PURIFICATION AND CHARACTERIZATION OF CATECHOL - 1, 2 - DIOXYGENASE FROM *Acinetobacter* sp. STRAIN Y64 AND *Escherichia.coli* TRANSFORMANTS

By

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ABSTRACT

Catechol 1, 2-dioxygenase (C1, 2O) is one of the enzymes in the rate determining steps of phenol biodegradation via ortho-pathway. Characteristics such as high stereo-, regio-selectivity and broad substrate specificity of these dioxygenases have made them potential candidate for biotechnological applications. The aim of this study was to purify and characterize C1, 2O of phenol-degrading *Acinetobacter* sp. strain Y64 and to clone C1, 2O for potential biotechnological application(s). *Acinetobacter* sp. strain Y64 was capable of degrading 1000 mg/L of phenol within 16±2 hours at 30°C, 160 rpm, and pH of 7. The optimal temperature of C1, 2O was 36°C. However, two pH optima (pH 4.5 and pH 7.5) were observed for C1, 2O activity in the crude extract of strain Y64. Two C1, 2O isomers were further confirmed by genome sequencing data. One C1, 2O of 36 kDa was induced by supplementing phenol throughout the study. The 36 kDa C1, 2O was purified using ammonium sulphate precipitation and Hitrap QFF column chromatography with 49% recovery and a 10.6-fold increase in purity. The optimal pH and temperature of the purified C1, 2O from *Acinetobacter* sp. strain Y64 was pH 7.5 and 36°C, respectively. The specific activity, K_m and V_{max} of the purified enzyme were 15.1 U/mg, 17.53 µM and 1.95 U/mg, respectively. The presence of Fe^{3+}, and Fe^{2+} respectively enhanced the activity of Y64 C1, 2O, while other compounds such as Ca^{2+}, and EDTA respectively had an inhibitory effect. Eighty percentages of C1, 2O activity remained using 4–nitrocatechols as a substrate while 2% remained using 3-methylcatechol. Y64 *cat*A gene encoded C1, 2O was amplified using PCR. The PCR product was cloned into pET22b vector and expressed in *Escherichia coli* BL21DE3(pLys) after transformation. The biochemical properties and kinetics of C1, 2O purified from *Acinetobacter* sp. strain Y64 and *E. coli* transformants show no significant differences. In conclusion, C1, 2O of *Acinetobacter* sp. strain Y64 has been purified, cloned and characterized. The potential biotechnological application(s) of C1, 2O may be explored.

DECLARATION

I, **MrRidwaan Nazeer Milase**, declare that:

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Ridwaan N. Milase (209503810)

Declaration Plagiarism - 2015

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For my daughter

Ms. LESEDI XOLISA MILASE

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Excessive amounts of aromatic compounds are available in the environment following plant extracts and anthropogenic activities or by-products (Briganti *et al*., 1997; Wang *et al*., 2006). The degradation of aromatic carbon compounds takes place aerobically and anaerobically (Bouwer and Zehnder., 1993; Warhurst and Fewson, 1994; Williams and Sayer., 1994; Wang *et al*., 2006). The aromatic compounds are usually degraded in two phases. Primarily, the degradation begins with hydroxylation by either dioxygenases or monooxygenases resulting in reactive, hydroxylated intermediates like gentisate, protocatechuate or catechol (Krug *et al*., 1985; Krug and Straube 1986; Briganti *et al*., 1997; Pessione *et al*., 2001). The intermediates are further metabolized via ring fission to yield metabolites for the tricarboxylic acid (TCA) cycle (Brigantie*t al*., 1997). The literature has shown that catechol and its derivatives play a significant role in the rate determining steps for the biodegradation of phenolic compounds (Krug *et al*., 1985; Krug and Straube 1986). Therefore, enzymes involved in the oxygenation of catechol have received special attentions.

Catechol is the frequently detected metabolite and the ring fission of the compound is done by dioxygenases. Generally, catechol dioxygenases are non-heme iron proteins involved in both the oxygenation of catechol and its derivatives, followed by cleavage of the benzyl rings (Kojima *et al*., 1961; Patel *et al*., 1976; Dorn *et al*., 1978; Nakai *et al*., 1988; Miguez *et al*., 1993; Travkin *et al*., 1997; Pessione *et al*., 2001). Dioxygenases are classified into two groups which are extradiol or intradiol dioxygenases based on whether they cleave substrate on the *meta* or *ortho* cleavage site (Briganti *et al*., 1997). Catechol 1, 2 dioxygenases catalyzes ortho cleavage while 2, 3 dioxygenases are known to catalyze *meta* cleavage

(Briganti *et al*., 1997). Extradioldioxygenases make use of Fe^{2+} or Mn^{2+} for a successful cleavage outside the two hydroxyl groups of the aromatic ring to form muconic-semialdehyde (Patel *et al*., 1976; Nakai *et al*., 1988; Miguez *et al*., 1993; Travkin*et al*., 1997; Pessione *et al*., 2001). On the other hand, intradiol dioxygenases make use of Fe^{3+} and cleave between the vicinal hydroxyl groups to form muconic acid (Nakai *et al*., 1988; Miguez *et al*., 1993;Travkin *et al*., 1997). Furthermore, *cis, cis* muconic acid has been reported to play a vital role in the regulation of *cat* genes during the catabolism of catechol in the *ortho*-pathway (Collier *et al*., 1998).

1.2 REGULATION OF *cat*A AND *cat*B GENES DURING THE CATABOLISM OF CATECHOL IN DIFFERENT MICROORGANISMS

The transcriptional regulators BenM and CatM with BenR and CatR were reported to be responsible for the regulation of genes involved in phenolic compounds degradation by *Acinetobacter* sp. and *Pseudomonas* sp., respectively (Jeffrey *et al*., 1992; Collier *et al*., 1998; McFall *et al*., 1998). In response to the presence of benzoate available as carbon source, the regulator BenM stimulates the transcription of *ben*ABCDE operon and the *cat* genes for the breakdown of catechol in response to the relevant compounds benzoate and *cis, cis* muconic acid in *Acinetobacter* sp. strain ADP1 (Collier *et al*., 1998). Additional stimulation is performed by CatM (75% similar to BenM) for the transcription of *cat* genes for further breakdown of catechol followedby the accumulation of *cis, cis* muconic acid (Collier *et al*., 1998). It remains an unusual feature in this system that both BenM and CatM recognize a similar inducer; *cis, cis* muconate, and regulate similar genes like *cat*A and *cat*B (Collier *et al*., 1998). Jeffrey *et al*. (1992) reported the transcription of the *ben*ABCD operon regulated by BenR in *Pseudomonas putida* when benzoate was the available substrate. CatR in *P. putida* has been reported to induce the transcription of the *cat*BCA operon following the

accumulation of *cis, cis* muconic acid (McFall *et al*., 1998). Hence, different microorganisms have different regulatory mechanisms for *cat* genes. Many microorganisms have been found to express catechol 1, 2 dioxygenase during the degradation of phenolic compounds (McFall *et al*., 1998). Although the regulatory mechanisms of the *cat* genes encoding catechol 1, 2 dioxygenase vary with different microorganisms, catechol 1, 2 dioxygenases have general characteristics.

1.3 GENERAL CHARACTERISTICS OF CATECHOL 1, 2 DIOXYGENASES

1.3.1 Induction of catechol 1, 2 dioxygenases

The Fe^{3+} enzyme; catechol 1, 2 dioxygenases have been purified from numerous bacterial species (Aoki *et al.*, 1984a); Nakai *et al.*, 1990; Murakami *et al.*, 1991; Sauret-Ignazi *et al.*, 1996; Kim and Kwoon-Soo, 1997; Strachan *et al.*, 1998). Catechol 1, 2 dioxygenase has been reported to be inducible by various aromatic compounds in different microorganisms. Catechol 1, 2 dioxygenase can be induced in the presence of aniline in *Rhodococcus erythropolis* 1CP and *Acinetobacter lwoffi* K24, respectively (Eulberg *et al.*, 1997; Kim *et al.*, 1997), benzoate in *Pseudomonas fluorescens* (Cenci and Caldini, 1997), benzene and phenol in different strains of *Acinetobacter calcoaceticus* (Winstanley *et al.*, 1987; Ehrt *et al.*, 1995; Paller *et al.*, 1995), benzamide in *R. erythropolis* AN-13 (Murakami *et al.*, 1991), and benzyl alcohol in *R. rhodochrous* NCIMB13259 (Strachan *et al.*, 1998). Reports indicate that among non-aromatic molecules, strong induction has been observed with muconate (Pessione *et al*., 2001). However, succinate (end-product) shows strong inhibition to the expression of catechol 1, 2 dioxygenase (Neidle and Ornston, 1987). The induction of catechol 1, 2 dioxygenase from *Acinetobacter* sp. strain ADP1 specifically, has been shown to respond to the presence of benzoate and muconate as a stimulator (Neidle and Ornston, 1987). Interestingly, benzoate is a precursor and muconate is a product of C1, 2O activity in

Acinetobacter sp. strain ADP1. Hence, participation of these two structurally different compounds (benzoate and muconate) during the induction of catechol 1, 2 dioxygenase in *Acinetobacter* sp. strain ADP1 may be due to the occurrence of many mechanisms that regulate the expression of catechol 1, 2 dioxygenase (Neidle *et al*., 1987; Pession *et al*., 2001).

1.3.2 Purification of catechol 1, 2 dioxygenase

Catechol 1, 2 dioxygenase is mainly purified from various microorganisms using three purification steps (Strachan *et al*., 1998; Chuan *et al*., 2006; Wang *et al*., 2006). The cells expressing intracellular C1, 2O are lysed and the contaminating proteins are basically precipitated using ammonium persulfate. The fraction containing C1, 2O activity is harvested, desalted and directly loaded to an affinity column and finally, size exclusion column (Briganti *et al*., 1997; Vijay *et al*., 2011). However, there are reports where the elimination of some protein purification steps has led to a successful purification of C1, 2O from some microorganisms (Briganti *et al*., 1997; Nadaf and Ghosh *et al*., 2011). A study conducted by Briganti *et al*. (1997) on the purification of catechol 1, 2 dioxygenase from *Acinetobacter radioresistens* was done by directly loading the crude extract of *Acinetobacter radioresistens* into DE52 cellulose column and finally Q Sepharose high performance column. Briganti *et al*. (1997) obtained pure C1, 2O with a specific activity of 24.5 U/mg after enriching 15.0 folds with a yield of 54%. Vijay *et al.* (2011) reported that hydrophobic interaction chromatography showed no efficiency in removing impurities during the purification of catechol 1, 2 dioxygenase. Difficulties in removing the impurities during the purification process led to gel permeation chromatography which increased the purity of catechol 1, 2 dioxygenase significantly.

Studies conducted by Nadaf and Ghosh *et al*. (2011) on the purification of catechol 1, 2 dioxygenase from *Rhodococcus* sp. NCIM 2891 used a one step purification procedure. The

C1, 2O of *Rhodococcus* sp. NCIM2891 was purified using only DEAE – Cellulose chromatography. C1, 2O purified from *Rhodococcus* sp. NCIM 2891 had a specific activity of 1.83 U/mg of protein after 2 folds of enrichment with a yield of 45%.

1.3.3 Effect of temperature and pH on the activity of catechol 1, 2 dioxygenase

The structure and characteristics of C1, 2Os from the genus *Arthrobacter*, *Geobacillus*, *Pseudomonas*, *Rhodococcus* and *Sphingomonas* (Strachan *et al*., 1998; Saxena and Thakur 2005; Giedraityte and Kalediene 2009) has been described. Most of these enzymes have a molecular mass of 30.5 – 36 kDa and consist of two identical or unidentical subunits (Kojima *et al*., 1961; Patel *et al*., 1976; Nakai *et al*., 1988; Miguez *et al*., 1993; Vijay *et al*., 2011). The largest molecular mass of C1, 2O reported in the literature is 109 kDa (Varga *et al*., 1970). While the smallest molecular mass reported is 22 kDa and the enzyme was a monomer (Guzik *et al*., 2011b).

The activity of catechol 1, 2 dioxygenase isolated from various microorganisms is highly affected by temperature and pH. A study by Guzik *et al*. (2011a) showed C1, 2O purified from *Stenotrophomonas. maltophilia* KB2 to have a maximum activity (3.062 U/mg of protein) at pH 8 and 40°C with a half-life of 3 hours. C1, 2O was reported to have lost 16.5% activity at 50°C after 20 minutes (Guzik *et al*., 2011a). A rapid decline on the activity of catechol 1, 2 dioxygenase was further observed at 55°C. Similar enzyme stability patterns were reported for C1, 2O purified from *Arthrobacter* sp. BA-5-17 and *P. aeruginosa*, respectively (Murakami *et al.*, 1998; Wang *et al*., 2006).

The activity of C1, 2Os purified from various microorganisms is generally stable at neutral pH and affected by strong acidic and strong basic pH (Nadaf and Ghosh, 2011; Saxena and Thakur 2005; Wang *et al*. 2006). On a contrary, a study conducted by An *et al*. (2000) showed that the C1, 2O from *Streptomyces setonii* is active in a broad temperature range (25-

65°C) and function optimally at 45°C. Similar to other C1, 2Os, this enzyme displayed optimum activity at a neutral pH (An *et al*., 2000).

1.3.4 Kinetic properties of catechol 1, 2 dioxygenase

The kinetic properties of C1, 2O vary significantly depending on the microorganism from which the enzyme originates from. The K_m values for catechol cleavage by C1, 2O purified from various microorganisms of the genus *Acinetobacter*, *Rhodococcus* and *Pseudomonas* range between 1 and 12 µM (Briganti *et al*., 1997; Chuan *et al*., 2006; Suvorova *et al*., 2006; Solyanikova *et al*., 2009; Vijay *et al*., 2011; Nadaf and Ghosh *et al*., 2011). Similarly, the V_{max} values of catechol cleavage by C1, 2Os isolated from different microorganisms vary (Briganti *et al*., 1997; Suvorova *et al*., 2006; Solyanikova *et al*., 2009; Chuan *et al*., 2006; Vijay *et al*., 2011; Nadaf and Ghosh *et al*., 2011; Guzik *et al*., 2011a). The smallest K_m value for catechol cleavage reported in the literature is 0.0011 mM for C1, 2O purified from *Rhodococcus rhodochrous* NCIMB 13259 strain (Strachan *et al*., 1998). Hence, C1, 2O purified from *R. rhodochrous* NCIMB 13259 strain has the highest reported affinity for catechol.

The K_m and V_{max} parameters for catechol cleavage at 35°C by catechol 1, 2 dioxygenase isolated from *S. maltophilia* KB2 were estimated to be 12.18 µM and 1 218.8 U/mg of protein, respectively (Guzik *et al*., 2011a). The V_{max} value of C1, 2O purified from *S.maltophilia* KB2 is approximately 20 – 100 folds higher in contrast to the other catechol 1, 2 dioxygenases reported in the literature (Guzik *et al*., 2011a). In a study conducted by Briganti *et al*. (2000) the V_{max} value of catechol 1, 2 dioxygenase from *Acinetobacter radioresistens* was 25.8 U/mg of protein. The V_{max} of catechol cleavage by C1, 2O isolated from *R.opacus* strains1 CP and 6a were 9.6 U/mg and 55.5 U/mg of protein, respectively

(Suvorova *et al*., 2006; Solyanikova *et al*., 2009). The comparison shows the K_m value obtained by Guzik *et al*. (2011a) to be 2 folds higher than those obtained by Wang *et al*. (2006) and Nadaf and Ghosh *et al.* (2011). Hence, C1, 2O isolated from *S.maltophilia* has low affinity for catechol. Substrate inhibition of catechol 1, 2 dioxygenase from *S. maltophilia* KB2 at >80 µM was observed (Guzik *et al*., 2011a). Similar substrate inhibition observations were made by Sauret-Ignazi *et al*. (1996), where inhibition activity was observed for *Alicaligenes eutrophus* CH34 1, 2 dioxygenase that specifically catalyzes the degradation of tetrachlorocatechol.

1.3.5 Substrate specificity of catechol 1, 2 dioxygenases

Catechol 1, 2 dioxygenases isolated from various microorganisms have been reported to possess relaxed substrate activity (Strachan *et al*., 1998; Ridder *et al*., 1998; Wang *et al*., 2006). Some microorganisms have been shown to utilize both halo- and methyl-catechols as a sole carbon source (Strachan *et al*., 1998; Ridder *et al*., 1998; Wang *et al*., 2006). However, the type of substrate cleavage depends on the micoroorgansism; *meta* cleavage of catechol was reported for C1, 2O purified from *Rhodococcus*, *Pseudomonas arvill* and *Ralstonia* sp. when 3-methoxy and 3-methylcatechols was used as substrates (Fujiwara *et al*., 1975; Dorn *et al*., 1978). Catechol 1, 2 dioxygenase purified from various microorganisms has shown > 50% relative activity when methoxy-substituted catechols and nitrocatechols, respectively are used as a substrate (Fujiwara *et al*., 1975; Dorn *et al*., 1978; Briganti *et al*., 1997; Wang *et al*., 2006; Vijay *et al*., 2011). However, there is a variation on the activity of C1, 2O depending on species) when methyl catechol is used as a substrate (Fujiwara *et al*., 1975; Vijay *et al*., 2011; Wang *et al*., 2006; van Duuren *et al*., 2011c). Usually, C1, 2O has been reported to show higher relative activity when pyrogallol (170 – 180%) is used as a substrate instead of catechol (Fujiwara *et al*., 1975; Vijay *et al*., 2011; Wang *et al*., 2006; van Duuren *et al*.,

2011a). Hence, there are similarities and variations on the specificities of C1, 2Os to other substrates as opposed to catechol.

1.4 APPLICATION OF CATECHOL 1, 2 DIOXYGENASES

General characteristics of catechol 1, 2 dioxygenases highlights the significance of catechol 1, 2 dioxygenases during the degradation of phenolic compounds via the *ortho* pathway. Furthermore, the purification of active catechol 1, 2 dioxygenases from various microorganisms suggest that the enzyme can be exploited for biotechnological application(s).

A few successful studies have been done on cloning and expression of C1, 2Os (Neidle *et al*., 1987; An *et al*., 2001). However, the cloning of *cat*A genes in relevant biochemical pathways to optimize the production of *cis, cis* muconic acid has been successful (Frost and Draths, 1995; 1996; 1997; Niu *et al*., 2002).

Catechol 1, 2 dioxygenases were recently employed in the synthesis of *cis, cis* muconic acid an essential precursor of adipic acid (Ran *et al*., 2008).

1.4.1 Relation of *cis, cis* muconic acid and catechol 1, 2 dioxygenases

Research which intends to find enzymes with biotechnological application(s); specifically chemical synthesis for industrial scale has been of importance for the past few decades. Enzymes with biotechnological importance have further gained interest following their role in the production of stereoisomers; as such enzymes exhibit stereoselectivity and regioselectivity (Ran *et al*., 2008).

Numerous studies have reported the microbial production of *cis, cis* muconic acid from benzene, benzoic acid, catechol or toluene (Bang and Choi 1995; Bang *et al*., 1996; Frost and Draths, 1996; 1997). Mizuno and Yoshikawa, (1990) reported a mutant strain of *Arthrobacter* sp. capable of producing up to 44 g/L of *cis, cis* muconic acid in a period of two days in batch

culture. Catechol 1, 2 dioxygenase is the key enzyme during the biosynthesis of *cis, cis* muconate; thus, finding highly active C1, 2Os improve and enhance the productivity of *cis, cis* muconic acid (Kim *et al*., 1998; Wu *et al*., 2006). The formation of *cis, cis* muconic acid has been reported to be highly dependent on substrate concentration.

Guzik *et al*., (2011a) estimated the molar conversion yield based on the amount of catechol consumed theoretically at a value of 100% (mol/mol). Kaneko *et al*. (2011) reported similar results for the production of *cis, cis* muconic acid using recombinant *cat*A derived from *Pseudomonas putida* mt-2.

1.4.2 Production and biotechnological perspective of muconic acid

Global pressures to reduce petroleum footprints have encouraged the research for alternative, renewable techniques for the production of almost all specialty chemicals (Curran *et al*., 2013). Thus far the biotechnological field with its molecular techniques and metabolic engineering has started to attend to this demand. The attempt has been via metabolically engineering organisms to excessively produce the chemicals in demand (Lee *et al*., 2011; 2012; Curran and Alper, 2012; de Jong *et al*., 2012; Jang *et al*., 2012). Particularly, the manufacture of bio-plastics has gained interest as indicated by the metabolic engineering of microbial strains with the interest to produce precursors. The produced precursors include, styrene, ethylene glycol (from bio-ethanol), succinic acid, 1,4-butanediol, r-hydroxy-styrene, 1,3-propanediol, polyhydroxyalkanoates and polylactides (Yumoto *et al*., 1995; Stols *et al*., 1997; Antoniewicz *et al*., 2007; Qi *et al*., 2007; Ikushima *et al*., 2009; Li *et al*., 2010; McKenna and Nielsen, 2011; Yim *et al.,* 2011; Zhou *et al*., 2005; 2011).

Muconic acid is another compound which has gained much attention as a precursor and a chemical platform for the generation of numerous bio-plastics (Mirasol, 2011). The

application of a hydrogenation process on muconic acid can easily lead to the formation of adipic acid (Mirasol, 2011). Additionally, muconic acid may go through a process of Diels–Alder reaction with the use of acetylene and successive oxidations to form terephthalic acid, which is one of the fundamental components in the plastic polyethylene terephthalate (Burridge, 2011) and it can be utilized during the manufacture of polyester (Burridge, 2011; Mirasol, 2011). The net production of terephthalic acid and adipic acid annually internationally is said to be over 71 and 2.8 million tones, respectively (Burridge, 2011; Mirasol, 2011). Despite the numerous environmental problems associated with the use of non-renewable petroleum based feedstock. It is unfortunate that muconic acid and adipic acid are currently produced primarily from toxic commodities and from non-renewable petroleum based feedstock (Burridge, 2011; Mirasol, 2011). This therefore calls for a biosynthetic and sustainable production platform.

No organism has been reported to endogenously produce muconic acid from carbohydrates. Conversely, muconic acid may be produced during the breakdown and detoxification of phenolic compounds by various microorganisms including yeast such as *Candida* sp., and bacterial species in the genera *Acinetobacter*, *Sphingobacterium*, and *Rhodococcus*, amongst others (Warhurst and Fewson, 1994; Wu *et al*., 2004; Tsai *et al*., 2005). Drath and Frost (1995) metabolically engineered a recombinant *E. coli* for the production of muconic acid from glucose (Figure 1.1) (Warhurst and Fewson, 1994; Wu *et al*., 2004; Tsai *et al*., 2005). The engineered recombinant *E. coli* strains possessed a heterologous synthetic pathway designed to draw from the natural intermediate in the shikimate pathway; 3-dehydroshikimate (DHS) to optimize the production of *cis, cis* muconic acid (Frost and Draths, 1995; Niu *et al*., 2002). The synthetic pathway operated in the manner where DHS is converted by DHS dehydratase (originally from *Klebsiella pneumoniae*) to protoctechuric acid (PCA).

Protoctechuric acid is then converted by PCA decarboxylase to catechol which is then acted upon by catechol 1, 2 dioxygenase (originally from *Acinetobacter bayiyi*) to *cis, cis* muconic acid (Frost and Draths, 1995; Niu *et al*., 2002). Following slight modifications, the synthetic pathway was successful in producing significant amounts of muconic acid (17 mM muconic acid) in supernatant during batch fermentation with 0.3 mol glucose as the sole carbon source in the *E. coli* strain (Frost and Draths, 1995). Niu *et al*. (2002) incorporated two copies of *aroZ* genes into the same *E. coli* chromosomal DNA. Following the incorporation of *aroZ* genes, *cat*A and *aro*Y were expressed in the plasmid (Niu *et al.,* 2002). This modification drastically increased the production of muconic acid to 260 mM after 48 hours in fed-batch conditions (Niu *et al*., 2002). After the removal of soluble protein and cells, hydrogenation of *cis, cis* muconic acid with Pt on carbon yielded 97% (mol/mol) formation of adipic acid at high pressure (3400 kPa) under ambient temperature for 2 and a half hours (Niu *et al*., 2002).

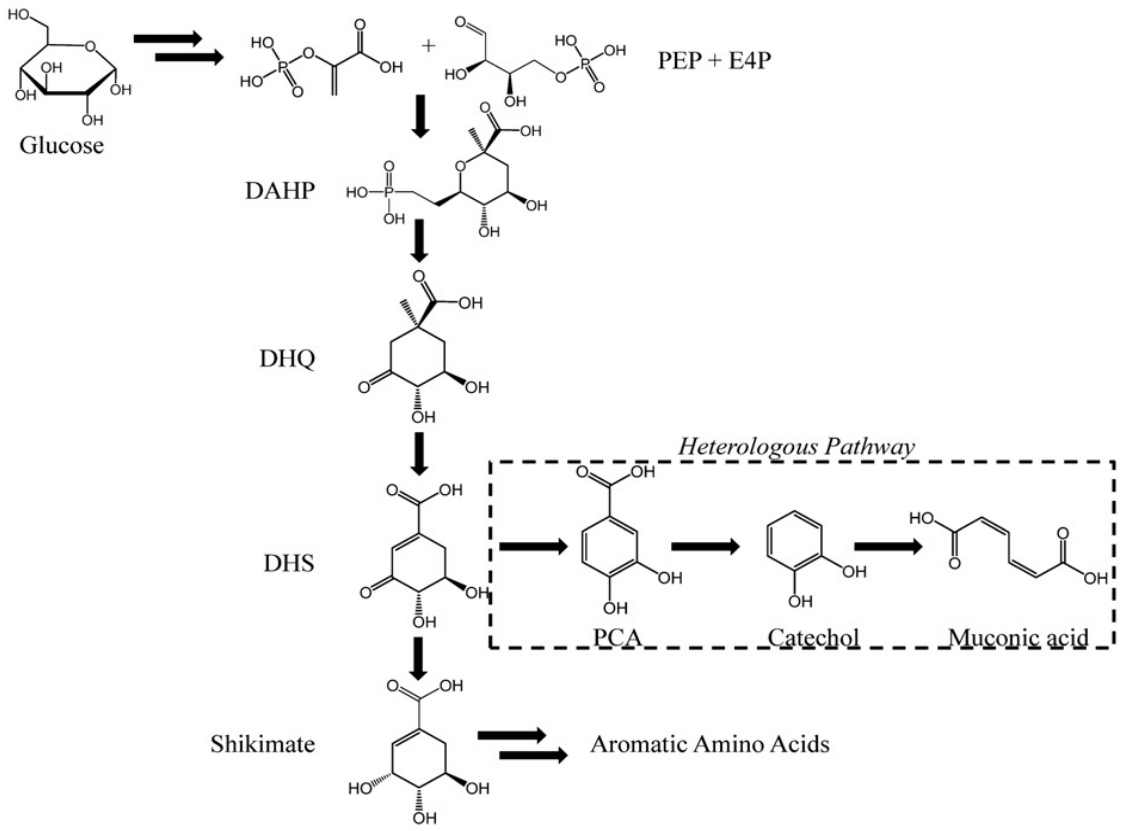

Figure 1.1 Composite heterologous pathway for muconic acid production. The synthetic pathway for muconic acid is depicted in the context of the shikimate pathway in yeast. The following metabolite abbreviations are used: PEP: phosphoenolpyruvate, E4P:erythrose-4-phosphate, DAHP: 3-deoxy-D-arabinoheptulosonate-7-phosphate, DHQ: dehydroquinate, DHS: dehydroshikimate, and PCA: protocatechuic acid (Frost and Draths, 1995; Niu *et al*., 2002).

1.4.3 Utilization of benzoate for the production of *cis, cis* muconic acid

The *ortho*-degradation pathway of phenol serves as an applicable pathway for the production of *cis, cis* muconic acid. The pathway proceeds via *cis, cis* muconic acid to succinyl-CoA within the ketoadipate pathway (Polen *et al*., 2013). The ketoadipate pathway is one of the major aerobic biodegradation pathways in bacteria dominating the soil habitat. Depending on bacteria species, the biodegradation pathway may either involve protocatechuate branch or catechol branch (Harwood and Parales, 1996). Primarily, species of the genera *Acinetobacter*, *Pseudomonas*, *Burkholderia* and *Rhodococcus* have been studied intensively for the corresponding genes, their gene expression and organization, the enzyme activities and

encoded enzymes which have been analyzed and characterized (Neidle *et al*., 1987; Jeffrey *et al*., 1992; Collier *et al*., 1998; McFall *et al*., 1998; Kitagawa *et al*., 2001; Takenaka *et al*., 2005; Denef *et al*., 2006; Yoon *et al*., 2007; Cao *et al*., 2008; Moreno and Rojo, 2008; Zhan *et al*., 2008). Relevant studies have indicated that the assimilation of benzoate and other derivatives into the cell is facilitated by transport systems (Nichols and Harwood, 1997; Clark *et al*., 2002; Chaudhry *et al.,* 2007; Nishikawa *et al*., 2008; Ledger *et al*., 2009). In the cell, catechol is a major intermediate within the *ortho* pathway and acted upon by catechol 1, 2 dioxygenase to form *cis, cis* mucnic acid (Figure 1.2). Muconate cycloisomerase acts on *cis, cis* muconic acid to form muconolactone and further broken down to acetyl-CoA and succinyl-CoA (Figure1.2).

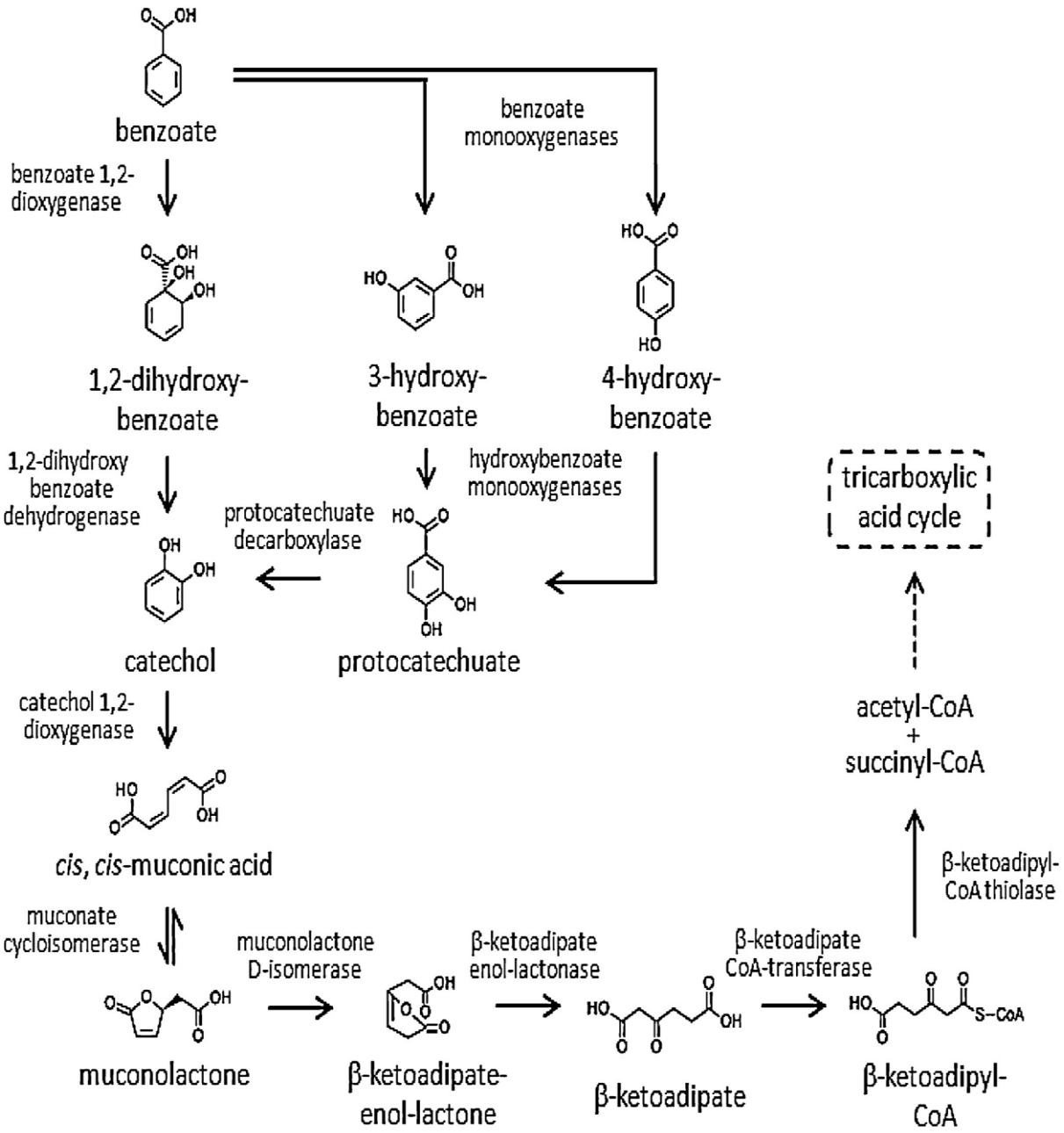

Figure 1.2 Metabolites and enzymes associated with the dissimilation of benzoate via the ortho-cleavage pathway of catechol, which proceeds via *cis, cis* muconic acid to succinyl-CoA and acetyl-CoA in the ketoadipate pathway (Polen *et al*., 2013).

To produce *cis, cis* muconic acid, benzoate is acted upon by a multicomponent system made up of benzoate 1, 2 dioxygenase, NADH-cytochrome c reductase, an iron-sulfur-containing terminal oxygenase and by 1, 2- dihydroxybenzoate dehydrogenase in the catechol branch (Polen *et al*., 2013).

In the protocatechuate pathway, benzoate is acted upon by benzoate monooxygenases to form 3- or 4-hydroxybenzoate; these intermediates are further altered by 3- or 4-hydroxybenzoate monooxygenases to form protecatechuate. Protocatechuate is converted to catechol by protecatechuate decarboxylase. Catechol is the essential intermediate and acted upon by catechol 1, 2 dioxygenases to form *cis*, *cis* muconic acid during the *ortho*-cleavage of the aromatic C6-ring (Polen *et al*., 2013).

The absence of muconate cycloisomerase results in the accumulation of *cis*, *cis* muconic acid (Polen *et al*., 2013). Likewise in the presence of muconatecycloisomerase, muconolactone is formed and converted to ketoadipate by muconolactone D-isomerase and ketoadipateenol-lactonase (Polen *et al*., 2013). Ketoadipate is transformed by ketoadipate CoA-transferase to ketoadipyl CoA (Polen *et al*., 2013). This CoA-activated intermediate is then converted to acetyl-CoA and succinyl-CoA by ketoadipyl-CoA thiolase (Polen *et al*., 2013).

Some microorganisms have been reported with the ability to produce the compound *cis, cis* muconic acid via fermentation (Choi *et al*., 1997; Polen *et al*., 2013). It was reported that *Pseudomonas* sp. B13 used for a 1 L minimal medium supplemented with 60 mM succinate in a controlled bioreactor could convert benzoate to *cis, cis* muconic acid (Polen *et al*., 2013). The addition of benzoate was done successively with concentrations ranging from 5 – 10 mM in growth medium (Polen *et al*., 2013). This resulted in a 91% yield (mol *cis, cis*-muconate/mol benzoate) of *cis, cis* muconic acid (Schmidt and Knackmuss, 1984). *Pseudomonas* sp. B13 was also reported to produce no muconatecycloisomerases as a response to *cis, cis* muconic acid titre reaching 52 mM (Schmidt and Knackmuss, 1984). Choi *et al.* (1997) reported the enhanced accumulation of *cis, cis* muconic acid via biocatalytic conversion from benzoate to be dependent on high cell density culture of a mutant *P. putida* strain BM014 within a cell-recycle bioreactor operated in fed-batch mode. In this study they reported a productivity of 38.7 mM/L/hour and a *cis, cis* muconic acid titre

of 95 mM within the culture broth (Choi *et al*., 1997). Product formation and optimum rate of cell growth was found to be positive with phosphate limitation, dissolved oxygen concentrations at least 20% saturation and cobalt supplementation (Polen *et al*., 2013). A strategy for controlling the oxygen supply to obtain high cell densities was applied, and gave a high *cis, cis* muconic acid titre of more than 225 mM (Bang and Choi, 1995). This strategy made use of a fed-batch process with automated dissolved oxygen-stat feeding (Bang and Choi, 1995). A microorganism of the genus *Arthrobacter* was devoid of muconatecycloisomerase activity via ultraviolet-irradiation (Mizuno *et al*., 1988). The mutant showed the capability to produce about 310 mM *cis, cis* muconic acid within 48 hours in a 30 L fermentation jar, these results were obtained by successive addition of small amounts of benzoate into the bioreactor (Mizuno *et al*., 1988).

van Duuren *et al*. (2011b) transformed *P*. *putida* KT2440 using random mutagenesis with N-methyl-N-nitro-N-nitrosoguanidine and 3-fluorobenzoate. The transformed strain (*P. putida* KT2440-JD1) and the parent strain (*P*. *putida* KT2440) were unable to grow on benzoate acting as a sole carbon source. However the KT2440-JD1 was able to co-metabolize benzoate to *cis, cis* muconic acid and yield 89% (mol *cis, cis*-muconate/molbenzoate) in the presence of glucose. The strain demonstrated a growth rate of 0.2/hour in continuous culture, moreover a specific *cis, cis* muconic acid production rate of around 1.3 mM/g dry cell weight/ hour and increases to 10 mM/g/h throughout the wash out propagation at a dilution rate of 1.3 /hour (μ_{max} = 0.6/hour). This was an indication that the *cis, cis* muconic acid productivity from *P. putida* KT2440-JD1 was reasonably higher under μ_{max} conditions (Myers *et al*., 2001; van Duuren *et al*., 2011b). This has been the highest reported production rate (eight times higher) in contrast to those reported for former *cis, cis* muconic acid generating strains (van Duuren *et al*., 2011b).

Using the fundamental tools of proteomics and transcriptomics it was discovered that the *cat*BCA operon in *P. putida* KT2440-JD1 showed mute response to the presence of 5 mM benzoate (van Duuren *et al*., 2011b, Polen *et al*., 2013). Furthermore, DNA sequencing of *cat*BCA-*cat*R region showed a single point mutation within the conserved DNA-binding domain of the transcriptional regulator CatR. It was postulated that the mutation on the transcriptional region inhibited every gene of the *cat*BCA operon (van Duuren *et al*., 2011b; Polen *et al*., 2013). The mutant and the parental strain showed significant expression levels for the ben-operon in the presence of benzoate. The strain was still capable of converting catechol to *cis, cis* muconic acid given that the operon contained *cat*A2 gene (encoding another catechol 1, 2 dioxygenase apart from *cat*A) (van Duuren *et al*., 2011b, Polen *et al*., 2013). Further work done on *P. putida* KT2440-DJ-1 included a fed-batch process where benzoate was added with acidification of the medium. The medium was continuously acidified during the conversion of *cis, cis* muconate. This further prevented undesired accumulation of benzoate via optimized feeding (van Duuren *et al*., 2011b; Polen *et al*., 2013). The results showed up to 130 mM *cis, cis* muconic acid accumulation in the medium under these conditions. This was equivalent to a molar product yield of close to 100% when *P. putida* KT2440-JD1 was propagated solely on glucose (van Duuren *et al*., 2011b).

To stabilize the pH which is coupled to the pH decrease occurring during benzoate conversion to *cis, cis* muconic acid in a fed-batch process, pH stat fed-batch process is a considerable option (van Duuren *et al*., 2011a, Polen *et al*., 2013). Conversely, similar to the process where chemicals are utilized for the production of adipic acid, bioconversion of petroleum-based benzoate to *cis, cis* muconic acid does not deal with the problem of toxic starting material, nor does it limit the utilization of petroleum-based feedstock in the long run (Polen *et al*., 2013). Perhaps a possible alternative might be to consider the utilization of bio-based benzoate or other closely related compounds like catechol, phenol, 4 hydroxybenzoic

acid and protocatechuric acid (Polen *et al*., 2013). The alternative compounds may be generated by either the degradation of lignocellulosic material by anintricate microbiota consisting of prokaryotes like *Actinomycetes*, eukaryotes or by technical solubilisation and aqueous phase restructuring of lignin material (Trigo and Ball, 1994; van Duuren *et al*., 2011a; Zakzeski and Weckhuysen, 2011).

The use of yeast as a new production biocatalyst in industrial biotechnology has gained much attention. Usually yeast, specifically *Saccharomycetes cerevisiae* has advantages such as the tolerance of low temperatures, completed phage-free processes, easier separations, sustain ability in large-scale fermentation processes, and high tolerance to lower pH ranges in general (Curran *et al*., 2013). These advantages have made *S. cerevisiae* a potential candidate for the production of heterologous models that make use of precursors such as vanillin, naringenin, r-hydroxycinnamic acid, r-amino benzoic acid, r-hydroxybenzonic acid, and resveratrol in aromatic amino acid and shikimate pathways (Jiang *et al*., 2005; Vannelli *et al*., 2007; Hansen *et al*., 2009; Naesby *et al*., 2009; Wang *et al*., 2011; Krömer *et al*., 2012; Wang and Yu, 2012).

Curran *et al*. (2012) exploited the similar advantages of *S. cerevisiae* for the generation of *cis, cis* muconic acid using the same system with slight modification. The significant feature of *S. cerevisiae* include that; *S. cerevisiae* naturally tolerates lower pH conditions than *E. coli* which is a preferred condition for the production of *cis, cis* muconic acid (Curran *et al*., 2013).

Unfortunately, aromatic amino acid based production in yeast showed significant metabolic engineering challenges (Curran *et al.,* 2013). However, the use of *E. coli* for amino acid based production may overcome metabolic engineering challenges by simple modifications such as alleviation of feedback inhibition in *E. coli*. Allevaition of feedback inhibition in *E. coli* may possibly lead to an increase in grams per litre quantities of aromatic amino acids and

their relative products (Patnaik and Liao, 1994; Berry *et al*., 2002; Lutke-Eversloh and Stephanopoulos, 2007). Hence, muconic acid levels can be increased in *E. coli* (Frost and Draths, 1995; Niu *et al*., 2002). The unsuccessful abilities to achieve greater production levels from logical metabolic engineering methods in *S. cerevisiae* implies that the flux in the aromatic amino acid and shikimate pathways is highly regulated possibly via both the local and global transcriptional machinery (Alper *et al*., 2006; Alper and Stephanopoulos, 2007). An inclusive omics study on the production of amino acids using yeast as biocatalyst showed significant transcriptional and allosteric regulation within the numerous amino acid pathways, moderately controlled by factors like Gcn4P (Curran *et al*., 2013). More studies using improved *S. cerevisiae* strains may assist in finding similar regulatory proteins responsible for regulating the overall flux through the shikimate pathway. Identification of these targets may be useful in application of techniques like global transcriptional machinery engineering (gTME) (Alper *et al*., 2006; Alper and Stephanopoulos, 2007) to increase the titre and yield in *S. cerevisiae*.

Santos *et al*. (2012) the application of gTME using global regulatory factors could improve the production of L-tyrosine in *E. coli*. Similar strategies are thought to assist in making yeast a potential platform for shikimate-based molecules, especially when providing processing advantages and tolerance at low pH levels over *E. coli* (Curran *et al*., 2013). The specificity of catechol 1, 2 dioxygenases and its corresponding genes likewise promise alternatives to the synthesis of adipic acid the precursor of nylon 6, 6 polyamide, a chemical established in the 1930s.

1.4.4 Synthesis and demand of adipic acid

The dioxygenases have catalytic characteristics which have drawn so much attention for regioselective and stereoselective addition of oxygen in a great range of organic substrates

(Ran *et al*., 2008). These processes shall allow the production of compounds which are unattainable via chemical convention methods (Gibson *et al*., 2000). Furthermore, other potential biotechnological applications of phenolic compound–degrading organisms and their constituent enzymes may include their incorporation into diagnostic systems, their uses in bioreactor systems to remove toxic pollutants and as biosensors (Eskinjia *et al*., 1995; Ali *et al*., 1998; Nakajima *et al*., 2002).

The compound *cis*, *cis* muconic acid is one of the major metabolites essential for the synthesis of benzene-free adipic acid (Kuwahara *et al*., 1977; Gomi *et al*., 1988; Mizuno and Yoshikawa, 1990). This process promises to be an undeniable environmentally friendly alternative in contrast to chemical conventional process currently used for the production of nylon (Niu *et al*., 2002). Adipic acid also known as hexaneliolic acid is an industrial important aliphatic dicarboxylic acid. The chemical is odourless and colourless with an acidic taste (Musser, 2005). The chemical is mainly required for the manufacture of nylon 6, 6, bactericide, insecticide and polyurethane (Yuan *et al*., 2004). The primary importance of adipic acid is during chemical production of nylon-6, 6 polyamide established during the 1930s by W.H. Carothers of DuPont (Luedeke, 1977). The international production of adipic acid has reported to be 2.6 million tons (equivalent to 2 358.6 million kg) annually during the year 2010 (Merchant Research and Consulting Ltd, 2011). Furthermore the figures are expected to increase to at least 3.3 million tons annually in 2016 (Merchant Research and Consulting Ltd, 2011). The amount of adipic acid utilized for the synthesis of nylon 6, 6 fibres and other resins accounts for about 65% of the total production. In addition adipic acid is utilized as one of the reactants in the synthesis of plasticizers, lubricant components and for the production of polyurethanes (Merchant Research and Consulting Ltd., 2011). Adipic acid is also used for foods that require acidulation (gelatins and desserts) (Merchant Research and Consulting Ltd., 2011).

Large scale chemical processes for the production of adipic acid have been created (Polen *et al*., 2013). Almost all successful processes for commercial production of adipic acid have been derived from benzene. The chemical process consists of benzene being reduced to cyclohexane which is further converted into a cyclohexanol and cyclohexanone mixture (Polen *et al*., 2013). The conversion makes use of two successive oxidations. The produced mixture is further reacted with air and nitric acid in the presence of copper or vanadium as a catalyst to produce adipic acid (Musser *et al*., 2005). Benzene is a volatile carcinogen, and other diseases like acute myeloid leukemia and malignant hematopoietic disease are highly linked to exposure to benzene (Galbraith *et al*., 2010). Another method used for the production of adipic acid is via the hydrocyanation of butadiene, then hydroisomerization to form adiponitrile which is further hydrolyzed to adipic acid.

Nitrous oxide is produced in abundance as a by-product during the oxidation of cyclohexanol and cyclohexanone mixture leading to a major environmental concern (Alini *et al*., 2007). Literature has shown nitrous oxide as an active gas which contributes directly to ozone depletion and global warming (Alini *et al*., 2007). Alini *et al.* (2007) has estimated at least 10% of emitted nitrous oxide to be from industrial processes, specifically during the production of adipic acid. Hence, alternatives or another environmentally friendly process is required for the production of adipic acid (Shimizu *et al*., 2000; Alini *et al*., 2007). A potential green alternative route to the production of adipic acid with nitric acid would be the direct cyclohexene oxidation with 30% H_2O_2 in organic solvent and halide free conditions (Sato *et al*., 1998; Blach *et al*., 2010). Nevertheless, the required materials to begin the adipic acid chemical synthesis are obtained from scarce non-renewable potentially harmful fossil fuels with high harmful potentials (Thiemens *et al*., 1991; Weber *et al*., 2012). On the other hand adipic acid has a very low toxicity (Kennedy, 2002).

Currently, clean technologies in industrial biotechnology are promising as keys to economic developments. The strategies used in these platforms provide production of pure chemicals in a more environmentally friendly and energy efficient manner from biorenewable resources (Cooke, 2008; Kircher, 2006). Despite the two markets built by the provision of biorenewables via green technology and the processing by white biotechnology, the oil-based chemistry has been leading the economic competition (Kircher, 2006). This follows the recognition of ecological benefits of white biotechnology by the market only when they provide a cost advantage (Kircher, 2006). Adipic acid obtained from renewable carbon sources was predicted to give a cost advantage of about 20-30% depending on the feedstock in a long term (Kircher, 2006). This turns out to be even advantageous when compared to petrochemical-based adipic acid production (De Guzman, 2010).

1.4.5 Adipic acid in cellular metabolism

Adipic acid is found naturally in juice of red beet and sugar beet. Furthermore adipic acid in cellular metabolism is found as an intermediate within the cyclohexane, cyclohexanone or cyclohexanol degradation pathways (Ulrich 1988; Weber *et al*., 2012). Adipic acid was found as an intermediate in some *Pseudomonas* strains that degrade caprolactam (Cheng *et al*., 2002; Steffensen and Alexander, 1995). Ideally, these degradation pathways form a common intermediate, adipate semialdehyde, which is oxidized by 6-oxo-hexanoate dehaydrogenase to form adipic acid. Iwaki *et al.* (1999) reported that 6-oxo-hexanoate dehydrogenase functions as a coenzyme in the presence of $NADP^+$ in *Acinetobacter* sp.

The study on the degradation pathway of cyclohexanol and cyclohexanone has been essential for decades (Norris and Trudgill, 1971; Donoghue and Trudgill, 1975; Cheng *et al*., 2002). Screening for genes and enzymes involved in the degradation of cyclohexanone and cyclohexanol from numerous species showed enzymes in gene clusters to be more efficacious;

these include cyclohexanol dehydrogenases, monooxygenases, caprolactone hydrolase, and 6-hydroxyhexanoate dehydrogenases (Brzostowicz *et al*., 2000; 2002; 2003; 2005; Cheng *et al*., 2000). Brzostowics *et al.* (2003) detected traces of adipic acid in *E. coli* transformants expressing distinct gene clusters from *Acinetobacter* sp., *Rhodococcus* sp. and, or *Arthrobater* sp. The detected traces of adipic acid were indicative of specific degradation of cyclohexanone and cyclohexanol. Furthermore, it was postulated that the high levels of adipic acid accumulation in *E. coli* than in the native *Arthrobacter* sp., was due to the *E. coli* strain being unable to further metabolize adipic acid (Brzostowics *et al*., 2003). Brzostowics *et al*., (2003) reported the conversion rates and adipic acid levels to be essentially low for production in *Arthrobacter* sp. (Polen *et al*., 2013).

Metabolic pathways for adipic acid biosynthesis which begin with intermediates of the central carbon metabolism have likewise been investigated as reported in the literature (Niu *et al*., 2002). The metabolic pathways involved in the biosynthesis of adipic acid have led to the formation of adipic acid as an intermediate. However, the pathways involved in the biodegradation of phenolic compounds failed to do so. Conversely, adipic acid could also be obtained by chemo-catalytic conversion of abio–based precursors, glucaric acid and *cis, cis* muconic acid. Glucaric acid or *cis, cis* muconic acid may be processed via hydrogenation in the presence of nanoparticles (Pt) on carbon as catalysts (De Guzman, 2010; Niu *et al*., 2002; Thomas *et al*., 2003). Renewables–based adipic acid production appears possible from combining biotechnological process for fermentative production of a bio-based precursor and an environmentally conscious chemo-catalytic process to convert the precursor to adipic acid (Figure 1.3).

The compound *cis, cis* muconic acid is a dicarboxylic acid consisting of two conjugated double bonds formed as an intermediate of phenol degradation via the *ortho*-pathway (Myers *et al*., 2001). The compound glucaric acid is formed as an intermediate during the metabolism

of ascorbate (Polen *et al*., 2013). The compound consists of four chiral centers and has been reported to have several potential applications, those including its participation as a building block for numerous polymers such as hyperbranched polyesters and nylons (Yu *et al*., 2011). The microbial production of the compounds glucaric acid and *cis, cis* muconic acid has been dealt with via metabolic engineering and fermentation approaches in various studies (Yu *et al*., 2011; Polen *et al*., 2013).

In summary the demand and, health implications associated with the production of muconic acid and adipic acid. Furthermore, the current biotechnological applications of catechol 1, 2 dioxygenases and their corresponding genes from various microorganisms have been outlined. Hence, catechol 1, 2 dioxygenases and their corresponding genes are promising alternatives to environmentally benign processes involved in the production of muconic acid and adipic acid.

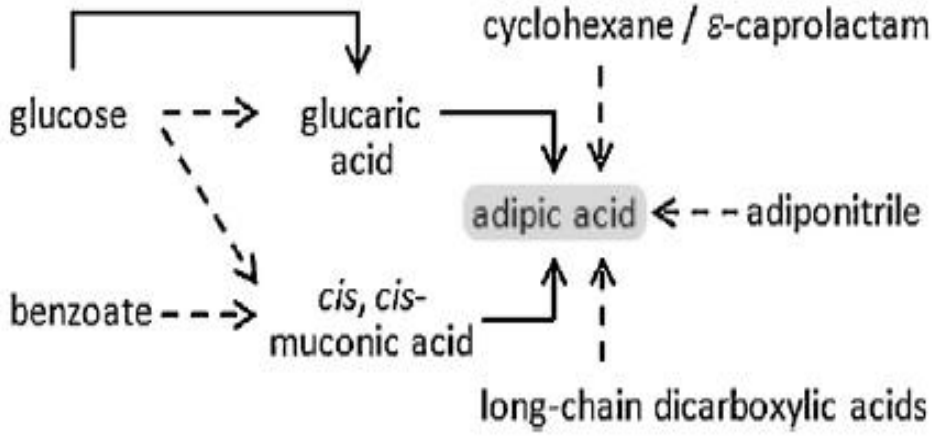

Figure 1.3 Flow chart of access to natural adipic acid. The precursors glucaric acid and *cis*, *cis*-muconic acid can be produced by fermentation (- - ->) from glucose and benzoate and chemo-catalytically. These precursors may be further converted (——>) to adipic acid. Furthermore, adipic acid is generated as an intermediate in the degradation pathways of cyclohexane and even-numbered n-alkyl derivatives there of, caprolactam and long-chain aliphatic dicarboxylic acids or aldehydes, and is formed from adiponitrile by aliphatic nitri-lases (Polen *et al*., 2013).

1.5 MOTIVATION OF STUDY

The parental strain *Acinetobacter* sp. V_2 obtained from the Discipline of Microbiology is capable of degrading up to 400 mg/L of phenol (24 hours) as a sole carbon source in Bushnell Hass (BH) medium. Lin *et al.,* 2014 (unpublished data) have shown that acclimatized mutation(s) in the genome of *Acinetobacter* sp. parental strain has increased the capacity of phenol degradation to 1400 mg/L of phenol within 16 hours. Hence, the adapted strain (*Acinetobacter* sp. strain Y64) is capable of degrading higher concentrations of phenol at a much faster rate, and possesses stable mutation(s). *Acinetobacter* sp. strain Y64 degrades phenol using catechol 1, 2 dioxygenase (C1, 2O) via the *ortho* pathway (also known as the β – ketoadipate pathway). Catechol 1, 2 dioxygenase is the key enzyme in the β – ketoadipate pathway and catechol is the primary substrate for C1, 2O. Improvement in the concentration of phenol degradation, and the rate of phenol degradation in strain Y64, may suggest an improvement in the activity C1, 2O activity expressed by strain Y64 during phenol degradation. Hence, C1, 2O purified from *Acinetobacter* strain Y64 may possess a greater potential for biotechnological application(s).

1.6 SIGNIFICANCE OF STUDY

To purify and evaluate the properties of catechol 1, 2 dioxygenase (C1, 2O) from *Acinetobacter* sp. strain Y64 for potential biotechnological application(s).

1.7 OBJECTIVES

- To purify catechol 1, 2 dioxygenase from *Acinetobacter* sp. strain Y64.
- To clone the catechol 1, 2 dioxygenase gene from *Acinetobacter* spp. into *E. coli*.
- To characterize the purified catechol 1, 2 dioxygenase from *Acinetobacter* sp. strain Y64 and transformants.
- To compare the biochemical properties of the purified catechol 1, 2 dioxygenase from *Acinetobacter* sp. strain Y64 and transformants.

CHAPTER 2

PARTIAL CHARACTERIZATION OF CATECHOL 1, 2 DIOXYGENASE EXTRACTED FROM *Acinetobacter* sp. STRAIN Y64

2.1 INTRODUCTION

Effluents from industries, essentially those involved in coal conversion, metal casting, wood preservation and pulp and paper manufacturing, are thought to be the main source of phenols and phenolic compounds in wastewater (Caposio *et al*., 2002). Scientific research has shown phenolic compounds to have serious health implications to humans and animals (Klibanov *et al*., 1980;; Yan *et al*., 2006).

Numerous methods such as natural oxidation, chemical and physical methods have been applied to try and eradicate phenolic compounds in wastewater. Yan *et al.* (2006) reported that the products of broken down phenolic compound degradations are even more hazardous. Hence, a serious demand for more convenient methods to completely eradicate phenol and phenolic compounds in domestic and industrial water bodies is essential to sustain biodiversity (Yan *et al*., 2006).

The biodegradation of phenol and phenolic compounds, either through aerobic or anaerobic pathways, has been shown to be an environmentally friendly solution. Intensive studies have been conducted on degradation via the aerobic pathways. Aerobic biodegradation may be accomplished via the *meta* or *ortho*-pathway (Caposio *et al*., 2002). In both pathways catechol dioxygenases play a vital role in the breakdown of phenol compounds especially in soil bacteria. The utilization of catechol and the pathway of oxidation depend on the bacterial species (Ngai *et al*., 1990; Caposio *et al*., 2002).

Catechol dioxygenases are non-heme iron enzymes that directly catalyze the addition of oxygen molecules to catechol (Johnson and Stanier, 1971; Houghton and Shanley, 1994; Yan *et al*., 2006). This is achieved via the subsequent cleavage of the benzene rings. The cleavage

of the phenol ring may be used to distinguish and characterize catechol dioxygenases into two groups, i.e. catechol 2, 3 dioxygenase in the *meta* pathway and catechol 1, 2 dioxygenase (C1, 2O) in the *ortho* pathway (Caposio *et al*., 2002). Catechol 1, 2 dioxygenases are one of the intensively studied intradiol dioxygenases. The enzyme has a high spin Fe^{+3} at the active site and is essentially responsible for enzyme activity (Caposio *et al*., 2002). Catechol is cleaved by catechol 1, 2 dioxygenase to form *cis, cis* muconate. Catechol is converted by catechol 2, 3 dioxygenase to 2-hydroxymuconic semialdehyde in the *meta* pathway. Descending from the cleavage this may further lead to the formation of metabolites such as; acetic acid, acetyl-CoA, pyruvatic acid and succinic acid which are essential metabolites of the Kreb cycle via the β-ketoadipate pathway (Johnson and Stanier, 1971; Houghton and Shanley, 1994; Caposio *et al*., 2003).

There are two types of C1, 2Os that can be distinguished by specificity to their substrates. C1, 2Os that show less or no activity with chlorocatechols are type-I enzymes while those that cleave chlorocatechols are type-II enzymes (Ngai *et al*., 1990; Caposio *et al*., 2002). The expression of C1, 2O enzymes indicates the ability of microorganisms to metabolize a variety of aromatic compounds (Caposio *et al*., 2002). Microorganisms expressing C1, 2O enzymes have been mainly found in sewage, water and soil (Eulberg *et al*., 1997; Sauret-Ignazi *et al*., 1996; Strachan *et al*., 1998). Bacteria of the genus *Acinetobacter* have obtained much attention due to their ability to utilize a variety of organic carbon sources (Kim *et al*., 1997).

Characteristics such as high stereo- and regio-selectivity and broad substrate specificity of dioxygenases have brought more focus due to potential biotechnological applications (Curran and Alper, 2012). Catechol 1, 2 dioxygenase has the ability to convert phenolic compounds based on stereo- and regio-selectively into numerous organic products such as *cis, cis* muconic acids and compounds that can be used as raw materials in plastic products (Curran and Alper, 2012). *Acinetobacter* sp. strain Y64 was obtained through a phenol-enrichment

process and found to degrade up to 1400 mg of phenol in less than 24 hours (Lin *et al.,* unpublished data). Phenol hydroxylase and C1, 2O are reported as the key enzymes in the degradation of phenol in BH medium. The C1, 2O of *Acinetobacter* sp. strain Y64 is suggested to possess a high phenol degradation rate and amenable for biotechnological application(s). Therefore, the study intends to characterize C1, 2O from *Acinetobacter* sp. strain Y64.

2.2 MATERIALS AND METHODS

2.2.1 Maintenance of *Acinetobacter* sp. strain Y64

Acinetobacter sp. Y64 mutant strain was obtained from the Discipline of Microbiology at the University of KwaZulu–Natal Westville Campus. *Acinetobacter* strain Y64 was cultivated at in Bushnell Haas medium (BH) (per litre: K_2HPO_4 1.0 g, KH_2PO_4 1.0 g, NH_4NO_3 1.0 g, $CaCl_22H_2O$ 0.02 g, $MgSO_47H_2O$ 0.2 g, $FeCl_3$ 0.05 g (Bushnell and Hass, 1941) and 1000 mg/L of phenol (as a carbon source) at pH 7, 30°C, and 160 r.p.m (Lin and collegues – personal communication, 2014) . The *Acinetobacter* sp. strain Y64 culture was preserved in Bushnell Haas medium supplemented with 1000 mg/L of phenol concentrations and 80% (v/v) glycerol and stored in the biofreezer at -80°C (Lin *et al*., 2008).

2.2.2 Determination of cell growth, enzyme activity of *Acinetobacter* sp. strain Y64 and phenol concentrations

Acinetobacter sp. was grown in 100 ml of Bushnel Haas (BH) medium supplemented with 0.5% sodium acetate in a 250 ml Erlenmeyer flask overnight. Approximately 5% of seed culture was transferred into 100 ml BH medium supplemented with 1000 mg/L of phenol. The incubation was set for 72 hours at 30°C, 160 rpm. Cell growth of *Acinetobacter* sp. strain

Y64 was determined spectrophotometrically (OD_{600}) with a Shumadzu UV-1800 Ultra Spectrophotometer as described by Lin *et al.* (2008).

The phenol concentration of the samples was monitored using the method of Klibanov *et al*. (1980). One ml of the sample was centrifuged at 13,000 ×g for 10 minutes. The obtained supernatant was mixed with 25 µl of 4–aminoantipyrine (2% aqueous solution), 25 µl of 6.0 M NH_4OH and 50 µl of potassium ferricyanide (8% w/v). The reaction mixture was allowed to react for 5 minutes and phenol was extracted with 2 ml of chloroform. The concentration of extracted phenol was determined by the optical density at 505 nm.

The catechol 1, 2 dioxygenase activity (intradiol cleavage) of the supernatant and sonicated cells at each sampling time was assayed spectrophotometrically (Nadaf and Ghosh *et al*., 2011). Five µl of the crude C1, 2O extract (standardized by volume) was added to 1 ml of 50 mM sodium phosphate buffer (pH 7.0) containing 20 µl of 10 mM catechol as a substrate. The activity was monitored by measuring the absorbance at 260 nm (Shumadzu UV-1800 Ultra Spectrophotometer) due to the formation of *cis, cis* muconic acid ($\boldsymbol{\varepsilon}_{260}$= 16 000 M^{-1} cm^{-1}) (Nadaf and Ghosh *et al*., 2011).

The concentration of cis, cis muconic acid was calculated using the following equations:

$$\text{Concentration of } cis, cis \text{ muconic acid} = \frac{\text{Abs}_{260nm}}{\varepsilon_{260nm}}$$

Where,

$_{260}$is the extinction coefficient of *cis, cis* muconic acid (16 000 M^{-1} cm^{-1}). One unit of enzyme activity is defined as the amount of the enzyme required to catalyze the formation of 1 µ mol of product per min at 30°C.

A *cis, cis* muconic acid standard curve was also used to confirm the concentration of *cis, cis* muconic acid. Specific activity was defined as µmole of *cis, cis* muconic acid formed per min per mg of protein.

The catechol 2, 3 dioxygenase activity (C2, 3O; extradiol cleavage) of both supernatant and sonicated cells was assayed spectrophotometrically by measuring the formation of 2-hydroxymuconic semialdehyde at 390 nm under similar conditions as reported for intradiol activity above (Briganti *et al*., 1997).

2.2.3 Preparation of crude extract

Acinetobacter sp. strain Y64 was grown as described in Section 2.2.2 in in which 500 ml of Bushnel Haas medium supplemented with 1000 mg/L of phenol until the highest activity of catechol 1, 2 dioxgenase was achieved. The cells were harvested by centrifugation at 8 000 x *g* at 4°C for 30 minutes. The cells were washed with 30 ml of 50 mM sodium phosphate buffer (pH = 7.0) and centrifuged twice, followed by resuspension in 20 ml of the same buffer. The cells were homogenized by sonication (Omni International Sonic Ruptor 400 Untrasonic homogenizer) at 50 amplitude for 10 minutes with 1 minutes pulse while the sample was kept on ice (Guzik *et al*., 2011b). The homoginized sample was centrifuged at 8 000 x *g* at -20°C. The pellets were stored at -20°C until required. All the experiments and assays were performed at least in triplicate and the results were presented as the mean ± standard deviation.

2.2.4 Determination of protein concentration

Protein concentration of the sample was determined by the Bradford method using bovine serum albumin (BSA) as a standard (Bradford, 1976). A standard curve was constructed using BSA (Sigma) of known concentrations. A ratio of 1:50 for sample to Bradford was kept for both the protein samples and the BSA. Following 5 minutes of incubation at room temperature, absorbance for the BSA and samples was measured at 595 nm with the

Shumadzu UV-1800 Ultra Spectrophotometer. Protein concentration (mg/ml) was estimated by extrapolation from the BSA standard curve.

2.2.5 Protein profile and estimation of catechol 1, 2 dioxygenase molecular weight

The lysate of strain Y64 obtained from actively growing cells (in 1 g/L of phenol) sampled every hour (for 9 hours) and standardized by volume were subject to 15% SDS-PAGE analysis (Laemmli *et al*., 1970). Strain Y64 cells grown in 0.5% sodium acetate were used as a control. The gels were stained with Coomassie Brilliant Blue G250. The gel was viewed on a transilluminator and digital photographs were taken using an Alpha Imager TM 3400.

2.2.6 Optimum pH and temperature of catechol 1, 2 dioxygenase in lysate

The pH optima were monitored by incubating the enzyme at different pH (Tsai *et al*., 2007) under the standard assay conditions as described in Section 2.2.2. The effect of pH on enzyme activity was studied at pH ranging from 4.0 to 10.0, using citrate phosphate buffer (pH 3.0-5.0), sodium- phosphate buffer (pH 6.0-8.0), glycine- NaOH buffer (pH 9.0-10.0). The optimum temperature of the enzyme was studied at temperatures ranging between 4 to 50ºC at the optimal pH under the standard assay conditions (Nadaf and Ghosh *et al*., 2011).

2.2.7 Determination of catechol 1, 2 dioxygenase kinetics

The K_m and V_{max} of catechol 1, 2 dioxygenase were determined by Lineweaver-Burk plots of reciprocal reaction velocities versus reciprocal substrate concentrations (Nadaf and Ghosh *et al*., 2011). Assays were performed in 50 mM sodium phosphate buffer pH 7.0, with increasing concentrations of catechol (up to1 400 µM).

2.3 RESULTS AND DISCUSSION

Acinetobacter sp. strain Y64 degraded 1 g/L of phenol completely in 16 ± 2 hours at pH 7 and at 30°C (Fig. 2.1). Phenol concentration in the medium decreased rapidly during the first 8 hours. Approximately 730 mg/L of phenol was degraded by *Acinetobacter* sp. Y64 within 4 hours, with approximately 1 U of catechol 1, 2 dioxygenase activity. The decrease of phenol concentration corresponded to a significant increases in cell growth and concentration of *cis, cis* muconic acid. A plateau on the cell growth curve and a decrease in rate of phenol degradation was observed after approximately 900 mg/L of phenol was degraded in 9 hours (Figure 2.1). However, a continuous increase in C1, 2O activity was observed after 9 hours. The increase in C1, 2O activity even after the cease in cell growth indicates a continuous production of C1, 2O in strain Y64. The excessively produced C1, 2O may provide the cell with a selective advantage such as degrading residual catechol. *Acinetobacter* sp. strain Y64 however showed no growth when catechol supplemented as a sole carbon and energy source in BH medium indicating sensitivity of this strain to the presence of catechol.

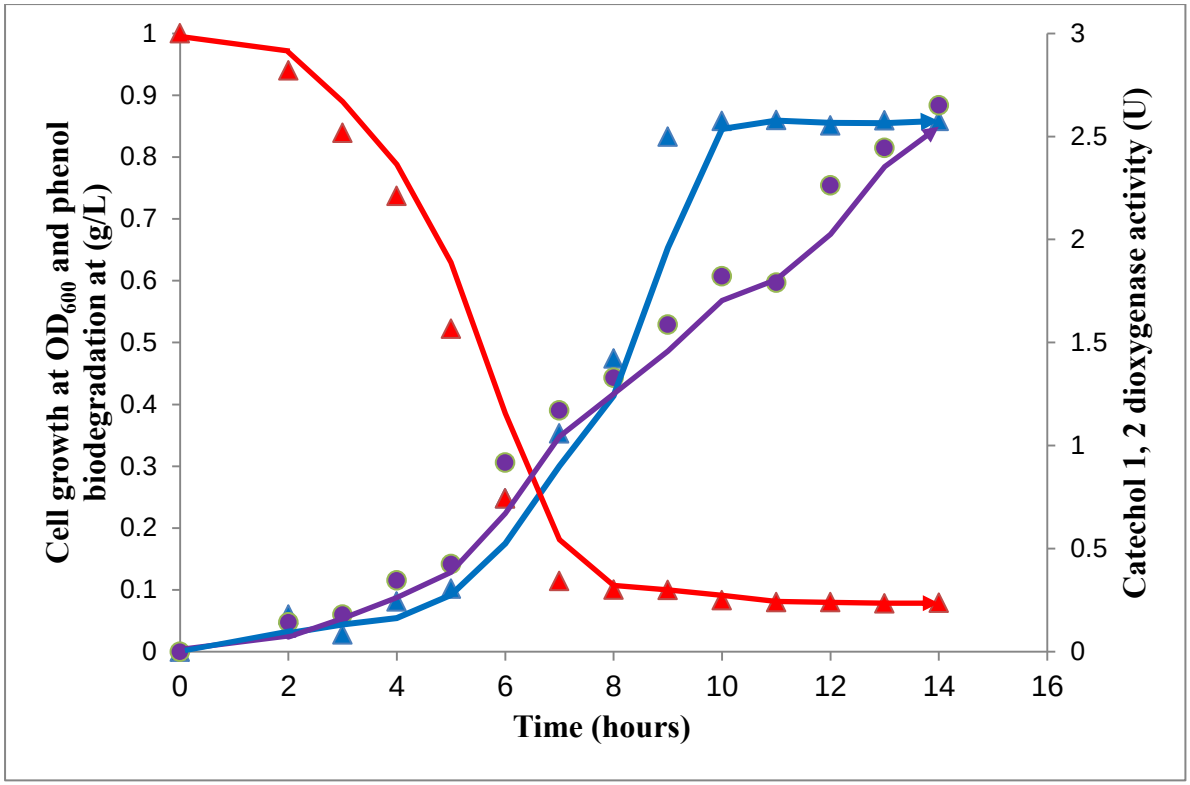

Figure 2.1 Growth curve of *Acinetobacter* sp. strain Y64, phenol degradation and the catechol 1, 2 dioxygenase activity monitored in a period of 16 hours when the strain was grown in BH-medium supplemented with 1 g/L of phenol concentration.

Acinetobacter sp. strain Y64 was more efficient at degrading higher concentrations of phenol when compared to *Acinetobacter radioresistens* which was reported to degrade up to 400 mg/L of phenol after 24 hours (Briganti *et al*., 1997). *Pseudomonas putida* N6 and *P. fluorescens* PU1 were shown to degrade up to 600 mg/L of phenol after 24 hours (Guzik *et al*., 2011b; Mahiudddin *et al*., 2012). These bacterial strains were also capable of using catechol as a sole carbon source. *Acinetobacter* sp. S13 can utilize acetate, phenol and benzoate respectively as a carbon source but not catechol (Caposio *et al*., 2002). Guzik *et al*. (2011b) further demonstrate that *P. putida* strain N6 was able to utilize up to 10 mM concentrations of catechol, hydroquinone, 4-hydroxybenzoic acid (4-HB), vanillic acid, protocatechuic acid (3, 4-DHB) and benzoic acid as a sole carbon source. Chacón *et al*. (2004)

showed that *Candida tropicalis* could grow on catechol (1160 mg/L), resorcinol (840 mg/L) and up to 800 mg/L of phenol, respectively.

After harvesting the cells by centrifugation, the supernatant did not show any C2, 3O activity as the absorbance at 390 nm did not increase during the assay period. However, a steady increase in the absorbance at 260 nm was observed using catechol as the substrate, thus indicating the conversion of catechol to *cis, cis* muconic acid. The time course of catechol oxidation by catechol 1, 2 dioxygenase in lysate in a period of 1 hour is shown in Figure 2.2. The time course curve shows that more *cis, cis* muconic acid was produced and the reaction is linear (Figure 2.2). The corresponding catechol is a key metabolite in the *ortho* pathway utilized by *Acinetobacter* sp. strain Y64 in the assimilation of phenol. Hence, the *Acinetobacter* sp. strain Y64 possesses intracellular catechol 1, 2 dioxygenase.

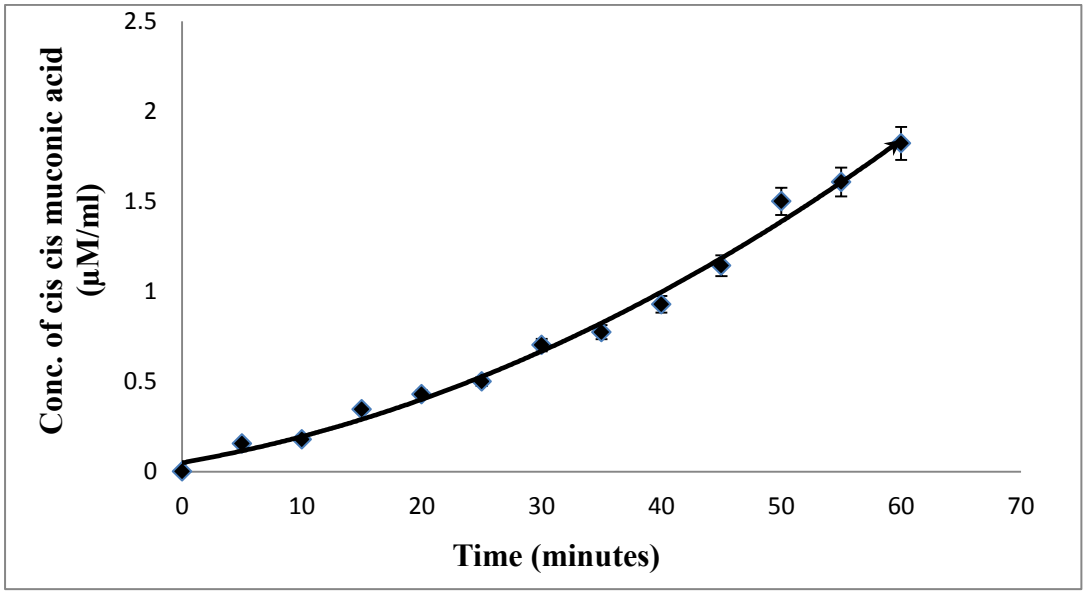

Figure 2.2 Progress curve of cathechol 1, 2 dioxygenase monitored for 1 hour after the enzyme in lysate was kept to react with 10 mM catechol in reaction buffer.

The total genomic sequencing data of *Acinetobacter* sp. strain Y64 shows that two isomers of C1, 2Os, encoded *iso*A and *iso*B (with the molecular weights of 36 kDa and 18 kDa) respectively, are involve during the biodegradation of phenol (Sharma and Lin – unpublished results). Thus to obtain a further insight into *Acinetobacter* sp. strain Y64 C1, 2O expression,

the proteins expressed during growth on acetate and phenol were subjected to SDS-PAGE analysis.

The lysate of *Acinetobacter* sp. strain Y64 obtained from actively growing cells (in 1 g/L of phenol) sampled every hour and standardized by volume were subjected to 15% SDS-PAGE analysis. The SDS-PAGE analysis electrophoretogram showed extra protein bands (circularized) induced by phenol (Figures 2.3B and 2.4) in contrast to the lysate of strain Y64 cells grown in acetate (Figure 2.3A). The most prominent protein band is 36 kDa, and likewise is induced in the presence of phenol (Figure 2.3). The intensity of the 36 kDa protein band was observed to have a direct increase with C1, 2O activity. Another protein band of 18 kDa was later observed in the lysate of strain Y64 after 11 hours of growth in phenol (Figure. 2.4). The intensity of the 18 kDa protein band increased and peaked after 14 hours. It is noteworthy that the two mentioned protein bands (at 36 kDa and 18 kDa) were not visible in the lysate of strain Y64 grown in acetate (Figure 2.3A). However, other extra protein bands circularized in Figures 2.3 and 2.4 are bigger than 36 kDa. The data on the expression of genes involved in phenol degradation has shown two C1, 2Os (termed *isoA and isoB*) present during the degradation of phenol by strain Y64 (Sharma and Lin – unpublished data). Furthermore, *iso A* and *isoB* were said to be expressed at different time intervals. Lack of these protein bands (at 36 kDa and 18 kDa) in the cell lysate of strain Y64 grown in acetate for 15 hours, strongly suggested that the expression of these two proteins were specifically regulated by the addition of phenol to the medium. The direct relationship between C1, 2O activity and the intensity of the protein bands at 36 kDa and 18 kDa, probably suggest the two protein bands to may be *isoA* and *isoB*, respectively. The data in Figures 2.3 and 2.4 shows that both *isoA* and *isoB* are induced by phenol or their derivatives and are not constitutive. However, *isoA* is expressed first, and is followed by *isoB*. We suggest that *isoA* and *isoB* may be presumably involved in different biological functions during phenol degradation. Our

findings agree with the data shown by Caposio *et al*. (2002) who showed that two C1, 2Os from *Acinetobacter radioresistens* S13 were also induced by phenol. However, *isoB* from *A. radioresistens* S13 was said to be expressed in the presence of acetate, phenol or benzoate, thus constitutive.

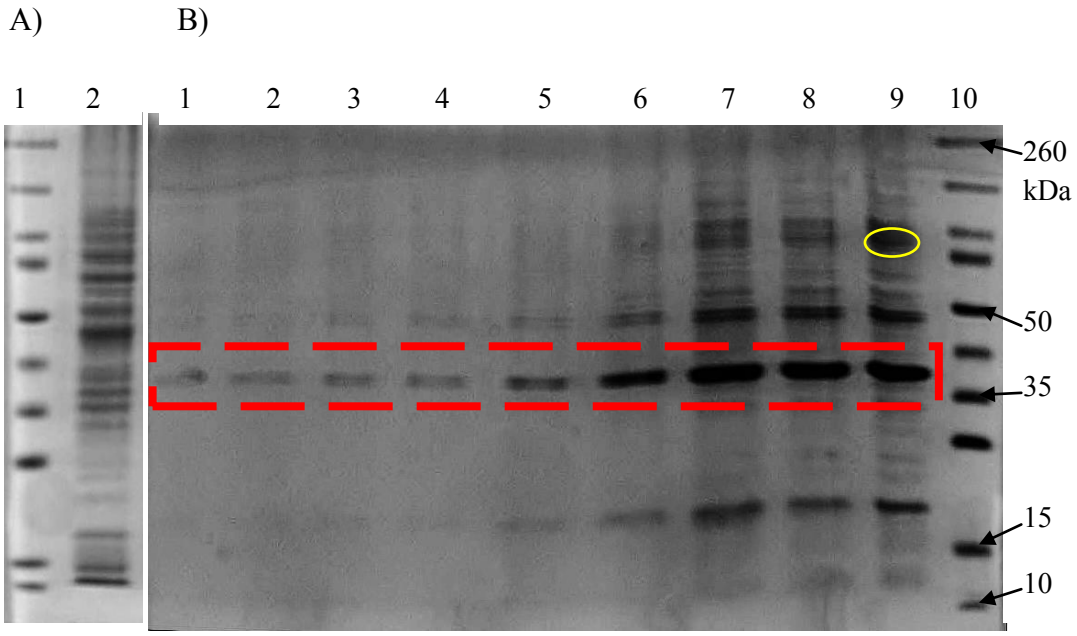

Figure 2.3 Protein expression profile of *Acinetobacter* sp. strain Y64 lanes; 1 - standard protein molecular weight marker (Thermo scientific) (10 – 260 kDa), lane 2; lysate of *Acinetobacter* sp. straina Y64 fter 9 hours in 0.5% of sodium acetate. Lanes 3 – 9 Protein expression profile of *Acinetobacter* sp. strain Y64 lysate of *Acinetobacter* sp. strain Y64 obtained after every hour of cell grown in phenol, lane 10; standard protein molecular weight marker. The red doted line highlights the expression of C1, 2O for 9 hours, while the circular yellow line highlights another protein of high molecular weight expressed in response to phenol after 9 hours of cell growth.

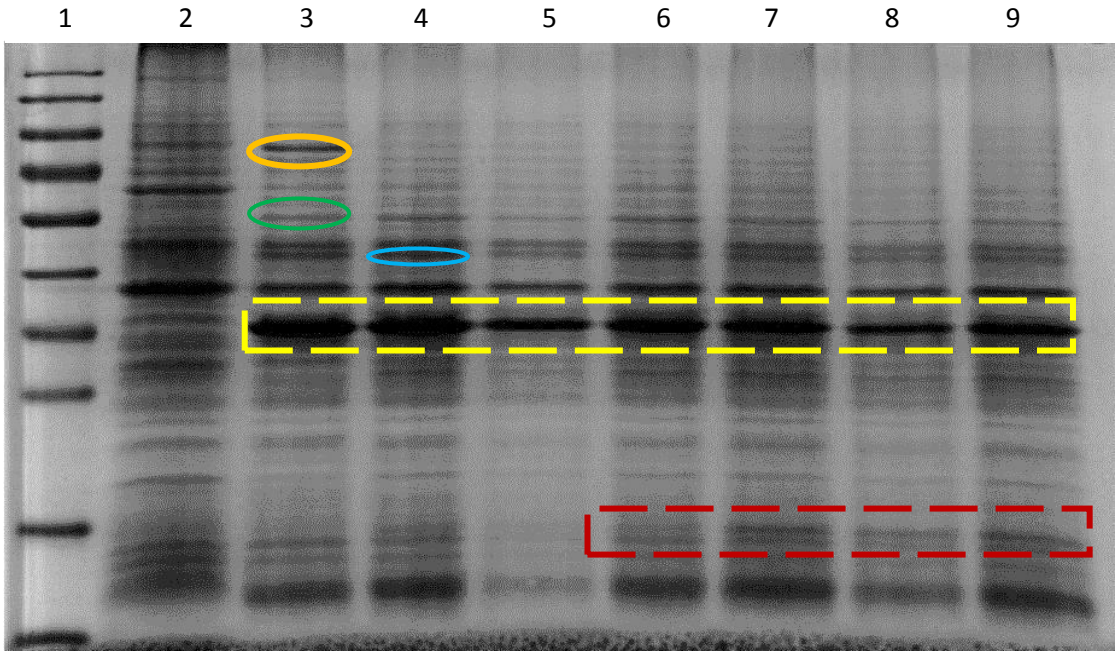

Figure 2.4 Protein expression profile of *Acinetobacter* sp. strain Y64 lanes; 1 – protein standards molecular weight marker (10 – 260 kDa), lane 2; lysate of *Acinetobacter* sp. strain Y64 with protein expression profile of *Acinetobacter* sp. strain Y64 in 0.5% acetate after 16 hours of growth. Lanes 3 – 9 lysate of *Acinetobacter* sp strain Y64 after every hour of growth in phenol, The yellow dotted line highlights the continuous and constant expression of the big C1, 2O produced by Strain Y64 from 10 hours to 16 hours. The red dotted line highlights the continuous expression of the big C1, 2O produced by Strain Y64 from 13 hours to 16 hours, while other circular lines indicate other proteins with higher molecular sizes after 10 hours of cell growth.

The enzyme activity is highly affected by temperature and pH. The optimal pH and temperature at which most of the enzyme activity is observed, corresponds directly with the point where the rate of reaction is at maximum value (Guzik *et al*., 2011b). The C1, 2O of *Acinetobacter* sp. strain Y64 showed enzyme activity between pH 3 and 10 (Figure 2.5). The optimum pH curve of C1, 2Os obtained from strain Y64 showed two pH optima; pH 4.5 and pH 7.5 (Figure 2.5). Approximately, 85% relative C1, 2O activity was observed at pH 4.5, comparing to the C1, 2O activity at pH 7.5. Less than 30% C1, 2O relative activity was observed at strong acidic pH ranges < 3 (data not shown). Similarly, at higher alkaline pH ranges (> 9), less than 50% relative activity was observed. A similar pattern has been reported for C1, 2Os purified from numerous microorganisms tested for activity at strong

acidic and basic pH ranges, respectively (Nadaf and Ghosh, 2011; Saxena and Thakur, 2005; Wang *et al*., 2006). The C1, 2O of strain Y64 showed less than 50% relative activity at pH 6 (Figure. 2.5). The loss of activity at pH 6 suggests inhibition of the C1, 2O activity extracted from strain Y64. This may be due to change in conformation of the active site or unavailability of Fe^{3+} to facilitate catalysis. The observed two peaks might be due to two isozymes of C1, 2Os from strain Y64. Isozyme activity of C1, 2Os was reported by Patel *et al*. (1976) in *A. calcoaceticus*. C1, 2Os of strain Y64 showed a broad range of activity between 25°C and 45°C. The optimum temperature of C1, 2O purified from strain Y64 is 36°C (Figure 2.6), in agreement with the findings reported by Guzik *et al*. (2011b). No C1, 2O activity was observed at 4°C and approximately 60% of C1, 2O activity was lost at 45°C (Figure 2.6). Furthermore, a complete loss of C1, 2O activity was observed at 50°C after 10 minutes (Figure 2.6) and similar findings were reported by Miguez *et al*. (1993).

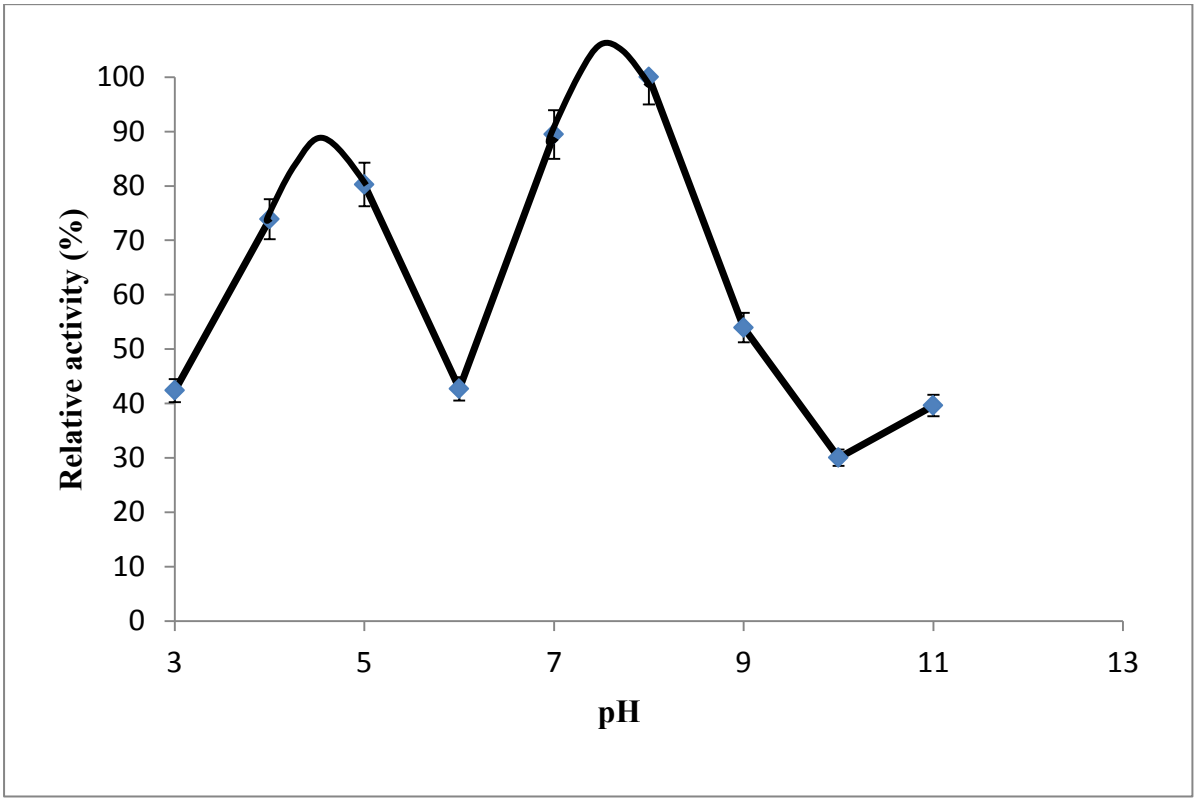

Figure 2.5 pH activity profile of catechol 1, 2 dioxygenase of strain Y64.

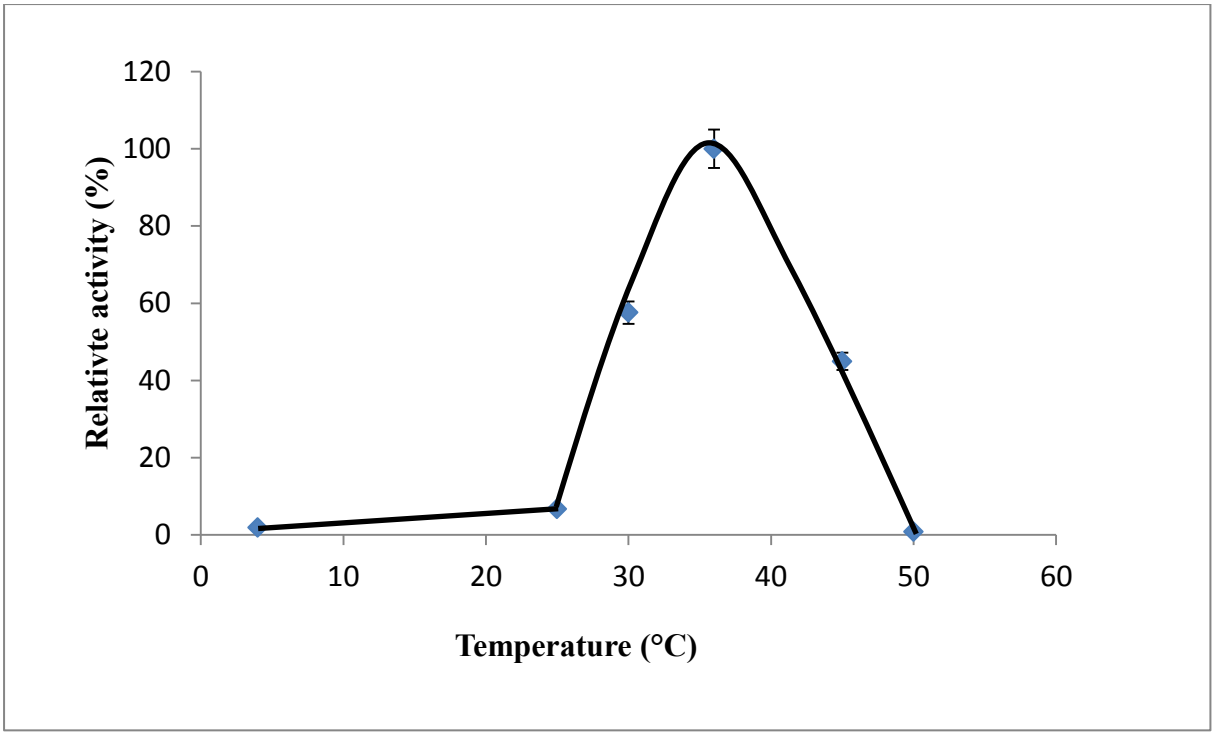

Figure 1.6 Temperature profile of catechol 1, 2 dioxygenase of strain Y64. The reaction was done at pH 7.5 for at least 10 min before analysis.

The existence of two isofunctional C1, 2Os involved in the biodegradation of aromatic compounds has been reported in other studies (Briganti *et al*., 2000). Whether such a trait provided the host with a selective advantage remains unclear. Perhaps studies on the identification of the promoter regions and elucidation of the transformational mechanisms giving rise to isoform specific expression of the two genes may provide an explanation.

A direct increase in the initial rate of C1, 2O reaction rate (V_0) with increasing concentration of catechol to 200 µM was observed (Figure 2.7). However, an increase in catechol concentration above 200 µM resulted in a level off of the reaction rate, suggesting substrate saturation in the active site of C1, 2O. Hence the kinetics of catechol 1, 2 dioxygenase were shown to fit the Michaelis – Menten mode of enzyme kinetics (Figures. 2.7 and 2.8). V_{max} and K_m were determined using the lineweaver – Burk double reciprocal plot (Figure 2.7). C1, 2O isolated from strain Y64 showed V_{max} = 5.05 U/mg of protein and K_m = 93.5 µM when

catechol was used as a substrate and there was no inhibition to the activity of C1, 2O in the lysate of *Acinetobacter* sp.strain Y64.

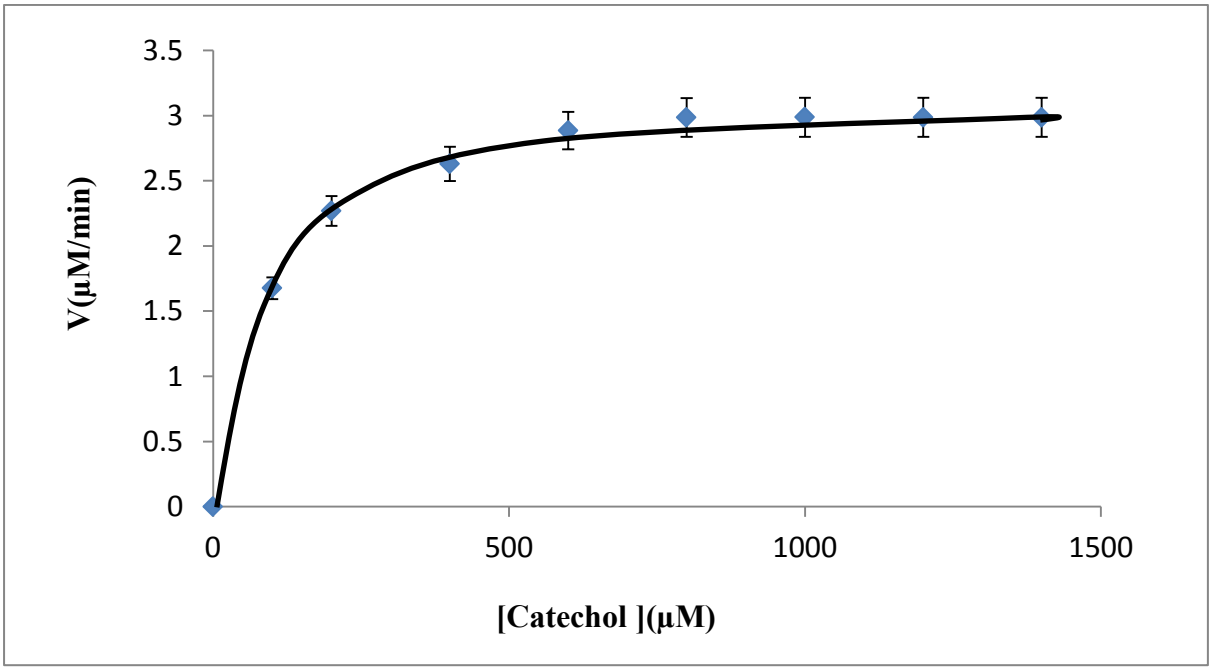

Figure 2.7 Kinetics of crude catechol 1, 2 dioxygenases with increasing substrate concentration (0 – 1500 µM catechol).

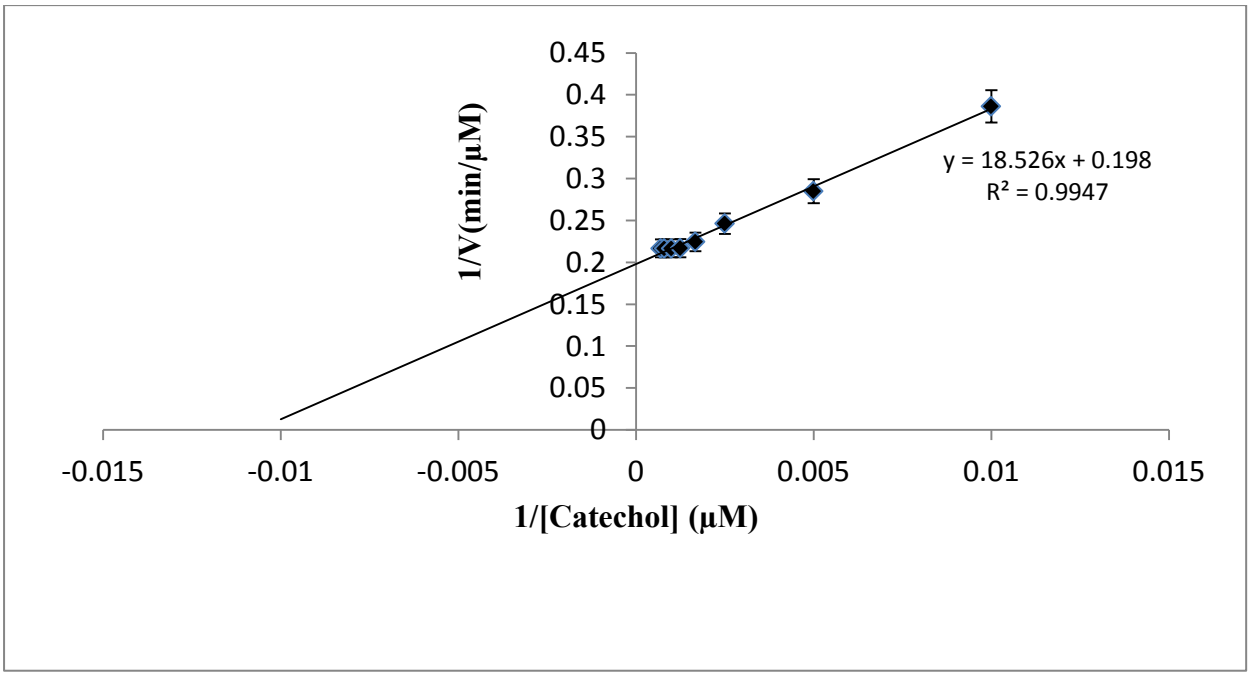

Figure 2.8 Lineweaver-Burk double reciprocal plot – determination of K_m and V_{max} of crude catechol 1, 2 dioxygenases of strain Y64.

Similar findings were reported for the kinetics of catechol 1, 2 dioxygenase of *S. maltophilia*, KB2, *P. putida* N6 (Guzik *et al.,* 2011a; 2011b), *R. opacus* 1CP (Suvorova *et al*., 2006) and *R. opacus* 6a (Solyanikova *et al*., 2009). The K_m value (93.53 μM) of C1, 2O of *Acinetobacter* sp. strain Y64 may be compared to C1, 2O isolated from *P. putida* N6 (K_m = 85.19 μM). However, the K_m value of C1, 2O of *Acinetobacter* sp. strain Y64 was high in contrast to that reported for *Rhodococcus rhodochrous* (K_m = 0.0011 mM) (Strachan *et al*., 1998). The purification of C1, 2O was demonstrated to improve the affinity of the enzyme for the substrate (catechol) (Briganti *et al*., 1997; Pessione *et al*., 2001; Wang *et al*., 2006). Furthermore purification of C1, 2O would assist in evaluating its potential biotechnological application(s). Therefore, the purification of C1, 2O and the characterization from *Acinetobacter* sp. strain Y64 was the main focus in the next chapter.

2.4 CONCLUSIONS

Acinetobacter sp. strain Y64 is capable of growing on phenol as a sole carbon source. Catechol 1, 2 dioxygenase (C1, 2O) is an enzyme at the rate determining step of phenol degradation strain Y64. Two C1, 2Os (*isoA* and *isoB*) with the molecular weights 36 and 18 kDa were induced by phenol in *Acinetobacter* sp. strain Y64. However, the 36 kDa protein was observed earlier in the presence of phenol, whilst the 18 kDa protein was observed after 11 hours. Isozyme activity was observed for C1, 2O isolated from *Acinetobacter* sp.strain Y64 with expected molecular weights of 36 kDa and 18 kDa, respectively. C1, 2O isolated from *Acinetobacter* sp. strain Y64 has two pH optima (4.5 and 7.5). However the optimum temperature of C1, 2 O isolated from strain Y64 was 36°C. Catechol 1, 2dioxygenase of strain Y64 shows affinity for catechol (K_m = 93.53 μM) and V_{max} = 5.05 U/mg before purification. Characteristics of C1, 2O motivates for the purification and characterization of C1, 2O from *Acinetobacter* sp. strain Y64.

CHAPTER 3

CLONING, PURIFICATION AND CHARACTERIZATION OF CATECHOL 1, 2 DIOXYGENASE FROM *Acinetobacter* sp. STRAIN Y64 AND *E. coli* TRANSFORMANTS

3.1 INTRODUCTION

Catechol 1, 2-dioxygenase (C1, 2O) is an enzyme at the rate determining step during the catabolism of numerous aromatic compounds (Pessione *et al*., 2001). Aromatic compounds are broken down to catechol which is further broken down by catechol 1, 2 dioxygenase to form *cis, cis* muconate. *Cis, cis* muconate is further metabolized to tricarboxylic acid cycle intermediates via the β-ketoadipate pathway (Nadaf and Ghosh *et al*., 2011).

Catechol 1, 2-dioxygenase has been purified from various microbial sources and consistently has been reported to contain trivalent nonheme iron (Kim *et al*., 2002; 2003; Nadaf and Ghosh *et al*., 2011). Diverse oligomeric structures and subunit sizes for C1, 2O have also been reported (Patel *et al.,* 1976; Nakai *et al.,* 1988; Miguez *et al.,* 1993; Vijay *et al.,* 2011), thus the properties of these proteins depend on the biological source.

Catechol 1, 2-dioxygenases have gained attention due to their regioselectivity and stereoselectivity which is essential for the production of stereoisomers. Moreover, C1, 2O has other biotechnological applications such as the removal of toxic pollutants in bioreactor systems and incorporation into diagnostics systems and biosensors (Ali *et al*., 1998; Nakajima *et al*., 2002). *Acinetobacter* sp. strain Y64 subject of the current investigation has been selected based on its fast phenol catabolism (Chapter 2; Figure 2.1). The structural gene for C1, 2O, *cat*A, has been shown to be expressed when *Acinetobacter* strain Y64 was grown on phenol. In the present study, the *cat*A gene has been inserted into pJET cloning vector and the resulting plasmid, pVSJ3, was transformed into *E. coli* BL21 DE3(pLys) to further increase the production of C1, 2O. The purification of catechol 1, 2 dioxygenase from a novel

Acinetobacter sp. strain Y64 and *E. coli* transformants and characterization of C1, 2O from *Acinetobacter* sp. strain Y64 and *E. coli* transformants are discussed.

3.2 MATERIALS AND METHODS

3.2.1 Purification and characterization of catechol 1, 2 dioxygenase from *Acinetobacter* sp. strain Y64

3.2.1.1 Preparation of crude extract

Acinetobacter sp. strain Y64 was grown overnight in 100 ml of Bushnell Haas medium supplemented with 0.5% sodium acetate in a 250 ml Erlenmeyer flask. Approximately 5% of overnight culture was transferred into 500 ml of fresh BH medium supplemented with 1000 mg/L of phenol as a sole carbon source. The culture was further grown at pH 7.0, 30°C and 160 rpm for a period of 15 hours when optimal C1, 2O activity (Chapter 2) was achieved. The cells were harvested by centrifugation at 8 000 × *g*, 4°C for 30 min and the pellet was resuspended in 50 ml of 20 mMTris-Cl buffer pH 7.5. The suspension was centrifuged at the above conditions, as described above. The final pellet was resuspended in 15 ml of 50 mM Tris-Cl buffer pH 7.5. The crude extract was obtained by lysing the cells using the sonication method (Omni International Sonic Ruptor 400 Untrasonic homogenizer) at 60W for 10 short burst of 10 seconds followed by intervals of 30 seconds cooling. The cell lysate was centrifuged at 8 000 × *g* for 30 min at 4°C to obtain clear supernatant containing the C1, 2O activity (Section 2.2.3). The supernatant was centrifuged further at 8 000 x *g* for 1 hour at 4°C to remove remaining contaminants. The final supernatant was used for purification of catechol 1, 2 dioxygenase.

3.2.1.2 Purification of catechol 1, 2 dioxygenase

Step 1: $(NH_4)_2SO_4$ precipitation – The crude extract after centrifugation was subjected to a stepwise (20 – 60% saturation) ammonium sulphate precipitation at 4°C. At 60% ammonium sulphate saturation, maximum enzyme activity was recovered. The solution was centrifuged at 9 800 × *g* for 20 minutes at 4°C and the pellet was resuspended in 1 equal volume of 50 mM Tris-Cl buffer pH 7.5. The solution containing the C1, 2O activity was subjected to dialysis (12 kDa molecular weight cut-off) against 50 ml of the same buffer for 3 hours at 4°C with subsequent change of buffer and left overnight.

Step 2: Anion-exchange chromatography - The dialyzed sample was subjected to separation using FPLC AKTA Purifier 450 system with a Hitrap QFF column (5 ml) previously equilibrated with buffer A (50 mM Tris-Cl pH 7.5 + 0.18 M NaCl). The column was washed with 5 column volumes of buffer A. The proteins were eluted out with a linear gradient 0 – 100% of buffer B (50 ml of 50 mM Tris-Cl pH 7.5 + 0.4 M NaCl) at a flow rate of 0.4 ml/min and the protein concentration of the eluent was monitored by the absorbance at 280 nm wavelength. The eluents containing the enzyme activity were collected and concentrated (using a Merck- Ultra spin column) to make up a final volume of 5 ml.

The enzyme activity, protein concentration and protein profile of the samples at each purification step were monitored as described below.

3.2.2 Characterization of purified catechol 1, 2 dioxygenase

3.2.2.1 Determination of enzyme activity and protein concentration

The catechol 1, 2 dioxygenase activity and protein concentration of the samples were monitored as described in Section 2.2.4 and 2.3.5 of Chapter 2.

3.2.2.2 Protein profile and estimation of catechol 1, 2 dioxygenase molecular weight

The purified fractions showing C1, 2O activity were assessed for purity using 12% SDS-PAGE (Laemmli *et al*., 1970). The gels were stained with Coomassie Brilliant Blue G250. The gel was viewed on a transilluminator and digital photographs were taken using an Alpha Imager TM 3400.

3.2.2.3 Calculation of specific activity and yield

The specific activity of the purified catechol 1, 2 dioxygenase was calculated using the following formula:

$$\text{Specific activity (U/mg)} = \frac{\text{mol of } \textit{cis},\ \textit{cis} \text{ muconic acid per minute}}{\text{total mg of purified C1,2O}}$$

The concentration of *cis, cis* muconic acid was calculated using the formula mentioned in Chapter 2 (Section 2.2.2).

3.2.2.4 Determination of optimum pH and temperature of the purified catechol 1, 2 dioxygenase

The pH and temperature optima of the purified C1, 2O were determined as described in Section 2.2.6 of Chapter 2.

3.2.2.5 Thermal and pH stability of the purified catechol 1, 2 dioxygenase

To evaluate the thermal stabilities of the purified C1, 2O, 5 µl of purified enzyme (about 1.3 µg) was mixed with 985 µl of 50 mM Tris-Cl (pH 7.5) and kept at 37°C, 40°C, 45°C, 50°C or 55°C for 60 minutes, respectively (Pessione *et al*., 2001). At different time intervals the catalytic activity of C1, 2O was determined at the optimal temperature of 30°C, using 20 µl of 10 mM catechol as substrate. This experiment was done in triplicates. To evaluate the pH stability of C1, 2O, 50 µl of the purified enzyme (about 1.3 µg) was mixed with 650 µl of

buffers with different pH (range from 3 - 11) (Pessione *et al*., 2001). The mixtures were incubated for an hour at 4°C. The residual enzyme activity was measured by withdrawing 100 µl of the mixture after every hour (for at least six hours) and mixing with 880 µl of the reaction buffer. An amount of 20 µl of 10 mM catechol as substrate was added to the mixture to activate the activity of C1, 2O, and the reaction mixture was incubated at 36°C. This experiment was done in triplicates.

3.2.2.6 Substrate specificity of catechol 1, 2 dioxygenase

To evaluate the effect of substituted derivatives of aromatic compounds on the activity of catechol 1, 2 dioxygenase, an enzyme reaction mixture was setup with a standardized concentration (1.3 µg) of purified C1, 2O in 1 ml of 50 mM Tris-Cl (pH 7.5) buffer. The reaction was initiated by adding 20 mM of catechol (or 3 methylcatechol; 4 methylcatechol; 4 nitrocatechol or 1, 2, 4 benzenetriol) as a substrate and incubating for 15 minutes at 30°C. Catechol 1, 2 dioxygenase activity was measured by monitoring the absorbance reading at 260 nm as mentioned in Section 2.2.2 of Chapter 2.

3.2.2.7 Effect of various metal ions on the activity of catechol 1, 2 dioxygenase

A standardized concentration (1.3 µg) of purified C1, 2O was incubated with 0.5 mM of metal salts in 1 ml of 50 mM Tris-Cl (pH 7.5) at 30°C for 15 minutes. Following the incubation of the mixture, 20 µl of 10 mM catechol as substrate was added to the mixture. The mixture with the substrate was then incubacted for another 20 minutes and the absorbance at 260 nm was measured including the incubation mixture without C1, 2O (Pessione *et al*., 2001).

3.2.2.8 The kinetics of the purified catechol 1, 2 dioxygenase

The K_m and V_{max} of the purified catechol 1, 2 dioxygenase was determined by double reciprocal Lineweaver-Burk plots of reciprocal reaction velocities versus reciprocal substrate concentrations. These reactions were conducted as reported in Section 2.2.6 of Chapter 2 (Nadaf and Ghosh *et al*., 2011).

3.2.3 Cloning and expression of catechol 1, 2 dioxygeanse of *Acinetobacter* sp. strain Y64 into *E. coli*

Chromosomal DNA from *Acinetobacter* sp. strain Y64 and pJET1.2/blunt vector (Thermo scientific) were purified using the instruction of the specific purification kits (Qiagen). All the other molecular biology tools such as agarose gel electrophoresis, SDS-PAGE, preparation of competent cells of *E. coli* and transformation were carried out as described by (Sambrook *et al*., 1989) unless specified otherwise.

3.2.1.1 PCR amplification of catechol 1, 2 dioxygenase gene from *Acinetobacter* sp. strain Y64

PCR amplification of *cat*A gene was carried out using *Acinetobacter* sp. strain Y64 genomic DNA as template. The C1, 2O encoding gene *cat*A was amplified using primer pair set: Cat12DOF1 (5' GGA TCCATG CCC GCA GTG CTG CCA 3') and Cat12DOR1 (5' GCGGCCGCT TAA GCG CTA GCG CGA CG 3') designed based on genomic sequence available (Sharma and Lin – unpublished data) with the help of online primer designing software Primer 3 (http://frodo.wi.mit.edu/) (Rozen and Skaletsky, 2000). The forward primer contained a *Bam*HI site while the reverse primer contained a *Not*I site for in frame directional cloning. The concentration of reagents for each PCR reaction (50 µl) was as follows: Primers, 1 µM each; Taq buffer 1×; dNTPs, 200 µM; $MgCl_2$, 1.5 mM, 10 ng of

template DNA and 1 U of Phusion High-Fidelity DNA polymerase (Thermo scientific). The PCR conditions included: 95°C for 5 min (1-Cycle); 95°C for 30 s, 65°C for 30 s, 72°C for 60 s (40-cycles) and final extension for 10 min at 72°C. The amplified product was confirmed using DNA gel electrophoresis (1.2% agarose gel) after staining with 0.5 µg/ml ethidium bromide and viewed on a transilluminator and digital photographs were taken using an Alpha Imager TM 3400.

3.2.1.2 Cloning of catechol 1, 2 dioxygenase gene into pJET cloning vector and transformation of *E. coli* DH5α

Cloning of the amplified 988 bp PCR product into pJET T/A cloning vector (Figure 3.1) (Fermentas, Germany) was carried out as follows. The 947 bp sized band was purified from the gel using the GeneJET Gel Extraction Kit (Thermo scientific) as directed by the manufacturer's protocol. The purified products were directly ligated into the 2974 bp T/A cloning vector pJET (Thermo Scientific) as directed by the manufacturer's protocol with the following modifications:

The ligation mixture (10 µl) consisted of 1 µl pJET vector (~ 200 ng DNA), 1 µl 10× ligase buffer, 1 U T4 DNA ligase, 2 µl PCR product (~ 1:1 molar ratio to vector DNA) and 4.7 µl ddH_2O. The DNA concentration was measured using Nanodrop (Thermo Scientific) and purity was measured using OD_{260}/ OD_{280} ratio. The mixture was incubated 22°C for 5 minutes. The map of pJET containing the *cat*A gene (designated as pVSJ3) is presented in Figure 3.2.

The ligation mixture was transformed into chemically competent *E. coli* DH5α using the method of Griffith, (1928). The transformants were plated on LB agar plates (containing 100 µg/ml ampicillin). Single colonies selected on ampicillin LB agar plates were picked and confirmed for the presence of *cat*A gene using colony PCR. The positive clones were further

confirmed by isolating the plasmids using mini plasmid isolation kit (Thermo Scientific) and restriction digestion of the plasmids with *Bam*HI and *Not*I.

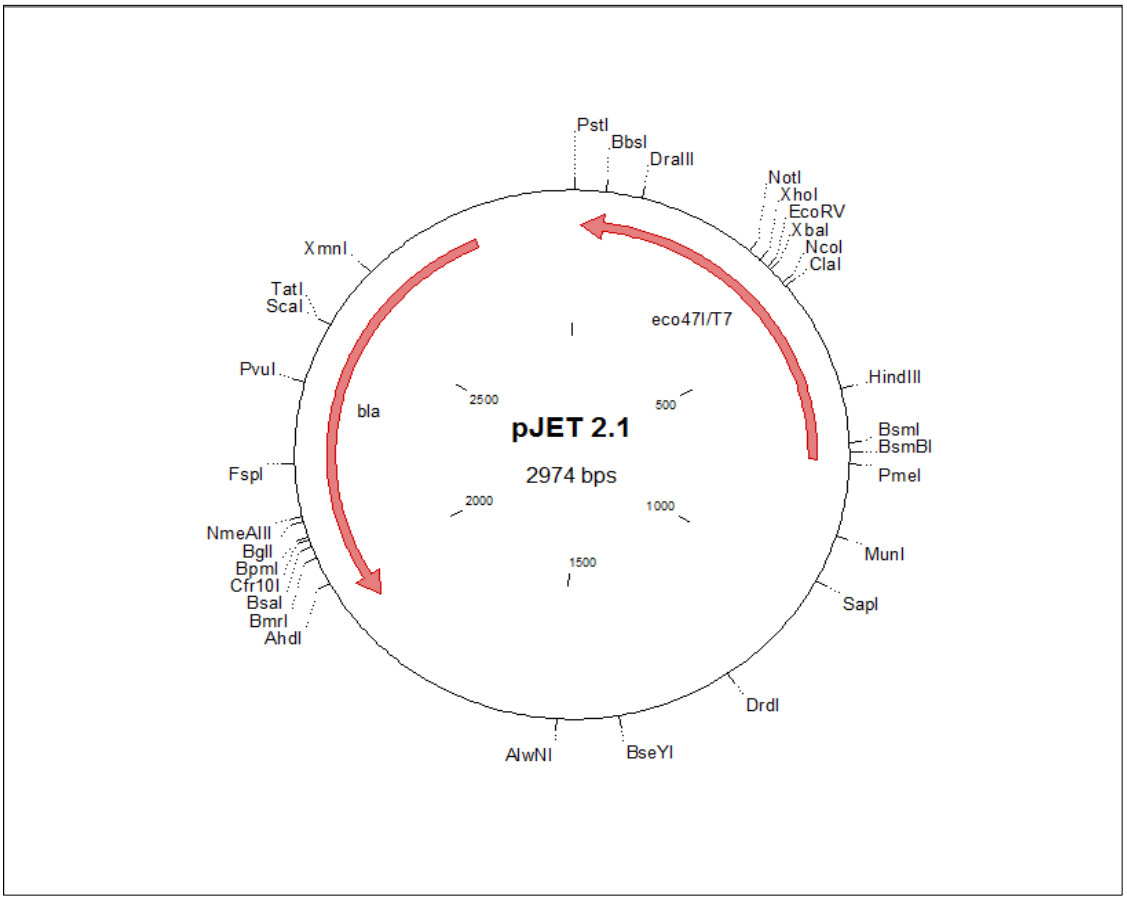

Figure 3.1 pJET1.2/blunt vector (2974 bp) map without the insert.

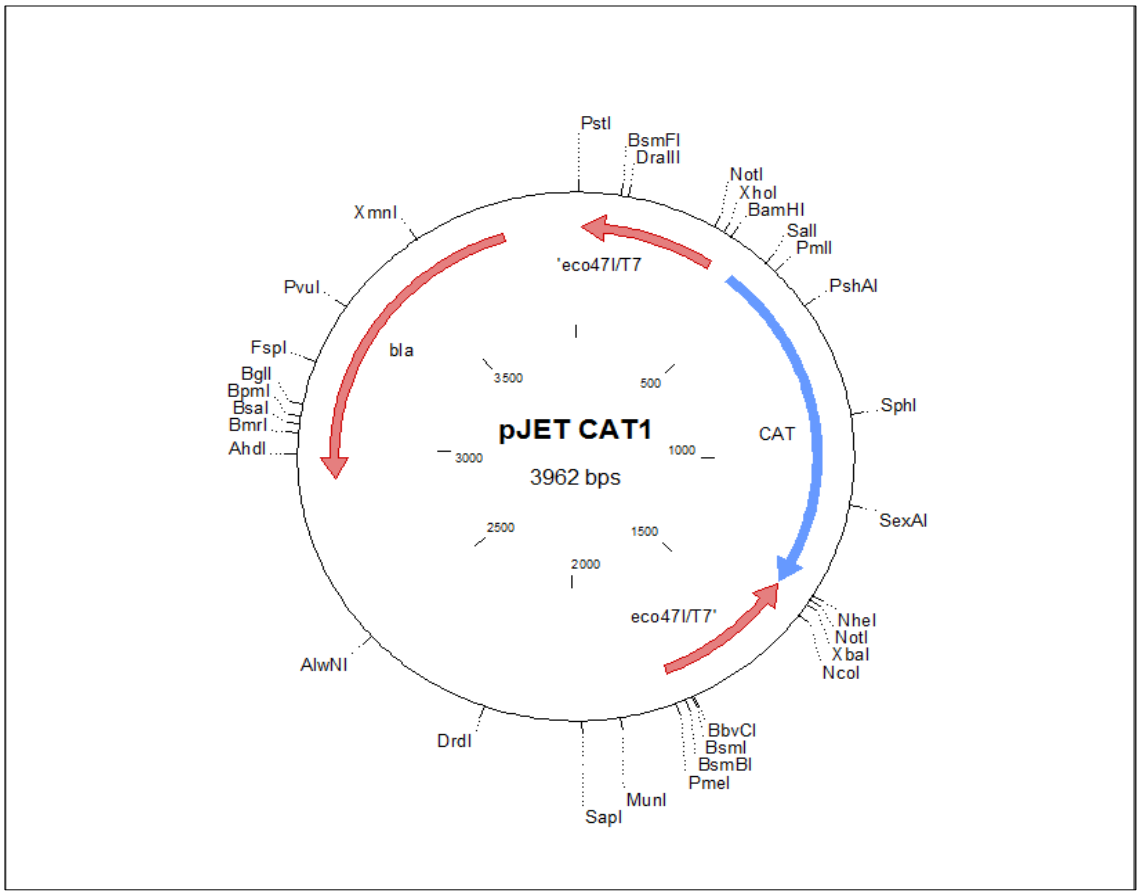

Figure 3.2 pJET containing (988 bp) *cat*A gene from *Acinetobacter* sp. strain Y64. The *cat*A gene of was ligated to *Bam*HI and *Not*I cohesive ends. The size of the plasmid with the insert is 3962 bp.

E. coli DH5α transformant containing the pJET-*cat*A plasmid was inoculated into 5 ml LB broth (containing 100 µg/ml ampicillin) and incubated overnight at 37°C. The pJET-*cat*A plasmid was purified using the mini-Plasmid isolation kit (Thermo Scientific).

3.2.1.3 Construction of pVSJ3 and transformation of *E. coli* BL21(DE3)pLys

Plasmid pJET-*cat*A was digested with *Bam*HI and *Not*I and 988 bp *Bam*HI/*Not*I fragment containing *cat*A gene was ligated with corresponding ends of pET22b expression vector (5493 bp) (Figure 3.3) for expression under T7 promoter. The pelB leader sequence guides

the protein to be secreted in the periplasmic space of the cell. The ligation mixture was then transformed in *E. coli* DH5α using a similar protocol described in Section 3.2.3.2. The transformants were then selected on LB ampicillin plates and confirmed using colony PCR. The plasmids (pVSJ3) (Figure 3.4) were then isolated using the mini plasmid isolation kit (Thermo Scientific) and then restriction digested with *Bam*HI and *Not*I to release 988 bp *cat*A gene. A typical double restriction digest reaction contained 3 U *Bam*HI, 3 U *Not*I, 1 µl 10× fast digest (Fermentas, Germany), 5 µl (300 ng) isolated pVSJ3 or pET22b vector and ddH2O to make the reaction volume to 10 µl. The double restriction digestion reaction mixture was incubated at 37°C for 5 minutes, and analyzed on 1.2% agarose gel stained with 0.5 µg/ml ethidium bromide. The agarose gel was viewed on a transilluminator and digital photographs were taken using an Alpha Imager TM 3400.

The plasmid pVSJ3 and pET22b was further transformed into *E. coli* BL21(DE3)pLys cells using a similar protocol described in Section 3.2.3.2. The transformants were selected on LB plates (containing 100 µg/ml ampicillin and 34 µg/ml Chloramphenicol) and the transformants were confirmed using colony PCR and plasmid endonuclease restriction analysis.

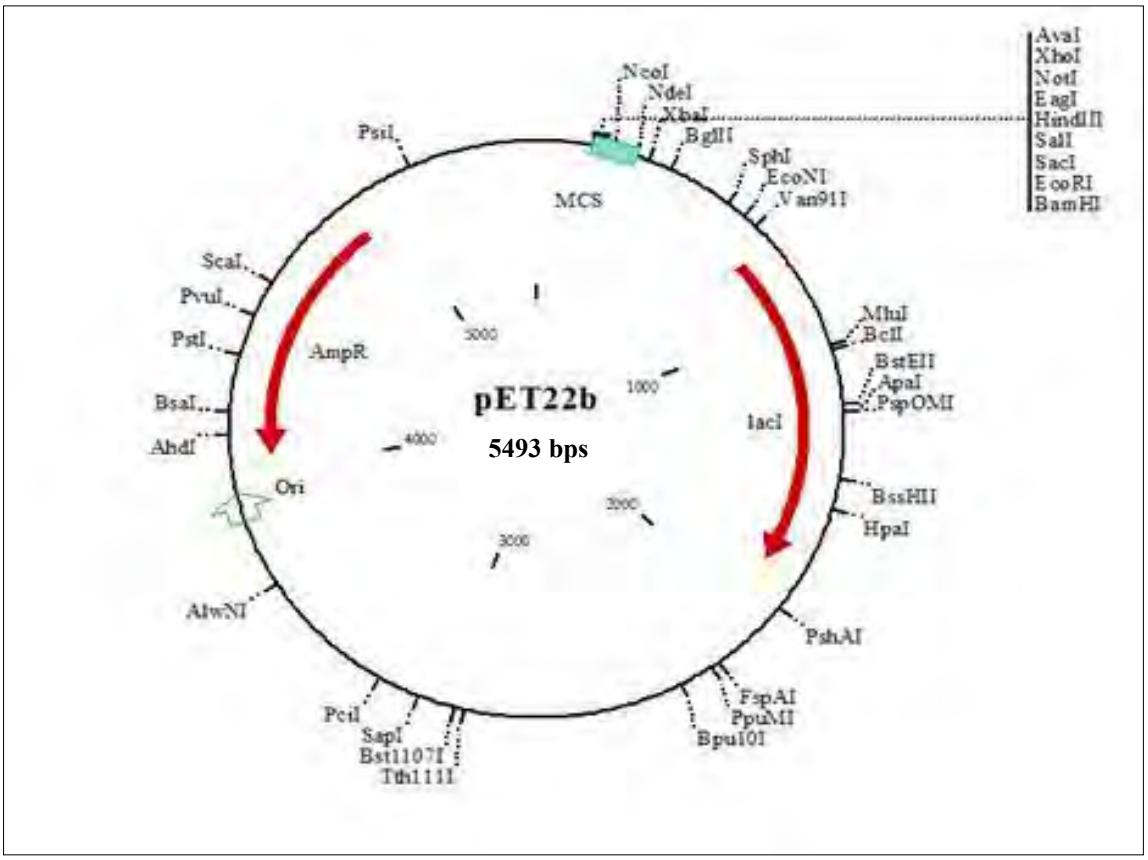

Figure 3.3 The 5493 bp pET22b expression vector. The pET22b expression vector contains *BamH*I and *Not*I restriction sites within the multiple cloning site (MCS) which is used for the insertion of *cat*A gene of *Acinetobacter* sp. strain Y64.

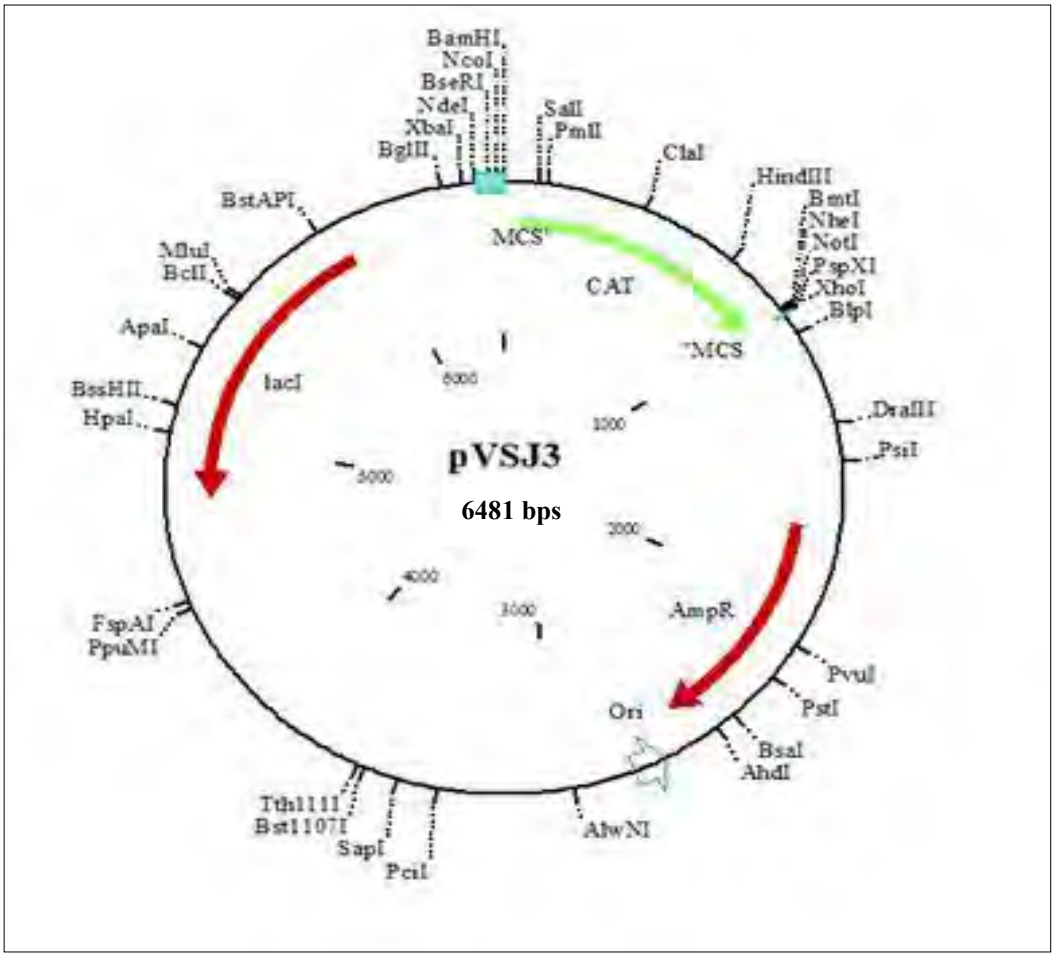

Figure 3.4 The 6481 bp pVSJ3 containing *cat*A gene containing insert ligated in *BamH*I and *Not*I region of the MCS.

3.2.1.4 Induction and expression of catechol 1, 2 dioxygenase in *E. coli* BL21(DE3)pLys

E. coli BL21(DE3)pLys cells harbouring the plasmids pVSJ3 and pET22b (as a control) were grown at 37°C in LB broth (containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol) and incubated overnight. The overnight culture was diluted 100-fold in LB broth containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol and grown for approximately 3 hours in a 2 L flask at 37°C to an optical density 0.4-0.5 at OD_{600}. The *cat*A gene in *E. coli* BL21(DE3) was induced with 1.5 mM of isopropyl-3-D-thiogalactopyranoside (IPTG) for 6 hours. The cells were harvested by centrifugation at 5 000 × *g* for 30 minutes, 4°C and stored at -20°C.

3.2.1.5 Purification and characterization of C1, 2O expressed by *E. coli* transformant

Catechol 1, 2 dioxygenase was harvested from the periplasmic space of the transformants by resuspending the pellet in ice cold sucrose solution (20 mM Tris-Cl pH 8.0; 25% (w/v) sucrose and 5 mM EDTA) for 15 minutes in ice (Vázquez-Laslop *et al*., 2001). The sample was centrifuged at 8 500 × *g* for 20 minutes. The supernatant was decanted while the pellet was saved in ice. The ice cold pellet (1 g) was dissolved in 4 ml of 5 mM $MgCl_2$ (containing 0.5 unit of protease inhibitor cocktail) and incubated for 30 minutes at room temperature. The sample was finally centrifuged at 8500 × *g* for 30 minutes and the supernatant was collected, and stored at – 20°C. The expression of the target protein was assessed by 12% SDS-PAGE stained with Coomasie blue R250 stain.

Further purification and characterization of C1, 2O harvested from *E.* coli BL21(DE3)pLys transformants was done using similar protocols to those described above in Sections 3.2.1.2 – 3.2.2.6 for C1, 2O from *Acinetobacter* sp. strain Y64.

3.3 RESULTS AND DISCUSSION

Catechol 1, 2 dioxygenase catalyzing catechol intradiol cleavage in phenol degrading *Acinetobacter* sp. strain Y64 was purified. The purification of C1, 2O was achieved by ammonium sulfate precipitation and anion exchange chromatography. The 60% saturated ammonium sulfate precipitation recovered 92% yield and increased the specific activity of C1, 2O by 4.8 folds (Table 3.1). After dialysis, the dialysate was loaded onto the Hitrap QFF anion exchange column. One protein peak was obtained at 100% NaCl gradient (Figure 3.5). However, the peak containing active C1, 2O was eluted out at 0.4 M NaCl (100% gradient) and fractions containing pure C1, 2O were carefully pooled from the peak indicated by a pointer in Figure 3.5 to avoid protein cross contamination. The elution of C1, 2O at 0.4 M NaCl (100% gradient) indicated strong binding of C1, 2O to the Hitrap QFF anion exchange column. Other proteins that did not bind strongly to the column were eluted before the NaCl gradient. The unbound proteins (eluted before NaCl gradient) showed no C1, 2O activity. The enzyme activity and protein concentration of the samples in each step were determined. The results are summarized in Table 3.1.

Table: 3.1 Purification of catechol 1, 2 dioxygenase from *Acinetobacter* sp. strain Y64.

Purification steps	Volume (ml)	Total Activity (U)	Total protein (mg/ml)	Specific activity (U/mg)	Yield (%)	Purification folds
Crude extract	**80**	**175.87**	**123.6**	**1.422**	**100**	**1.0**
60% Ammonium sulphate Saturation precipitation	**45**	**161.30**	**23.7**	**6.812**	**92**	**4.8**

Hitrap QFF	**20**	**85.40**	**5.6**	**15.125**	**49**	**10.6**

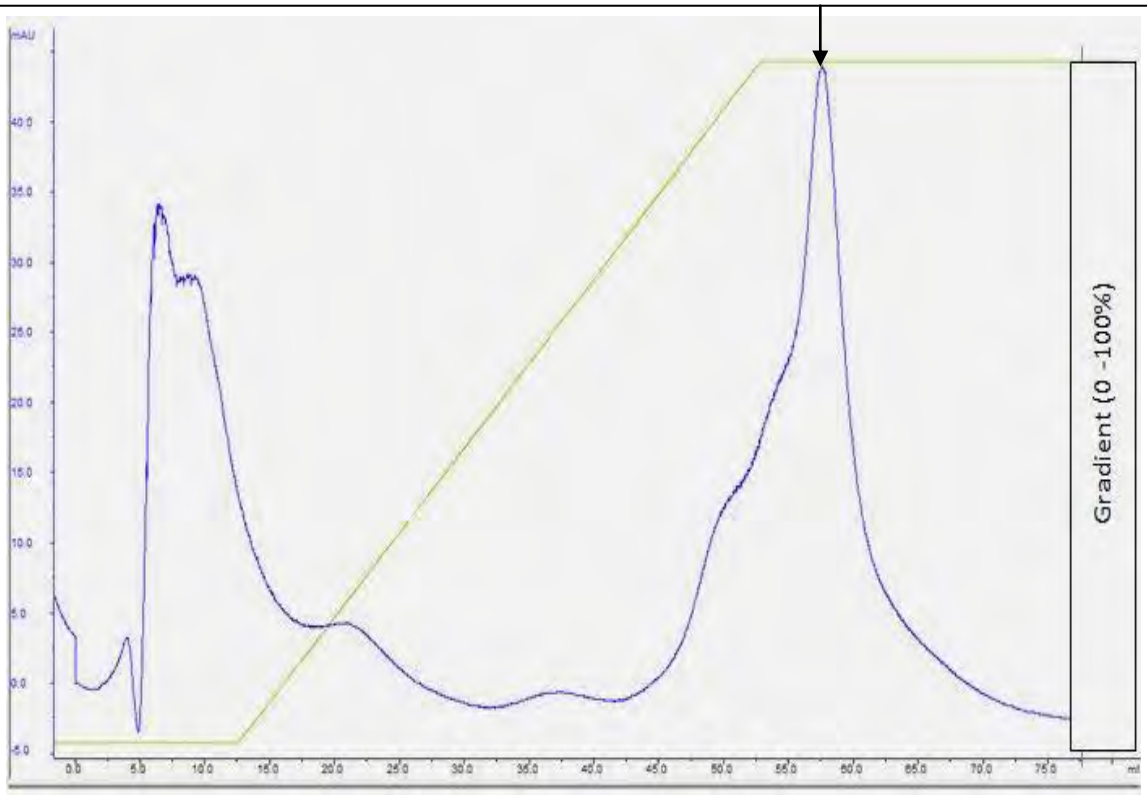

Figure 3.5 Hitrap QFF Anion exchange chromatography of catechol 1, 2-dioxygenase from *Acinetobacter* sp. strain Y64.The arrow indicates the peak containing the C1, 2O activity. The 0.4 M NaCl gradient is also shown (100% B: 0.4 M NaCl, 50 mMTris-Cl pH 7.5).

A study conducted by Wang *et al*. (2006) recovered 27% yield after ammonium sulfate precipitation during the purification of C1, 2O from *Pseudomonas aeruginosa* TKU002. The purification procedure used in the current study resulted in a 49% yield and an 11 fold increase in specific activity of C1, 2O purified from strain Y64 (Table 3.1).

The purification of C1, 2O was further analyzed on 15% SDS – PAGE (Figure. 3.6) and characterized. The purified protein was approximately 36 kDa as estimated from the SDS-

PAGE gel (Figure. 3.6) and confirmed with the genomic data (Sharma and Lin unpublished data) and equivalent to the molecular weight reported by Aoki *et al*. (1984)(b) which was 36 kDa. Patel *et al* (1976) reported an 80 kDa dimer for C1, 2O purified from *Acinetobacter calcoaceticus*. Pessione *et al* (2001) purified two C1, 2Os of the molecular weight 38.7 kDa and 37.6 kDa, respectively from *Acinetobacter radioresistens*.

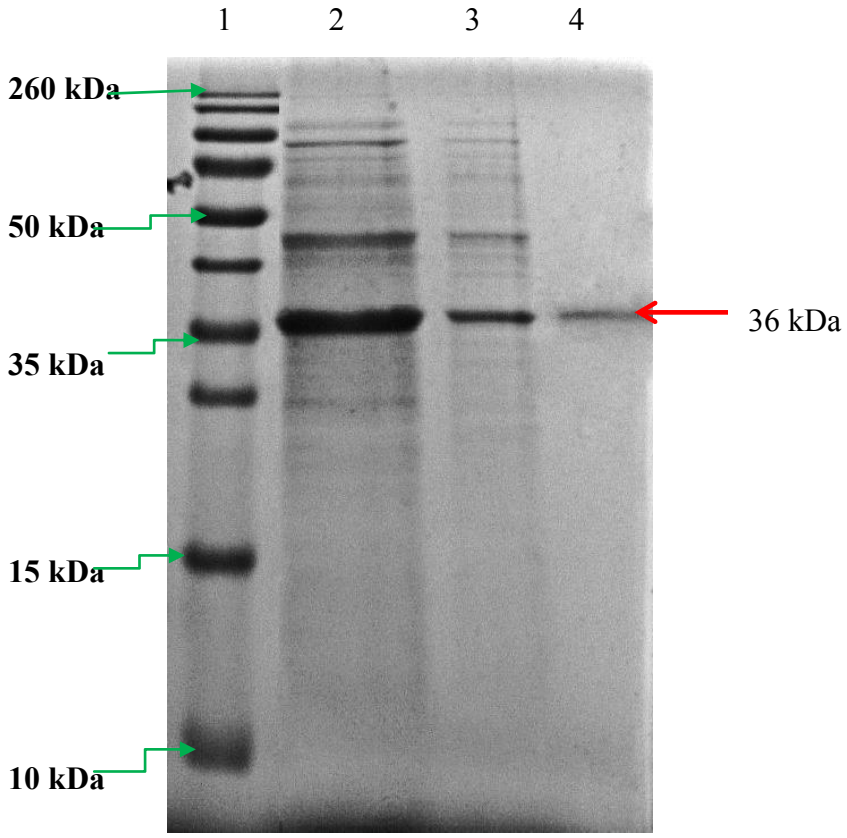

Figure 3.6 SDS-PAGE (15%) of the purified catechol 1, 2-dioxygenase from *Acinetobacter* sp. strain Y64 Lane 1: protein standards, molecular masses (10 – 260 kDa) are indicated on the left; lanes 2 and 3: lysate and 60% saturated ammonium sulfate precipitation of cell debris, respectively; lane 4: purified C1, 2O (1.5 µg) after anion exchange chromatography.

The purified catechol 1, 2 dioxygenase activity increased significantly with increasing temperature from 25 to 36°C (Figure 3.7). However, a significant loss (80%) in enzyme activity was observed when the temperature was raised to 45°C, while approximately 20% activity was observed at 50°C. Thus, C1, 2O purified from *Acinetobacter* sp. strain Y64 has

maximum activity of 36°C. The optimum temperature curve of the purified C1, 2O is similar to that observed for the crude extract in Chapter 2 (Figure 2.6).

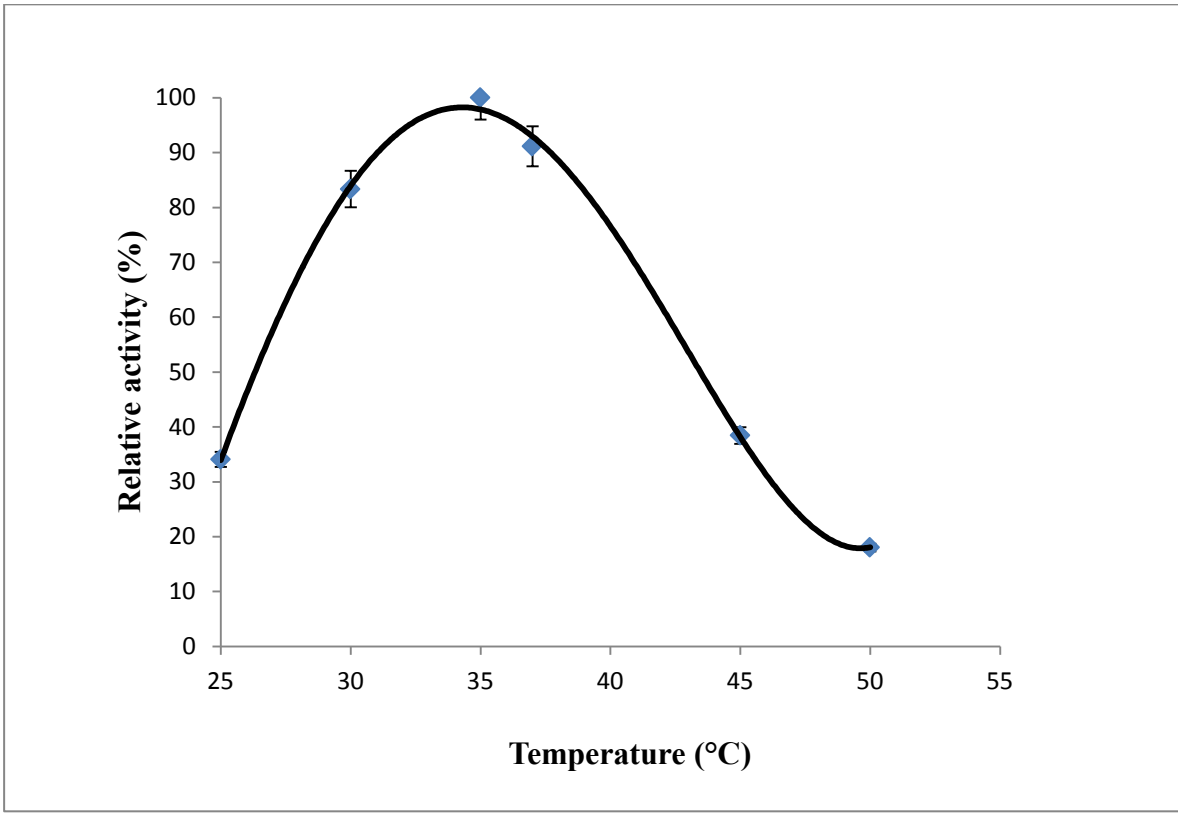

Figure 3.7 Optimum temperature curve of C1, 2O purified from *Acinetobacter* sp. strain Y64.

Catechol 1, 2 dioxygenase purified from *Acinetobacter* sp. strain Y64 showed optimal activity at pH 7.5 (Figure 3.8). Less than 5% activity was detected when pH was less than 6 or higher than 11. On the contrary, the optimal pH curve is different from the curve of the crude enzymes observed in Chapter 2 (Figure 2.5) suggesting two C1, 2Os produced during phenol degradation. While the purified C1, 2O performs optimally at pH 7.5, perhaps the other C1, 2O performs optimally at pH 4.5 specifically. Many other catechol 1, 2 dioxygenases have been reported with optimal pH 7.5 (Patel *et al*., 1976; Akoi *et al*., 1984; Nakai *et al*., 1990; Wang *et al*., 2006). Higher optimal pHs (pH 8.6) have also been reported by Murakami *et al* (1998), while pH 9 has been the highest reported optimal pH (Strachan *et al*., 1998). No optimal pH lower than pH 6 has yet been reported.

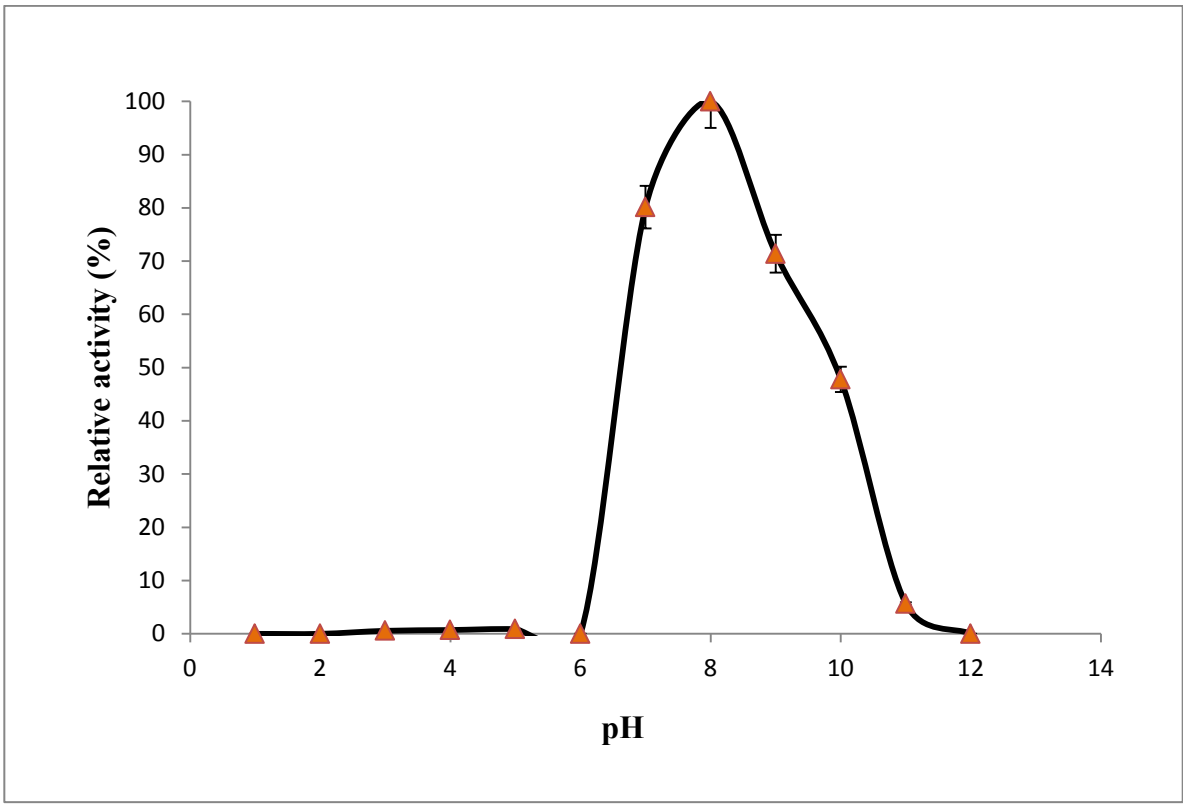

Figure 3.8 Optimum pH curve of the purified C1, 2O from *Acinetobacter* sp. strain Y64.

Catechol 1, 2 dioxygenase purified from *Acinetobacter* sp. strain Y64 lost about 10% activity in the first hour and remained stable throughout the experiment at 37°C (Figure 3.9). However, at 40°C, C1, 2O purified from strain Y64 lost approximately 20% activity every hour for the first 3 hours throughout the experiment. At 45°C C1, 2O lost 50% activity in the first 2 hours and a complete loss in enzyme activity was observed after 3 hours. Catechol 1, 2 dioxygenase purified from strain Y64 lost approximately 90% activity in an hour at 50°C and 55°C, respectively. Catechol 1, 2 dioxygenase isolated from *S. maltophilia* was shown to be unstable at 40°C with a half-life of 3 hours and lost 16.5% of its enzyme activity at 50°C, and a drastic decrease in activity was reported at 55°C (Guzik *et al*., 2011a). Thus, the data obtained on the thermal stability of purified C1, 2O from strain Y64 is comparable to those of

catechol 1, 2-dioxygenase isolated from *Arthrobacter* species BA-5-17 and *Pseudomonas aeruginosa* (Murakami *et al*., 1998; Wang *et al*., 2006).

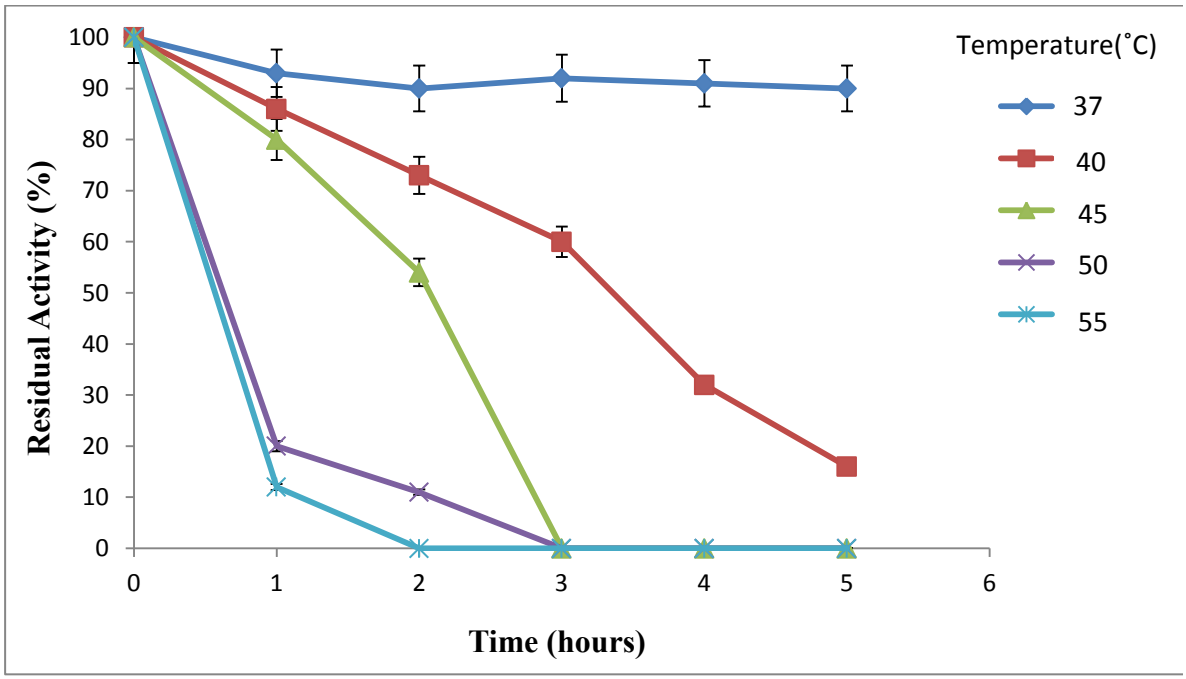

Figure 3.9 Thermostability of C1, 2O purified from *Acinetobacter* sp. strain Y64 evaluated as residual percent of activity *versus* time (hours). The activity was determined at 37 °C in 0.05 M Tris-HCl buffer, pH 8, using 3 µM of purified C1, 2O, where, each point of the curve is the result of average of three experiments.

The purified C1, 2O from strain Y64 was found to be stable at pH 7 and pH 8 (Figure 3.10). The purified C1, 2O lost 20% activity at pH 8 after 4 hours and remained stable for 2 hours of the experiment, while at pH 9 approximately 30% activity was lost every hour. C1, 2O of strain Y64 lost approximately 40% activity after 6 hours while a similar activity trend was observed for pH 3 and pH 10; where approximately 80% C1, 2O activity was lost in the first 2 hours followed by a quick complete loss of enzyme activity after 3 hours. No C1, 2O activity was observed at pH 6 and the results is reproducible (n = 3) and different buffers were tested. The activity of C1, 2O is affected at strong acidic and basic pH ranges, respectively. However, at strong acidic pH ranges amino acids such as aspartic acid and/or glutamic acid are perhaps available at the active site of C1, 2O of strain Y64 and neutralized

at strong acidic pH ranges. Similar pH stability results were observed for catechol 1, 2 dioxygenase isolated from *S. maltophilia* KB2 which lost all of its enzymatic activity at pH 2.2 and around 83% of the activity was lost a pH 12.0 (Guzik *et al*., 2011a; Nadaf and Ghosh 2011; Saxena and Thakur 2005; Wang *et al*. 2006). Studies conducted by Pessione *et al*. (2001) showed a variation on the sensitivity of C1, 2Os to different pH ranges (4 – 10).

Broad substrate specificity of the enzyme is another interesting character noted amongst the isofunctional C1, 2Os from various sources. The relative activities (in percentage) of C1, 2O purified from *Acinetobacter* sp. strain Y64 towards various substrates are presented in Table

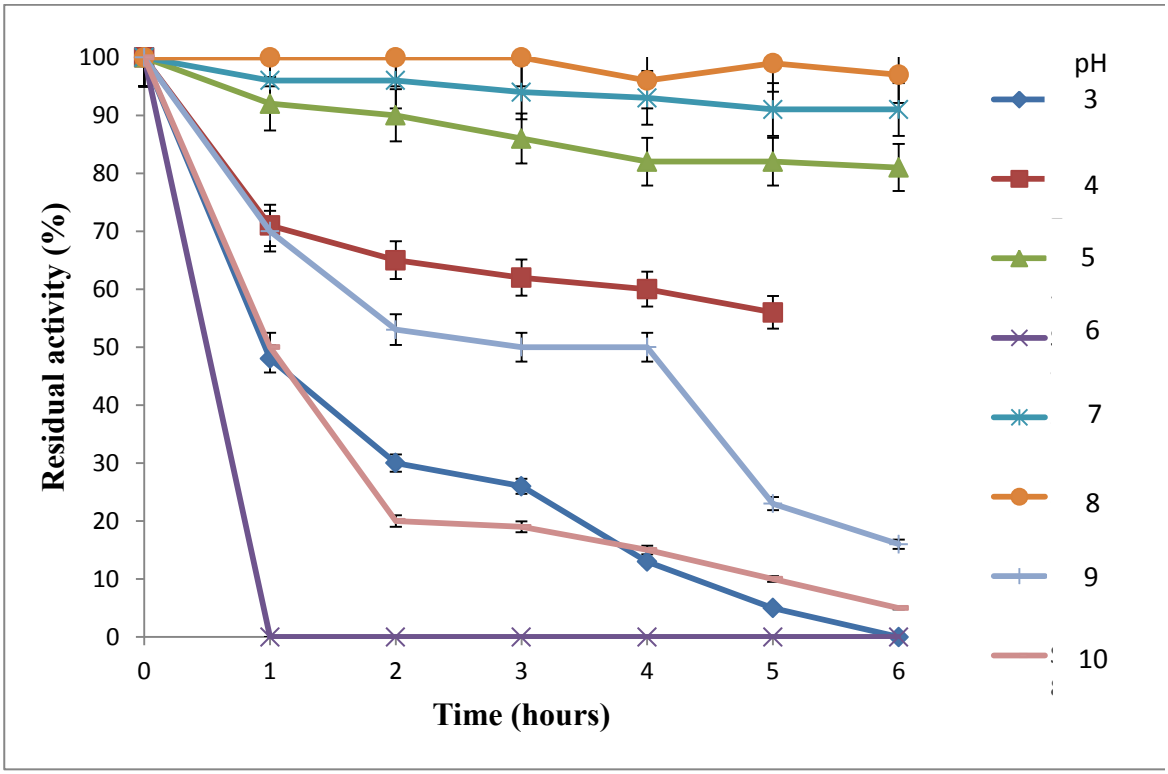

Figure 3.10 pH stability of C1, 2O purified from *Acinetobacter* sp. strain Y64 expressed as residual percent of activity *versus* time (hours) each point of the curve is the result of an average of three experiments. The standard deviation does not exceed 5% and the results are reproducible.

3.2. Intradiol cleavage by the purified C1, 2O was observed on catechol (100%), 4 – methycatechol (61%), 4 – nitrocatechol (80%) and 1, 2, 4 Benzenetriol (62%) respectively. Interestingly, less relative activity was observed for 3 – methylcatechol (2%) (Table 3.2). These findings importantly confirm the stereoselectivity of C1, 2O purified from

Acinetobacter sp. strain Y64. Catechol 1, 2 dioxygenase of strain Y64 is specific for phenolic compounds with groups attached at carbons 1, 2 and 4 of the benzene ring. However, the attachment of a group on carbon 3 of the benzene ring does not fit to the active site of the purified C1, 2O perhaps due to sterric hindrance. Briganti *et al*. (1997) showed intradiol cleavage of both methyl substituted catechols; 3 – methylcatechol (14.4%) and 4 – methylcatechol (16.5%), respectively with C1, 2O purified from *A. radioresistens*. However, Wang *et al*. (2006) showed 43%, 12% and 10% of C1, 2O activity for 4 – methylcatechol, 3 – methylcatechol, 4 – chlorocatechol (10%), respectively when evaluating the substrate specificity of C1, 2O purified from *P. aeruginosa* TKU002. C1, 2O purified from *P. aeruginosa* TKU002 showed no activity for 4 – nitrocatechol. On the contrary, C1, 2O purified from *Acinetobacter* sp strain Y64 has higher activity for 4 – substituted catechol in contrast to C1, 2Os reported by Briganti *et al*. (1997) and Wang *et al*. (2006).

Table: 3.2 Substrate specificity of catechol 1, 2 dioxgenase from *Acinetobacter* sp. strain Y64.

Substrate	Relative activity (%)
Catechol (control)	100
3 – methylcatechol	2
4 – methylcatechol	61
4 – nitrocatechol	80
1, 2, 4 Benzenetriol	52

Metal ions may cause alterations in enzyme conformation such as a reduction in β sheets and α-helices. These conformational alterations may lead to substitution of original metal ions

with other metal ions at the enzyme's active site, leading to partial or a total loss of enzyme activity, depending upon metal ion (Wang *et al*., 2006). Conversely, the loss of C1, 2O activity could be due to the binding of thiol groups of the enzyme to transition metals which in turns inactivate the enzyme. Hence it is necessary to evaluate the resistance of C1, 2O to inhibitors such as metal ions. The effect of various metal salts, on the activity of C1, 2O was tested using catechol as the substrate (Table 3.3). The activity of C1, 2O purified from *Acinetobacter* sp. strain Y64 was increased in the presence of Fe^{2+} and Fe^{3+}. However, the purified enzyme was sensitive to EDTA and Ca^{2+} while Hg^{2+} had less effect on the purified enzyme. Fe^{2+} and Fe^{3+} were suggested to be essential for C1, 2O activity, while EDTA was suggested to remove metal at the active of purified C1, 2O (Miyazawa *et al*., 2004). In contrast Ca^{2+} competes with Fe^{2+} and Fe^{3+} at the metal binding site of C1, 2O (Miyazawa *et al*., 2004).

Table: 1.3 Effect of various compounds on enzyme activity of catechol 1, 2 dioxygenase.

Compound	Concentration (mM)	Relative activity (%)
None (Control)	0.5	100
Hg^{2+}	0.5	95
Fe^{2+}	0.5	144
Ca^{2+}	0.5	58
EDTA	0.5	50
Fe^{3+}	0.5	164

The activity of purified C1, 2O is not strictly dependent on the presence of the cofactor Fe^{2+} or Fe^{3+}, however the presence of the cofactor enhances the activity of C1, 2O purified from

Acinetobacter sp. strain Y64. Guo *et al*. (2009) showed that the activity of C1, 2O isolated from *Sphingomonas xenophaga* QYY was increased in the presence of Pb^{2+}, Mg^{2+}, K^{+}, Fe^{3+} and Ca^{2+}, while the addition of Mn^{2+}, Hg^{2+}, Ni^{2+} and Co^{2+} decreased the enzyme activity. However, a complete loss of C1, 2O activity was seen in the presence of Cu^{2+}, Zn^{2+} and Fe^{2+} ions (Guo *et al*., 2009). C1, 2O isolated from *Pseudomonas aeruginosa* was reported sensitive to the presence of Mg^{2+}, Fe^{2+}, and Ca^{2+}, while the presence of Mn^{2+}, Cu^{2+}, and Ag^{+} inhibited the enzyme (Wang *et al*., 2006). In a study conducted by Nadaf and Ghosh (2011) and Li *et al*. (2012) C1, 2O isolated from *Rhodococcus* sp. NCIM 2891 was completely inhibited by the presence of Fe^{3+}, Cu^{2+} and Hg^{+}. In contrast, C1, 2O from *Gordonia polyisoprenivorans* was resistant to Fe^{3+}, Cu^{2+} and Hg^{+} (Camargo *et al*. (2012).

A direct increase in the initial rate of C1, 2O reaction (V_0) with increasing concentration of catechol to 100 µM was observed (Figure 3.11). However, an increase in concentration of catechol after 100 µM lead to a a constant $V_{0,}$ suggesting a saturation in the active site of C1, 2O. Hence the kinetics of catechol 1, 2 dioxygenase has been shown to fit the Michaelis – Menten mode of enzyme kinetics and similar to the finding in Chapter 2 (Figures. 2.7 and 2.8). The K_m and V_{max} parameters were calculated by monitoring the activity of catechol 1, 2 dioxygenase purified from *Acinetobacter* sp. strain Y64 at various substrate concentrations (Figure. 3.12). The K_m and V_{max} values of C1, 2O purified from strain Y64 for catechol were found to be 17.53 µM and 1.95 U/mg of protein, respectively. Guzik *et al*. (2011a) showed K_m and V_{max} values to be 12.18 µM and 1 218.8 U/mg of protein, respectively, for *S. maltophilia* KB2. The V_{max} value of *S. maltophilia* is remarkably higher than the activity of strain Y64 and other reported catechol 1, 2 dioxygenases in the literature. Briganti *et al*. (2000) reported V_{max} of 25.8 U/mg of protein for catechol 1, 2 dioxygenase isolated from *Acinetobacter radioresistens*, while Suvorova *et al*. (2006) and Solyanikova *et al*. (2009)

showed V_{max} values of 9.6 and 55.5 U/mg of protein for catechol 1, 2-dioxygenase isolated from *Rhodococcus opacus* 1CP and *R. opacus* 6a, respectively. On the contrary the K_m value of *S. maltophilia* was comparable to C1, 2O purified from *Acinetobacter* sp. strain Y64; hence the affinity of C1, 2O purified from strain Y64 may be compared to that of *S. maltophilia*. However, C1, 2O purified from *Acinetobacter* sp. strain Y64 has high K_m value when compared to the K_m value was reported by Wang *et al*. (2006) and Nadaf and Ghosh (2011).

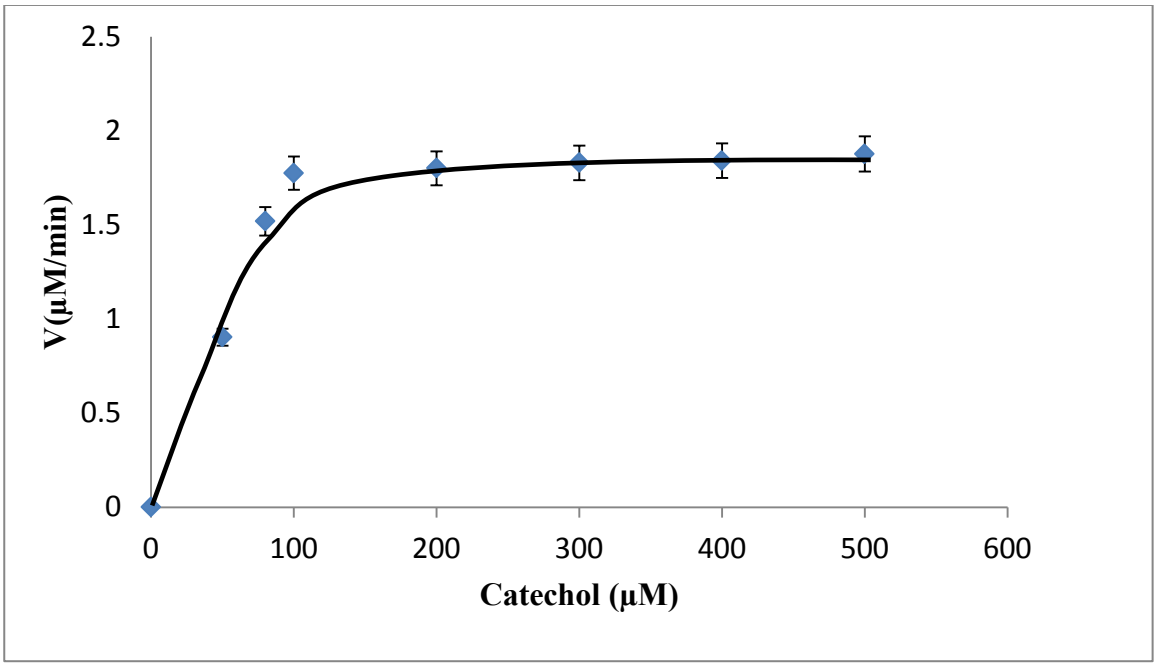

Figure 3.11 Kinetics of the purified catechol 1, 2 dioxygenases with increasing substrate concentration (0 – 600 µM catechol).

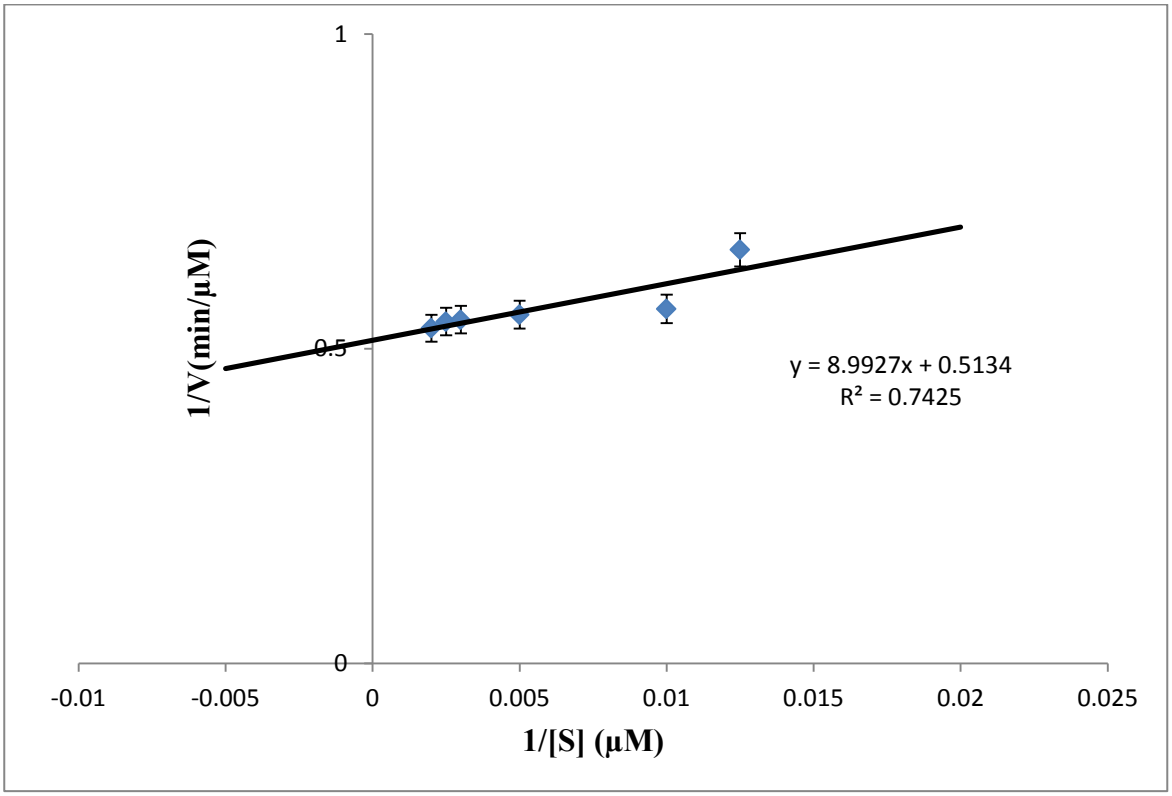

Figure 3.12 Determination of K_m and V_{max} on Lineweaver-Burk double reciprocal plot.

Table 3.4 Summary of K_m and V_{max} of catechol 1, 2 dioxygenase after purification from *Acinetobacter* sp. strain Y64 and *E. coli* transformants.

Source of Catechol 1. 2-dioxygenase	**Substrate**	**K_m(µM)**	**V_{max}(U/mg)**
Crude extract of *Acinetobacter* sp. strain Y64	Catechol	93.53	5.05
Purified from *Acintebacter* sp. strain Y64		17.53	1.95
Purified from *E. coli* transformants		17.49	1.91

Cloning of C1, 2O into pJET 1.2/Blunt vector (2974 bp) was confirmed via amplification of the *cat*A gene using PCR. The 988 bp product was in approximate according to the number

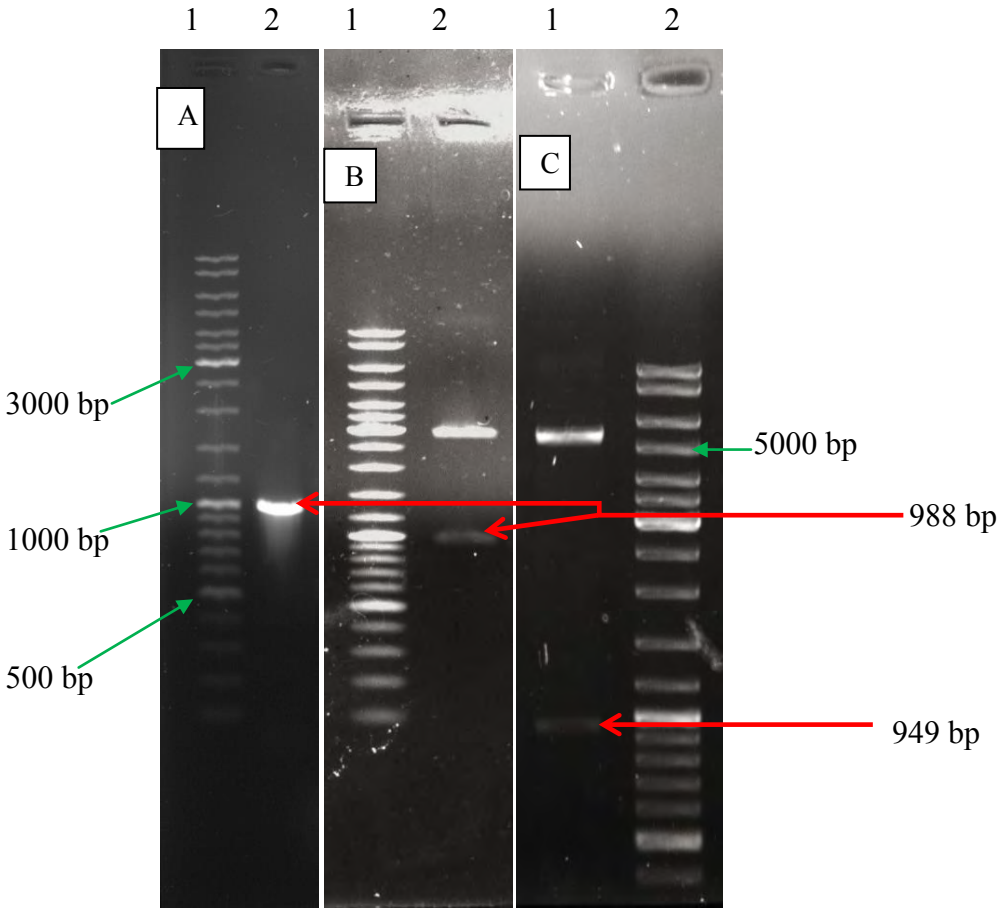

Figure 3.13 The visualization of product of PCR amplification of the entire C1, 2O gene from *Acinetobacter* sp. strain Y64, Lanes: 1A - Molecular size standard (10 kbp DNA ladder), 2A – Product of amplification (988 bp); Lanes: 1B - Molecular size standard (10 kbp DNA ladder), 2B – pJET cloning vector (2974 bp) and *BamH*I – *Not*I released product (988 bp); Lanes:1C – pVSJ3 (5493 bp) and *Bam*HI – *Hind*III release (949 bp), 2C – Molecular size standard (3 kbp DNA ladder).

of base pairs predicted from genomic sequences available (Sharma and Lin – unpublished data). The PCR product obtained was subsequently cloned into pET22b expression vector (designated pVSJ3) (5493 bp). The PCR amplification product of the *cat*A gene (988 bp) is shown in Figure 3.13 A. Figure 3.13 B and C shows the release of the cloned inserts (988 bp and 949 bp) from pJET 1.2/Blunt vector and pET22b expression vector, respectively. The restriction enzyme *Hind*III was used as an alternative to confirm the cloning of the *cat*A gene

in pET22b when *Not*I was scarce in the research laboratory. Further confirmation of clones with colony PCR revealed 10 recombinant strains, each containing the 988 bp insert. The

Figure 3.14 Catechol 1, 2 dioxygenase gene sequence of *Acinetobacter* sp. strain Y64.

plasmid with the insert was sequenced and the results are shown in Figure 3.14 and DNA sequence of the *cat*A gene of pVSJ3 campared with the same DNA sequence of strain Y64 showed 100% match. The sequencing data revealed in-frame initiation and termination codons, confirming that the entire gene has been successfully cloned.

The amino acid sequence of cloned C1, 2O form *Acinetobacter* strain Y64 alignment with other non-heme iron intradiol cleaving enzymes is shown in Figure 3.15. The closest alignment was observed with the catechol 1, 2 dioxygenase from Gram-negative bacteria such as *Burkholderia* sp. strain TH2 (Suzuki *et al*., 2001) and *A. radioresistens* (Briganti *et al*., 1997), having 262 and 251 amino acid residues respectively, and are 60% and 80% identical with *Acinetobacter* sp. strain Y64.

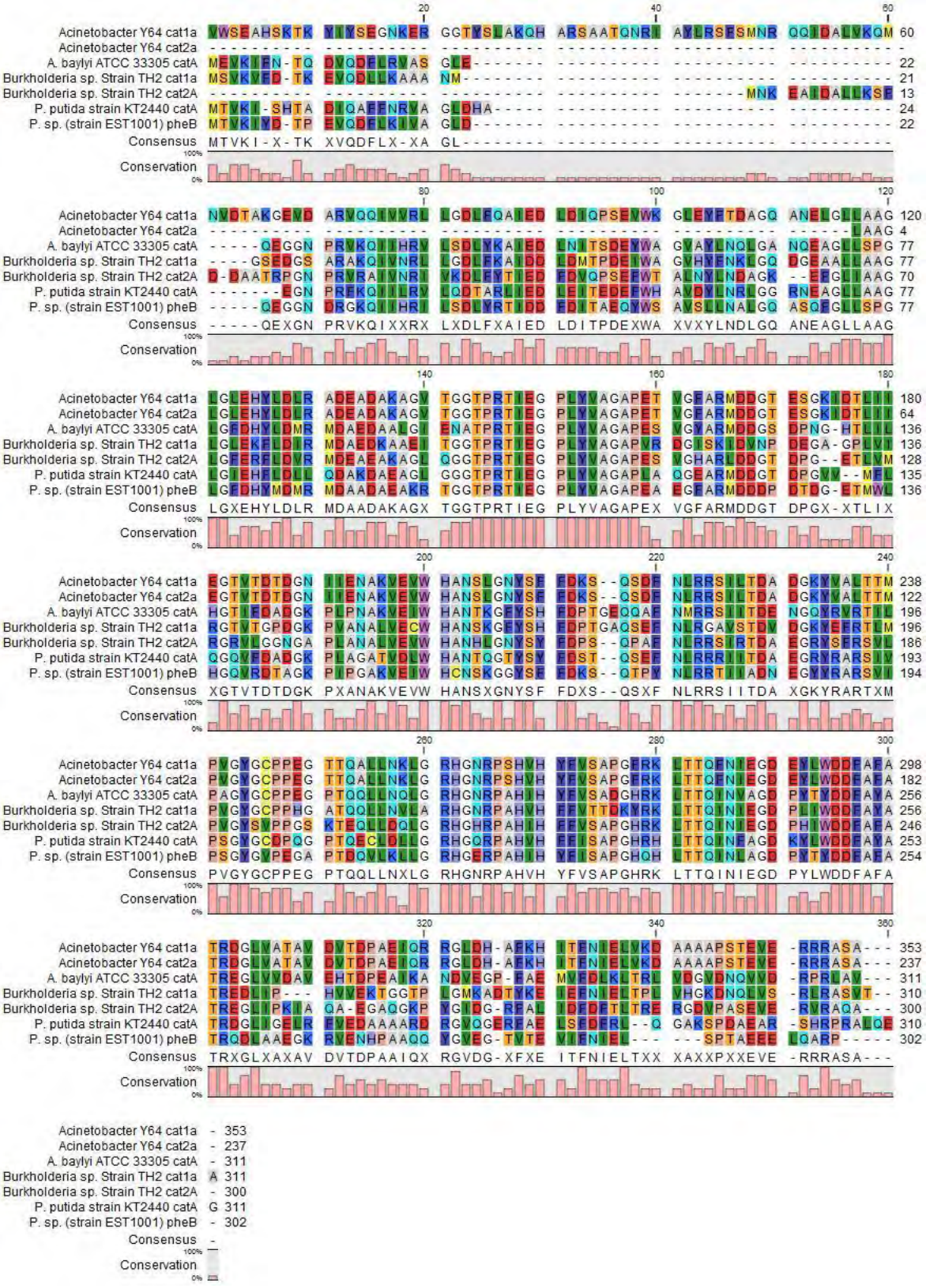

Figure 3.15 Amino acid sequence of C1, 2O derived from the cloned DNA sequence of *Acinetobacter* sp. strain Y64 and its alignment with other C1, 2O amino acid sequences from other bacteria.

Catechol 1, 2 dioxygenase of *Acinetobacter* Y64 was expressed in *E. coli* found along with the periplasmic space proteins and referenced by the purified C1, 2O of strain Y64 (Figure 3.16). The periplasmic space proteins were harvested and C1, 2O was purified using similar protocols described above in Section 3.2.1.2. Catechol 1, 2 dioxygenase was expressed

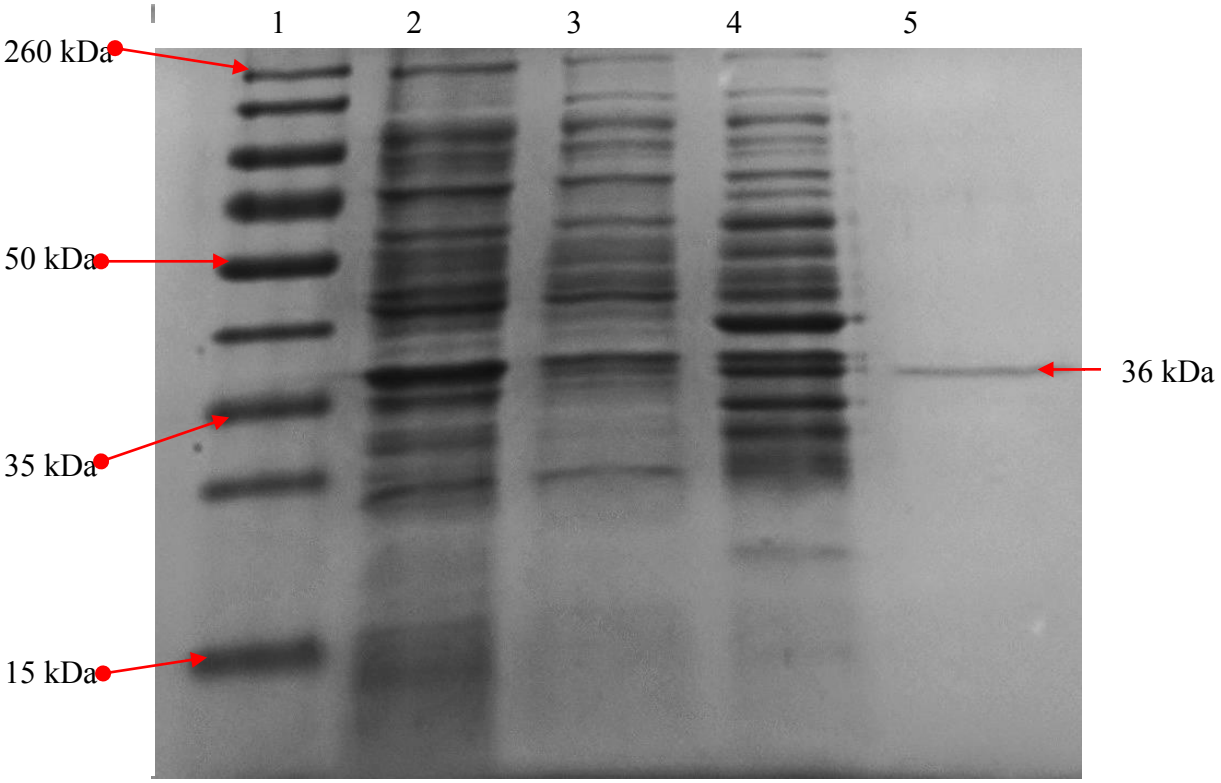

Figure 3.16 Expression of C1, 2O in *E. coli* BL 21 DE3 (pLys); Lanes: 1 – (Thermo Scientific Broard Spectrum) protein standards molecular weight marker (15 – 260 kDa), lane 2 – lysate of transformed *E. coli* before induction (Control), lane 3 – lysate of transformants after induction and extraction of periplasmic space proteins; lane 4 – Proteins extracted from periplasmic space of transformants, lane 5 – purified enzyme of *Acinetobacter* sp. strain Y64.

poorly in *E. coli* BL21DE3(pLys) while the expressed enzyme had the same specific activity as the wild-type enzyme. The K_m and V_{max} values of C1, 2O purified from *E. coli* were found to be 17.49 µM and 1.91 U/mg of protein respectively, similar to that of strain Y64.

The optimal pH and temperature of C1, 2O purified from *E. coli* transformants was 7.5 and 36°C, respectively; similar to results obtained for C1, 2O purified from strain Y64. Furthermore C1, 2O purified from *E. coli* transformants has a molecular weight of 36 kDa

suggesting a successful cloning and expression of C1, 2O of *Acinetobacter* sp. strain Y64 in *E. coli* transformants.

Enzymes with potential biotechnological applications, specifically for industrial scale chemical synthesis have been under investigation for a few decades, since these enzymes exhibit stereoselectivity and regioselectivity (Ran *et al*., 2008). Stereoselectivity and regioselectivity characteristics of the enzymes (such as C1, 2Os) are essential during the production of stereoisomers (like *cis, cis* muconic acid) unattainable by conventional chemical methods (Gibson *et al*., 2000). Other potential biotechnological applications of constituent enzymes obtained from phenol degrading microorganisms include their use in diagnostic systems and in bioreactors for the removal of toxic pollutants (Eskinjia *et al*., 1995; Ali *et al*., 1998; Nakajima *et al*., 2002). *Cis, cis* muconic acid is an essential metabolite required for the synthesis of benzene – free adipic acid (Kuwahara *et al*., 1977; Gomi *et al*., 1988; Mizuno and Yoshikawa, 1990). However, the production of *cis, cis* muconic acid and its further conversion to adipic acid for the production of nylon is achieved by unfriendly conventional chemical processes (Niu *et al*., 2002). Thus, C1, 2Os provides a promising environmentally friendly alternative to the synthesis of muconic acid from phenolic compounds (Niu *et al*., 2002).

3.4 CONCLUSIONS

Catechol 1, 2 dioxygenase (C1, 2O) of *Acinetobacter* sp. strain Y64 with the molecular weight of 36 kDa has been purified. The optimum pH and optimum temperature of purified C1, 2O is 8 and 36°C, respectively. Furthermore, the purified C1, 2O of strain Y64 has high specific activity and a K_m value (17.53 μM) for catechol. The activity of purified C1, 2O of strain Y64 is affected by methyl substitution at the third carbon of the benzene ring. C1, 2O purified from strain Y64 further shows at least 50% activity at 45°C for 2 hours. Fe^{2+} and Fe^{3+} were observed to enhance the activity of the purified C1, 2O, whilst resistance to other respective metals ions decreasing C1, 2O activity was observed. Hence, purified C1, 2O possess applicable features for potential biotechnological application(s) in the industry such as the production of *cis, cis* muconic acid. The properties of the purified C1, 2O from the strain Y64 and transformants were similar, suggesting a successful cloning. However, an increase in the quantity of C1, 2O expressed in *E. coli* requires improvement.

CHAPTER 4

CONCLUSIONS

Acinetobacter sp. strain Y64 is capable of growing on 1000 mg/L of phenol as a sole carbon source. Phenol hydroxylase and catechol 1, 2 dioxygenase is the key enzyme at the rate determining step during phenol degradation. *Acinetobacter* sp. strain Y64 possesses two C1, 2Os (*iso*A and *iso*B) molecular weights 36 kDa and 18 kDa, respectively. The two C1, 2Os were induced in the presence of phenol. The expression of the two C1, 2Os in strain Y64 is not simultaneous, rather *iso*A is expressed earlier and later on followed by the expression of *iso*B after 11 hours of growth in phenol. The optimum pH curve for C1, 2O isolated from strain Y64 showed two peaks suggesting isozymes. Two pH optima were observed for crude extracts containing C1, 2O from *Acinetobacter* sp. strain Y64, pH 4.5 and 7.5, respectively. However the optimum temperature of C1, 2O isolated from strain Y64 is 37°C. Catechol 1, 2 dioxygenase of strain Y64 has affinity for catechol (K_m = 93.53 μM; V_{max} = 5.05 U/mg) before purification.

Catechol 1, 2 dioxygenase (36 kDa molecular weight) of *Acinetobacter* sp. strain Y64 has been purified. The specific activity of C1, 2O the purified from strain Y64 is 15.125 U/mg of protein. While the K_m value (17.53 μM) of purified C1, 2O suggests a strong affinity for catechol. The optimum pH and temperature of the purified C1, 2O is 8 and 36°C, respectively. Methyl substitution at the third carbon of the benzene ring has a negative effect on the activity of the purified C1, 2O. Catechol 1, 2 dioxygenase purified from strain Y64 shows at least 50% activity at 45°C for 2 hours. However, purified C1, 2O was not stable at 50°C. Fe^{2+} and Fe^{3+} were observed to enhance the activity of purified C1, 2O. Furthermore, the removal of metal ions at the active site of purified C1, 2O with 0.5 mM EDTA left 50% activity. Hence, purified C1, 2O possess features that may be applicable in the industry for activities such as environmentally friendly production of *cis, cis* muconic acid. C1, 2O purified from

the transformants possessed similar properties to C1, 2O purified from *Acinetobacter* sp. strain Y64, suggesting a successful cloning and expression. However, future work would require an increase in the production of C1, 2O expressed in *E. coli*.

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