CHARACTERIZATION OF THE DEGRADATION PATHWAY OF PHLOROGLUCINOL (1,3,5-TRIHYDROXYBENZENE) BY A *RHODOCOCCUS* sp. BPG-8

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STEPHEN MARK ARMSTRONG

CHARACTERIZATION OF THE DEGRADATION PATHWAY OF PHLOROGLUCINOL (1,3,5-TRIHYDROXYBENZENE) BY A *RHODOCOCCUS* sp. BPG-8

BY

©STEPHEN MARK ARMSTRONG (B.SC, M.SC)

A thesis submitted to the school of Graduate Studies in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Abstract

Rhodococcus sp. BPG-8 was isolated from oil rich soil in Newfoundland and found to utilize numerous aromatic compounds as sources of carbon and energy. Chemical analysis of cell wall composition which included amino acids, sugars, and fatty acids showed complete homology with Rhodococcus erythropolis. Identical profiles for acid production and growth on various substrates occurred. Growth of the isolate on phloroglucinol occurred in the pH range 5-8; with substrate and temperature optima of 8.0 mM and 25°C, respectively. Phloroglucinol induced cells when fed phloroglucinol or resorcinol produced 1,2,3,5-tetrahydroxybenzene and 1,2,4-trihydroxybenzene, respectively. Cell-free extracts of cells grown on phloroglucinol contained a phloroglucinol hydroxylase that also hydroxylated resorcinol. Dioxygenases present in the induced cells carried out the ortho-cleavage of 1,2,3,5-tetrahydroxybenzene while meta-clevage of 1,2,4-trihydroxybenzene appeared to be constitutive. Cell-free extracts also showed inducible activity for the metabolism of acetopyruvate with the accumulation of formate in the supernatant. Tentative degradative pathways for phloroglucinol and fortuitous resorcinol metabolism are discussed. This is the first reported case in which phloroglucinol is metabolized by an oxidative rather than a reductive pathway. The

oxidations of 1,2,3,5-tetrahydroxybenzene and 1,2,4-trihydroxybenzene produce superoxide radicals that may have potential deleterious effects on cellular integrity. It has been shown that both superoxide dismutase and catalase retard the auto-oxidation of these molecules by hindering their free radical reaction mechanism with superoxide. A non-inducible NAD(P)H dependent reductase appeared to convert the 2-hydroxy-1,4-benzoquinone back to 1,2,4-trihydroxybenzene; although similiar effects were not found for 1,2,3,5-tetahydroxybenzene. These novel findings suggest that constitutive non-pathway enzymes may participate in stabilization of intermediates. Partial purification of the phloroglucinol hydroxylase was performed using ammonium sulfate percipitation, ion exchange chromatography, and gel filtration. The pH, temperature, and substrate optima for phloroglucinol hydroxylase were 7.0, 25°C, and 68.0 μM for the substrates phloroglucinol and resorcinol. NADH+H$^+$ was the primary reductant and FAD stimulated the hydroxylase activity by 300 %. The enzyme had a native molecular weight of 155,000 daltons and an apparent Km of 8.3 μM and 12.5 μM for phlorglucinol and resorcinol respectively. Chloride ion along with numerous metal ions appeared to inhibit phloroglucinol and resorcinol hydroxylase activities. This is the first reported case for the partial purification of a phloroglucinol hydroxylase.

Acknowledgements

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Chapter I: Review of the literature

1.1 General

Microbial degradation of aromatic compounds is very important to the earths' carbon cycle. The pyrolysis of organic material from natural or anthropogenic sources produces many aromatic compounds that are relatively recalcitrant to degradation (Dagley, 1971). If the carbon became locked inside these products eventually it would be exhausted from the biosphere. The benzene nucleus of many of these products furnishes some of this inertness because of its resonance structure stability (Gibson and Subramanian, 1984). Some soil microbes can produce mono- and dioxygenases that are able to insert oxygen within this benzene nucleus, thus making it more amenable to degradation and subsequent utilization as a carbon and energy source (Gibson and Subramanian, 1984).

The importance which microbes play in the economy of the earths carbon may be circumvented by man-made compounds. The problems of microbial fallibility and molecular recalcitrance have serious consequence to the health of the global ecosystem and its inhabitants. Halogenation of the benzene nucleus, a feature of many modern pesticides, presents a novel situation for many soil microbes (Cork and Krueger, 1991). It is important to realize that production and application of such chemicals should be shaped by its biodegradability in the prevailing environment.

It has been shown that many of these man-made compounds may be degraded partially or completely by both microbial consortia and single microbes. This is a testament of the versatility and power microbes employ when presented with a novel carbon source. This is not to concede that microbes are infallible, since there are numerous cases in which degradation of toxic compounds is incomplete, producing intermediates that may be more toxic than the original compound (Reineke, 1984).

An overview of the degradatory processes will be presented to illustrate our current understanding of microbial degradation as it pertains to aromatic metabolism.

1.2 Degradation of Benzenoid Compounds

1.2.1 Aromatics, Polycyclic Aromatics and Lignin

The aerobic degradation of the aromatic nucleus whether it is unsubstituded or substituted with various groups (aliphatic, hydroxyl, amino, or halogen) involves two basic mechanisms. The first mode of oxygenated cleavage of the bond between adjacent carbon atoms that carry hydroxyl groups is known as ortho-cleavage and the associate metabolic pathway is recognized as the β-ketoadipate pathway (Stanier and Ornston, 1973) (Fig. 1.1). The second mode of oxygenated cleavage known as meta-cleavage and it occurs between two carbon atoms, only one of which carries a hydroxyl group; the other carbon may be

unsubstituted or substituted with anything but a hydroxyl group (Bayly and Barbour, 1984). Meta-cleavage occurs when the hydroxyl groups are ortho or para to each other with the respective sequences called the meta and gentisate pathways (Bayly and Barbour, 1984).

Many aromatics are metabolically altered to produce two common intermediates such as catechol and protocatechuate. Ortho-cleavage produces cis,cis-muconate and β-carboxy-cis,cis-muconate from catechol and protocatechuate, respectively (Stanier and Ornston, 1973). Metabolic convergence of the separate branches leads to three common intermediates: β-ketoadipate enol-lactone, β-ketoadipate, and β-ketoadipyl-CoA which undergoes thiolytic cleavage to produce succinate and acetyl-CoA (Stanier and Ornston, 1973) (Fig. 1.2). Catechol 1,2-dioxygenase and protocatechuate 3,4-dioxygenase have molecular weights of 95,000 daltons (two associated ferric ions) and 700,000 daltons (eight associated ferric ions) respectively (Gibson and Subramanian, 1984; Stanier and Ornston, 1973). The lactonizing enzymes for cis,cis-muconate and β-carboxy-cis,cis-muconate which eventually produce β-ketoadipate are subject to independent regulatory control and are not antigenically related. They have sharp enzymatic specificity, are stable at 60°C, and have

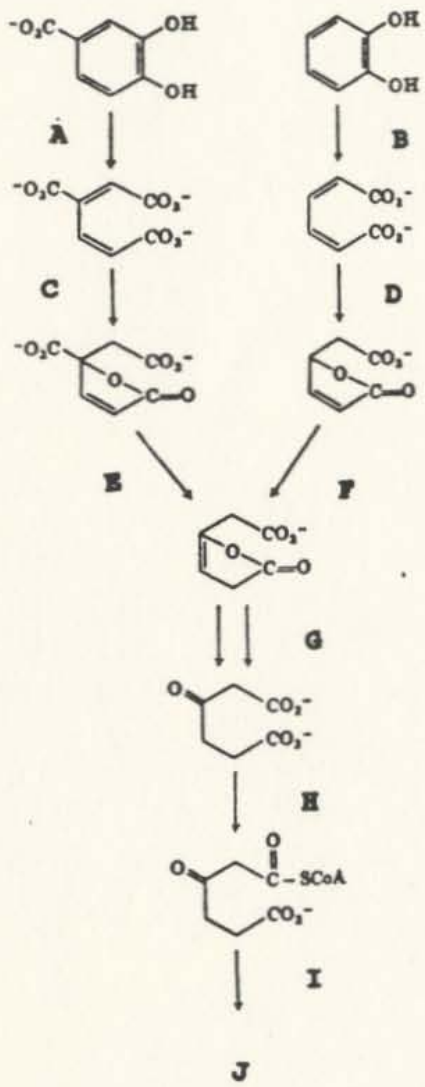


Fig. 1.1. The central reactions of the β-ketoadipate pathway in bacteria. A, protocatechuate oxygenase; B, catechol oxygenase; C, β-carboxymuconate lactonizing enzyme; D, muconate lactonizing enzyme; E, γ-carboxymuconolactone decarboxylase; F, muconolactone isomerase; G, β-ketoadipate enol-lactone hydorolase; H, β-ketoadipate succinyl-CoA transferase; I, β-ketoadipyl-CoA thiolase; J, succinate and acetyl-CoA (modified from: Stanier and Ornston, 1973).

equal molecular weights of 190,000 daltons (Stanier and Ornston, 1973). Muconolactone isomerase and γ-carboxymuconolactone decarboxylase have narrow enzyme specificity, differ in external charges, are subject to independent controls, are antigenically unrelated, and both have a molecular weight of 93,000 daltons (Stanier and Ornston, 1973).

The β-ketoadipate enol-lactone hydrolase induced by protocatechuate and catechol have molecular weights of 21,000 and 24,000 daltons, respectively; however the existence of mutants of Acinetobacter in which either the synthesis of one or the other enzymes is specifically affected suggest that the enzymes are coded by different structural genes. β-ketoadipate succinyl-CoA transferase are induced by growth with β-ketoadipate, aromatic acids, and saturated dicarboxylic acids (Stanier and Ornston, 1973).

Regulation of the β-ketoadipate pathway and the ancillary initial reactions all seem to be inducible. During the metabolism of catechol in Alcaligenes eutrophus the primary aromatic compound, cis,cis-muconate, or muconolactone act as inducers; whereas protocatechuate, β-ketoadipate or β-ketoadipyl-CoA act as inducers during protocatechuate degradation (Stanier and Ornston, 1973).

Meta-cleavage of catechol and protocatechuate produce α-hydroxy-muconate semialdehyde and α-hydroxy-γ-carboxy-muconic

semialdehyde respectively (Dagley, 1975; Dagley, 1971). The end products from meta-cleavage of catechol are formate, acetaldehyde and pyruvate, while formate and two molecules of pyruvate are produced from meta-cleavage of protocatechuate under similiar conditions (Bayly and Barbour, 1984) (Fig. 1.2).

The catechol and protocatechuate ortho- and meta-cleavage pathways differ in the mode of enzyme induction, number of substrate inducers, and substrate specificity (Bayly and Barbour, 1984; Stanier and Ornston, 1973). The meta pathway is regarded as being able to degrade a wider spectrum of aromatic compounds obligatory to enzymes with broader substrate specificity and induction patterns (Bayly and Barbour, 1984). The meta and the gentisate pathway degrade a wider spectrum of aromatics that include phenolics, polycyclic aromatics, steroids, and alkylbenzoic sulfonates with Pseudomonas, Acinetobacter, Bacillus, Alcaligenes, and Nocardia being the predominant genera (Bayly and Barbour, 1984).

Degradation of various aromatics such as m-cresol, 3-hydroxybenzoic acid, salicylic acid, anthranilic acid, β-naphthol, and xylenol produce alkyl substituted gentisate and gentisate as intermediates (Bayly and Barbour, 1984; Chapman, 1972) (Fig. 1.3). The important aspect of the gentisate pathway are cleavage of the aromatic ring by a dioxygenase to form maleylpyruvate which is isomerized to fumarylpyruvate

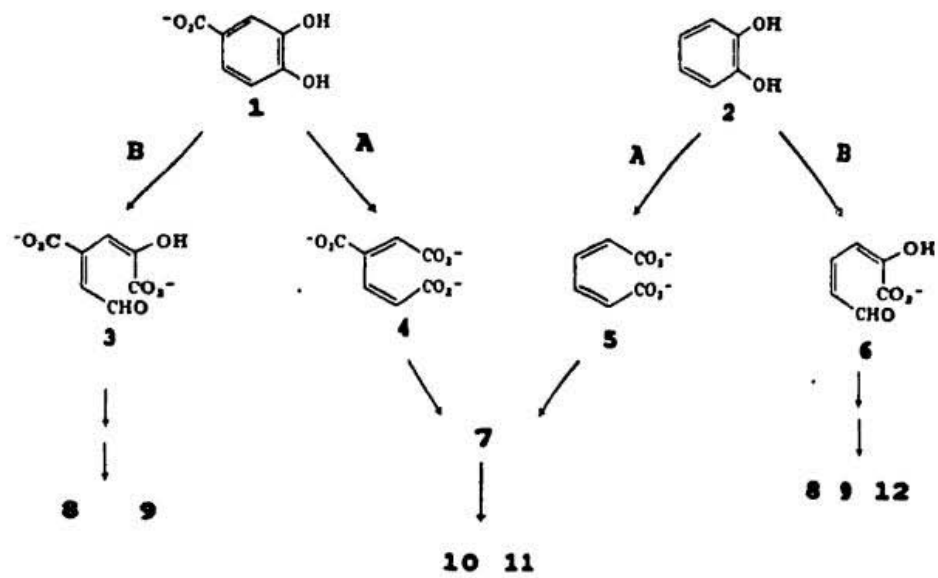


Fig. 1.2. The ortho- and meta-cleavage pathways for dissimilation of protocatechuate (1) and catechol (2). The structure intermediates 3-6 are shown: 3, α-hydroxy-γ-carboxymuconic semialdehyde; 4, β-carboxy-cis,cis-muconate; 5, cis,cis-muconate; and 6, α-hydroxymuconic semialdehyde. The stuctures of β-ketoadipate and the end products are not shown: 7, β-ketoadipate; 8, formate; 9, pyruvate; 10, succinate; 11, acetyl-CoA; 12, acetylaldehyde; A, ortho pathway; B, meta pathway. (modified from: Stanier and Ornston, 1973).

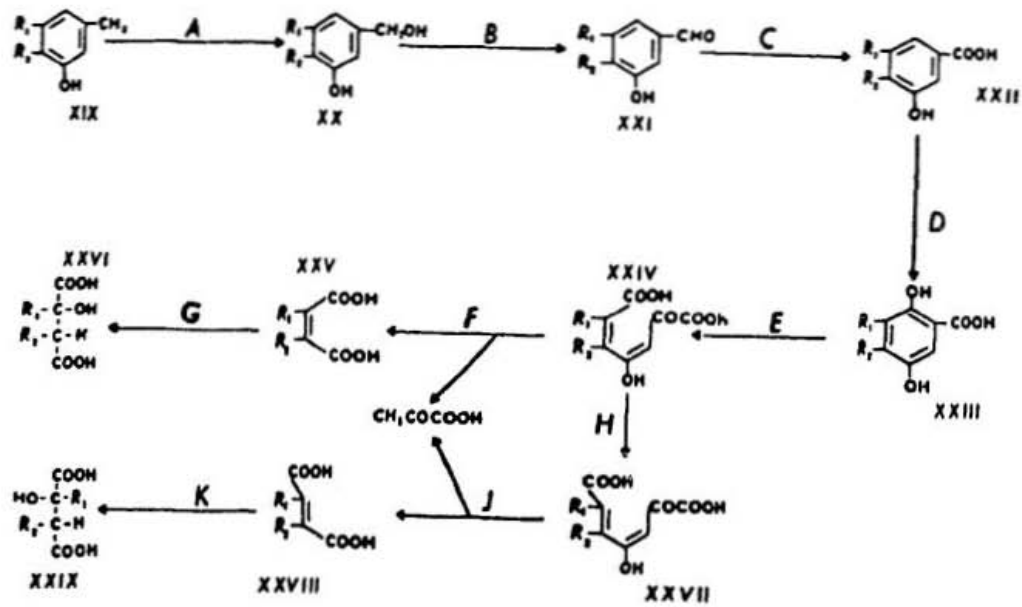


Fig. 1.3. The gentisate pathway for the degradation of m-cresol, and 2,5- and 3,5-xylenol.

Key to compounds: R_1, R_2 = H; XIX, m-cresol; XX, 3-hydroxybenzylalcohol; XXI, 3-hydroxybenzaldehyde; 3-hydroxybenzoate; XXIII, gentisate; XXIV, maleylpyruvate; XXV, maleic acid; XXVI, D-malic acid; XXVII, fumarylpyruvate; XXVIII, fumarate; XXIX, L-malic acid.

R_1 = H, R_2 = CH_3; XIX, 2,5-xylenol; XX, 3-hydroxy-4-methylbenzylalcohol; XXI, 3-hydroxy-4-methylbenzaldehyde; XXII, 3-hydroxy-4-methylbenzoate; XXIII, 4-methylgentisate; XXIV, 5-methyl-maleylpyruvate; XXV, citraconic acid.

R_1 = CH_3, R_2 = H; XIX, 3,5-xylenol, XX, 3-hydroxy-5-methylbenzylalcohol; XXI, 3-hydroxy-5-methylbenzyaldehyde; XXII, 3-hydroxy-5-methylbenzoate; XXVIII, 3-methylgentisate; XXIV, 6-methyl-maleylpyruvate; XXV, citraconic acid.

Key to enzymes: A, methylhydroxylase; B, alcohol dehydrogenase; C, aldehyde dehydrogenase; D, 6-mono-oxygenase; E, gentisate 1,2,-dioxygenase; F, maleylpyruvate hydrolase; G, maleate hydratase; H, isomerase; J, fumarylpyruvate hydrolase; K, fumarase (Reproduced with permission from Marcel Dekkar Inc., Bayly and Barbour, 1984).

which is then hydrolysed to fumarate and pyruvate (Bayly and Barbour, 1984). Fumarate is subsequently converted into L-malic acid by a fumarase. Maleylpruvate may also be converted into maleic acid and pyruvate by a maleylpyruvate hydrolase. Maleic acid is subsequently converted by a maleate hydratase into D-malic acid (Bayly and Barbour, 1984).

Bacteria, filamentous fungi, yeast, cyanobacteria, diatoms and other eukaryotic algae have been shown to oxidize polycyclic aromatics (PAH) that range in size from naphthalene to benzo[a]pyrene (Cerniglia, 1984). The fate of PAH's in the environment is influenced by physicochemical factors of the PAH's, environmental factors of the biota, and microbial factors (Cerniglia, 1984). It has been recognized that high PAH concentrations have been associated with higher levels of cancer in humans (Leahy and Colwell, 1990; Cerniglia, 1984).

Difference between prokaryotic and eukaryotic transformation of PAH's are quite significant (Gibson and Subramanian, 1984; Jerina and Daly, 1974). Prokaryotes produce a cis-dihydrodiol from the incorporation of two atoms of oxygen within the PAH's, whereas fungal enzymes produce an arene oxide through the action of the cytochrome P-450 system and the incorporation of one atom of oxygen within the PAH's (Gibson and Subramanian, 1984) (Fig. 1.4). The arene oxide can either undergo an non-enzymatic rearrangement (NIH shift) to a phenol which is later conjugated, or reacted with an epoxide

hydrolase to form the trans-dihydrodiol (Gibson and Subramanian, 1984; Cerniglia et al., 1982). The cis-Napthalene dihydrodiol produced by bacteria is oxidized by a NAD+-dependent dehydrogenase to produce a catechol-type molecule which then undergoes either ortho- or meta-fission, which eventually lead to complete mineralization of the PAH (Gibson and Subramanian, 1984) (Fig. 1.5).

Lignin biodegradation is important to the earth's carbon cycle since lignin is second only to cellulose in abundance (Kirk and Farrell, 1987). Lignin also protects most of the earths cellulose and hemicellulose from enzymatic hydrolysis (Kirk and Farrell, 1987). Lignin is found in higher plants including ferns, and biochemically arises from the free radical copolymerization of three precursors: p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol which are β-O-4 linked (Kirk and Farrell, 1987) (Fig. 1.6). The structural features of lignin suggest that the degradation must be extracelluar, nonspecific, and nonhydrolytic (Blanchette, 1991; Kirk and Farrell, 1987).

Lignin is not biodegraded anaerobically and it appears that neither rapid nor extensive bacterial degradation occurs under aerobic conditions. The degradation of lignin by Streptomycetes spp. has been suggested (Kirk and Farrell, 1987; Kirk, 1984); however numerous studies seem to suggest that this may be incomplete or nil (Pometto and Crawford,

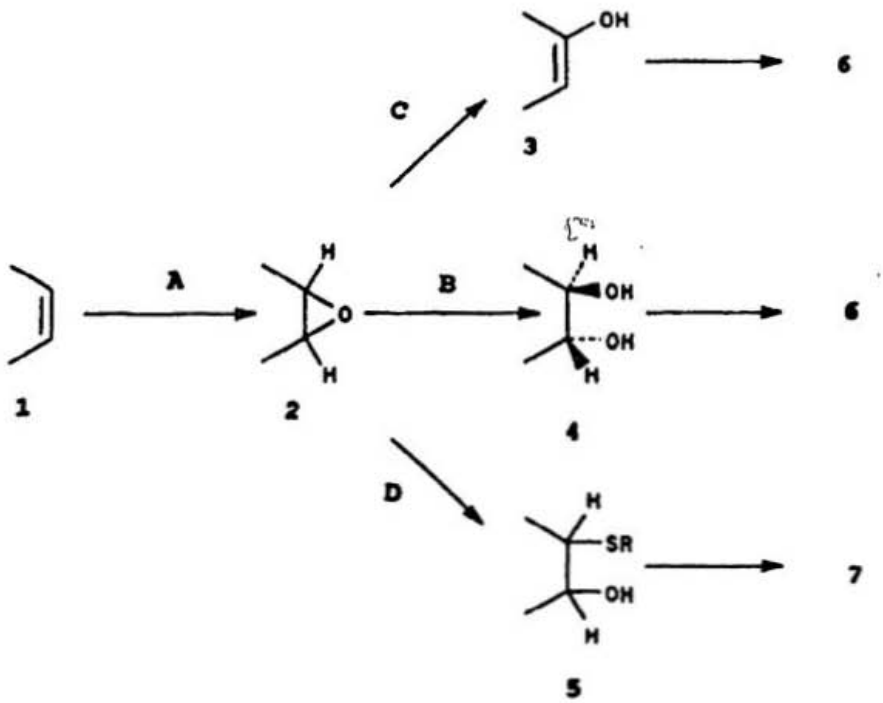


Fig. 1.4. Reactions utilized by mammals for the transformation of aromatic hydrocarbons: 1, aromatic hydrocarbon; 2, arene oxide; 3, phenol; 4, <u>trans</u>-dihydrodiol; 5, glutathione conjugate; 6, sulfates and glucuronides; 7, mercapturic acids; A, cytochrome P-450; B, epoxide hydratase; C, NIH shift; D, glutathione transferase (Reproduced with permission from Marcel Dekkar Inc., Gibson and Subramanian, 1984).

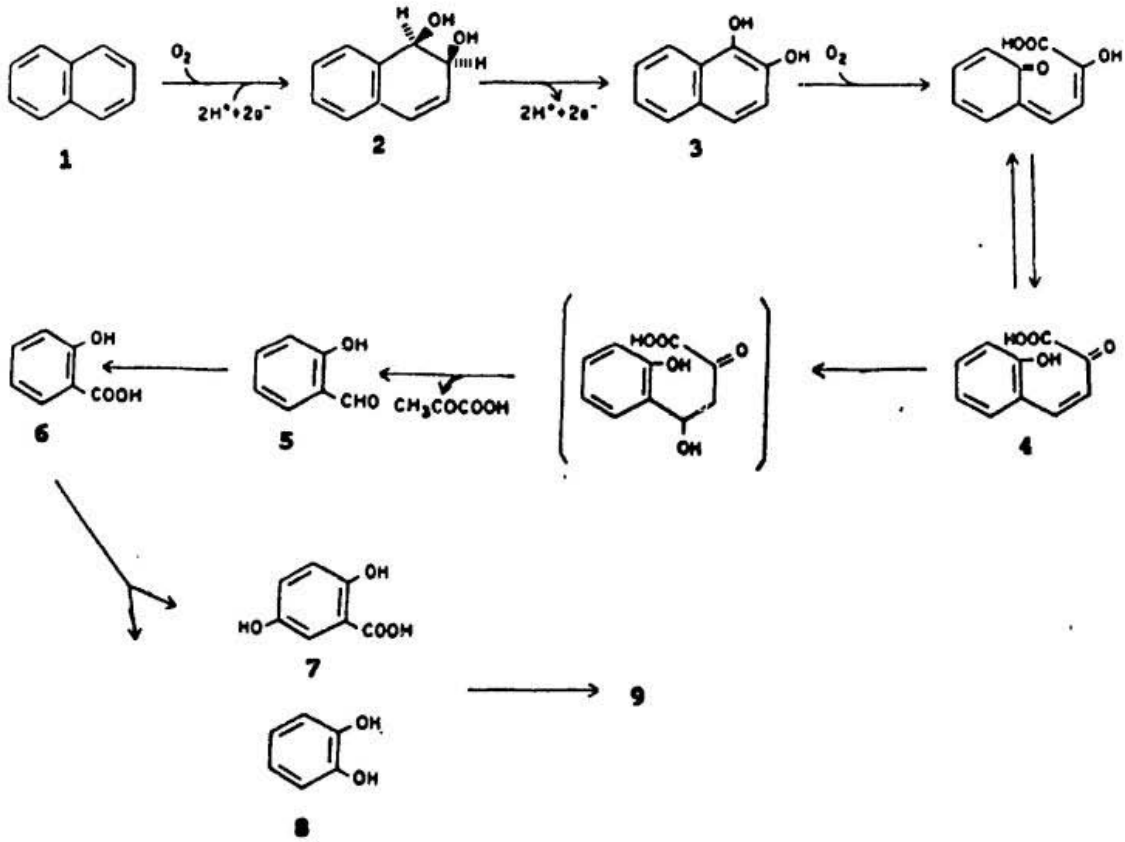


Fig. 1.5. Proposed pathway for the degradation of naphthalene (a polycyclic aromatic) by bacteria: 1, naphthalene; 2, cis-naphthalene dihydrodiol; 3, 1,2-dihydroxynaphthalene; 4, cis-o-hydroxybenzalpyruvate; 5, salicylaldehyde; 6, salicylic acid; 7, gentisic acid; 8, catechol; 9, respective ring fission products (Reproduced with permission from Marcel Dekkar Inc., Gibson and Subramanian, 1984).

1986; Petty and Crawford, 1985; Crawford et al., 1983).

The white-rot basidiomycetes, notably Phanerochaete chrysosporium degrade lignin more completely and rapidly than any other microbial group (Kirk and Farrell, 1987; Kirk, 1984). They seem to invade the lumens of wood cells where they secrete enzymes such as ligninase, Mn-peroxidase, phenol-oxidizing, and H_2O_2-producing enzymes. The lignin degradation process seems to follow an enzymatic combustion process which resembles a nonspecific enzyme-catalyzed burning (Blanchette, 1991; Kirk and Farrell, 1987). This leads to a potpourri of divergent reactions that is unmatched by any other enzyme system (Kirk and Farrell, 1987). Depolymerization is kinetically favoured because ligninase oxidizes its substrate by one electron and subsequently produces unstable cation radicals which elicit a variety of non-enzymatic reactions (Kirk and Farrell, 1987) (Fig. 1.7). It is also clear from the structure of lignin that its conversion to water and carbon dioxide is thermodynamically favourable.

1.2.2 Anaerobic degradation of Aromatics

Ecosystems are common in sediments, alimentary tract of animals, and in industrial activities that produce anoxic systems (Evans and Fuchs, 1988). Therefore different mechanisms for the detoxification of aromatics must exist because oxygen is limiting (Young, 1984). The anaerobic

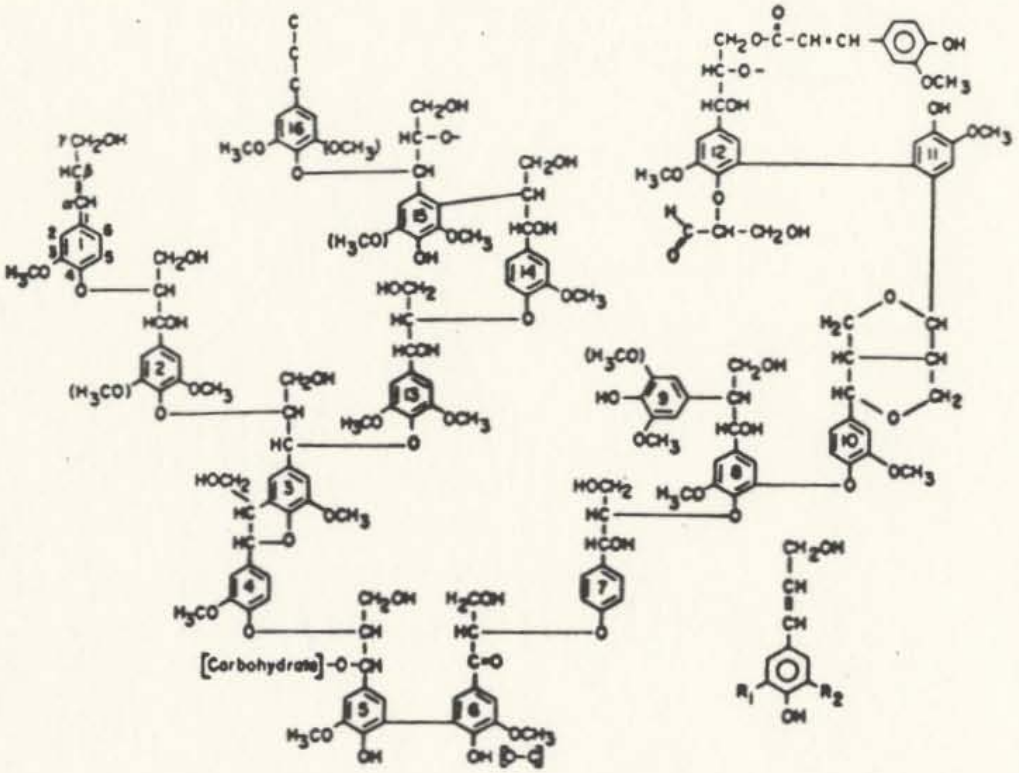


Fig. 1.6. Schematic structural formula of lignin. Polymerization of the three precursor alcohols (shown at the lower right) produces lignin. The precursor alcohols have the various R-groups: $R_1=R_2=H$: p-coumaryl alcohol; $R_1=OCH_3$, $R_2=H$: coniferyl alcohol; $R_1=R_2=OCH_3$: sinapyl alcohol. The numbers 2-16 refer to the number of component alcohols (Reproduced with permission from Ann. Rev. Microbiol., Kirk and Farrell, 1987).

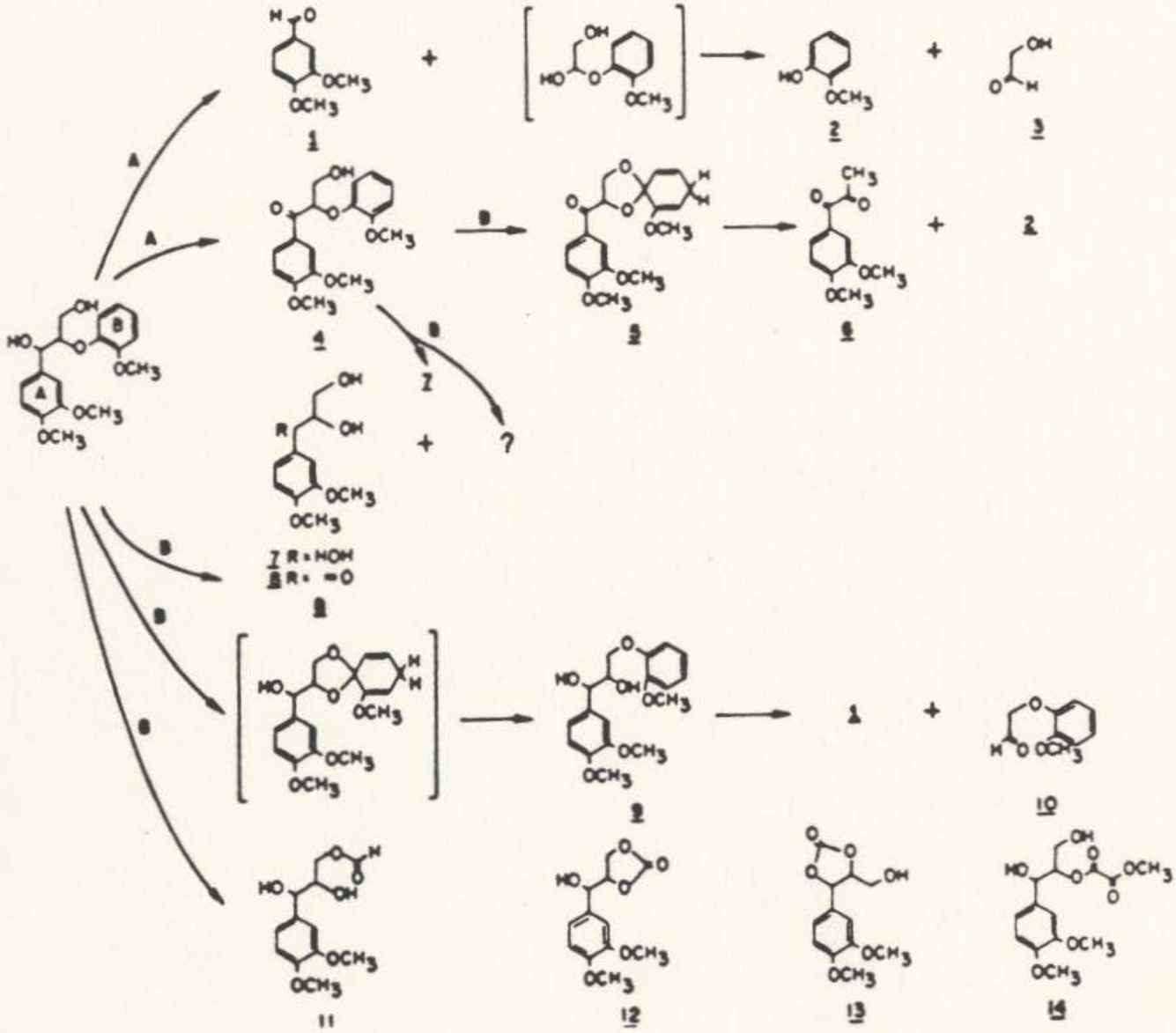


Fig. 1.7. Plethora of products produced during the oxidation of β-O-4 model compound by ligninase/H_2O_2. A, A-ring cleavage; B, B-ring cleavage, while numbers refer to products produced during oxidation of β-O-4 model compound by ligninase/H_2O_2. (Reproduced with permission from Ann. Rev. Microbiol., Kirk and Farrell, 1987).

metabolism of aromatic compounds may occur by photosynthetic phosphorylation, denitrification, sulfate reduction, fermentation, and methanogenic fermentation (Harwood and Gibson, 1988; Nozama and Maruyama, 1988; Krumholz et al., 1987; Bak and Widdle, 1986; Healy and Young, 1978).

Photosynthetic phosphorylation occurs in several species of the purple nonsulphur Rhodospirillaceae family (Evans and Fuchs, 1988). Species such as Rhodopseudomonas palustris and Rhodopseudomonas gelatinosa can metabolize compounds such as benzoate, m- and p-hydroxybenzoate, and phloroglucinol (Evans and Fuchs, 1988; Harwood and Gibson, 1988). These species obtain their energy from light and use simple aromatic compounds as carbon sources. The photometabolism of benzoate suggests that the aromatic ring becomes fully reduced with the incorporation of six hydrogen equivalents to form cyclohexanecarboxylate. The subsequent reactions would be analogous to fatty acid β-oxidation (Evans and Fuchs, 1988). During the photometabolism of phloroglucinol, it is reduced to dihydrophloroglucinol and subsequently cleaved to 2-oxo-4-hydroxyadipate (Evans and Fuchs, 1988; Whittle et al., 1976). The ring cleavage mechanism and enzymatic reactions of this pathway are still uncertain.

The metabolism of nitrate-reducing bacteria suggests that the oxidation of aromatic compounds is coupled with the exergonic reduction of nitrate to nitrogen or ammonia (Evans

and Fuchs, 1988). Energy is derived mainly from electron transport phosphorylation, and carbon is supplied from the aromatic degradation. The anaerobic nitrate metabolism of phthalate and other aromatics suggests that there may be a reduction and hydrolytic cleavage of the aromatic ring followed by β-oxidation (Aftring and Taylor, 1981)

Sulfate reducers couple the oxidation of organic compounds with water to the exergonic reduction of sulfate to sulphide. Electron transport phosphorylation supplies the energy for the sulphate reducers, while the carbon is derived from aromatic degradation. The genera associated with sulfate metabolism of aromatics include Desulfovibrio, Desulfococcus, Desulfonema, and Desulfosarcinia (Evans and Fuchs, 1988). Although there have been reports of aromatic degradation by sulfate reducers, along with the associated sulfate reduction to hydrogen sulphide, no pathway has been published (Bak and Widdle, 1986)

Microorganisms that derive their energy from substrate level phosphorylation, and employ organic compounds as electron donors and acceptors, are fermentative. Some genera include Coprococcus, Streptococcus, Pelobacter, and Eubacterium all of which degrade compounds such as phloroglucinol and various phenolic compounds (Krumholz and Bryant, 1986; Schink and Pfenning, 1982; and Patel et al., 1981). Krumholz et al., (1987) isolated an Eubacterium

oxidoreducens that degrades gallate, pyrogallol, and phloroglucinol to acetate, butyrate, and carbon dioxide. Formate or hydrogen was used as an electron donor to catabolize these aromatic substrates (Evans and Fuchs, 1988).

The degradation of aromatic compounds also occurs in methanogenic consortia which include fermentative, acetogenic, and methanogenic bacteria. Methanogenic consortia depend on a syntrophic relationship in which the fermentors degrade aromatics into metabolizable products for the methanogens (Evans and Fuchs, 1988). Numerous aromatic compounds seem to be degraded by a reduction of the aromatic ring followed by a hydrolytic cleavage and β-oxidation to aliphatic compounds such as acetate, formate, and various carboxylic acids (Evans and Fuchs, 1988).

1.2.3 Halogenated Aromatics and Pesticide Compounds

The emergence of the chemical industry during this century has lead to the introduction of many halogenated compounds into the environment either through point source or dispersed pollution (Haggblom, 1990). The relative novelty of these compounds in the environment and the lack of time for microorgansims to develop adequate degradative enzymes through a evolutionary process has lead some to believe that this may be the reason for their recalcitrance (Haggblom, 1990; Reineke and Knackmuss, 1988). There is also an argument that numerous

halogenated compounds have been exposed to microbial populations since early in the earths history and therefore halogenated compounds should be degradable (Haggblom, 1990; Reineke and Knackmuss, 1988). Some halogenated compounds have been shown to be totally degraded or transformed while others are not degradable suggesting that microorganisms may have not had enough time to utilize these specific compounds. The biodegradation of the halogenated arenes can only be considered complete when the carbon skeleton is converted into intermediary metabolites and its organic halogen is returned to the mineral state (Haggblom, 1990; Reineke and Knackmuss, 1988; Reineke, 1984). Incomplete biodegradation may lead to dead-end metabolites that are sometimes more toxic than the initial substrate.

The biodegradation may occur by cometabolism or by modified basic metabolic sequences such as the β-ketoadipate (Haggblom, 1990). Elimination of the halogen may occur before or after ring cleavage by aerobic and anaerobic processes (Reineke and Knackmuss, 1988).

Displacement of the halogen by a hydrogen may occur anaerobically in a methanogenic consortia that consist of a dechlorinating bacterium, benzoate oxidizing bacterium, two butyrate-oxidizing bacteria, two H_2-consuming methanogens (Methanospirillum hungatei, Methanobacterium sp.), and a sulfate-reducing bacterium (Desulfovibrio sp.) (Haggblom,

1990; Reineke and Knackmuss, 1988; Reineke, 1984). It appears that one or more of these organisms cross-feed the dechlorinating bacterium. The reducing power required for reductive dechlorination may be provided by acetogenic oxidation of benzoate. One third of this hydrogen was shown to be consumed by reductive dechlorination, while two thirds was utilized by the methanogen. These communities may degrade mono- and polysubstituted chlorinated aromatics to methane and carbon dioxide. Reductive dechlorination was observed with 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), chlorophenols, and 1,2,4-trichlorobenzene. Even pentachlorophenol (PCP) was completely dechlorinated by a mixture of 2-chlorophenol (2-CP), 3-CP, and 4-CP-acclimated sludges (Reineke and Knackmuss, 1988) (Fig. 1.8).

Aerobic dehalogenation of the halogenated aromatics may occur by displacement of the halogen by a hydroxy group, oxygenolytic cleavage of the halogen-carbon bond, and chlorine elimination from nonaromatic intermediates (Haggblom, 1990; Reineke and Knackmuss, 1988; Reineke, 1984) (Fig. 1.9). Displacement of the halogen by hydrogen utilizes water instead of oxygen as the hydroxyl donor, with dechlorination proceeding via hydrolytic cleavage of the carbon-chlorine bond. This has been reported for numerous genera which include Arthrobacter, Nocardia, Micrococcus, Pseudomonas, Flavobacterium, and Rhodococcus. Pentachlorophenol, 4-

iodobenzoate, and 4-bromobenzoate have been dehalogenated by this process (Reineke and Knackmuss, 1988; Reineke, 1984).

Oxygenolytic cleavage of the halogen-carbon bond has initiated fortuitous dehalogenation by specific dioxygenases.

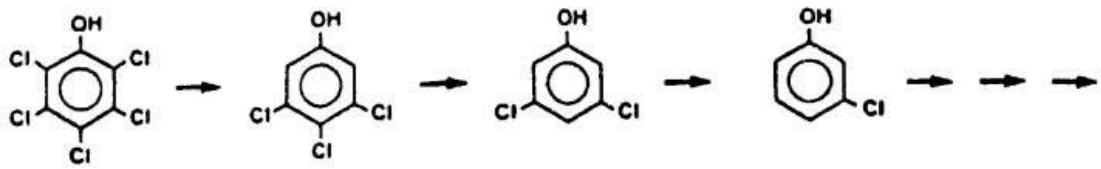


Fig. 1.8. Proposed pentachlorophenol degradation pathway by a mixture of halogenated acclimate sludges (Reproduced with permission from Ann. Rev. Microbiol., Reineke and Knackmuss, 1988).

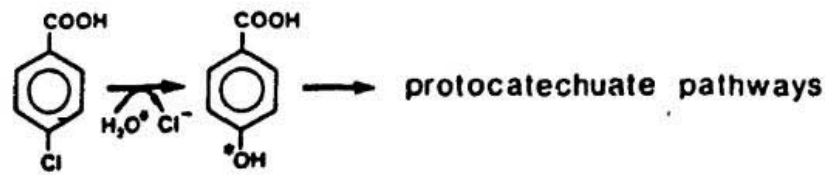


Fig. 1.9. Hydrolytic dechlorination of 4-chlorobenzoate by a Micrococcus spp. (Reproduced with permission from Ann. Rev. Microbiol., Reineke and Knackmuss, 1988).

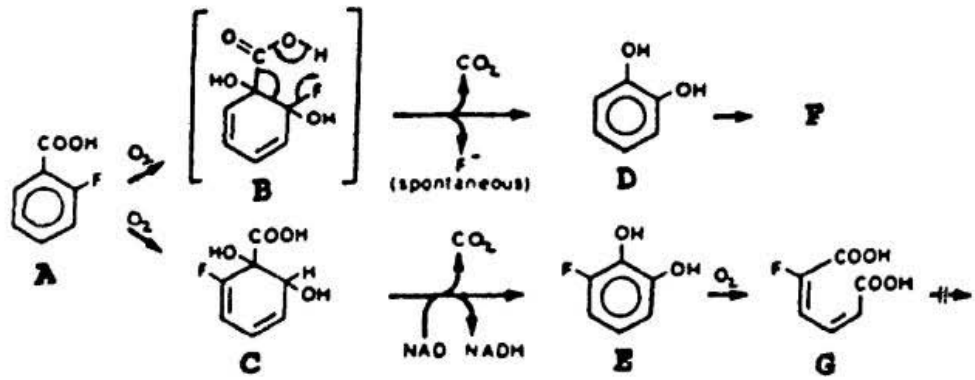


Fig. 1.10. Degradation pathway for 2-fluorobenzoate by a pseudomonad. A, 2-fluorobenzoate; B,C, 2- and 6-fluoro-1,2-dihydro-1,2-dihydroxybenzoate, respectively; D, catechol; E, 6-fluorocatechol; F, 3-oxoadipate pathway; G, 2-fluoro-cis,cis-muconate (dead-end product) (Reproduced with permission from Ann. Rev. Microbiol., Reineke and Knackmuss, 1988).

Dehalogenation of 2-fluorobenzoate occurred when fluorine was non-enzymatically removed from the 2-fluoro-1,2-dihydro-1,2-dihydroxybenzoate to produce catechol (Reineke and Knackmuss, 1988) (Fig. 1.10). Oxygenolytic elimination from a cis-dihydrodiol produced by dioxygenation was shown to account for the initial dehalogenation of 4-halophenylacetates by a Pseudomonas sp. strain CBS3 (Reineke and Knackmuss, 1988).

Chlorine elimination may occur in non-aromatic

intermediates after ortho-cleavage of the chlorocatechol. Chloride is eliminated spontaneously after the carbon-halogen bond has been labilized through isomerases or reductases to form maleylacetate (Haggblom, 1990; Reineke and Knackmuss, 1988) (Fig. 1.11).

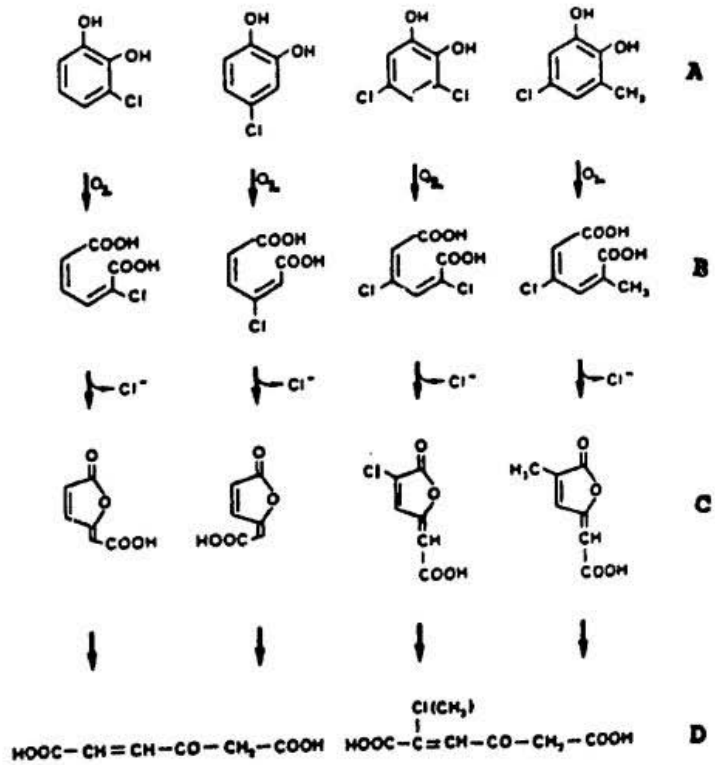


Fig. 1.11. Degradation of chlorocatechols to maleylacetates by a Pseudomonas strain B13. A, chlorocatechols; B, chloro-cis-cis-muconates; C, cycloisomerization products; D, maleylacetates and chloro-maleylacetates (Reproduced with permission from Ann. Rev. Microbiol., Reineke and Knackmuss, 1988).

1.3 Phloroglucinol

1.3.1 General Description

Anhydrous and dihydrated phloroglucinol have melting points of 219°C and 115°C, respectively. Phloroglucinol is a colourless, odourless, sweet tasting compound with a pKa of 7.97 and 9.23 at 20°C (Robern, 1965). Its aqueous solution gives a violet colour with ferric chloride, reduces Fehlings' solution and precipitates gold, silver, and platinum from solutions of their salts (Robern, 1965). Alkaline solutions of phloroglucinol absorb oxygen from air but not as pronounced as pyrogallol or 1,2,4-trihydroxybenzene. The ultraviolet, infrared, and nuclear magnetic resonance spectra are consistent with the phenolic structure of phloroglucinol at neutral pH; however it as been shown to have six interchangeable protons (Erlenmyer et al., 1936).

Phloroglucinol was first prepared by the heating of the monosodium derivative of ethylicmalonate, forming an ester of phloroglucinol which on hydrolysis yielded phloroglucinol (Jordan, 1897). Subsequent synthesis involves the reduction of trinitrobenzene with tin and hydrochloric acid, with the amine being neutralized by boiling in water for one day (Clarke and Hartman, 1929).

Phloroglucinol occurs in most plants as part of the complex tannin molecule or as the dihydrochalcine glycoside phloridizin (Robern, 1965). It does not exist in the free

form, but a significant component of seeds, leaves and bark of many trees contains the phloroglucinol moiety (Robern, 1965; Robinson, 1962). It naturally exists as a component of several plant polymers such as flavones, anthocyanins, catechins, lignin precursors and their intermediate degradation products (Krumholtz and Byrant, 1986; Robinson, 1962). Phloroglucinol occurs in nature in the A-ring of flavonoid compounds and other plant phenolic compounds and may arise from the microbial degradation of these compounds (Walker and Taylor, 1983). It has also been suggested that the breakdown of natural products such as coal yield phloroglucinol (Robern, 1965; Mathur, 1971). The pentahydroxy flavone quercetin was also shown to be biotransformed to phloroglucinol and protocatechuate by Rhizobium loti and Bradyrizobium strains (Rao et al., 1991).

1.3.2 Aerobic and Anaerobic Degradation

The degradation of phloroglucinol may be accomplished by aerobic and anaerobic microorganisms. Wagner (1914), and Gray and Thornton (1928) isolated microorganisms from soil and feces that could utilize phloroglucinol. Species of Brevibacterium, Candida, Debaromyces, Pseudomonas, Arthrobacter and Penicillium have been shown to degrade phloroglucinol (Nakagawa and Takeda, 1962; Harris and Rickets, 1962; Robern, 1965; Mathur, 1971). Jamieson et al. (1970),

and Robern (1965) suggested that the phloroglucinol molecule is converted into a dihydrophloroglucinol (1,3-dioxo-5-cyclohexane) which later spontaneously loses a water to form a resorcinol molecule (Fig. 1.12). This resorcinol molecule is subsequently converted into a 1,2,4-trihydroxybenzene which later undergoes ortho-cleavage to produce β-ketoadipate. Interestingly resorcinol hydroxylation to 1,2,4-trihydroxybenzene by a Pseudomonas putida suggested that both ortho- and meta-cleavage enzymes may operate in the cleavage of 1,2,4-trihydroxybenzene (Chapman and Ribbons, 1976 a; Chapman and Ribbons, 1976 b).

Phloroglucinol has also been shown to produce pyrogallol through a hypothetical resorcinol epoxide (Walker and Taylor, 1983) (Fig. 1.13). The pyrogallol then undergoes ortho-cleavage to produce a 2-hydroxymuconate. Similarly, Groseclose and Ribbons (1981) stated that resorcinol may be hydroxylated to a pyrogallol intermediate which is later cleaved by ortho-enzymes to produce oxalocrotonate. Phloroglucinol has also been suggested to act as a substrate/effector for the purified orcinol and resorcinol hydroxylases from a Pseudomonas putida ORC (Ohta et al., 1975). This has interesting implication because it suggests that phloroglucinol may be metabolized through a 1,2,3,5-tetrahydroxybenzene intermediate.

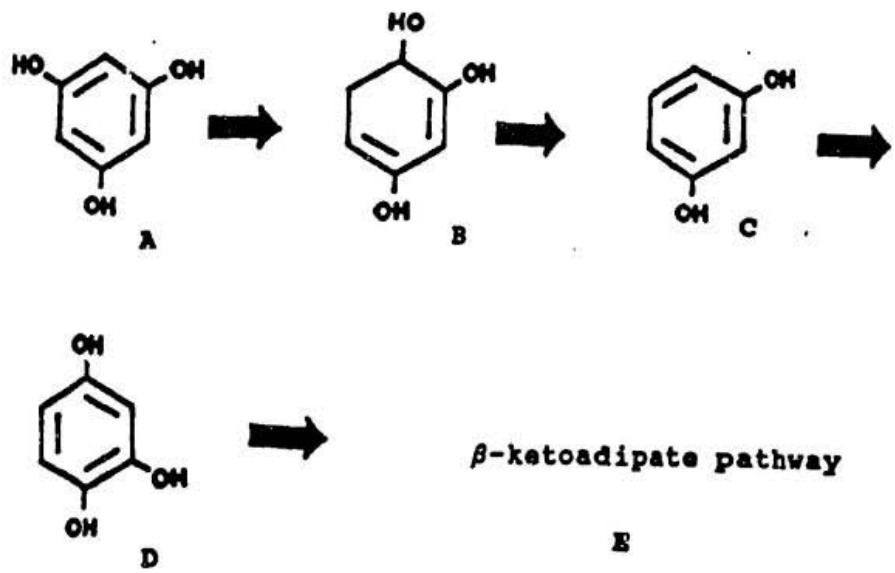


Fig. 1.12. Aerobic degradation of phloroglucinol by a Pseudomonas species. The pathway includes: A, phloroglucinol; B, dihydrophloroglucinol; C, resorcinol; D, 1,2,4-trihydroxybenzene; E, β-ketoadipate pathway (from: Blackwood et al., 1970).

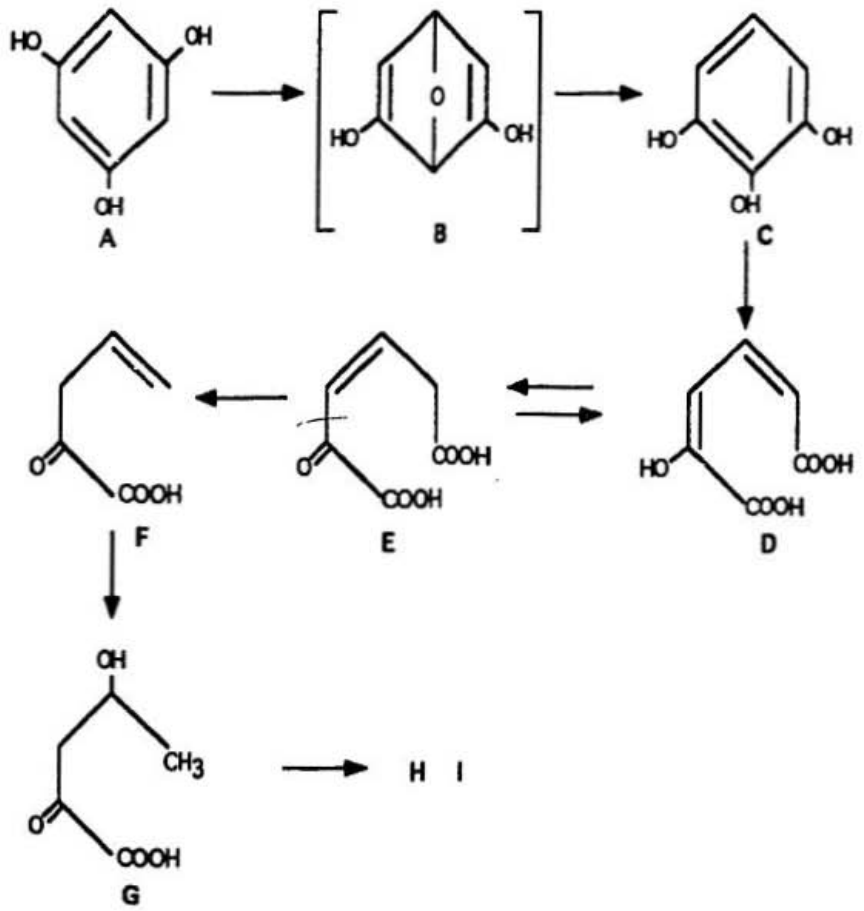


Fig. 1.13. Proposed pathway for the catabolism of phloroglucinol by Fusarium solani. The pathway includes: A, phloroglucinol; B, resorcinol epoxide; C, pyrogallol; D, 2-hydroxymuconate; E, oxalocrotonate; F, vinylpyruvate; G, 4-hydroxy-2-oxovalerate; H, pyruvate; I, acetaldehyde (modified from: Walker and Taylor, 1983).

Leatham et al. (1983), also showed that a Phanerochaete chrysosporium may degrade numerous mono-, di-, triphenols and cause the ring cleavage of the catechol intermediates. Phloroglucinol was not shown to be degraded by this pathway; however it has been suggested that the symmetrical meta substituted hydroxyl groups make this molecule relatively recalcitrant to degradation (Chambers et al., 1963). Trichosporon cutaneum has also been implicated in the degradation of many phenolic compounds, however phloroglucinol metabolism was not suggested (Neujahr and Varga, 1970).

Krumholz and Bryant (1988), Krumholz et al., (1987), Krumholz and Bryant, (1986) have shown that Eubacterium oxidoreducens anaerobically reduces phloroglucinol to the dihydrophloroglucinol, which subsequently undergoes hydrolytic cleavage to produce a 3-hydroxy-5-oxohexanoate (Fig. 1.14). The phloroglucinol reductase was later purified and characterized (Haddock and Ferry, 1989). Eubacterium oxidoreducens was also shown to contain an inducible pyrogallol-phloroglucinol isomerase (Krumholz and Bryant, 1988). Samain et al., (1986) stated that Pelobacter acidigallici may convert numerous trihydroxylated aromatic monomers into phloroglucinol as a transient intermediate from which three moles of acetate are formed. The degradation of phloroglucinol by Pelobacter acidigallici has been shown to follow a similiar pathway as that of Eubacterium oxidoreducens

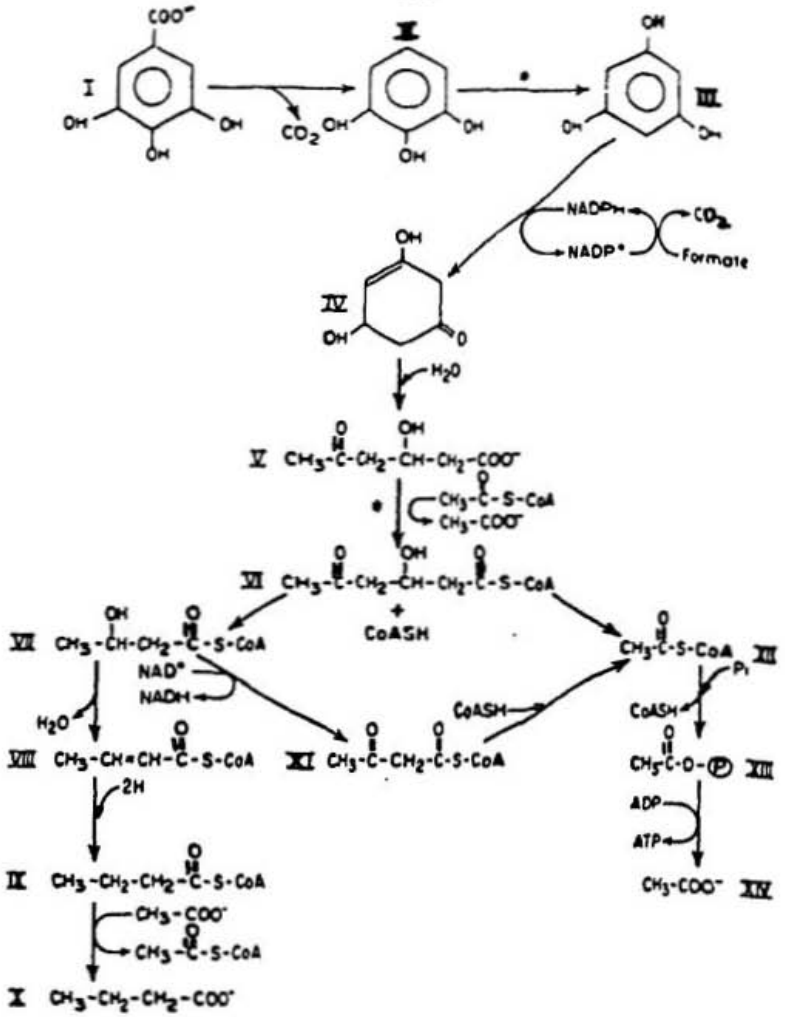


Fig. 1.14. Proposed pathway for energy metabolism of gallate and phloroglucinol by Eubacterium oxidoreducens. The compounds represented are gallate (I), pyrogallol (II), phloroglucinol (III), dihydrophloroglucinol (IV), HOHN (V), 3-hydroxy-5-oxohexanoyl-CoA (VI), 3-hydroxybutyryl-CoA (VII), crotonyl-CoA (VIII), butyryl-CoA (IX), butyrate (X), acetoacetyl-CoA (XI), acetyl-CoA (XII), acetyl phosphate (XIII), and acetate (XIV). (Reproduced with permission from ASM, Krumholz et al., 1987).

however the associated pyrogallol-phloroglucinol isomerase was shown to be stimulated and restored by a 1,2,3,5-tetrahydroxybenzene (Brune and Schink, 1990) (Fig. 1.15).

A Coprococcus species Pe15, which is a rumen strain, has been shown to possess a phloroglucinol reductase with a molecular weight of 130,000 (Patel et al., 1981). However, the phloroglucinol from Eubacterium oxidoreducens had a molecular weight of 78,000 (Haddock and Ferry, 1989).

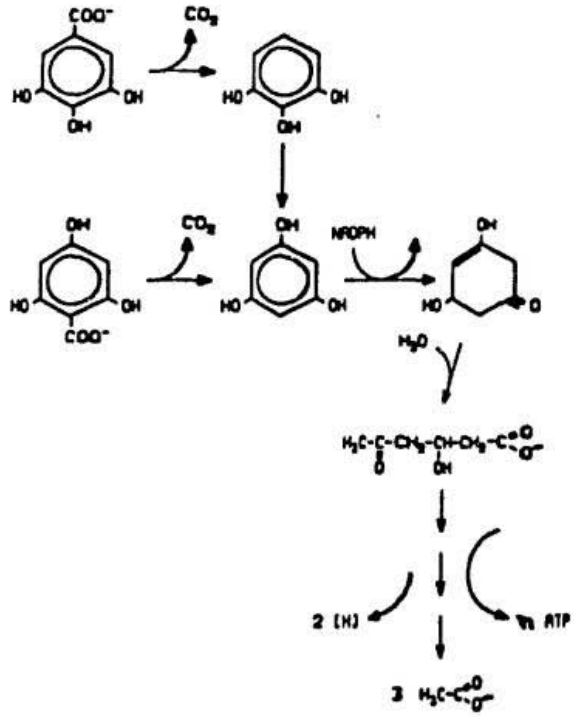


Fig. 1.15. Proposed phloroglucinol pathway for acetate formation from trihydroxybenzenoids in Pelobacter acidigallici. (Reproduced with permission from ASM, Brune and Schink, 1990).

Numerous other rumen strains, belonging to the genera Streptococcus and Coprococcus have been shown to anaerobically degrade phloroglucinol (Tsai and Jones, 1975). Anaerobic photometabolism of phloroglucinol by the purple non-sulphur bacterium Rhodopseudomonas gelantinosa also occurs (Evans 1977; Whittle et al., 1976). It appears that the phloroglucinol is reduced to the dihydrophlorglucinol, in which the subsequent ring cleavage reaction involves hydratation and oxidation to 2-oxo-4-hydroxyadipate. This organism was also shown to degrade diverse aromatic compounds by both aerobic and anaerobic metabolism, although the authors do not mention phloroglucinol as a possible metabolite (Harwood and Gibson, 1988). Similiar pathways for the degradation of dihydroxybenzoates through resorcinol, its reduced product 1,3-cyclohexanediol, and ring cleavage metabolite 5-oxocaproic acid have been suggested (Kluge et al., 1990).

1.3.3 Pharmaceutic Effects of Phloroglucinol Derivatives

Phloroglucinol and its derivatives have been reported to have both antiviral and antimicrobial activity. Tada et al. (1990), have reported the isolation and structure of antimicrobial compounds chinesin I and chinesin II from flowers of Hypericum chinense L. Numerous antimicrobial acylphloroglucinols such as aspidin, uliginosin, humulon, and

lupulon have also been isolate from higher plants (Tada et al., 1990). A 2,4-diisobutyrylphloroglucinol was shown to inhibit numerous pathological bacteria such as Staphlylococcus aureus, Staphylococcus epidermis, Mycobacterium smegmatis, Bacillus subtilus, Micrococcus luteus, and yeast such as Candida albicans. This compound was also shown to be active against vesicular stomatitis virus (VSV), and herpes simplex virus type I (HSV-I), but was not active against polio virus type I (Tada et al., 1990).

Chan et al. (1989), isolated novel phloroglucinol derivatives from the plant Melicope sessiliflora with inhibitory activity against HSV-I and HSV-II in vitro. The active component was a diacylphloroglucinol named sessiliflorene, which lost inhibitory activity during in vivo studies. Phloroglucinol derivatives from Mallotus japonicus were shown to have strong activity against HSV-I during in vitro studies (Arisawa et al., 1990). Ishiguro et al. (1990), isolated a new antibiotic sarothralin from Hypericum japonicum Thunb. which was shown to contain phloroglucinol and fulicinic acid moieties.

Three new euglobals isolated from juvenile leaves of Eucalyptus grandis were shown to inhibit the activation of Epstein-Barr virus (Takasaki et al., 1990). The structure of the euglobals was based on an acylphlorglucinol-monoterpene structure. Inhibitory effects of a series of 3-nitro-2,4,6-

trihydroxybenzamides on the Epstein-Barr virus early antigen induction suggests that these compounds might be novel inhibitors of tumor production (Honda et al., 1991).

Chan and Westley (1991), have recently shown that phloroglucinol and its derivatives either synthesized or isolated from Medicosma sessiliflora may be useful in the treatment of human immunodeficiency virus (HIV). The phloroglucinol derivatives were shown to have the core phlorglucinol trihydroxylic structure, with either an increase in protons in the hydroxyl group or in the nonsubstituted positions. Nakane et al. (1991), suggest that the phloroglucinol derivatives mallotochromene and mallotojaponin inhibit reverse transcriptase in HIV. The mode of inhibition was competitive and non-competitive with the template primer and the triphosphate substrate dTTP respectively.

Arisawa et al. (1990), showed that two phloroglucinol derivatives isomallotolerin and isomallotochromanol were cytotoxic. Arisawa et al. (1990), have shown that a variety of phloroglucinol derivatives, isolated from the pericarps of Mallotus japonicus were cytotoxic against human larynx, lung, and carcinoma cells. They were also toxic towards mouse melanoma, and leukaemia cells.

1.4 Aromatic Degradation and Transformation by the Genus Rhodococcus.

The genus Rhodococcus has a nutritional spectrum and catabolic flexibility that is as diverse as the pseudomonads. The nocardioforms which include Rhodococcus, assimilate aromatic compounds by progressive hydroxylation of the aromatic ring followed by cleavage by specific dioxygenases to central metabolic intermediates (Cain, 1988). Sometimes they resemble eukaryotes in the ability to oxidize by epoxidation, however they do not conjugate metabolites.

Hensel and Straube (1983) have isolated a Rhodococcus sp. PI that utilizes phenol as a carbon and energy source via the β-ketoadipate pathway. Straube (1987) characterized the phenol hydroxylase which was found to have a pH optimum of 7.9 and temperature optimum of 20°C. This enzyme may hydroxylate numerous aromatics. The metabolism of lignin-related compounds and the bioconversion of anison (4,4-dimethoxybenzoin) was shown for a Rhodococcus rhodochrous (Andreoni et al., 1991). In the presence of the yeast extract the (R,S)-anison was converted into the pure (1R,2R)-1,2-bis(4-methoxyphenyl)ethane-1,2-diol. The presence of this highly steroselective dehydrogenase may have industrial applications in that pure compounds may be obtained from racemic mixtures.

The degradation of non-aromatics such as dioxane, tetrahydrofuran, and numerous other cyclic ethers by

Rhodococcus species suggest that the catabolic pathway consists of an initial 2-hydroxylation followed by several oxidations (Bernhardt and Diekmann, 1991). Dickel and Knackmuss (1991) have shown a Rhodococcus sp. QT-1, isolated from contamination resulting from explosives production could utilize both 1,3-dinitrobenzene (1,3-DNB) and 4-nitrocatechol (4-NC) as a source of nitrogen. They state that 1,3-DNB is hydroxylated to the dihydrodiol by an initial 3,4-dioxygenation, followed by the spontaneous loss of nitrite by rearomatization. The 4-NC may lose another nitrate via an oxygenolytic reaction to form 1,2,4-trihydroxybenzene which may be subject to dioxygenase cleavage (Chapman and Ribbons, 1976 a; Chapman and Ribbons, 1976, b). Grund et al. (1992), suggests that during naphthalene degradation in Rhodococcus sp. strain B4, salicylate and gentisate are produced.

The degradation of 2-methylaniline and its chlorinated isomers by Rhodococcus rhodochrous strain CTM was co-metabolized in the presence of ethanol (Fuchs et al., 1991). The degradation of 2-methylaniline proceeds through a dihydrodiol with subsequent elimination of ammonia to produce 2-methylcatechol. Ortho- and meta-cleavage of 2-methylcatechol produces 2-methylmuconic acid and 2-hydroxy-6-oxoheptadienoic acid, respectively. The 3-chloro-2-methylaniline and 4-chloro-2-methylaniline proceeded through a similiar pathway except that the meta-cleavage of 4-chloro-3-methylcatechol produced

a dead end metabolite 2-hydroxy-5-chloro-6-oxoheptadienoic acid. Ortho-cleavage of 4-chloro-3-methylcatechol and 5-chloro-3-methylcatechol produced a 3- or 4-chloro-2-methylmuconic acid with subsequent elimination of chlorine (Fuchs et al., 1991).

Janke et al. (1988), suggested that the degradation of various monochlorinated aromatics in pre-adapted cells of different aromatic utilizing Rhodococcus species occurs in the presence of glucose. Ihn et al. (1989), and Janke et al. (1989), showed that non-growth substrates such as 2-chloroaniline, 3-chloroaniline, 3-chlorophenol, 4-chlorophenol, and 3-chlorobenzoic acid proceeded through the 3- or 4-chlorocatechol with the production of dead end products such as 2-chloro-cis,cis-muconate or cis-4-carboxymethylene-but-2-en-4-olide.

Engresser et al. (1988), state that 3-trifluoromethyl-benzoate may be co-metabolized by a Rhodococcus rubropertinctus N657. Other halogenated and methylated 1,2-dihydroxy-2-hydrobenzoates (DHB) exhibited activity as measured by Michaelis constants (K_m) and relative maximum turnover velocities ($V_{max\text{-}rel}$) of a DHB-dehydrogenase. The K_m and $V_{max\text{-}rel}$ for catechol-1,2-dioxygenase also showed similiar activity, however the 3-trifluoromethyl-(TFM)-catechol had a K_i of 0.02 μM , even though 3-TFM-catechol bound tightly to the enzyme. It apparently did not cleave the 3-TFM-catechol.

Pentachlorophenol (PCP) and other polychlorinated phenols are utilized by the genus Rhodococcus. Rhodococcus chlorophenolicus PCP-1 dechlorinates PCP through hydroxylation and reductive dechlorination (Haggblom et al., 1989; Apajalahti and Salkinoja-Salonen, 1987). Haggblom et al. (1988), also state that polychlorinated phenols may be dechlorinated by the Rhodococcus species CP-2 and Rhodococcus species CG-1 through hydroxylation and reductive dechlorination.

Rhodococcus species which are widely distributed throughout nature may play a role not only in biodegradation of chlorophenols but also biotransformation. Transformations of chlorinated-phenolic compounds through O-methylation has been shown for the genus Rhodococcus. Allard et al. (1985) showed that a Rhodococcus species methylated 3,4,5-trichloroguaiacol (3,4,5-TCG) and 4,5,6-trichloroguaiacol (4,5,6-TCG) to 3,4,5-trichloroveratrole (3,4,5-TCV) under low substrate conditions. At high substrate conditions 3,4,5-TCG was transformed to 3,4,5-trichloro-2,6-dimethoxyphenol and subsequently methylated to 1,2,3-trichloro-4,5,6-trimethoxybenzene. Rhodococcus chlorophenolicus PCP-1 selectively methylate the hydroxyl group flanked by two chlorine substituents (Haggblom et al., 1988). The molecules 2,3,5,6-tetrachlorohydroquinone,2,3,5-trichlorohydroquinone, and 3,5-dichlorohydroquinone were methylated to 2,3,5,6-

tetrachloro-4-methoxyphenol,2,3,5-trichloro-4-methoxyphenol, and 3,5-dichloro-4-methoxyphenol respectively. It appears that the methyl donor is probably S-adenosylmethionine with O-methylation being constitutively expressed (Haggblom et al., 1988; Neilson et al., 1988). Haggblom et al. (1989) found several strains of the genus Rhodococcus which first hydroxylated trichlorophenols to chlorocatechols, which were subsequently methylated to chloroquaiacols and chloroveratroles.

The reaction rates of these transformations are significantly slower than the degradation rates, and it has been suggested that the methylation reaction could act as a detoxification mechanism (Haggblom et al., 1988; Allard et al., 1985). Perhaps methylation in the genus Rhodococcus is the start of a rudimentary conjugation process so often found in eukaryotic organisms.

1.5 Research Objectives

Research on PG metabolism was initiated to elucidate if PG could be metabolized by a novel mechanism. Phloroglucinol seems to be metabolized predominantly by the reductive pathway in which dihydrophloroglucinol and resorcinol (1,3-dihydroxybenzene) are produced (Blackwood et al., 1970). The conversion of dihydrophloroglucinol to resorcinol by the spontaneous loss of water seemed unrealistic because the chemical conversion rate is too slow to account for the efficient utilization of PG. Hydroxylation of the phloroglucinol would produce a 1,2,3,5-tetrahydroxybenzene that may be cleaved by ortho- or meta-cleavage enzymes. The reductive pathway may therefore be eliminated saving the microorganism the energy expense of producing a phloroglucinol reductase and resorcinol hydroxylase.

The objects of my research were: (1) to characterize the unknown strain and identify it to the genus level; (2) to investigate the metabolic pathway for the degradation of phloroglucinol and compare it to other published pathways; (3) to examine any additional pathways utilized by this isolate that may be associated with the degradation of phloroglucinol; (4) to attempt to isolate and characterize the first enzyme in the phloroglucinol degradation pathway.

Chapter II: Taxonomic Characteristics of the BPG-8 Isolate as a Rhodococcus species and its Utilization of Various Aromatics.

2.1 Introduction

Similiarities between Brevibacterium, Arthrobacter, and Rhodococcus make their identification extremely difficult. Inconsistencies exist in the identification scheme for coryneform and nocardioform bacteria (Seiler, 1983; Goodfellow and Pirouz, 1982); however various studies have been undertaken to clarify this problem (Goodfellow et al., 1982; Suzuki and Komagata, 1983).

The taxonomic characterization of the genus Rhodococcus also has a long history of a confused and redefined pedigree (Goodfellow and Cross, 1984). Previous studies characterized the strain as a Bacillus sp. BPG-8 (Acharya, 1986). The Rhodococcus genus encompasses a wide variety of morphological traits, but the defining principles are based on the cell envelope composition (Goodfellow et al., 1982). Rhodococcus spp. have no distinct morphological features other than the ability of many strains to form hyphae and fragment into rods and cocci (Goodfellow, 1986). The timing of the fragmentation process is influenced by environmental parameters that affect the growth rate. Characterization by genetic studies has been hindered by the slow growth of the genus Rhodococcus and their

tendency to clump and form coenocytic structures (Goodfellow, 1986).

The catabolic diversity of the Rhodococcus genus includes compounds such as alicyclics, aromatics, polyaromatics, pryridines, steroids, and numerous carbohydrates (Goodfellow, 1986). Rhodococcus spp. also has the ability to transform numerous xenobiotics, pesticides, and detergents (Goodfellow, 1986). These abilities may be useful in the characterization of the isolate.

In this chapter I describe a Rhodococcus BPG-8 species isolated from soil that is able to mineralize numerous aromatics. Emphasis is on phloroglucinol (PG; 1,3,5-trihydroxybenzene) metabolism. Detailed aspects of PG metabolism by this species have not been studied previously. This chapter describes the taxonomic characterization of the strain and initial studies on its metabolic characteristics. These studies were pertinent before detailed metabolic studies could be implemented.

2.2 Materials and Methods

2.2.1 Source of organism. A loop of soil suspension in physiological saline was streaked on agar plates containing basal salts. PG crystals placed in the lid of the inverted petri dish formed the sole source of carbon and energy. Pure cultures were obtained by subculturing isolated colonies on

the same medium (Dr. Patel, unpublished work). Pure colonies were then transferred to trypticase soy agar (TSA) plates and slants for storage at 4°C.

2.2.2 Growth conditions. The basal salt medium contained (g/l): $(NH_4)_2SO_4$, 0.5; $MgSO_4.7\ H_2O$, 0.1; KH_2PO_4, 6.8; 0.1 ml/l trace elements according to (Bushwell and Clark, 1976); and 0.001% yeast extract. The pH was adjusted with 1 N NaOH to the required values. The concentration of the PG utilized for growth ranged between 0-16 mM while the other aromatics were tested at 8 mM. The inoculum was prepared by growing the bacterium in basal salts medium containing 8.0 mM PG for 18 hours. Culture flasks (250 ml) containing 50 ml of basal salt medium was inoculated to give an initial optical density reading of 0.2 at 600nm. The bacterium were grown in 250 ml dark brown coloured flasks to minimize photodegradation of the PG. Controls for the photodegradation were similar to experimental treatments except the inoculum consisted of bacterial cells boiled for 5 min.

Colony characteristics and physiological tests, which included acid production and carbohydrate utilization, were performed according to O'Brien and Colwell (1987), and Smibert and Krieg (1981). Sensitivity to antibiotics was analyzed using antibiotic discs (Oxoid Ltd., Basingstoke, Hampshire, England) placed on nutrient agar plates. The following

antibiotics were employed: penicillin G, 10 U; streptomycin, 10 μg; chloramphenicol, 30 μg; erythromycin, 5 μg (LD), 15 μg (HD); tetracycline, 30 μg; rifampin, 5 U; methicillin, 5 U; and bactracin, 10 U.

2.2.3 Preparation of cell free extracts. Two grams of pelleted and washed cells (3X) were suspended in 3 ml of 20 mM phosphate buffer containing 1 mM EDTA, 1 mM 2-mercaptoethanol, and 15% glycerol. The suspension was ice cooled and sonicated (Braunsonic 2000, Canlab) at maximum power for 3 minutes with gaps of 30 seconds to cool the probe and samples. The disrupted cells were then centrifuged (20,000 x g, 30 min) in a Sorvall RC-3 centrifuge. The supernatant formed the source of cell-free extract and was free of any cells as determined by light microscopy and plating 0.1 ml of extract onto TSA plates.

2.2.4 Enzyme assay. PG hydroxylase was assayed according to the method of Patel et al., (1981) under aerobic conditions. One enzyme unit was defined as the amount of enzyme required to oxidize 1 μmole of NAD(P)H per min. Specific activity was defined as enzyme units per mg protein.

2.2.5 Analytical methods. Protein in cell extracts was determined by the methods of Lowry et al. (1951) or Bradford

(1976). PG concentrations were determined by the colorimetric method of Jayasankar and Bhat (1966). The absorbance of the solution containing PG was read at 534 nm and compared against a standard curve of PG. Microscopic investigation, which included light, scanning electron microscopy (SEM), and transmission electron microscopy (TEM) of the growth cycle of the bacterium was carried out (Krulwich and Pate, 1971; Krulwich et al., 1967). Bacterial cells were fixed for 1 hour at 4°C in Karnovsky's fixative in 0.1 M Sorensen's phosphate buffer, washed and post-fixed in osmium tetroxide in Sorenson's buffer for 1 hour at 4°C, and dehydrated through a graded series of ethanol. TEM preparations were embedded in Spurr's resin, and the thin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 9A TEM. SEM samples were grown on glass coverslips, washed in phosphate buffer (pH 7.0, 50 mM), and critical point dried from liquid carbon dioxide in a Polaron E 3000 apparatus. The coverslips were attached to aluminum stubs, gold coated, and examined with a Hitachi S570 SEM at an accelerating voltage of 20 KV.

Cell wall analysis for sugars, amino sugars, and amino acids were prepared according to the method of Bousefield et al. (1985); with the exception that sugars that were acetylated were detected by gas chromatography and mass spectrophotometry (GC-MS). Amino sugars and amino acids were

detected using a Beckman amino acid analyzer. A comparison of the fatty acid profile of the unknown to those in a database which included numerous species of Corynebacterium, Mycobacterium, Nocardia, Caseobacter, Brevibacterium, Arthrobacter, and Rhodococcus was performed using gas chromotography with a flame ionization detector, autosampler, integrator, and a computer database. This was done by Microcheck Inc. Northfield, Vermont, USA.

2.3 Results

2.3.1 Bacterial isolate. On trypticase soy agar the isolate produced circular, raised, white mucoid opaque colonies that contained Gram positive, nonmotile, strict aerobic bacteria. The bacterium was catalase positive and oxidase negative. Both the strain and Rhodococcus erythropolis ATCC #4277 were susceptible to all the antibiotics except methicillin. When the unknown was compared with a standard R. erythropolis there was 100% homology for acid production and growth on the numerous substrates routinely examined for the identification of an organism (Table 2.1 and Table 2.2).

The organism had a distinctive rod and coccus growth cycle that was dependent on the nutrient status of the organism. Coccal growth occurred during nutrient limiting conditions while rod growth was associated with nutrient rich conditions (Fig. 2.1, Fig. 2.2, and Fig. 2.3). To

differentiate the Rhodococcus sp. BPG-8 from other genera exhibiting similar characteristics, a cell wall analysis showed that the Rhodococcus sp. BPG-8 had meso-diamino-pimelic acid and arabinose in the cell wall (Table 2.3). Comparison between the fatty acid profile with the best match from the 7000 computer profiles suggested that the unknown was probably R. erythropolis (Table 2.4). No match could be found for any species of Corynebacterium, Mycobacterium, Nocardia, Caseobacter, Brevibacterium, and Arthrobacter.

2.3.2 Growth conditions. The optimal conditions for growth of the Rhodococcus sp. BPG-8 in the basal salt medium were 25°C, pH 5.0-8.0, and 8.0 mM PG (Fig. 2.4). Darkened brown flasks helped to minimize the PG photodegradation and no growth occurred when the substrate was eliminated from the media. PG was almost completely utilized when the increasing biomass of the isolate reached approximately 15 μg protein/ml (Fig. 2.5). Values were corrected for PG photo-oxidation (maximum 1.5 mM).

2.3.3 Growth on different substrates. The strain could also utilize various other aromatic compounds when grown at a concentration of 8.0 mM (Table 2.5). Maximum biomass occurred with 1,2,4-trihydroxybenzene and protocatechuate (3,4-dihydroxybenzoic acid), while numerous other aromatics failed to promote growth of the strain or caused inhibition.

Experiments were performed to find out if degradation of PG was inducible in the isolate. Trypticase soy broth or PG induced cells were washed and later suspended in a medium containing PG. Only the PG induced cells had increased biomass yields suggesting that induction may be important to PG utilization. PG hydroxylase was detected in the PG grown cell extract by the method of Patel et al. (1981). When the cells were grown on substrates such as glucose, succinate, and pyruvate no induction of PG hydroxylase was detected by the enzymatic assay (Table 2.6).

Table 2.1. Comparison of acid production by the isolate Rhodococcus sp. BPG-8 and R. erythropolis (RE) ATCC #4277.

	Acid Production				
Substrate	BPG-8	RE	Substrate	BPG-8	RE
D-glucose	+	+	maltose	-	-
glycerol	+	+	adonitol	-	-
sorbitol	+	+	arabinose	-	-
trehalose	+	+	cellibiose	-	-
ribose	+	+	galactose	-	-
fructose	+	+	glycogen	-	-
inositol	+	+	inulin	-	-
saccharose	+	+	melezitose	-	-
D-arabitol	+	+	rhammose	-	-
esculin	+	+	xylose	-	-
mannitol	+	+	erythritol	-	-
starch	-	-	gluconate	-	-
lactose	-	-	salicin	-	-
melibiose	-	-	D-raffinose	-	-
L-sorbose	-	-	D-mannose	-	-

Substrate concentration 8 mM; pH 7.0; 25°C; 24 hours growth; N=3. + : acid production; - : no acid production.

Table 2.2. Comparison of substrate utilization by the isolate Rhodococcus sp. BPG-8 and R. erythropolis (RE) ATCC #4277.

	Substrate Utilization				
Substrate	BPG-8	RE	Substrate	BPG-8	RE
D-glucose	+	+	lactose	-	-
glycerol	+	+	glycine	-	-
sorbitol	+	+	malonate	-	-
trehalose	+	+	mannitol	+	+
GLAmine	+	+	rhammose	-	-
D-salicin	-	-	benzoate	-	-
inositol	+	+	citrate	+	+
D-alanine	+	+	melezitose	-	-
L-asparagine	+	+	testost.	+	+
L-PHE	+	+	ethanol	+	+
L-proline	+	+	sucrose	+	+
L-arabinose	-	-	lactate	+	+
galactose	-	-	acetate	+	+
L-serine	+	+	pyruvate	+	+
gluconate	+	+	fumurate	+	+

GLAmine, _glucosamine; L-PHE, phenylalanine; testost., testosterone; substrate concentration 8 mM; pH 7.0; 25°C; 24 hours growth; N=3. + : acid production; - : no acid production.

Table 2.3. Qualitative identification of soil isolates using cell wall analysis for the determination of the meso diamino-pimelic acid and arabinose.

Isolates	Rod/Cocci cycle	Meso-diamino pimelic acid	Arabinose present
Soil isolate	+	+	+
Rhodococcus erythropolis	+	+	+
Brevibacterium linens	+	+	nd
Arthrobacter globiformis	+	nd	nd

+, present; nd, not detected. See Materials and Methods for details.

Table 2.4. Fatty acid profile of the Rhodococcus sp. BPG-8 with the best match R. erythropolis as determined from a computer profile of 7000 isolates.

Fatty acid	BPG-8 soil isolate	R. erythropolis isolates
	Mean %	Mean % (range)
14:0	5	6 (5-8)
15:0	4	4 (3-6)
16:1	1	3 (3-4)
16:0	25	22 (19-25)
17:1 iso-9 *	1	1 (1-2)
17:1 B	5	3 (2-5)
17:0	1	1 (1-2)
18:1 iso F	2	2 (1-3)
18:1 cis-9	23	19 (15-25)
18:0	1	1 (1-2)
19:0	5	4 (0-8)
20:0	4	3 (0-5)

* iso-9,iso-F, B, cis-9 are derivatives of the specific fatty acid type. See Methods and Materials for details.

Table 2.5. Growth of Rhodococcus sp. BPG-8 on various aromatic substrates as determined by biomass yield.

	Biomass yield (protein, μg/ml)	
	Substrate	Sub. + Succ.
Pyrogallol	15 ± 0.5	NT
1,3,5-trichlorbenzene	4 ± 3.0	20 ± 2.4
1,4-dichlorobenzene	5 ± 3.0	20 ± 3.5
Phloroglucinol	18 ± 0.8	NT
Resorcinol	6 ± 0.3	NT
1,2,4-trihydroxybenzene	21 ± 2.0	NT
Protocatechuate	25 ± 1.6	NT
Acetylsalicylate	6 ± 2.5	21 ± 1.5
3-HydroxyBenzoate	25 ± 3.0	NT
Quinate	28 ± 2.5	NT
Orcinol	8 ± 2.0	NT
Naphthalene	6 ± 2.5	23 ± 1.7
2,4-dichlorophenoxyacetate	2 ± 1.5	2 ± 1.8
Venezuelan crude oil	30 ± 4.5	NT
Para-aminobenzoic acid	2 ± 1.0	20 ± 2.7
2,4-dichloronaphthalene	0	0
2.4-dichlorophenol	0	0
4-chlororesorcinol	0	0

Reaction conditions: 8.0 mM substrate (sub.) supplemented with 8.0 mM succinate (succ.), 25°C, pH 7.0, Mean ± st. dev., N=3. NT: not tested.

Table 2.6. Inducibility of Phloroglucinol hydroxylase in the soil isolate Rhodococcus sp. BPG-8.

Growth Substrate	Specific Activity
PG	0.052 ± 0.05
PG + succinate	0.048 ± 0.03
PG + pyruvate	0.056 ± 0.05
PG + glucose	0.047 ± 0.06
Succinate	0.0
Pyruvate	0.0
Glucose	0.0

Specific activity: μmoles of NADH oxidized/min/mg protein. Growth conditions: substrate concentration 8 mM, pH 7.0, 25°C, Mean ± st. dev., N=3.



Fig. 2.1. Micrographs A,B,C illustrating the rod/cocci cycle of the Rhodococcus sp. BPG-8 at 0, 28, & 48 hours. The initial micrograph (A) are Rhodococcus sp. BPG-8 in nutrient deprived conditions. Peptone (0.5%) was added at 24 hours with rod formation completed at 28 hours (B). The rod shaped bacteria subsequently returned to their cocci form by 48 hours (C) with complete utilization of the peptone.

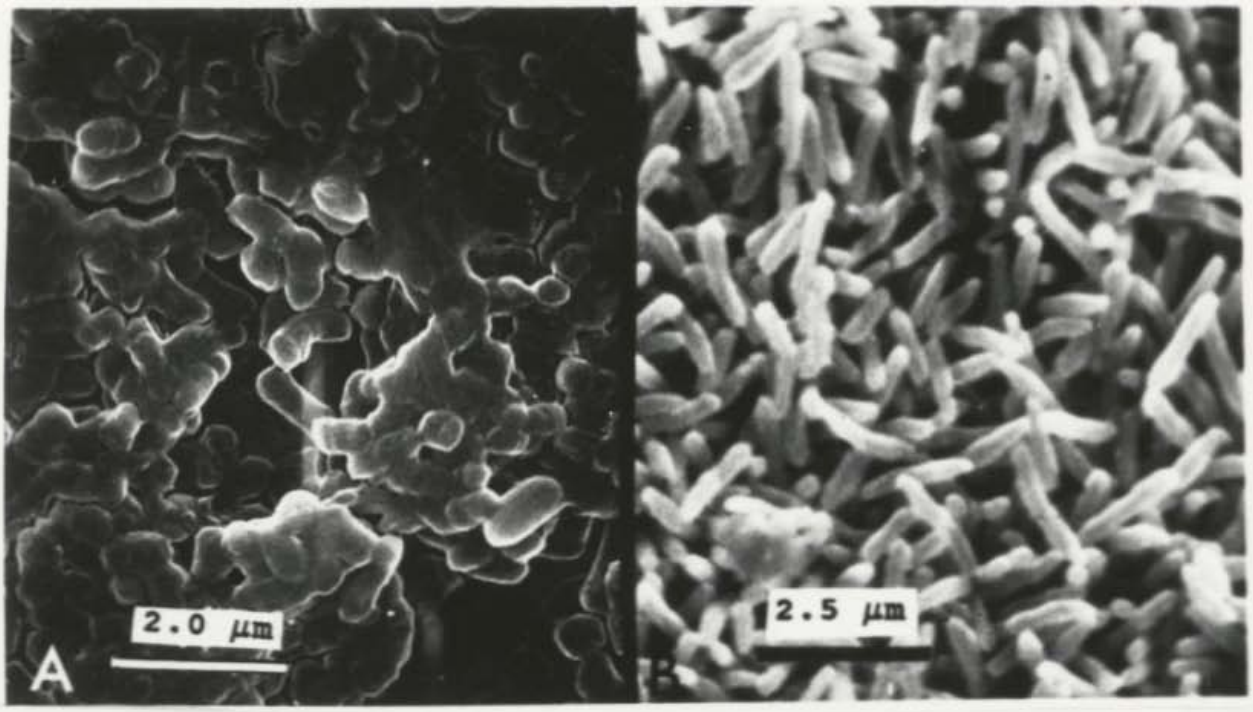


Fig. 2.2. Scanning electron micrographs (SEM) of the morphological types of the Rhodococcus sp. BPG-8 isolate. Micrographs A and B illustrate the coccal and rod shape under nutrient poor and rich conditions. See Materials and Methods for details concerning preparation.

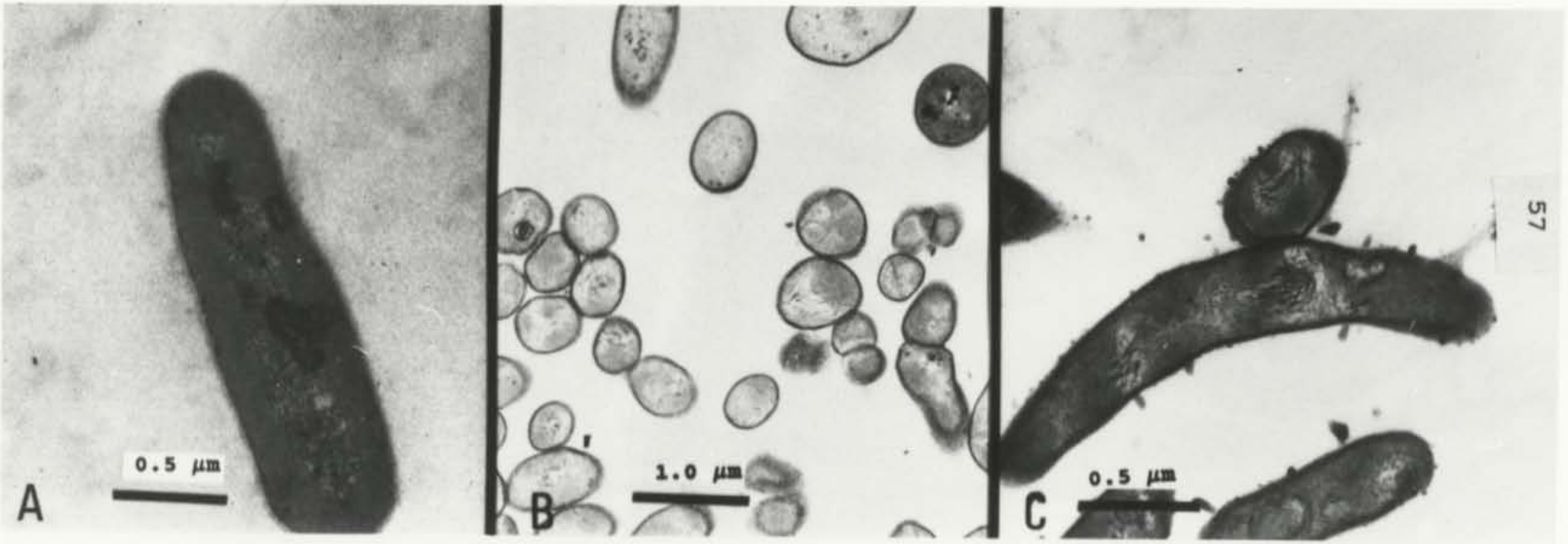


Fig. 2.3. Transmission electron micrographs (TEM) of the morphological types of the Rhodococcus sp. BPG-8. Micrographs A, B, and C illustrate the rod shape, coccal shape and the transition state between rod and coccus, respectively. See Materials and Methods for details concerning preparation.

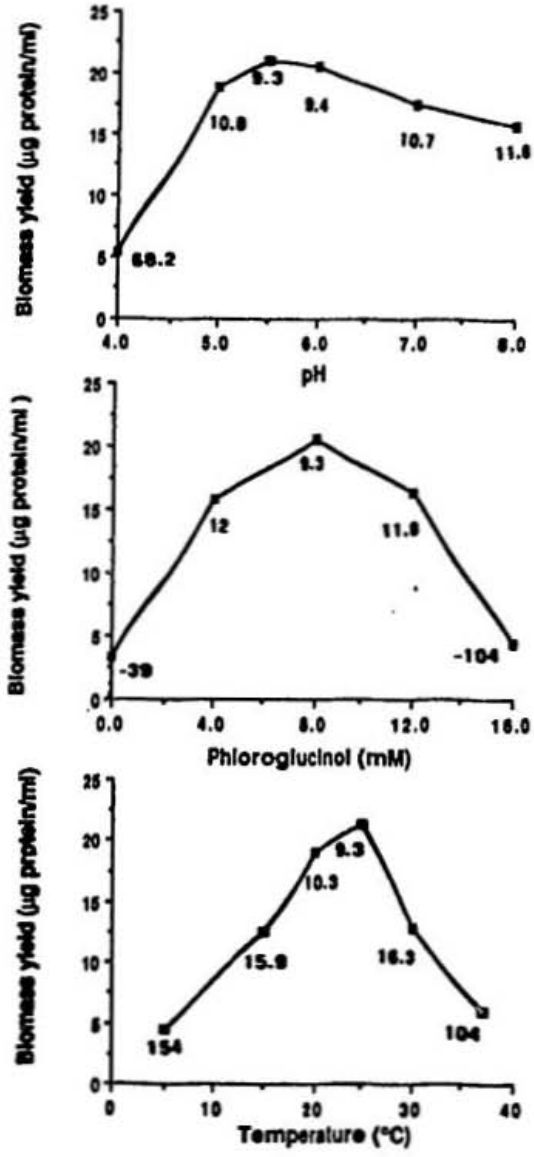


Fig. 2.4. Effect of pH, phloroglucinol concentration, and temperature on the growth of the Rhodococcus sp. BPG-8 (µg protein/ml). Numbers refer to generation time in hours. Values are an average of three determinations with maximum standard deviation less that 5%.

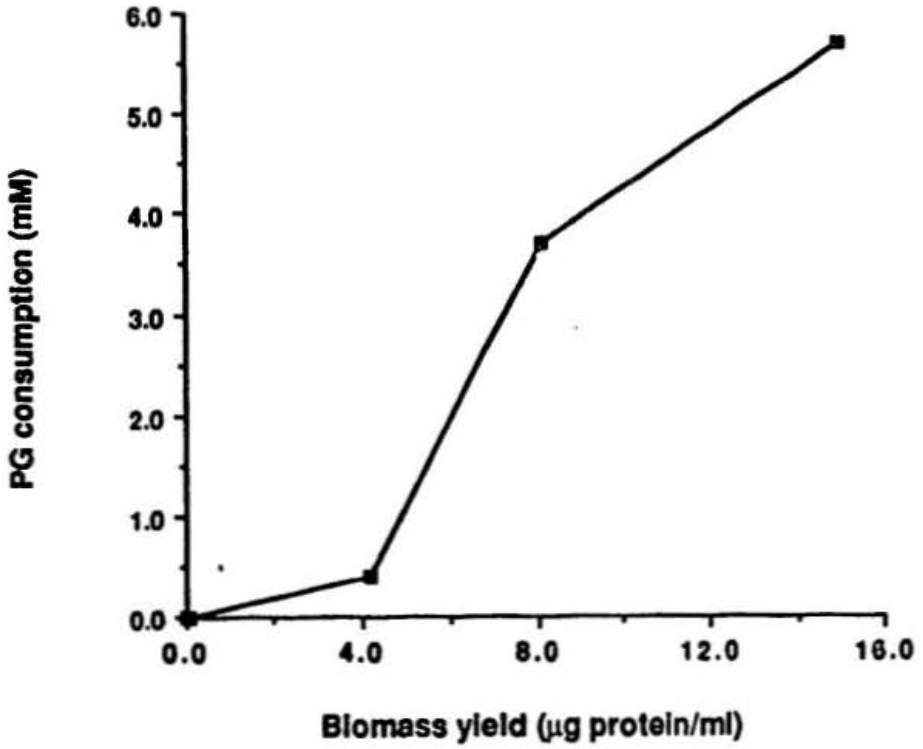


Fig. 2.5. Consumption of PG by a growing population of the Rhodococcus sp. BPG-8 (µg protein/ml). Values were corrected for PG photodegradation. Experiments were performed at pH 7.0, 25°C with 8.0 mM PG initially added. Values are an average of three values with maximum standard deviation less that 5%.

2.4 Discussion

The distinctive rod and coccus cycle suggested that the isolate could belong to one of a limited number of genera. The identification of meso-diaminopimelic acid and arabinose in the cell wall, along with the fatty acid comparison profile, suggest that the unknown is a Rhodococcus species. The 100% phenotypic homology between control R. erythropolis ATCC #4277 and the unknown for both acid production and growth with the pertinent carbon sources showed that the unknown was probably R. erythropolis.

Numerical analysis of the accumulated data seemed to confirm the classical chemical analysis that suggested that the isolate belongs to the genus Rhodococcus (Whalen, 1991). In the simple matching coefficient and Jaccard coefficient analysis the isolate clustered with R. erythropolis at 90% and 84% similiarity respectively (Whalen, 1991).

The isolate was shown to degrade numerous aromatic compounds which is characteristic of some Rhodococcus species (Janke et al., 1988; Nagasawa et al., 1990). The ability of the genus Rhodococcus to utilize numerous aromatic compounds, notably PG has not been thoroughly studied. Gram positive cells utilizing PG, seem to be limited to a small number of organisms which include Mycobacterium species (Brune and Schink, 1990), Streptococcus bovis, Coprococcus (Tsai and Jones, 1975), and Pelobacter acidigallici (Samain et al.,

1986). Physical parameters such as temperature, pH, and substrate concentration seemed to have a significant effect on the PG metabolism by the Rhodococcus sp. BPG-8 isolate. Growth was optimal at 8.0 mM PG, however higher concentrations were increasingly toxic to the organism, while lower concentrations decreased growth by limiting the carbon source. Rhodococcus sp. BPG-8 had a wide pH range between 5-8 and a temperature optimum of 25°C. The decrease in growth at higher pH's is probably caused by susceptibility of the PG molecule to alkaline conditions since some yellowing of the media occurred. This color change was minimized by excluding manganese (which catalyses PG oxidation) from the trace element solution. The temperature optimum probably reflects the enzyme stability of the degradating enzymes in the metabolic pathway of PG degradation. However, the rumen strain Coprococcus sp. is known to have a temperature optimum of 30°C (Patel et al., 1981), while the enzyme PG reductase from Eubacterium oxidoreducens G-41 has a temperature optimum of 40°C (Haddock and Ferry, 1989).

PG was shown to disappear as growth increased. The growth on PG seemed to be inducible since trypticase soy broth grown cells, and not PG grown cells, showed lower growth values. This was verified by using a cell-free extract of the Rhodococcus sp. BPG-8 strain grown on various substrates and assaying for PG hydroxylase. The organism was able to grow on

a number of other aromatic substrates when induced on PG. This showed that the enzymes pathways associated with their degradation may be related. However numerous substrates, notably catechol which is a common intermediate of numerous pathways of aromatic metabolism, did not support growth. This could possibly be caused by an inefficient uptake system (Groenewegen et al., 1990; Thayer and Wheelis, 1982) or from a lack of an adequate enzyme system. Other possible intermediates could include: 1,2,4-trihydroxybenzene, 1,2,3-trihydroxybenzene, or even a 1,2,3,5-tetrahydroxybenzene (1,2,3,5-THB).

The metabolic pathway of PG biodegradation by this strain will be further investigated in later chapters. Molecular mechanisms involved in this process will be compared to known aerobic and anaerobic mechanisms of PG utilization.

Chapter III: Metabolic Pathway for Phloroglucinol Degradation and Resorcinol Biotransformation by Rhodococcus sp. BPG-8.

3.1 Introduction

Phloroglucinol (PG) is a constituent of flavonoids such as anthocyanins, catechins, quercetin, and chalcones (Rao et al., 1991; Robinson, 1962). It is released from these compounds by fungi and bacteria that contain adequate enzymatic systems for cleaving PG from these complex structures.

The degradation of PG may occur aerobically and anaerobically in prokaryotes, as well as in eukaryotes. Bacteria such as Pseudomonas (Hang, 1967), Arthrobacter (Mullakhanhai and Bhatt, 1966), Flavobacterium (Bennetti and Schlesser, 1950) and Mycobacterium (Bernheim, 1956) have been shown to aerobically utilize PG. It has also been reported that a marine alga may degrade PG (Craigie et al., 1965) as well as fungi such as Penicillium simplicissimum (Patel et al., 1990), Fusarium solani (Walker and Taylor, 1983), and yeast such as Trichosporon cutaneum (Neujahr and Varga, 1970).

Anaerobic degradation of PG can occur in photosynthetic organisms such as Rhodopseudomonas gelantinosa (Evans, 1977) as well as anaerobes such as Streptococcus bovis (Tsai and Jones, 1975), Butyrivibrio species (Krishnamurthy et al., 1970), Eubacterium oxidoreducens (Krumholtz et al., 1987),

Pelobacter acidigallici (Brune and Schink, 1990; Samain et al., 1986), and Coprococcus species (Patel et al., 1981).

This chapter reports a Rhodococcus sp. BPG-8 isolate that is able to utilize PG as a source of carbon and energy. Resorcinol also seems to be transformed by the PG grown cells. This is believed to be the first reported case of PG degradation by a Rhodococcus species, and a novel tentative pathway for the degradation of PG and the biotransformation of resorcinol is described.

3.2 Materials and Methods

3.2.1 Source of organism. The organism tentatively named Rhodococcus sp. BPG-8 was isolated by the procedure outlined in Chapter 2. PG induced cultures were obtained by subculturing isolated colonies on both liquid and solid minimal salts medium supplemented with PG. Pure PG induced cultures were lyophilized in 20 % glycerol and stored at -20°C.

3.2.2 Growth conditions. The minimal salt medium contained (g/L): $(NH_4)_2SO_4$, 1.0; $MgSO_4.7\ H_2O$, 0.5; KH_2PO_4, 6.8, 0.1 ml/L trace elements (Bushwell and Clark 1976), and 0.001% yeast extract. The Rhodococcus sp. BPG-8 isolate was grown under the optimal conditions of 8 mM PG, 25°C, and pH 7.0 (Armstrong and Patel, 1992). The inoculum was prepared by growing and

subculturing Rhodococcus sp. BPG-8 cells in PG and suspending them into 4 X 250 ml of minimal salt media to an optical density of 0.2 at 600 nm. The bacterium was grown in one litre dark brown flasks to an optical density of approximately 0.7 at 600 nm and aseptically transferred to 18 liters of sterile minimal salts media in a fermenter. The fermenter was sparged with sterile air (200 litres/ hour) during the growth process.

3.2.3 Preparation of cell free extracts. Two grams of washed pelleted cells were resuspended in 3 ml of 20 mM phosphate buffer containing 1 mM EDTA (ethylenediaminetetracetic acid), 1 mM 2-mercaptoethanol, and 15% glycerol. The suspension was ice cooled and sonicated (Braunsonic 2000, Canlab) at maximum power for 3 minutes with gaps of 30 seconds to cool the probe. The cells were subsequently broken by a French pressure cell (SLM Aminco Inc.) at 846 kg/cm^2. The disrupted cells were then centrifuged (20,000 x g, 30 min) in a Sorvall RC-5 centrifuge (Dupont instruments). The supernatant formed the source of cell-free extract and was free of any viable cells as determined by light microscopy and by plating a loopful onto trypticase soy agar plates (TSA).

3.2.4 Analytical methods. PG hydroxylase and resorcinol hydroxylase were assayed according to the methods of Patel et al., (1981), and Neujahr and Gaal, (1975). A total of 3 ml

reaction mixture contained (in μmoles): potassium phosphate buffer, pH 7.2, 270; NAD(P)H, 0.6; PG, 1.0; and dilute cell-free extract, 0.2 ml or partially purified extract 0.1 ml. The reaction was initiated by adding PG to the reaction mixture while the blank contained no substrate. Appropriate controls were included to check for endogenous oxidation of NAD(P)H. Disappearance of NAD(P)H at 340 nm and 25°C was measured using a Shimadzu UV-260 Recording Spectrophotometer. One enzyme unit was defined as the amount of enzyme required to oxidize 1 μmole of NAD(P)H per min. Specific activity was defined as enzyme units per mg protein. Protein was measured according to the method of Bradford (1976).

The 1,2,3,5-tetrahydroxybenzene (1,2,3,5-THB) and 1,2,4-trihydroxybenzene dioxygenase activities were illustrated spectroscopically by the disappearance of their λ-max at 283 and 290 nm, respectively. Appropriate controls were performed with substrate alone and boiled enzyme extract with the assay system consisting of a total of 3 ml containing (in μmoles): potassium phosphate buffer, (pH 7.0), 270; 1,2,3,5-THB, 0.02; 1,2,4-trihydroxybenzene, 0.1; and dilute cell-free extract, 0.2 ml.

The acetopyruvate hydrolysis (total volume 3 ml) reaction mixture contained (in μmoles): potassium phosphate buffer, pH (7.0), 270; acetopyruvate, 1.0; and cell-free extract, 0.2 ml (1.43 mg/ml protein). The reaction was initiated by adding

acetopyruvate to the reaction mixture, while the blank contained no substrate. Utilization of substrate was shown by the decrease in absorbance at 287 nm (Chapman and Ribbons, 1976 a)

Oxygen consumption by cell-free extracts and whole cells in the presence of different substrates was determined using a Clark oxygen electrode (Patel et al., 1990). Formate production was measured by the method of Barker and Somers, (1966); while pyruvate was measured as its 2,4-dinitrophenylhydrazone derivative (Friedemann and Haugen, 1943). Acetic acid was measured enzymatically with the Boehringer Mannheim kit (Cat. No. 148 261).

3.2.5 Isolation of metabolic intermediates. Metabolite intermediates (51) were obtained by growing BPG-8 in 8 mM PG and by centrifuging the cells (20,000 x g, 30 min) in a Sorvall RC-5 centrifuge. The supernatant was acidified to pH 2.0 with concentrated HCl. The clear acidified liquid was evaporated to 100 ml and extracted in four volumes of ethyl acetate. The organic phase was evaporated to dryness and acetylated in acetic anhydride and pyridine at room temperature for 12 hours (Hellou et al., 1989; Hellou and Payne, 1987). The acetylated products were compared against the authentic acetylated standards and analyzed by GC-MS. GC-MS was performed using a Hewlett-Packard mass selective

detector, series 5970, a Hewlett-Packard 5890 gas-liquid chromatograph, and a Hewlett-Packard Series 300 data system, using a CP-Sil 5CB column (25 m X 0.2 mm i.d.) (Hellou et al., 1989). A temperature program, starting at 100°C for 1 min and then increasing to 250°C at 5°C/min, was employed.

Induced cells (50 mg dry wt.) were uniformly suspended into 10 ml of KH_2PO4 buffer (50 mM, pH 7.0, 25°C) and inoculated with PG, and resorcinol at a concentration of 40 mM and allowed to react for approximately 20 min. The cells were removed by centrifugation (20,000 x g, 30 min) using a Sorvall RC-5 centrifuge and the supernatant acidified to pH 2.0 with concentrated HCl. The clear liquid was subsequently extracted with ethyl acetate and dissolved into 0.5 ml of methanol. The methanol solution (50 μl) was spotted on Kodak chromatogram sheets (13181 silica gel with fluorescent indicator, Eastman Kodak Co. Rochester N.Y. 14650) and developed in a benzene: methanol: acetate (45:8:4) solvent system (Randerath, 1963). The spots were visualized under ultraviolet light and compared against authentic standards. The spots were then gently scraped off the chromatograms and again extracted into ethyl acetate. Then it was evaporated and dissolved into methanol. The methanol extract was then analyzed by ultraviolet spectrophotometry and compared against the authentic standards.

The 1,2,3,5-THB was synthesized by the method of Baxter

and Brown (1967), its structure confirmed by melting point, MS, FT-IR, UV, and carbon NMR (Ragan, 1978).

3.3 Results

3.3.1 Isolated Intermediates. The isolated intermediates as determined by GC-MS showed that the acetylated aromatic compound of 1,2,4-trihydroxybenzene was detected in the acidified supernatant of the resorcinol fed PG induced Rhodococcus sp. BPG-8 cells (Table 3.1). GC-MS spectra of the PG extract had high molecular weight molecules that could not be discerned. However, these may have been the polymerization products of the quinones of 1,2,3,5-THB which readily form polymers (Corbett, 1970). Interestingly, a product was formed with the identical GC-MS spectral pattern for both the acetylated 1,2,3,5-THB standard and experimentally detected product. Catechol, another possible intermediate, could not be detected by GC-MS.

Experiments using PG induced Rhodococcus sp. BPG-8 cells detected 1,2,4-trihydroxybenzene when PG induced cells were fed resorcinol. However, a peculiar product appeared with peaks at 283 nm and 273 nm when these cells were fed PG (Table 3.2). The Rf value and UV profile was identical for a 1,2,3,5-THB that was in equilibrium with its oxidized derivative 2,6-dihydroxy-1,4-benzoquinone.

Conversion of resorcinol into 1,2,4-trihydroxybenzene was

detected by ultraviolet spectrophotometry (Fig. 3.1). This was confirmed by GC-MS analysis of the acetylated product (Table 3.1).

3.3.2 Oxygen Utilization. Substrate oxidation as measured by oxygen utilization by PG induced cell-free extract or whole cells was performed. Oxygen consumption was observed in the presence of PG, 1,2,3,5-THB, 1,2,4-trihydroxybenzene, resorcinol, acetopyruvate, acetate, and pyruvate (Table 3.3). Intermediates such as catechol and β-ketoadipate showed no oxygen consumption.

3.3.3 Enzymatic Studies. PG hydroxylase and resorcinol activities were detected in both the cell-free and partially purified extract (Table 3.4). The hydroxylation of PG did not occur until air was introduced into the cuvette with the partially purified enzyme (Fig. 3.2). When cell-free extract replaced the partially purified extract a peak formed at 285 nm and then disappeared subsequently (Fig. 3.3). When PG was added to PG induced whole cells (1.43 mg protein), an extracted product exhibited the same spectral pattern as the authentic 1,2,3,5-THB and its oxidized product (Fig. 3.4). Dihydrophloroglucinol was never detected by UV-spectrophotometry or by GC-MS as its 2,4-dinitrophenyl-hydrazone in the medium, nor could this reaction be shown to

be reversible.

The spectral peak at 283 nm for 1,2,3,5-THB shifted to 288 nm with the subsequent extinction of this peak occurring over 30 minutes (Fig. 3.5). The 1,2,3,5-THB (0.2 μmol) utilized approximately equimolar amounts of oxygen (0.18 ± 0.04 μmol, N=3). This reaction could only be shown with PG induced cell-free extract. When 1,2,4-trihydroxybenzene was reacted with cell-free extracts or whole cells of PG induced Rhodococcus sp. BPG-8, a peak was formed at approximately 308 nm (Fig. 3.6). The 1,2,4-trihydroxybenzene had an initial peak at 290 nm with a peak forming at 345 nm for both the 1,2,4-trihydroxybenzene alone and with boiled extract controls. This peak is due to a chemical reaction between the 2-mercaptoethanol component in the enzyme extract and the 2-hydroxy-1,4-benzoquinone (the oxidation product of 1,2,4-trihydroxybenzene) (Redfearn 1965).

Attempts to synthesize 2,4-dihydroxy-2,4-hexadiene-1,6-dioic acid and 2,4-dihydroxy-6-oxo-2,4-hexadienoic acid, the cleavage products of 1,2,3,5-THB and 1,2,4-trihydroxybenzene respectively, were unsuccessful due to the extreme lability of these molecules.

The formation of equimolar amounts of formate (8 mM) was detected for the PG-grown cells, but not for cells grown on succinate (Fig. 3.7). Activity for acetopyruvate was detected from PG induced cell-free extract as opposed to glucose grown

or boiled cell-free extracts (Fig. 3.8). Acetate (1.02 μM) and pyruvate (1.03 μM) were detected in approximately equimolar concentrations when acetopyruvate (1.0 μM) was added to the cell-free extracts. Acetate and pyruvate were not detected with the boiled or succinate cell-free extracts.

Pyruvate as its dinitrophenylhydrazone was detected in culture filtrates of PG, but not in filtrates of succinate grown Rhodococcus sp. BPG-8. The succinate control produced no pyruvate while PG grown cells showed significant increases in pyruvate at 24 hours growth (0.6 mM) (Fig. 3.9). The Rf values (0.23) and UV peaks (455 nm) were identical for both the authentic and detected derivatized pyruvate (Fig. 3.9).

The novel tentative degradation pathway for PG and the fortuitious biotransformation of resorcinol are illustrated for the Rhodococcus sp. BPG-8 (Fig. 3.10).

Table 3.1. Detection of intermediates in culture filtrates of Rhodococcus sp. BPG-8 grown on PG or resorcinol.

Metabolites	Retention time		Mass spectra	
	Standard	Isolated	Standard	Isolated
1,2,3,5-THB	16.2	16.2	270,227,171 143,129,87 74	270,227,171 143,129,87 74
Resorcinol	7.8	7.8	194,152,110 82,53	194,152,110 82,53
1,2,4-Btriol	15.1	15.1	252,210,168 151,126,97 69	252,210,168 151,126,97 69
Catechol	6.8	N/D	N/D	N/D

N/D: not detected; 1,2,3,5-THB: 1,2,3,5-tetrahydroxybenzene: 1,2,4-Btriol: 1,2,4-trihydroxybenzene; Retention time (min).

Table 3.2. Comparison of the aromatic standards and extracted intermediates of PG and resorcinol metabolism by induced Rhodococcus sp. BPG-8.

	Controls		Detected	
Intermediate	Rf's	Peak(nm)	Rf's	Peak(nm)
Phloroglucinol	0.50	268	0.50	268
Resorcinol	0.78	275	0.78	275
1,2,3,5-THB	0.13	283	0.13	283
2,6-D-1,4-Bq	0.00	273	0.00	273
1,2,4-Btriol	0.65	290	0.65	290
2-H-1,4-Bq	0.00	260	0.00	260

1,2,4-Btriol: 1,2,4-trihydroxybenzene; 1,2,3,5-THB: 1,2,3,5-tetrahydroxybenzene. 2,6-D-1,4-Bq: 2,6-dihydroxy-1,4-benzoquinone; 2-H-1,4-Bq: 2-hydroxy-1,4-benzoquinone. TLC was performed using a benzene:methanol:acetate (45:8:4) solvent system. Adsorption spectra of the compounds were obtained in methanol.

Table 3.3. Oxygen consumption by cell-free extracts and whole cells of PG-grown Rhodococcus sp. BPG-8.

	Specific Activity	
Substrate	Cell-free extract	Whole cells
Phloroglucinol	0.026 ± 0.006	0.190 ± 0.035
Resorcinol	0.014 ± 0.002	0.093 ± 0.020
1,2,3,5-THB	0.034 ± 0.003	NT
1,2,4-Btriol	0.008 ± 0.001	NT
Catechol	0.00	0.00
Acetopyruvate	0.025 ± 0.07	0.089 ± 0.006
β-ketoadipate	0.00	0.00
Acetate	0.048 ± 0.07	0.110 ± 0.010
Pyruvate	0.029 ± 0.03	0.080 ± 0.010

Specific Activity: μmol O_2 utilized/min/mg protein. N=3, Mean ± st. dev., NT: not tested. 1,2,4-Btriol: 1,2,4-trihydroxybenzene; 1,2,3,5-THB: 1,2,3,5-tetrahydroxybenzene.

Table 3.4. Specific activity of phloroglucinol hydroxylase and resorcinol hydroxylase in cell-free extracts of Rhodococcus sp. BPG-8 grown on PG.

	Specific Activity	
Enzyme	NADPH	NADH
PG-hydroxylase	0.052 ± 0.03	0.069 ± 0.05
Resorcinol hydroxylase	0.026 ± 0.05	0.030 ± 0.06

Specific Activity: μmol NAD(P)H oxidized/min/mg protein.

N=3, Mean ± st. dev.

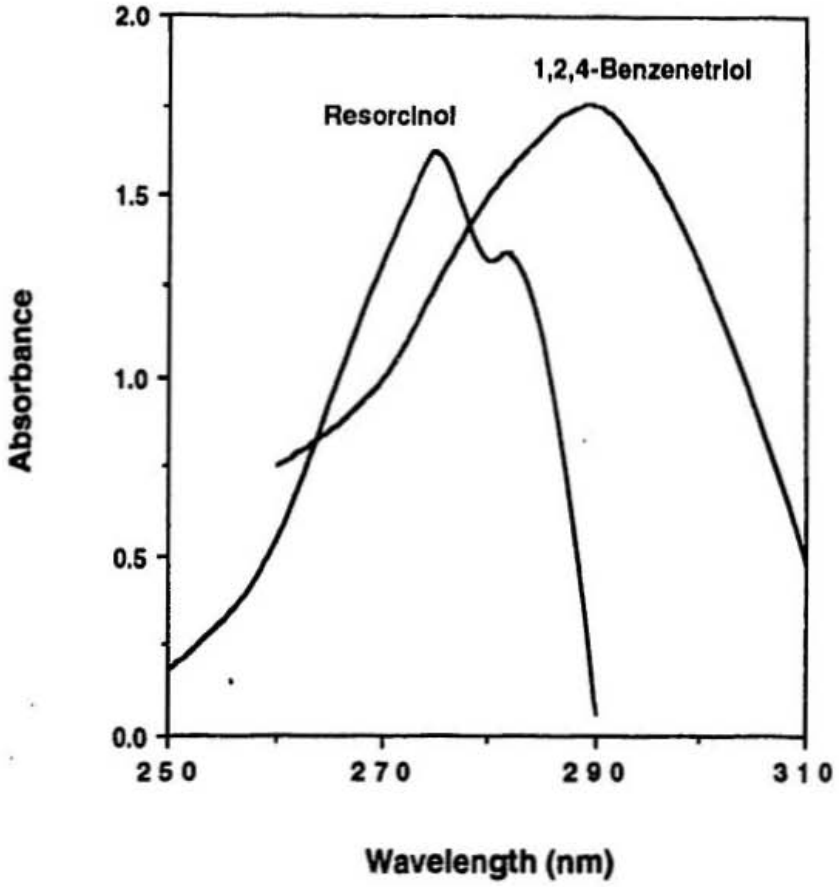


Fig. 3.1. Spectral changes observed during enzymatic conversion of resorcinol into 1,2,4-benzenetriol by phloroglucinol induced Rhodococcus sp. BPG-8 cell-free extracts (0.15 mg protein).

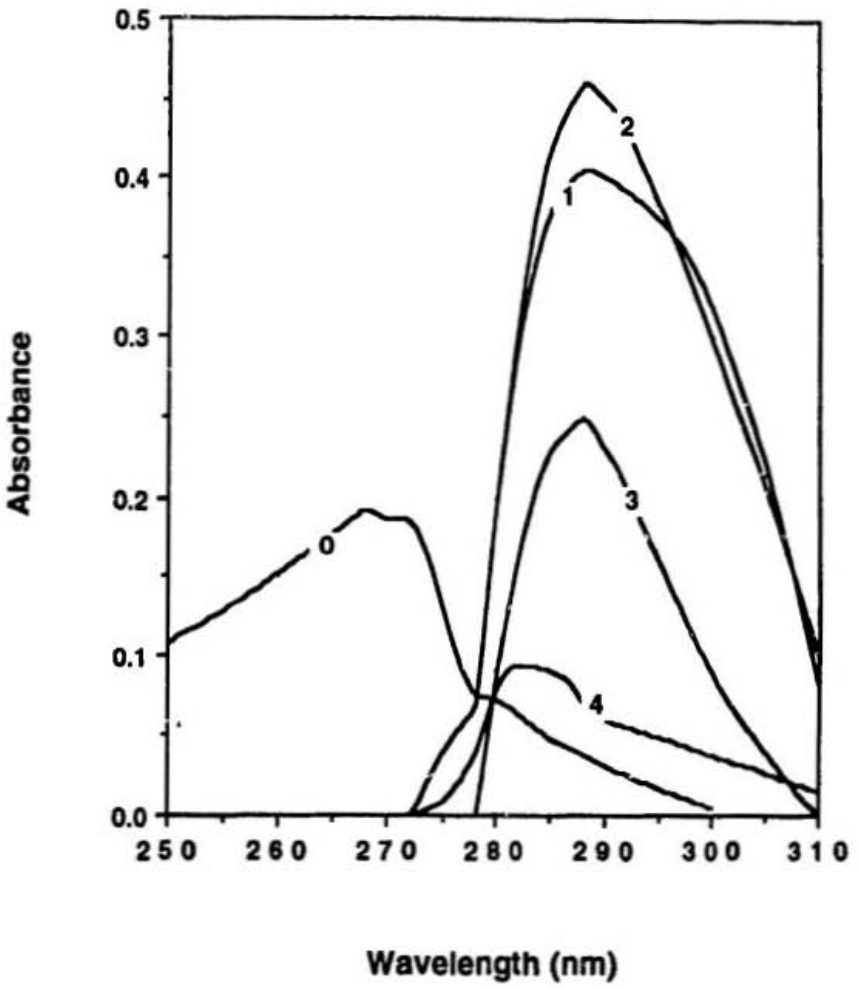


Fig. 3.3. Spectral changes observed during enzymatic oxidation of phloroglucinol by cell-free enzyme extract (0.15 mg protein) from Rhodococcus sp. BPG-8. Reaction mixture as outlined in Materials and Methods. The numbers 0,1,2,3,4 correspond to 0,2,5,10,15 minutes, respectively.

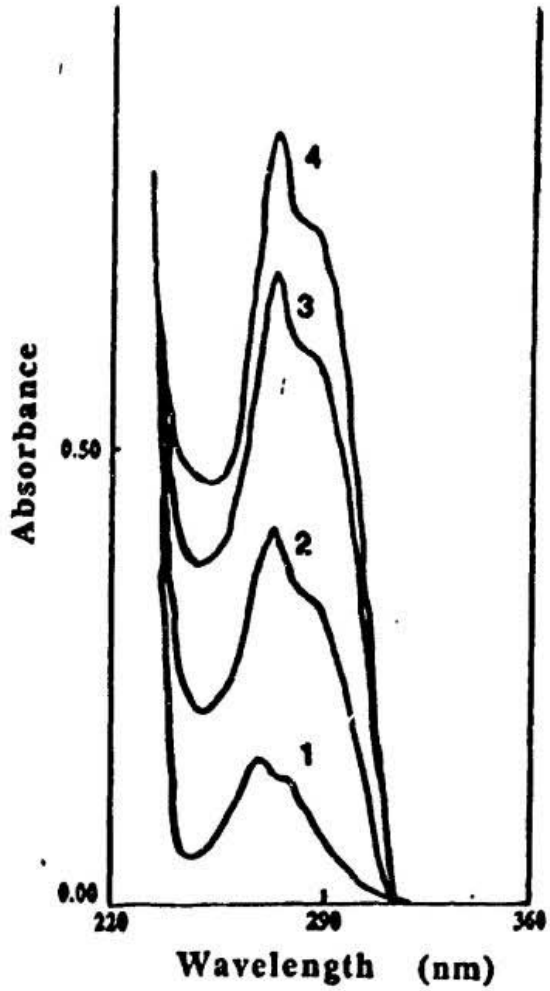


Fig. 3.2. Spectral changes observed during enzymatic oxidation of phloroglucinol by partially purified enzyme extract (0.15 mg protein) from Rhodococcus sp. BPG-8. Reaction mixture as outlined in Materials and Methods except 2 μM FAD, and 50 ng of catalase were added to the partially purified enzyme extract. The numbers 1,2,3,4 correspond to 0,1,3,5 minutes, respectively.

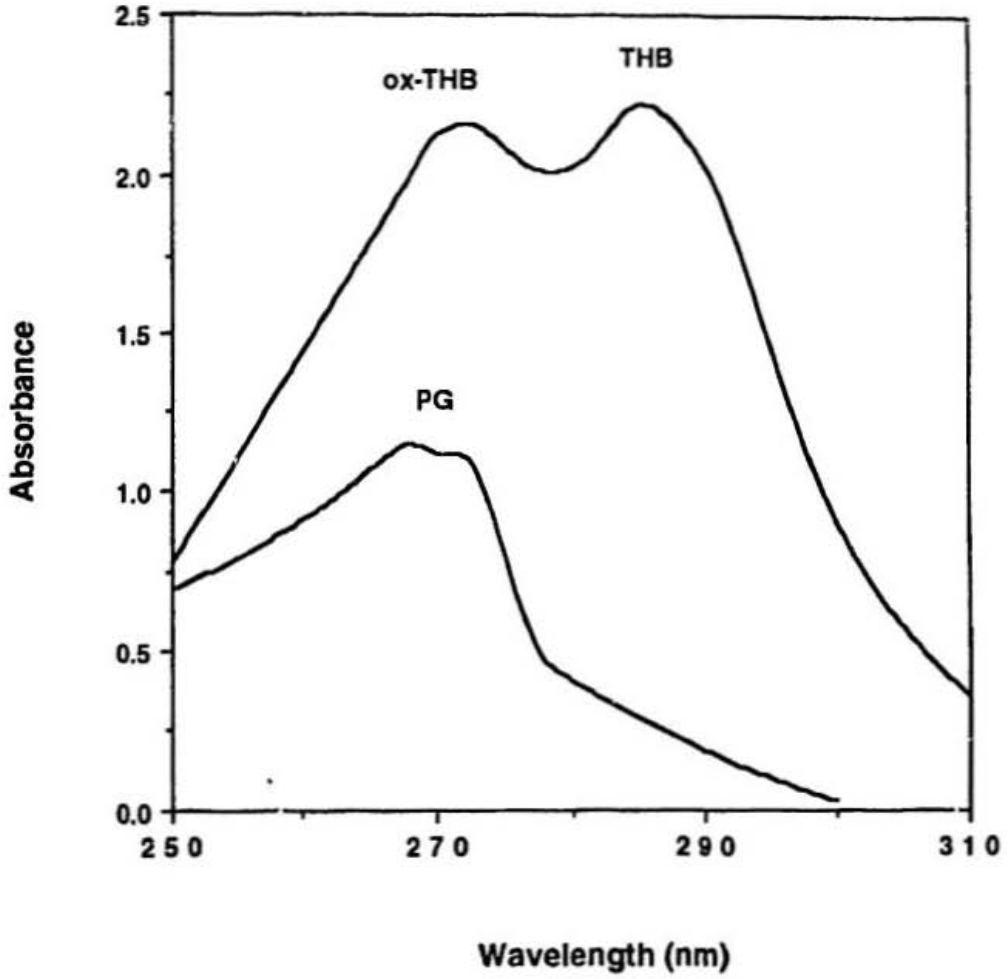


Fig. 3.4. Spectral changes observed during enzymatic oxidation of phloroglucinol by phloroglucinol induced whole cells of the Rhodococcus sp. BPG-8. Peak at 283 nm and 273 nm correspond to the 1,2,3,5-tetrahydroxybenzene and the 2,6-dihydroxy-1,4-benzoquinone respectively. Boiled and succinate grown cells showed no spectral change.

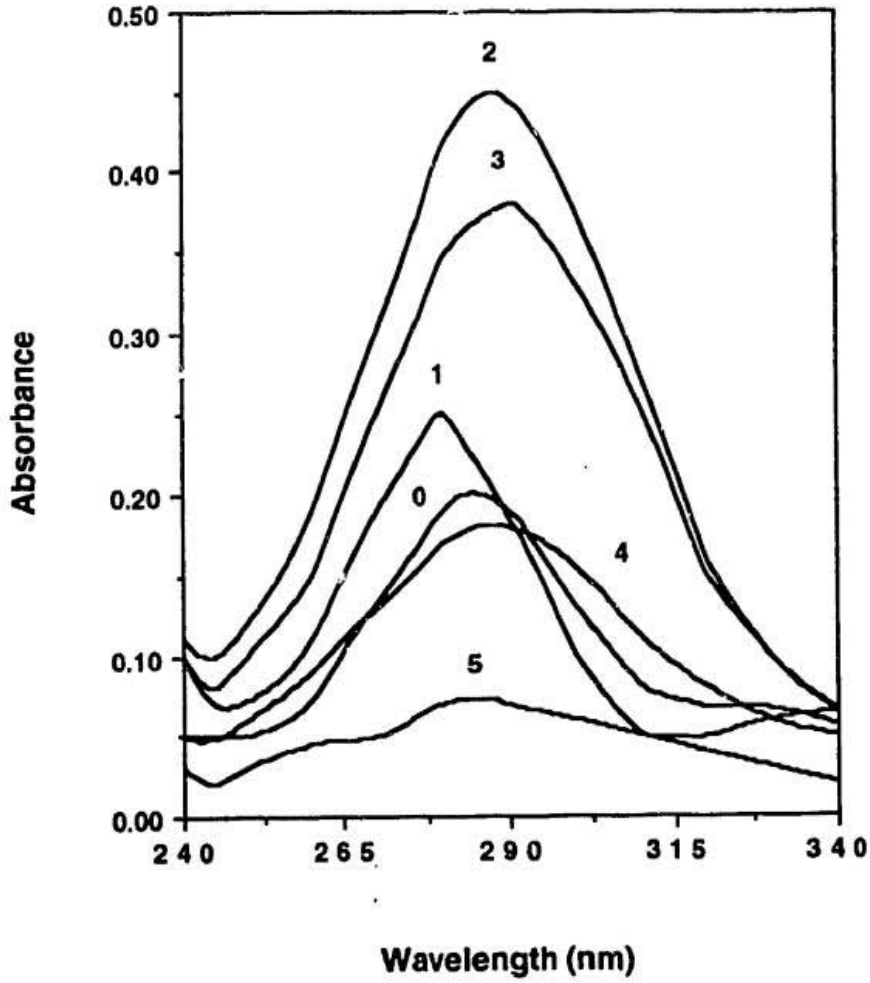


Fig. 3.5. Spectral changes observed during enzymatic oxidation of 1,2,3,5-tetrahydroxybenzene by cell-free enzyme extracts (0.15 mg protein) from Rhodococcus sp. BPG-8. The numbers 1,2,3,4,5 correspond to 0,0.5,5,10,20 minutes, respectively.

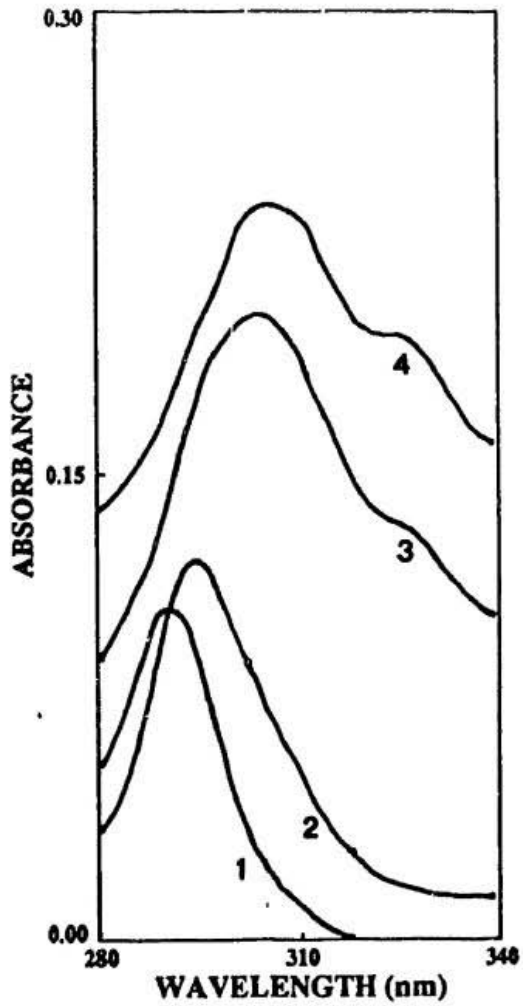


Fig. 3.6. Spectral changes observed during enzymatic oxidation of 1,2,4-benzenetriol by cell-free extract (1.43 mg protein) from Rhodococcus sp. BPG-8. Reaction mixture as outlined in Materials and Methods. The numbers 1,2,3,4 correspond to 0,5,10,20 minutes, respectively.

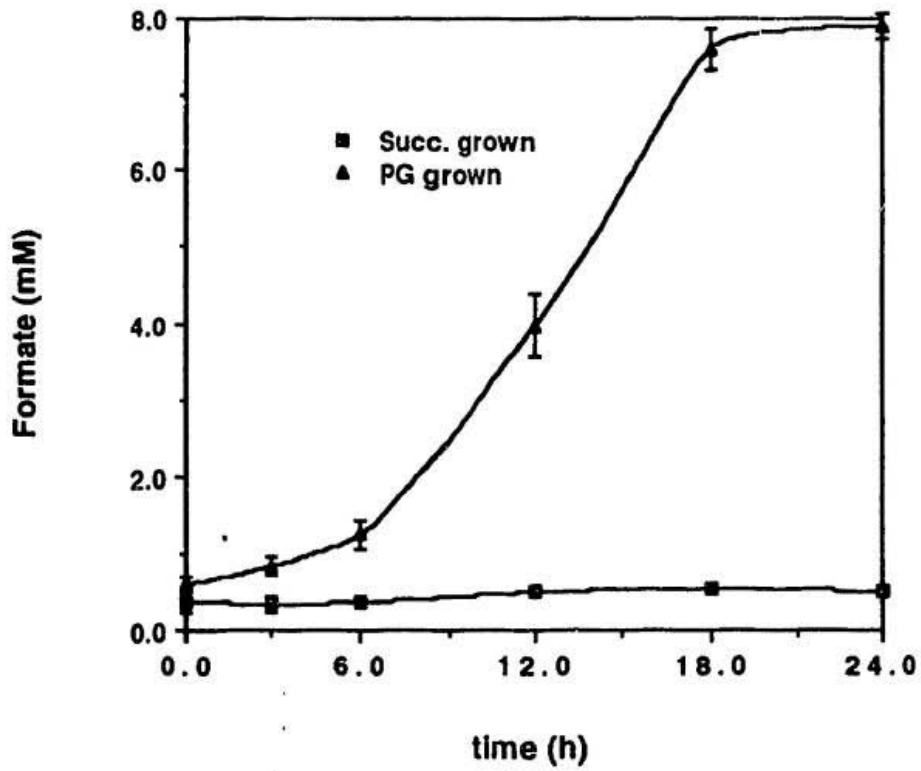


Fig. 3.7. Accumulation of formate in the supernatant of whole cells of Rhodococcus sp. BPG-8 grown on phloroglucinol and succinate. Washed phloroglucinol induced cells (4.5 mg protein/ml) were initially suspended to give an absorption at 600 nm of 0.20.

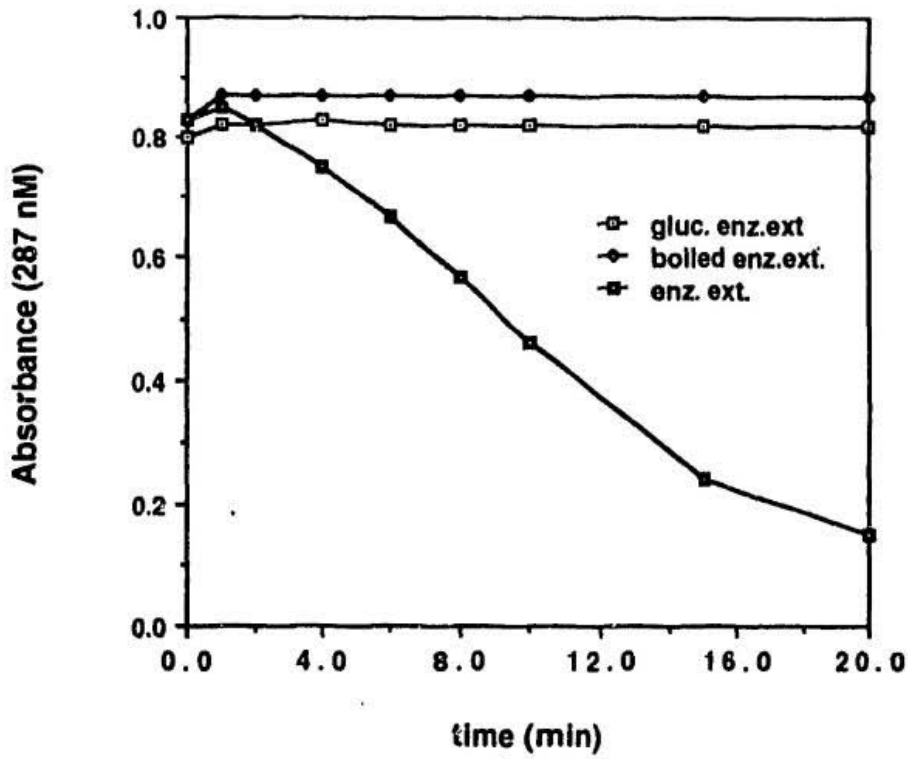


Fig. 3.8. Enzymatic breakdown of acetopyruvate by cell-free extracts (1.43 mg/ml protein) of Rhodococcus sp. BPG-8 grown on PG and glucose. Reaction mixture as outlined in Materials and Methods.

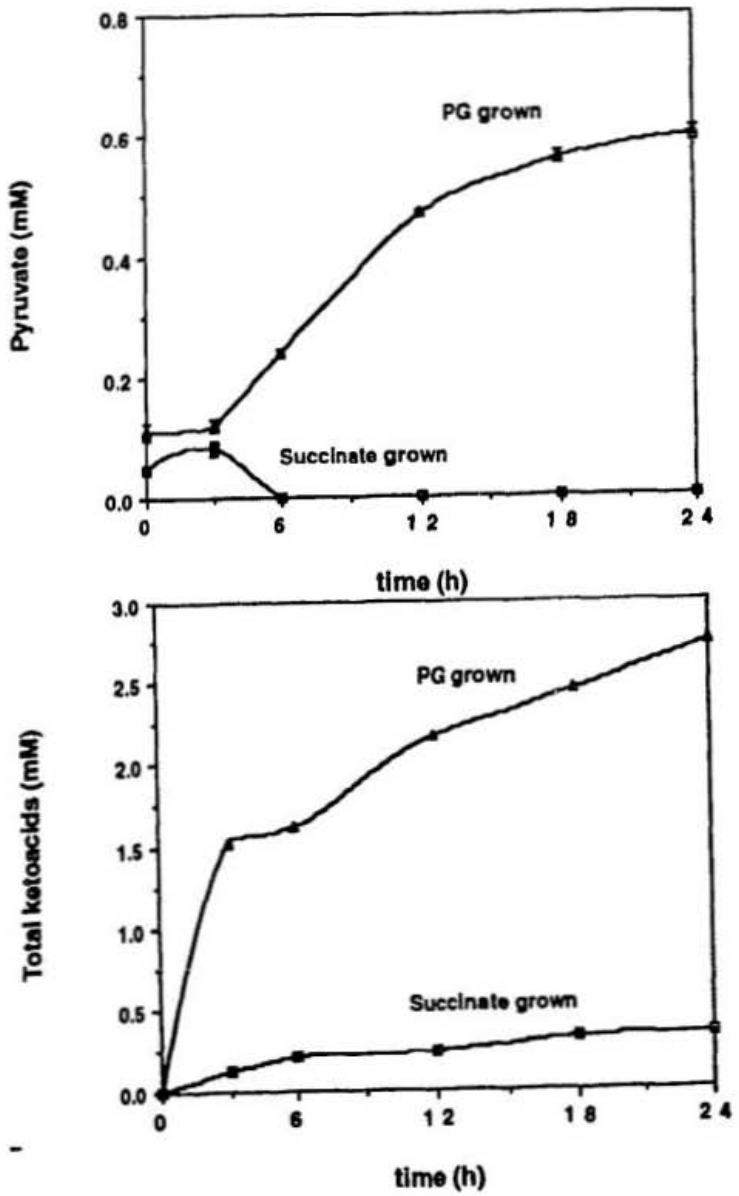


Fig. 3.9. Accumulation of pyruvate and total ketoacids in the supernatant of whole cells of Rhodococcus sp. BPG-8 grown on phloroglucinol and succinate. Washed phloroglucinol induced cells are initially suspended to give an absorption at 600 nm of 0.20. In both cases 4.5 mg protein per assay was used.

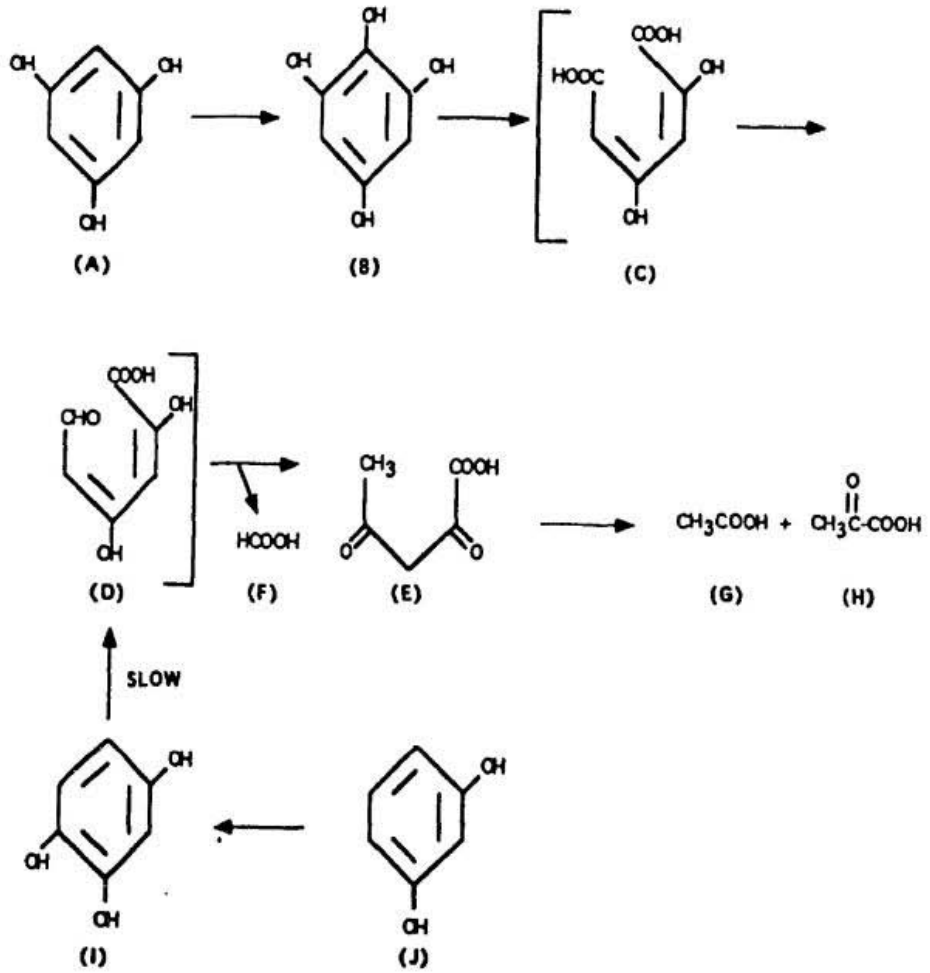


Fig. 3.10. Proposed pathway for phloroglucinol catabolism and the fortuitous resorcinol biotransformation by Rhodococcus sp. BPG-8. The pathway is: (A) phloroglucinol → (B) 1,2,3,5-tetrahydroxybenzene → (C) (2,4-dihydroxy-2,4-hexadiene-1,6-dioic acid) → (D) (2,4-dihydroxy-6-oxo-2,4-hexadienoic acid) → (E) acetopyruvate + (F) formate → (G) acetate + (H) pyruvate. (J) Resorcinol → (I) 1,2,4-trihydroxybenzene → (D) → (E) + (F) → (G) + (H).

3.4 Discussion

The product detected by GC-MS and TLC and UV spectophotometry when PG was fed to induced whole cells, was probably 1,2,3,5-THB. Interestingly, Patel et al. (1990), suggested that a fungus Penicillium simplicissimim may degrade PG through a 1,2,4-trihydroxybenzene intermediate, while Walker and Taylor (1983), suggested a 1,2,3-trihydroxybenzene (pyrogallol) intermediate was involved in the aromatic cleavage of PG. Brune and Schink (1990), stated that in Pelobacter acidigallici, PG is reduced to the dihydrophloroglucinol which then undergoes hydrolytic cleavage to form 3-hydroxy-5-oxohexanoate.

Activity for PG hydroxylase and resorcinol hydroxylase were also detected using cell-free extract. The PG hydroxylase suggests that the PG is hydroxylated to 1,2,3,5-THB and not reduced to the dihydrophloroglucinol (1,3-dioxo-5-hydroxycyclohexane) (Patel et al., 1981). The 1,2,3,5-THB and its oxidized derivative were detected in cell-free extracts incubated in the presence of PG using TLC and UV spectrophotometry. The partially purified NAD(P)H dependent PG hydroxylase, in the presence of PG, formed a peak at 275 nm which is characteristic of 2,6-dihydroxy-1,4-benzoquinone, not dihydrophloroglucinol. Apparently PG is hydroxylated to a 1,2,3,5-tetrahydroxybenzene which will be readily oxidized to

its quinone if the 1,2,3,5-THB dioxygenase is absent. The PG reductase that was partially purified by Patel et al. (1981) would function both aerobically and anaerobically, however the partially purified PG hydroxylase isolated from Rhodococcus BPG-8 would only function aerobically. Brune and Schink (1990) suggest that 1,2,3,5-THB acts as a cosubstrate rather than an intermediate in the anaerobic metabolism of PG in Pelobacter acidigallici; however this was not observed in this study.

When resorcinol was fed to the PG induced Rhodococcus sp. BPG-8 the first product detected was 1,2,4-trihydroxybenzene. Chapman and Ribbons (1976 a) and Chapman and Ribbons (1976 b) also showed that when Pseudomonas putida was grown on resorcinol the first product detected was 1,2,4-trihydroxybenzene. This is believed to be a fortuitous because Rhodococcus species portrayed ubiquitous biotransformation, growth on resorcinol was small, and resorcinol was not part of the Rhodococcus sp. BPG-8 PG degradation pathway. Interestingly, phenolic hydroxylases may hydroxylate numerous substrates at reduced efficiency (Straube, 1987; Ohta and Ribbons, 1976).

Rhodococcus sp. BPG-8 ortho-dioxygenase activity for 1,2,3,5-THB was dependent on PG induction. The characteristic increase and subsequent decrease in UV spectra was similiar to the ortho-cleavage of pyrogallol by Azotobacter vinelandii (Groseclose and Ribbons, 1981). This is further supported by

the equimolar consumption of oxygen and 1,2,3-THB, similiar specific activities, and the formation of ketoacids hypothesized to be 2,4-dihydroxy-2,4-hexadiene-1,6-dioic acid and 2,4-dihydroxy-6-oxo-2,4-hexadienoic acid (Walker and Taylor, 1983; Groseclose and Ribbons, 1981; Chapman and Ribbons, 1976 a; Chapman and Ribbons, 1976 b).

The cleavage of 1,2,4-trihydroxybenzene by cell-free extract of PG induced Rhodococcus sp. BPG-8, with the formation of a product with a peak at 308 nm is in some respects similiar to the meta-cleavage of 2,3,5-trihydroxytoluene by P. putida (Chapman and Ribbons, 1976 a). The absence of the ortho-cleavage enzymes and the lack of formation of maleylacetate from 1,2,4-trihydroxybenzene suggests that PG-induced Rhodococcus sp. BPG-8 may carry the meta-cleavage enzyme for 1,2,4-trihydroxybenzene. The latency of this conversion as compared to 1,2,3,5-THB dioxygenase suggests a fortuitous transformation. It can be speculated that because Rhodococcus sp. BPG-8 grows well on 1,2,4-trihydroxybenzene there may be some basal meta- dioxygenase activity for this intermediate. Interestingly the Rhodococcus sp. BPG-8 seemed to have both ortho- and meta-cleavage enzymes which is comparable to P. putida which carries out both ortho- and meta-cleavage of 1,2,4-trihydroxybenzene (Ohta et al., 1975; Chapman and Ribbons, 1976 a, b).

No oxygen uptake occurred in the presence of β-

ketoadipate for both whole cells and cell-free extracts of PG induced Rhodococcus sp. BPG-8. The accumulation of formate in the supernatant of PG grown cells of Rhodococcus sp. BPG-8 along with the enzymatic activity for acetopyruvate metabolism suggest that the meta-cleavage product of 1,2,4-trihydroxybenzene (notably 2,4-dihydroxy-6-oxo-2,4-hexadienoic acid) loses a formate to form the product acetopyruvate. The products of the hydrolysis of acetopyruvate gave equimolar concentration of both pyruvate and acetate similiar to P. putida (Chapman and Ribbons, 1976 a; Chapman and Ribbons, 1976 b). Accordingly pyruvate was shown to accumulate in PG, and not succinate grown or boiled Rhodococcus sp. BPG-8 cells.

Proposed is the following tentative pathway: phloroglucinol -> 1,2,3,5-tetrahydroxybenzene -> (2,4-dihydroxy-6-oxo-2,4-hexadienoic acid) -> (2,4-dihydroxy-6-oxo-2,4-hexadienoic acid) -> acetopyruvate + formate -> acetate + pyruvate -> TCA (Fig. 3.10). Proposed is the tentative resorcinol biotransformation pathway: Resorcinol -> 1,2,4-trihydroxybenzene -> (2,4-dihydroxy-6-oxo-2,4-hexadienoic acid) -> acetopyruvate + formate -> acetate + pyruvate -> TCA (Fig. 3.10).

Chapter IV: Protective Mechanisms Employed by the Rhodococcus sp. BPG-8 to minimize quinone formation

4.1 Introduction

The metabolism of non-toxic aromatic compounds has the potential to produce toxic intermediates that may be lethal to the integrity of cellular components. Some of these toxic intermediates include quinones that may be formed from the auto-oxidation of the parent hydroquinone (Miller et al., 1986). The auto-oxidation of hydroquinones produces superoxide anion radicals ($O_2^{\cdot -}$) that may lead to conditions of oxidative stress (Chesis et al., 1984).

The cellular activation of quinoid compounds is usually centered on redox processes which include electron transfer from cellular flavoproteins to produce superoxide radicals. This process is known as redox cycling (Gant et al., 1986; Gant et al., 1988). Also a 1,4-reductive addition to quinoid compounds by cellular nucleophiles such as glutathione produces glutathionyl-quinone conjugates which also participate in redox cycling (Ollinger et al., 1990; Miller et al., 1986). Redox cycling and reductive addition may cause changes in cellular biochemical pathways which lead to cellular deficiency and death (Ollinger et al., 1990; Miller et al., 1986).

It has also been implicated that deoxyribonucleic acid (DNA) may be damaged by the auto-oxidation of polyphenols such

as 1,2,3,5-THB and 1,2,4-trihydroxybenzene through some unknown reactive species (Kawanishi et al., 1989; Kawanishi et al., 1986). This interaction has been implicated in the mutagenic and carcinogenic activities of benzene and its metabolites such as phenol, catechol, hydroquinone, and 1,2,4-trihydroxybenzene (Schwartz et al., 1985; Chesis et al., 1984; Greenlee et al., 1981; Timbrell and Mitchell, 1977).

In chapter three it has been shown that 1,2,3,5-THB and 1,2,4-trihydroxybenzene are formed during the respective transformation of PG and resorcinol by PG induced cell-free extracts of a Rhodococcus sp. BPG-8. This chapter investigates the potential protective mechanisms this organism may employ to reduce the toxicity of the oxidation product of 1,2,3,5-THB and 1,2,4-trihydroxybenzene.

4.2 Materials and Methods

4.2.1 Materials. Superoxide dismutase (SOD), catalase, bovine serum albumin (BSA), 1,4-benzoquinone, 1,2,4-trihydroxybenzene, and NAD(P)H were obtained from Sigma Chem. Co. (St. Loius, MO, USA). Solvents and thin layer chromatograpy plates (TLC, silica gel G) were purchased from Fisher Scientific (Pittsburgh, PA, USA). The 2,6-dihydroxy-1,4-benzoquinone and 2-hydroxy-1,4-benzoquinone was produced from the oxidation of 1,2,3,5-THB and 1,2,4-trihydroxybenzene respectively, and the purity of 1,2,4-benzene was determined by TLC and UV (ultraviolet) spectrophotometry.

4.2.2 Source of organism. Optimal growth parameters were described earlier (Chapter 2). The organism was taxonomically characterized and subsequently identified as a Rhodococcus sp. BPG-8 (Chapter 2) (Armstrong and Patel, 1992).

4.2.3 Analytical Methods. Detection of 1,2,3,5-THB and 1,2,4-trihydroxybenzene was performed using TLC and ultraviolet (UV) spectrophotometry. The solvent system for TLC detection of intermediates was benzene:dioxane:acetic acid (60:36:4) (Randerath, 1963). Acetylation of 1,2,4-trihydroxybenzene was performed in acetic anhydride and pyridine at 25°C for 12 hours. Gas chromatography-mass spectra (GC-MS) analysis was performed on a Hewlett-Packard mass selective detector as outlined in chapter 3 (Hellou et al., 1989). The oxidized product of 1,2,4-trihydroxybenzene (2-hydroxy-1,4-benzoquinone) was detected by UV-spectrophotometry (Kawanishi et al., 1989; Corbett, 1970). The 2-hydroxy-1,4-benzoquinone was also reduced to the 1,2,4-trihydroxybenzene by the addition of equimolar amounts of sodium dithionate. Protein was measured according to the method of Bradford (1976), using bovine serum albumin as a protein standard.

A Clark oxygen electrode was employed to examine the auto-oxidation of 1,2,3,5-THB and 1,2,4-trihydroxybenzene in the presence of cell-free extract, SOD, and catalase. The reaction mixture contained 1.7 ml of KH_2PO_4 (50 mM, pH 7.0, 25°C), 0.1 ml of 1,2,3,5-THB (1.42 mg/ml) or 1,2,4-

trihydroxybenzene (1.25 mg/ml), 0.1 ml of cell-free extract (4.8 mg/ml protein); 0.1 ml of SOD or catalase (1.0 mg/ml protein).

4.2.4 Preparation of cell-free extract. Cell-free extract was prepared from PG and succinate grown Rhodococcus sp. BPG-8 according to the earlier report (Patel et al., 1990). Cells were broken using a French pressure cell (SLM Aminco Inc.) at 846 kg/cm^2, washed (3X) and centrifuged (20,000 x g, 30 min, 5°C). Approximately 2.0 g of wet packed cells was added to 3 ml of enzyme stabilization buffer, minus 2-mercaptoethanol (Patel et al., 1990).

4.2.5 Biochemical assays. Activities of catalase and SOD were determined according to Worthington (1988). Catalase specific activity was defined as 1 μmol of H_2O_2 decomposed per minute per mg protein at 25°C and pH 7.0. SOD specific activity was defined as the amount of enzyme causing half the maximum inhibition of nitroblue tetrazolium (NBT) reduction per mg protein at 25°C and pH 7.8. Reductase assay was similiar to the diaphorase assay except that 3 mM N-ethylmaleimide, a known inhibitor of diaphorase was added (Worthington 1988). Reductase specific activity was defined as the reduction of 1 μmol of 2,6-dichlorophenolindophenol (DCPIP) per minute per mg protein at 25°C and pH 8.5.

The effects of cell-free extract on the auto-oxidation of

1,2,3,5-THB (λ max 273 nm, estimated extinction coefficient: 15.0 x 10^3 $M^{-1}cm^{-1}$) and 1,2,4-trihydroxybenzene (λ max 487 nm, estimated extinction coefficient: 1.02 x 10^3 $M^{-1}cm^{-1}$) were also examined using a Shimadzu spectrophotometer. The 487 nm peak was more amenable to measure oxidation rates because interference due to UV-absorbing materials such as DNA or protein was minimized. The conversion of 1,2,3,5-THB and 1,2,4-trihydroxybenzene to their respective oxidized products were defined as the increase in μmoles of 2,6-dihydroxy-1,4-benzoquinone or 2-hydroxy-1,4-benzoquinone per min per mg protein. The reaction mixture contained 2.8 ml KH_2PO_4 (50 mM, pH 7.0, 25°C), 0.1 ml of 1,2,3,5-THB (1.42 mg/ml) or 1,2,4-trihydroxybenzene (1.25 mg/ml), or 0.1 ml of cell-free extract (4.8 mg/ml protein), SOD (1 mg/ml), catalase (1 mg/ml), BSA (1 mg/ml).

The reduction of 2,6-dihydroxy-1,4-benzoquinone, 2-hydroxy-1,4-benzoquinone and 1,4-benzoquinone by a NAD(P)H dependent reductase was also performed. Specific activity was defined as 1 μmol of NAD(P)H oxidized per min per mg protein at 25°C and pH 7.0.

4.3 Results

The 2,6-dihydroxy-1,4-benzoquinone and 2-hydroxy-1,4-benzoquinone were shown to absorb maximally at 273 nm (Fig. 4.1, Fig. 4.2) and 260, respectively. The 2-hydroxy-1,4-benzoquinone had another major peak at 487 nm. The auto-

oxidations of 1,2,3,5-THB and 1,2,4-trihydroxybenzene were retarded by cell-free extract of Rhodococcus sp. BPG-8 grown either on PG or succinate (Table 4.1, Table 4.5). In contrast, boiled cell-free extract had no effect. Moreover, SOD and catalase significantly lowered the auto-oxidation rate of 1,2,4-trihydroxybenzene to almost zero (Table 4.2). The specific activities of SOD, catalase, and quinone reductase in the cell-free extract were quite high (Table 4.3).

Reductase activity was quantified because a NAD(P)H dependent reductase was found to convert 2-hydroxy-1,4-benzoquinone to 1,2,4-trihydroxybenzene (Table 4.4). Cell-free extracts of PG grown cells exhibited oxidation of both NADPH and NADH in the presence of 1,4-benzoquinone and 2-hydroxy-1,4-benzoquinone. Cell-free extract of succinate grown cells showed similar results.

The formation of 1,2,4-trihydroxybenzene as a reduction product of 2-hydroxy-1,4-benzoquinone was demonstrated by spectral changes (Fig. 4.2) as well as by isolation and confirmation of the acetylated 1,2,4-trihydroxybenzene by GC-MS analysis (Retention time: 15.1 min, mass spectrum: 252,210,168,151,126,97,69).

In a reaction mixture containing SOD, catalase, and cell-free extract the reduction of 2-hydroxy-1,4-benzoquinone did not occur (Fig. 4.3). However, when either NADPH or NADH were added immediate conversion of the 2-hydroxy-1,4-benzoquinone to 1,2,4-trihydroxybenzene occurred. The conversion of 2,6-

dihydroxy-1,4-benzoquinone to 1,2,3,5-THB never occurred.

When catalase and SOD, individually or in combination were added to the reaction mixture, containing 1,2,4-trihydroxybenzene reduced oxygen consumption occurred (Fig. 4.4). The formation of H_2O_2 during the auto-oxidation of 1,2,4-trihydroxybenzene was also investigated using catalase or cell-free extract. Oxygen was released when catalase or cell-free extract was added, suggesting that H_2O_2 may be formed during this reaction. SOD released negligible amounts of oxygen (Fig. 4.4). Results presented in Fig 4.4 shows separate experiments to measure the auto-oxidation of 1,2,4-trihydroxybenzene. The oxidation of 1,2,4-trihydroxybenzene was slowed with PG grown BPG-8 cell-free extract, SOD, catalase, or SOD and catalase; in which each treatment lowered the oxidation rate of 1,2,4-trihydroxybenzene to 0.02, 0.003, 0.038 and 0.00 μmoles of oxygen/min, respectively. The oxidation rate of 1,2,4-trihydroxybenzene alone was 0.28 μmoles oxygen/min. The oxidation of 1,2,3,5-THB to 2,6-dihydroxy-1,4-benzoquinone showed a similiar, but weaker response (Table 4.5). The oxidations of 1,2,3,5-THB and 1,2,4-trihydroxybenzene were strongly dependent upon the pH of the reaction mixture (Fig. 4.5).

Table 4.1. Effect of Rhodococcus sp. BPG-8 cell-free extract on the oxidation of 1,2,4-trihydroxybenzene to 2-hydroxy-1,4-benzoquinone.

	Specific Activity	
Cell-free ext.	*Succinate	*Phloroglucinol
Boiled	2.01 ± 0.12	1.79 ± 0.12
Native	0.32 ± 0.06	0.42 ± 0.03

*Cell-free extracts of cells grown on succinate or PG. Specific activity: Increase in μmol 2-hydroxy-1,4-benzoquinone per min per mg protein. Mean ± st. dev., N=3. Reaction conditions outlined in Materials and Methods.

Table 4.2. Oxidation of 1,2,4-trihydroxybenzene to 2-hydroxy-1,4-benzoquinone in the presence of different proteins.

	Specific Activity			
Protein	BSA	SOD	CAT	SOD/CAT
Boiled	3.18 ± 0.06	1.32 ± 0.12	1.02 ± 0.03	1.09 ± 0.09
Native	2.46 ± 0.03	0.21 ± 0.06	0.38 ± 0.03	0.00

Specific activity: Increase in μmol 2-hydroxy-1,4-benzoquinone per min per mg protein. BSA:bovine serum albumin; SOD:superoxide dismutase; CAT:catalase. Mean ± st. dev., N=3. See Materials and Methods for reaction conditions.

Table 4.3. The detection of different enzyme activities in phloroglucinol induced cell-free extract of Rhodococcus sp. BPG-8.

	Specific Activity		
Enzyme	CAT	SOD	Reductase
Boiled	0.0	0.0	0.0
Native	467	100	0.14 *(0.07)

Specific activity of catalase (CAT) was defined as one μmol of H_2O_2 decomposed per min per mg protein at 25°C and pH 7.0 Specific acitivity of superoxide dismutase (SOD) was defined as the amount of enzyme causing half the maximum inhibition of NBT reduction/mg protein at 25°C and pH 7.8. Specific activity of the reductase was defined as the reduction of one μmol of DCPIP per min per mg protein at 25°C and pH 8.5. * Activity was determined with NADPH as opposed to NADH. See Materials and Methods for reference to reaction conditions.

Table 4.4. Reduction of 1,4-benzoquinone, 2-hydroxy-1,4-benzoquinone, 2,6-dihydroxy-1,4-benzoquinone by cell-free extracts of Rhodococcus sp. BPG-8.

	Specific Activity		
	B-quinone	H-quinone	THBox-quinone
NADPH	0.20 ± 0.04	0.48 ± 0.12	N.D.
NADH	1.08 ± 0.22	3.77 ± 0.14	N.D.

Specific Activity was defined as one μmol of NAD(P)H oxidized per min per mg protein at 25°C and pH 7.0. B-quinone (1,4-benzoquinone); H-quinone (2-hydroxy-1,4 benzoquinone); THBox-quinone (2,6-dihydroxy-1,4-benzoquinone). N.D. not detected. Mean ± st. dev., N=3. See Materials and Methods for reference to reaction conditions.

Table 4.5. The effects of various treatments on the auto-oxidation of 1,2,3,5-THB to 2,6-dihydroxy-1,4-benzoquinone.

Treatment	Quinone Conversion Rate (μmol/min)	Total time (min)
pH 4	0.12	694.0
pH 5	9.3	9.0
pH 7, C/S	13.3	6.3
pH 7, A	20.0	4.2
pH 7	29.0	2.9

Catalase (100 μg) and SOD (100 μg) were added to the reaction mixture (C/S). The reaction mixture was made anaerobic (A) by bubbling nitrogen within the cuvette for 2 min. Reaction conditions as outlined in Materials and Methods.

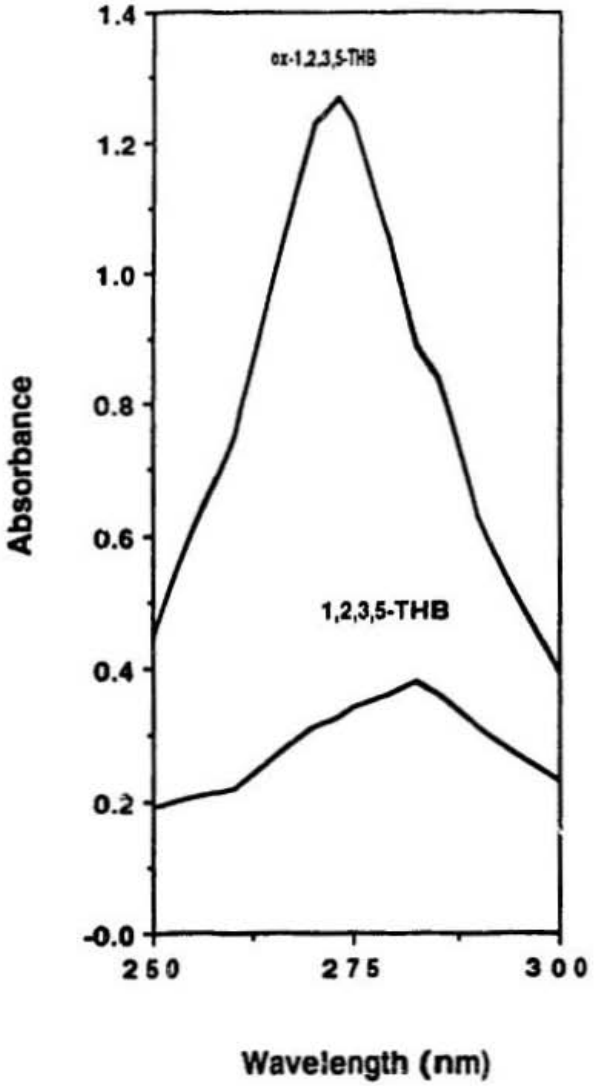


Fig. 4.1. Spectral pattern of the auto-oxidation of 1,2,3,5-THB to 2,6-dihydroxy-1,4-benzoquinone (ox-1,2,3,5-THB).

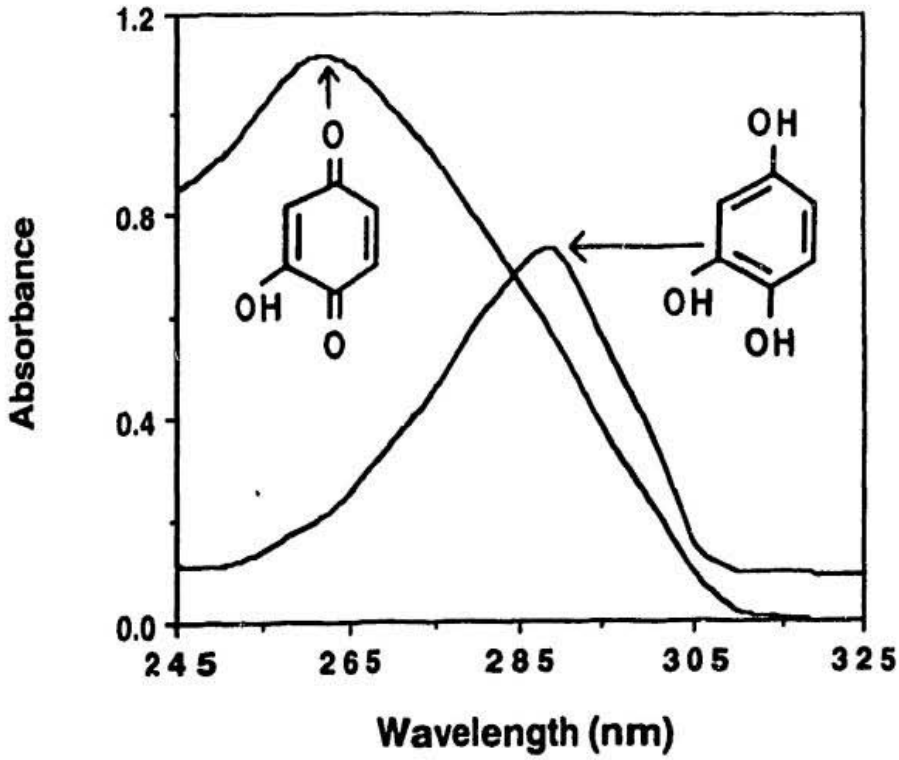


Fig. 4.2. Ultraviolet spectrophotometric examination of the NAD(P)H dependent cyclic conversion of 2-hydroxy-1,4-benzoquinone to 1,2,4-trihydroxybenzene bycell-free extracts of Rhodococcus sp. BPG-8 (0.48 mg protein). The purity of the 1,2,4-trihydroxybenzene was checked by TLC-UV and GC-MS.

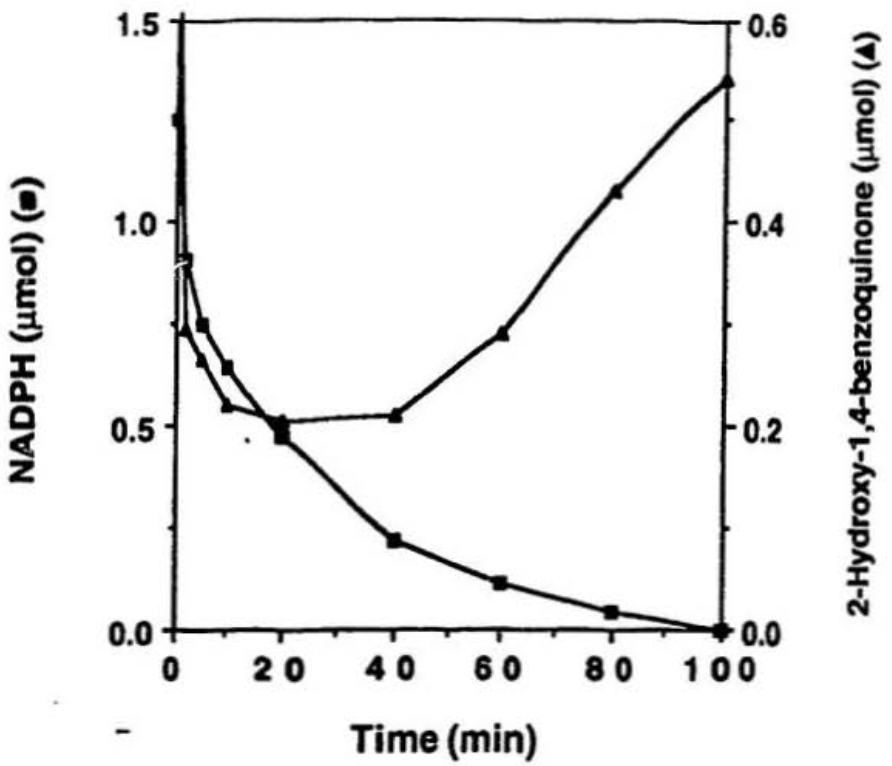


Fig. 4.3. NADPH oxidation with the concurrent cyclic oxidation-reduction of 2-hydroxy-1,4-benzoquinone by the cell-free extract (0.48 mg protein) of Rhodococcus sp. BPG-8.

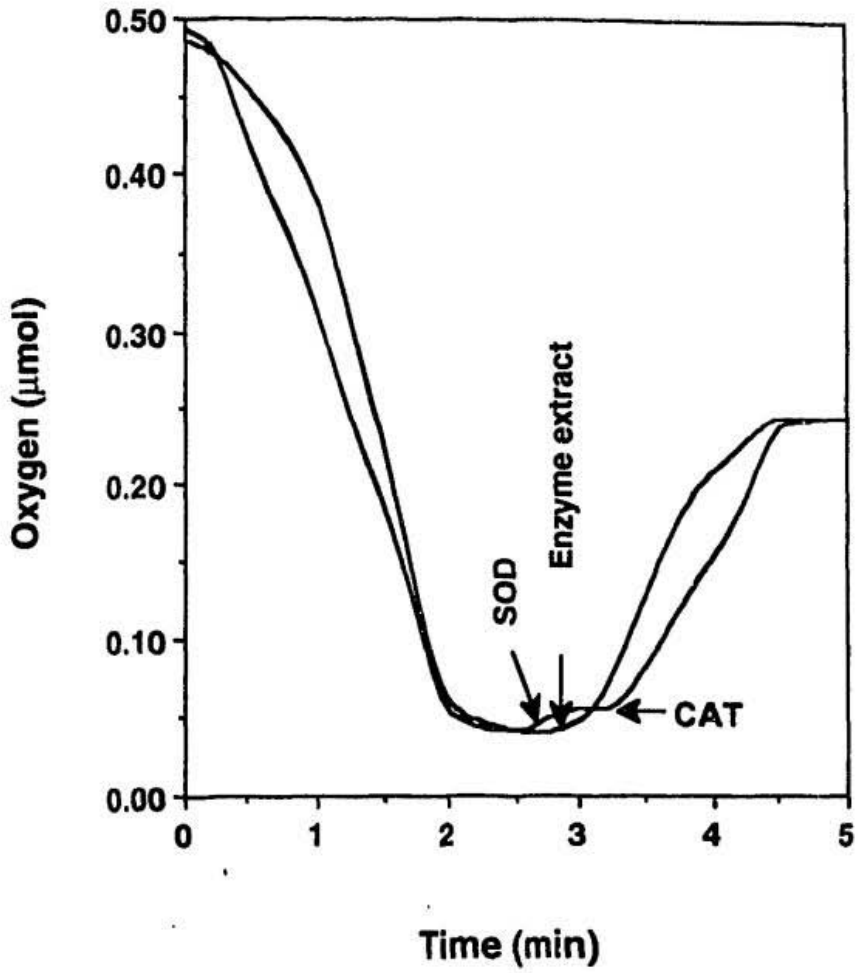


Fig. 4.4. Auto-oxidation of 1,2,4-trihydroxybenzene to 2-hydroxy-1,4-benzoquinone and the release of oxygen upon addition of catalase and enzyme extracts (4.8 mg protein/ml) of PG grown Rhodococcus sp. BPG-8. All treatments were standardized with 0.1 mg protein. Mean ± st.dev., N=3. Reaction conditions as outlined by Materials and Methods.

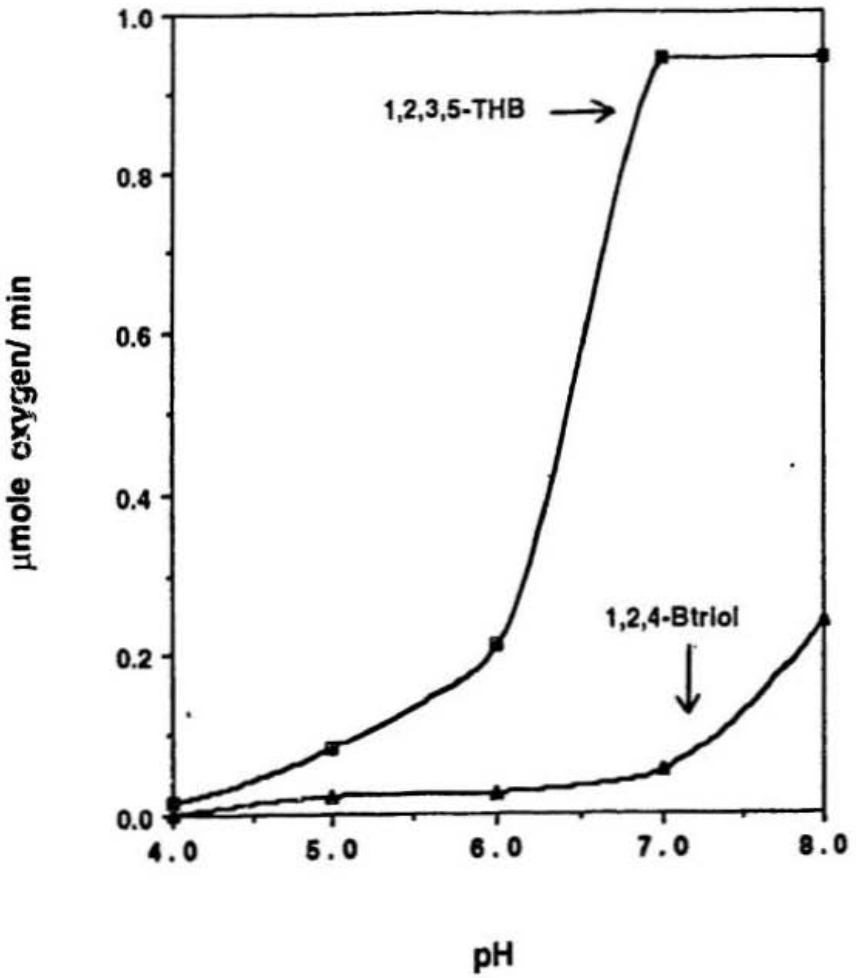


Fig. 4.5. Effect of pH on the auto-oxidation rate of 1,2,3,5-THB and 1,2,4-trihydroxybenzene.

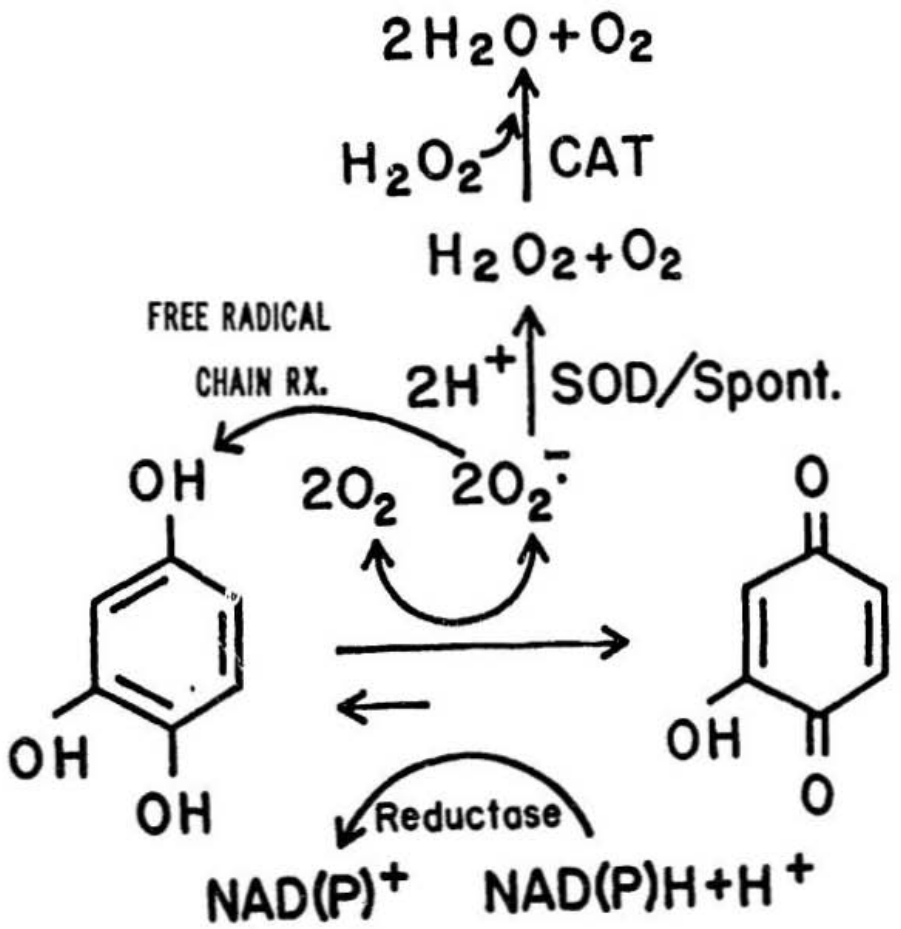


Fig. 4.6. Simplified schematic diagram of the protective mechanisms employed by Rhodococcus sp. BPG-8 during 1,2,4-trihydroxybenzene metabolism. Ortho-cleavage enzymes provided protection for the auto-oxidation of 1,2,3,5-tetrahydroxybenzene.

4.4 Discussion

The metabolism of PG and resorcinol by PG induced Rhodococcus sp. BPG-8 produce 1,2,3,5-THB and 1,2,4-trihydroxybenzene as intermediates, respectively. It has been suggested that polyphenol auto-oxidation may cause DNA damage through the production of a superoxide radical (Kawanishi et al., 1989; Chesis et al., 1984). The auto-oxidation of 1,2,3,5-THB and 1,2,4-trihydroxybenzene appears to be catalysed by the production of superoxide radicals during their conversion to their respective quinones (Ollinger et al., 1990; Buffington et al., 1989). Therefore, Rhodococcus sp. BPG-8 must employ protective mechanisms to prevent the auto-oxidation of 1,2,3,5-THB and 1,2,4-trihydroxybenzene.

Meta- or ortho-cleavage of 1,2,3,5-THB or 1,2,4-trihydroxybenzene is one potential method employed to alleviate the potential toxicity of the 1,2,4-trihydroxybenzene (Chapman and Ribbons, 1976 a, Chapman and Ribbons, 1976 b). Low efficiency constitutive meta-cleavage appeared to function for 1,2,4-trihydroxybenzene; while 1,2,3,5-THB elicited a high efficiency ortho-cleavage pattern. Cell-free extracts of either PG-grown or succinate grown Rhodococcus sp. BPG-8 cells inhibited the formation of 2-hydroxy-1,4-benzoquinone from 1,2,4-trihydroxybenzene. Chapman and Ribbons (1976, a) and Chapman and Ribbons (1976, b) also suggested this for P. putida, but they provided no explanation. Similiar but less pronounced effects occurred

during the auto-oxidation of 1,2,3,5-THB. SOD and catalase together perhaps hinder the auto-oxidation by the conversion of the superoxide radicals to oxygen and hydrogen peroxide. SOD and catalase activities were quite high in the cell-free extracts of Rhodococcus sp. BPG-8. Ollinger et al. (1990) suggest that superoxide radical removal by SOD may prevent the initiation of a free radical chain reaction between various hydroquinones and the superoxide radical.

The spontaneous production of H_2O_2 upon auto-oxidation of 1,2,3,5-THB and 1,2,4-trihydroxybenzene can be demonstrated by the release of oxygen upon introduction of pure catalase or cell-free extract. However, introduction of SOD did not bring about the release of oxygen. Fridovich (1972), Fridovich (1975), and Fridovich (1977) suggested that superoxide radicals may spontaneously dismutate because the superoxide radical is not stable relative to the products of hydrogen peroxide and triplet oxygen. SOD mediated and spontaneous dismutation of the superoxide radical probably occurs simultaneously (Winterbourn et al., 1989; Winterbourn et al., 1981).

Other investigators (Butler and Hoey, 1986; Fridovich 1976) have suggested that the removal of the superoxide radical causes the equilibrium to shift towards the oxidized product. The opposite phenomena occurred for the auto-oxidation of 1,2,4-trihydroxybenzene resulting from the removal of the superoxide radical which eliminates a possible

free radical chain reaction with 1,2,4-trihydroxybenzene (Ollinger et al., 1990; Buffington et al., 1989). The same effects may have occurred during the auto-oxidation of 1,2,3,5-THB; however the decreased stability of this molecule relative to 1,2,4-trihydroxybenzene may have produced copious amounts of superoxide radicals which, in essence, swamped the SOD/catalase protective mechanism. Brune et al. (1992), suggest that 1,2,3,5-THB oxidizes to its quinone immediately upon exposure to air. The increased stability of 2,6-dihydroxy-1,4-benzoquinone relative to 2-hydroxy-1,4-benzoquinone is a result of a lower reduction potential to its parent hydroquinone. The increase in the hydroxyl electron donating groups (inductive effect) of the 2,6-dihydroxy-1,4-benzoquinone causes these differential stabilities.

The 1,2,3,5-THB and 1,2,4-trihydroxybenzene are intermediates that are cleaved in the degradation sequence of PG and resorcinol, respectively. The auto-oxidation products of 1,2,3,5-THB and 1,2,4-trihydroxybenzene, respectively 2,6-dihydroxy-1,4-benzoquinone and 2-hydroxy-1,4-benzoquinone are dead end metabolites (Chapman and Ribbons, 1976 a, Chapman and Ribbons, 1976 b). Conversion of 2-hydroxy-1,4-benzoquinone to 1,2,4-trihydroxybenzene is dependent upon a specific NAD(P)H-dependent reductase. Both 1,4-benzoquinone and 2-hydroxy-1,4-benzoquinone had greater activity for NADH which suggest that it may be a NADH dependent reductase. Spain and Gibson (1991) suggest that 1,4-benzoquinone is converted to the hydroquinone

by a NADPH-dependent reductase. Moreover, 2-hydroxy-1,4-benzoquinone could be reduced to 1,2,4-trihydroxybenzene by sodium dithionite or sodium borohydride (Chapman and Ribbons, 1976 a). Enzymes that might be involved in this reduction of 2-hydroxy-1,4-benzoquinone include diaphorase, NADH cytochrome b_5 reductase, NADH-ubiquinone oxidoreductase, or NAD(P)H-cytochrome P-450 reductase (Chesis et al., 1984; Winterbourn 1981). This chapter suggests that there is a reductase that will convert the 2-hydroxy-1,4-benzoquinone to 1,2,4-trihydroxybenzene. Interestingly, no reductase specific for 2,6-dihydroxy-1,4-benzoquinone could be detected. Apparently 1,2,3,5-THB, as opposed to 1,2,4-trihydroxybenzene, is efficiently removed by a specific ortho-cleavage diooxygenase.

It appears that 1,2,4-trihydroxybenzene may be prevented or retarded from being oxidized to 2-hydroxy-1,4-benzoquinone by SOD and catalase or by the interaction with cell-free extract (Fig. 4.6) (Chapman and Ribbons, 1976 a). The 2-hydroxy-1,4-benzoquinone that is formed may be converted back to the 1,2,4-trihydroxybenzene by a specific reductase. Cleavage enzymes of 1,2,4-trihydroxybenzene may also alleviate this potential toxicity by removal of this intermediate, however 2-hydroxy-1,4-benzoquinone may still be seen (Chapman and Ribbons 1976 a).

Chapter V: Partial Purification and Enzymatic Characteristics of the Rhodococcus sp. BPG-8 Phloroglucinol Hydroxylase.

5.1 Introduction

Oxygenases participate in numerous degradation and biosynthesis pathways which catalyze the incorporation of oxygen into the substrate. The major classes of oxygenases, such as the monooxygenases (mixed-function oxygenases) and the dioxygenases, catalyze the incorporation of one and two atoms of dioxygen within the substrate, respectively (van Berkel and Muller, 1991). The monooxygenases reduce the other atom of dioxygen into water.

Monooxygenases that contain flavin as a prosthetic group may be divided into internal and external categories. Internal flavin-dependent monooxygenases transform the substrate by hydroxylation, followed by decarboxylation in which the substrate serves as the electron donor (van Berkel and Muller, 1991; Flashner and Massey, 1974; Nakazawa et al., 1972).

External flavoprotein monooxygenases are a class of inducible enzymes that utilize pyridine nucleotides as external electron donors by the insertion of one atom of dioxygen within the substrate. Approximately 25 external flavin-dependent enzymes have been isolated and (partially) characterized (van Berkel and Muller, 1991; Ohta et al., 1975). They can be divided into two subclasses which either: (1) cause hydroxylation of substituted aromatic substrates

(van Berkel and Muller, 1991; Krug and Straube, 1986; Ohta et al., 1975); (2) or the peroxide oxygenation of either nucleophilic and electrophilic substrates (van Berkel and Muller, 1991; Itagaki, 1986; Trower et al., 1985).

Reductive attack of PG seemed to be favoured by aerobic (Blackwood, et al., 1970, Jamieson et al.,1970), anaerobic (Patel et al., 1990, Krumholz et al., 1987, Patel et al., 1981, Schink and Pfenning, 1982) and phototrophic bacteria (Whittle et al., 1976) with no mention of the possible hydroxylation of PG by a specific monooxygenase.

In the previous chapter evidence was presented for the hydroxylation of PG by the novel PG hydroxylase isolated from a Rhodococcus sp. BPG-8. In this chapter the partially purified PG hydroxylase will be compared to similiar hydroxylases as well as to PG reductases.

5.2 Materials and Methods

5.2.1 Cell Material. Rhodococcus sp. BPG-8 was isolated and stored by the procedures outlined in chapters two and three. The minimal salt medium contained (g/l): $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 6.8, 0.1 ml/l trace elements, and 0.001% yeast extract. The Rhodococcus sp. BPG-8 isolate was grown under the opcimal conditions of 8 mM PG, 25°C, and pH 7.0. Growth conditions were similiar to those described in chapters two and three. Thirty grams of pelleted and washed cells were suspended in 60 ml of 20 mM phosphate buffer containing 1 mM

EDTA, 1 mM 2-mercaptoethanol, 15% glycerol, and 0.1 mM phenylmethylsulfonyl -fluoride (PMSF) (pH 7.4). The suspension was ice cooled and sonicated at minimum power to uniformly disperse the cells (Braunsonic 2000, Canlab). After sonification the cells were lysed by a French pressure cell (SLM Aminco Inc.) at 846 kg/cm^2. The disrupted cells were then centrifuged (20,000 x g, 40 min) in a Sorvall RC-5 centrifuge (Dupont instruments). The supernatant obtained formed the source of the hydroxylase.

5.2.2 Partial Purification of Phloroglucinol Hydroxylase. All procedures were performed under aerobic conditions at 5°C. All buffers contained 20 mM potassium phosphate, 1 mM EDTA, 1 mM 2-mercaptoethanol, 15% glycerol, and 0.1 mM PMSF (pH 7.4) (buffer A). Buffer A was found to stabilize and optimize PG hydroxylase activity (Acharya, 1986). After centrifugation of the disrupted cells, the cell-free extract (25 ml) was precipitated with 1.2 ml of 10 % polyethyleneimine (PEI) which was efficient at removing nucleic acid and residual lipids. The PEI was added slowly over 15 min, then centrifuged (20,000 x g, 20 min).

The clear supernatant (26.2 ml) was used directly for ammonium sulfate fractionation from 0-40 % and 40-80 %. The active fraction which was contained in the 40-80 % fraction was resuspended in 15 ml of buffer A and dialyzed for 12 hours in 5 l of buffer A at 5°C. The dialysate (15 ml) was applied

to a 1.5 x 30 cm DEAE-Sephacel column, equilibrated and then washed at 15 ml/h for 20 h with buffer A. The PG hydroxylase was eluted using a 500 ml linear gradient of potassium phosphate buffer (20 mM-800 mM) containing 1 mM EDTA, 1 mM 2-mercaptoethanol, 15% glycerol, and 0.1 mM PMSF (pH 7.4) at 20 ml/h. The most active fractions (37 ml) were pooled and precipitated with a 40-80 % ammonium sulfate fraction and resuspended in 15 ml of buffer A and dialyzed for 12 hours in 5 l of buffer A at 5°C.

The dialysate (15 ml) was sequentially applied to a Sephadex G-200 and G-100 column (1.5 x 40 cm) equilibrated with potassium phosphate buffer A. Approximately 2.0 ml fraction were collected. Active fractions (#26-34) and (#5-11) from G-200 and G-100 columns respectively were pooled and the protein was precipitated by ammonium sulfate. The precipitated protein was redissolved in 15 ml of buffer A, and dialyzed against buffer A (5l) for 12 h. The dialysate was applied to a column (1 X 5 cm) packed with reactive Red Agarose 120 type-1000 CL (Sigma) and equiilibrated with buffer A. Various treatments which included adding of FAD (2μM), catalase and/or SOD (50 ng), and PG/resorcinol (1 μmole) were performed.

5.2.3 Analytical methods. Phloroglucinol hydroxylase and resorcinol hydroxylase were assayed according to modification of the methods of Patel et al., (1981), and Neujahr and Gaal (1975) as outlined in chapter three. The assay buffer also

contained FAD (2 μM), and catalase (50 ng).

Native molecular weight was determined using a 1.5 X 40 cm Sephadex G-200 and G-100 (superfine) gel filtration columns (Pharmacia). The Sephadex G-200 column was calibrated with ovalbumin (M_r= 43,000), bovine serum albumin (M_r= 68,000), collagenase (M_r= 101,000), alcohol dehydrogenase (M_r= 150,000), and catalase (M_r= 230,000). The Sephadex G-100 column excluded the standards alcohol dehydrogenase and catalase.

Physical parameters such as temperature (10°C-37°C), pH (6.0-8.0), substrate concentration (0-1.0 μm), FAD concentration (0-4 μM), and the amount of protein (0-25 μg) were examined for PG hydroxylase and resorcinol hydroxylase. The effect of various substrates (8 mM) on PG and resorcinol hydroxylase activity were examined (Table 5.7). The effects of the inhibitors, sodium azide (1 mM) and arsenic (1 mM) were also examined. The following reaction mixture (3 ml) contained (in μmoles): potassium phosphate buffer, pH 7.2, 270; NAD(P)H, 0.6; PG, 1.0; and partially purified extract (0.1 ml). All experiments except those involving FAD or otherwise mentioned had 2 μM of FAD, and 50 ng of catalase added to the reaction mixture.

Metal ions such as zinc, nickel, ferrous and ferric ions, magnesium, calcium, copper, manganese, cobolt, and mercury (0.5 mM) were examined for the ability to inhibit the PG hydroxylase and resorcinol hydroxylase using the optimal conditions determined from experiments described above.

Various cofactors (cyt.c, FAD, FMN, ATP, ADP, AMP) at a concentration of 2 μM and enzymes such as SOD (50 ng) and catalase (50 ng) were added to the reaction mixtures. The effect of storage temperature (-20°C, 4°C, 25°C) along with the thermostability (5°C-60°C) of the PG and resorcinol hydroxylases were examined.

5.3 Results

5.3.1 Partial Purification of Phloroglucinol Hydroxylase. The purification procedure for PG hydroxylase from extracts of Rhodococcus sp. BPG-8, after growth on PG, is summarized in Table 5.1. Activity for resorcinol hydroxylase mirrored the elution patterns for PG hydroxylase. At no point could these two activities be separated. The highest specific activity obtained for PG hydroxylase with PG and resorcinol as substrates were 0.48 and 0.16 μmole of NADH oxidized per min per mg protein, respectively. The elution profiles for PG hydroxylase for DEAE-Sephacael, Sephadex G-200, and Sephadex G-100 columns are shown in Fig. 5.1, Fig. 5.2, and Fig. 5.3 respectively. Affinity chromatography seemed to have no effect on the binding of the PG or resorcinol hydroxylases (Table 5.2). Factors such as SOD, catalase, and FAD were utilized to enhance the binding of the hydroxylase to the affinity column.

5.3.2 Enzyme characteristics. Gel filtration chromatography of the native PG hydroxylase was performed and gave an estimated

molecular weight of 155,000 daltons (Fig. 5.4). This seems to verify previous molecular weight estimates utilizing crude enzyme extracts (Acharya, 1986). The PG hydroxylase was eluted in the void volume when applied to superfine Sephadex G-100.

PG hydroxylase activity, with substrates PG or resorcinol was adversely affected by increases in temperature as determined by their storage temperature and thermostability profiles (Table 5.3, Table 5.4). The effect of various cofactors on the activity of the partially purified extract was examined (Table 5.5). FAD and cytochrome c seemed to enhance the PG and resorcinol hydroxylase activities. Increasing the amount of FAD affected the specific activity of the partially purified PG hydroxylase and resorcinol hydroxylase (Fig. 5.5, Fig. 5.6).

Most metal ions seemed to suppress the activity of PG and resorcinol hydroxylase (Table 5.6). The effect of various substrates on the activity of the PG and resorcinol hydroxylase was investigated (Table 5.7). Apparently numerous phenolic aromatics, chlorinated aromatics, benzoquinones, and metabolic poisons had no effect on the activity of these hydroxylases. Inhibitors such as sodium arsenic and sodium azide did not affect the hydroxylase activity. The effect of increasing concentration of chloride ion on the specific activity of the partially purified PG and resorcinol hydroxylase is shown in Fig. 5.7, and Fig. 5.8. Apparently increasing chloride ion concentration seemed to decrease the

activity of the hydroxylases.

The specific activity of PG hydroxylase in a growing culture of Rhodococcus sp. BPG-8 peaked at approximately 18 hours, however activity significantly decrease after this time (Fig. 5.9). The pH, substrate, temperature optimum for both the PG and resorcinol hydroxylase from Rhodococcus sp. BPG-8, when 2 μM of FAD and 50 ng of catalase was added to the reaction mixture were 7.0, 68 μM, and 25°C respectively (Fig. 5.10, Fig. 5.11, Fig. 5.12). The apparent V_{max} values for PG and resorcinol were 0.48 and 0.28 μmole of NADH oxidized per minute per mg protein respectively (Fig. 5.13). The apparent K_m values were 8.3 μM and 12.5 μM for PG and resorcinol respectively (Fig. 5.13).

When FAD was excluded from the reaction mixture, identical optimal pH (7.0) and temperature (25°C) profiles occurred. The optimal substrate concentration for PG and resorcinol were 68 μM, and 125 μM respectively (Fig. 5.14). The apparent K_m values, when PG and resorcinol were added to the reaction mixture, were 12.5 μM and 16.7 μM respectively, while the apparent V_{max} values were 0.16 and 0.09 μmole of NADH oxidized per minute per mg protein respectively (Fig. 5.15). The oxidation of NADH increased arithmetically with increasing protein concentration (Fig. 5.16).

Table 5.1. Purification steps for the partially purification of phloroglucinol hydroxylase.

Treatment	Total volume	Total protein	Total E.U.	Total Activity	Purification fold
Crude extract	25.0	245	7.23	0.030	1.0
PEI ppt.	26.2	241	7.58	0.031	1.03
NH_4SO_4 ppt.(40-80%)	15.0	105	6.95	0.065	2.2
DEAE-Sephacel	37.0	11.8	5.35	0.450	15.0
NH_4SO_4 ppt.(80%)	15.0	11.2	5.30	0.470	15.7
Sephadex G-200	15.0	2.29	1.09	0.48	16.0
NH_4SO_4 ppt.(80%)	15.0	2.21	1.03	0.47	15.7
Sephadex G-100	12.5	2.20	1.05	0.48	16.0

Polyethyleneimine: PEI.

Table 5.2. Ability of reactive red agarose to bind the partially purified Rhodococcus sp. BPG-8 phloroglucinol hydroxylase and resorcinol hydroxylase.

Treatment	Phloroglucinol hydroxylase	Resorcinol hydroxylase
Enz. alone	-	-
Enz.+FAD	-	-
Enz.+PG/RES	-	-
Enz.+PG/RES+FAD	-	-
Enz.+PG/RES+FAD+CAT	-	-
Enz.+PG/RES+FAD+CAT+SOD	-	-

Partially purified cell-free extract (Enz.). Various treatments included adding FAD (2μM), catalase and/or SOD (50 ng), and PG/resorcinol (1 μmole).

Table 5.3. Effect of storage temperature on the stability of phloroglucinol hydroxylase and resorcinol hydroxylase from Rhodococcus sp. BPG-8.

Time (days)	Percentage Activity					
	PG hydroxylase			RES hydroxylase		
	-20°C	4°C	25°C	-20°C	4°C	25°C
0	100	100	100	100	100	100
1	100	95	0	100	85	0
2	100	80	0	100	85	0
5	100	28	0	100	30	0
10	100	0	0	100	0	0
20	100	0	0	98	0	0
30	98	0	0	94	0	0
60	93	0	0	92	0	0
90	95	0	0	93	0	0

Enzyme was stored in a -20°C, and 5°C fridge, and a water bath at 25°C.

Table 5.4. Thermostability of the phloroglucinol hydroxylase and resorcinol hydroxylase from Rhodococcus sp. BPG-8.

Temp. (°C)	PG-hydroxylase		RES-hydroxylase	
	%	Activity	%	Activity
5	100	0.48	100	0.28
15	100	0.48	100	0.28
20	100	0.48	100	0.28
25	100	0.48	100	0.28
28	96	0.46	93	0.26
30	73	0.35	57	0.16
35	54	0.26	39	0.11
37	15	0.07	0	0
40	4	0.02	0	0
45	0	0	0	0
60	0	0	0	0

The enzyme was equilibrated for two minutes at the desired temperature using a Shimadzu water bath connected to the Shimadzu spectrophotometer. Specific activity was then measured at 25°C as described in Materials and Methods. It was also expressed as the percentage of total specific activity.

Table 5.5. Effect of various cofactors on the activity of the partially purified Rhodococcus sp. BPG-8 phloroglucinol hydroxylase and resorcinol hydroxylase.

Treatment	PG Hydroxylase		RES Hydroxylase	
	Activity	%	Activity	%
none	0.16	100	0.09	100
FAD	0.48	300	0.28	311
FMN	0.16	100	0.08	89
CAT	0.16	100	0.09	100
SOD	0.16	100	0.09	100
FAD+CAT	0.48	300	0.27	300
FAD+CAT+SOD	0.48	300	0.27	300
cyt. c	0.19	119	0.10	111
β-carotene[A]	0.16	100	0.09	100
ATP	0.17	105	0.09	100
ADP	0.16	100	0.09	100
AMP	0.16	100	0.09	100

FAD: flavin adenine dinucleotide (2μM); FMN: flavin mononucleotide (2μM); CAT: catalase (50ng); SOD: superoxide dismutase (50ng); cyt.c: cytochrome-c (2μM); ATP, ADP, AMP: adenosine triphosphate, adenosine diphosphate, and adenosine monophosphate respectively (2μM each). [A] β-carotene was isolated from Rhodococcus sp. BPG-8. N=3.

Table 5.6. Effect of metal ions on the specific activity of the partially purified phloroglucinol hydroxylase and resorcinol hydroxylase.

	PG hydroxylase		RES hydroxylase	
Metal ion	Activity	%	Activity	%
none	0.48	100	0.28	100
Zinc sulfate	0.19	40	0.10	36
Nickel sulfate	0.26	54	0.12	43
Ferric sulfate	0.26	54	0.15	53
Ferrous sulfate	0.22	46	0.13	46
Magnesium sulfate	0.22	46	0.23	82
Calcium sulfate	0.45	94	0.26	93
Copper sulfate	0.13	27	0.05	18
Manganese sulfate	0.38	79	0.21	75
Cobolt sulfate	0.32	66	0.16	57
Mercury acetate	0.00	00	0.00	00

Specific activity was measured as described in Materials and Methods. It was also expressed as a percentage of the total specific activity. All metal ions were added at a concentration of 0.5 mM. N=3.

Table 5.7. Effect of various substrates on the activity of the partially purified hydroxylase from Rhodococcus sp. BPG-8.

	Specific Activity		
Substrate	Alone	PG+substrate	Res+substrate
Phloroglucinol	0.48	NA	NA
Resorcinol	0.28	NA	NA
4-Chlororesorcinol	0.10	0.48	0.28
1,3,5-TCB	0.00	0.48	0.28
2,4-DCP	0.00	0.45	0.28
3,5-DCP	0.00	0.45	0.26
3,4-DCP	0.00	0.45	0.26
B-quinone	0.00	0.48	0.28
1,2,4-Btriol	0.00	0.48	0.28
Pyrogallol	0.00	0.48	0.28
4-methylcatechol	0.00	0.48	0.26
catechol	0.00	0.48	0.26
3-hydroxybenzoate	0.00	0.48	0.28
3,5-di-OH-Bz	0.00	0.48	0.27
O-cresol	0.00	0.48	0.28
M-cresol	0.00	0.48	0.28
P-cresol	0.00	0.48	0.28
1,3,5-TMeB	0.10	0.48	0.28
1,2,3,5-THB	0.00	0.48	0.28
Quinate	0.00	0.48	0.28

1,3,5-TCB, 1,3,5-trichlorobenzene; DCP, dichlorophenol; B-quinone, 1,4-benzoquinone; 1,2,4-btriol, 1,2,4-trihydroxybenzene; 3,5-di-OH-Bz, 3,5-dihydroxy benzoate; 1,3,5-TMeB, 1,3,5-trimethyoxylbenzene; 1,2,3,5-THB, 1,2,3,5-tetrahydroxybenzene. NA, not applicable. All substrates were tested at an 8 mM concentration.

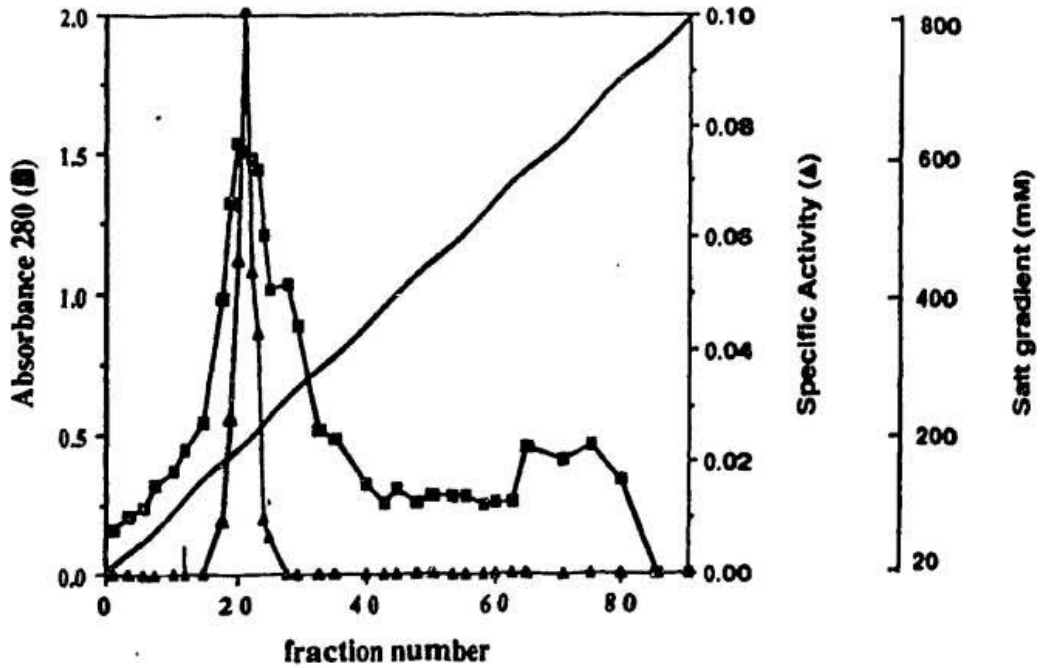


Fig. 5.1. Linear gradient elution of phloroglucinol hydroxylase from DEAE-Sephacel column. The gradient, containing 20 mM (250 ml) to 800 mM KH_2PO_4 (250 ml) (pH 7.4), 1 mM EDTA, 1 mM 2-mercaptoethanol and 0.1 mM PMSF was used to elute the phloroglucinol hydroxylase from the column.

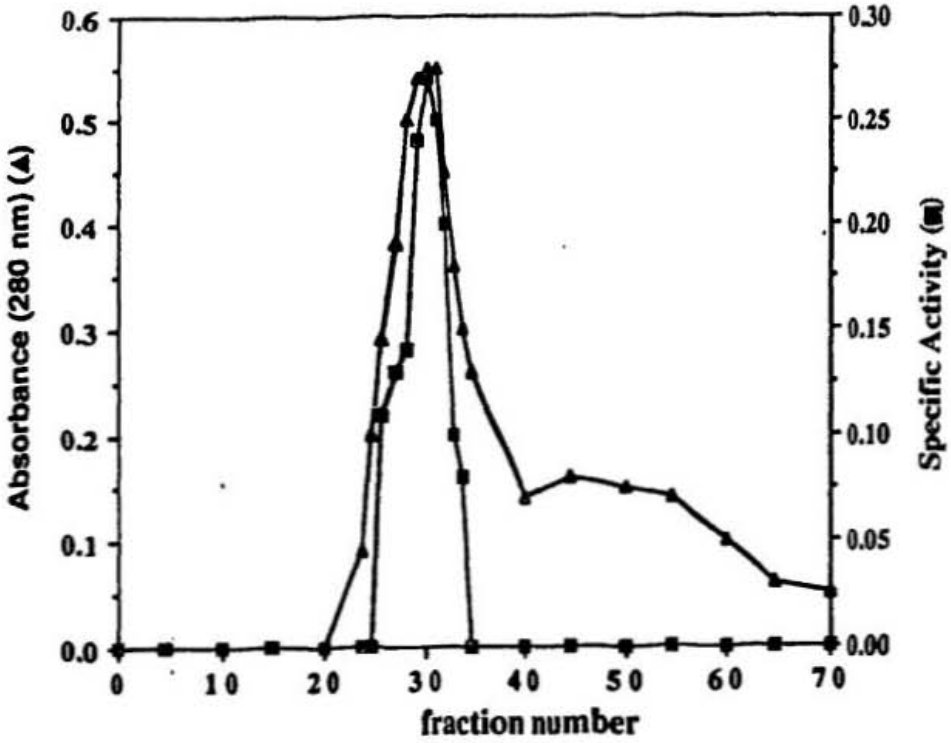


Fig. 5.2. Elution pattern of the phloroglucinol hydroxylase from a Sephadex G-200 column using a 20 mM KH_2PO_4 buffer (pH 7.4) containing 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.1 mM PMSF.

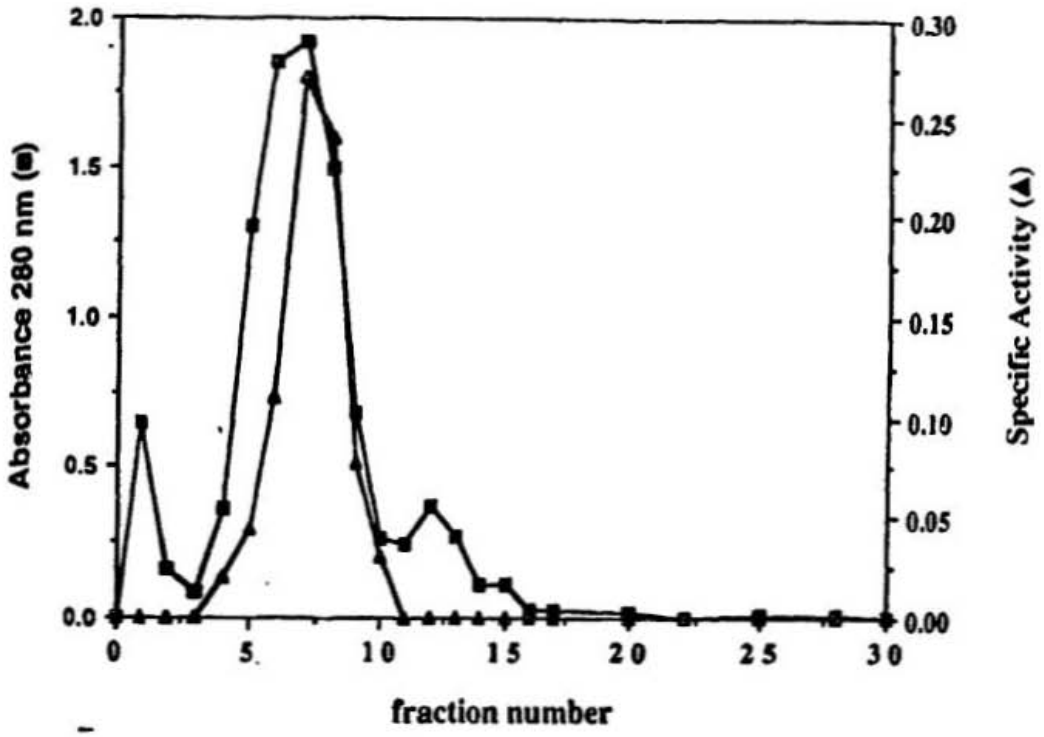


Fig. 5.3. Elution pattern of the phloroglucinol hydroxylase from a Sephadex G-100 column using a 20 mM KH_2PO_4 buffer (pH 7.4) containing 1 mM EDTA, 1 mM 2-mercaptoethanol and 0.1 mM PMSF.

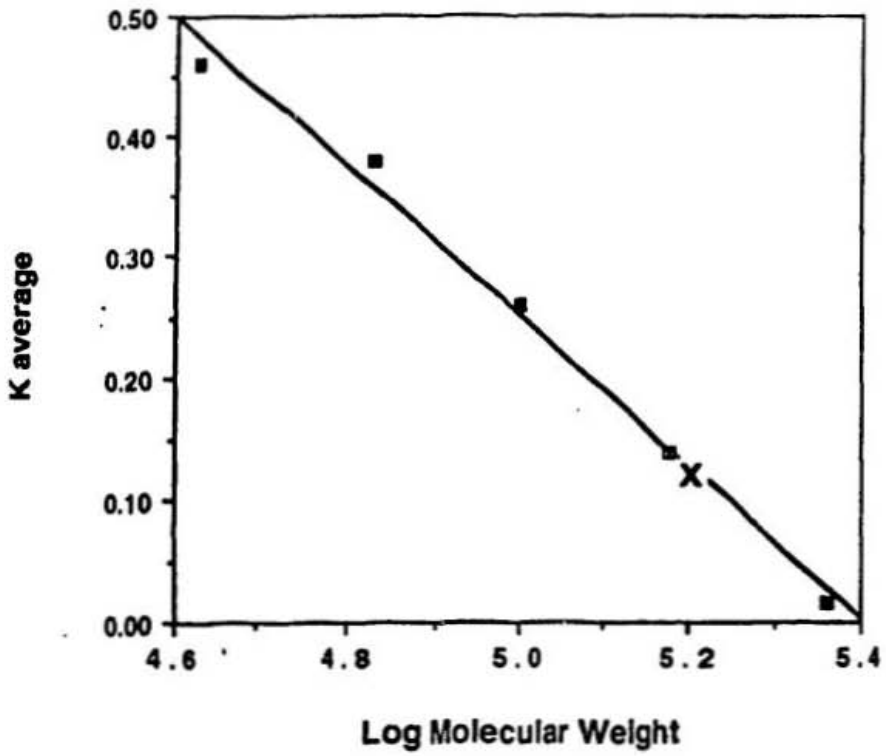


Fig. 5.4. Native molecular weight of the phloroglucinol hydroxylase (X) was determined using a 1.5 X 40 cm Sephadex G-200 superfine gel filtration column (Pharmacia). The column standards were ovalbumin (M_r= 43,000), bovine serum albumin (M_r= 68,000), collagenase (M_r= 101,000), alcohol dehydrogenase (M_r= 150,000), and catalase (M_r= 230,000). Linear regression analysis was performed to obtain the plot.

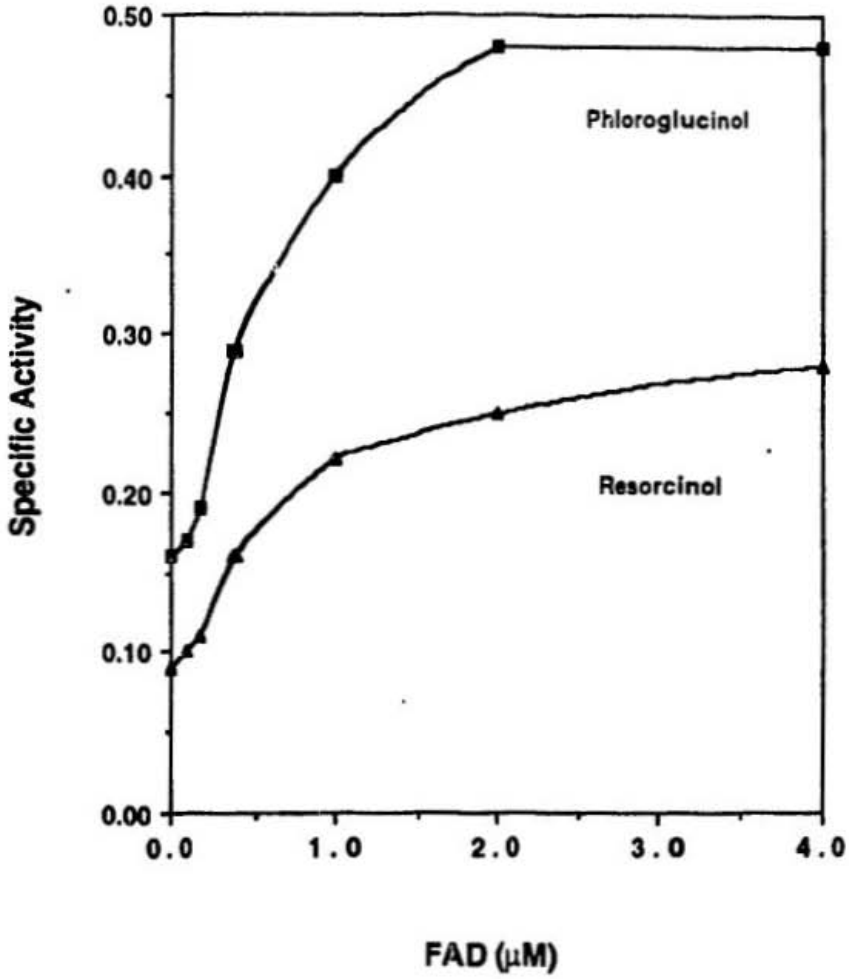


Fig. 5.5. Effect of increasing amounts of flavin adenine dinucleotide (FAD) on the specific activity of the partially purified phloroglucinol hydroxylase and resorcinol hydroxylase isolated from Rhodococcus sp. BPG-8.

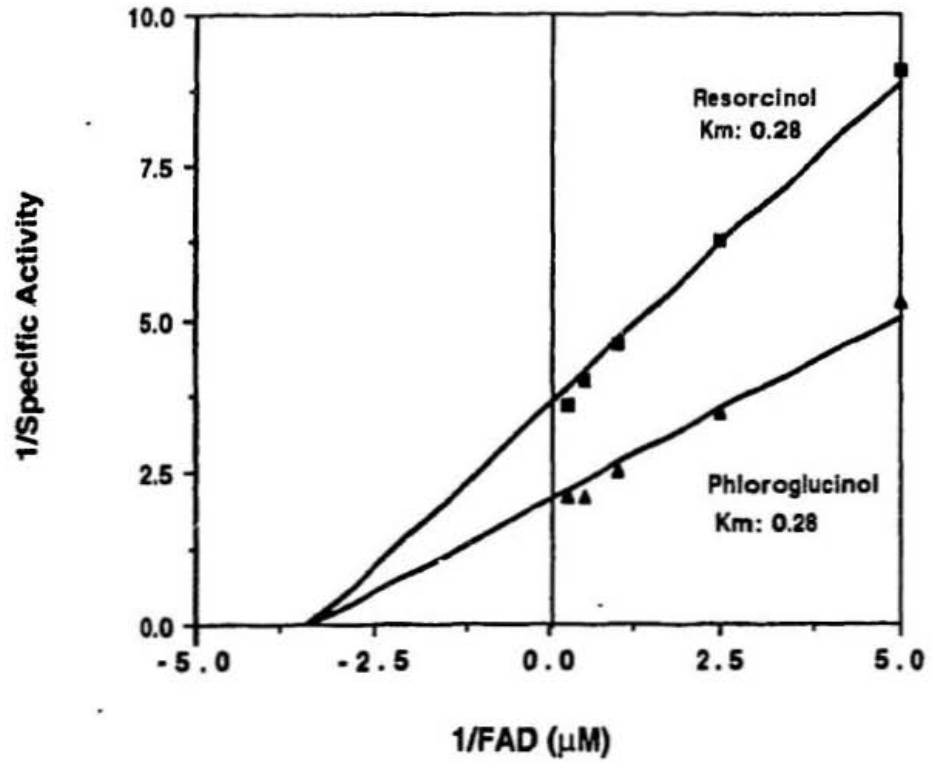


Fig. 5.6. Lineweaver-Burk plot of the effects of increasing amounts of flavin adenine dinucleotide (FAD) on the specific activity of the partially purified phloroglucinol hydroxylase and resorcinol hydroxylase isolated from Rhodococcus sp. BPG-8.

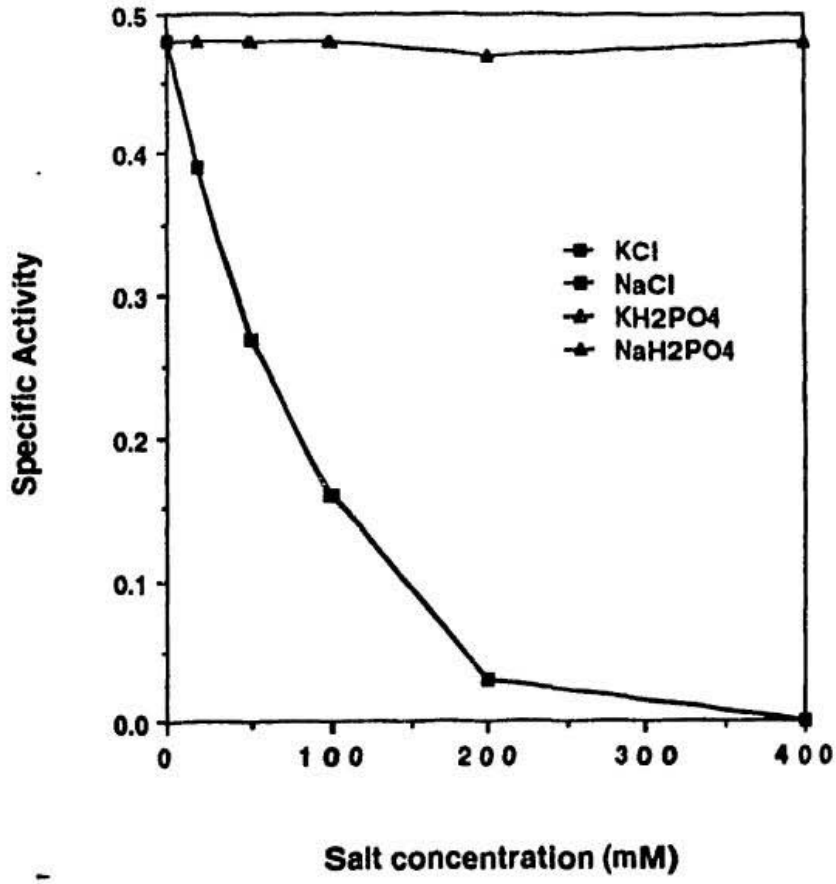


Fig. 5.7. Effect of increasing concentration of chloride ion on the specific activity of the partially purified phloroglucinol hydroxylase isolated from Rhodococcus sp. BPG-8.

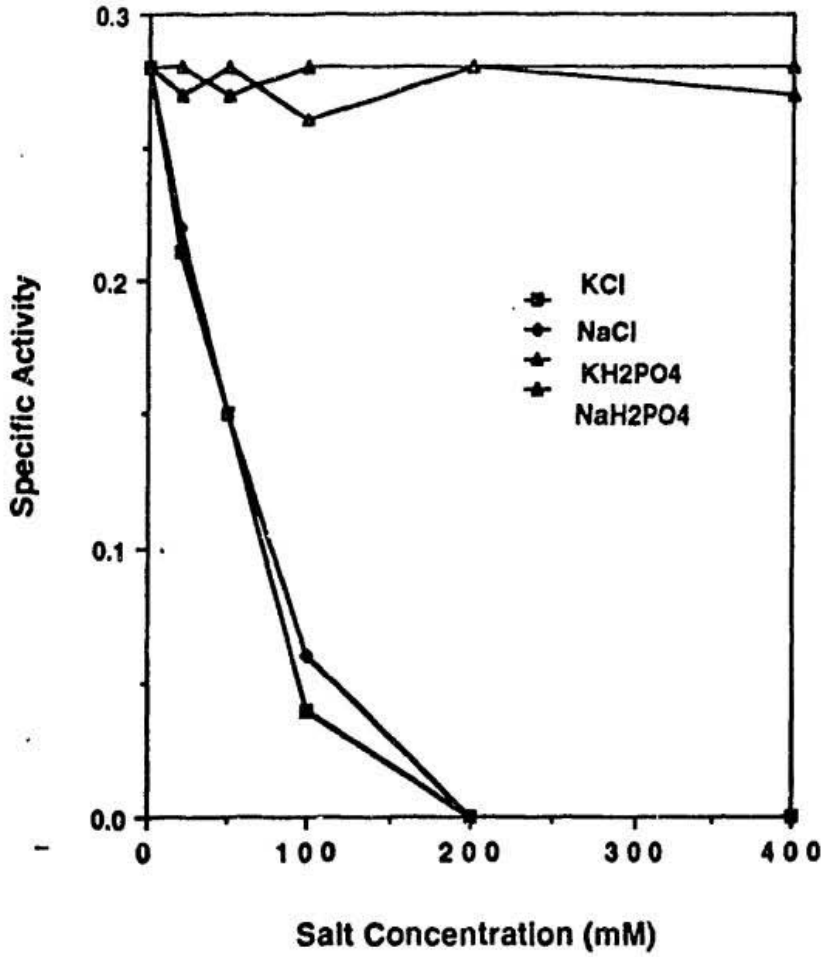


Fig. 5.8. Effect of increasing concentration of chloride ion on the specific activity of the partially purified resorcinol hydroxylase isolated from Rhodococcus sp. BPG-8.

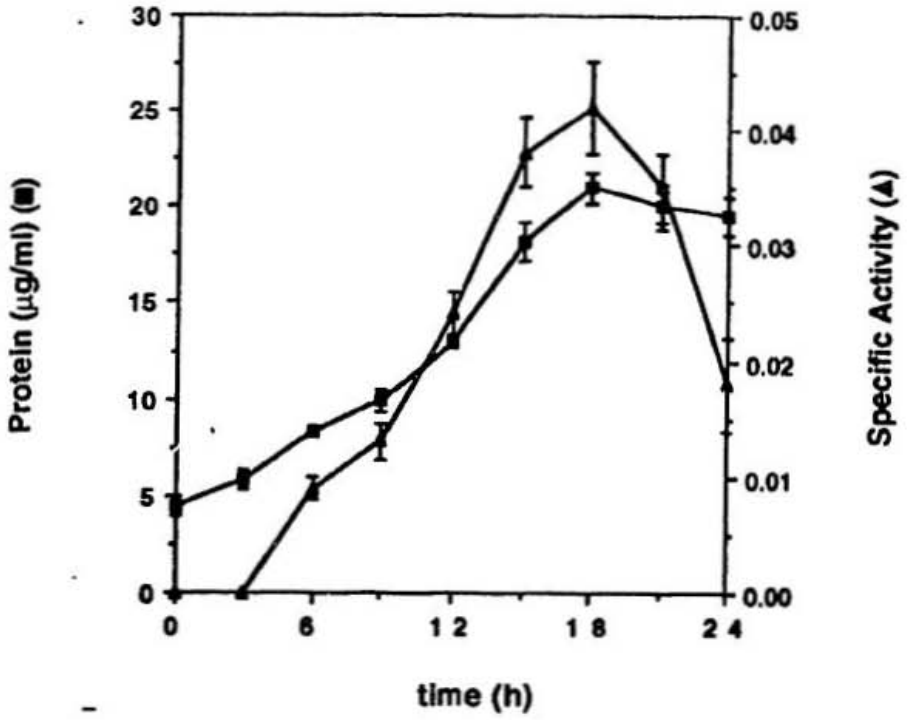


Fig. 5.9. Phloroglucinol hydroxylase activity in the cell-free extract of Rhodococcus sp. BPG-8 grown on PG during its growth cycle. Each extract was treated with PEI (0.5 %) to precipitate lipids and DNA before enzyme assays were performed.

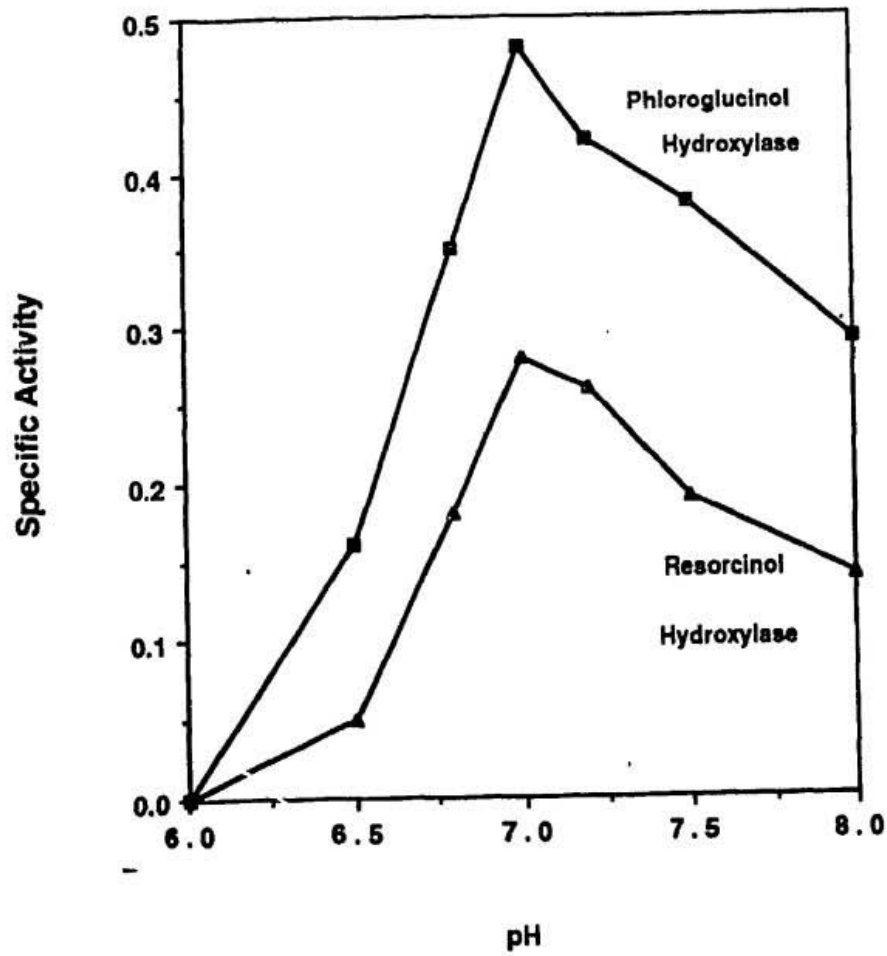


Fig. 5.10. Effect of pH on the specific activity of the partially purified phloroglucinol hydroxylase and resorcinol hydroxylase isolated from Rhodococcus sp. BPG-8.

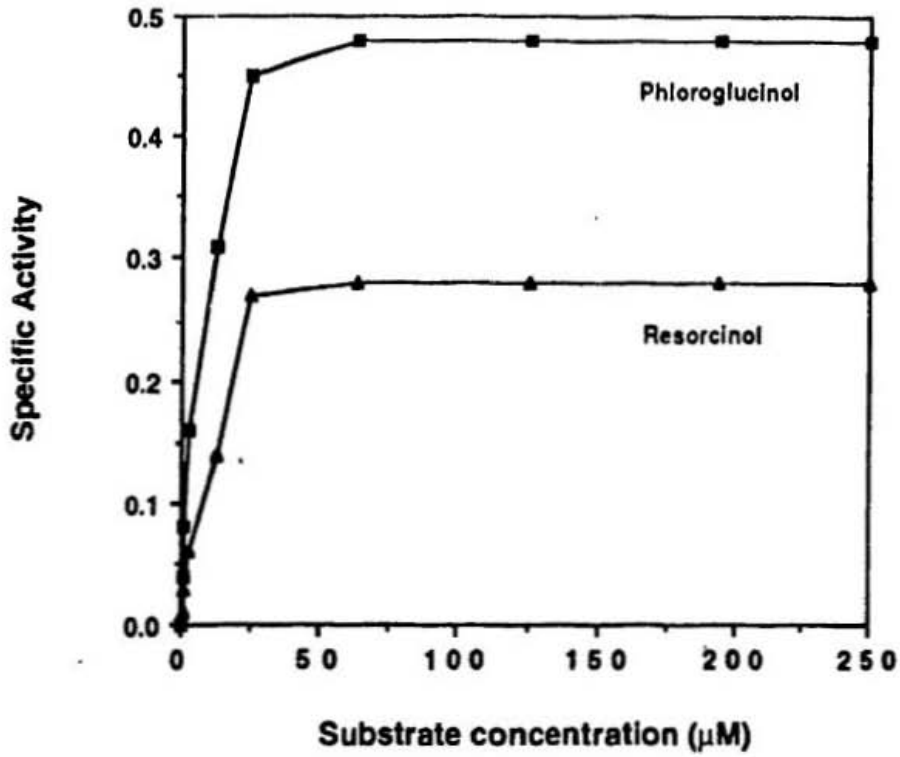


Fig. 5.11. Effect of substrate concentration on the specific activity of the partially purified phloroglucinol hydroxylase and resorcinol hydroxylase isolated from Rhodococcus sp. BPG-8. The enzyme assay contained 2 μM FAD, and 50 ng of catalase.

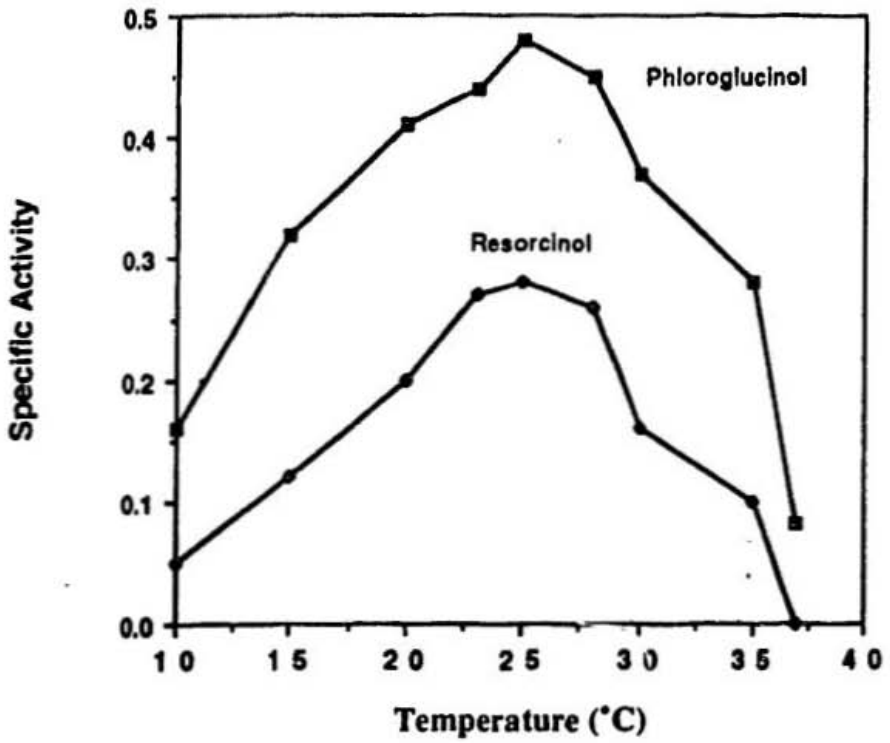


Fig. 5.12. Effect of temperature on the specific activity of the partially purified phloroglucinol hydroxylase and resorcinol hydroxylase isolated from Rhodococcus sp. BPG-8.

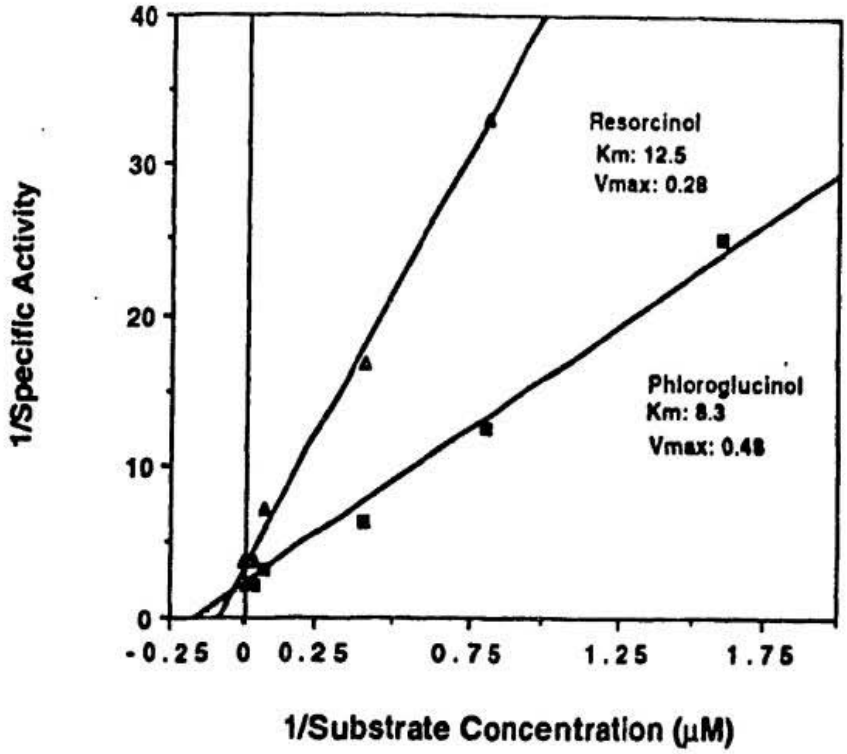


Fig. 5.13. Lineweaver-Burk plot of the effects of increasing phloroglucinol and resorcinol concentrations on the specific activity of the partially purified phloroglucinol hydroxylase isolated from Rhodococcus sp. BPG-8. The enzyme assay contained 2 μM FAD, and 50 ng of catalase.

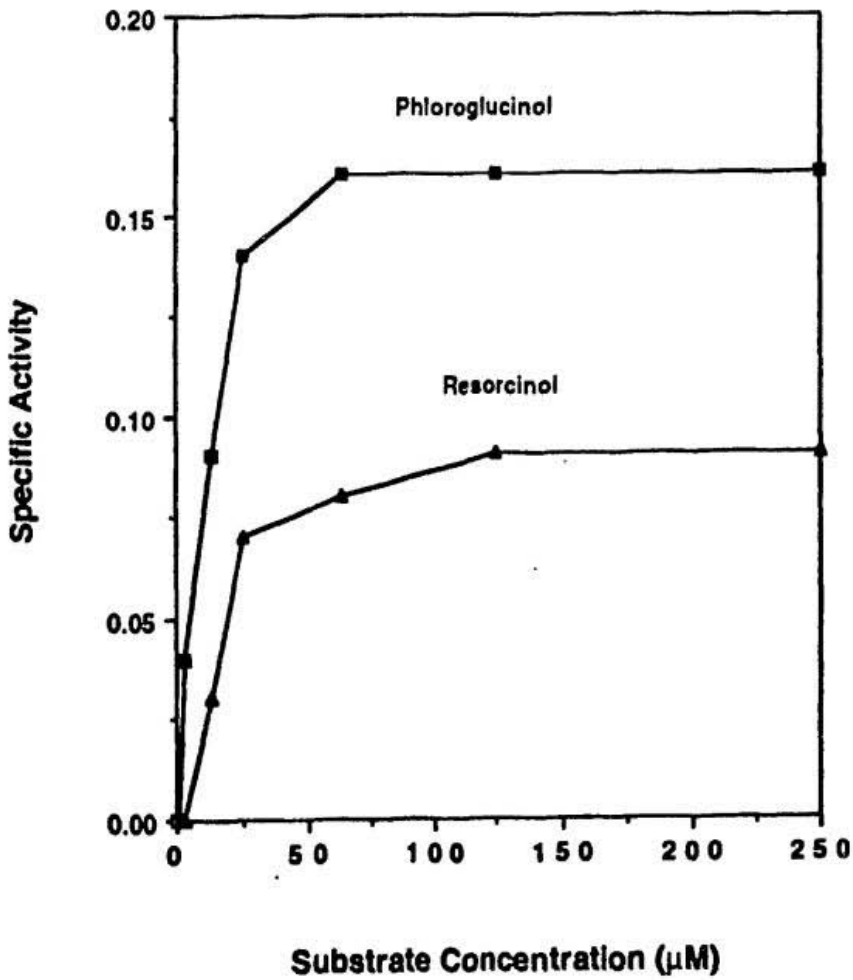


Fig. 5.14. Effect of substrate concentration on the specific activity of the partially purified phloroglucinol hydroxylase isolated from Rhodococcus sp. BPG-8. The enzyme assay did not contain 2 μM FAD, but contained 50 ng of catalase.

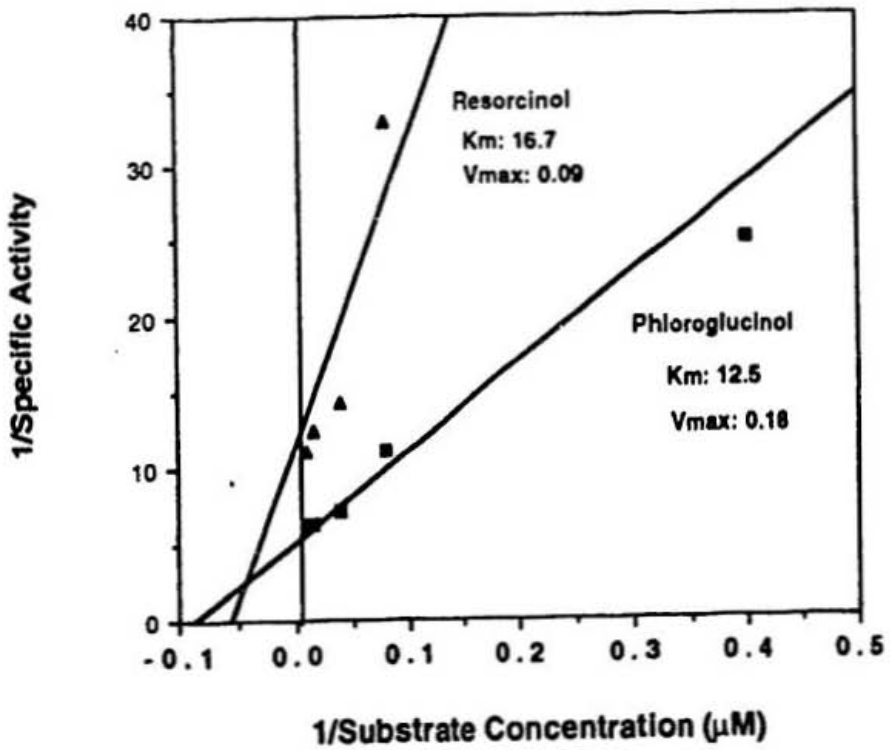


Fig. 5.15. Lineweaver-Burk plot of the effects of increasing phloroglucinol and resorcinol concentrations on the specific activity of the partially purified phloroglucinol hydroxylase isolated from Rhodococcus sp. BPG-8. The enzyme assay did not contain 2 μM FAD, but contained 50 ng of catalase.

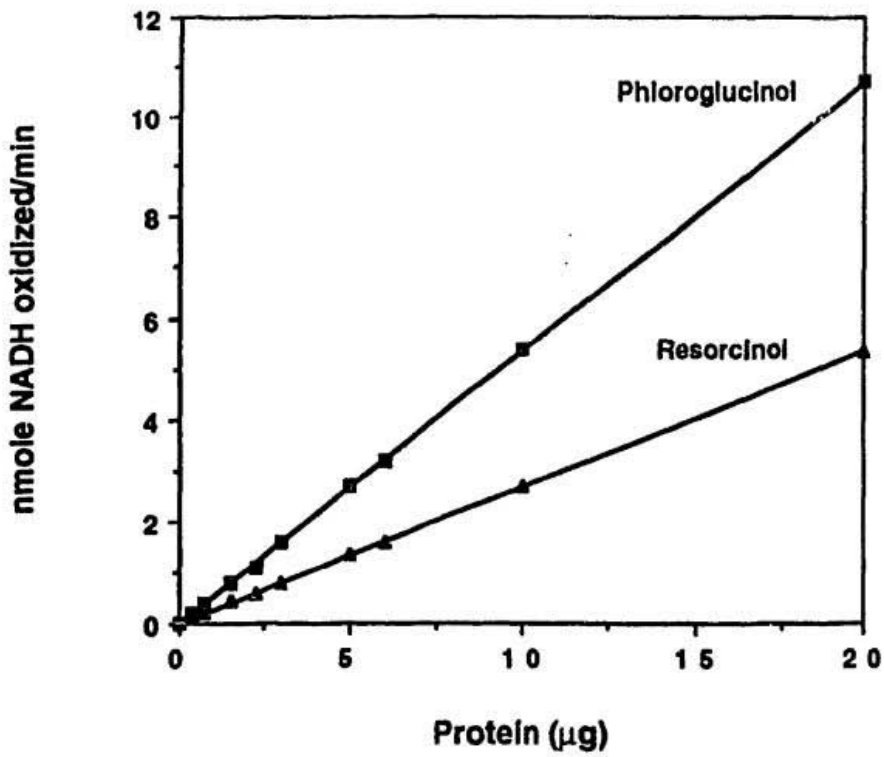


Fig. 5.16. Effect of increasing protein concentration on the enzyme activity of the partially purified phloroglucinol hydroxylase for the substrates phloroglucinol and resorcinol. See Material and Methods for details.

5.4 Discussion

The mineralization of PG involves the reduction to the dihydrophloroglucinol via a PG reductase in many organisms (Krumholz et al., 1987; Schink and Pfenning, 1982; Patel et al., 1981; Mathur, 1971). The evidence for a possible hydroxylation of PG to 1,2,3,5-THB has received little attention. Partial purification of the PG utilizing oxygen dependent enzyme suggests that the enzyme is a hydroxylase and not a reductase. It has been suggested that phenol hydoxylase, resorcinol hydroxylase, and orcinol hydroxylase may accept PG as a substrate / effector molecule in Rhodococcus sp. P1 and Pseudomonas putida ORC (Straube, 1987; Ohta and Ribbons, 1976). However, during their studies, their organisms never utilized PG as the growth substrate.

The purification of PG reductase from Eubacterium oxidoreducens G-41 suggested that a NADPH-dependent forward and a $NADP^+$-dependent reverse reaction of PG to dihydrophloroglucinol occurred (Haddock and Ferry, 1989). The inducible partially purified PG hydroxylase would only function with a NAD(P)H-dependent forward reaction and no reverse reaction was observed. The preferred energy cofactor was NADH, not NADPH, as suggested for other isolated PG reductases (Haddock and Ferry, 1989; Patel et al., 1981).

The PG reductase purified from Eubacterium oxidoreducens G-41 was shown to be colorless and free of any flavins

(Haddock and Ferry, 1989), while the partially purified PG and resorcinol hydroxylase from Rhodococcus sp. BPG-8 was stimulated in the presence of FAD. Neujahr (1991) suggests that FAD is noncovalently linked to a Trichosporon cutaneum phenol hydroxylase and therefore is easily displaced during purification. This could explain the differences in the apparent V_{max} and K_m values when FAD was excluded from the reaction mixture containing the partially purified hydroxylase from Rhodococcus sp. BPG-8.

The temperature and pH optima of the purified PG reductase from Eubacterium oxidoreducens G-41 were 40°C and 7.3 (Haddock and Ferry, 1989). This differed significantly from the partially purified PG hydroxylase from Rhodococcus sp. BPG-8.

Chloride anions were shown to inhibit activity of the partially purified PG hydroxylase. This is consistent with similiar results for other phenolic hydroxylases (van Berkel and Muller, 1991; Neujahr, 1991). Halogen ions may be competitive inhibitors of the enzyme NAD(P)H binding site (van Berkel and Muller, 1991; Neujahr, 1991). Patel et al., (1981) suggest that PG reductase from Coprococcus sp. Pe15 is stimulated in the presence of chloride anion.

Phloroglucinol hydroxylase was extremely thermo-unstable with an apparent native molecular weight of 155,000 daltons which is similiar to phenol hydroxylase from Trichosporon cutaneum (148,000 daltons) (Neujahr, 1991; Neujahr and Gaal,

1973). Interestly the native molecular weight of resorcinol and orcinol hydroxylase from Pseudomonas putida ORC was 68,000 daltons (van Berkel and Muller, 1991; Neujahr, 1991; Ohta and Ribbons, 1976). Haddock and Ferry (1989) found that the purified PG reductase had a native molecular weight of 78,000 daltons.

The PG and resorcinol hydroxylase in the present case could not be separated, suggesting that they are the same enzyme with broad substrate specificity (van Berkel and Muller, 1991; Neujahr, 1991; Batie et al., 1987; Straube, 1987; Ohta and Ribbons, 1976). Both the PG and resorcinol probably behaved as substrate/effectors, with PG hydroxylated at a higher efficiency. Effectors convert the oxygen into H_2O_2, instead of hydroxylating the substrate. This was verified by the detection of the uncoupling effector product H_2O_2; and the increased efficency of NADH oxidation wnen catalase was added to the reaction mixture (van Berkel and Muller, 1991; Neujahr, 1991). The catalase converted the H_2O_2 to oxygen and H_2O; thus rejuvenating the expended oxygen and allowing it to again react with the PG hydoxylases.

Stadtman (1992) suggests that H_2O_2 production and ferric ions are catalysed by mixed function oxygenases (MFO) and metal catalysed oxidation (MCO) systems which react and oxidize enzymes making them more amenable to intracellular proteases. The introduction of substrates, EDTA, catalase/SOD, may inhibit this mechanism by blocking this interaction.

Chapter VI: General Discussion and Conclusions

The metabolism of PG and the fortuitous metabolism of resorcinol may be helped by constitutive enzymes such as catalase, SOD, and NAD(P)H specific quinone reductases located within the Rhodococcus sp. BPG-8. These enzymes appear to stabilize intermediates and also increase enzyme efficiency during the mineralization of PG and resorcinol.

The effects are more pronounced in the conversion of resorcinol to 1,2,4-trihydroxybenzene by the PG induced Rhodococcus sp. BPG-8. Interestingly resorcinol was never found to be an intermediate during PG mineralization. The efficiency of the conversion of resorcinol to 1,2,4-trihydroxybenzene in the presence of catalase was increased because of the elimination of H_2O_2 and the regeneration of oxygen (Fig. 6.1) (Dagley, 1971). The same effect could apply to PG; however it has been shown that PG is converted into 1,2,3,5-THB at a greater effiency. This increased effiency suggests that PG may be the preferred substrate, while resorcinol may behave as an effector and a substrate.

The conversion of resorcinol to 1,2,4-trihydroxybenzene presents another problem to the PG induced Rhodococcus sp. BPG-8. The stability and equilibrium of 1,2,4-trihydroxybenzene favours the 2-hydroxy-1,4-benzoquinone, a potential toxic dead-end product that produces superoxide radicals during its formation. Therefore the Rhodococcus sp.

BPG-8 must employ various protective mechanisms to first stabilize 1,2,4-trihydroxybenzene and also convert the 2-hydroxy-1,4-benzoquinone back to 1,2,4-trihydroxybenzene.

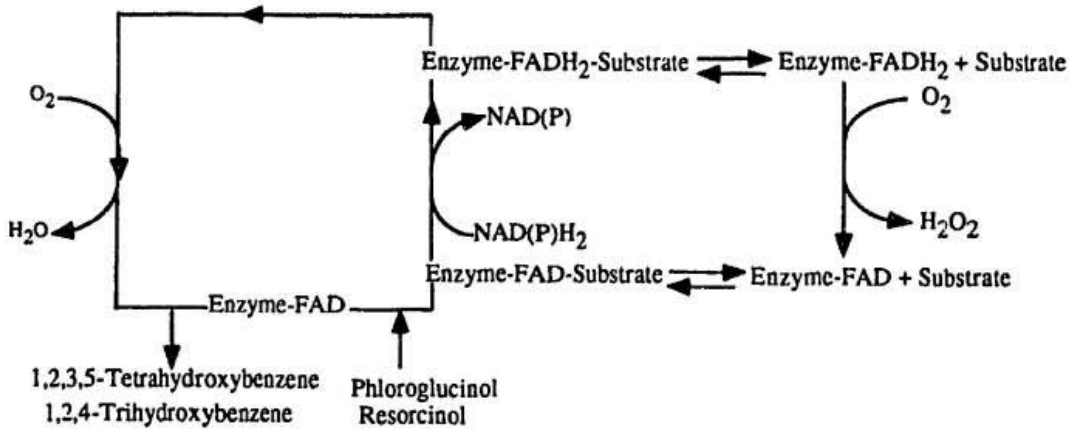


Fig. 6.1. Schematic diagram of the substrate/effector function of phenolic hydroxylases (modified from: Dagley, 1971).

It was shown that a non-inducible NAD(P)H 2-hydroxy-1,4-benzoquinone reductase converted the quinone back to 1,2,4-trihydroxybenzene. Catalase and SOD within the Rhodococcus sp. BPG-8 seemed to stabilize 1,2,4-trihydroxybenzene by the elimination of a possible free radical reaction with the superoxide radical produced during the formation of 2-hydroxy-1,4-benzoquinone. The crude cell-free extract had the same effect as adding catalase and SOD to the partially purified

cell-free extract.

Stabilization of the 1,2,4-trihydroxybenzene only to produce a product that would not be further metabolized would in essence create a situation no better that the quinone formation scenario. Rhodococcus sp. BPG-8 would deplete both energy and carbon, with the inevitable production of the daughter quinone and superoxide radical.

It was shown that Rhodococcus sp. BPG-8 may grow on 1,2,4-trihydroxybenzene, and thus may have enzymatic capacity to utilize such a substrate. The slow conversion of 1,2,4-trihydroxybenzene to a product that resembles the meta-cleavage product 2,4-dihydroxy-6-oxo-2,4-hexadienoic acid may reflect a constitutive activity for 1,2,4-trihydroxybenzene. It has been shown that 2,4-dihydroxy-6-oxo-2,4-hexadienoic acid may be converted into acetopyruvate and subsequently acetate and pyruvate during the mineralization of PG. Albeit the slow conversion of 1,2,4-trihydroxybenzene into 2,4-dihydroxy-6-oxo-2,4-hexadienoic acid allows the PG induced, resorcinol fed Rhodococcus sp. BPG-8 to fortuitously utilize resorcinol through the PG pathway. However, this is quite an expensive energy expenditure because the slower the conversion of 1,2,4-trihydroxybenzene to 2,4-dihydroxy-6-oxo-2,4-hexadienoic acid, the more NAD(P)H must be utilized to stabilize the 1,2,4-trihydroxybenzene. This is reflected in the low growth yields for resorcinol as opposed to PG fed Rhodococcus sp. BPG-8.

The conversion of PG to 1,2,3,5-THB also presents possible problems for the Rhodococcus sp. BPG-8. The 1,2,3,5-THB also has the tendency to form various quinones with the subsequent production of superoxide radicals. The production of a possible 2,6-dihydroxy-1,4-benzoquinone specific reductase was not found suggesting that conversion back to 1,2,3,5-THB may not be possible. The effect of catalase and SOD seemed to have some effect on the oxidation rate of 1,2,3,5-THB to a possible 2,6-dihydroxy-1,4-benzoquinon, thus appearing to minimally stabilize the 1,2,3,5-THB. The 1,2,3,5-THB was relatively more unstable as compared to the 1,2,4-trihydroxybenzene. Perhaps the increased production of superoxide radicals during 1,2,3,5-THB oxidation swamped the SOD and catalase protective mechanisms. This would, in essence, stimulate the free radical chain reaction between 1,2,3,5-THB and the superoxide radical.

The protective mechanisms employed to stabilize 1,2,3,5-THB in contrast to 1,2,4-trihydroxybenzene did not seem to be as critical. When 1,2,3,5-THB was added to the crude cell-free extract, cleavage to 2,4-dihydroxy-6-oxo-2,4-hexadienoic acid occurred with such high efficiency that the production of the oxidized product was never detected. This suggests that PG may be the preferred substrate for hydroxylation, while resorcinol may be another substrate PG hydroxylase utilizes.

The application of catalase and SOD seemed to stabilize both the PG and resorcinol hydroxylases. Stadtman (1992)

suggests that enzyme degradation follows a two-step mechanism in which the enzyme is oxidized to a catalytically inactive form by H_2O_2 or ferric ions, which make it more amenable to intracellular proteases (Fig.6.2). He suggests that MFO and MCO systems are involve in the production of H_2O_2 and ferric ions. It appears that introduction of catalase and SOD may not only stabilize the polyhydroxylated intermediates such as 1,2,3,5-THB and 1,2,4-trihydroxybenzene, but also help prevent the oxidation and subsequent proteolysis of the BPG-8 PG and resorcinol hydroxylase.

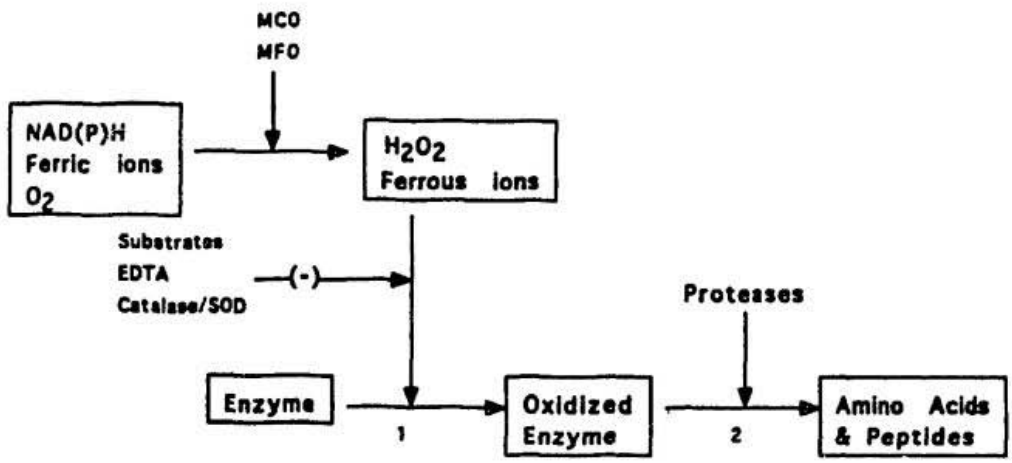


Fig. 6.2. The two-step mechanism of enzyme degradation (Reproduced with permission from American Academy for the Advancement of Science, Stadtman, 1992).

The production of superoxide radicals by the polyphenols 1,2,3,5-THB and 1,2,4-trihydroxybenzene suggests possible pharmalogical applications. It would be interesting to see if 1,2,3,5-THB would have anti-viral applications during in vitro and in vivo studies. Similiar phloroglucinol derivatives have been implicated as having anti-viral and even anti-tumor function as outlined in chapter 1. I would speculate that such a function is a product of superoxide production with its associate deleterious affects on cellular material. Perhaps viral infected cells or tumor cells, as compared to normal cells, have weakened protective mechanisms against superoxide generating molecule such as 1,2,3,5-THB. It would be interesting to test this hypothesis.

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