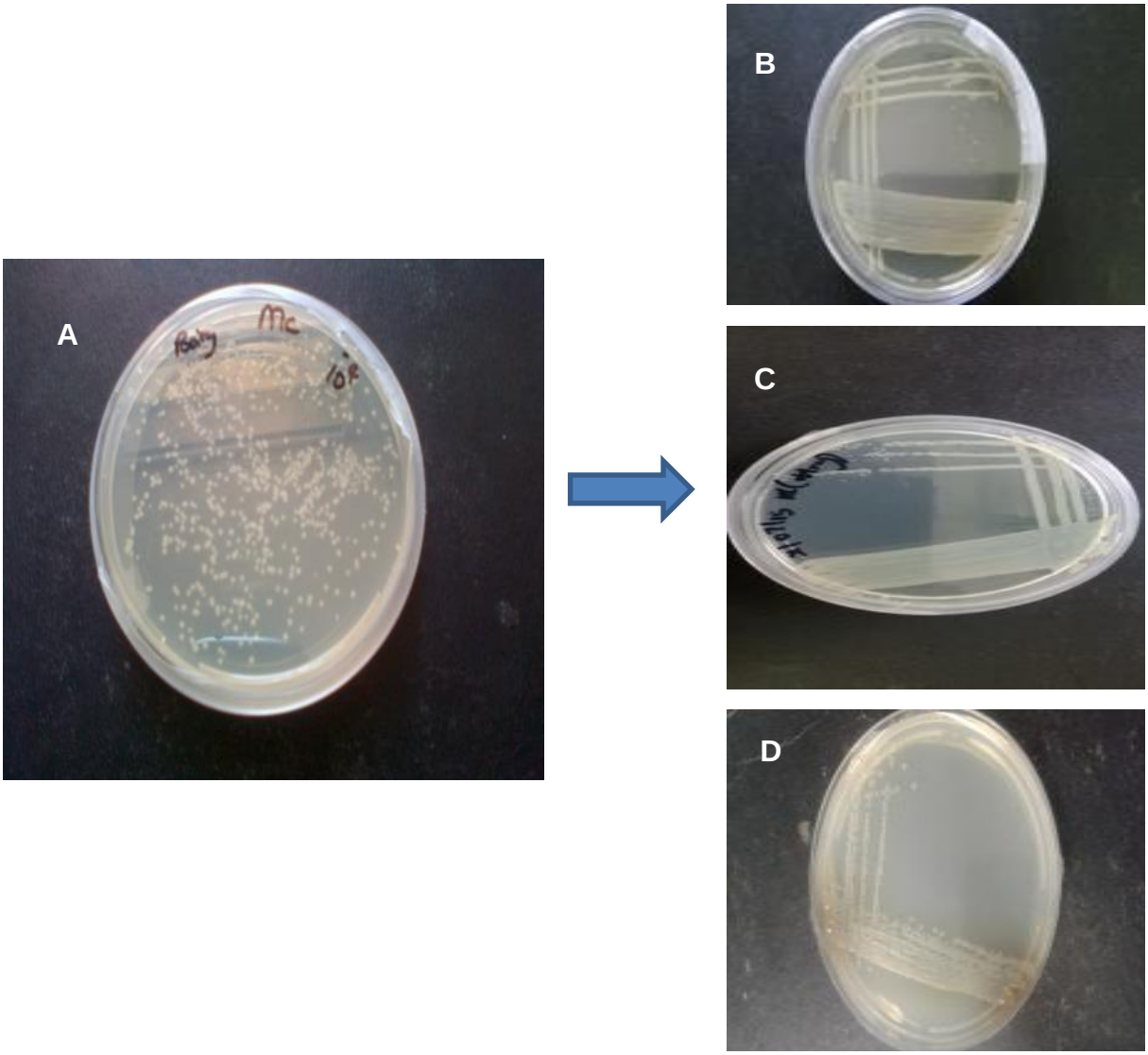


Figure 2.2: Culture plates showing growth of bacteria capable of growth on 2,4-DCP, following enrichment of MSM with activated sludge sample (A) and subsequent isolation of pure cultures (B, C and D).

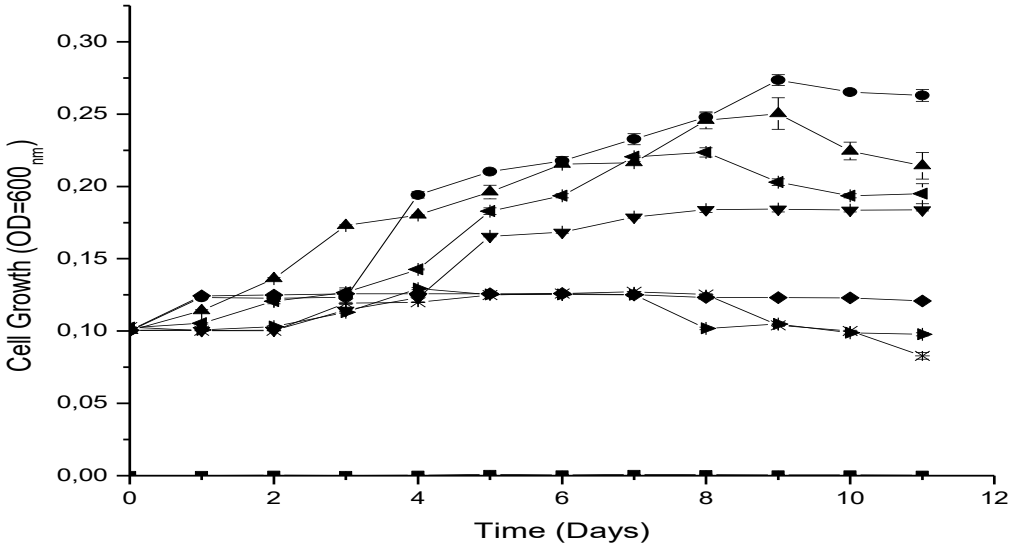


Figure 2.3: Growth profiles of the isolates in 40 ppm 2,4-DCP. (■) Negative control (MSM and 2,4-DCP); (•) Positive control (*Cupriavidus necator* JMP134); (▲) YR; (▼) MC; (♦) MCsr; (◄) GW; (►) GWbr; (ж) GWtr.

The concentration of 2,4-DCP during growth of the bacterial isolates was also determined by extrapolation from the standard curve (Figure 2.9). Of the six bacteria isolated in this study, the highest 2,4-DCP degradation was obtained for isolate YR at 70%, while 52% and 40% degradation was obtained for isolate GW and MC, respectively after 11 days of incubation. Low degradation ranging from 30 to 5% was obtained for the rest of the isolates, while *Cupriavidus necator* JMP134 demonstrated 80% degradation, value higher than those obtained for the bacteria isolates in this study.

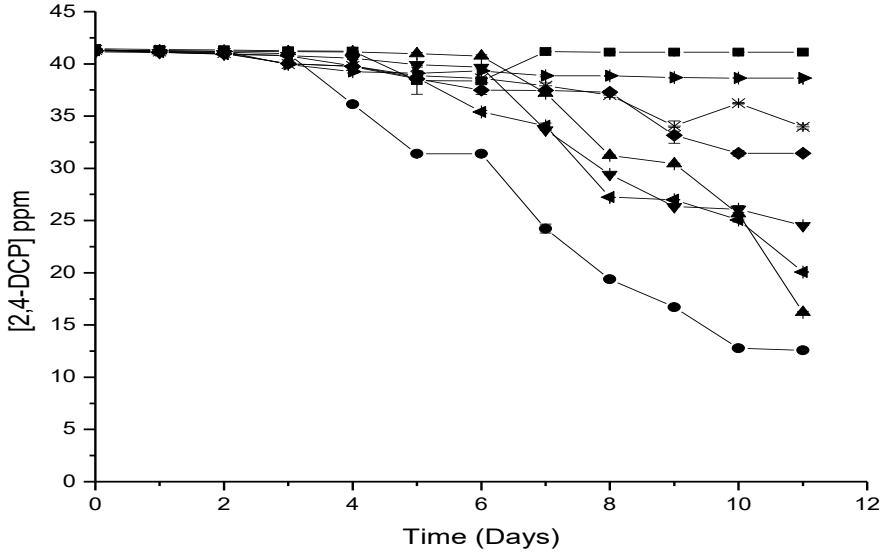


Figure 2.4: Concentration of 2,4-DCP (40 ppm) degraded by isolates. (■) Negative control; (•) Positive control; (▲) YR; (▼) MC; (♦) MCsr; (◄) GW; (►) GWbr; (ж) GWtr.

2.3.3 Identification of selected 2,4-DCP degrading bacterial isolates

PCR assay using the DNA isolated from the isolates as template and universal 16S rRNA primers resulted in the amplification of the expected 800 bp gene (Figure 2.5). Sequencing and analysis of the PCR product revealed the identities of YR as *Pseudomonas chlororaphis* strain UFB2 (accession number: CP011020.1), MC as *Klebsiella pneumoniae* strain DHQP1002001 (accession number: CP016811.1) and GW as *Klebsiella pneumoniae* strain KPNIH39 (accession number: CP014647.1) as shown in Table 2.3

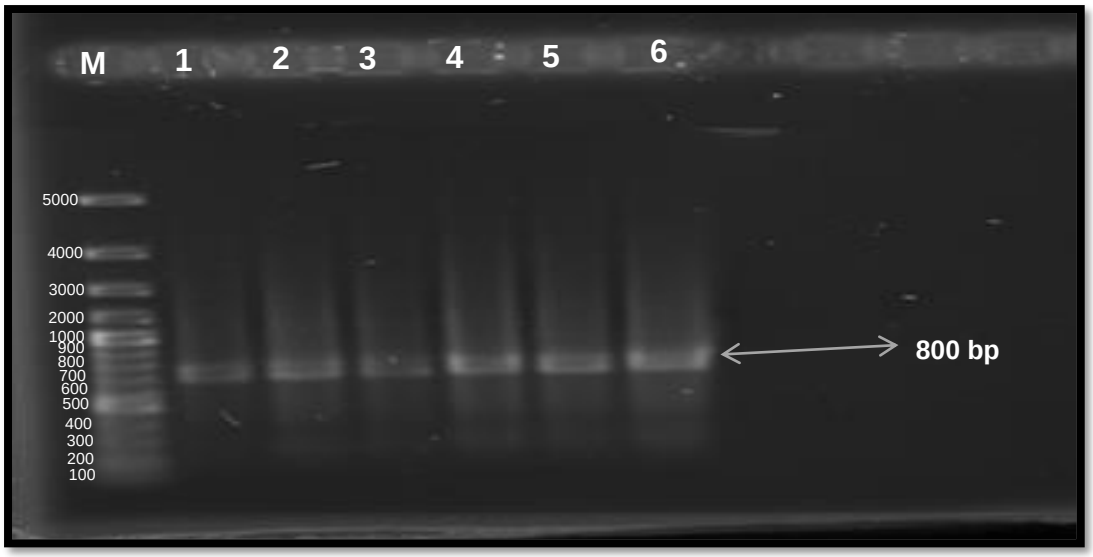


Figure 2.5: Amplicons of the PCR amplified 16S rRNA genes of the bacterial isolates. Isolate YR **(Lane 1 and 2),** isolate MC **(Lane 3 and 4),** and isolate GW (**Lane 5 and 6).** Lane **M** shows 100 bp DNA marker.

Table 2.3: Identification of 2,4-DCP degrading bacterial isolates based on the analysis of their 16S rRNA gene sequences

Culture	Name	Accession Number	Identity
YR	*Pseudomonas chlororaphis* strain UFB2	CP011020.1	89%
MC	*Klebsiella pneumoniae* strain DHQP1002001	CP016811.1	97%
GW	*Klebsiella pneumoniae* strain KPNIH39	CP014647.1	98%

2.3.4 Phylogenetic analysis of strains based on the 16S rRNA gene sequences

16S rRNA gene sequence-based phylogenetic tree (Figure 2.6) showed that *Pseudomonas chlororaphis* strain UFB2 shared more than 90% sequence similarity with other *Pseudomonas*

spp. in the neighbouring-joint phylogenetic tree. *Pseudomonas putida* strain Cal (Accession Number: AY962603.1), *Pseudomonas* sp. SJ08 (Accession Number: KF312469.1), *Pseudomonas* sp. AD3.12 (Accession Number: KP267839.1), *Pseudomonas* sp. RHQ12 (Accession Number: GQ375795.1), *Pseudomonas gessard*ii strain M-B5C (Accession Number: KJ806381.1), *Pseudomonas* sp. P97.38 (Accession Number: DQ453823.1), *Pseudomonas* sp. F96.27 (Accession Number: DQ453817.1) showed 100% homology with *Pseudomonas chlororaphis* strain UFB2. Other *Pseudomonas* strains showed 98% homology with *Pseudomonas chlororaphis* strain UFB2 and they were *Pseudomonas* sp. SAP195.3 (Accession Number: JX067660.1), *Pseudomonas fluorescence* A506 (Accession number: CP003041.1), *Pseudomonas fluorescens* strain LMG 5329 (Accession number: JQ974027.1). While *Pseudomonas azotoformans* strain F77 (Accession number: CP019856.1) showed around 90% homology with *Pseudomonas chlororaphis* strain UFB2

Pseudomonas putida strain Cal (Accession Number: AY962603.1*) and Pseudomonas fluorescence* A506 (Accession number CP003041.1) were reported to degrade 2,4-DCP and other phenolic compounds. The phylogenetic tree for *Pseudomonas chlororaphis* UFB2 showed that all the isolates that relate to it and can degrade 2,4-DCP are from the genus *Pseudomonas*.

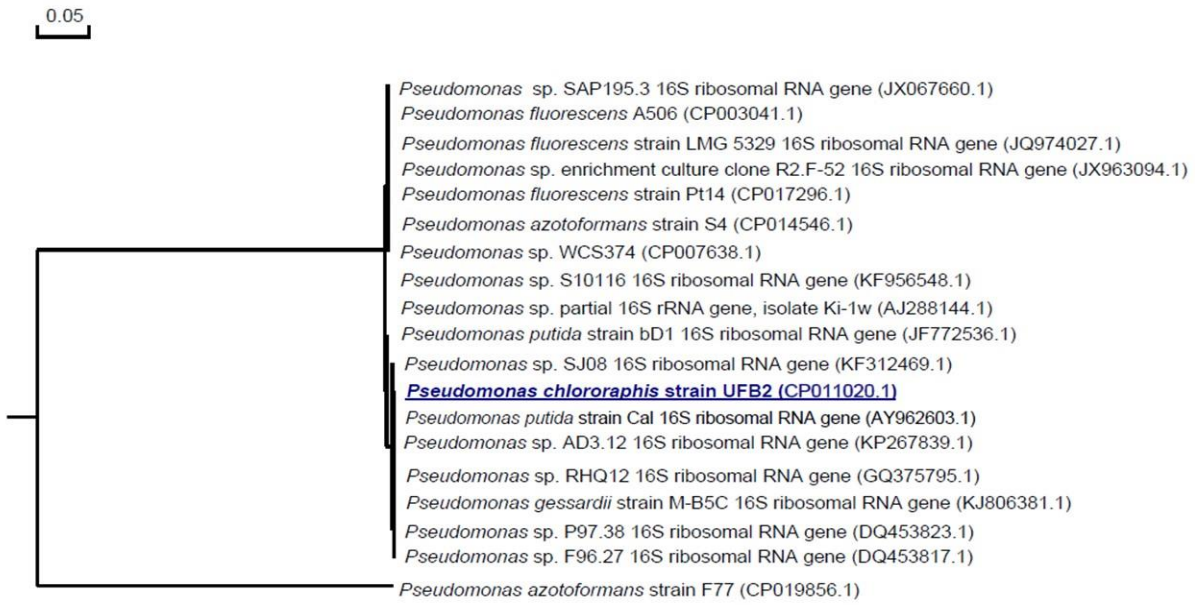


Figure 2.6: Phylogenetic tree showing relatedness of *Pseudomonas chlororaphis* UFB2 isolated in this study to other reported 2,4-DCP and other phenolic compounds degrading *Pseudomonas* spp. The 16S rRNA sequences were retrieved from NCBI and the phylogenetic tree was constructed by rooted neighbour-joining method using DNAMAN (version 7), Lynnon Corporation, CA, USA (Demo version). The numbers on branching points are bootstrap values with 1000 replicates (values <95% were not included).

The phylogenetic tree constructed for *Klebsiella pneumoniae* strain DHQP1002001 (Accession number: CP016811.1) and *Klebsiella pneumoniae* strain KPNIH39 (Accession number: CP014762.1) also resulted in the detection of *Klebsiella pneumoniae* isolates which can degrade 2,4-DCP (Figure 2.7). *Klebsiella pneumoniae* strain KPNIH39 (This study) showed 100% homology with *Klebsiella pneumoniae* strain CAV1596 (Accession number: CP011647.1), *Klebsiella pneumoniae* strain UHKPC33 (CP0119891), *Klebsiella pneumoniae* strain ATCC 35657 (Accession number: CP015134.1). *Klebsiella pneumoniae* strain DHQP1002001 (This study) showed 80% homology with *Klebsiella pneumoniae* strain

KPNIH39. Some *Klebsiella pneumoniae* strains that showed 80% homology with *Klebsiella pneumoniae* strain KPNIH39 and 90% homology with *Klebsiella pneumoniae* strain DHQP1002001 were *Klebsiella pneumoniae* strain XH209 (Accession number: CP009461.1), *Klebsiella pneumoniae* strain KPNIH36 (Accession number: CP014647.1), *Klebsiella pneumoniae* strain KpN01 (Accession number: CP012987.1), *Klebsiella pneumoniae* UHKPC07 (Accession number: CP011985.1), *Klebsiella pneumoniae* strain CAV1344 (Accession number: CP011624.1), *Klebsiella pneumoniae* strain 34618 (Accession number: CP010392.1).

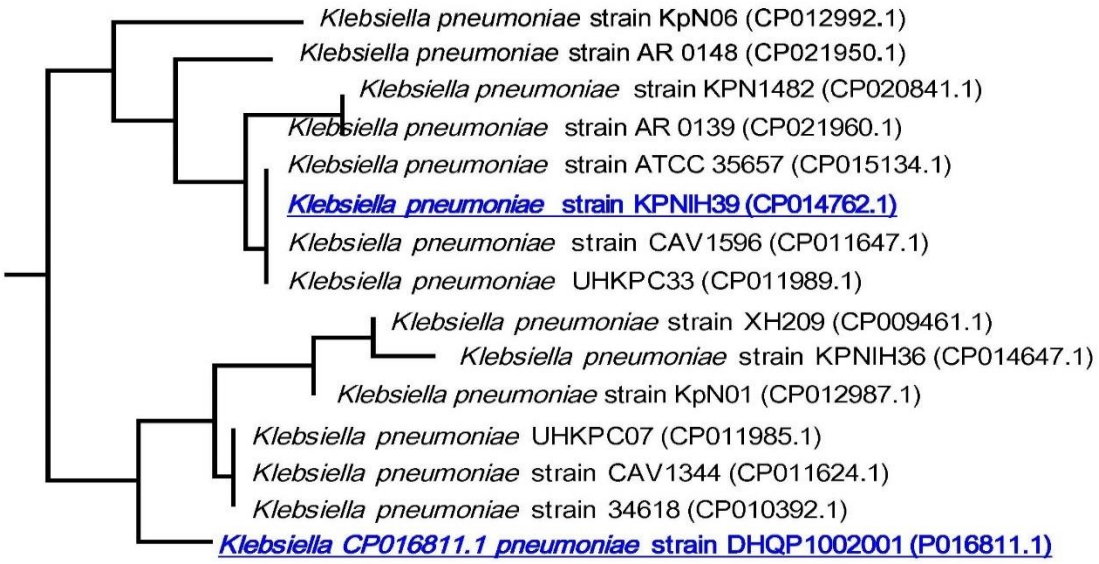


Figure 2.7: Phylogenetic tree of *Klebsiella pneumoniae* strain DHQP1002001 and *Klebsiella pneumoniae* strain KPNIH39 and its relatedness with other *Klebsiella pneumonia* strains can degrade the 2,4-DCP and other phenolic compounds. The 16S rRNA sequences were retrieved from NCBI and the phylogenetic tree was constructed by rooted neighbour-joining method using DNAMAN (version 7), Lynnon Corporation, CA, USA (Demo version). The numbers on branching points are bootstrap values with 1000 replicates (values <95% were not included).

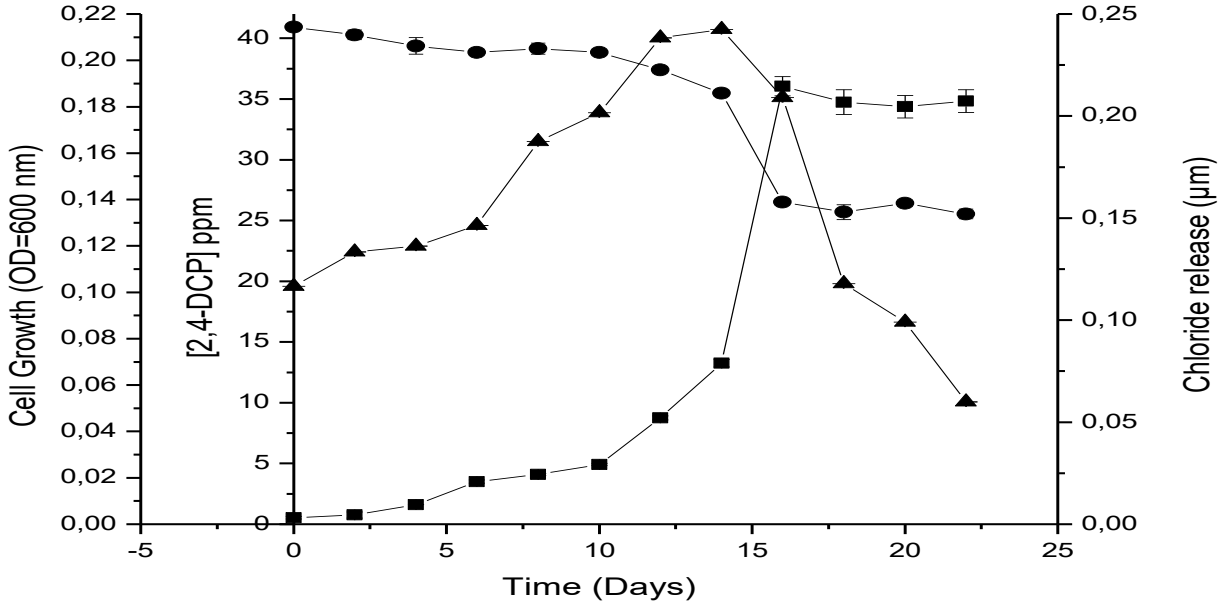
2.3.5 Growth and Degradation studies of selected bacterial isolates on 2,4-DCP

Three identified strains: *Pseudomonas chlororaphis* strain UFB2, *Klebsiella pneumoniae* strain DHQP1002001 and *Klebsiella pneumoniae* strain KPNIH39 were further used for growth and degradation kinetic studies by monitoring cell growth, chloride release and 2,4-DCP degradation.

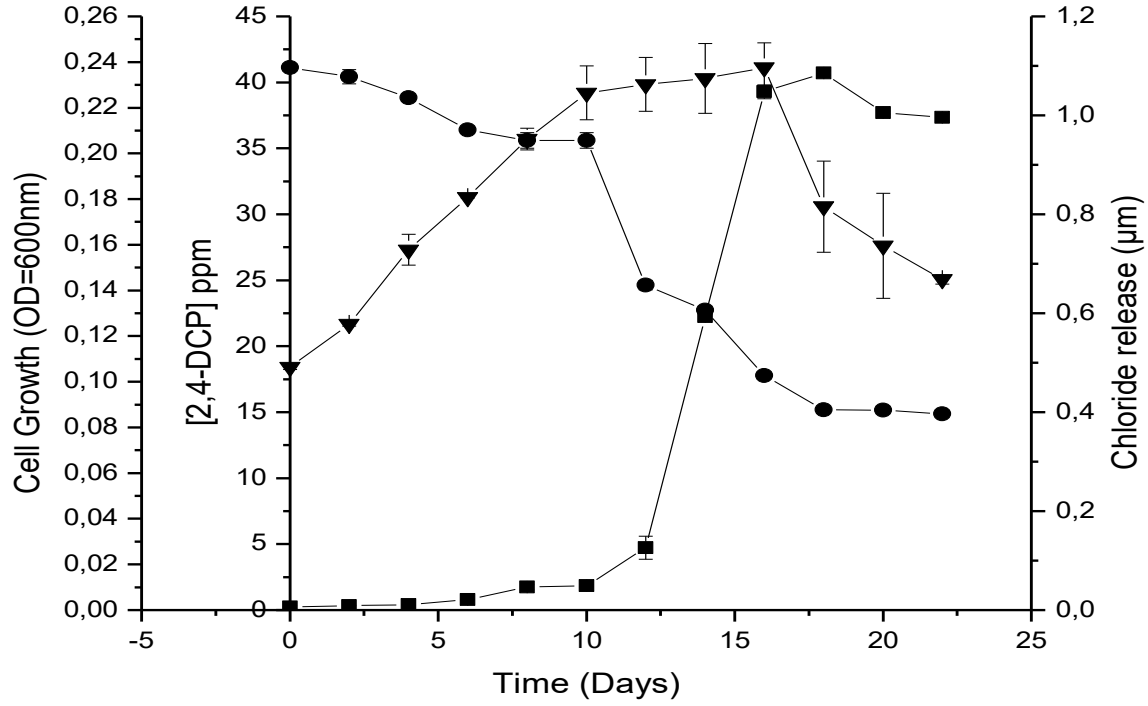
Klebsiella pneumoniae strain DHQP1002001 isolated from activated sludge demonstrated an increase in OD during growth in MSM supplemented with 2,4-DCP as the sole carbon source, attaining a peak OD of 0.21 after 13 days, after which a decline in growth was observed. The organism was able to degrade 49% of 2,4-DCP after 16 days, with a peak chloride release of 0.2 µm after 16 days (Figure 2.8a). *Pseudomonas chlororaphis* strain UFB2 isolated from activated sludge demonstrated an increase in OD during growth in MSM supplemented with 2,4-DCP as the sole carbon source, attaining a peak OD of 0.23 after 13 days, after which a decline in growth was observed. The organism was able to degrade 75% of 2,4-DCP after 18 days, with a peak chloride release of 1.1 µm after 16 days (Figure 2.8b).

Klebsiella pneumoniae strain KPNIH39 showed almost the same pattern as *P. chlororaphis* strain UFB2 to degrade 2,4-DCP (Figure 2.8c). *Klebsiella pneumoniae* strain KPNIH39 isolated from groundwater demonstrated an increase in OD during growth in MSM supplemented with 2,4-DCP as the sole carbon source, attaining a peak OD of 0.21 after 13 days, after which a decline in growth was observed. The organism was able to degrade 52% of 2,4-DCP after 16 days, with a peak chloride release of 0.55 µm after 18 days (Figure 2.6c).

(a)


Cell Growth (OD=600 nm)
[2,4-DCP] ppm
Chloride release (µm)
Time (Days)

(b)


Cell Growth (OD=600nm)
[2,4-DCP] ppm
Chloride release (µm)
Time (Days)

(c)

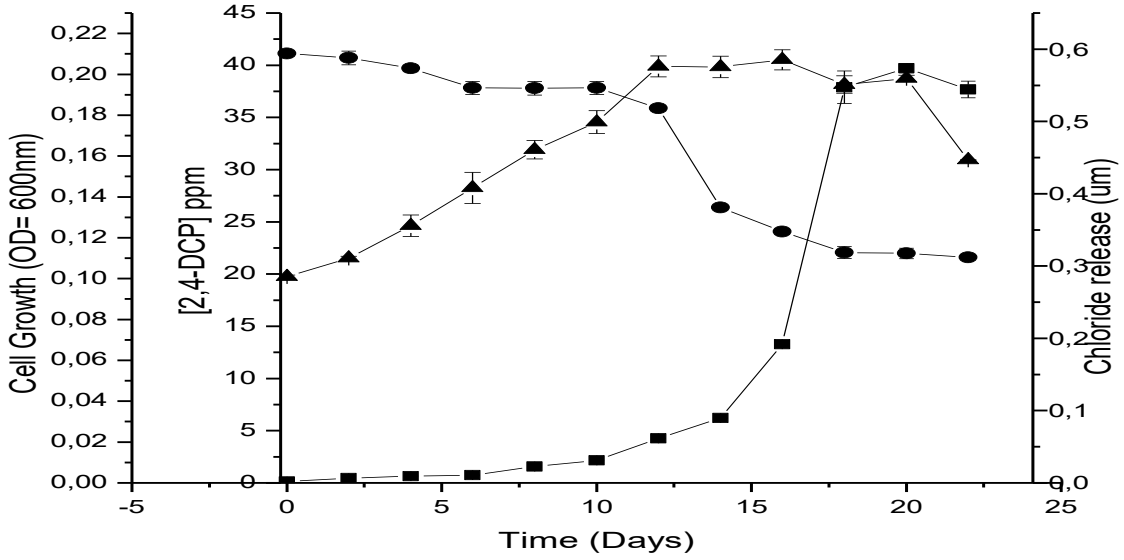


Figure 2.8: Cell Growth (▲), [2,4-DCP] (●) and Chloride release (■) by (a) *Klebsiella pneumoniae* strain DHQP1002001, (b) *Pseudomonas chlororaphis* strain and (c) *Klebsiella pneumoniae* strain KPNIH39 over a period of 22 days.

Pseudomonas chlororaphis strain UFB2, *Klebsiella pneumoniae* strain DHQP1002001 and *Klebsiella pneumoniae* strain KPNIH39 showed the ability to degrade the 2,4-DCP gradually. *P. chlororaphis* UFB2 showed a specific growth rate of 0.05 d^{-1} and doubling time of 0.007 days, with the highest degradation rate constant of 0.14 mg/L/d, compared to the other isolates (Table 2.4). *Klebsiella pneumoniae* strain DHQP1002001 showed a specific growth rate of 0.02 d^{-1} and doubling time of 0.03 days. The strain degraded 2,4-DCP with degradation rate constant of 0.14 mg/L/d, the lowest compared to the other isolates. *Klebsiella pneumoniae strain* KPNIH39 had a specific growth rate of 0.003 d^{-1} and doubling time of 0.004 days, the degradation rate constant of the strain for 2,4-DCP degradation was 0.03 mg/L/d (Table 2.4).

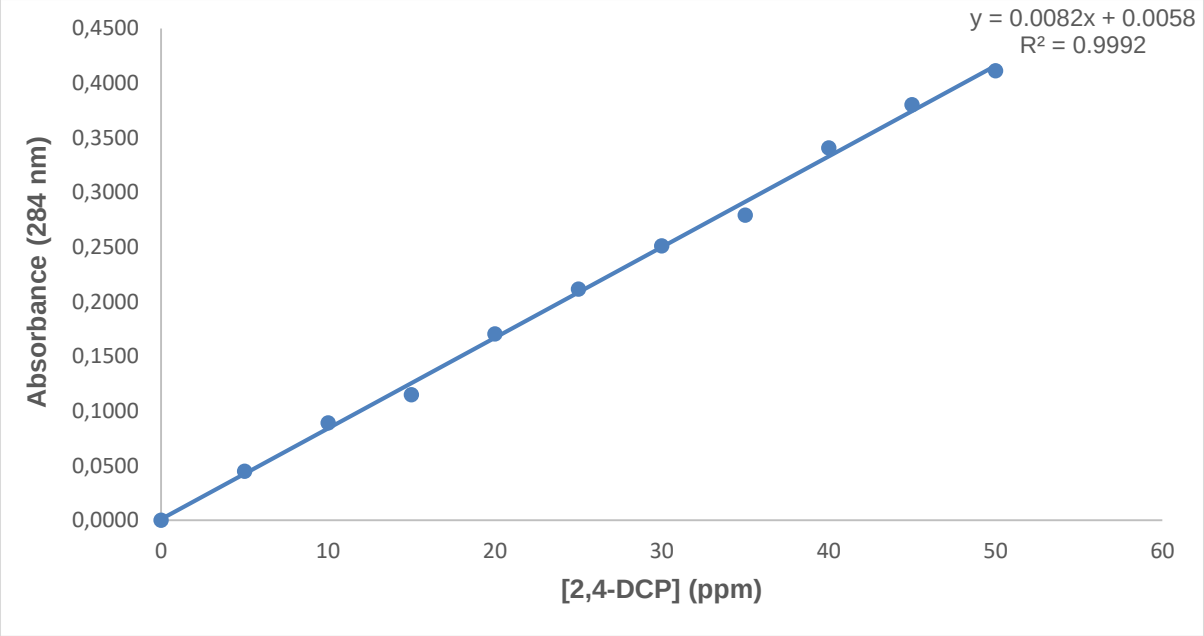


Figure 2.9: 2,4-dichlorophenol standard curve.

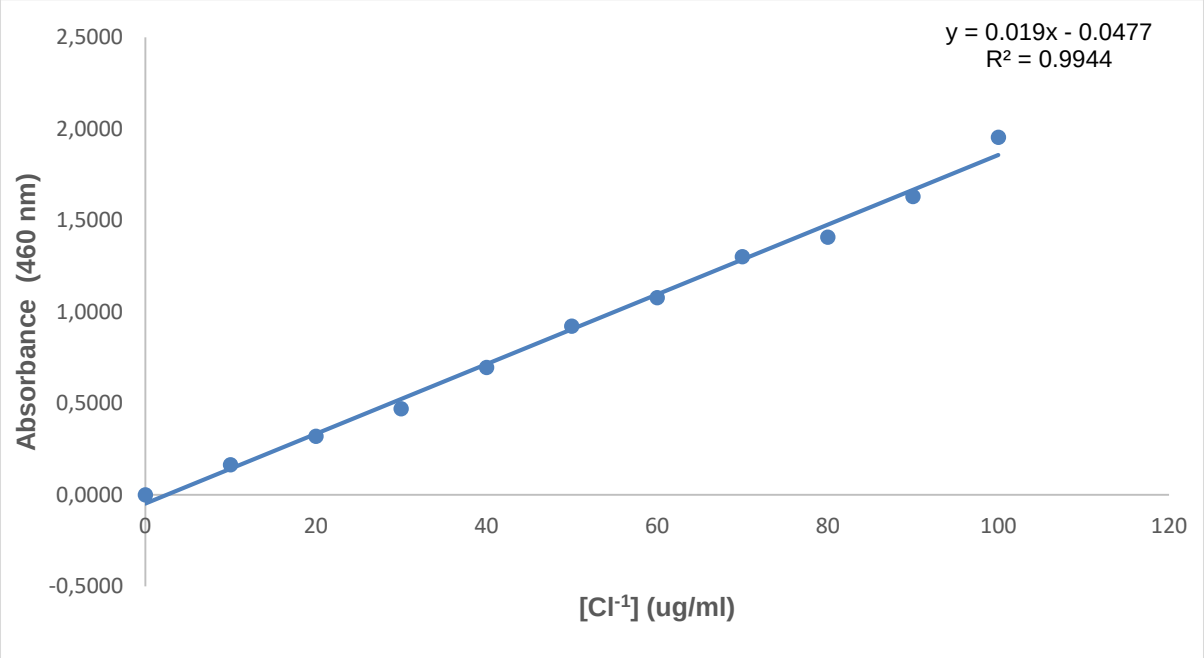


Figure 2.10: Chloride release standard curve.

Table 2.4: Specific growth rate, doubling time and 2,4-DCP degradation rate constant of the bacterial isolates

Rate constants	Specific growth rate (d^{-1})	Doubling Time (d)	Degradation rate constant (mg/L/d)
Pseudomonas chlororaphis strain UFB2	0.05	0.07	0.14
Klebsiella pneumoniae strain DHQP1002001	0.02	0.03	0.07
Klebsiella pneumoniae strain KPNIH39	0.003	0.004	0.03

2.3.6 Profiling of enzymes involved in 2,4-DCP metabolic pathways in the bacterial isolates

The level of 2,4-DCP catalyzing enzymes in the three selected bacterial isolates was studied by expression of enzymes induced by phenol (600 ppm) and 2,4-DCP (40 ppm). The isolates showed a higher level of enzyme expression with phenol as inducer compared to 2,4-DCP (Tables 2.5).

Crude extracts from all three isolates showed the highest for Phenol hydroxylase activity followed by catechol 1,2-dioxygenase activity. The isolates did not show any catechol 2,3-dioxygenase activity when grown on 2,4-DCP or phenol, suggesting that the three isolates follow the *ortho*-pathway instead of *meta*-pathway for 2,4-DCP degradation. The isolates also showed affinity to the *trans*-dienelactone substrate and no activity on *cis*-dienelactone when isolates were grown on 2,4-DCP. There was a little activity detected when both *trans*- and *cis*-dienelactone were used with phenol as an inducer. However, data indicate that microorganisms prefer utilizing *cis*-dienelactone but may express both types of dienelactone hydrolase i.e. type 1 and type 2 (Table 2.5). When phenol (600 ppm) was used as a substrate. The activity of phenol hydroxylase in *Pseudomonas chlororaphis* strain UFB2, *Klebsiella pneumonia* strain KPNIH39 and *Klebsiella pneumoniae* strain DHQP1002001 was 21.58, 0.610 and 0.017 U/mg,

respectively, when phenol (600 ppm) was used as a substrate. The activity of phenol hydroxylase in *Pseudomonas chlororaphis* strain UFB2, *Klebsiella pneumonia* strain KPNIH39 and *Klebsiella pneumoniae* strain DHQP1002001 was 21.58, 0.610 and 0.017 U/mg, respectively. The isolates also showed presence of catechol 1,2-dioxgenase with activity of 15.63, 0.031 and 0.013 and no activity for catechol 2,3-dioxygenase in the three isolates. Induction of muconate isomerase was observed with activity of 1.48, 0.020 and 0.025 U/mg in *Pseudomonas chlororaphis* strain UFB2, *Klebsiella pneumonia* strain KPNIH39 and *Klebsiella pneumoniae* strain DHQP1002001 respectively. *Pseudomonas chlororaphis* strain UFB2 showed induction of *cis*- and *trans*-dienelactone hydrolase, while both *klebsiella pneumonia* strains showed a higher activity towards the *trans*-dienelatone hydrolase.

Table 2.5: Enzyme Activities showed by three strains grown in presence of 600 ppm of phenol.

Enzymes (U/mg)	***Pseudomonas chlororaphis* strain UFB2**	***Klebsiella pneumoniae* strain KPNIH39**	***Klebsiella pneumoniae* strain DHQP1002001**
Phenol hydroxylase	21.58±0.19	0.610±0.01	0.017±0.00
Catechol 1,2- dioxygenase	15.63±0.89	0.031±0.00	0.013±0.00
Catechol 2,3- dioxygenase	0.000±0.00	0.000±0.00	0.000±0.00
Muconate isomerase	1.48±0.12	0.029±0.00	0.020±0.01
Cis-Dienelactone Hydrolase	2.34±0.58	0.000±0.00	0.005±0.00
Trans-Dienelactone Hydrolase	1.49±0.15	0.84±0.09	0.016±0.00
Maleylacetate reductase	0.000±0.00	0.000±0.00	0.000±0.00

When 2,4-DCP was used as a substrate for induction of the metabolic enzymes, the activity of phenol hydroxylase was 5.07, 0.013 and 0.003 for *Pseudomonas chlororaphis* strain UFB2, *Klebsiella pneumoniae* strain KPNIH39 and *Klebsiella pneumoniae* strain DHQP1002001 respectively. Catechol 1,2-dioxygenase activity was 0.196, 0.256 and 0.005 U/mg for the isolates, incontrast there was no activity for catechol 2,3-dioxygenase. The isolates showed presence of muconate isomerase with activities of 0.124, 0.091 and 0.020, however only *Pseudomonas chlororaphis* strain UFB2 showed the ability of utilizing *Cis*-dienelactone hydrolase at activity of 0.015 while *klebsiella pneumonia* isolates showed none. When *trans*-dienelactone hydrolase was used all three isolates showed activity of 0.120, 0.097 and 0.030 U/mg. Maleylacetate reductase was not induced in the three isolates and there was no activity of the enzyme.

Table 2.6: Enzyme Activities of enzymes of the catabolic pathway when 40 ppm of 2,4-DCP is used as the substrate

Enzymes (U/mg)	***Pseudomonas chlororaphis* strain UFB2**	***Klebsiella pneumoniae* strain KPNIH39**	***Klebsiella pneumoniae* strain DHQP1002001**
Phenol hydroxylase	5.07±0.19	0.013±0.00	0.003±0.00
Catechol 1,2- dioxygenase	0.196±0.02	0.256±0.01	0.005±0.00
Catechol 2,3- dioxygenase	0.000±0.00	0.000±0.00	0.000±0.00
Muconate isomerase	0.124±0.02	0.091±0.01	0.020±0.01
Cis-Dienelactone Hydrolase	0.015±0.01	0.000±0.00	0.000±0.00
Trans-Dienelactone Hydrolase	0.120±0.001	0.097±0.01	0.030±0.00
Maleylacetate reductase	0.000±0.00	0.000±0.00	0.000±0.00

2.3.7 Profiling of 2,4-DCP catabolic genes in the bacterial isolates based on PCR detection of the genes

The primers specifically designed to detect genes involved in 2,4-DCP degradation successfully amplified the fragments for phenol hydroxylase, catechol 1,2-dioxygenase and dienelactone hydrolase genes using genomic DNA from *Pseudomonas chlororaphis* UFB2 as a template (Figure 2.11). However, PCR amplification detected neither catechol 2,3-dioxygenase (the enzyme that facilitates *meta*-pathway) nor maleyacetate reductase (the enzyme that is involved in funneling the compounds to the Krebs cycle).

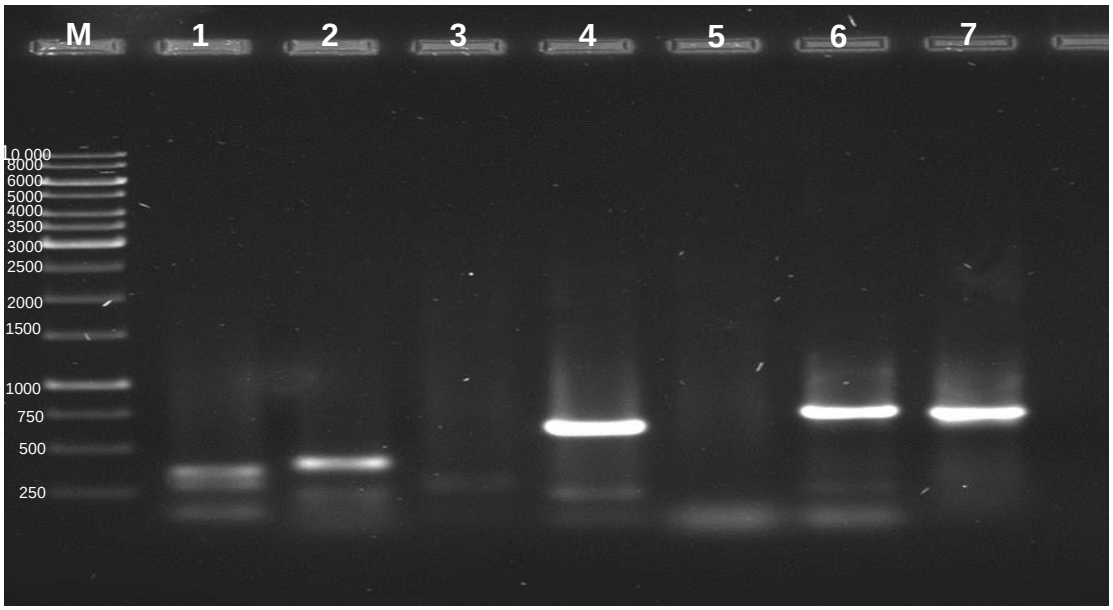


Figure 2.11: Amplification of genes involved in the biodegradation of 2,4-DCP in *Pseudomonas chlororaphis* UFB2: M=1Kb DNA Marker, Lane 1= Catechol 1,2-dioxygenase gene (467 bp); Lane 2= Dienelactone hydrolase gene (491 bp); Lane 3= Dienelactone hydrolase (2) gene=429 bp; Lane 4= Phenol hydroxylase gene (600 bp); Lane 5= Phenol hydroxylase (2) gene= (587 bp); Lane 6 and 7= Positive control (750 bp).

Klebsiella pneumoniae showed the presence of genes that code for the enzyme phenol hydroxylase, catechol 1,2-dioxygenase, dienelactone hydrolase. The genes amplified were

found to be of the same size. However, there was no amplification detected for catechol 2,3-dioxygenase and maleyacetate reductase in this strain as well (Figure 2.12).

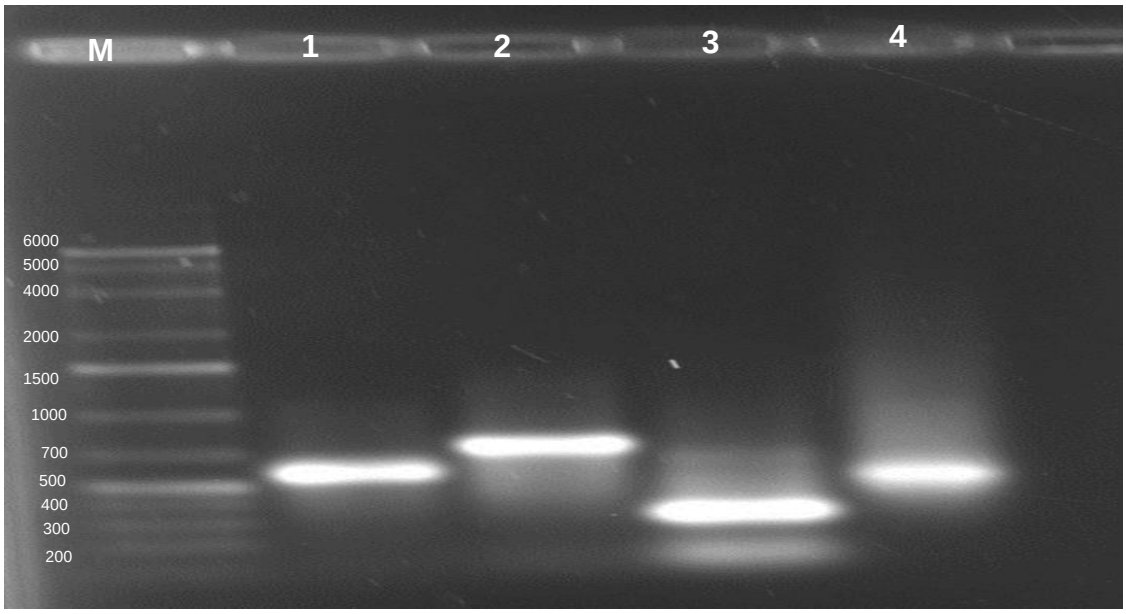


Figure 2.12: Amplification of genes involved in the biodegradation of 2,4-DCP in *Klebsiella pneumoniae* strain DHQP1002001 and *Klebsiella pneumoniae* strain KPNIH39: M= 1Kb Plus DNA Marker, Lane 1= Dienelactone hydrolase gene (567 bp); Lane 2= Phenol hydroxylase1 (715 bp); Lane 3= Phenol hydroxylase 2 (304 bp); Lane 4= Catechol 1,2-dioxygenase (507 bp).

Both strains of *Klebsiella pneumoniae* and *Pseudomonas* showed the amplification of muconate isomerase genes. The genes codes for muconate isomerase enzyme involved in *ortho*-pathway that is facilitated by catechol 1,2-dioxygenase (Figure 2.13).

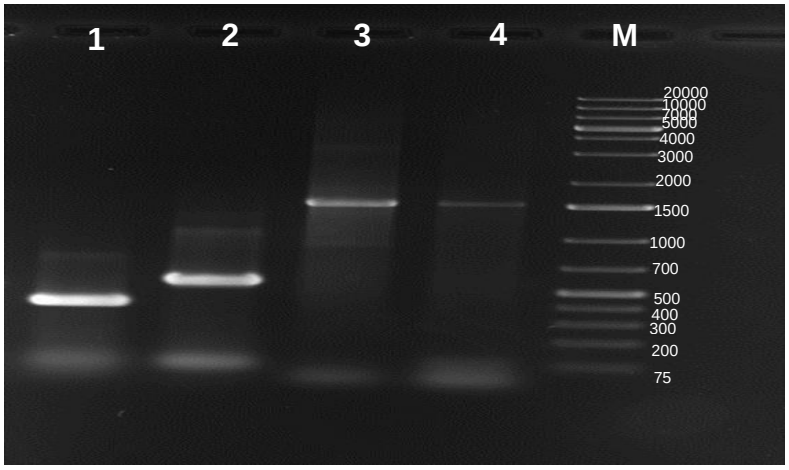


Figure 2.13: Detection of Muconate isomerase genes in the bacterial isolates. **Lane 1**= *Klebsiella pneumoniae* (494 bp); **Lane 2**: *Pseudomonas chlororaphis* (651 bp), **Lane 3** and **Lane 4**= Positive controls (1500 bp) and **M**= 1Kb Plus DNA Marker.

2.4 Discussion

In this study, indigenous bacteria capable of degrading 2,4-DCP were isolated from contaminated groundwater and activated sludge samples in Durban, South Africa. Studies have reported that microorganisms isolated from the groundwater (Tiirola *et al*., 2002; Arora and Bae, 2014; El-Naas *et al*., 2017) and activated sludge (Bae *et al*., 2002; Quan *et al*., 2004; Onya *et al*., 2007; Durruty *et al*., 2011; Arora and Bae *et al*., 2014) are capable of degrading chlorophenols. A total of six isolates showed the capability to grow in MSM supplemented with 2,4-DCP (40 ppm). Of the six isolates screened for their potential for growth and degradation of 2,4-DCP, only three were capable to effectively degrade 2,4-DCP by showing more than 40% degradation and were selected for further studies. However, none of the isolates could completely mineralize 2,4-DCP, possibly due to substrate inhibition (Andrews, 1968; Kargi and Eker 2005; Patel and Kumar, 2016). The amplification, sequencing and analysis of 16S rRNA genes identified the isolates as *Klebsiella pneumoniae* strain DHQP1002001 (MC), *Pseudomonas chlororaphis* strain UFB2 (YR) and *Klebsiella pneumoniae* strain KPNIH39

(GW). *Klebsiella pneumoniae* strain DHQP1002001, *Pseudomonas chlororaphis* strain UFB2 and *Klebsiella pneumoniae* strain KPNIH39 could degrade 49, 75 and 51% of 2,4-DCP, respectively after 11 days (Figure 2.6a, 2.6b and 2.6c). *Pseudomonas chlororaphis* strain UFB2 was the highest degrader amongst the isolates. To the best of our knowledge, this is the first report on the degradation of 2,4-DCP by *Pseudomonas chlororaphis*. However, *Pseudomonas chlororaphis* has been reported to degrade the most toxic compound, viz. 1,2,3,4-tetrachlorobenzene (Potrawfke *et al*., 1998). *Pseudomonas* species have been shown to degrade other less toxic compounds (Stoilova *et al*., 2006; Elkarmi *et al*., 2008; Seo *et al*., 2009; Arora and Bae, 2014; El-Naas *et al*., 2017). A previous study showed *Pseudomonas putida* strain DSM 6978 to degrade 27% of 577 ppm of 2,4-DCP at 192 hours (Kargi and Eker, 2005). Another study conducted by Elkarmi *et al*. (2009) showed *Pseudomonas alcaligenes* to degrade 50% of 220 ppm 2,4-DCP within 96 hours. *Pseudomonas* spp. have shown the ability to use a broad range of organic and inorganic compounds and to survive under different detrimental environmental conditions. They can grow fast and utilize a huge array of substrates such as chlorophenol as their carbon and energy sources (Mannisto *et al*., 1999; Moore *et al*., 2006; Wang *et al*., 2007; Cao *et al*., 2009; Afifi *et al*., 2015; El-Naas *et al*., 2017). *Klebsiella pneumoniae* has also been shown to degrade phenolic compounds (Michałowicz and Duda, 2007; Lin *et al*., 2010; Kadakol *et al*., 2011).

Although *Pseudomonas chlororaphis* strain UFB2 showed the ability to degrade 75% of 2,4-DCP within 21 days, the growth of the isolates started to decline at day 15 showing that the compound becomes lethal to the microorganism. Chloride released during the degradation of the compound by the isolate also showed that the compound is utilized by the microorganism. However, at day 15 there is a stationary phase of the released chloride, which correlates with the growth rate of the isolates. *Klebsiella pneumoniae* strain DHQP1002001 could degrade 51% of 2,4-DCP as illustrated in Figure 2.8; the isolate was able to utilize and grow on the

compound although there was a longer lag phase. A previous study showed *Bacillus cereus* GN1 to degrade 77.6% of 2,4-DCP (32 ppm) compound within 48 hours (Matafonova *et al*., 2006). Another study showed that *Cupriavidus necator* JMP 134 was able to degrade 10 ppm of 2,4-DCP within 16 hours (Wu *et al*., 2017) and *Alcaligenes eutrophus* JMP222 degraded 69% of 2,4-DCP (50 ppm) within 47 hours (Koh *et al*., 1997). *Micrococcus* showed the degradation rate of 0.28 mg/dm/h and the growth was low at 0.011 within 10 days, when 100 ppm of 2,4-DCP was degraded (Gallizia *et al*., 2003). A fungi, *Pleurotus ostreatus* degraded 54% of 20 ppm of 2,4-DCP within 96 hours with the degradation rate of 0.47 mg/L^{-1} h^{-1} and a low growth which was inhibited by 2,4-DCP (Silva *et al*., 2009).

Microorganisms have enzymes that facilitate the degradation of chlorophenolic compounds (karigar *et al*., 2011). In this study, *Pseudomonas chlororaphis* UFB2, *Klebsiella pneumoniae strain* KPNIH39 and *Klebsiella pneumoniae* strain DHQP1002001 showed production of the enzyme phenol hydroxylase, catechol 1,2-dioxygenase, catechol 2,3-dioxygenase, muconate isomerase, *cis*-dienelactone hydrolase, *trans*-dienelactone hydrolase and maleylacetate reductase activities when either phenol or 2,4-DCP was used as their sole carbon and energy sources. The results revealed there was higher activity when phenol is used as a carbon source in contrast to 2,4-DCP. *Pseudomonas chlororaphis* UFB2 had the highest activity amongst the isolates and had a higher production of phenol hydroxylase.

A previous study showed *Pseudomonas fluorescens* PU1 to follow the *meta*-pathway during the degradation phenol, with a specific activity of 429 mU/mg for catechol 2,3-dioxygenase and 9 mU/mg (Mahudddin *et al*., 2012).

A previous study of phenol hydroxylase and catechol 1,2-dioxygenase from *Candida tropicalis,* showed specific activities of to be 10540 mU/mg and 23540 U/mg respectively when induced with 540 ppm of phenol (Ahuatzi-Chacon *et al*., 2004). A *Bacillus* strain reported

in a study by Đokić *et al*. (2011) had specific activities of 590 mU/mg, 139 mU/mg and 92 mU/mg for phenol hydroxylase, catechol 1,2-dioxygenase and catechol 2,3-dioxygenase, respectively. In a study conducted using the isolate *Cupriavidus necator* JMP134, the activity of catechol 1,2 dioxygenase, muconate isomerase, *cis*-dienelactone hydrolase and maleylacetate reductase were reported to be 4300 mU/mg, 5 mU/mg, 1,050 mU/mg and 750 mU/mg, respectively (Plumeier *et al*., 2002). The activity of muconate isomerase was found to be 87 mU/mg from the crude extract of *Arthrobacter* sp. BA-5-17 (Murakami *et al*., 2003).

The phylogenetic tree shows that the isolates are related to the microorganisms that have been used over the years to degrade highly toxic chlorophenol compounds. In the case of *Pseudomonas chlororaphis*, the two microorganisms that were in close relation (*Pseudomonas fluorescens* and *Pseudomonas putida*) have been used for the degradation of aromatic compounds. Many authors have reported the potential of *Pseudomonas fluorescens* to degrade a range of chlorophenols (Goswami *et al*., 2005; Kargi and Eker, 2005; Mahiudddin *et al*., 2012; Arora and Bae, 2014). Similarly, *Pseudomonas putida* has also been reported by several authors for its potential to mineralize 2,4-DCP and related chlorophenolic compounds (Kargi and Eker, 2004; Elkarmi *et al*., 2009; Liu *et al*., 2009; Durruty *et al*., 2011; Bhatkal *et al*., 2012; Arora and Bae, 2014). In case of *Klebsiella pneumoniae* strain KPNIH39 and *Klebsiella pneumoniae* strain DHQP1002001, the isolates had a close relation with other *Klebsiella pneumoniae* strains. *Klebsiella pneumonia* has been reported to degrade phenolic compounds (Michałowicz and Duda, 2007). Analysis of the gene sequences of the isolates led to the detection of genes that encode the different enzymes of the catabolic pathway (Figure 2.11, 2.12 and 2.13). The absence of catechol 2,3-dioxygenase suggests that the microorganisms follow the *ortho*-pathway for the degradation of the compound. Most of the organisms follow the *ortho*-pathway for the degradation of chlorophenols compounds because during the *meta*-pathway there is extradiol suicidal inactivation of the microorganism, therefore, there are many

challenges for the microorganisms for degrading the compounds using this pathway (Kaschabek *et al*., 1998; Schmidt *et al.,* 2003; Brenner *et al*., 2004; Camera *et al*., 2007).

2.5 Conclusion

In this study, *Pseudomonas chlororaphis* strain UFB2, *Klebsiella pneumoniae strain* DHQP1002001 and *Klebsiella pneumoniae* strain KPNIH39 were isolated and identified. The strains could use 2,4-DCP as their sole carbon and energy source. *Pseudomonas chlororaphis* was the highest degrader at 75%, with the highest degradation rate constant of 0.14 mg/d/L and growth rate of 0.05 d^{-1}. However, it could not completely degrade the compound likely due to lack of catabolic genes that are involved in the degradation of the compound. The results show that all the three bacterial isolates did not possess the genes that encode for the enzyme catechol 2,3-dioxygenase, an enzyme which facilitates the *meta*-pathway, showing that they only use the *ortho*-pathway for 2,4-DCP degradation. The strains also did not possess genes that encode the enzyme maleyacetate reductase, which is involved in funneling the last intermediate in the pathway into the Krebs cycle. Further work should focus on improving the degradation of the compound by microorganisms. *Pseudomonas chlororaphis* UFB2 was chosen as a model for the purification and characterization of the enzymes phenol hydroxylase and catechol 1,2-dioxygenase.

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CHAPTER THREE: PURIFICATION, CHARACTERIZATION AND THREE-DIMENSIONAL STRUCTURAL PREDICTION OF PHENOL HYDROXYLASE FROM *PSEUDOMONAS CHLORORAPHIS* STRAIN UFB2 ISOLATED FROM ACTIVATED SLUDGE

Abstract

Microorganisms isolated from sites contaminated with xenobiotic compounds generally possess enzymes that facilitate the degradation of the compounds. Phenol hydroxylase is a monooxygenase enzyme that is involved in hydroxylating the attachment of a hydroxyl group at the *ortho*-position of phenol, converting it to catechol. The aims of the study were to purify and characterize phenol hydroxylase enzyme from the indigenous *Pseudomonas chlororaphis* strain UFB2 isolated from a contaminated site in Durban as well as to predict template based three-dimensional structure of the enzyme. Cell free extract of the isolate was prepared by growing the organism in Mineral Salt Media (MSM) supplemented with 600 ppm phenol as an inducer for 36 h. Harvested cells were lysed by sonication and centrifuged to obtain the cell lysate and assayed for phenol hydroxylase activity. The cell lysate showed 21.58 U/mL of phenol hydroxylase activity with a specific activity of 7.85 U/mg. A 61 kDa phenol hydroxylase from crude extract was successfully purified to 1.6-fold and with a yield of 33.6% using anion exchange chromatography (1 mL HiTrap ANX). The enzymes were optimally active at pH 8 and 30 °C, with stability at pH 7.5 and 30°C. The Lineweaver-Burk plot showed the v_{max} and K_m values of 4.04 µM/min and 4.0334 µM, respectively. Characterization and optimization of enzyme activity can assist in understanding the metabolic pathways followed by this bacterium for chlorophenol degradation and could also be useful in optimizing the potential industrial use of this enzyme.

3.1 Introduction

Globally, environmental pollution caused by recalcitrant xenobiotics is due to industrial, agricultural, medical and municipal waste streams (Wells and Ragauskas, 2012). Chlorophenols are one of the leading xenobiotics due to their toxicity and recalcitrant nature (Igbinosa *et al*., 2013; Arora and Bae, 2014). The detrimental effects of these compounds on the environment necessitates the development of methods for their removal from the environment (Krastanov *et al*., 2013). Bioremediation continues to be the preferred method for remediating the environment from the toxic compounds; it involves the use of microorganisms to degrade the pollutants (Olukunle *et al*., 2015; Krastanov *et al*., 2013). A microorganism facilitates the degradation using specific enzymes that are involved in each step of the degradation pathway (Sridevi *et al*., 2012; Krastanov *et al*., 2013).

Phenol hydroxylase is the monooxygenase enzyme involved in hydroxylating the attachment of a hydroxyl group at the *ortho*-position of phenol, converting it to catechol (Ahuatzi-Chacòn *et al*., 2004; Solyanikova and Golovleva, 2004; Gerginova *et al*., 2007; Vilímková *et al*., 2008; Đokić *et al*., 2011; Krastanov *et al*., 2013; Long *et al*., 2014; Hasan *et al*., 2015). Phenol hydroxylase assimilates one oxygen atom in the aromatic ring of phenol and reduces the other oxygen atom to H_2O by a hydrogen atom (Solyanikova and Golovleva, 2004; Nolan and Kevin, 2008; Vilímková *et al*., 2008, Krastanov *et al*., 2013) (Figure 3.1). Phenol hydroxylase has FAD binding pocket and need NADH or NADPH for its activity, thus having a wide substrate specificity (Solyanikova and Golovleva, 2004; Karigar *et al*., 2011).

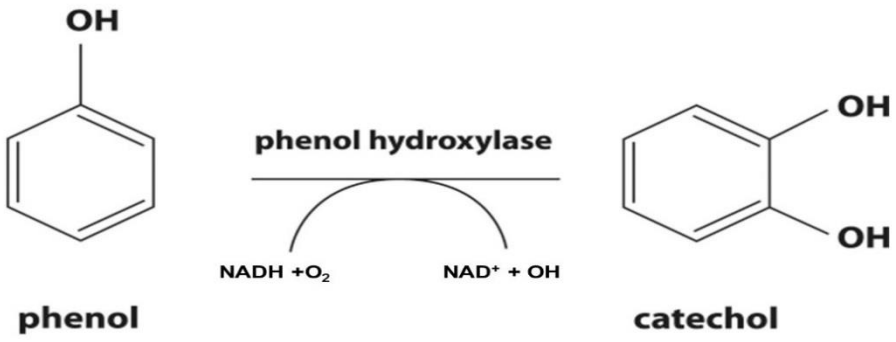

Figure 3.1: Phenol degradation facilitated by the enzyme phenol hydroxylase (Vilímková *et al*., 2008).

Studies showed that phenol is the highly preferred substrate catalyzed by phenol hydroxylase (Ahuatzi-Chacòn *et al*., 2004; Wojcieszyńska *et al*., 2011; Long *et al*., 2014). However, other substrates catalyzed by the enzyme include; hydroxyl-, amino-, halogen-, or methyl-substituted phenols (Solyanikova and Golovleva, 2004; Đokić *et al*., 2011; Krastanov *et al*., 2013). Phenol hydroxylases include monomers, homodimers, and homotetramers with a molecular mass that range from 57-73 kDa (Krastanov *et al*., 2013).

Studies have shown diverse types of phenol hydroxylases (single component or multicomponent) in microorganisms. Multi-component phenol hydroxylase is comprised of a family of non-heme and di-iron enzymes that use oxygen to hydroxylate different types of organic compounds (Notomista *et al*., 2003; El-Sayed *et al*., 2014). The first component of the multicomponent phenol hydroxylase is the one that is responsible for hydroxylating the oxygenase that binds oxygen and substrate while the second component is involved in the reduction of NADH (Powlowski and Shingler, 1990; El-Sayed *et al*., 2014). Microorganism with single component phenol hydroxylases include *Pseudomonas pickettii*, *Trichosporon cutaneum* (Gerginova *et al*., 2007); while microorganisms that have been characterized by multicomponent phenol hydroxylases include *Pseudomonas* spp. CF600 and *Acinetobacter radioresistens* S13 (Powlowski and Shingler, 1994; Cafaro *et al*., 2004; El-Sayed *et al*., 2014).

The aims of this study were to purify and characterize phenol hydroxylase from *Pseudomonas chlororaphis* strain UFB2, to understand the catalytic mechanism of the enzyme and predict the three-dimensional structure of the protein.

3.2 Materials and Methods

3.2.1 Preparation of crude extracts

The crude extracts were prepared by growing the bacterial cell for 24 h in Mineral Salt Medium (MSM) comprising (g/L): K_2HPO_4, 2.75; KH_2PO_4, 0.1; NH_4Cl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.01; $CaCl_2 \cdot 2H_2O$, 1.0; NH_4Cl, 0.5, and Yeast extract, 1.0. The pH was adjusted to 7.0 with 2 M NaOH prior to autoclaving at 121°C for 15 minutes. 1 mL of trace metal solution composed of (mg/L): $FeSO_4 \cdot 7H_2O$, 5; $ZnSO_4 \cdot 7H_2O$, 4; $MnSO_4 \cdot 4H_2O$, 0.2; $NiCl \cdot 6H_2O$, 0.1; H_3BO_3, 0.15; $CoCl_2 \cdot 6H_2O$, 0.5; $ZnCl_2$, 0.25; EDTA, 2 was added by syringe filter (0.2 µL pore) into IL of the MSM. *Pseudomonas chlororaphis* strain UFB2 cells were grown in nutrient broth overnight at 30°C and the culture was standardized to OD=1 at 600 nm. Ten percent of the standardized culture was inoculated into above described MSM supplemented with 600 ppm of phenol as a sole carbon and energy source. The inoculated medium was incubated at 30°C for 36 h shaking at 150 rpm. The cells were harvested at the late exponential phase of growth by centrifugation at 10000 ×*g* for 15 min at 4°C. The cells were washed twice with 50 mM Sodium phosphate buffer, pH 7.5 (containing 1 mM EDTA and 1mM β-Mercaptoethanol to halt the proteases activity). A total of 24 g of the cell pellet was collected and re-suspended in 100 mL of the same buffer. Cell-free extracts were prepared by lysing the pellet by sonication with 400 Ultrasonicator (OMNI International) 8 cycles each with a pulse of 30s on/off for 4 min. The cell lysed cell extract was centrifuged at 20000× *g* for 30 min at 4°C. The clear supernatant was kept on ice to prevent inactivation of the enzymes and used as a crude extract for enzyme

assays, while the remaining extract was kept in -20°C for further studies (Mahiudddin *et al*., 2011).

3.2.2 Enzyme assay

Phenol hydroxylase activity was measured by the modified method of Kotresha and Vidyasagar (2012). One milliliter reaction mixture contained 2.5 µM phenol, 1.0 mM NADH in 50 mM Sodium phosphate buffer (pH 8.0). The reaction was initiated by adding 100 µl of enzyme extract to the reaction mixture and incubated for 30 min at 30°C. The initial and final absorbance was measured at 340 nm using Shimadzu UV-1800 UV-Vis Spectrophotometer fitted with temperature controller CPS-240A unit set at 30°C. The proper controls for each reaction were conducted along with the assay. One unit of enzyme activity was defined as the amount of the enzyme that reduced 1 µM of NADH per minute under standard assay conditions. The OD at 340 nm representing the reduction of NADH was converted to the micromole of product by using extinction coefficient of NADH i.e 6225 µM/min/cm. The unit of enzyme activity was calculated by the equation below:

$$\textit{Enzyme activity} = (\Delta \text{ Abs}/ \text{ E} \times \text{L})/ \text{ time (min)}$$

Where Abs=Optical density at the different wavelengths; E= molar extinction coefficient and L =path length.

3.2.3 Determination of protein concentration

The total protein concentration was determined by Bradford method (Bradford, 1976), using Bovine Serum Albumin (BSA) as a standard. The standard curve was plotted by using 0 to 2.0 mg/mL BSA (Appendix C, 7.3). The curve was fitted to the linear equation y= mx+c to determine the protein concentration of unknown sample.

3.2.4 Purification of phenol hydroxylase

The enzyme was purified using anion exchange chromatography column. For anion exchange, 1 mL anion exchange HiTrap ANX column was equilibrated with 5 column volumes (CV) (1-CV=5 mL) of 20 mM Sodium sulfate buffer (pH 8) and 1 mL of the sample was loaded onto the column. The unbound proteins were washed 5-CV of 20 mM Sodium sulfate buffer (pH 8). The proteins bound to the matrix were eluted with 10 CV of a 0-1.0 M linear gradient of NaCl in 20 mM Sodium sulfate buffer (pH 8). The eluted proteins were collected as 1 mL fractions using the AKTA purifier 100-P950 automated fraction collector at a flow rate of 1 mL/min. The fractions showing phenol hydroxylase activity were concentrated chilled with acetone (200 µL of fractions and 800 µL acetone) for 2 h and the sample was loaded onto 12% SDS-PAGE to confirm purity and homogeneity (Kumar *et al*., 2014).

3.2.5 Determination of optimum pH and temperature

The optimum pH of purified phenol hydroxylase was determined by setting up a reaction in different buffers as follows: 50 mM Citrate-phosphate buffer (pH 4-6.5), Sodium phosphate buffer (pH 7.0-8.0) and Tris-phosphate buffer (pH 8.5-10) (Long *et al.,* 2014). The optimum temperature of phenol hydroxylase was determined by incubating the reaction mixture for 30 min at temperatures of 20, 25, 30, 35, 40, 45, 50 and 60°C. The reaction was set up as described in Section 3.2.2.

3.2.6 pH and temperature stability of phenol hydroxylase

To determine the pH stability of phenol hydroxylase, an adequate volume of the enzyme was incubated in buffers: 50 mM Citrate-phosphate buffer (pH 4 and 6), 50 mM Sodium phosphate buffer (pH 7.0 and 8.0) and 50 mM Tris-phosphate buffer (pH 10) at a designated time (0-2 h).

One hundred microliters (0.26 µg) of the enzyme aliquots were withdrawn at different time intervals and the enzyme reaction assay was set up as described in Section 3.2.2. The relative enzyme activity at different pHs was represented related to the initial activity. To determine the temperature stability, an adequate volume of the enzyme (0.26 µg) was incubated at the following temperatures (30, 50 and 70°C) in Sodium phosphate buffer (pH 8.0) for (0-2 hours). One hundred microliters (0.26 µg) of the enzyme aliquots were withdrawn at different time intervals and the reaction was set up as described in Section 3.2.2. The relative enzyme activity at different temperatures was represented related to the initial activity.

3.2.7 Determination of the enzyme kinetic parameters

The kinetic parameters for substrate hydrolysis by phenol hydroxylase were determined by measuring the initial rate of enzymatic activity. One hundred microliters (0.26 µg) of the enzyme was incubated with sodium phosphate buffer (pH 8) containing phenol (0.5-10 µM) at 30°C for 30 min. The Lineweaver-Burk plot was constructed by plotting the reciprocal of the rate of substrate hydrolysis (1/V) against the reciprocals of the substrate concentrations (1/[S]). The v_{max} and K_m values were determined by fitting the data in Michaelis-Menten equation using ORIGIN 8 pro (Evaluation version). The catalytic constant (k_{cat}) of the enzyme substrate reaction also known as a turnover number (the number of reactions catalysed per unit time by each active site) was determined by using the equation $k_{cat} = v_{max} /[E]_t$, where v_{max} is the maximum velocity and $[E]_t$, is the total enzyme concentration. Catalytic efficiency was calculated by dividing k_{cat} with K_m (Nadaf and Ghosh, 2011).

3.3.8 Effects of metals and inhibitors on phenol hydroxylase activity

One hundred microliters (0.26 μg) of purified phenol hydroxylase was incubated separately with 0.1 mM of β-Mercaptoethanol, EDTA, $CUSO_4$, $HgCl_2$, $FeSO_4$, Tween 20, Tween 80 and Sodium Dodecyl Sulfate (SDS) and the enzyme assay was performed as described in Section 3.2.2.

3.3.9 Substrate specificity of phenol hydroxylase

To determine the substrate specificity of phenol hydroxylase, a stock solution of various substrate: Phenol, 4-Nitrocatechol, 3-Methylcatechol, 4-Methylcatechol, 1,2,4-Benzenetriol, catechol and 2,4-dichlorophenol were prepared in 50 mM Sodium phosphate buffer (pH 8). One hundred microliters (0.26 μg) enzyme was added to 0.1 mM of the substrates except for addition to 0.2 mM phenol to initiate the reaction and the assay was performed as described in Section 3.2.2.

3.2.10 Determination of amino acid sequences of the purified phenol hydroxylase

The pure protein (10 μg) was loaded onto 12% SDS–PAGE and stained with coomassie blue R250. The protein band was excised carefully and digested with trypsin and fragments analyzed by electrospray mass spectrometry (at CAF, Stellenbosch University, Stellenbosch, South Africa). The raw files generated by the mass spectrometers were imported into Proteome Discoverer v1.3 (Thermo Scientific, USA) and SearchGUI v. 3.2.18 and processed using the Mascot 1.3. Algorithm (Matrix Science) as well as the Sequest algorithm to get the peptides generated from the enzyme. The database from tryptic digestion was analyzed by PeptideShaker (version 1.16.9) for homology of phenol hydroxylase, the sequence was then

used for the determination of the functions and the FASTA sequence of the protein by using UniProtKB (http://www.uniprot.org/). Homology search for the phenol hydroxylase from *Pseudomonas chlororaphis* strain UFB2 was carried out using Basic Local Alignment Search Tool (BLAST) protein blast at National Center for Biotechnology Information (NCBI).

3.2.11 Prediction of biophysical properties and three-dimensional structure

The protein and DNA homology search were carried out using PSI-and nucleotide BLAST in NCBI (non-redundant) database using Open Reading Frame (ORF) of the protein and DNA sequence as the query sequence. The multiple sequence alignments for protein and DNA were performed using DNAMAN (version 7), Lynnon Corporation, CA, USA (Demo version). Biophysical properties of the protein were determined using ExPASy server, while structure prediction of the enzymes was carried out using SWISS-MODEL workspace (http://swissmodel.expasy.org). The default parameters used for performing the automated SWISS-MODEL were used as explained previously (Kumar *et al*., 2016) and elaborated at (http://swissmodel.expasy.org/workspace/index%20.php?func=special_help) webpage. The modeled PDB files were submitted to online tool RAMPAGE for Ramachandran plot analysis to check the quality and validation of the predicted models (Kumar *et al*., 2016).

3.3 Results

3.3.1 Purification of phenol hydroxylase

Phenol hydroxylase activity of 21.58 U/mg was measured in the crude extract of *Pseudomonas chlororaphis* UFB 2 cells induced with 600 ppm of phenol with a specific activity of 7.67 U/mg of protein. The crude cell extract was concentrated using Millipore ultrafiltration spin tubes and loaded in ANX purification column attached to AKTA. The proteins peak observed at 280

nm as shown in the chromatogram (Figure 3.2) were collected in 1mL fraction and assayed for phenol hydroxylase activity. The fractions (A13 and A14) showing phenol hydroxylase activity were concentrated and checked for purity of the protein. Fractions (A13 and A14) showed the presence of single band protein in Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Further, the fraction (A13 and A14) were concentrated using 10 kDa Millipore ultrafiltration membrane and again assayed for phenol hydroxylase activity. The purified protein showed the specific activity of 12.45 U/mg of protein, indicating a 1.6-fold purification of the enzyme (Table 3.1).

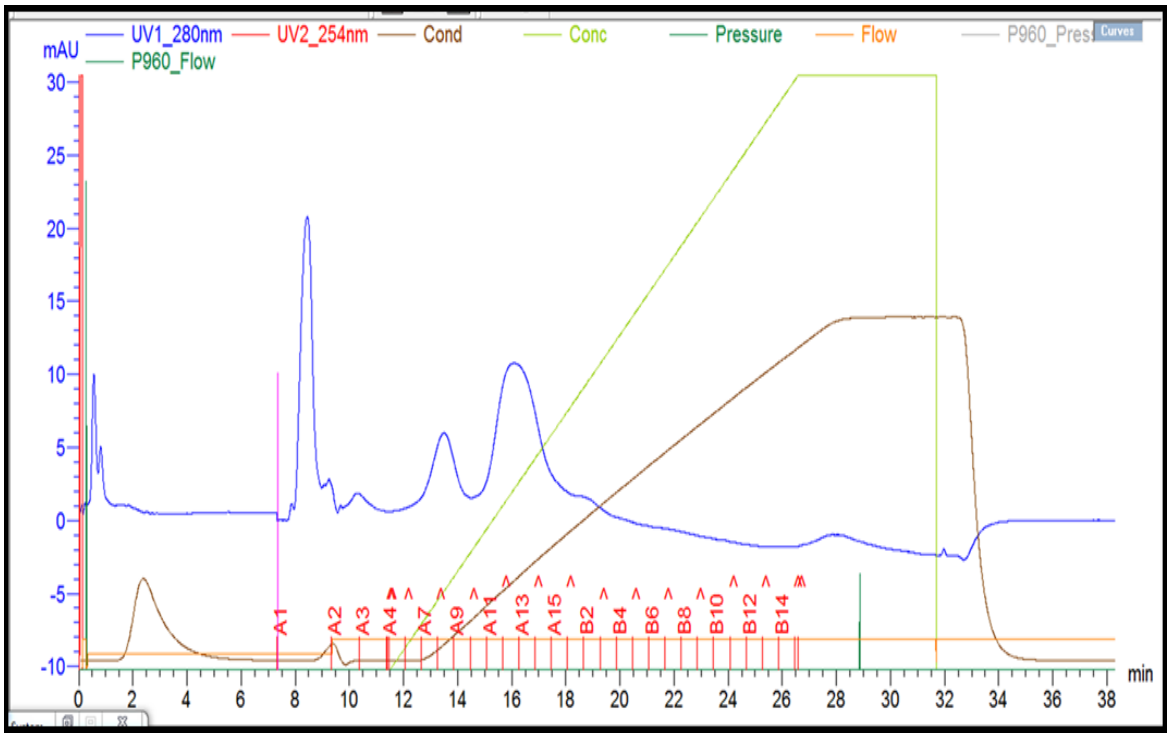

Figure 3.2: Anion Exchange Chromatography purification of crude extract loaded on ANX anion column.

Table 3.1: Purification table for phenol hydroxylase from *Pseudomonas chlororaphis* UFB2,

Step	Total activity (U/mL)	Total protein (mg/mL)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude	21.58	3.03	7.67	100	1
Anion Exchange Chromatography	12.45	0.99	12.57	57.69	1.6

Phenol hydroxylase enzyme was successfully purified to homogeneity with a band size of 61 kDa on 12% SDS-PAGE (Figure 3.3).

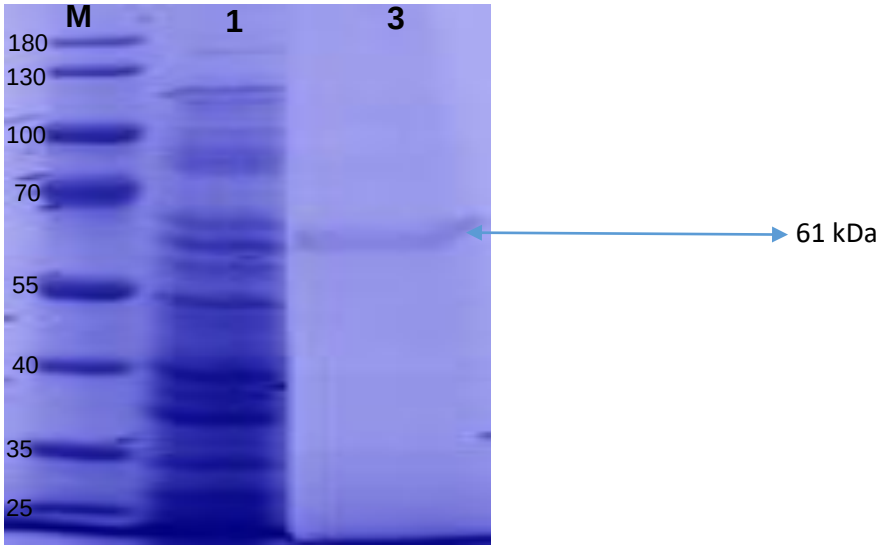

Figure 3.3: 12% SDS-PAGE for crude and purified phenol hydroxylase from *Pseudomonas chlororaphis* strain UFB2. Lane M= Protein Marker (180 kDa Marker), Lane 1= Crude cell extract; Lane 2= Purified phenol hydroxylase showing a band at 61 kDa.

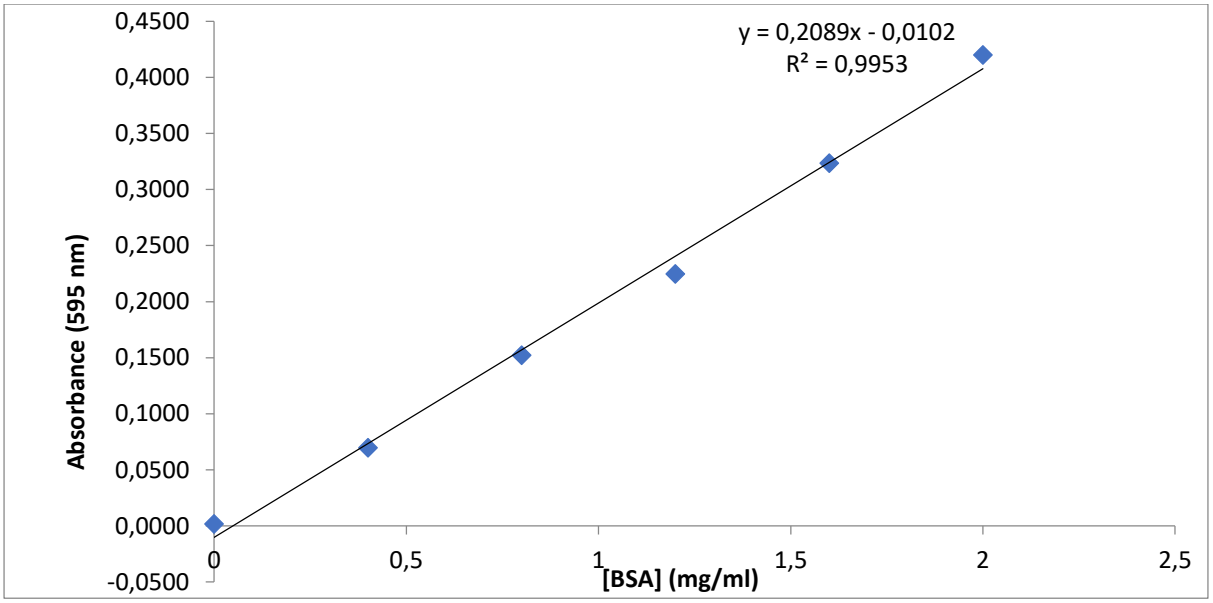

Figure 3.4: Standard Curve of Bovine Serum Albumin (BSA)

3.3.2 Optimum pH and pH stability of purified phenol hydroxylase

Purified phenol hydroxylase from *Pseudomonas chlororaphis* strain UFB 2 showed optimum activity at pH 8 (Figure 3.5), retaining 65% and 81% of its activity at pH 7.5 and 8.5, respectively. The enzyme was incubated for a period of time (0-240 minutes) to determine its stability and was found to be stable until 240 minutes at pH 7, with only 10% loss in activity after 240 min. Phenol hydroxylase was highly unstable at pH 4 where it lost its 50% activity within 10 min and up to 90% reduction in activity after 240 min. At pH 6 there was 50% loss of activity after 60 min and gradually the activity decreased in 240 minutes. There was a gradual decrease in activity of the enzyme at pH 8 from 60 min to 240 min (Figure 3.6).

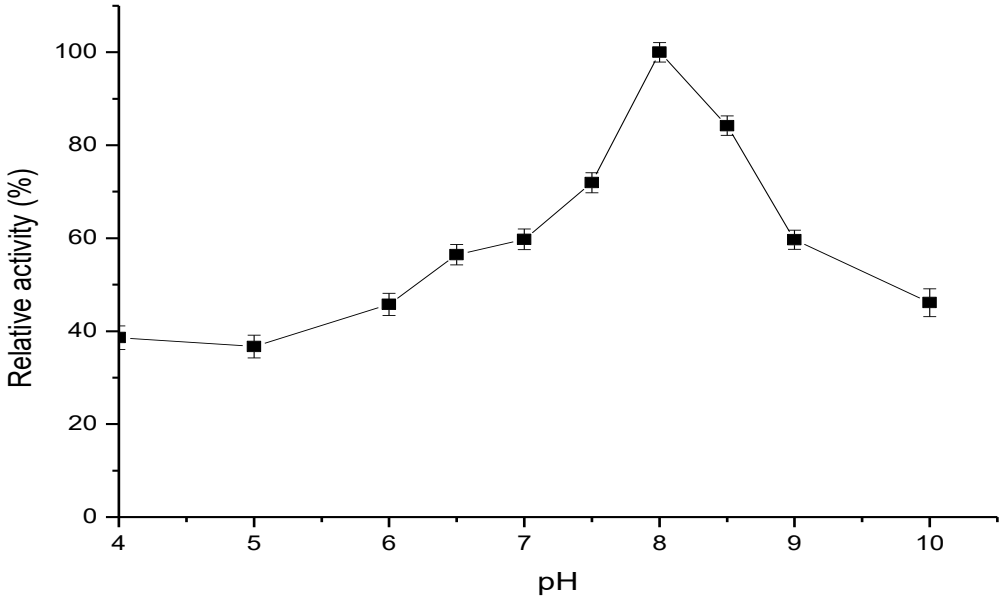

Figure 3.5: Optimum pH of phenol hydroxylase from *Pseudomonas chlororaphis* strain UFB2.

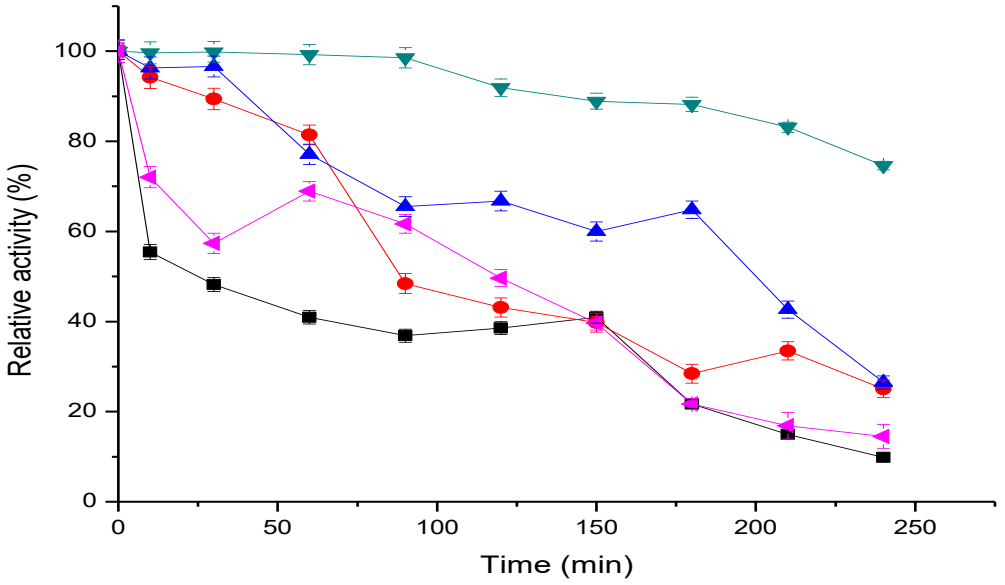

Figure 3.6: pH stability of phenol hydroxylase from *Pseudomonas chlororaphis* strain UFB2: pH 4 (■), pH 6 (●), pH 7 (▲), pH 8 (▲), pH 10 (▲).

3.3.3 Optimum temperature and temperature stability

Purified phenol hydroxylase from *Pseudomonas chlororaphis* strain UFB 2 showed optimum activity at 30 °C, with 55% and 80% of its activity retained at 25°C and 35°C (Figure 3.7). The enzyme was incubated for a certain time (0-240 min) to determine its stability. The enzyme was stable for 120 min at 30°C and the activity gradually decreased with time, retaining 80% of its activity after 240 minutes. Phenol hydroxylase was highly unstable at 70°C where it lost over 60% activity within 30 min and about 90% activity after 240 min. At 50°C there was 50% loss of activity after 60 minutes and drastically the activity decreased in 240 min (Figure 3.8).

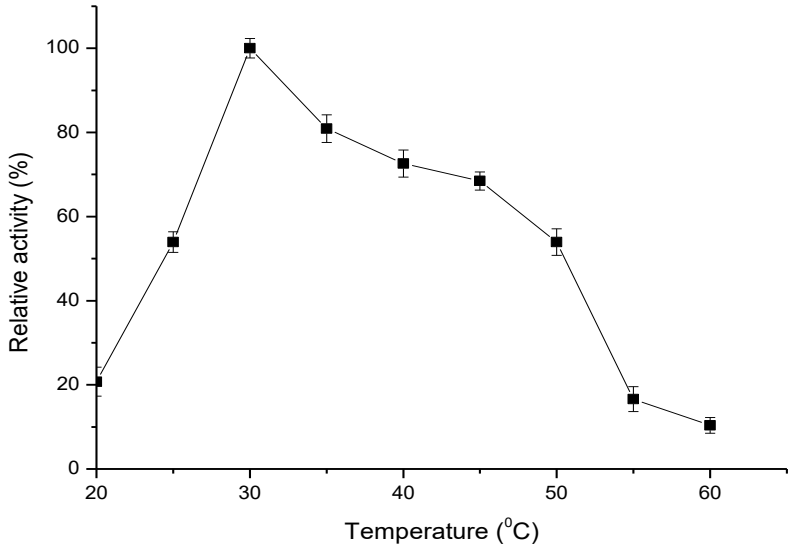

Figure 3.7: Optimum temperature on phenol hydroxylase from *Pseudomonas chlororaphis* strain UFB2.

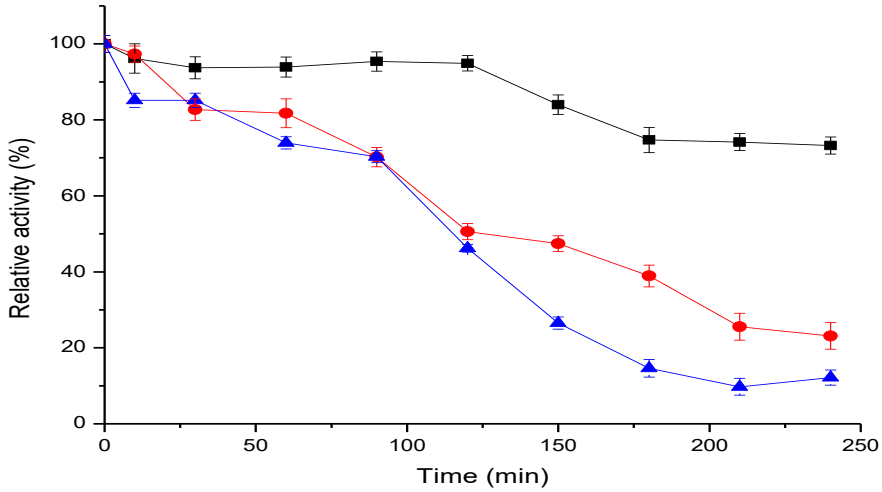

Figure 3.8: Temperature stability of phenol hydroxylase from *Pseudomonas chlororaphis* strain UFB2: 30°C (■), 50°C (●), 70°C (►).

3.3.4 The kinetic properties for phenol hydroxylase

The Lineweaver – Burk plots fitted in Michaelis-Menten equation showed the K_m value of 4.03 µm of phenol and v_{max} of 4.04 µm/ min (Figure 3.9).

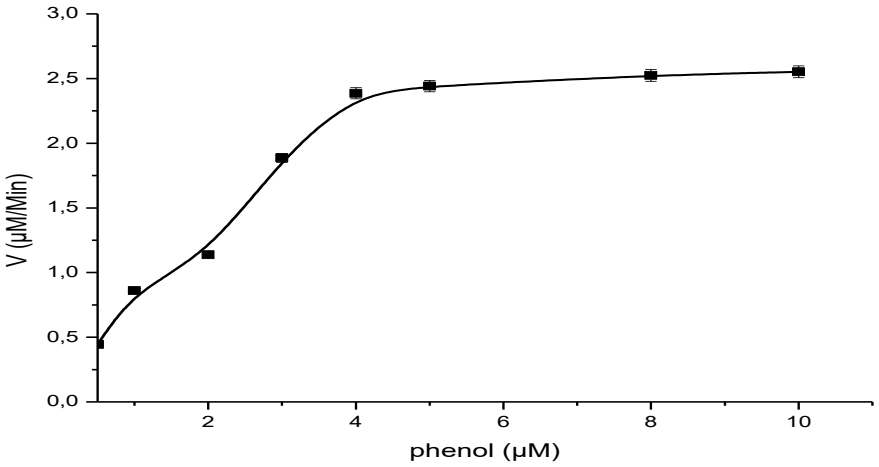

Figure 3.9: Substrate Saturation curve for phenol hydroxylase from *Pseudomonas chlororaphis* strain UFB2. The assays were performed by incubating 0.26 µg of the enzyme with 0.5-10 µM phenol.

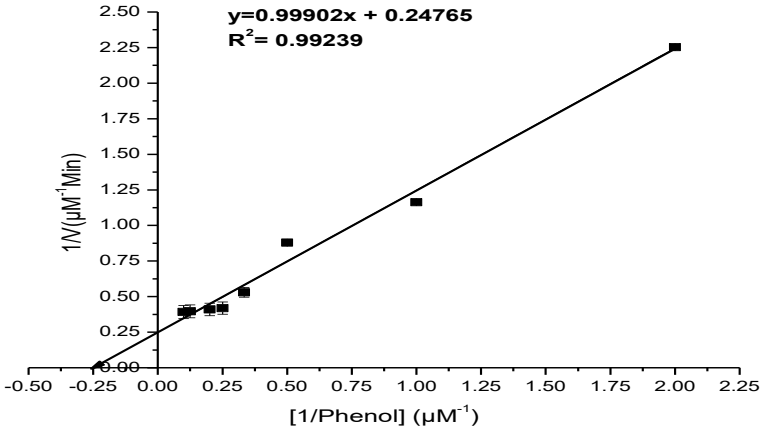

Figure 3.10: Lineweaver-Burk double reciprocal plot of substrate saturation curve of phenol hydroxylase from *Pseudomonas chlororaphis* strain UFB2. The double reciprocal plot was fitted to Michaelis-Menten equation to determine the values of K_m and v_{max}.

3.3.5 Effects of Metals and inhibitors on phenol hydroxylase activity

The effect of metals and inhibitors on purified phenol hydroxylase activity were evaluated by incubating 100 µl (0.26 µg) enzyme with a specific concentration of metals and inhibitors (Table 3.2). In the presence of *β*-Mercaptoethanol, phenol hydroxylase showed 70% residual activity, EDTA was able to reduce the activity to 55%. The phenol hydroxylase activity was drastically inhibited in the presence of metal ions like $CuSO_4$, $HgCl_2$ and $FeSO_4$ with a relative activity of 34, 47 and 48%, respectively. The surfactants, Tween 20 and Tween 80 reduced the activity by 40 and 48%, respectively. The protein denaturing agent SDS reduced activity to 20% almost deactivating the enzyme.

Table 3.2: Effects of Metals and inhibitors (1 mM) on the purified phenol hydroxylase from *Pseudomonas chlororaphis* strain UFB2

Inhibitor/detergent	***Residual activity (%)**
None (Control)	100±0.035
β-Mercaptoethanol	70±0.025
EDTA	55±0.019
$CUSO_4$	34±0.012
$HgCl_2$	47±0.017
$FeSO_4$	48±0.017
Tween 20	60±0.021
Tween 80	52±0.018
Sodium Dodecyl Sulfate (SDS)	20±0.007

*Residual activity of phenol hydroxylase represented the percentage of activity (U/mL) in the presence of various substrates (metals and inhibitors) as compared to the activity measured in the presence of phenol.

Phenol hydroxylase showed more affinity to phenol as compared to other substrates. Table 3.3, shows the phenol hydroxylase activity in the presence of other substrates such as 2,4-dichlorophenol, catechol, 4-nitrocatechol, 3-methylcatechol, 4-methylcatechol and 1,2,4-Benzenetriol. The enzyme showed only 25, 29 and 51% of residual activity in the presence of 4-nitrophenol, catechol and 2,4-DCP, respectively. Phenol hydroxylase did not show any activity when 3-methylcatechol, 4-methylcatechol and 1,2,4-Benzenetriol were used as substrates.

Table 3.3: Substrate specificity of purified phenol hydroxylase

Substrates	***Residual Activity (%)**
Phenol	100±0.005
4-Nitrocatechol	25±0.001
4-methylcatechol	0.00±0.000
3-methylcatechol	0.00±0.000
1,2,4-Benzenetriol	0.00±0.000
Catechol	29±-0.022
2,4-dichlorophenol	51±0.001

*Residual activity of phenol hydroxylase represented the percentage of U/mL activity in the in the presence of various substrates as compared to the activity measured in the presence of phenol.

3.3.6 ES-MS and amino acid sequence determination

The pure protein band from SDS-PAGE was incised, cleaned and digested with trypsin to generate small peptides to be analyzed on ES-MS. The raw data generated was analyzed in SearchGui 3.2.18 and peptide shaker (version 1.16.9). The FASTA files for the mass spectrum pf proteins from *Pseudomonas chlororaphis* was downloaded from Uniprot protein database. PeptideShaker data showed the presence of 6 spectra matching to the peptide spectra of probable protein kinase ubiB. Probable protein kinase ubiB (2-octaprenylphenol hydroxylase) is a hydroxylating enzyme (accession number, A0A1H2C6D1), required, probably indirectly, for the hydroxylation of 2-octaprenylphenol to 2-octaprenyl-6-hydroxy-phenol, the fourth step

in ubiquinone biosynthesis. In *Pseudomonas chlororaphis*, the enzyme is specific for aerobically grown log-phase cells. Accession number A0A1H2C6D1 searched on www.Uniprot.org lead to the depiction of an amino acid sequence of ubiB (Figure 3.11).

```
        10         20         30         40         50
MKLLAVRRLL RIQRVVIRYR LDDLLFALPL PWFLLALRYA LPWRWFPRKQ
        60         70         80         90        100
LELTRGARLR LALQDLGPIF IKFGQILSTR RDLLPEDIAD ELMLLQDRVP
       110        120        130        140        150
PFDSKKSVAL IEEQLGKKIS DVFSRFDIEP LASASVAQVH AAQLKSGEEV
       160        170        180        190        200
VVKVIRPGLK PVIAQDLAWL FILARAAEKV SADARLLHPV DVVSDYEKTI
       210        220        230        240        250
YDELDLLREA ANASQLKRNF EGSPLLYVPQ VYWDWCRPKV LVMERIYGIQ
       260        270        280        290        300
VTDLATLADQ RTDMKMLAER GVEIFFTQVF RDSFFHADMH PGNIFVSTVQ
       310        320        330        340        350
PWSPQYIAID CGIVGSLTPE DQDYLARNLF AFFKRDYRRV AQLHIDSGWV
       360        370        380        390        400
PAETKLNEFE AAIRTVCEPI FEKPLKDISF GQVLMRLFQT ARRFNMEVQP
       410        420        430        440        450
QLVLLQKTLL NIEGLGRQLY PELDLWNTAQ PFLERWMRER VSPKALLGNL
       460        470        480        490        500
HSQVEQLPHL ANMTRDLLER MSQPHAQDPS PPWKRRKDDW FLRLLGAAHL
       510        520        530
AGGAILAAGG PLNGLGHWPA GIMMAVGLYL VVRR
```

Figure 3.11: Amino acid sequence of phenol hydroxylase from *Pseudomonas chlororaphis* UFB 2.

3.3.7 Template based structure of UbiB

The structure of phenol hydroxylase from *P. chlororaphis* UFB 2 was used as a template for homology modeling. Two models were predicted, the sequence identity for Model 1 and 2 with the purified phenol hydroxylase were 19.18% and 19.53% (Figure 3.12 a-d).

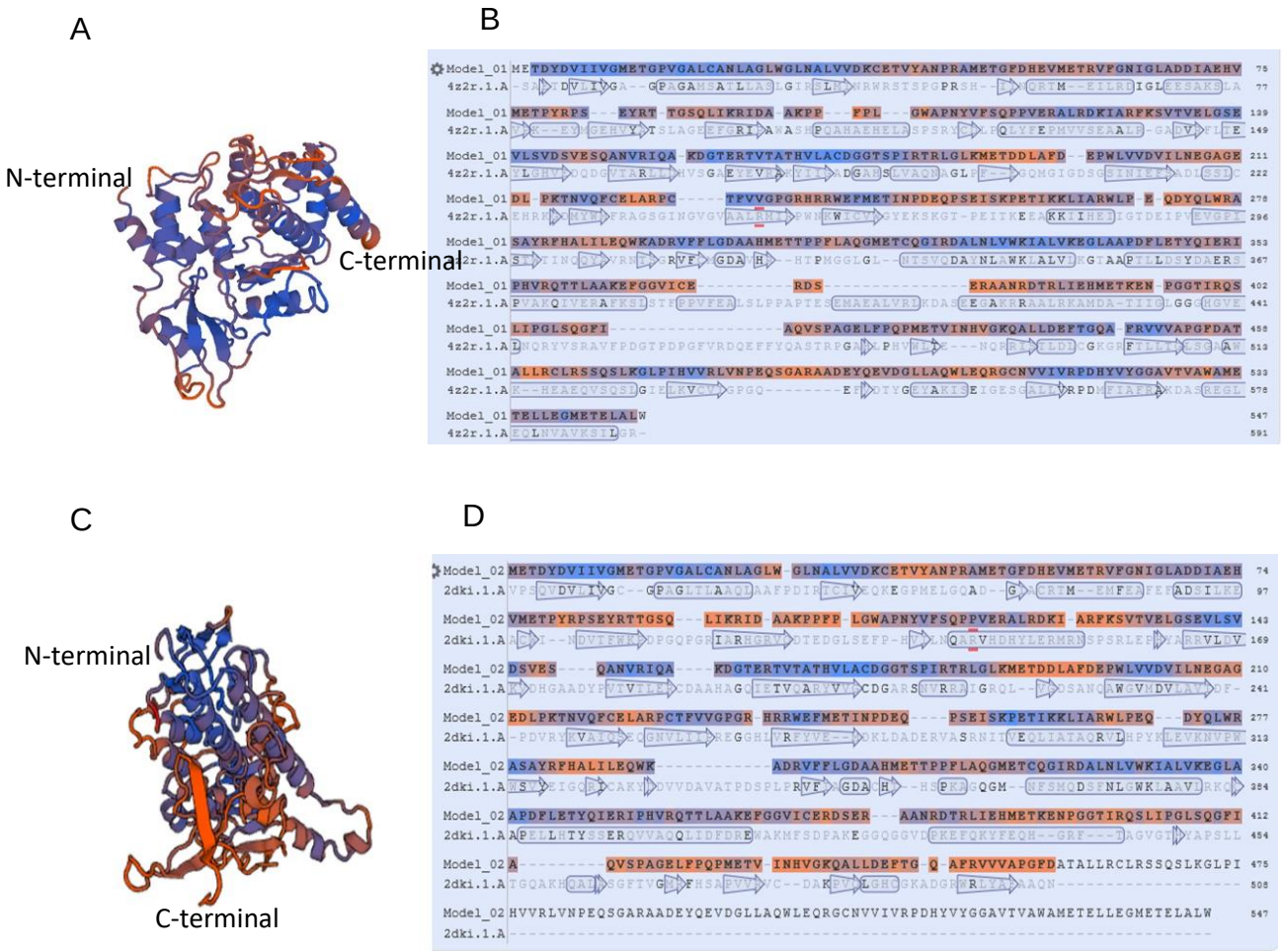

Figure 3.12: The model constructed by homology modeling at SWISS-MODEL workspace using probable protein kinase ubiB as the template and predicted tertiary structure of probable protein kinase ubiB deduced from 4z2r.1.A (A) and 2dki.1.A (C). The N-terminus and C-terminal of the enzyme are marked. (B) Homology alignment of probable protein kinase ubiB (Model_1) with 2-hydoxybinyl-3-monooxygenase (4z2r.1.A) (D) Homology alignment of probable protein kinase ubiB (Model_2) with 3-hydroxybenzoate hydroxylase (2dki.1.A).

Model_1 described as 2-hydoxybinyl-3-monooxygenase, is an NADH-dependent enzyme that hydroxylase 2-hydroxybiphenyl into 2,3-dihydroxybiphenyl and was discovered in *Pseudomonas* sp. strain HBP1 (Kohler *et al*., 1988). The enzyme has been reported to be FAD-dependent monooxygenase that catalyzes a wide range of 2-substituted phenols in the presence of NADH and molecular oxygen (Kanteev *et al*., 2015). The 2-hydroxybiphenyl is found in the hydrophobic region of the active site, above the isoalloxazine ring (which is above *ortho* position of 2-hydroxybiphenyl) of the FAD molecule to form a polar interaction His48. Kanteev *et al.* (2015) reported that there is a similarity between the Tyr201 in 2-hydoxybinyl-3-monooxygenase and His213 in 3-hydroxybenzoate 6-hydroxylase.

Model_2 described as 3-hydroxybenzoate hydroxylase is an NADH-dependent enzyme involved in hydroxylation of 3-hydroxybenzoate to 2,5-dihydroxybenzoate, the enzyme was induced in *Pseudomonas aeruginosa* (Groseclose *et al*., 1973). The enzyme was active in homodimer form with two domains comprising of the active site. The C-terminal domain involved in oligomerization and catalytic domains of the active site were similar with other phenol hydroxylases. Hiromoto *et al*. (2017) reported the same findings where 3-hydroxybenzoate hydroxylase was similar to 4-hydroxybenzoate hydroxylase and phenol hydroxylase due to the same catalytic and active sites.

3.3.8 Predicted biophysical properties of probable protein kinase ubiB and phenol hydroxylase expressed in other *Pseudomonas* strains

Physiochemical parameters of the enzyme were determined by using ExPaSy server. The parameters on the ExPaSy server included the following: Number of amino acids, Molecular Weight (MWt), theoretical pI, extinction coefficients (M^{-1} cm-1), estimated half-life, Instability Index (II), Aliphatic Index (AI) and grand average of hydropathicity (GRAVY) (Table 3.4). The number of amino acids for phenol hydroxylase from *Pseudomonas chlororaphis* strain

UFB 2 was 576, the highest compared to the number of amino acids reported for phenol hydroxylase enzymes from other *Pseudomonas* sp. The predicted molecular weight for phenol hydroxylase from *Pseudomonas chlororaphis* strain UFB2 was computed to be 63.96 kDa with an Isoelectric point (pI) of 4.93 showing that phenol hydroxylase from this isolate is acidic in nature. Extinction coefficients of the enzyme were 77390 M^{-1} cm^{-1}. The estimated half-life of phenol hydroxylase in *Escherichia coli in vitro* was greater than 10 hours, showing it can be stable in prokaryotic cells. The Instability index of the enzyme was 43.20 and showed that phenol hydroxylase was unstable *in vitro*. Aliphatic index of the enzyme of 85.54, was above 70 indicating that the enzyme is thermophilic. Grand average of hydropathicity (GRAVY) of the enzyme was -0.243 which was negative, and an indication of the hydrophobicity of phenol hydroxylase to water.

The number of amino acids reported for phenol hydroxylase from *Pseudomonas* species range from 400-576, with the lowest 497 amino acids reported for *Pseudomonas putida F1*. The molecular weight of phenol hydroxylase from *Pseudomonas* species ranged from 54.60-64.76 kDa, the molecular weight of phenol hydroxylase from *Pseudomonas chlororaphis* strain UFB2 was computed to be 63.96 kDa. The Isoelectric point (pI) of *Pseudomonas* species was from 4.93-6.03, which was less than 7 showing that phenol hydroxylase is acidic in nature. Extinction coefficients of the enzyme were between 52495-89505 M^{-1} cm^{-1}, the lowest extinction coefficient was from *Pseudomonas fluorescens* PICF7 at 52495 M^{-1} cm^{-1}. *Pseudomonas anartica* strain PAMC 27494, *Pseudomonas chlororaphis* PA23**,** *Pseudomonas simiae* strain WCS417 had an extinction coefficient of 89505 M^{-1} cm^{-1}, which was the highest in this study. The estimated half-life of phenol hydroxylase in *Escherichia coli in vitro* was greater than 10 hours in all the phenol hydroxylase from *Pseudomonas* species, showing it can be stable in prokaryotic cells. The instability index of the enzyme showed that most phenol

hydroxylases form the genus are unstable in the test tubes, only *Pseudomonas fluorescens* PICF7 at 28.02, *Pseudomonas azotoformans* strain S4 at 27.17 had a stable index whilst *Pseudomonas chlororaphis* strain UFB2 and other isolates had an unstable index. Aliphatic index of the enzyme ranged from 85.54-103.82 indicating that phenol hydroxylase from the strains have a wide range of temperature as the aliphatic index was above 70. Grand average of hydropathicity (GRAVY) of the enzyme was negative; low GRAVY is an indication of hydrophobicity of the protein with water. *Pseudomonas chlororaphis* strain UFB2 had GRAVY-0.243, which was very low.

Table 3.4: Phenol hydroxylase physiochemical properties from different *Pseudomonas* spp.

Organism name	**Number of amino acids**	**Molecular weight**	**Theoretical pI**	**Extinction coefficients (M^{-1} cm^{-1})**	**Estimated half-life**	**Instability index**	**Aliphatic index**	**Grand average of hydropathicity (GRAVY)**
Pseudomonas chlororaphis strain UFB2	576	63.96	4.93	77390	>10 hours	43.20 (unstable)	85.54	-0.243
Pseudomonas fluorescens strain FW300-N2C3	558	64.22	5.88	88015	>10 hours	45.86 (unstable)	103.82	-0.121
Pseudomonas antarctica strain PAMC 27494	562	64.76	5.61	89505	>10 hours	42.68 (unstable)	100.62	-0.149
Pseudomonas azotoformans strain S4	525	57.14	5.24	59610	>10 hours	27.19 (stable)	86.23	-0.237
Pseudomonas brassicacearum strain DF41	556	64.03	6.03	88015	>10 hours	45.30 (unstable)	104.17	-0.124
Pseudomonas chlororaphis isolate 189	558	64.02	6.03	89380	>10 hours	46.87 (unstable)	102.78	-0.138

Pseudomonas chlororaphis strain PA23	560	64.31	5.93	89505	>10 hours	47.22 (unstable)	101.89	-0.149
Pseudomonas fluorescens A506	514	59.73	5.34	77015	>10 hours	46.66 (unstable)	95.23	-0.260
Pseudomonas fluorescens PICF7	504	54.60	5.43	52495	>10 hours	28.02 (stable)	90.18	-0.220
Pseudomonas fluorescens SBW25	566	65.34	5.67	89505	>10 hours	42.53 (unstable)	98.71	-0.181
Pseudomonas putida F1	497	57.38	5.64	73005	>10 hours	41.54 (unstable)	99.07	-0.196
Pseudomonas putida SJTE-1	503	57.99	5.41	71515	>10 hours	42.24 (unstable)	99.64	-0.176
Pseudomonas simiae strain WCS417	566	65.25	5.67	89505	>10 hours	43.27 (Unstable)	99.24	-0.161
Pseudomonas putida strain 1A00316	503	57.99	5.41	71515	>10 hours	42.24 (Unstable)	99.64	-0.176

3.4 Discussion

Phenol hydroxylase from *Pseudomonas chlororaphis strain* UFB2 was successfully purified to 1.6 fold and 57% yield with a molecular weight of 61 kDa. A previous study on phenol hydroxylase from *Rhodococcus* sp. strain FP1 reported a molecular weight of 61 kDa (Duque *et al*., 2012). The computational studies for phenol hydroxylases from *Pseudomonas* spp. indicated that molecular weight for phenol hydroxylase fall in the range of 30- 40 kDa (Hasan *et al*., 2015). Some phenol hydroxylases existing are homodimers and homotetramers with a molecular mass that ranges from 57-73 kDa (Krastanov *et al*., 2013).

In this study, the optimum pH and temperature for phenol hydroxylase were found to be pH 8 and 30°C, respectively. Phenol hydroxylase from *Flavobacterium* showed optimum activity at pH 8.3 and 30°C (Wojcieszyńska *et al*., 2011). Phenol hydroxylase from *Candida tropicalis* strain JH8 showed optimum activity at pH 7.5 and temperature 30°C (Long *et al*., 2014), *Acinetobacter radioresistens* S13 phenol hydroxylase had optimum activity at pH 7.5 and temperature 25°C (Divari *et al*., 2003). Phenol hydroxylase reported from *Stenotrophomonas maltophilia* KB2 was optimum at pH 7.2 and temperature 30°C (Wojcieszyńska *et al*., 2011). *Bacillus* sp. is reported to produce thermophilic phenol hydroxylase showing optimum activity at 70°C.

Eisenthal *et al*. (2006) reported that most of the enzymes have optimum activity at 25-30°C, and are unstable at higher temperatures. Straube (1987), reported that phenol hydroxylase from *Rhodococcus* was optimally active at 35°C, while *Bacillus thermoglucosidasius, a* thermophilic microorganism was reported to produce phenol hydroxylase optimally active at a 55°C (Kirchner *et al*., 2003).

Wojcieszyńska *et al*. (2011) reported phenol hydroxylase from *Stenotrophomonas maltophilia* strain KB2 to be optimally active at 30°C. Long *et al*. (2014) also reported phenol hydroxylase

enzyme that had an optimum activity at 30°C. In the literature, the optimum pH for these enzymes ranges from 5.5 to 8.2 (Ahuatzi Cacon *et al*., 2004; Kirchner *et al*., 2003; Viggor *et al*., 2008).

Lineweaver-Burk plot showed the K_m and v_{max} values of 4.03 µm and 4.04 µm/min, respectively. The values of K_m and v_{max} were lower than what has been previously reported (Wojcieszyńska *et al*., 2011). The lower K_m and v_{max} values showed that the enzyme has a high affinity to the substrate (Wojcieszyńska *et al*., 2011). Similar findings were reported by Long *et al*. (2014) with low values of K_m and v_{max}. Phenol hydroxylase from *Candida tropicalis* strain JH8 (Paca *et al*., 2007) and *Stenotrophomonas maltophilia* KB2 (Wojcieszyńska *et al*., 2011) showed higher K_m and v_{max} for phenol.

Phenol hydroxylase activity from *Pseudomonas chlororaphis* strain UFB2 was inhibited severely by heavy metals ($CuSO_4$ and $HgCl_2$). However, EDTA, Tween 20, Tween 80 and β-mercaptoethanol showed no inhibitory effect. Similar findings where $CuSO_4$ and $HgCl_2$ showed the highest inhibitory effect on phenol hydroxylase have been reported (Wojcieszyńska *et al*., 2011; Long *et al*., 2014). Phenol hydroxylase seems to be active on a wide range of substrates. The enzyme had high affinity for catechol, 2,4-dichlorophenol, 4-Nitrocatechol and phenol. Long *et al*. (2014) reported that phenol hydroxylase was induced by catechol and phenol showing a higher relative activity as compared to the other substrates tested. However, Wojcieszyńska *et al*. (2011) reported a phenol hydroxylase from *Stenotrophomonas maltophilia* that was not induced by catechol but had a higher relative activity for phenol. Several studies have proven that phenol is the best inducer for phenol hydroxylase (Gerginova *et al*., 2007; Wojcieszyńska *et al*., 2011; Long *et al*., 2014).

The purified protein band was incised from 12% SDS-PAGE and trypsin digested generated peptides were analyzed on ES-MS. The data analyzed by peptide search (version 1.16.9) showed that the protein band corresponded to enzyme 2-octaprenylphenol hydroxylase (probable protein kinase ubiB) in *Pseudomonas chlororaphis* strain UFB2. 2-octaprenylphenol hydroxylase has been implicated in *Pseudomonas aeruginosa* and *E. coli* for the degradation of aromatic compounds (Diaz *et al*., 2001). The prediction showed two models (4z2r.1. A and 2dki.1. A), built in relation to the probable protein kinase ubiB. Model_1 (4z2r.1.A) had 19.18% sequence similarity with probable protein kinase ubiB. The protein was described as 2-hydoxybinyl-3-monooxygenase, an NADH-dependent enzyme that hydroxylase 2-hydroxybiphenyl into 2,3-dihydroxybiphenyl discovered in *Pseudomonas* sp. strain HBP1 (Kohler *et al*., 1988***)***. Model_2 (2dki.1. A) had 19.53% sequence similarity with probable protein kinase ubiB. The enzyme was described as 3-hydroxybenzoate hydroxylase, an NADH-dependent enzyme involved in hydroxylation of 3-hydroxybenzoate to 2,5-dihydroxybenzoate, induced in *Pseudomonas aeruginosa* (Groseclose *et al*., 1973).

Biophysical properties showed *Pseudomonas chlororaphis* strain UFB2 had the molecular weight and pI of 61 kDa and 4.93 respectively, suggesting that phenol hydroxylase from this study is a large protein and acidic in nature due to the low pI value. The enzyme had extinction coefficients of 77390 M^{-1} cm^{-1} while its estimated half-life of in *Escherichia coli in vitro* was greater than 10 hours, showing that it can be stable in prokaryotic cells. The enzyme showed the instability index of 43.20 which is characterized as unstable protein. The aliphatic index of 85.54 from the enzyme, was above 70 showing that the globular protein is thermostable. Grand average of hydropathicity (GRAVY) of the protein was negative, which showed that the protein is hydrophilic. Hasan *et al*. (2015) reported similar results with phenol hydroxylase from *Pseudomonas chlororaphis*, with pI of 5.25 and the protein was unstable with an instability index 39.77. The aliphatic index of the enzyme was 109.76 showing its thermostability and the

enzyme was shown to be hydrophilic with a low Gravy of 0.294. However, there was difference in its molecular weight which was lower at 30 kDa, this is as a result of different types of phenol hydroxylases.

3.5 Conclusion

This study covered the purification and characterization of phenol hydroxylase enzyme from *Pseudomonas chlororaphis* strain UFB2. The enzyme was successfully purified with a molecular weight of 61 kDa confirmed by sequence from tryptic digestion which classified the enzyme as multicomponent phenol hydroxylase. The enzyme was identified as probable protein kinase ubiB (2-octaprenylphenol hydroxylase) which hydroxylates 2-octaprenylphenol to 2-octaprenyl-6-hydroxy-phenol in the ubiquinone biosynthesis. The enzyme was optimum at pH 8 and 30°C, and had a stable activity at pH 7 and 30°C for 240 minutes. The kinetics study of this phenol hydroxylase showed it has high affinity to phenol as compared to other substrates, with a K_m and v_{max} of 4.03 µm and 4.0334 µm/min, respectively. This enzyme may play a vital role in biotechnological applications due to ease of handling as compared to whole cells of microorganism. The optimization of the enzyme production potentially can lead to improvement in eco-friendly agricultural and industrial applications. The enzyme can be used in treatment of wastewater where the temperatures are not higher than 50°C to prevent denaturation of the enzyme.

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CHAPTER FOUR: PURIFICATION, CHARACTERIZATION AND THREE-DIMENSIONAL STRUCTURAL PREDICTION OF CATECHOL 1,2-DIOXYGENASE FROM *PSEUDOMONAS CHLORORAPHIS* STRAIN UFB2 ISOLATED FROM ACTIVATED SLUDGE

Abstract

Microbial enzymes are studied extensively due to their potential to form toxic compounds into less harmful substances. Catechol dioxygenases cleave catechol into *cis*-*cis*-muconic acid or 2-hydroxymuconic semialdehyde via the *ortho*- or *meta*-pathway, respectively. The aims of the study were to purify, characterize and predict template based structure of catechol 1,2-dioxygenase. Preliminary studies showed that *Pseudomonas chlororaphis* strain UFB2 isolated from a contaminated site in Durban can degrade 40 ppm of 2,4-DCP. The isolate was then used for the preparation of the crude cell extract induced with 600 ppm phenol and grown in Mineral salt Media (MSM) for 36 h. The crude cell lysate showed 10.34 U of catechol 1,2-dioxygenase with a specific activity of 2.23 U/mg of protein. A 61 kDa catechol 1,2-dioxygenase was successfully purified to 1.5 fold and with a yield of 13.02% by applying anion exchange and gel filtration chromatography. The enzyme was optimally active at pH 7.5 and temperature 30 °C. The Lineweaver-Burk plot showed the v_{max} and K_m values of 16.67 µM/min and 35.76 µM, respectively. Characterization and optimization of catechol 1,2-dioxygenase activity can assist in understanding the metabolic pathways of the microorganisms and their possible application in bioremediation techniques.

4.1 Introduction

Aromatic compounds and their distribution in the environment has led to an increase in pollution, which affects the health and quality of living organisms (Igbinosa *et al*., 2013). Microorganisms have developed mechanisms to degrade the compounds with an aid of enzymes (Das and Chandran *et al*., 2011; Karigar and Rao, 2011). Enzymes from microorganisms have been studied extensively for their ability to convert aromatic compounds into non-toxic compounds.

During aerobic biodegradation of aromatic compounds, there are different central intermediates such as catechols, protocatechuates, gentisates and (hydroxy) benzoquinols formed by the introduction of hydroxyl groups. This is usually facilitated at *ortho* position of catechol to yield intermediates of the Krebs cycle (Vetting *et al*., 2000; Perez *et al*., 2008**;** Karigar and Rao, 2011; Nadaf and Ghosh, 2011; Guzik *et al*., 2013; Long *et al*., 2016) (Figure 1.4).

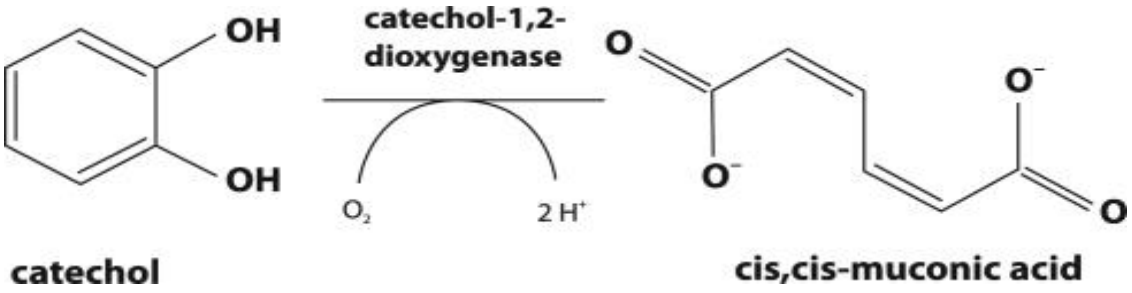

Figure 4.1: Catalysis of catechol degradation by catechol 1,2-dioxygenase via *Ortho*-pathway of 2,4-DCP degradation (Vilímková *et al*., 2008).

The enzymes catechol dioxygenases have potential to be used in the process of remediating wastewater contaminated with phenol, benzoate, fluorocatechol, bromocatechol, cholorocathecol, methylcathecol, herbicides (diuron), polychlorinated biphenyls and chloroethanes (Shunkova *et al*., 2009; Silva *et al*., 2012). Catechol 1,2-dioxygenase has the

Fe^{2+} prosthetic group involved in cleaving catechol as intradiol (through the *ortho*-pathway) producing *cis*-*cis*-muconic acid (Marín *et al*., 2010; Đokić *et al*., 2011; Silva *et al*., 2012; Lin and Milase, 2015).

Krastanov *et al*. (2013) reported that the enzyme catechol 1,2-dioxygenase was first isolated and purified in *Pseudomonas* species. The enzyme incorporates oxygen atom into the catechol resulting in the formation of *cis*-*cis*-muconic acid (Sridevi *et al*., 2012; Krastanov *et al*., 2013). In *Pseudomonas*, the enzyme is Fe^{2+} and Fe^{3+} ions dependent and has high substrate specificity (Krastanov *et al*., 2013; Hasan *et al*., 2015). Hasan *et al*. (2015) studied *In-silico* physiochemical properties of catechol 1,2-dioxygenase in different *Pseudomonas* spp. and concluded that the molecular weight ranges from 22-35 kDa.

Pseudomonas aeruginosa TKU002 strain capable of mineralizing benzoic acid is reported to produce a low molecular weight of catechol 1,2-dioxygenase showing highest activity against pyrogallol, making it different from another catechol 1,2-dioxygenase (Krastanov *et al*., 2013; Hasan *et al*., 2015). Catechol 1,2-dioxygenase was isolated from *Trichosporon* species and reported to produce a high molecular weight of 100 kDa, stable at pH 8 and optimally activate at pH 6.2 (Krastanov *et al*., 2013). The aim of this study was to purify and characterize catechol 1,2-dioxygenase in *Pseudomonas chlororaphis* strain UFB2, to understand the catalytic mechanism of the enzyme and predict the three-dimensional structure of the protein.

4.2 Materials and Methods

4.2.1 Preparation of crude extracts

The crude extracts were prepared by growing the bacterial cell for 24 h in Mineral Salt Medium (MSM) comprising (g/L): K_2HPO_4, 2.75; KH_2PO_4, 0.1; NH_4Cl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.01;

$CaCl_2 \cdot 2H_2O$, 1.0; NH_4Cl, 0.5, and Yeast extract, 1.0. The pH was adjusted to 7.0 with 2 M NaOH prior to autoclaving at 121°C for 15 minutes. 1 mL of trace metal solution composed of (mg/L): $FeSO_4 \cdot 7H_2O$, 5; $ZnSO_4 \cdot 7H_2O$, 4; $MnSO_4 \cdot 4H_2O$, 0.2; $NiCl \cdot 6H_2O$, 0.1; H_3BO_3, 0.15; $CoCl_2 \cdot 6H_2O$, 0.5; $ZnCl_2$, 0.25; EDTA, 2 was added by syringe filter (0.2 µL pore) into IL of the MSM. *Pseudomonas chlororaphis* strain UFB2 cells were grown in nutrient broth overnight at 30°C and the culture was standardized to OD=1 at 600 nm. Ten percent of the standardized culture was inoculated into above-described MSM supplemented with 600 ppm of phenol as a sole carbon and energy source. The inoculated medium was incubated at 30°C for 36 h shaking at 150 rpm. The cells were harvested at the late exponential phase of growth by centrifugation at 10000 ×*g* for 15 min at 4°C. The cells were washed twice with 50 mM Sodium phosphate buffer, pH 7.5 (containing 1 mM EDTA and 1 mM β-Mercaptoethanol to halt the proteases activity). A total of 24 g of the cell pellet was collected and re-suspended in 100 mL of the same buffer. Cell-free extracts were prepared by lysing the pellet by sonication with 400 Ultrasonicator (OMNI International) 8 cycles each with a pulse of 30s on/off for 4 min. The cell lysed cell extract was centrifuged at 20000× *g* for 30 min at 4°C. The clear supernatant was kept on ice to prevent inactivation of the enzymes and used as a crude extract for enzyme assays, while the remaining extract was kept in -20°C for further studies (Mahiudddin *et al*., 2011).

4.2.2 Enzyme assay

Catechol 1,2-dioxygenase activity was measured by the method described by Kotresha and Vidyasagar (2012). One milliliter reaction mixture contained 10 mM of substrate catechol, in 50 mM Sodium phosphate buffer (pH 8.0). The reaction was initiated by adding 100 µl of enzyme extract 100 µg of total protein to the reaction mixture and incubated for 30 min at

30°C. The initial and final absorbance at 260 nm and 375 nm were measured using Shimadzu UV-1800 UV-Vis Spectrophotometer fitted with temperature controller CPS-240A unit set at 30°C. The proper controls for each reaction were conducted along with the assay. One unit of enzyme activity was defined as the amount of the enzyme forming 1 µm either *cis-cis*-muconic acid at 260 nm or 2-hydroxymuconic semialdehyde at 375 nm under standard assay conditions. The enzyme activity was calculated by using the following equation:

$$\textit{Enzyme activity}\ (\mu m \text{ of product formed/min}) = [(E \times L/v)\ (\Delta OD\ /min)$$

Where ΔOD = Optical density at the different wavelengths; E = molar extinction coefficient, V= volume and L = path length. Molar extinction coefficient of 16 800 µM/min/cm (muconic acid) and 14 700 µM/min/cm (2-hydroxysemialdehyde) were used for catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activity, respectively.

4.2.3 Determination of protein concentration

The total protein concentration was determined by Bradford method (Bradford, 1976), using Bovine Serum Albumin (BSA) as a standard. The standard curve was plotted by using 0 to 2.0 mg/mL BSA (Appendix C, 7.3). The curve was fitted to the linear equation y = mx+c to determine the protein concentration of unknown samples.

4.2.4 Purification of catechol 1,2-dioxygenase

The enzyme was purified by using anion exchange and gel filtration chromatography. For anion exchange, 1 mL anion exchange HiTrap ANX column was equilibrated with 5 column volumes (CV) (1-CV=5 mL) of 20 mM Sodium sulfate buffer (pH 8) and 1 mL (200 µg total protein) of the sample was loaded onto the column. The unbound proteins were washed 5-CV of 20 mM Sodium sulfate buffer (pH 8). The proteins bound to the matrix were eluted with 10-CV of a 0-1.0 M linear gradient of NaCl in 20 mM Sodium sulfate buffer (pH 8). The eluted proteins were collected as 1 mL fractions using the AKTA purifier 100-P950 automated

fraction collector at a flow rate of 1 mL/min. The fractions showing the catechol 1,2-dioxygenase activity were concentrated with chilled acetone (200 µl of fractions and 800 µl acetone) for 2 hours at -70°C and the sample was loaded on 12% SDS-PAGE to confirm purity and homogeneity. The fractions showing the activity of catechol 1,2-dioxygenase activity were pulled together and concentrated using an Amicon Ultra-15 Centrifugal Filter Unit (MW cut off 10 kDa). One milliliter of the 0.36 mg of total protein was again loaded on a 35 mL (1-CV) gel filtration column manually packed with Sephacryl HR 100 matrix (from Sigma-Aldrich, St Louis, MO, USA) and equilibrated with 2-CV of 20 mM Sodium sulphate buffer (pH 8) and collected as 2 mL fractions using the AKTA purifier 100-P950 automated fraction collector at a flow rate of 0.5 mL/min. The fractions showing the activity of catechol 1,2-dioxygenase activity were pulled together and concentrated using an Amicon Ultra -15 Centrifugal Filter Unit (MW cut off 10 kDa).

The fractions were concentrated with chilled acetone (200 µl of fractions and 800 µl acetone) for 2 hours at -70°C and the sample was loaded on 12% SDS-PAGE to confirm purity and homogeneity (Kumar *et al*., 2014).

4.2.5 Determination of optimum pH and Temperature

The optimum pH of catechol 1,2-dioxygenase was determined by setting up a reaction in different buffers as follows: 50 mM Citrate-phosphate buffer (pH 4-6.5), Sodium phosphate buffer (pH 7.0-8.0) and Tris-phosphate buffer (pH 8.5-10) (Long *et al*, (2016). The optimum temperature of catechol 1,2-dioxygenase was determined by incubating the reaction mixture for 30 min at 20, 25, 30, 35, 40, 45, 50 and 60°C. The reaction was set up as described in Section 3.2.2.

4.2.6 pH and Temperature stability catechol 1,2-dioxygenase

To determine the pH stability of catechol 1,2-dioxygenase, an adequate volume of the enzyme was incubated in buffers: 50 mM Citrate-phosphate buffer (pH 4 and 6), 50 mM Sodium phosphate buffer (pH 7.0 and 8.0) and 50 mM Tris-phosphate buffer (pH 10) at a designated time (0-2 h). One hundred microliters (0.13 µg) of the enzyme aliquots were withdrawn at different time intervals and the reaction was set up as described in Section 4.2.2. The relative enzyme activity at different pHs was represented related to the initial activity. To determine the temperature stability, an adequate volume of the enzyme (0.13 µg) was incubated at the following temperatures (30, 50 and 70°C) in Sodium phosphate buffer (pH 8.0) for (0-2 h). One hundred microliters (0.13 µg) of the enzyme aliquots were withdrawn at different time intervals and the reaction was set up as described in Section 4.2.2. The relative enzyme activity at different temperatures was represented related to the initial activity.

4.2.7 Determination of kinetic parameters

The kinetics parameters for substrate hydrolysis by catechol 1,2-dioxygenase were determined by measuring the initial rate of enzymatic activity. One hundred microliters (0.13 µg) of the enzyme was incubated with Sodium phosphate pH 8 containing phenol (0.5-10 µM) at 30°C for 30 min. The Lineweaver-Burk plot was constructed by plotting the reciprocal of the rate of substrate hydrolysis (1/V) against the reciprocals of the substrate concentrations (1/[S]). The v_{max} and K_m values were determined by fitting the data in Michaelis-Menten equation using ORIGIN 8 pro (Evaluation version). The catalytic constant (k_{cat}) of the enzyme substrate reaction also known as a turnover number (the number of reactions catalysed per unit time by each active site) was determined by using the equation $k_{cat} = v_{max} /[E]_t$, where v_{max} is the

maximum velocity and $[E]_t$, is the total enzyme concentration. Catalytic efficiency was calculated by dividing k_{cat} with K_m (Nadaf and Ghosh, 2011).

4.2.8 Effects of metals and inhibitors on catechol 1,2-dioxygenase activity

One hundred microliters (0.13 µg) of purified catechol 1,2-dioxygenase was incubated with 0.1 mM of β-Mercaptoethanol, EDTA, $CUSO_4$, $HgCl_2$, $FeSO_4$, Tween 20, Tween 80 and Sodium Dodecyl Sulfate (SDS) and the assay was performed as described in Section 4.2.2.

4.2.9 Substrate specificity of catechol 1,2-dioxygenase

To determine the substrate specificity of catechol 1,2-dioxygenase, a stock solution of various substrate: Phenol, 4-Nitrocatechol, 3-Methylcatechol, 4-Methylcatechol, 1,2,4-Benzenetriol, catechol and 2,4-dichlorophenol were prepared in 50 mM Sodium phosphate buffer (pH 8). One hundred microliters (0.13 µM) enzyme was added to the 0.1 mM of the substrates except addition to 0.2 mM catechol to initiate the reaction, and the assay was performed as described in Section 4.2.2.

4.2.10 Determination of amino acid sequencing

The pure protein (10 µg) was loaded onto 12% SDS–PAGE and Coomassie blue R250 stained band were incised carefully and digested with trypsin and fragments analyzed by electrospray mass spectrometry (at CAF, Stellenbosch University, Stellenbosch, South Africa). The raw files generated by the mass spectrometers were imported into Proteome Discoverer v1.3 (Thermo Scientific, USA) and SearchGUI v. 3.2.18 and processed using the Mascot 1.3. Algorithm (Matrix Science) as well as the Sequest algorithm to get the peptides generated from

the enzyme. The database from tryptic digestion was analyzed by PeptideShaker (version 1.16.9) for homology of catechol 1,2-dioxygenase, and the sequence was then used for the determination of the function and the FASTA sequence of the protein by using UniProtKB ((http://www.uniprot.org/). The catechol 1,2-dioxygenase from *Pseudomonas chlororaphis* strain UFB2 homology search was carried out using Basic Local Alignment Search Tool (BLAST) protein blast at National Center for Biotechnology Information (NCBI).

4.2.11 Prediction of Biophysical properties and Three-dimensional structure

The protein and DNA homology search were carried out using PSI-and nucleotide BLAST in NCBI (non-redundant) database using ORF of the protein and DNA sequence as the query sequence. The multiple sequence alignments for protein and DNA were performed using DNAMAN (version 7), Lynnon Corporation, CA, USA (Demo version). Biophysical properties of the protein were determined using ExPASy server, while structure prediction of the enzymes was carried out using SWISS-MODEL workspace, (http://swissmodel.expasy.org). The default parameters used for performing the automated SWISS-MODEL were used as explained previously (Kumar *et al*., 2016) and also elaborated at (http://swissmodel.expasy.org/workspace/index%20.php?func=special_help) webpage. The modeled PDB files were submitted to online tool RAMPAGE for Ramachandran plot analysis to check the quality and validation of the predicted models (Kumar *et al*., 2016).

4.3 Results

4.3.1 Production and purification of catechol 1,2-dioxygenase

A total of 5 L production media was inoculated with 10% culture inoculum and induced with 600 ppm phenol for the production of catechol 1,2-dioxygenase enzyme. The crude extract

incubated with substrate catechol did not show any absorbance at OD375 nm (detection of 2-hydroxymuconic semialdehyde) incontrast there was an increased absorbance at OD260 nm (detection of *cis*-*cis*-muconic acid). The results concluded that the crude cell extract exhibit catechol 1,2-dioxygenase activity. Using the extinction coefficient of 14 800 µM/min/cm for muconic acid described in Section 4.2.2 of materials and methods, the OD at 260 nm was converted to µM of product released.

Thus, the specific activity of catechol 1,2-dioxygenase from the crude extract was 2.23 U/mg of protein. The crude cell extract was concentrated using Millipore ultrafiltration spin tubes and loaded in ANX anion exchange purification column attached to AKTA 100 purifier system. The peaks were observed at OD=280 nm, assayed for enzyme activity and loaded on SDS-PAGE (Figure 4.2). The fractions showing enzyme activity were pooled together, concentrated using spin column and loaded on gel filtration chromatography column packed with Sephacryl HR100 matrix. The fractions were collected and assayed for catechol 1,2-dioxygenase activity, with fractions A1 and A2, showing catechol 1,2-dioxygenase activity, were concentrated and checked for the purity of the protein (Figure 4.3). Fractions A1 and A2 showed the presence of single band protein SDS-PAGE (Figure 4.4). The purified protein showed the specific activity of 2.02 U/mg of protein. The enzyme was purified to 1.5 fold (Table 4.1).

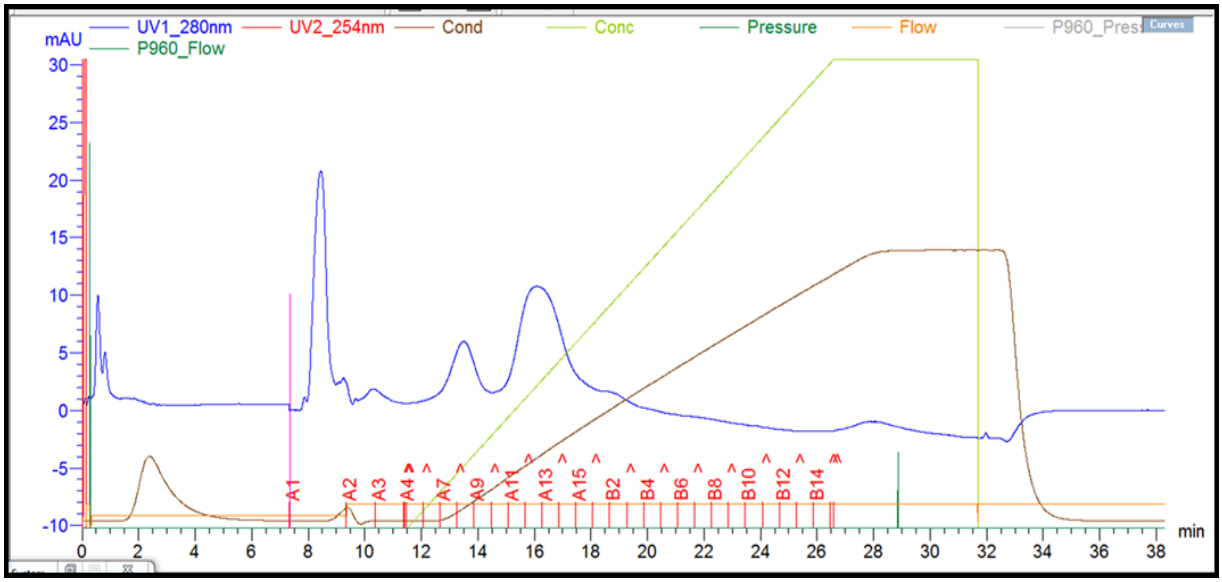

Figure 4.2: Anion Exchange Chromatogram of crude extract loaded on ANX anion exchange column connected to AKTA100 purification system. Labels from A1-A14 shown the number of fractions collected.

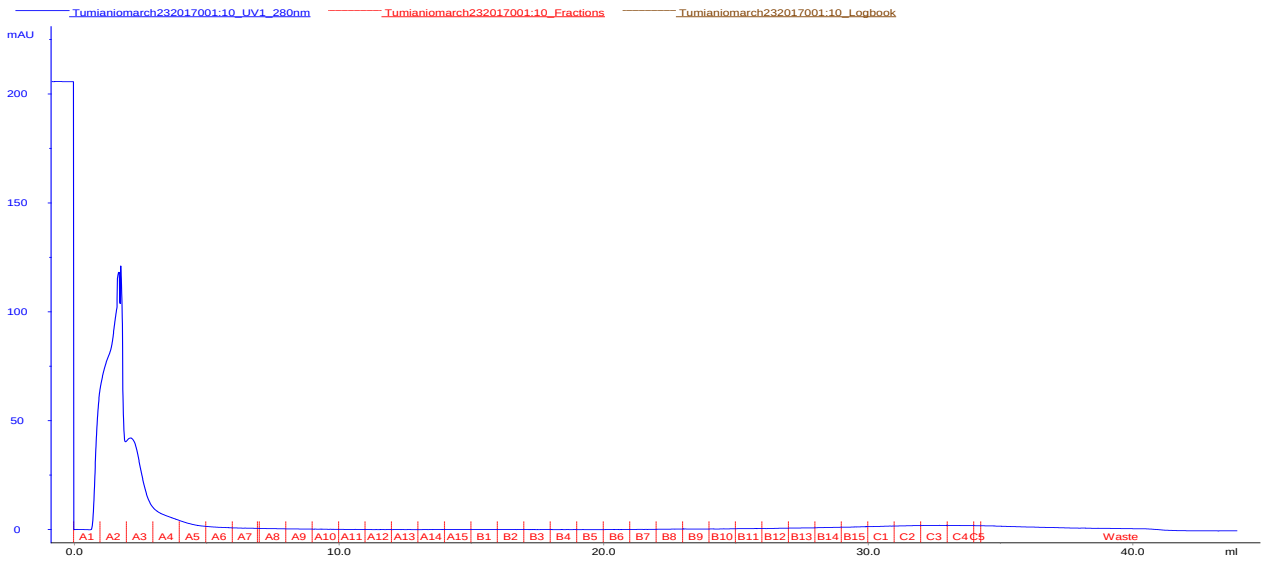

Figure 4.3: Gel Filtration Chromatogram of fraction collected after ANX anion exchange chromatography, pooled, concentrated and loaded on a column packed with Sepharyl 100 matrix. Labels from A1-C5 shows a number of the fraction collected.

Table 4.1: Purification of catechol 1,2-dioxygenase from *Pseudomonas chlororaphis* UFB2

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude	10.34	4.64	2.23	100	1
Anion Exchange Chromatography	5.16	1.75	2.91	57.7	1.3
Gel Filtration Chromatography	2.02	0.59	3.42	13.02	1.5

The purified protein loaded on 12% SDS-PAGE, stained with Coomassie Blue (CBB) showed a band size of 35 kDa confirming the purity and homogeneity of the enzyme (Figure 4.4).

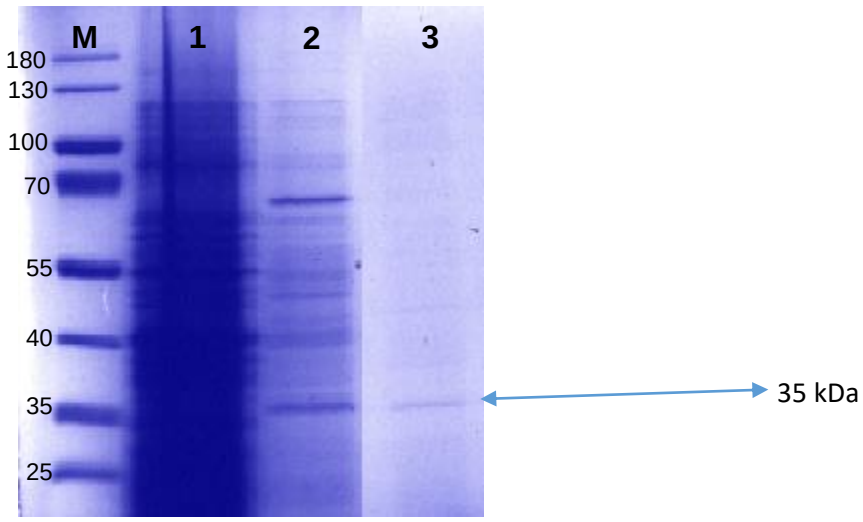

Figure 4.4: 12% SDS-PAGE for crude and purified catechol 1,2-dioxygenase. Lane M= Protein Marker (180 kDa Marker), Lane 1= Crude cell extract; Lane 2= 100 µg total protein colleted and loaded after ANX column; Lane 3= Purified catechol 1,2-dioxygenase after Gel filtration chromatography .

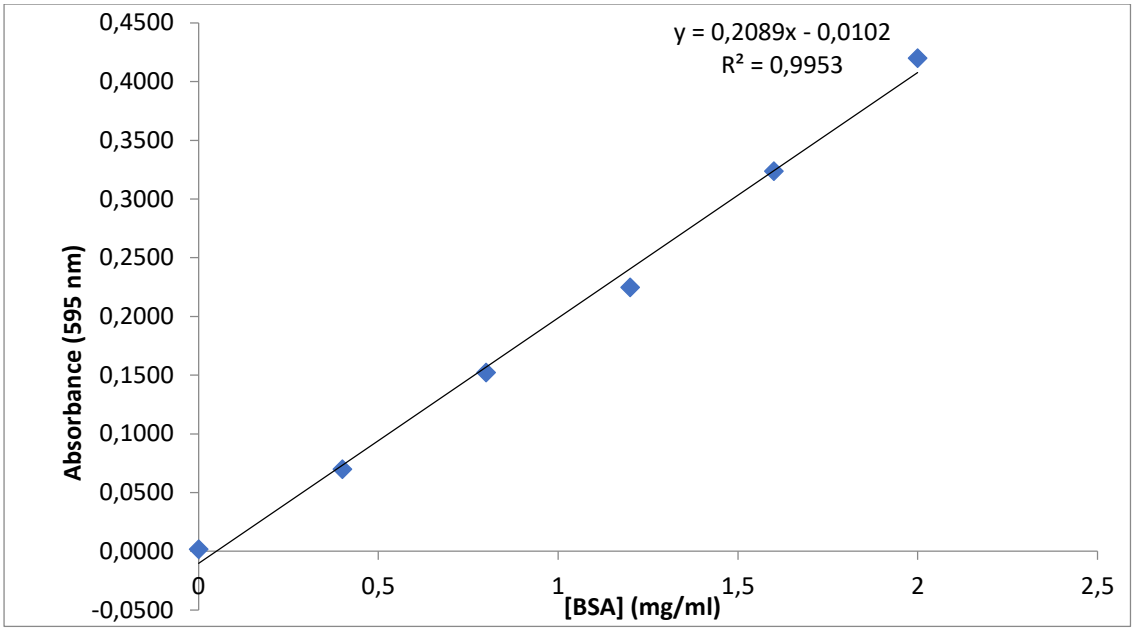

Figure 4.5: Standard Curve of Bovine Serum Albumin (BSA).

4.3.2 Optimum pH and pH stability of purified catechol 1,2-dioxygenase

Purified catechol 1,2-dioxygenase showed the optimum of pH 7.5 for its activity (Figure 4.6), retaining 84% and 80% of its activity at pH 7 and 8, respectively. The enzyme incubated for a period (0-240 min) of time, assayed at its optimum conditions showed that the enzyme was stable until 240 min at pH 8 retaining its 90% activity. At pH 4 the enzyme lost 50% activity within 10 min and about 90% activity was lost after 240 min. At pH 6, the enzyme lost 30% of activity after 180 min of incubation and about 50% activity was lost gradually in 240 min. The enzyme was quite stable at pH 7 retaining 90% after 240 min and a sudden decrease in activity at 140 min losing 10% (Figure 4.7).

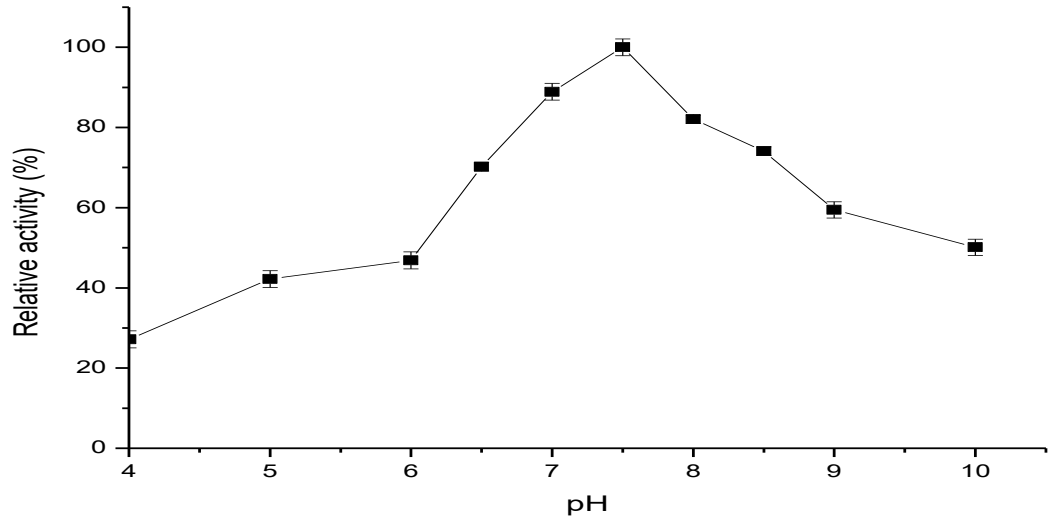

Figure 4.6: Optimum pH profile of catechol 1,2-dioxygenase from 0.13 µg of enzyme incubated at different and assayed for enzyme activity.

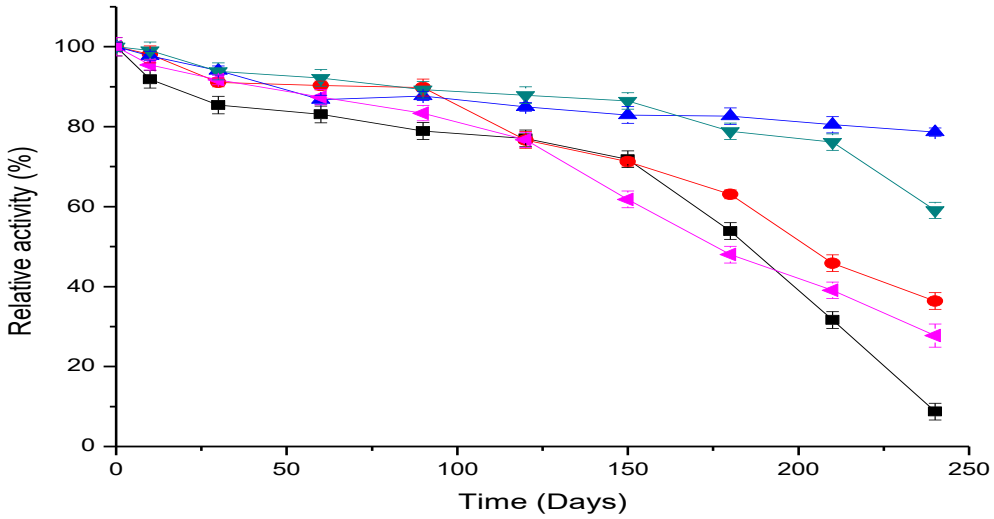

Figure 4.7: pH stability profile of catechol 1,2-dioxygenase at pH 4 (■), pH 6 (●), pH 7(▲), pH 8 (▲) and pH 10 (▲).

4.3.3 Optimum temperature and temperature stability

Purified catechol 1,2-dioxygenase showed an optimum temperature of 30°C for its activity (Figure 4.8), with 81% and 82% of its activity retained at 25°C and 35°C. The enzyme was incubated for a certain time (0-240 min) to determine its stability. The enzyme was stable until 120 min at 30°C and lost only 5% activity even after incubation at 240 min. The enzyme was found to be unstable at 70°C with 60% activity loss within 125 min and about 90% activity was lost after 240 min. At 50°C there was 50% loss of activity after 100 min and the activity drastically decreased in 240 min remaining 10% of activity (Figure 4.9).

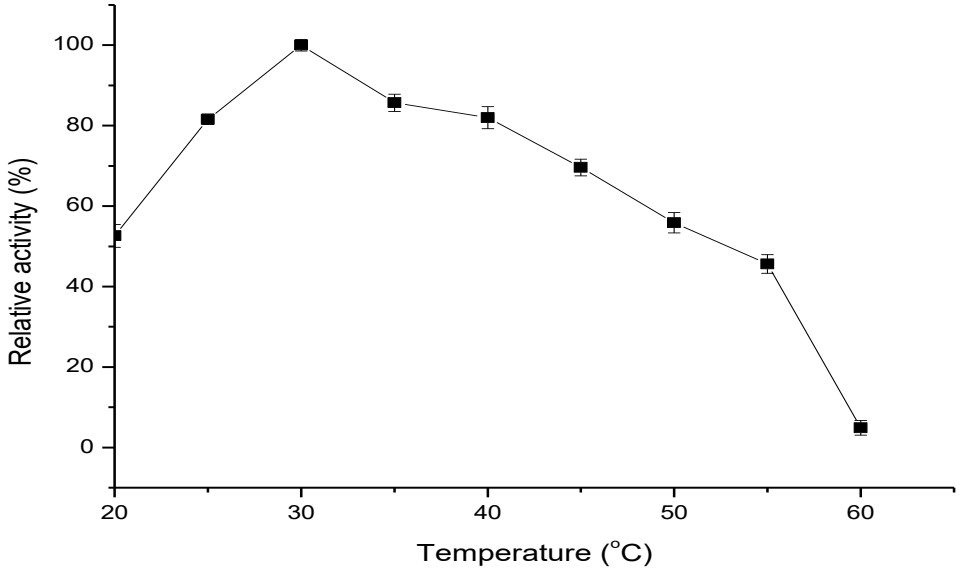

Figure 4.8: The optimum temperature of catechol 1,2-dioxygenase from 0.13 µg of enzyme incubated at a different temperature and the activity was measured at optimum pH 7.5.

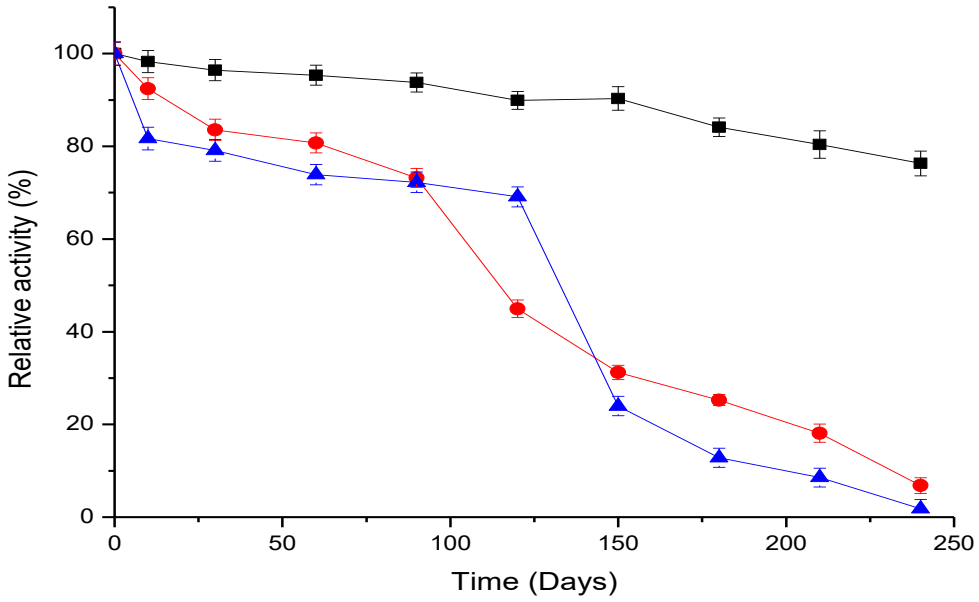

Figure 4.9: The temperature stability of catechol 1,2-dioxygenase at 30°C (■), 50°C (●) and 70°C (►).

4.3.4 The kinetic properties for catechol 1,2-dioxygenase

The Lineweaver–Burk plots fitted in Michaelis-Menten equation showed the K_m and v_{max} values of 35.76 µM and 16.67 µM/min, respectively (Figure 4.10 and 4.11). Figure 4.10 shows that the 0.13 µg of enzyme get saturated with the 200 µM of catechol keeping the maximum reaction Velocity constant till 500 µM of catechol. The curve does not indicate any type of substrate inhibition of the enzyme.

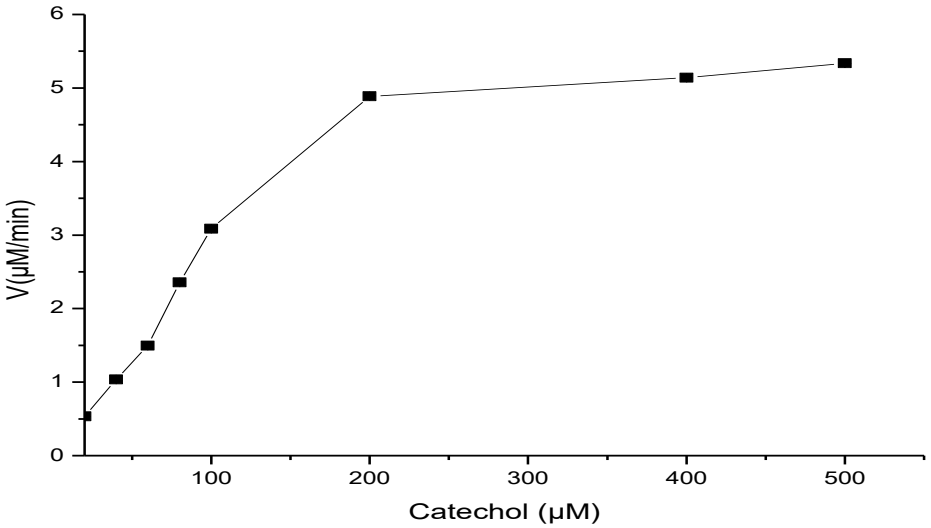

Figure 4.10: Substrate Saturation curve for catechol 1,2-dioxygenase. The assays were performed by incubating 0.13 µg of the enzyme with 20-500 µM catechol.

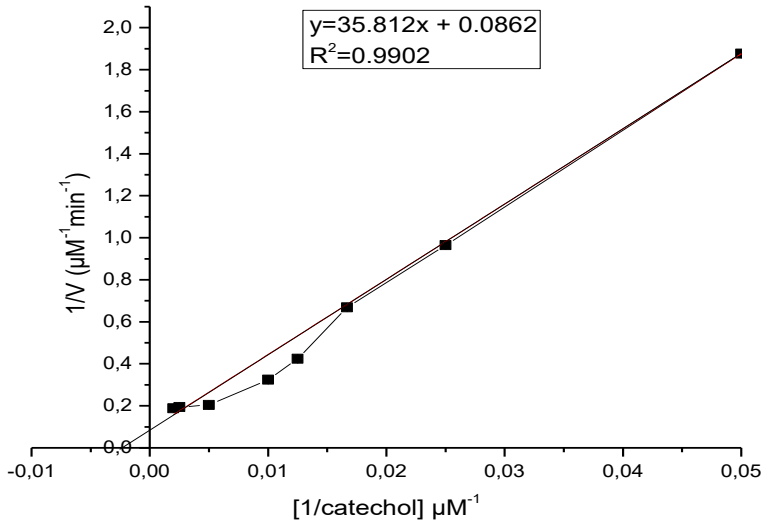

Figure 4.11: Lineweaver-Burk double reciprocal plot of substrate saturation curve of catechol 1,2-dioxygenase. The double reciprocal plot was fitted to Michaelis-Menten equation to determine the values of v_{max} and K_m.

4.3.5 Effects of Metals and inhibitors on catechol 1,2-dioxygenase activity

The effects of metals and inhibitors on purified catechol 1,2-dioxygenase activity were evaluated by incubating 100 microliters (0.13 µg) enzyme with a specific concentration of metals and inhibitors (Table 4). In the presence of *β*-Mercaptoethanol and EDTA, the enzyme showed 59% and 58% residual activity, respectively. Catechol 1,2-dioxygenase activity was drastically inhibited by the presence of metal ions like $CuSO_4$, $HgCl_2$ and $FeSO_4$ with residual activity of 33, 15 and 12%, respectively. The surfactants: Tween 20 and Tween 80 reduced the activity to 80 and 96% respectively. The protein denaturating agent SDS reduced activity to 10% almost deactivating the enzyme.

Table 4.2: Effects of Metals and inhibitors (1 mM) on the purified catechol 1,2-dioxygenase from *Pseudomonas chlororaphis* strain UFB2

Inhibitor/detergent	***Residual activity (%)**
None (Control)	100±0.035
β-Mercaptoethanol	59±0.025
EDTA	58±0.019
$CUSO_4$	33±0.012
$HgCl_2$	15±0.017
$FeSO_4$	12±0.017
Tween 20	80±0.021
Tween 80	96±0.018
Sodium Dodecyl Sulfate (SDS)	10±0.007

* Residual activity of catechol 1,2-dioxygenase represented the percentage of activity (U/mL) in the presence of various substrates (metals and inhibitors) as compared to the activity measured in the presence of catechol.

4.3.6 Substrate specificity of catechol 1,2-dioxygenase

The enzyme catechol 1,2-dioxygenase showed more affinity to catechol as compared to other substrates (Table 5). The enzyme could catalyze phenol efficiently showing 72% of residual activity as compared to catechol. The enzyme showed only 25, 21, and 51% of residual activity in the presence of 4-nitrophenol, 1,2,4-benzenetriol and 2,4-DCP, respectively. Catechol 1,2-dioxygenase did not show any activity in the presence of 3-methylcatechol and 4-methylcatechol (Table 5).

Table 4.3: Substrate specificity of purified catechol 1,2-dioxygenase

Substrates	*** Residual activity (%)**
Catechol	100±0.005
4-Nitrocatechol	25±0.001
4-methylcatechol	0.00±0.000
3-methylcatechol	0.00±0.000
1,2,4-Benzenetriol	21.00±0.015
Phenol	72±0.022
2,4-dichlorophenol	51±0.001

*Residual activity of catechol 1,2-dioxygenase represented the percentage of U/mL activity in the in the presence of various substrates as compared to the activity measured in the presence of catechol.

4.3.7 ES-MS and amino acid sequence determination

The pure protein band from SDS-PAGE was incised, cleaned and digested with trypsin to generate small peptides to be analyzed on ES-MS. The raw data generated was analyzed in SearchGui 3.2.18 and peptide shaker (version 1.16.9). The FASTA files for the mass spectrum pf proteins from *Pseudomonas chlororaphis* was downloaded from Uniprot protein database. Peptide Shaker data showed the presence of 6 peptide spectra matching to the peptide spectra and homogentisate 1,2-dioxygenase (accession number, A0A0G3GN46). This enzyme catalyzes homogentisate to maleylacetoacetate. Accession number A0A0G3GN46 searched on www.Uniprot.org lead to the depiction of an amino acid sequence of homogentisate 1,2-dioxygenase (Figure 4.12).

```
        10         20         30         40         50
MNLDSPASDL AYQSGFGNEF ASEALPGALP VGQNSPQKAP YGLYAELFSG
        60         70         80         90        100
TAFTMARSEA RRTWMYRIRP SANHPAFTPL ERQLVGGPLG EVTPNRLRWN
       110        120        130        140        150
PLENPAQPTD FLDGLVRMAA NAGMDKPAGI SIFHYCANRS MERVFFNADG
       160        170        180        190        200
EWLIVPQLGR LRIITELGVL ELAPLEIAVL PRGLKFRVEL LDAQARGYLA
       210        220        230        240        250
ENHGAPLRLP DLGPIGSNGL ANPRDFLTPV AHYENLAQPT TLVQKFLGQL
       260        270        280        290        300
WACELGHSPF DVVAWHGNNV PYKYDLRRFN TIGTVSFDHP DPSIFTVLTS
       310        320        330        340        350
PTSVHGLANL DFVIFPPRWM VAEKTFRPPW FHRNLMNEFM GLIQGAYDAK
       360        370        380        390        400
AEGFVPGGAS LHSCMSAHGP DGETCTKAIN AELQPAKIDH TMAFMFETSQ
       410        420        430
VLRPSRFAMD CPQLQTDYDG CWASLPVTFD PTRR
```

Figure 4.12: Amino acid sequence of homogentisate 1,2-dioxygenase protein *Pseudomonas chlororaphis* UFB 2.

4.3.8 Template based structure of homogentisate 1,2-dioxygenase

The structure of catechol 1,2-dioxygenase from *P. Chlororaphis* UFB 2 was used as a template for homology modeling. There were two models that were predicted, Model_1 (3zds.1.A) had 81.06% and Model_2 (2uyb.1.A) had 14.62% sequence identity with the purified catechol 1,2-dioxygenase (Figure 4.13 a-d).

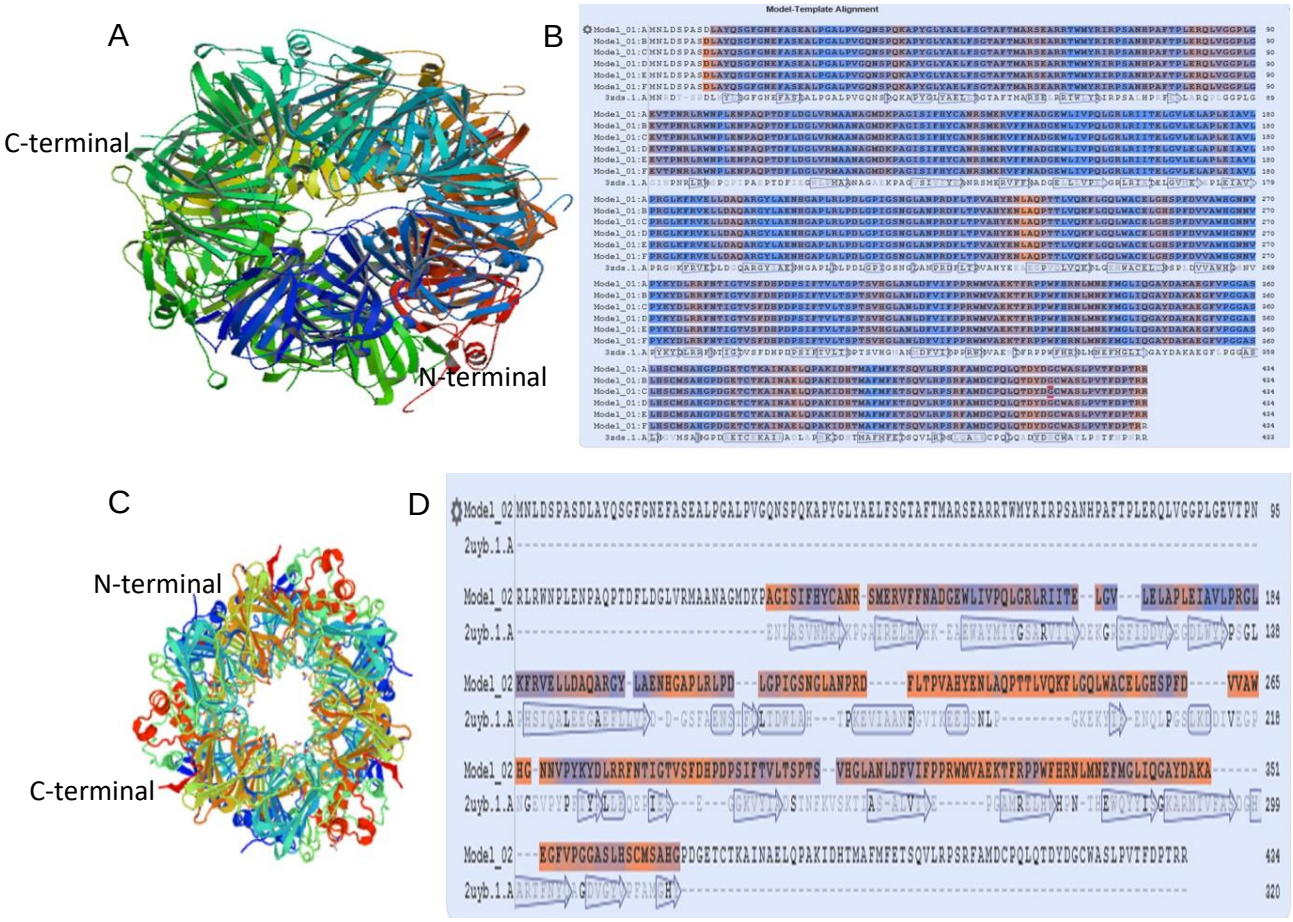

Figure 4.13: The model constructed by homology modeling at SWISS-MODEL workspace using homogentisate 1,2-dioxygenase as the template and predicted tertiary structure of homogentisate 1,2-dioxygenase deduced from 3zds.1.A (A) and 2uyb.1.A (C). The N-terminus and C-terminal of the enzyme are marked. (B) Homology alignment of homogentisate 1,2-dioxygenase (Model_1) homogentisate 1,2-dioxygenase (3zds.1.A) (D) Homology alignment of homogentisate 1,2-dioxygenase (Model_2) with oxalate decarboxylase oxdc (2uyb.1.A).

Model_1 (3zds.1.A) was described as homogentisate 1,2-dioxygenase, is involved in the catabolism of aromatic compounds, this enzyme converts homogentisate to 4-maleylacetoacetate facilitated by the amino acid phenylalanine and tyrosine in an aerobic microorganism (bacteria) found in the soil. The active site of homogentisate 1,2-dioxygenase comprises of the following residues; His292, His335, His365, His371, and Glu341. The active sites such as Glu341, His335, and His371 are bound by homogentisate via the Fe^{2+} atom. His 292 binds the hydroxy group of the aromatic ring and His365 bonds to Glu341 via hydrogen bonding for amino acid stability (Titus *et al*., 2000).

Model_2 (2uyb.1.A) was described as 3 oxalate decarboxylase oxide, the enzyme was described as oxalate decarboxylase oxide. Oxalate decarboxylase is a manganese and dioxygenase dependent enzyme that catalyzes oxalate into carbon dioxide and formate. In *Bacillus subtilis*, the enzyme has two manganese-binding sites (site 1 and site 2), site 1 is for the catalytic active site and site 2 for structural purpose (Just *et al*., 2007).

4.3.9 Predicted biophysical properties of homogentisate 1,2-dioxygenase and catechol 1,2-dioxygenase expressed in other *Pseudomonas* strains.

Physiochemical parameters of the enzyme were determined by using ExPaSy server. The parameters on the ExPaSy server included the following: Number of amino acids, Molecular Weight (M. Wt), theoretical pI, extinction coefficients (M^{-1} cm^{-1}), estimated half-life, Instability Index (II), Aliphatic Index (AI) and grand average of hydropathicity (GRAVY) (Table 2). The number of amino acids for enzyme catechol 1,2-dioxygenase from *Pseudomonas chlororaphis* strain UFB 2 was 314. The predicted molecular weight for catechol 1,2-dioxygenase from *Pseudomonas chlororaphis* strain UFB2 was computed to be 34.58 kDa with an Isoelectric point (pI) of 5.18 showing that catechol 1,2-dioxygenase from the isolate is acidic in nature. Extinction coefficients of the enzyme were 30035 M^{-1} cm^{-1}. The estimated

half-life of catechol 1,2-dioxygenase in *Escherichia coli in vitro* was greater than 10 hours, showing it can be stable in prokaryotic cells. The Instability index of the enzyme showed that catechol 1,2-dioxygenase from the strain is 39.92, the protein was stable in invitro. Aliphatic index of the enzyme was 85.54, which was above 70 showing its thermostability. Grand average of hydropathicity (GRAVY) of the enzyme was -0.477 which was negative, and it's an indicating that the enzyme is hydrophilic.

The number of amino acids for enzyme catechol 1,2-dioxygenase in *Pseudomonas* species were ranging from 314-327. The molecular weight of catechol 1,2-dioxygenase from *Pseudomonas* species ranged from 34-36 kDa. The Isoelectric point (pI) of *Pseudomonas* species was from 4-11, showing that catechol 1,2-dioxygenase from the isolates is acidic in nature. An exception was the enzyme from *Pseudomonas chlororaphis* strain PCL1606 which had a pI of 11.37, showing that it is basic in nature. Extinction coefficients of the enzyme were between 30035-87595 M^{-1} cm^{-1}, some of the *Pseudomonas* had the same extinction coefficients. The estimated half-life of catechol 1,2-dioxygenase in *E. coli in vitro* was greater than 10 hours in all the catechol 1,2-dixoxygenase in *Pseudomonas* species, except the enzyme from *Pseudomonas chlororaphis* strain PCL1606 had a half- life of 3 min showing it is unstable in prokaryotic cells. Aliphatic index of the enzyme ranged from 52-79, *Pseudomonas chlororaphis* strain PCL1606 was the lowest at 52.57. Catechol 1,2-dioxygenase from the isolates is thermostable, with aliphatic index above 70. Grand average of hydropathicity (GRAVY) of the enzyme was negative; an indication that the enzyme is hydrophillic (Table 4.4).

Table 4.4: Catechol 1,2-dioxygnase physiochemical properties from different *Pseudomonas* spp.

Organism name	**Number of amino acids**	**Molecular weight**	**Theoretical pI**	**Extinction coefficients (M^{-1} cm^{-1})**	**Estimated half-life**	**Instability index**	**Aliphatic index**	**Grand average of hydropathicity (GRAVY)**
Pseudomonas chlororaphis UFB2	314	34.58	5.18	30035	>10 hours	39.92 (stable)	77.74	-0.477
Pseudomonas antarctica PAMC 27494	317	35.43	4.68	32890	>10 hours	37.57 (stable)	75.80	-0.598
Pseudomonas simiae WCS417	317	35.47	4.85	31400	>10 hours	35.50 (stable)	77.63	-0.573
Pseudomonas fluorescens PICF7	317	35.49	4.77	31400	>10 hours	36.76 (stable)	77.95	-0.570
Pseudomonas trivialis IHBB745	317	35.35	4.79	31400	>10 hours	35.00 (stable)	79.21	-0.529
Pseudomonas chlororaphis PCL1606	327	36.65	11.37	87595	3 min	72.35 (unstable)	52.57	-0.469
Pseudomonas fluorescens Pf0-1	324	undefined	undefined	30035	>10 hours	39.29 (stable)	77.74	-0.462
Pseudomonas fluorescens Pt14	317	35.45	4.70	31400	>10 hours	37.07 (stable)	77.16	-0.578
Pseudomonas fluorescens KENGFT3	319	35.71	4.84	31400	>10 hours	34.29 (Stable)	75.34	-0.557

4.4 Discussion

Catechol 1,2-dioxygenase from *Pseudomonas chlororaphis* strain UFB2 was successfully purified to 1.5 fold with a 13.02% yield. The enzyme showed a molecular weight of 35 kDa. The reports for catechol 1,2-dioxygenase from *Pseudomonas* spp. indicated that molecular weight falls in the range of 20- 40kDa (Hasan *et al*., 2015). Catechol 1,2-dioxygenases include homodimers and homotetramers with molecular mass that ranges from 20-40 kDa (Chang-Jun *et al*., 2006; Krastanov *et al*., 2013).

In this study, the optimum pH and temperature for catechol 1,2-dioxygenase from *Pseudomonas chlororaphis* strain UFB2 were found to be pH 7.5 and 30°C, respectively. The catechol from *Rhodococcus* sp. NCIM 2891 also showed optimum pH 7.5 and temperature 30°C (Nadaf and Ghosh, 2011). The enzyme from other microorganisms such as *Acinetobacter sp.* Y64 strain had optimum pH 8 and optimum temperature 37°C (Lin and Milase, 2015), *Gordonia polyisoprenivorans* showed an optimum pH 7 and 30°C (Silva *et al*., 2012). While the enzyme from *Pseudomonas putida* strain N6 had optimum pH 7.5 and temperature 35°C (Guzik *et al*., 2011). Some catechol 1,2-dioxygenases are reported to have optimum temperature at 40°C as reported in *Pseudomonas aeruginosa* KB2 and *Candida albicans* TL3 strains (Wang *et al*., 2006; Tsai and Li, 2007). Catechol 1,2-dioxygenase from *Mycobacterium fortuitum* immobilized on different surfaces are reported to have elevation in optimum temperature of 45°C.

The Lineweaver-bluk plot showed the K_m and v_{max} values of 35.76 µM and 16.67 µM/min, respectively for the enzyme. The v_{max} value for catechol 1,2-dioxygenase was 100-fold lower than v_{max} value of 1.218 U/mg reported (Guzik *et al*., 2013) with a v_{max} for catechol 1,2-dioxygenase from *Stenotrophomonas maltophilia* KB2. The v_{max} values for catechol 1,2-dioxygenase from *Rhodococcus opacus*1CP and *Rhodococcus opacus* 6a were found to be

9.6 U/mg and 55.5 U/mg of protein, respectively (Solyanikova *et al*., 2009). The K_m and v_{max} values were high, showing that the enzyme has less affinity to the substrate, the K_m value in this study the was higher than what was reported (Wang *et al*., 2006; Nadaf and Ghosh, 2011; Guzik *et al*., 2013).

Various potential inhibitors/ detergents were examined for their effect on catechol 1,2-dioxygenase activity. The enzyme activity was inhibited significantly by heavy metals like $CuSO_4$, $HgCl_2$, $FeSO_4$ and Sodium Dodecyl Sulfate (SDS). However, EDTA, Tween 20, Tween 80 and β-mercaptoethanol showed no inhibitory. Nadaf and Ghosh, (2011) reported similar results with a catechol 1,2-dioxygenase from *Rhodococcus* sp. NCIM 2891 which was inhibited by $CuSO_4$, $HgCl_2$ and $FeSO_4$. However, Tsai and Li, (2007) reported a different trend of the effect of inhibitors and metals from catechol 1,2-dioxygenase isolated from *Candida albicans* TL3, where $CuSO_4$ and $FeSO_4$ did not affect the activity of the enzyme as opposed to our study.

Catechol 1,2-dioxygenase has a wide range of substrate specificity. In this study, the enzyme had high affinity for catechol, and showed low affinity for 2,4-dichlorophenol, 1,2,4-Benzenetriol 4-nitrocatechol and phenol relatively. Studies have reported that catechol 1,2-dioxygenase from different microorganisms have different substrate specificity. Pandeeti and Siddavattam, (2011) reported the same findings as this study where catechol 1,2-dioxygenase from the *C120* of *Acinetobacter* sp. DS002 was induced by catechol, 1,2,4-Benzenetriol and 4-Nitrocatechol, while 3-methylcatechol and 4-methylcatechol could not induce the expression of enzyme. Lin and Milase, (2015) had reported same findings where 3-methylcatechol resulted in lower activity as compared to catechol, 1,2,4-Benzenetriol and 4-Nitrocatechol, however 4-methylcatechol was able to induce the enzyme in *Acinetobacter* sp. Y64.

Guzik *et al*. (2013) showed that enzyme from *Stenotrophomonas maltophilia* KB2 catalysed 3-methylcatechol and 4-methylcatechol with 50% less efficiency as compared to catechol,

while 2,4-dichlorophenol showed 74% relative activity. Stoilova *et al*. (2006) showed that 2,4-dichlorophenol was not an inducer of catechol 1,2-dioxygenase incontrast to phenol in *Aspergillus awamori* cells. Studies show that *Rhodococcus*, *Ralstonia* and *Pseudomonas arvilla* catechol 1,2-dioxygenases have a broad substrate specificity (Suresh *et al*., 2006; Pandeeti and Siddavattam, 2011). Catechol 1,2-dioxygenase from *Pseudomonas chlororaphis* UFB2 was induced more efficiently by catechol when used as a substrate (Wang *et al*., 2006; Pandeeti and Siddavattam, 2011; Guzik *et al*., 2011; Lin and Milase, 2015).

The purified protein band was incised from 12% SDS-PAGE and followed by trypsin digestion and ES-MS analysis. The protein band corresponded to enzyme homogentisate 1,2-dioxygenase. The structure prediction showed that this enzyme has good homology with two models (3zds.1.A and 2uyb.1.A). Model_1 (3zds.1.A) had 81.06% sequence similarity with homogentisate 1,2-dioxygenase (Figure 12). It was also confirmed that the enzyme is an intradiol enzyme and it catalyses homogentisate to maleylacetoacetate. It has been reported that the ring cleavage in homogentisate is a multiple step process. The initial step is coordination of carbonyl and *ortho* phenol oxygens by Fe^{2+}, the Fe^{2+} is therefore coordinated to His335, His371 and Glu341 (Guzik *et al*, 2013). The structure shows an octahedral coordination for Fe^{2+} with two histidine residues (His331 and His367), a bidentate carboxylate ligand (Glu337), and two water molecules. Homogentisate binds as a monodentate ligand to Fe^{2+}, and its interaction with Tyr346 result in the folding of a loop over the active site, effectively shielding it from solvent (Jeoung *et al*., 2013). Model_2 (2uyb.1.A) had 14.62% sequence similarity with homogentisate 1,2-dioxygenase. The enzyme was described as oxalate decarboxylase oxdc, oxalate decarboxylase is a manganase and dioxygenase dependent enzyme that catalyses oxalate into carbon dioxide and formate. In *Bacillus subtilis,* the enzyme has two manganese-binding sites (site 1 and site 2), site 1 is for catalytic active site and site 2 for structural purpose (Just *et al*., 2007). In *Pseudomonas putida* strain, homogentisate (HMG)

cleavage is facilitated by homogentisate 1,2-dioxygenase producing maleylacetoacetate. The maleylacetoacetate is isomerized to fumarylacetoacetate by maleylacetoacetate isomerase. Fumarate and acetoacetate are then formed from catalyzes of fumarylacetoacetate by fumarylacetoacetate hydrolase (Méndez *et al*., 2011). Oxygen atom (O_2) therefore binds to the iron atom and reacts with the aromatic ring *(*Borowski *et al*., 2005; Guzik *et al*, 2013). Guzik *et al*, (2013) reported that most intradiol dioxygenases enzymes have N-terminal domain with five α-helix and C-terminal domain consisting of β- sheets, a similar structure of catechol 1,2-dioxygenase from *Pseudomonas putida* N6 has been reported by Guzik *et al*, (2011). Physiochemical parameters of the enzyme were determined by using ExPaSy server.

The biophysical parameters calculated on ExPaSy showed the enzyme to be 314 amino acid long. The predicted molecular weight for enzyme was computed to be 34.58 kDa which corresponded to the one calculated by SDS-PAGE (Figure 4). The Isoelectric point (pI) of the enzyme was found to be 5.18. Extinction coefficients of the enzyme was found to be 30035 M^{-1} cm^{-1} showing high number of aromatic ring amino acid in the sequence. Estimated half-life of catechol 1,2-dioxygenase was greater than 10 hours when overexpressed in *Escherichia coli*, showing it can be stable in prokaryotic cells. The Instability index of the enzyme is shown as 39.92, considered stable *in vitro*. Aliphatic index of the enzyme 85.54, which was above 70 showing that it has a range of temperatures. Grand average of hydropathicity (GRAVY) of the enzyme was -0.477 which was negative, and it is an indication of the hydrophobicity of the enzyme to water. Most physiochemical properties were similar in the isolates the same findings were reported by Hasan *et al*, (2015) in *Pseudomonas aeruginosa*, with a molecular weight that ranged from 22 to 40 kDa and pI that ranged from 4 to 8. The instability index differed and ranged from to 35 to 47, which denotes that the enzyme can be stable or unstable depending on its type. The aliphatic index ranging from 70 to 85 showed that the enzyme can be thermostable and its hydrophilic with as a result of the negative value of the Gravy.

4.5 Conclusion

This Chapter covered the purification and characterization of enzyme catechol 1,2-dioxygenase from *Pseudomonas chlororaphis* strain UFB 2. The enzyme was successfully purified with a molecular weight of 35 kDa and confirmed by sequence from tryptic digestion which has been shown to be part of the intradiol dioxygenase enzyme. The enzyme was identified as the homogentisate 1,2-dioxygenase which hydroxylate of homogentisate to maleylacotetate in the ubiquinone biosynthesis. The enzyme was optimally active at pH 7.5 and temperature 30 °C. The enzyme was not stable at elevated temperatures. The kinetics parameters of the enzyme show that it has high affinity to catechol and phenol as a substrate and it has a strict substrate specificity. The characteristics of the purified catechol 1,2-dioxygenase, shows that the enzyme can have application in remediation of pollutants in the waste water treatment plants.

4.6 References

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CHAPTER FIVE: GENERAL DISCUSSION AND CONCLUSION

5.1 Research in perspective

Industrial revolution has improved the lives of humans over the years by production of chemicals that are used daily. However, it has equally resulted in damage to the environment by production of toxic substances. Chemical compounds are manufactured and used daily for domestic, industrial and agricultural use (García-Peña *et al*., 2012). Chlorophenols, in particular, are the leading toxic chemical compounds, and includes; 2,4-dichlorophenol (2,4-DCP), the derivative of the 2,4-Dichlorophenoxyacetic acid, the mostly used herbicide (Igbinosa *et al*., 2013; Arora and Bae, 2014). 2,4-DCP is a major health concern due to its carcinogenic, mutagenic and teratogenic properties (Olaniran and Igbinosa, 2011). Physical and chemical methods for 2,4-DCP removal are expensive and produce harmful secondary metabolites. Therefore, bioremediation is considered a suitable alternative due to its potential to form the compound into non-toxic end products (Wang *et al*., 2000; Wei *et al*., 2010). Microorganisms have been studied intensively due to their potential to utilize chemical pollutants as their carbon and energy sources (Elkarmi *et al*., 2009; Sridevi *et al*., 2012). Most of the catabolic pathways involved in the microbial degradation of the compounds are facilitated by microbial enzymes which can rapidly form the pollutants into innocuous form (Karigar and Rao, 2011; El-Naas *et al*., 2017).

The current study focused on the isolation of bacteria indigenous to contaminated sites in Durban, South Africa and their potential to degrade 2,4-DCP. The catabolic genes involved in the degradation process were detected and the enzymes encoded by these genes were assayed and characterized. Groundwater sample with known history of contamination with chlorinated organic compounds, was collected from an environmental site, while activated sludge sample was collected from New Germany wastewater treatment plant, both located in Durban, South Africa. The samples were enriched separately for the presence of bacteria with the potential to

utilize 2,4-DCP as their only carbon and energy source in MSM supplemented with 40 ppm of 2,4-DCP. A total of six bacteria were isolated (GW, GWbr, GWtr, MC, MCsr and YR) and screened, with only three isolates MC, YR and GW able to degrade 51%, 75% and 49% of 2,4-DCP, respectively after 18 days. The isolates were identified by 16S rRNA gene sequence analysis as *Klebsiella pneumoniae* strain DHQP1002001 (MC), *Klebsiella pneumoniae* strain KPNIH39 (GW) and *Pseudomonas chlororaphis* strain UFB2 (YR). The phylogenetic analysis revealed the relatedness of these isolates to other *Pseudomonas* and *Klebsiella* strains previously reported to degrade 2,4-DCP. A study conducted by Potrawfke *et al*. (1998) showed *Pseudomonas chlororaphis* was able to degrade 1,2,3,4-tetrachlorobenzene, a very toxic inorganic compound. Other studies also showed that *Pseudomonas* spp. have the potential to degrade 2,4-DCP and other chlorophenolic compounds (Kargi and Eker 2005; Stoilova *et al*., 2006; Elkarmi *et al*., 2008; Arora and Bae *et al*., 2014). It is therefore not surprising that the *Pseudomonas chlororaphis* strain isolated in this study demonstrated the highest potential to degrade 2,4-DCP. *Klebsiella pneumoniae* degraded 2,4-DCP at very low concentrations as previously reported (Perez-Pantoja *et al*., 2009; Michałowicz and Duda, 2007).

To detect the genes involved in 2,4-DCP degradation pathway, degenerate primers were designed for the presence of phenol hydroxylase, catechol 1,2-dioxygenase, muconate isomerase and dienelactone genes. PCR amplification of the catabolic genes involved in 2,4-DCP degradation revealed the presence of the correct amplicons for phenol hydroxylase (600 and 715 bp), catechol 1,2-dioxygenase (467 and 507 bp), muconate isomerase (651 and 494 bp), *cis*-dienelactone hydrolase (491 and 567 bp), and *trans*-dienelactone hydrolase (491 and 567 bp) in *Pseudomonas chlororaphis* and *Klebsiella pneumoniae*, respectively. However, the gene that encode for the enzyme catechol 2,3-dioxygenase, an enzyme which facilitates the *meta*-pathway, was not detected in all the three bacterial isolates suggesting that they only use the *ortho*-pathway for 2,4-DCP degradation. This was further confirmed by the absence of

catechol 2,3-dioxygenase enzyme in the crude extracts of the organisms. The crude extracts of the isolates, showed the enzyme activity of 21840 mU/mg, 15630 mU/mg, 1480 mU/mg, 2340 mU/mg and 1490 mU/mg for phenol hydroxylase, catechol 1,2-dioxygenase, muconate isomerase, *cis*-dienelactone hydrolase and *trans*-dienelactone hydrolase, respectively. Most of the 2,4-DCP degrading microorganisms have been reported to follow the *ortho*-pathway for the degradation of the compound. This has been attributed to the extradiol suicidal inactivation of the microorganism during *meta*-pathway (Kaschabek *et al*., 1998; Schmidt *et al*., 2003; Camera *et al*., 2007).

Based on the preliminary studies, *Pseudomomas chlororaphis* strain UFB2 showed high potential to degrade 2,4-DCP and was chosen as a model organism to use for the purification and characterization of phenol hydroxylase and catechol 1,2-dioxygenase enzymes. Crude cell extract of *P. chlororaphis* was prepared by growing it on MSM supplemented with 600 ppm of phenol, for the induction of the enzymes. The enzymes were purified by applying the crude cell extract to anion exchange chromatography and gel filtration chromatography. Phenol hydroxylase was successfully purified to 1.6-fold with a yield of 57%. The purified phenol hydroxylase showed the molecular weight of 61 kDa, similar to the size of phenol hydroxylase from *Rhodococcus* sp. strain FP1 reported by Duque *et al.* (2012). The optimum temperature and optimum pH for phenol hydroxylase from *Pseudomonas chlororaphis* strain UFB2 in this study was found to be 30°C and pH 8, respectively. Phenol hydroxylase from *Flavobacterium* showed an optimum pH 8.3 and temperature of 30°C (Wojcieszyńska *et al*., 2011). The Lineweaver-bluk plot fitted Michaelis and Menten equation showed the K_m and v_{max} values were 4.03 µM and 4.04 µM/min, respectively, when enzyme was incubated with phenol. Phenol hydroxylase from *Candida tropicalis* strain JH 8 showed lower K_m and v_{max} values (Long *et al*., 2014). The purified protein band was incised from 12% SDS-PAGE and trypsin digested generated peptides were analyzed on ES-MS. The data analyzed by peptide search

(version 1.16.9) showed that the protein band corresponded to enzyme 2-octaprenylphenol hydroxylase (probable protein kinase ubiB) in *Pseudomonas chlororaphis* strain UFB2. 2-octaprenylphenol hydroxylase has been implicated in *Pseudomonas aeruginosa* and *E. coli* for the degradation of aromatic compounds (Diaz *et al*., 2001). The prediction showed two models (4z2r.1.A and 2dki.1.A) that were built in relation with probable protein kinase ubiB. Model_1 (4z2r.1.A) had 19.18% sequence similarity with probable protein kinase ubiB. The protein was described as 2-hydoxybinyl-3-monooxygenase, an NADH dependent enzyme that hydroxylase 2-hydroxybiphenyl into 2,3-dihydroxybiphenyl discovered in *Pseudomonas* sp. strain HBP1 (Kohler *et al*., 1988***)***. Model_2 (2dki.1.A) had 19.53% sequence similarity with probable protein kinase ubiB. The enzyme was described as 3-hydroxybenzoate hydroxylase, an NADH dependent enzyme involved in hydroxylation of 3-hydroxybenzoate to 2,5-dihydroxybenzoate, induced in *Pseudomonas aeruginosa* (Groseclose *et al*., 1973). Biophysical properties showed that phenol hydroxylase from *Pseudomonas chlororaphis* strain UFB2 investigated in this study had a molecular weight and pI of 61 kDa and 4.93 respectively, indicating that this enzyme is a large protein and acidic in nature. The enzyme had an extinction coefficient of 77390 M^{-1} cm^{-1} and its estimated half-life in *Escherichia coli in vitro* was greater than 10 hours, suggesting that it can be stable in prokaryotic cells. The enzyme showed the instability index of 43.20 which is characterized as unstable protein. The aliphatic index of 85.54 from the enzyme, was above 70 showing that the globular protein is thermostable. Grand average of hydropathicity (GRAVY) of the protein was negative, which showed that the protein is hydrophilic.

Catechol 1,2-dioxygenase was also successfully purified from the crude extract of *Pseudomonas chlororaphis* UFB2 to homogentisate using anion exchange and gel filtration chromatography, with molecular weight of 35 kDa, a purification fold of 1.5 and a yield of 13.02%. Computational study on catechol 1,2-dioxygenase revealed that molecular weight of

catechol 1,2-dioxygenase from *Pseudomonas* spp. range between 20 and 40 kDa (Hasan *et al*., 2015). The optimum temperature and optimum pH for catechol 1,2-dioxygenase from *Pseudomonas chlororaphis* strain UFB2 characterized in this study was 30°C at pH 7.5, respectively. Catechol 1,2-dioxygenase from *Rhodococcus* sp. NCIM 2891 strain showed optimum activity at pH 7.5 and temperature 30°C (Nadaf and Ghosh, 2011). The Lineweaver-Burk plot fitted Michaelis and Menten equation showed the K_m and v_{max} were 35.76 µm and 16.67 µm/min, respectively, when enzyme was incubated with phenol. The value of K_m was higher than what has been reported in other microorganisms for catechol 1,2-dioxygenase, showing less affinity for the substrate (Nadaf and Ghosh, 2011; Guzik *et al*., 2013). The purified protein band was incised from 12% SDS-PAGE and trypsin digested generated peptides were analyzed on ES-MS. The prediction showed two models (3zds.1.A and 2uyb.1.A) that were built in relation to homogentisate 1,2-dioxygenase. Model_1 (3zds.1.A) had 81.06% sequence similarity with homogentisate 1,2-dioxygenase. This was also confirmed by the protein band that corresponded to homogentisate 1,2-dioxygenase, an intradiol enzyme that catalyses homogentisate to maleylacetoacetate. It has been reported that the ring cleavage in homogentisate is a multiple step process. The initial step is coordination of carbonyl and *ortho* phenol oxygens by Fe^{2+}, the Fe^{2+} is therefore coordinated to His335, His371 and Glu341 (Guzik *et al*, 2013). The structure shows an octahedral coordination for Fe^{2+} with two histidine residues (His331 and His367), a bidentate carboxylate ligand (Glu337), and two water molecules. Homogentisate binds as a monodentate ligand to Fe^{2+}, and its interaction with Tyr346 result in the folding of a loop over the active site, effectively shielding it from the solvent (Jeoung *et al*., 2013). Model_2 (2uyb.1.A) had 14.62% sequence similarity with homogentisate 1,2-dioxygenase. The enzyme was described as oxalate decarboxylase oxdc. Oxalate decarboxylase is a manganase and dioxygenase dependent enzyme that catalyses oxalate into carbon dioxide and formate. In *Bacillus subtilis*, the enzyme has two manganese-

binding sites (site 1 and site 2); site 1 for catalytic active site and site 2 for structural purpose (Just *et al*., 2007). The biophysical parameters calculated on ExPaSy showed the enzyme to be 314 amino acid long. The predicted molecular weight for enzyme was computed to be 34.58 kDa with an Isoelectric point (pI) of 5.18, showing the size and acidity of the protein respectively. Extinction coefficients of the enzyme was found to be 30035 M^{-1} cm^{-1} showing high number of aromatic ring amino acid in the sequence. Estimated half-life of catechol 1,2-dioxygenase was greater than 10 hours when overexpressed in *Escherichia coli*, showing it can be stable in prokaryotic cells. The aliphatic index of 77.74 from the enzyme, was above 70 showing that the globular protein is thermostable. Grand average of hydropathicity (GRAVY) of the protein was negative, which showed that the protein is hydrophilic.

5.2 Conclusion

This study showed that *Pseudomonas chlororaphis* strain UFB2, *Klebsiella pneumoniae* strain DHQP1002001 and *Klebsiella pneumoniae* strain can utilize 2,4-DCP as sole carbon and energy source. *Pseudomonas chlororaphis* strain UFB2 could degrade 75% of 2,4-DCP added in MSM after 18 days, with the degradation rate constant of 0.14 mg/d/L and growth rate of 0.05 d^{-1}. The microorganism used the *ortho*-pathway for the degradation of the compound as confirmed by the presence catechol 1,2-dioxygenase and absence of catechol 2,3-dioxygenase. The presence of phenol hydroxylase, muconate isomerase and dienelactone hydrolase encoding genes was confirmed in all the three organisms. The maleyacetate reductase gene was found to be absent from the whole genome of the isolate. Purified phenol hydroxylase and catechol 1,2-dioxygenase showed a molecular weight of 61 kDa and 35 kDa, respectively. Further biochemical studies concluded that purified enzyme showing phenol hydroxylase activity was 2-octaprenylphenol hydroxylase. The purified catechol 1,2-dioxygenase was found to be homogentisate 1,2-dioxygenase. Therefore, the enzymes involved in the degradation of 2,4-

DCP were found to be 2-octaprenylphenol hydroxylase (working as phenol hydroxylase), homogentisate 1,2-dioxygenase (working as catechol 1,2-dioxygenase), muconate isomerase and dienelactone hydrolase.

5.3 Recommendations and future work

A deeper understanding of 2,4-DCP degradation by the organisms isolated in this study will be obtained upon completion of the following additional investigations:

5.3.1 Optimization of the growth parameters for the microorganisms during the degradation of 2,4-DCP.

5.3.2 Detection of metabolite formed during 2,4-DCP degradation of the compound using Gas chromatography mass spectrometry (GC/MS).

5.3.3 Further Characterization of the other enzymes (muconate isomerase and dienelactone hydrolase) that are involved in the degradation of 2,4-DCP.

5.3.4 Immobilization and application of the enzymes for 2,4-DCP degradation.

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Appendix

Appendix A: 2,4-dichlorophenol standard curve.

Tab**le 6.1:** Standard curve for 2,4-dichlorophenol (Absorbance at 284 nm in triplicates)

Concentration (ppm)	**1**	**2**	**3**	**Average**
0	0.0001	0.0003	0.0001	0.0002
5	0.1688	0.1635	0.1599	0.1641
10	0.3312	0.3012	0.3316	0.3213
15	0.4992	0.4236	0.4897	0.4708
20	0.6789	0.7008	0.7126	0.6974
25	0.9256	0.9156	0.9237	0.9216
30	1.1002	1.1235	1.0090	1.0776
35	1.3167	1.2996	1.2897	1.3020
40	1.4236	1.4007	1.3998	1.4080
45	1.6358	1.6300	1,6298	1.6319
50	1.9562	1.9503	1,9600	1.9555

Appendix B: Chloride release assay reagents used comprised of the following reagents:

Reagent A: 0.25 M [NH4Fe(SO4)2.12H2O] in 9M nitric acid [HNO3] Ammonium Ferrous sulfate (60.25 g) dissolved in 30 mL ddH2O.

Reagent B: 2.5 g of C2HgN2S2 dissolved in 250 mL of 95% ethanol, brought up to 500 mL with 95% ethanol. Mix and filter through a filter paper and cover with foil.

Table 6.2: Chloride release standard curve (Absorbance at 460 nm in triplicates)

Concentration (µg/mL)	**1**	**2**	**3**	**Average**
0	0.0001	0.0003	0.0001	0.0002
10	0.1688	0.1635	0.1599	0.1641
20	0.3312	0.3012	0.3316	0.3213
30	0.4992	0.4236	0.4897	0.4708
40	0.6789	0.7008	0.7126	0.6974
50	0.9256	0.9156	0.9237	0.9216
60	1.1002	1.1235	1.0090	1.0776
70	1.3167	1.2996	1.2897	1.3020
80	1.4236	1.4007	1.3998	1.4080
90	1.6358	1.6300	1,6298	1.6319
100	1.9562	1.9503	1,9600	1.9555

Appendix C: Bovine Serum Albumin (BSA) Standard Curve

The standard curve for Bovine Serum Albumin (BSA) was constructed using a BSA concentration of 0-2 mg/mL. Bradford method was used to determine the protein concentration by adding a ratio of 1:50 of Bradford reagent to the sample and incubating the solution at room temperature for 5 minutes prior to reading the absorbance at 595 nm using Shimadzu UV-1800 UV-Vis Spectrophotometer. The BSA standard was used to extrapolate protein concentration in the sample.

Table 6.3: Standard Curve for Bovine Serum Albumin (BSA) (Absorbance at 595 nm in triplicates)

BSA(mg/ml)	**1**	**2**	**3**	**Average**
0	0.0001	0.0000	0.0001	0.0001
0,4	0.0659	0.0685	0.0751	0.0698
0,8	0.1233	0.2285	0.1051	0.1523
1,2	0.2967	0.1883	0.1895	0.2248
1,6	0.3336	0.3712	0.2660	0.3236
2	0.4247	0.4150	0.4205	0.4201