

BIODEGRADATION OF PHLOROGLUCINOL  
AND GALLIC ACID BY THE SOIL FUNGUS  
PENICILLIUM SIMPLICISSIMUM

CENTRE FOR NEWFOUNDLAND STUDIES

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NUZHAT S. HAMEED









BIODEGRADATION OF PHLOROGLUCINOL AND GALLIC ACID

BY

THE SOIL FUNGUS PENICILLIUM SIMPLICISSIMUM

BY,

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## ABSTRACT

Studies were undertaken to define the biodegradative pathway of aromatic compounds namely phloroglucinol and gallic acid by the soil fungus Penicillium simplicissimum. Optimal growth conditions were determined for the utilization of phloroglucinol and gallic acid as sole sources of carbon. P. simplicissimum grew best at pH 5.5 and the optimal temperature was about 27-29°C. The optimal concentration of substrate was 1.5 % (w/v) in the case of phloroglucinol and 0.2 % (w/v) for gallic acid.

The metabolic pathways of these aromatic compounds were studied in crude extracts from mycelia grown on the respective substrates. P. simplicissimum mycelia used for enzymatic studies were 24 hours and 30 hours old. The biodegradation of phloroglucinol was initiated by phloroglucinol reductase (PG-R), an  $\text{NADPH}+\text{H}^+$  dependent enzyme, forming dihydrophloroglucinol as the first intermediate. The second intermediate detected and isolated in crude extract was resorcinol and the third one, possibly a ring cleavage substrate, was 1,2,4-benzenetriol. This intermediate was detected and isolated from the culture medium containing phloroglucinol as a substrate. The enzymes responsible for ring cleavage, catechol 1,2 oxygenase and catechol 2,3 oxygenase, were found to be present in the crude extract.

The utilization of gallic acid followed a different metabolic pathway. The initial step was catalyzed by gallic acid reductase (GA-R) in the presence of  $\text{NADPH}+\text{H}^+$ . The ring fission enzymes, catechol 1,2 oxygenase and catechol 2,3 oxygenase, were again found to be present in the crude extract. Catechol 1,2

oxygenase was more active and stable than catechol 2,3 oxygenase. The possible substrate for the oxygenases was 1,2,4-benzenetriol which was detected and isolated from the culture medium. The product of ring cleavage was shown to be maleylacetate by the enzymatic breakdown with maleylacetate reductase in the presence of reduced NADPH<sup>+</sup>. The end product of enzymatic reduction of maleylacetate was  $\beta$ -ketoadipate, which was detected and isolated from the reaction mixture containing maleylacetate, NADPH+H<sup>+</sup> and maleylacetate reductase.

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# Chapter 1

## INTRODUCTION

### 1.1. General Introduction.

Biodegradation refers to the ability of an organism to catabolise or biochemically modify substances or compounds into smaller metabolites for its own metabolism. Many biogeochemical cycles maintain the life on earth through continuous cyclic operation and, to keep these cycles turning, energy is provided by solar radiation. By the process of oxygenic photosynthesis, autotrophic organisms harness the solar radiation and convert simple inorganic chemicals into compounds of high energy content and low thermodynamic stability. These compounds in turn help to synthesize molecules that are required for the production of biomass, which then enters the carbon-oxygen cycle. Ultimately living organisms die and the carbon-oxygen that had been temporarily sequestered into organic molecules are mineralized again to the level of simple inorganic substances.

It is generally accepted that all biosynthetic products are subjected to microbial degradation. Most biodegradation occurs as an unnoticed process in and on soil, in the grasslands and in the water of rivers, lakes and oceans where vast quantities of plant and animal wastes and the dead bodies of both macroscopic

and microscopic organisms are broken down to simple molecules. Some of the organic waste which may produce toxic effects in the environment, also enters the biogeochemical cycles.

### 1.1.1. Role of Microbes in Biogeochemical Cycles.

Microorganisms play a key role in biogeochemical cycles possessing a metabolic potential of impressive versatility for the breakdown of a wide variety of natural and xenobiotic products. In the course of this century, microorganisms have been made to face the novel challenge of thousands of new man-made chemicals which have been released into the environment, either deliberately via agricultural applications or through industrial and domestic wastes. These synthetic chemicals include pesticides, herbicides, detergents and coolants and their number is ever expanding. For almost a century studies about microbial biodegradation of aromatic compounds have been carried out. Biochemical assets of microbes lie in their ability to catalyze initial steps in the degradation of aromatic and polyaromatic compounds which other organisms cannot accomplish and to form metabolites that can enter the common pathways of metabolism.

### 1.1.2. Sources of Aromatic Compounds.

All possible chemical compounds found in the environment can be classified into two categories. One, in which the compounds are added by the man, is synthetic and xenobiotic. These compounds are often produced by industrial processes involving synthetic chemicals. Their production does not involve any natural enzymatic process. They bear little structural relationship to naturally occurring compounds. The second category contains the natural products which

are not man made. These compounds may be the products of living organisms or may be formed by other natural processes (Hutzinger and Veerkamp, 1981). These naturally occurring compounds become the components of biomass occurring in some environmental compartments.

The recycling of various biologically important elements is of prime importance. Vast quantities of insoluble aromatic macromolecules biodegrade very slowly (Evans, 1977). Members of the plant kingdom synthesize great quantities of natural products that are biochemically inert and are degraded by microbial enzymes. Most of these products are benzene derivatives, which are chemically stable and inert. If these are not degraded by soil microbes, vast quantities of carbon locked up in stable rings of six carbon atoms would be taken out of circulation when plants die (Dagley, 1971). It is true that large amounts of rather inert non-aromatic biochemicals are also synthesized by plants that also enter the carbon cycle through the action of microbes which initiate their metabolic degradation. For this reason the process of biodegradation has universal significance.

## **1.2. Metabolism of Aromatic Compounds by Microbes.**

For the obligatory maintenance of biochemical cycles, micro-organisms play an important role in nature. Many catabolic pathways have been suggested for the degradation of simple aromatic compounds by micro-organisms in soil.

### 1.2.1. Aerobic Metabolism.

It is about thirty years since the salient chemical features of the aerobic pathways of bacterial aromatic ring metabolism were elucidated (Hayaishi and Hashimoto, 1950). These aerobic pathways are initiated by microbial mono and di-oxygenases (Hayaishi, 1964). Natural selection among micro-organisms has been explored to convert stable aromatic structures into useful metabolites. Benzene was the first aromatic hydrocarbon tested for its utilization by bacteria (Stormer, 1908). Sohngen (1913) reported the utilization of benzene and other aromatic hydrocarbons by bacteria. Subsequently Wieland *et al.*, (1958) isolated strains of Nocardia corallina that were capable of oxidizing benzene to catechol. Ayenger *et al.* (1959) suggested the oxidation of benzene into catechol instead of phenol, as was assumed earlier. Claus and Walker, (1964) isolated a Pseudomonas sp. and an Achromobacter sp. that utilized toluene as the sole source of carbon and energy. The cells grown on toluene were able to oxidize toluene, benzene, catechol and 3-methylcatechol (Nozaka and Kusunose, 1969).

Polycyclic aromatic compounds were also investigated for their bacterial degradation (Strawinski and Stone, 1943). The bacterial oxidation of aromatics involves the formation of dihydrodiol intermediates, for example the oxidation of naphthalene by the bacteria utilizes both atoms of oxygen which are incorporated into a dihydrodiol. The available evidence suggests that bacteria utilize a dioxygenase reaction to initiate the degradation of aromatics. Further oxidation of dihydrodiols is catalyzed by dehydrogenases (Treccani *et al.*, 1954). Certain bacteria are also capable of growth on polyaromatics such as phenanthrene and anthracene (Strawinski and Stone, 1943).

There are limited reports on the degradation of aromatic hydrocarbons by fungi. Many fungal species have been reported for to utilize aromatic compounds. Studies have shown that the aryl-oxidative enzymes of fungi are similar to those found in mammalian liver (Auret *et al.*, 1971; Jerina and Daly, 1974). Enzymes found in fungi and mammalian liver have broad specificity with respect to substrates. Studies over the past several years have shown that fungi are remarkably similar to mammals in terms of their ability to oxidize aromatic hydrocarbons and related compounds. Smith and Rosazza (1974) suggested that fungi may serve as a valuable model for studies about mammalian metabolism. Gibson and Subramanian (1984) described fungi that oxidized carcinogenic hydrocarbons to metabolites which gave spectra similar to metabolites that are formed in mammals. Thus fungi provide a model system for reactions in higher organisms and can also provide vital information on the fate and reactivity of these molecules in higher organisms.

Reports about the oxidation of aromatic compounds by algae are limited and only a few compounds like catechol and phenol have been tested. Gibson and Subramanian, (1984) reported that aromatic hydrocarbons appear to inhibit the growth and photosynthetic activity of most algae. He also noted that algal metabolism does occur at very low substrate concentrations of aromatic hydrocarbons, but the oxygenated metabolites formed were often more toxic than the parent compounds (Gibson and Subramanian, 1984).

The mechanism of utilization of aromatic compounds by higher organisms is generally accepted to be through the cytochrome P450 enzyme system. The arene

epoxides formed by the microsomal enzymes can isomerize to phenols or undergo enzymatic hydration by epoxide hydratase to yield dihydrodiols in which the hydroxyl groups have trans relative stereochemistry. Further metabolism of trans-dihydrodiols results in the formation of catechol and together with other hydroxylated products these can undergo conjugative reactions with sulphate and glucuronate to form secondary metabolites (Daly *et al.*, 1972; Jerina and Daly, 1974). These secondary metabolites are eliminated in urine as detoxified compounds.

### 1.2.2. Anaerobic Metabolism.

The conventional view has been that most primitive organisms are anaerobic heterotrophs. Tarvin and Buswell (1934) reported ring fission of aromatic compounds by microorganisms in the absence of oxygen. They showed that carbon dioxide and methane were produced when benzoate, phenylacetate, phenylpropionate and cinnamate were completely utilized by a sewage-sludge inoculum in strictly anaerobic conditions. In anaerobic conditions, oxygenative ring-cleavage is prohibited. The only option available to an anaerobic organism is to metabolize aromatic compounds by hydrogenation (Evans, 1977). Hydrogen saturates the benzene nucleus and subsequently hydrolytic enzymes cleave the ring non-oxidatively (Guyer and Hegeman, 1969; Whittle *et al.*, 1976). It has been established, that under anaerobic conditions, aromatic substrates are cleaved by a remarkable biochemical device of ring reduction where no participation of oxygen exists (Dutton and Evans, 1968).



### Photosynthetic Bacteria.

Several species of the purple non-sulphur bacteria like, Rhodospseudomonas palustris (Dutton and Evans, 1969), Rhodospirillum rubrum (Pfennig *et al.*, 1965), and Rhodocyclus purpureus (Pfennig, 1978) are able to grow at the expense of simple aromatic compounds as sole carbon sources both anaerobically in the light by photosynthetic means and aerobically in the dark by respiration (Proctor and Scher, 1960). Dutton and Evans (1967) reported that Rhodospseudomonas palustris grown photosynthetically on benzoate or hydroxybenzoate showed no respiration under aerobic conditions with these substrates. Furthermore, this organism lacked enzymes of aerobic pathways when grown photosynthetically on benzoate and hydroxybenzoate. An inhibitory effect of oxygen and an obligatory requirement of light for the photometabolism of substrate was also reported.

### Oxidation by Nitrate Reducers.

A bacterial culture containing two different organisms from soil, which could utilize a variety of aromatic substrates in combination but not separately in the obligatory presence of nitrates under anaerobic conditions, was described by Oshima (1965). In such anaerobic cleavage of the aromatic ring, the oxygen atoms of nitrate were used in a manner similar to that of molecular oxygen. Williams and Evans (1973; 1975) isolated Moraxella cultures which metabolized benzoate anaerobically in the presence of nitrate which was reduced to nitrogen gas. Bakker (1977) reported a mixed bacterial culture which degraded phenol

under anaerobic conditions in a nitrate-mineral salt medium. A liquid culture of Gram-negative bacteria and Spirillum utilized benzoate, monohydroxybenzoate, protocatechuate and cresols under similar conditions producing carbon dioxide.

#### **Oxidation by Methanogens.**

The formation of methane and carbon dioxide from aromatic natural products was the first observation to be made involving the biological destruction of benzenoid structures under strictly anaerobic conditions. The reaction can occur in the absence of nitrate, sulphate and light through the action of an adapted microbial community (Tarvin and Buswell, 1934). Mixed cultures of methanogens which ferment aromatic compounds into methane and carbon dioxide, utilize the activity of gram-negative organisms to reduce the benzene ring which is followed by ring cleavage to form aliphatic acids. These acids are then converted to suitable substrates for various methane bacteria to complete the process.

### **1.3. Key Enzymes Involved in the Biodegradation of Aromatic Compounds.**

Knowledge of general mechanisms of benzene ring degradation by microorganisms has led to an understanding of the mechanisms employed by microorganisms to degrade benzene rings in aromatic compounds. The simplest hydrocarbons like benzene and monosubstituted derivatives of benzene such as toluene, offer microorganisms a choice for initial enzymatic attack through the aromatic ring or the methyl group. However, oxygenation of the ring seems to be

the most common mechanism. The metabolism of toluene and isopropyl benzene by Pseudomonas putida showed that many aromatic compounds undergo enzymatic hydroxylation of the aromatic ring in preference to degradation of the aliphatic side chain (Gibson, 1977). Initial steps in the oxidation of aromatic and polyaromatic hydrocarbons involve di-oxygenases in the case of procaryotes and mono-oxygenases in the case of eucaryotes.

### Di-oxygenases.

Di-oxygenases are responsible for incorporating both atoms of molecular oxygen into the substrate. Although the exact mechanism is not known, it is postulated that an intermediate such as dioxetane is formed initially and further reduction gives the dihydrodiols which have the relative stereochemistry of the *cis, cis* type, (Fig. 1-1). The dioxigenases involved in the formation of *cis*-dihydrodiols appear to be unique to procaryotic organisms.

### Mono-oxygenases.

The mono-oxygenases introduce only a single atom of molecular oxygen into the substrate to give an epoxide and the second atom of oxygen contributes to the formation of a molecule of water. Further transformation of epoxide is carried out by another enzyme called epoxide hydrase which forms a dihydrodiol with *trans* type stereochemistry (Fig. 1-1). The diols formed in both systems utilize dehydrogenases in the presence of  $\text{NADH} + \text{H}^+$  or  $\text{NADPH} + \text{H}^+$  and converge to form a common intermediate catechol, ready for ring cleavage.

*Trans*-dihydrodiols of aromatic compounds can be formed in bacteria, but probably only as anabolic intermediates (Young, 1984).

### 1.3.1. Ring Fission Reactions.

It is generally accepted that dihydroxylation is a prerequisite for enzymatic fission of the benzene ring. Either catechol or protocatechuic acid are formed as intermediates of several aromatic compounds. These hydroxylated intermediates are targets of ring fission enzymes. Generally monosubstituted aromatic substrates such as phenols, benzoic acid and mandelic acid are catabolized via catechol, while *para* disubstituted aromatic compounds such as parahydroxybenzoic acid, parahydroxymandelic acid and paracresol are degraded via protocatechuic acid, (Table 1-1). The two major pathways involved in ring fission are *ortho* and *meta* pathway systems. Both of these pathways are involved in aromatic hydrocarbon degradation (Gibson and Subramanian, 1984). Ring cleavage of initial metabolites occur by one of the two pathways depending on species and substrate.

#### Ortho-Pathway of Ring Fission.

The enzymes involved in the *ortho* pathway, cleave the ring structure between the hydroxyl groups (Hopper, 1977). Catechol and protocatechuic acid are metabolized either by the *ortho* pathway or by the *meta* pathway, although some organisms have the genetic capability to degrade these substrates by both pathways. When the organism has the capability of metabolizing a common intermediate by either *meta* or *ortho* pathway, the pathway used will be dependent primarily on the substrate used for the growth.

**Figure 1-1:** Generalized metabolic route to catechol from aromatic hydrocarbon in eucaryotic and procaryotic organisms. Gibson, 1972.

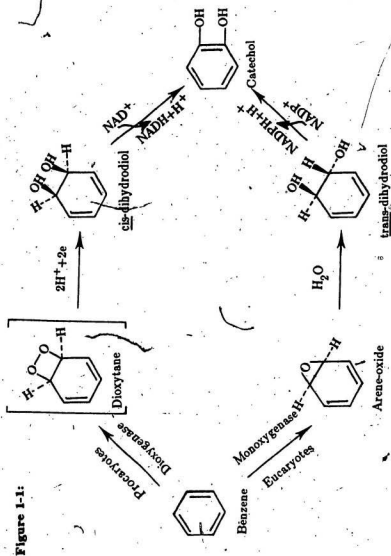


Table 1-1: Aromatic hydrocarbons showing catechol and protocatechuic acid as intermediates for ring cleavage. Gibson, 1977.

Compounds metabolized via	
Catechol	Protocatechuic acid
Phenol	m-Cresol
Benzene	p-Cresol
o-Cresol	Tyrosine
Tryptophan	Phthalic acid
Anthracene	p-Aminobenzoic acid
Naphthalene	p-Hydroxybenzoic acid
Phenylalanine	p-Hydroxymandelic acid
Benzoic acid	
Phenanthrene	
Salicylic acid	

Until the beginning of this decade, the most common type of ring cleavage known was shown to involve oxidative fission of the bond between carbon atoms bearing hydroxyl groups. Catechol and protocatechuic acid, both undergo cleavage to form *cis,cis* muconic acid (Hayaishi and Hashimoto, 1950; Evans *et al.*, 1951), and *cis,cis*  $\beta$ -carboxymuconic acid (MacDonald *et al.*, 1954; Gross *et al.*, 1956), respectively (Fig. 1-2). The enzymes involved in the ortho pathway are designated as catechol 1,2 oxygenases. Nozaki *et al.*, (1970) suggested an alternative nomenclature for ortho fission enzymes and called them intradiol dioxygenases.

#### Meta-Pathway of Ring Fission.

The enzymes utilized in the *meta* pathway are called catechol 2,3 oxygenases (Dagley, 1960) and metapyrocatechase (Kojima *et al.*, 1961). Nozaki *et al.*, (1970) named these enzymes as extradiol dioxygenases. The enzymes involved in *meta* fission cleave the ring between a carbon atom bearing a hydroxyl and an adjacent non-hydroxylated carbon atom (Hopper *et al.*, 1968). Substituted catechols are generally degraded via the *meta* pathway (Gibson and Subramanian, 1984). These oxygenases oxidize diols to semialdehydes rather than muconic acids. When *Pseudomonas* sp. was grown on *o*-cresol as sole carbon source, the oxidized product was  $\alpha$ -hydroxymuconic semialdehyde and the enzyme involved was catechol 2,3-dioxygenase which oxidized catechol (Dagley and Stopher, 1959) (Fig. 1-2). Growth of different *Pseudomonas* sp. on *p*-cresol induced the formation of the enzyme, protocatechuic acid-4,5-dioxygenase, which

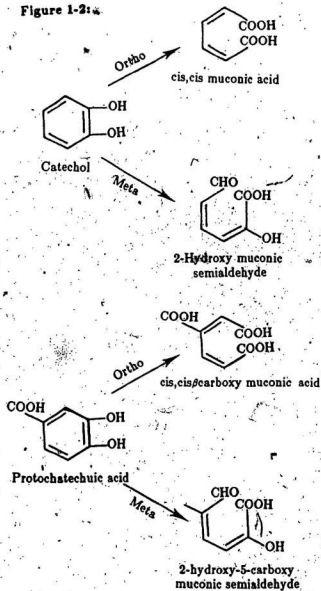


**Figure 1-2:** Postulated pathways of *ortho* and *meta* ring fission for catechol and protocatechuate.

Ortho ring fission:- (Hayaishi and Hashimoto, 1950; Evans, 1951; Macdonald *et al.*, 1954; Gross *et al.*, 1956).

Meta ring fission:- (Dagley and Stopher, 1959; Dagley *et al.*, 1963).

Figure 1-2:



oxidized protocatechuic acid to  $\alpha$ -hydroxy- $\gamma$ -carboxymuconic semialdehyde (Dagley *et al.*, 1964) (Fig. 1-2).

#### 1.4. Phloroglucinol.

Phenolic compounds like tannins, lignins and flavonoids of plant origin contain aromatic compounds such as anthocyanins, catechins, phloridzin and cholorés with complex structures. Phloroglucinol forms a part of such phenolic compounds and is released when these phenolic compounds are decomposed by microbes. Waage (1890) (cited from Robern, 1965) reported the synthesis of phloroglucinol from carbohydrates of leaves in the presence of sunlight and that phloroglucinol exists free in plants. Later tannins and complex molecules containing tannic acid extracted from the plants were shown to contain phloroglucinol, but free phloroglucinol could not be extracted (Moeller, 1897; Hartwich and Winckel, 1904). Presence of free phloroglucinol in soil, waste water and in spent gasses is the result of biological and chemical decomposition of more complex molecules such as phloridzin and tannins found in the soil. Chemical decomposition of crude oil or the breakdown of natural products such as coal yield phloroglucinol as one of the products (Pankhurst, 1959).

Besides its natural occurrence, phloroglucinol was also prepared synthetically by Jordan (1897). Phloroglucinol was synthesized from the reduction of trinitrobenzoic acid by Clark and Hartman (1929). Phloroglucinol acts as a tautomer and may exist in the ketonic and enolic forms depending on conditions of the reaction. The normal stability order of tautomers is reversed by the resonance stabilization of aromatic rings so that the enolic forms are favoured.

Phloroglucinol forms white rhombic crystals in nature, which have a sweet taste, sublimate upon decomposition, decolourize in light and have a melting point of 219°C. It is sparingly soluble in water and acts as a weak acid. Investigations on phloroglucinol revealed its economic importance. Phloroglucinol has been observed for its bactericidal and fungicidal properties due to its phenolic structure. It has been investigated for its pharmaceutical purposes as well as agricultural benefits. Phloroglucinol is mostly used in the printing, textile and dyeing industries and as a reagent for microscopy. It is also used in prevention of sludge formation, as an excellent decalcifier of bone suspension, and as an antimicrobial agent (Robern, 1965).

#### 1.4.1. Metabolism of Phloroglucinol by Microbes.

##### Aerobic Metabolism.

Utilization of phloroglucinol by microorganisms was first reported by Wagner (1914), who isolated bacteria from soil and faeces which were capable of utilizing phloroglucinol. He noted that the organisms capable of growing in a phloroglucinol medium could not assimilate benzene, resorcinol, hydroquinol and pyrogallol. Gray and Thornton (1928) isolated pure cultures of microorganisms from soil which were capable of utilizing phloroglucinol, resorcinol, cresol and other aromatic compounds as the sole carbon source. A partially purified enzyme of Brevibacterium fuscum oxidized phloroglucinol, resorcinol, para-, meta- and ortho-cresol, and orcinol only in the presence of  $\text{NADPH} + \text{H}^+$  (Nakagawa and Takeda, 1962). Further studies indicated that phenol was converted to  $\beta$ -ketoadipic acid by disruption of the ring. Several yeast strains of Candida

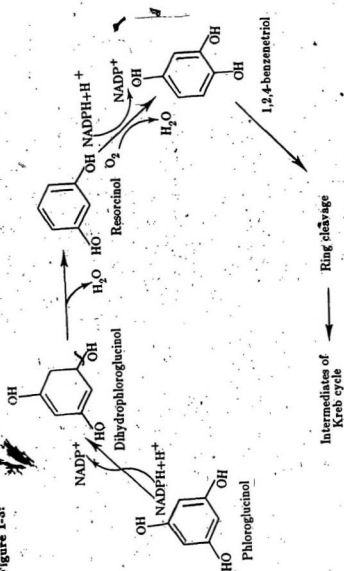
tropicalis and Debaromyces subglucosus were demonstrated to metabolize catechol and most of these had also the ability to metabolize phenols (Harris and Rickets, 1962).

Oxidation of phloroglucinol, resorcinol, catechol and pyrogallol by a fungus was reported by Higuchi (1953), who purified an enzyme from the mycelial mat of Coriolus hirsutus which had the capability to utilize these aromatic compounds. Aspergillus sp. was able to utilize 2,4, dihydroxybenzoic acid and resorcinol as sole carbon source (Halvorson, 1963). It was postulated that decarboxylation of 2,4, dihydroxybenzoic acid occurs prior to ring fission. Roberson (1965) isolated Penicillium sp. from soil that was able to utilize phloroglucinol as a sole carbon source. The metabolic pathway of phloroglucinol degradation by Penicillium sp. Mac M-47 was elucidated by Hang (1967), which involves the reduction of phloroglucinol into dihydro-phloroglucinol. Further dehydration of this intermediate produced resorcinol and substrate for ring cleavage. Utilization of phloroglucinol by isolated Penicillium sp. was demonstrated but no intermediates were detected. Subsequently, Mathur (1971) used Penicillium sp. Mac M-47 to study metabolic pathways and he proposed a theoretical pathway (Fig. 1-3).

All members of Athiorhodaceae examined utilize several aromatic acids photosynthetically but some can also metabolize phloroglucinol under anaerobic conditions. Using Rhodospseudomonas gelatinosa, dihydrophloroglucinol and 2-oxo-4-hydroxyadipate were detected in photosynthetic cultures growing on phloroglucinol as sole carbon source.

**Figure 1-3:** Proposed pathway for the biodegradation of phloroglucinol by Penicillium sp. Mac M-47. Mathur, 1971.

Figure 1-3:



## Anaerobic Metabolism.

Anaerobic photoassimilation of phloroglucinol by pure cultures of Rhodospseudomonas gelatinosa indicated that the degradation mechanism involves  $\text{NADPH} + \text{H}^+$ -dependent reduction to dihydrophloroglucinol followed by enol-ketone-tautomerization, saturation of the ring and subsequent hydrolytic cleavage of intermediates for further processing. Fermentative degradation of phloroglucinol was reported in pure cultures of Streptococcus bovis and Caprococcus sp. (Tsai and Jones, 1975; Tsai *et al.*, 1976).


### 1.5. Gallic Acid.

Aromatic acids, such as gallic acid also play an important role in the carbon-oxygen cycle. It forms a part of tannins, which are esters of sugars and phenolic acids or their derivatives. These tannins are present in condensed form and are hydrolysable in nature. Gallotannins on hydrolysis yield gallic acid as the only phenolic moiety. Gallic acid is usually obtained by alkaline or acidic hydrolysis of tannins from nutgalls. It is also synthesized by enzymatic hydrolysis using spent broths from Penicillium glaucum or Aspergillus niger which contain tannic acid. It decomposes at  $235\text{--}240^\circ\text{C}$  and in pure form exists as needles. It has very low systemic toxicity and causes mild local irritation in humans. Gallic acid is used in the manufacturing of gallic acid esters, anti-oxidants like propyl gallate, pyrogallol and ink dyes, as photographic developer, in tanning, in testing for free mineral acids, dihydroxy acetone and alkaloids. For therapeutic purposes gallic acid is used as an astringent and styptic agent.



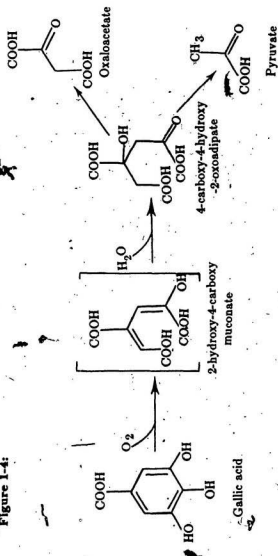
### 1.5.1. Metabolism of Gallic acid.

Gallic acid has mostly been used as a fermentable substrate (Schink and Pfennig, 1982). It has more importance as a precursor for the synthesis of pyrogallol than as a carbon source. Beveridge and Hugo (1965) studied the metabolism of gallic acid in Pseudomonas convexa X-1. Pseudomonas sp. was studied for metabolizing gallic acid into pyruvate with the consumption of oxygen and evolution of carbon dioxide (Dagley, 1971) (Fig. 1-4). Stanier and Ornston, (1973) utilized gallic acid for the elucidation of the  $\beta$ -ketoadipic acid pathway in Pseudomonas and Acinetobacter sp.



**Figure 1-4:** Biodegradation of gallic acid by bacteria.  
Dagley, 1971.

Figure 1-4:



## Objectives.

The main objectives of this research were:-

- i. To study the degradation of phloroglucinol and gallic acid by the soil fungus Penicillium simplicissimum.
- ii. To establish the optimal growth conditions under which these carbon sources were utilized by the organism.
- iii. To gain some information regarding enzymes involved in biodegradation of phloroglucinol and gallic acid.
- iv. To detect and characterize some of the enzymes involved in the metabolism of these substrates.
- v. To examine the metabolic pathway by detecting the intermediates produced in the biodegradation of phloroglucinol and gallic acid.

## Chapter 2

### MATERIALS AND METHODS

#### 2.1. Penicillium simplicissimum and its Culture Conditions.

Penicillium simplicissimum was collected from the southside hills of St. John's, Newfoundland and was isolated using an enrichment technique. Partial characterization and identification was done by Patel *et al.* (unpublished). Identification to the species level was performed at the Biosystematic Research Institute, National Identification Service, Ottawa.

The cultures of P. simplicissimum were maintained on potato-dextrose agar plates.

The organism was cultured in 2000-ml Erlenmeyer flasks containing 500 ml of minimal salt media (Roberts, 1965):

1. Stock solution - A.  $(\text{NH}_4)_2\text{SO}_4$ , 0.1g;  $\text{KH}_2\text{PO}_4$ , 0.5g;  $\text{K}_2\text{HPO}_4$ , 0.5g.
2. Stock solution - B.  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5g, in one litre of deionized water.
3. Stock solution - C.  $\text{FeCl}_3$ , 0.1g, in one litre deionized water.

The first two stock solutions were autoclaved separately and solution - C was filter sterilized. A litre of medium contained solution-A, 994-ml; solution-B, 5-ml; solution-C, 1-ml, which were added aseptically. The pH of the medium was adjusted to 5.5 using 0.1N HCl. The concentration of phloroglucinol in the medium was 1.5 % (w/v).

### 2.1.1. Preparation of Inoculum.

The inoculum was prepared in 1 litre of medium using a 100-ml spore suspension of P. simplicissimum in physiological saline containing 33% Triton X-100 (1-ml Triton X-100 + 2-ml saline). The flask was covered with aluminium foil to protect from light. The culture was incubated for 24 hours with constant shaking at 110-120 rpm in the dark at 27°C in a Psycrotherm incubator shaker (New Brunswick, N.J.).

### 2.2. Growth Conditions of the Organism.

The inoculum was used in proportion of one to ten (1:10) volumes of the media for further growth of the organism. The optimal growth conditions, pH, temperature, time of incubation and substrate concentration were determined separately.

#### 2.2.1. Effect of pH on Growth.

Two sets of nine flasks were prepared containing 100 ml of minimal salt media each. The pH was adjusted aseptically using either 1N HCl or 1N NaOH to 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. Phloroglucinol (1.5 g) was then added to each flask. Inoculum (10-ml) grown for 24 hours was added to each flask and incubated for 30 hours at 27°C in a Psycrotherm (New Brunswick, N.J.). The mycelia from each flask were collected by filtration through pre-weighed Whatman No. 1 paper, washed with deionized water and weighed. The filtered mycelia were transferred into clean 10-ml glass tubes and 2 ml of 1N NaOH was added to each tube, which was then immersed in boiling water for 30 minutes. The evaporated volume of 1N NaOH in the tubes was adjusted back to

2-ml using deionized water. These solutions were centrifuged at  $14,000 \times g$  for 15 minutes at  $4^{\circ}\text{C}$  in a Sorvall RC-5 Super Speed Centrifuge. The clear supernatant solution obtained was tested for protein content following the method of Lowry *et al.* (1951). The contents of the other set of culture flasks were filtered through pre-weighed Whatman No. 1 and washed with deionized water. The mycelia on the filter paper were dried at  $50^{\circ}\text{C}$  in a Precision oven (GCA corporation) until constant weight was achieved.

The effect of pH was also studied using gallic acid as a growth substrate and the procedure used was similar to the one outlined above.

#### 2.2.2. Effect of Temperature on Growth.

Eight flasks in two sets were prepared containing 100 ml of minimal salt media at pH 5.5. Phloroglucinol (1.5 g) was added to each flask. To each flask 10 ml of the prepared inoculum was added. For each temperature there were two flasks. Temperatures used were  $21^{\circ}\text{C}$ ,  $23^{\circ}\text{C}$ ,  $25^{\circ}\text{C}$ ,  $27^{\circ}\text{C}$ ,  $29^{\circ}\text{C}$ ,  $31^{\circ}\text{C}$  and  $33^{\circ}\text{C}$ . The flasks were incubated in an Orbit Environ-Shaker (Lab-Line Instruments Inc.) for 30 hours. Growth was determined by measuring the dry weight of filtered and washed mycelia from one flask. The second flask was used to determine the total protein by digesting the mycelia in 1N NaOH as described above.

The optimal temperature for growth of the organism utilizing gallic acid as a substrate was determined employing similar procedures.

### 2.2.3. Effect of Incubation Time on Growth.

A 10 ml of inoculum was added to two sets of ten flasks, each containing 100-ml minimal salt medium (pH 5.5), and 1.5g phloroglucinol. The flasks were incubated at 27°C in an Psycrotherm incubator shaker (New Brunswick, N.J.) with shaking (120 rpm) for different periods of time. The mycelia were collected from two flasks at a time starting from 12 hours of incubation to every 6 hours ending at 66 hours. Growth was determined by the dry weight of mycelia collected as well as by the total protein content. The procedure for determination of dry weight and protein contents were the same as described above.

The above experiment was repeated using gallic acid as substrate (0.2% w/v).

### 2.2.4. Effect of Substrate Concentration on Growth.

Effect of substrate concentration was studied in two sets of eight flasks containing 100 ml of minimal salt medium by inoculating with 10 ml of inoculum. The pH was adjusted to 5.5 with 1N NaOH. Different concentrations of phloroglucinol, 0.2%, 0.5%, 0.75%, 1.0%, 1.25%, 1.50%, 1.75%, and 2.0% (w/v) were added. All flasks were incubated in the dark for 30 hours in an Orbit Environ-Shaker at 27°C with constant shaking (120 rpm). Mycelia were collected from each flask. Mycelia from one flask were used to determine the dry weight and from the second flask to determine the total protein after digestion with 1N NaOH.

The experiment described above was also repeated to examine the effect of



gallic acid on the growth of P. simplicissimum. The concentrations of gallic acid used were 0.01%, 0.05%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5% and 0.6% (w/v), respectively.

### 2.3. Growth of the Organism on Different Substrates.

Capability of P. simplicissimum to utilise different organic substrates as sole carbon sources was determined by adding 0.5% of either phloroglucinol, orcinol, pyrogallol, gallic acid, hydroxyquinone, resorcinol, p-hydroxy benzoic acid, 1,2,4-benzenetriol, catechol or 2,4-dihydroxybenzoic acid to the culture medium. The inoculated flasks containing different substrates were incubated at 27°C for 30 hours as described above. The growth was determined by examining the cultures using the light microscope. The presence of extensive mycelia indicated growth.

### 2.4. Growth Curve.

Growth of P. simplicissimum utilizing phloroglucinol as the sole carbon source was determined in terms of total protein production. Flasks (44) were prepared each containing 50 ml of minimal salt medium and inoculated with 5 ml of the inoculum of (24 hours old) spore suspension. The flasks were incubated in the dark at 27°C with constant shaking (110-120 rpm) in an Orbit Environ-Shaker. After every 5 hours two flasks were taken out and the mycelia were filtered separately through Whatman No 1 filter paper. The first set of mycelia were washed with deionized water and stored in a small test tube at -20°C. The second set was washed with 0.1M phosphate buffer containing 1mM EDTA and 1mM 2ME, weighed and stored at -20°C. Similarly all the tubes were incubated for the desired time period and stored. The mycelia washed with deionized water

were used for total protein determination after digestion with 1N NaOH, as described previously. The mycelia washed with buffer were used for the preparation of the crude extract for enzyme assays. A plot correlating the growth with the enzyme activity was established.

The experiment was repeated using gallic acid as a growth substrate.

## **2.5. Preparation of Cell Free Crude Extract.**

The mycelia washed in 20mM potassium-phosphate buffer containing 1mM 2-mercaptoethanol (2ME) and 1mM EDTA was frozen in a pre-cooled X-Press (AB Biox. Nacka Sweden) chamber by immersing the whole cell assembly into a mixture of 60% ethanol and dry ice for 30 minutes. The frozen mycelia were subjected to hydraulic pressure of 12,000 lbs/sq inch using a Carver Laboratory Press - model C (Fred S. Carver Inc., USA). The recovered mycelial powder was suspended in 20mM phosphate buffer (pH 7.2), containing 1mM 2ME, 1mM EDTA and 15% glycerol. The proportion of mycelial powder and buffer was 1g of wet weight crushed mycelia and 2.5 ml of buffer. The suspension was centrifuged at 14,000 x g for 30 minutes in a Sorvall RC-3 Centrifuge. The pellet was discarded and the supernatant was used as an enzyme source.

### **2.5.1. Determination of Protein.**

The amount of protein present in cell free extracts or other solutions (alkaline digest of mycelia) was determined following the method of Lowry *et al.* (1951). Crystalline bovine serum albumin was used as a standard. The optical density of the coloured solutions was measured at 600 nm using a Shimadzu Spectronic UV-210 spectrophotometer.

### 2.5.2. Oxygen Uptake.

A Clark electrode was used to determine changes in oxygen concentration during enzymatic reaction. A circulating water bath was connected to the reaction vessel to maintain a constant temperature of 25°C. All solutions used were kept at 25°C. Oxygen consumption in the presence of three different substrates namely phloroglucinol, pyrogallol and resorcinol was measured by using crude extracts of P. simplicissimum.

### 2.6. Enzyme Assays.

The method of Mathur (1971)\* was employed to measure phloroglucinol reductase (PG-R) and resorcinol hydroxylase (R-H) activities. All enzymatic assays were run at room temperature in 3 ml of reaction mixture consisting of 2.7 ml of 0.1 M phosphate buffer (pH 7.2), containing 1 mM EDTA, 4 mM 2ME, 0.5  $\mu$ mole NADPH+H<sup>+</sup> or NADH+H<sup>+</sup>, 1.0  $\mu$ mole substrate and 0.2 ml of enzyme extract.

PG-R activity was determined by measuring the disappearance of NADPH in the reaction mixture at 340 nm using a Shimadzu UV-260 Recording Spectrophotometer. The reaction was initiated by the addition of phloroglucinol. The enzyme unit was defined as the amount of crude extract that oxidized 1  $\mu$ mole of NADPH+H<sup>+</sup> per min per ml under standard assay conditions. Specific activity was defined as enzyme units per mg protein.

R-H activity was determined spectrophotometrically by monitoring the disappearance of NADPH+H<sup>+</sup> in crude extracts. The reaction mixture contained 2.7-ml assay buffer, 1  $\mu$ mole of NADPH+H<sup>+</sup>, 0.2-ml enzyme and 1  $\mu$ mole

resorcinol. The decrease in optical density at 340 nm was monitored using a Gilford Spectrophotometer.

Catechol 1,2 oxygenase activity in the crude extract was determined by the method of Hayaishi (1964). The reaction mixture contained 2.8 ml of 0.01 M phosphate buffer pH (7.0), 0.05  $\mu$ mole catechol, 0.1  $\mu$ mole EDTA and 0.2 ml of crude extract. Oxygenase activity was determined by measuring the increase in optical density at 260 nm using a Shimadzu UV-260 Recording Spectrophotometer. One enzyme unit was defined as the amount of extract required to produce 1  $\mu$ mole of the product (*cis, cis* muconic acid) per min per ml. Specific activity was defined as enzyme units per milligram protein.

Catechol 2,3 oxygenase activity was measured by the method of Dagley and Stopher (1959). The reaction mixture contained 2.8 ml of 0.05 M phosphate buffer (pH 7.0), 0.1  $\mu$ mole catechol and 0.2 ml of crude extract. Activity was determined by measuring the increase in optical density at 375 nm using a Shimadzu UV-260 Recording Spectrophotometer. One enzyme unit was defined as the amount of extract producing 1.0  $\mu$ mole of the product ( $\alpha$ -hydroxymuconic semialdehyde) per min per ml. Specific activity was determined as enzyme units per milligram of protein.

Maleylacetate reductase activity was measured by the method of Gaal and Neujahr (1979). The reaction was carried out in a 1-ml volume containing 0.05 M Tris-HCl buffer (pH 7.6), 0.2  $\mu$ mole of maleylacetate, 0.2  $\mu$ mole of NADPH+H<sup>+</sup>, and 0.2 ml of crude extract. The enzymatic reduction of maleylacetate was

determined by measuring the decrease in optical density using a Shimadzu UV-260 Recording Spectrophotometer at 340 nm. One enzyme unit was defined as the amount of the extract oxidizing 1  $\mu$ mole of NADPH+H<sup>+</sup> per min per ml. The specific activity was defined as enzyme units per milligram protein.

## 2.7. Partial Purification of PG-R.

Crude extract (20-ml) was concentrated by filtering through a YM-10 membrane in a Diaflo Ultra-Filtration Cell-model 12 (Amnicon Corp., Lexington, Mass., USA), following the manufacturer's manual. The operation was carried out at 0-4°C under a flow of N<sub>2</sub> gas at 40 lbs/sq.in. pressure with constant stirring. The concentrated extract reduced to 3-ml was used for column chromatography.

### 2.7.1. Column Chromatography on Sephadex Gels.

Five grams of Sephadex G-200 was suspended with constant stirring in about 500 ml of 0.1 M phosphate buffer (pH 7.2), containing 1 mM of EDTA, 1 mM of 2ME, and 15% glycerol. The suspension was allowed to swell for 48 hours at 4°C and the buffer was changed 2-3 times in between by decantation. The slurry was then deaerated by using a freeze drying assembly and stored at 4°C. Degassed slurry was used to pack a column 30 cm x 2.5 cm. Small amounts of slurry were poured into the column and allowed to settle before adding more slurry. Concentrated enzyme extract (3-ml, 56 mg protein) was applied to the column and eluted using the above buffer. About 3-ml fractions were collected and the proteins in these fractions were determined by recording the absorption at 280 nm on Shimadzu Spectronic 210-UV (Bausch and Lomb).

### 2.7.2. Column Chromatography on DEAE-Sephadex.

DEAE-Sephadex A-50 was precycled and equilibrated following instructions by the manufacturer. Two grams of the exchanger gel were soaked in 200 ml of deionized water and allowed to swell for 24 hours. The supernatant containing the fine particles was decanted and the swollen gel was washed with 0.1 M potassium phosphate buffer (pH 7.2). The washed gel was resuspended in 0.1 N NaOH. After three changes in NaOH the gel was allowed to equilibrate. The gel was washed with 0.1 M phosphate buffer until the effluent reached pH 7.2. The gel was then washed in 0.1 N HCl followed by phosphate buffer washing until the pH was 7.2. Finally the treated gel was suspended in 200 ml of phosphate buffer and deaerated using a freeze drying assembly.

### 2.8. Enzyme Stabilization.

#### 2.8.1. Effect of Metal Ions on Enzyme Activity.

Ferric Chloride, Cobaltous Chloride, Zinc Sulphate, Calcium Chloride dihydrate, Ferrous Sulphate, Magnesium Sulphate and Ferrous Ammonium Sulphate were tested for their effect on PG-R. The concentration of metal ions in the reaction mixture was 1  $\mu$ mole per assay mixture of 3 ml.

#### 2.8.2. Effect of Buffers on PG-R Activity.

PG-R activity was tested using different buffers in standard assay reactions.

The buffers used were:-

- i. 0.1M potassium phosphate buffer (pH 7.2), containing 0.1mM EDTA, 20% glycerol and 0.6% (wt/vol) sodium cholate.

- ii. 0.1M potassium phosphate buffer (pH 7.2), containing mono and dibasic potassium phosphate.
- iii. Sorensen buffer. 0.1M sodium phosphate and 0.1M potassium phosphate (pH 7.2).
- iv. 0.1M potassium phosphate buffer (pH 7.2), containing 1 mM monobasic potassium phosphate 1 mM EDTA, 1 mM 2ME and 15% glycerol (Acharya, personal communication).
- v. 0.1M Tris HCl buffer (pH 7.2), containing 1 mM 2ME, 1 mM EDTA and 15% glycerol.
- vi. 0.1M Hepes buffer (pH 7.2), containing 1 mM 2ME, 1 mM EDTA and 15% glycerol.

### 2.8.3. Stability of PG-R at Storage Temperatures.

To test the stability of PG-R, freshly prepared crude extracts were incubated in ice for 24 hours and PG-R and R-H activities were monitored every hour.

Similarly freshly prepared crude extracts were frozen and kept at  $-20^{\circ}\text{C}$ . The extract was frozen in 0.6-ml aliquots in separate tubes. One tube was removed every hour and allowed to thaw before carrying out the assay for PG-R and R-H.

#### 2.8.4. Effect of Potassium Chloride on PG-R Activity.

Effect of KCl on PG-R was tested with an idea to use a KCl gradient for enzyme elution from an ion exchange column. Concentrations of 1, 2, 3, and 4  $\mu$ moles of KCl were used in enzyme assays. The PG-R activity was calculated by using the standard assay procedure.

The effect of potassium chloride on G-R was also tested by using freshly prepared crude extracts of the P. simplicissimum grown on gallic acid.

#### 2.8.5. Effect of Enzyme Concentration on PG-R Activity.

Different concentrations of freshly prepared crude extract were used to measure PG-R activity using standard assay conditions.

Similarly the experiment was repeated with the extract made from mycelia grown on gallic acid.

#### 2.8.6. Effect of Substrate Concentration on PG-R activity

Several concentrations of phloroglucinol, 1, 2, 3, 4 and 5  $\mu$ moles, were tested in standard assays for PG-R. The effect of substrate concentration on change in absorbance at 340 nm was noted using a Shimadzu UV-260 Recording Spectrophotometer. The enzyme units and specific activity were determined.

The same method was used to test the effect of different concentrations of the co-substrate, NADPH+H<sup>+</sup> on activity. The NADPH+H<sup>+</sup> concentration in the reaction mixture were 0.1, 0.2, 0.3, 0.4 and 0.5  $\mu$ moles.



## 2.9. Isolation and Detection of Metabolic Products.

The reaction products of phloroglucinol were detected, spectrophotometrically and by thin layer chromatography (TLC). Standards were prepared at a concentration of  $10\text{-}\mu\text{M}$  in anhydrous methanol, and usually  $20\text{ }\mu\text{l}$  were spotted on the plates.

### Preparation of samples for Spectrophotometry and Chromatography:-

Five litres of minimal salt medium containing 1.5% (w/v) of phloroglucinol were inoculated with preprepared inoculum. The flasks were incubated at  $27^{\circ}\text{C}$  in a Psycrotherm Incubator Shaker for 24 hours. Mycelia were filtered through Whatman No. 1 paper. A sample of 1 ml of filtrate was taken and scanned for maximum absorbance between 350 nm to 190 nm. A small amount of filtrate was also kept for TLC. The remaining filtrate was acidified with concentrated hydrochloric acid to pH 2.0 and was reduced in volume to 50-ml in a flash evaporator at  $35^{\circ}\text{C}$ . The concentrated filtrate was extracted with six volumes of ethyl ether. The ether fraction was evaporated to dryness and the residue was redissolved in 2 ml of methanol. This sample was tested for maximum absorbance from 350-190 nm and  $50\text{ }\mu\text{l}$  of the sample was also spotted on TLC plates. The spotted plates were developed in a solvent system (benzene, 45 : methanol, 8 : acetate, 4) (Randerath, 1963). The developed chromatograms were sprayed with colour developing reagents. The same method was used to make the samples of crude mycelial extract and of the incubated reaction mixture for spectrophotometry and chromatography. Organic solvent extracts were tested for maximum absorbance from 350-190 nm scanning and  $50\text{ }\mu\text{l}$  were also spotted on the TLC plate.

The intermediates produced in gallic acid metabolism were isolated and detected by ethyl ether extraction of five litres of culture medium which was prepared by inoculating five litres of medium containing 0.2% (w/v) of gallic acid with preprepared inoculum. The flasks were incubated for 30 hours at 27°C in a Psycrotherm Incubator Shaker. Mycelia were filtered through Whatman No. 1 paper. The filtrate (1-ml) was scanned for maximum absorbance between 350 nm to 190 nm. The remaining filtrate was treated the same way as outlined for phloroglucinol containing medium. The spectra were run for the maximum absorbance from 350-190 nm on a Shimadzu UV-260 Recording Spectrophotometer. Organic solvent extracts (50  $\mu$ l) were spotted on TLC plates and developed in the solvent system of benzene, 45 : methanol, 8 : acetic acid, 4 (Randerath, 1963). Developed chromatograms were sprayed with colour developing reagents.

#### 2.9.1. Solvent Systems used in Thin Layer Chromatography.

Several solvent systems and colour developing reagents were used for the resolution and detection of intermediates. All chromatograms were run at room temperature using the ascending technique in rectangular glass tanks. The atmosphere in the tanks was saturated by covering the walls with filter paper which was soaked with solvent by swirling the vessel before putting in the plates. Two kind of plates were used. One was paper back Kodak Chromatogram, 13181 Silica Gel with Fluorescent indicator (Eastman Kodak Company, N.Y.). The other was glass back chromatogram, Baker Si250F-PA Thin Layer Chromatography (J.T. Baker Chemical Co. Phillipsburg N.J.).

A

The chromatograms were prepared by applying 20  $\mu$ l of standard solutions prepared in anhydrous methanol and 50  $\mu$ l of samples on the starting line of the plates and allowing them to dry. The plates were then placed in chromatography tanks filled to a depth of 0.5 cm with the solvent. Plates were removed from the tank as soon as the solvent front travelled about 15 cm. The chromatograms were dried at room temperature. Solvents were of reagent grade and used without further purification.

The solvent systems used were as follows:

- i. Ethyl ether: Acetic acid: Water. To 13 parts of ethyl ether, 3 parts of acetic acid and 1 part of deionized water was added (v/v) (Denison and Pares, 1952).
- ii. Ethyl acetate: Acetic acid: Water. To 3 parts of ethyl acetate, 1 part of acetic acid and 1 part of deionized water was added (v/v) (Loffler and Reichl, 1953).
- iii. Benzene: Acetic acid: Water. Six parts of benzene was added to 7 parts of acetic acid and 3 parts of deionized water (v/v) (Griffith, 1957).
- iv. n-Butanol: Ethanol: Ammonium Hydroxide. To 7 parts of n-butanol, 1 part of 95% ethanol and 2 parts of 0.5 N ammonium hydroxide were added (v/v) (El Hawary *et al.*, 1953).

- v. Isopropanol: Water: Ammonium Hydroxide. To 20 parts of isopropanol 2 parts of deionized water and 1 part of concentrated ammonium hydroxide were added (v/v) (Smith and Smith, 1961).
- vi. Benzene: methanol: acetic acid. To 45 parts of benzene, 8 parts of methanol and 4 parts of acetic acid were added (v/v) (Randerath, 1963).
- vii. 95 % Ethanol: Conc. Ammonium Hydroxide. To 100 ml of 95 % ethanol 1 ml of conc. ammonium hydroxide was added (Kennedy and Barker, 1951).
- viii. Benzene: Dioxane: Acetic acid. To 90 parts of benzene 25 parts of dioxane and 4 parts of acetic acid were added (v/v) (Randerath, 1963).

The colour developing reagents used for the detection of the chromatograms

were:

- i. Tetrazotized benzidine (Randerath, 1963):- Solution A. 5 gm of benzidine was dissolved in 14 ml of concentrated hydrochloric acid and diluted with deionized water to 1000-ml. Solution B. Aqueous 10% sodium nitrite solution. Equal volumes of the two solutions were mixed just before use. After spraying, the plates were placed in a Precision Gravity Convection oven (GCA Corporation), at 105°C for a few minutes until the spots were clearly visible.
- ii. Vanillin toluene p-sulphonic acid (Roux and Maiss, 1960):- 2 gm vanillin and 1 gm toluene p-sulphonic acid were dissolved in 100 ml of absolute ethanol. After spraying, the chromatograms were placed in a Precision Gravity Convection oven, at 105°C for a few minutes until the spots were clearly visible.

iii. Bromocresol purple (Reid and Lederer, 1951):- 40 gm of bromocresol purple were dissolved in 100 ml of a 1:5 dilution of formalin in ethanol and the pH was adjusted to 5.0 with 0.1 N NaOH. After spraying, the chromatograms were dried in a Precision Gravity Convection oven at 105° C.

iv. Sucrose reagent (Roux, 1951):- 2 gm of sucrose were mixed with 10 ml of concentrated hydrochloric acid and 90 ml of absolute ethanol. Complete uniform solution was not achieved. The suspension was sprayed on the chromatograms.

## 2.10. Ultraviolet Absorption Spectrophotometry.

UV-absorption spectra were obtained using a Shimadzu UV-260 Recording Spectrophotometer and 3-ml cuvette with a 1-cm light path. The sample cuvette contained 2.5 ml of deionized water plus 0.5-ml sample. The reference cuvette contained 3 ml of deionized water. Authentic compounds were included as controls. Compounds isolated on TLC plates were extracted by ethyl ether before running their UV-spectra.

## 2.11. Enzymatic Synthesis of Maleylacetate.

Maleylacetate was prepared enzymatically by using crude extracts of Pseudomonas NCIB9816. The reaction mixture contained 150 enzyme units of catechol 1,2-oxygenase in 25 ml of 0.05 M potassium phosphate buffer (pH 6.9). Crystalline 1,2,4-Benzenetriol (66.5) was added in 3 mg portions over a period of 1 hour. The reaction mixture was kept at a temperature of 4°C with constant stirring. The pH of the reaction mixture was held between 6.5 to 6.9 with 1.0 N NaOH. When the reaction was completed, the pH was adjusted to 7.5 and the mixture was filtered through a Diaflo YM 10 membrane (Gaal and Neujahr,

1979). The filtrate was tested for maleylacetate reductase by the assay system described earlier.

## Chapter 3

# RESULTS

### 3.1. Growth Conditions of P. simplicissimum.

#### 3.1.1. Effect of pH on Growth.

Figure 3-1 illustrates the effect of pH on the growth of P. simplicissimum measured in terms of total dry weight per flask and total protein. The organism grown on 1.5% phloroglucinol as substrate, showed two peaks of optimal growth at pH 5.5 and 7.0.

When the organism was grown on 0.2% gallic acid at pH 5.5 the maximum dry weight recovered was 175 mg and a protein content of 280  $\mu$ g. At pH 7.0 the yield was 175 mg and the protein was 148  $\mu$ g (Fig. 3-2). Thus the organism showed two optimal growth peaks at pH 5.5 and 7.0.

#### 3.1.2. Effect of Temperature on the Growth of P. simplicissimum.

Effect of temperature on growth of the organism was calculated in terms of dry weight and the amount of protein produced in each flask. Maximum growth occurred at 27° C as illustrated in figure 3-3. Similarly when the organism was grown on gallic acid, optimal growth took place at 27° C.

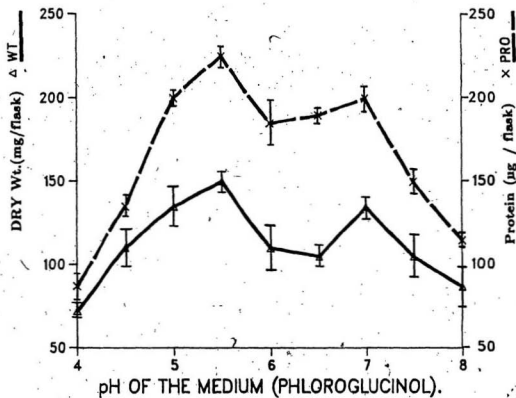
**Figure 3-1:** Effect of pH on the growth of P. simplicissimum, utilizing phloroglucinol.

P. simplicissimum was grown in 500-ml Erlenmeyer flasks containing 100 ml of minimal salt medium (of different pH) with 1.5 gm of phloroglucinol. The flasks were incubated at 27°C for 30 hours. Growth was determined per flask in terms of dry weight (mg) mycelia recovered and total protein ( $\mu$ g) recovered by alkali digestion.

(means and standard deviations, n=4).



Figure 3-1:

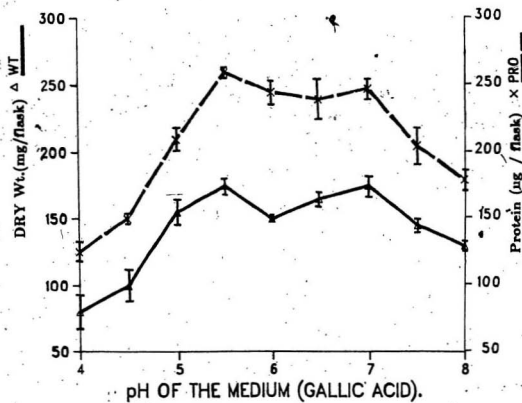


**Figure 3-2:** Effect of pH on growth of P. simplicissimum, utilizing gallic acid.

P. simplicissimum was grown in 500-ml Erlenmeyer flasks containing 100 ml of minimal salt medium (of different pH) with 0.2 gm of gallic acid. The flasks were incubated at 27°C for 30 hours. Growth was determined per flask in terms dry weight (mg) of mycelia recovered and total protein ( $\mu$ g) recovered by alkali digestion.

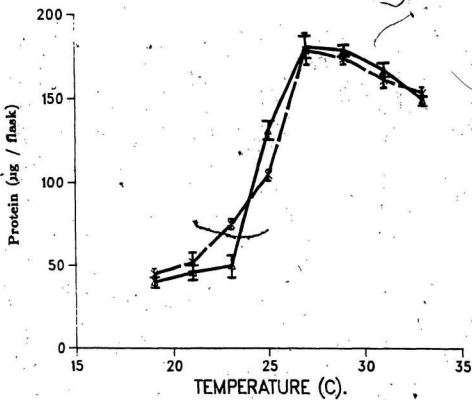
(means and standard deviations, n=4).

Figure 3-2:



**Figure 3-3:** Effect of temperature on growth of P. simplicissimum. P. simplicissimum was grown in 500-ml Erlenmeyer flasks containing 100 ml of minimal-salt medium (of different pH) with 1.5 gm of phloroglucinol and 0.2 gm gallic acid. The flasks were incubated at different temperatures for 30 hours. Growth was determined in terms of total protein ( $\mu$ g) recovered by alkali digestion. (means and standard deviations, n=4).

Figure 3-3:



Legend

△ PROPG× PROG

### 3.1.3. Effect of Substrate Concentration on the Growth of P. simplicissimum.

When grown on different concentrations of the substrate, maximum dry weight recovered was 110 mg at 1.5 % of phloroglucinol concentration and the protein amount was 147  $\mu$ g (Fig. 3-4). In the case of gallic acid utilization the maximum dry weight and protein contents were 126 mg and 149  $\mu$ g, respectively, when the concentration was 0.2 % (Fig. 3-5).

### 3.2. Utilization of Substrates.

P. simplicissimum utilized phloroglucinol, gallic acid, 1,2,4 benzenetriol, para-hydroxybenzoic acid and 2,4-dihydroxy benzoic acid as sole sources of carbon and energy (Table 3-1).

### 3.3. Growth Curve.

P. simplicissimum grown on 1.5% phloroglucinol showed three distinct phases namely lag, logarithmic and stationary phase. The lag phase remained for 18 hours from the time of inoculation. The logarithmic state lasted for 24 hours ending at about 40 hours of incubation. The stationary phase continued for almost 60 hours. The maximum PG-R activity detected was about 0.49 enzyme units/ml after 27 hours of incubation (Fig. 3-6). PG-R activity showed a rapid decline after 40 hours of incubation.

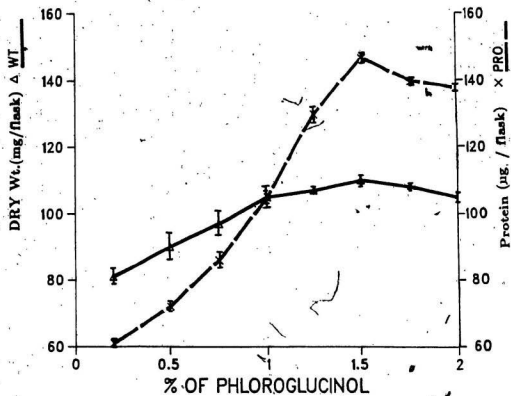
The growth curve for P. simplicissimum utilizing 0.2% of gallic acid also showed a lag phase of about 10 hours and the logarithmic phase lasted till the 65th hour. G-R activity was maximum (0.701 enzyme units per ml) during the

**Figure 3-4:** Effect of phloroglucinol concentration on growth of P. simplicissimum.

P. simplicissimum was grown in 500-ml Erlenmeyer flasks containing 100 ml of minimal salt medium with different concentrations of phloroglucinol. The flasks were incubated at 27°C for 30 hours. Growth was determined in terms of dry weight (mg) of mycelia recovered and total protein ( $\mu$ g) recovered by alkali digestion.

(means and standard deviations, n=4).

Figure 3-4:





**Figure 3-5:** Effect of gallic acid concentration on growth of P. simplicissimum.

P. simplicissimum was grown in 500-ml Erlenmeyer flasks containing 100 ml of minimal salt medium of with different concentrations of gallic acid. The flasks were incubated at 27°C for 30 hours. Growth was determined in terms of dry weight (mg) of mycelia recovered and total protein ( $\mu$ g) recovered by alkali digestion.  
(means and standard deviations, n=4).

Figure 3-5:

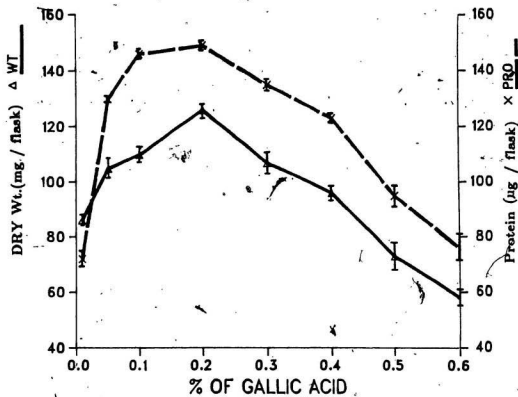


Table 3-1: Growth of P. simplicissimum on various substrates.

No.	Substrate	Growth
1.	Ofetinol	-
2.	Catechol	-
3.	Pyrogallol	-
4.	Resorcinol	-
5.	Gallic acid	+
6.	Phloroglucinol	+
7.	Hydroxyquinone	-
8.	1,2,4-Benzenetriol	+
9.	p-Hydroxybenzoic acid	+
10.	2,4-Dihydroxybenzoic acid	+

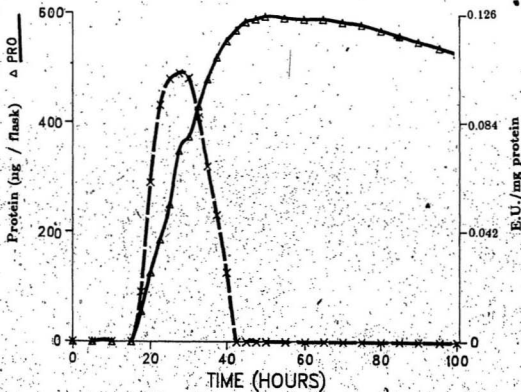
Growth is indicated by + and absence by - symbol.

0.5% of all the above mentioned substrates were added to the culture medium of P. simplicissimum and incubated at 27° C for 30 hours. Growth was determined by the presence of extensive mycelia.

Figure 3-6: Growth of P. simplicissimum utilizing phloroglucinol and the levels of PG-R activity.

P. simplicissimum was grown in 500-ml Erlenmeyer flasks containing 100-ml of minimal salt medium with 1.5 gm of phloroglucinol. The flasks were incubated at 27°C for different time intervals. Growth was determined in terms of total protein ( $\mu$ g) recovered by alkali digestion. Enzyme activity was determined by using crude extracts of mycelia. Enzyme assays were performed under standard conditions.

Figure 3-8:



late logarithmic phase at 36-40 hours of incubation. This activity declined with increase in incubation time (Fig. 3-7).

### 3.4. Detection of Different Enzymes in the Crude Extract of P. simplicissimum.

The supernatant solution recovered after extraction of mycelia grown on phloroglucinol was analyzed for protein concentration as well as enzyme activities. The four enzymes detected in the crude extract are shown in the table 3-2. The relative activities of PG-R, catechol 1,2 oxygenase and catechol 2,3 oxygenase were easily measurable, whereas that of resorcinol hydroxylase was present only in trace amounts.

The crude extract of mycelia grown on gallic acid showed GA-R, catechol 1,2 oxygenase, catechol 2,3 oxygenase and maleylacetate reductase activities (Table 3-8).

### 3.5. Enzymes Involved in Phloroglucinol Metabolism.

PG-R specific activity was 0.145 E.U./mg protein in the extract. R-H activity was negligible in crude extracts of the organism grown on phloroglucinol. Specific activity recovered was 0.005 E.U./mg protein in the presence of NADPH+H<sup>+</sup>. When NADH+H<sup>+</sup> was tested for the reduction of substrate, no activity was found. Catechol 1,2 oxygenase was active in freshly prepared crude extracts and the specific activity was 0.097 E.U./mg protein. Catechol 2,3 oxygenase had a specific activity of 0.082 E.U./mg protein (Table 3-2).

**Figure 3-7:** Growth of P. simplicissimum utilizing gallic acid, and the levels of QA-R activity.

P. simplicissimum was grown in 500-ml Erlenmeyer flasks containing 100 ml of minimal salt medium with 0.2 gm of gallic acid. The flasks were incubated at 27°C for different time intervals. Growth was determined in terms of total protein ( $\mu$ g) recovered by alkali digestion. Enzyme activity was determined by using crude extracts of mycelia. Enzyme assays were performed under standard conditions.

Figure 3-7:

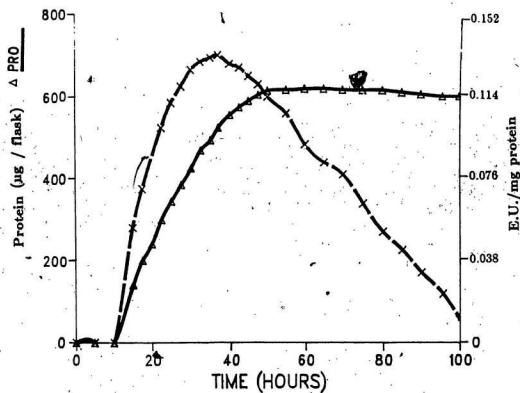




Table 3-2: Levels of enzymes involved in cells grown on phloroglucinol.

No.	Enzyme	Specific Activity E.U./mg.protein
1.	PG-R	0.145±0.04
2.	R-H	0.005±0.01
3.	Catechol 1,2 oxygenase	0.097±0.02
4.	Catechol 2,3 oxygenase	0.082±0.03

One enzyme unit in the case of PG-R and R-H was defined as the amount of extract required to oxidize 1  $\mu$ mole of NADPH+H<sup>+</sup> per min per ml under standard assay conditions. In the case of oxygenases one enzyme unit was defined as the amount of extract required to consume 1  $\mu$ mole of oxygen per min under standard assay conditions. Specific activity was defined as enzyme units per mg of protein. Enzyme assays were performed on freshly prepared crude extract of mycelia.

### **3.6. Properties of Phloroglucinol Reductase (PG-R).**

#### **3.6.1. Effect of Enzyme Concentration on PG-R.**

The PG-R activity was dependent on the concentration of extract in the reaction mixture as indicated in figure 3-8. It reached a plateau when the protein concentration in the reaction mixture was about 1.7 mg.

#### **3.6.2. Effect of Substrate Concentration on PG-R Activity.**

Figure 3-9 illustrates the effect of phloroglucinol concentration on the rate of the substrate utilization. In case of phloroglucinol a concentration of 0.67 mM reached saturation whereas in the case of the second substrate ( $\text{NADPH} + \text{H}^+$ ) the saturation point was not reached until 0.167 mM substrate was used (Fig. 3-10).

#### **3.6.3. Effect of Potassium Chloride on the PG-R Activity.**

The effect of potassium chloride on PG-R activity was inhibitory. A 1.66 M concentration of KCl caused 93.4 % inhibition when compared with the control (Table 3-3).

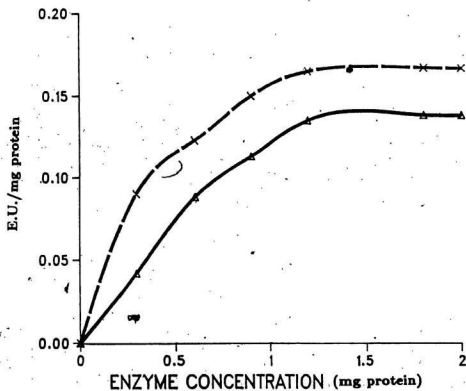
#### **3.6.4. Effect of Metal Ions on Enzyme Activity.**

Compounds listed in table 3-4 were tested for their effect on the activities of PG-R and resorcinol hydroxylase. Freshly prepared crude extract and partially purified enzymes were used for the experiment. All the metal ions tested neither activated nor inhibited the enzyme activities.

**Figure 3-8:** Effect of enzyme concentrations on PG-R and GA-R activities.

Different amounts of crude extracts of mycelia grown on phloroglucinol were used for the enzyme assays under standard conditions; the protein concentration of the crude extract was 4.8 mg /ml. Similarly crude extracts of mycelia grown on gallic acid were used for the enzyme assays and the protein concentration was 5.2 mg/ml.

Figure 3-8:

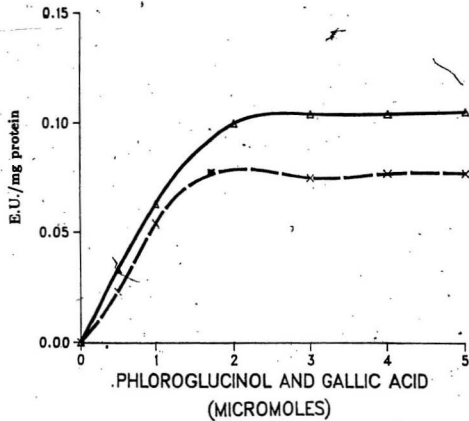


Legend

 $\Delta$  EUPG $\times$  EUG

**Figure 3-9:** Effect of substrates phloroglucinol and gallic acid on PG-R and GA-R activities respectively. Crude extracts of mycelia grown on phloroglucinol and gallic acid were used in enzyme assays; different concentrations of substrate (phloroglucinol and gallic acid) were used in each case.

Figure 3-9:



Legend

 $\Delta$  EUG $\times$  EUPG

**Figure 3-10:** Effect of  $\text{NADPH} + \text{H}^+$  on PG-R and GA-R activities. Crude extracts of mycelia grown on phloroglucinol and gallic acid were used in enzyme assays; different concentrations of  $(\text{NADPH} + \text{H}^+)$  were used in each system.

Figure 3-10:

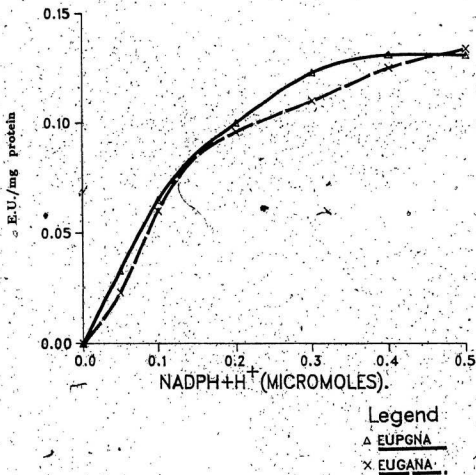




Table 3-3: Effect of potassium chloride on PG-R and GA-R activities.

No.	KCl $\mu$ moles/Assay	(%) Enzyme Activity	
		PG-R	GA-R
1.	0.00	100	100
2.	0.33	22.8	44.6
3.	0.66	16.3	35.8
4.	0.99	13.0	26.8
5.	1.33	9.3	18.5
6.	1.66	5.4	9.6

All enzyme assays were done under standard conditions. 100 % of enzyme activity was considered when no KCl was present in the reaction mixture. The protein concentration in the mycelial crude extract grown on phloroglucinol was 4.8 mg/ml and in gallic acid grown mycelia was 5.2 mg/ml.

Table 3-4: Effect of metal ions on PG-R activity.

No.	Metal Ions	Effect (PG-R)
1.	$\text{FeCl}_3$	
2.	$\text{CoCl}_2$	
3.	$\text{ZnSO}_4$	
4.	$\text{CaCl}_2$	
5.	$\text{FeSO}_4$	
6.	$\text{MgSO}_4$	
7.	$\text{NiCl}_2$	
8.	$\text{CuCl}_3$	
9.	$\text{Cu}_2(\text{SO}_4)_3$	
10.	$\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_3$	

No effect is indicated by (-) symbol.

In every assay mixture  $1 \mu\text{mole}$  of above mentioned solution was added and the oxidation of  $\text{NADPH} + \text{H}^+$  was monitored.

### 3.6.5. Stability of Enzymes in Different Buffer Systems-

The results of phloroglucinol reductase activity tested in different buffers showed that maximum enzyme stability was with 0.1M potassium phosphate buffer containing 1.0 mM EDTA, 1.0 mM 2ME and 15% glycerol (Acharya, personal communication). Other buffers did not have any protective effect on enzyme stability (Table 3-5).

### 3.6.6. Stability of PG-R at Storage Temperatures.

PG-R activity was tested in freshly prepared crude extracts of P. simplicissimum grown on phloroglucinol. The enzyme was active only in fresh extracts. It lost its activity within 5 hours when stored at 0-4°C and at -20°C it retained its activity for 18 hours (Table 3-6).

### 3.7. Oxygen Uptake by Crude Extracts.

Oxygen consumed by freshly prepared crude extract of catechol 1,2 oxygenase was  $0.81 \pm 0.13$   $\mu\text{moles/min/ml}$ . In case of catechol 2,3 oxygenase  $0.27 \pm 0.06$   $\mu\text{moles/min/ml}$  were consumed. Oxygen utilized by the extract in the presence of phloroglucinol was  $0.96 \pm 0.002$   $\mu\text{moles/min/ml}$  and when pyrogallol was present in the system the oxygen consumed was  $0.075 \pm 0.01$   $\mu\text{moles/min/ml}$ . Resorcinol was also tested for the utilization of oxygen but no oxygen consumption was recorded (Table 3-7).

Table 3-5: Effect of different buffers on the stability of PG-R activity.

No.	Buffers	% Activity
1.	Hepes	
2.	Tris HCl	
3.	Sorensen	
4.	0.1M Potassium phosphate	
5.	0.1M Potassium phosphate (Sodium cholate)	
6.	0.1M Potassium phosphate (15% glycerol)	100

All the above mentioned buffers were separately used for washing and extraction of mycelia. Enzyme assays were also done with the same buffer.

Hepes (N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid).  
Sorensen buffer is 0.1M sodium-potassium phosphate buffer.

Table 3-6: Stability of PG-R at different storage temperatures.

Incubation Hours	% PG-R Activity after storage at	
	0-4°C	-20°C
0	100	100
1	85.9	92.8
2	84.2	89.6
3	65.0	72.2
4	22.9	69.4
5	1.8	60.4
6	0	51.4
7	0	40.0
8	0	28.6
9	0	20.1
16	0	3.6
17	0	2.3
18	0	0

A freshly prepared crude extract was kept in ice (0-4°C) and 0.2-ml. were drawn out at 1 hour intervals for enzyme assays. Similarly fresh crude extract was frozen at -20°C in 0.6-ml aliquots, and at 1 hour intervals, tubes were removed, allowed to thaw and assayed for PG-R activity.

Table 3-7: Oxygen uptake by the crude extract of P. simplicissimum grown on phloroglucinol.

No.	Substrate	Oxygen Uptake μmoles/min/ml
1.	Catechol	0.27±0.06
2.	Resorcinol	none
3.	Pyrogallol	0.07±0.00
4.	Catechol+EDTA	0.72±0.13
5.	Phloroglucinol	0.96±0.00

A Clark electrode was used to determine changes in oxygen concentration during enzymatic reaction between crude extract of P. simplicissimum and the different substrates at 25° C.

### 3.8. Separation of PG-R and R-H Activities by Gel Filtration.

Concentrated crude extract was run through columns of Sephadex G-75, G-150 and G-200. Fractions collected from Sephadex G-75, G-150 and G-200 did not show sharp peaks at 280 nm. When tested for enzyme activities, none of the fractions had any measurable activity. Fractions from DEAE-Sephadex had a sharp protein profile but lacked enzyme activity.

### 3.9. Chemical Reduction of Phloroglucinol by Sodium Borohydride.

The ultraviolet spectrum of phloroglucinol gives maximum absorbance at 271 nm (Campbell and Coppinger, 1953). Chemical reduction of phloroglucinol with sodium borohydride produced a product which had maximum absorbance at 278 nm (Fig. 3-11). Enzymatic reduction of phloroglucinol also gave a product with a similar maximum absorbance at 278 nm.

### 3.10. Detection of Enzymes Involved in Gallic Acid Metabolism.

Crude extracts of P. simplicissimum grown on 0.2% gallic acid were analysed for enzymes involved in the metabolism of the substrate. Gallic acid reductase (GA-R) was the initial enzyme acting on the substrate. This enzyme was dependent on  $\text{NADPH} + \text{H}^+$  as an electron donor. The specific activity of GA-R was calculated to be 0.154 E.U./mg protein. The enzyme catalyzed the oxidation of  $\text{NADPH} + \text{H}^+$  in equimolar amounts. The spectrum of the product formed by the enzymatic reduction of gallic acid is shown in figure 3-12.

R-H was not detected in the crude extract of P. simplicissimum grown on

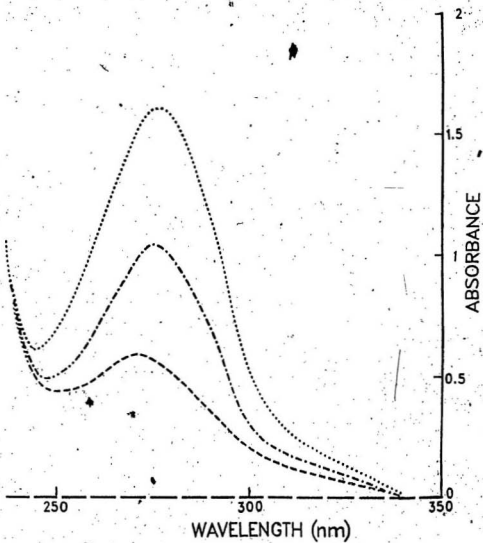
**Figure 3-11:** UV-Spectra of phloroglucinol and chemically\* reduced phloroglucinol.

Maximum UV-absorption of 1  $\mu$ mole of phloroglucinol was recorded from 350 nm to 190 nm, 1  $\mu$ mole of sodium borohydride was added and the UV-spectrum was recorded at 1 minute and 5 minutes of incubation.

----- Phloroglucinol  
-.-.-.-.- Dihydrophloroglucinol 1 minute  
..... Dihydrophloroglucinol 5 minutes



Figure 3-11:



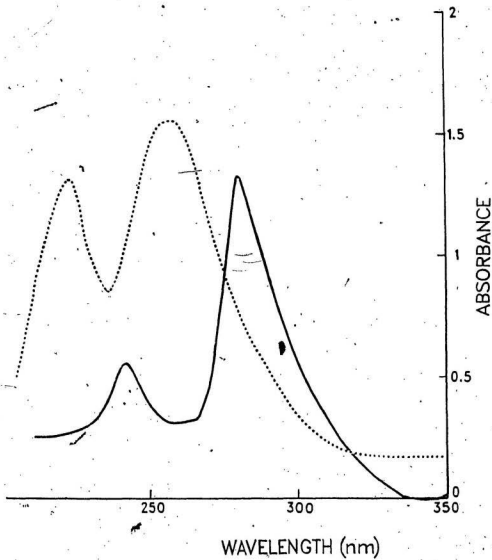
**Figure 3-12:** UV-Spectra of gallic acid and enzymatically reduced gallic acid.

Maximum UV-absorption of 1  $\mu$ mole of gallic acid was recorded from 350 nm to 190 nm. The UV-spectrum of the product produced by enzymatically reduced gallic acid was also recorded.

..... gallic acid

———— enzymatically reduced  
gallic acid

Figure 3-12:



gallic acid. However catechol 1,2 oxygenase and catechol 2,3 oxygenase were active in the crude extract with specific activities of  $0.129 \pm 0.21$  and  $0.087 \pm 0.06$  respectively. Maleylacetate reductase was also  $\text{NADPH} + \text{H}^+$  dependent and had a specific activity of  $0.085 \pm 0.06$ . No maleylacetate reductase activity was found when  $\text{NADH} + \text{H}^+$  was used as an electron donor (Table 3-8).

### **3.11. Properties of Gallic Acid Reductase (GA-R).**

#### **3.11.1. Effect of Potassium Chloride on GA-R.**

Potassium chloride had an inhibitory effect on GA-R. The control without KCl showed 100 % activity. Maximum inhibition was 90.4 % with  $1.66 \mu\text{M}$  KCl in the assay system (Table 3-3). A similar pattern of inhibition was observed for PG-R when KCl was used in the test.

#### **3.11.2. Effect of Enzyme Concentration on GA-R.**

The GA-R activity was found to be dependent on enzyme concentration as shown in figure 3-8. The substrates used were gallic acid and  $\text{NADPH} + \text{H}^+$ . The reaction rate reached a plateau when the concentration of the crude extract was 0.186 mg.

#### **3.11.3. Effect of Substrate Concentration on GA-R.**

Gallic acid reductivity was dependent on substrate as well as co-substrate ( $\text{NADPH} + \text{H}^+$ ) as illustrated in figure 3-9 and figure 3-10 respectively.

Table 3-8: Enzymes detected in cells grown on gallic acid.

No.	Enzyme	Specific Activity E.U./mg.protein
1.	GA-R	0.154 $\pm$ 0.32
2.	Maleylacetate reductase	0.085 $\pm$ 0.04
3.	Catechol 1,2 oxygenase	0.129 $\pm$ 0.21
4.	Catechol 2,3 oxygenase	0.087 $\pm$ 0.06
5.	R-H	not detected.

One enzyme unit in the case of GA-R, R-H and maleylacetate reductase was defined as the amount of extract required to oxidize 1 $\mu$ mole of NADPH+H<sup>+</sup> per min per ml under standard conditions. In the case of the oxygenases, one enzyme unit was defined as the amount of extract required to consume 1 $\mu$ mole of oxygen per min under standard assay conditions. Specific activity was defined as enzyme units per mg protein.

### 3.12. Isolation and Detection of Metabolic Products.

#### 3.12.1. TLC of Culture Media of Mycelia Grown on Phloroglucinol.

Samples of culture media of mycelia grown on phloroglucinol for chromatography were prepared as described in the materials and methods. The spotted sample on TLC plates resolved into three spots with RF values of  $0.21 \pm 0.04$ ,  $0.47 \pm 0.04$  and  $0.52 \pm 0.05$ . The RF value of the second band corresponded to authentic phloroglucinol (Table 3-9). The band which corresponded to the standard 1,2,4-benzene triol was removed from the chromatogram, extracted in ethyl ether (described in materials and methods) and redissolved in 1 ml of methanol. The methanol solution (50  $\mu$ l) was again spotted on a TLC plate and chromatographed as before. A sample of standard 1,2,4-benzene triol was also spotted. The plates were developed using solvent system C. The RF value of the sample was identical to that of authentic 1,2,4-benzene triol.

### 3.13. Ultra-Violet Absorption Spectrophotometry of Culture Medium.

A culture medium of a 24 hours old flask culture, when examined on a spectrophotometer showed the presence of a compound that absorbed maximally at 288 nm (Mason, 1949). In order to further identify this compound the culture medium was extracted with ethyl ether as discussed in materials and methods. The ethyl ether extract was subjected to thin layer chromatography using the same solvent system as showed before. The band corresponding to authentic 1,2,4-benzene triol was removed and reextracted in ethyl ether. When examined

Table 3-9: Thin layer chromatography of an ethyl ether extract of culture medium grown on phloroglucinol.

Samples	Compounds	RF Values (cm)
1.	Pyrogallol	$0.35 \pm 0.04$
2.	Gallic acid	$0.19 \pm 0.01$
3.	Phloroglucinol	$0.21 \pm 0.04$
4.	p-Hydroxyquinone	$0.66 \pm 0.02$
5.	p-Hydroxybenzoate	$0.40 \pm 0.04$
6.	1,2,4-Benzenetriol	$0.47 \pm 0.04$
7.	A	$0.21 \pm 0.03$
8.	B	$0.47 \pm 0.03$
9.	C	$0.52 \pm 0.05$

The samples and standards were spotted on TLC plates and developed in a solvent system consisting of benzene, 45: methanol, 8: acetic acid, 4. The spots were visualized by vanillin toluene p-sulphonic acid and UV-light.

on a spectrophotometer the compound gave maximum absorbance at 288 nm (Fig. 3-13).

### 3.13.1. Detection of Metabolites from Culture Medium of Mycelia Grown on Gallic Acid.

Thirty hours old culture media of mycelia grown on gallic acid were extracted in ethyl ether, and subjected to thin layer chromatography and ultraviolet absorption spectrophotometry. Three bands were observed, one of them corresponded to the authentic 1,2,4-benzene triol (Table 3-10). The corresponding band was removed and extracted in ethyl ether. The ethyl ether extract was taken to dryness and the residue was dissolved in methanol. The UV-spectrum was recorded using a spectrophotometer. The compound exhibited maximum absorption at 288 nm and the overall spectrum was similar to the authentic 1,2,4-benzene triol (Fig. 3-14).

### 3.13.2. Detection of Intracellular Resorcinol in Mycelia Grown on Phloroglucinol.

A crude extract of the organism grown on phloroglucinol was extracted as described in materials and methods. A 50  $\mu$ l of the prepared sample was spotted on TLC plates and developed in the solvent system: benzene, 90; dioxane, 25; acetic acid, 4. The sample separated into four bands. The  $R_f$  value of one of the bands corresponded to the  $R_f$  value of standard resorcinol ( $0.51 \pm 0.01$ ) spotted on the same plate. The band was removed from the plate, reextracted with ethyl ether and taken to dryness. The residue was redissolved in methanol and spotted on a TLC plate with authentic resorcinol. The  $R_f$  values observed were similar for the sample and the standard (Table 3-11).



**Figure 3-13:** UV-spectrum of culture medium of P. simplicissimum grown on phloroglucinol.

UV-spectra of culture filtrate of mycelia grown on phloroglucinol and 1,2,4-benzene triol were recorded. The filtrate was extracted using ethyl ether and redissolved in methanol. It was plotted on TLC plates and tested for UV-absorption. The spots recovered from TLC plates were extracted again with ethyl ether, dissolved in methanol, plotted on TLC plates and tested for UV-absorption.

..... spots recovered from TLC plates

----- ethyl ether extract of cultural medium

..... 1,2,4-Benzene triol

----- culture filtrate

Figure 3-13:

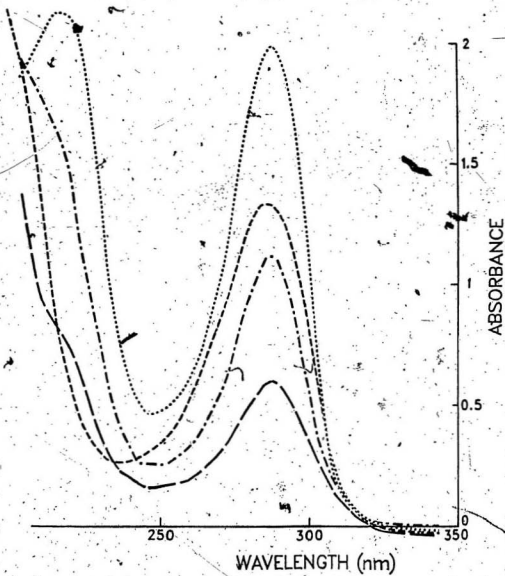


Table 3-10: Thin layer chromatography of an ethyl ether extract of culture medium grown on gallic acid.

Samples.	Compounds	RF Values (cm)
1.	Pyrogallol	$0.38 \pm 0.01$
2.	Gallic acid	$0.10 \pm 0.02$
3.	Phloroglucinol	$0.31 \pm 0.11$
4.	p-Hydroxybenzoate	$0.27 \pm 0.04$
5.	1,2,4-Benzenetriol	$0.22 \pm 0.02$
6.	A	$0.22 \pm 0.01$
7.	B	$0.36 \pm 0.05$
8.	C	$0.44 \pm 0.07$

The samples and standards were spotted on TLC plates and developed in a solvent system consisting of benzene, 45: methanol, 8: acetic acid, 4. The spots were visualized by vanillin toluene p-sulphonic acid and by UV-light.

Figure 3-14: UV-spectrum of culture medium of P. simplicissimum grown on gallic acid.

- UV-spectra of culture filtrate of mycelia grown on gallic acid and 1,2,4-benzene triol were recorded. The filtrate was extracted using ethyl ether and redissolved in methanol. It was plotted on TLC plates and tested for UV-absorption. The spots recovered from TLC plates were extracted again with ethyl ether, dissolved in methanol, plotted on TLC plates and tested for UV-absorption.

----- spots recovered from TLC plates

----- ethyl ether extract of cultural medium

..... 1,2,4-Benzene triol

----- culture filtrate

Figure 3-14:

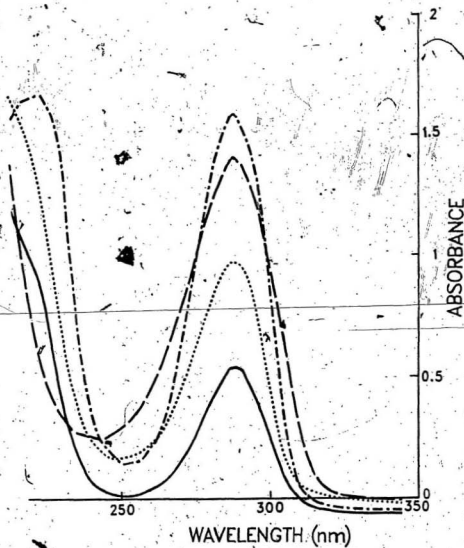


Table 3-11: Thin layer chromatography of the ethyl ether extract of mycelia-grown on phloroglucinol.

Samples	Compounds	RF Values (cm)
1.	Resorcinol	$0.51 \pm 0.01$
2.	Pyrogallol	$0.91 \pm 0.01$
3.	Phloroglucinol	$0.36 \pm 0.11$
4.	p-Hydroxyquinone	$0.67 \pm 0.02$
5.	A	$0.51 \pm 0.01$
6.	B	$0.49 \pm 0.01$
7.	C	$0.41 \pm 0.03$
8.	D	$0.38 \pm 0.05$

A, B, C and D represent spots obtained on TLC plates developed using solvent system benzene, 90: dioxane, 25: acetic acid, 4. The spots were visualized by bromocresol purple and UV-light.

An ethyl ether extract of the crude extract was examined using a spectrophotometer. A UV-absorbing compound with maximum absorption at 290 nm was detected (Fig. 3-15). For further identification, the ethyl ether extract of the TLC band was subjected to thin layer chromatography and UV-spectrophotometry.

### 3.13.3. Detection of Maleylacetate Reductase and its Product.

Maleylacetate was prepared using the method described by Gaal and Neujahr (1979). This substrate was then used to detect maleylacetate reductase in the crude extract of P. simplicissimum. NADPH+H<sup>+</sup> was oxidized in the presence of maleylacetate and crude extract. The reaction mixture was extracted and chromatographed on TLC plates. The ethyl ether extract showed a spot which had a R<sub>f</sub> value similar to that of authentic  $\beta$ -ketoadipate. The ethyl ether extract of the corresponding spot had maximum absorption at 275 nm, which was similar to authentic  $\beta$ -ketoadipate (Fig. 3-16).

Figure 3-15: UV-spectrum of crude extract when phloroglucinol was utilized by the organism.

A crude extract of mycelia grown on phloroglucinol was extracted with ethyl ether, redissolved in methanol and plotted on TLC plates. The spot recovered was again extracted in ethyl ether and checked for UV-absorption; 1  $\mu$ mole of resorcinol was also tested for maximum absorption.

— resorcinol  
- - - spot recovered from TLC plate  
..... Base line



Figure 3-15:

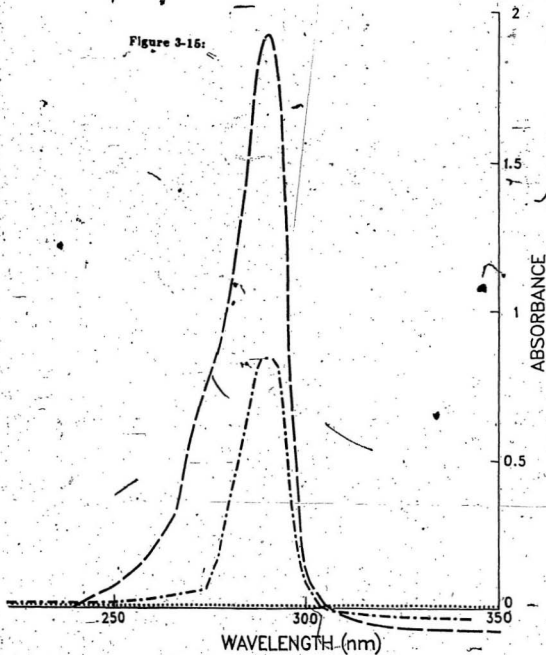
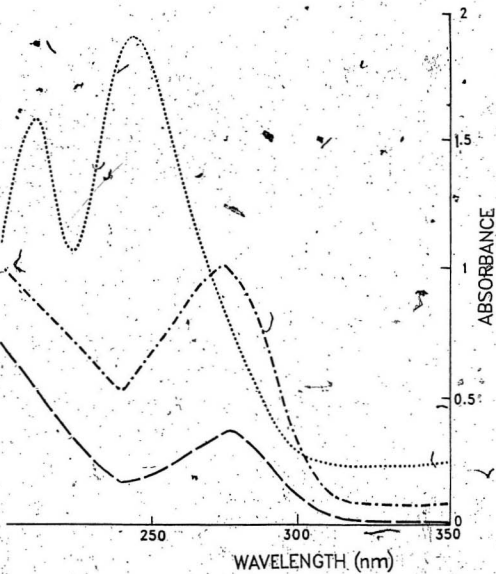


Figure 3-16: UV-spectrum of synthesized maleylacetate, and the product of its enzymatic reduction. Maleylacetate was tested for UV-absorption; similarly, 1  $\mu$ mole of  $\beta$  keto adipate was scanned. The product of enzymatic reduction of maleylacetate was extracted and plotted on TLC plates; the spot obtained was reextracted and tested for UV-absorption.

..... synthesized maleylacetate  
- - - - -  $\beta$ . keto adipate  
— — — — — extract of reaction mixture

Figure 3-16:



## Chapter 4

### DISCUSSION.

#### 4.1. Growth Conditions.

The primary objective of this research was to study the physical conditions under which Penicillium simplicissimum, a soil fungus, utilizes phloroglucinol and gallic acid as sole source of carbon and energy. The second objective was to detect and identify some intermediates involved in the degradative pathway of these compounds.

The utilization of phloroglucinol by microorganisms was first shown by Wagner (1914), and the studies were followed by Gray and Thornton (1928). In 1965 Roberson did detailed research about the metabolism of phloroglucinol by Pseudomonas sp. and Penicillium sp. After that, many scientists worked on the utilization of aromatics by microorganisms. Penicillium sp. Mac M-47 was used by Mathur (1971) to study the enzymatic degradation of phloroglucinol.

The soil fungus used in this research belongs to the class *Deuteromycetes* (Fungi Imperfecti), which is differentiated by the appearance of spore bearing structures, conidiophores (Frazier and Westhoff, 1978). P. simplicissimum utilized various aromatic compounds. Of all the aromatic compounds tested,

phloroglucinol and gallic acid were the two that showed maximum growth in terms of dry weight, protein contents and enzymes produced. These two compounds were used throughout in this research.

Robern (1965) reported that Penicillium sp. Mac M-47 grew best at neutral pH, when P. simplicissimum was grown on phloroglucinol and gallic acid separately. The optimum pH for growth was 5.5, and the second best pH was 7.0 which agree with the results of Robern (1965) and Mathur (1971). The optimum temperature for the growth of the organism was observed to range from 27°-29° C. Penicillium sp. Mac M-47 also showed optimum temperature of 30° C (Mathur, 1971).

A number of soil organisms were tested for utilization of phenols by Gray and Thornton (1928), who used 0.1 % of phloroglucinol (w/v) and reported that 100 % decomposition of the substrate took place in 4-28 days, depending upon the organism used. Wagner (1914) reported a bacterium isolate which utilized 0.18 % phloroglucinol in 34 days. Senning (1963) showed that pronounced inhibition in the growth of Sporocytophaga myxococcoides occurred at concentration higher than 0.1 %. In Pseudomonas sp. the optimum concentration of substrate was reported to be 1 % (Robern, 1965). The optimum for Penicillium sp. Mac M-47 was not reported but the organism grew well in the presence of 0.25 % phloroglucinol (Mathur, 1971). The optimum concentration of phloroglucinol for the growth of P. simplicissimum was 1.5 % (w/v), whereas higher concentrations inhibited growth. When the organism was grown on gallic acid as a sole source of carbon and energy the optimum pH and temperature were found to be similar to

those obtained with the utilization of phloroglucinol but the concentration of gallic acid was much lower than that of phloroglucinol. Concentrations of gallic acid higher than 0.2 % (w/v) were inhibitory.

#### 4.2. Enzymes Involved in Metabolism of Substrate.

Robern (1965) reported that cell-free extracts of Pseudomonas sp. required  $\text{NADPH} + \text{H}^+$  for the decomposition of phloroglucinol and substitution of  $\text{NADH} + \text{H}^+$  caused 50 % reduction in the rate of enzyme activity. Similar results were reported in the case of Pseudomonas sp. by Hang (1967), who showed that the partially purified enzyme was  $\text{NADPH} + \text{H}^+$ -dependent and substitution of  $\text{NADH} + \text{H}^+$  inhibits enzyme activity. He explained this by the possibility of the absence of  $\text{NADH} + \text{H}^+$ -oxidase and  $\text{NADH} + \text{H}^+ \rightleftharpoons \text{NADPH} + \text{H}^+$ -transhydrogenases from the partially purified enzyme preparation.

##### 4.2.1. Properties of PG-R and Metabolic Intermediates.

The present study showed that the cell-free extract is  $\text{NADPH} + \text{H}^+$ -dependent. No PG-R or GA-R activity was noted by the substitution of  $\text{NADH} + \text{H}^+$ . PG-R and GA-R were able to catalyse a rapid oxidation of  $\text{NADPH} + \text{H}^+$  in the presence of phloroglucinol and gallic acid respectively. Various other phenols like resorcinol, pyrogallol and catechol were found unable to act as hydrogen acceptor from  $\text{NADPH} + \text{H}^+$  in the presence of PG-R and G-R. Dagley and Patel (1957) isolated a compound from the oxidation of protocatechuic acid by Pseudomonas sp. having maximum absorption at 276 nm. In 1965 Robern investigated the fermentative pathway of phloroglucinol metabolism by resting cells of Penicillium sp. Mac M-47, and reported the accumulation of a compound

with maximum absorption at 278 nm, in culture medium. The compound was not further metabolized by the fungus, and he was unable to isolate the compound. Hang (1967) also reported a compound with maximum absorption at 278 nm in a culture medium of Pseudomonas sp. He identified the compound by stoichiometric studies. He proved that the oxidation of  $\text{NADPH} + \text{H}^+$ , in the presence of PG-R, was equivalent to the disappearance of phloroglucinol. Hang (1967) confirmed the earlier findings of the product of PG-R reaction by the addition of two hydrogen atoms to the phloroglucinol with  $\text{NADPH} + \text{H}^+$  as donor. He identified the compound as dihydrophloroglucinol.

Further confirmation was done by mass spectroscopic analysis (Jamieson *et al.*, 1970). They prepared the sample by ethyl ether extraction of ten litres of cell-free culture medium. The residue of ether extract was column chromatographed and the fractions having 278 nm absorption were pooled, passed through the Sephadex column again and a single fraction was selected. Further purification of the selected fraction was done by TLC. The prepared sample was used for mass spectroscopy. The results revealed that the parent ion in the product of phloroglucinol reduction had a molecular formula of  $\text{C}_6\text{H}_8\text{O}_3$  which corresponds to dihydrophloroglucinol. The product of enzymatic reduction of phloroglucinol with  $\text{NADPH} + \text{H}^+$  as hydrogen donor, was also identified as dihydrophloroglucinol.

In the present study it was possible to show that equivalent amounts of  $\text{NADPH} + \text{H}^+$  were oxidized in the presence of phloroglucinol and gallic acid by fresh extracts of P. simplicissimum. In case of phloroglucinol the absorption peak

shifted from 271 to 278 nm, which was further confirmed by the chemical reduction of phloroglucinol by sodium borohydride. These results agree with the results of Robern (1965), Hang (1967) and Patel *et al.*, (1981). Mathur (1971) reported that the new compound formed had maximum absorption at 285 nm in the case of Penicillium sp. Mac M-47. Robern (1965) reported that the first enzymatic reaction of phloroglucinol degradation was NADPH+H<sup>+</sup>-dependent and reductive in nature. This initial step was thought to be the result of removal of one hydroxyl group from phloroglucinol. No evidence was obtained to confirm the findings of Robern that the dehydroxylation occurred before the reduction of phloroglucinol in Pseudomonas sp. Mac M-471.

The initial enzymatic reaction for gallic acid decomposition was also reductive and NADPH+H<sup>+</sup>-dependent. The shifting of absorption peaks from 262.5 to 280 nm were due to product formation. Yashida *et al.*, (1985) gave the scheme for the production of pyrogallol from gallic acid by an isolated bacterium Citrobacter sp. Dagley (1971) reported that Pseudomonas sp., grown on syringic acid, had the ability to metabolize gallic acid. The utilization of gallic acid by microorganisms has not been established so far.

In the present study, *ortho* and *meta* fission enzymes were detected by specific assays described for catechol 1,2-oxygenase and catechol 2,3-oxygenase. The presence of these enzymes in microbial cells grown on phloroglucinol has not been previously reported. Both activities, in cell-free extract of P. simplicissimum grown on phloroglucinol were found to be dependent on enzyme concentration as demonstrated in spectrophotometric studies and by oxygen uptake experiments.



The cell-free extract of Penicillium sp. Mac M-47 used by Mathur (1971) retained its PG-R and resorcinol hydroxylase activities for 15 days at 4° C and for 75 days at -20° C. Cell-free extracts of mycelia grown on the phloroglucinol lost its PG-R activity within 5 hours at 4° C and 18 hours at -20° C. Resorcinol hydroxylase was never detected in these cell-free extracts of P. simplicissimum. All the attempts to purify the PG-R from the cell-free extract failed due to the unstability of the enzyme. PG-R activity in crude extracts could not be stabilized by metal ions or by different buffer systems.

Many bacteria and fungi can utilize cyclic compounds as sources of carbon and energy, a nutritional character which reflects the operation of specialized metabolic pathway through which these substrates are converted into aliphatic cellular intermediary metabolites. One of the major microbial pathways for the utilization of aromatics is the  $\beta$ -ketoadipate pathway. The prerequisite structures for ring cleavage are hydroxylated intermediates such as catechol and protocatechol. This is usually achieved either by elimination of substituent groups from the benzene nucleus (Dagley and Patel, 1957; Ribbons and Evans, 1960) or by the hydroxylation of benzene nucleus. Oxygenases are capable of breaking these compounds into aliphatic substrates for further metabolism.

Hang (1967) showed that dehydration of dihydrophloroglucinol, formed from phloroglucinol in reaction with the purified PG-R, resulted in the formation of resorcinol. Mathur (1971) also reported a conjugated phenolic compound which yielded resorcinol by hydrolysis, suggesting that dehydroxylation of phloroglucinol results in the formation of this compound.

In the present study two intermediary compounds were detected and isolated during the metabolism of phloroglucinol by P. simplicissimum. The first compound was resorcinol which was detected in the cell-free crude extracts, but the related enzyme was not demonstrated in the extract. Presence of resorcinol hydroxylase was reported by Māthur (1971), which hydroxylated resorcinol at C-4 position to yield 1,2,4-benzenetriol. The second intermediate was 1,2,4-benzenetriol from the cultural medium. This compound is proposed to be the substrate for the cleavage of benzene nucleus. Enzymatic, spectrophotometric and chromatographic data gave the evidence, that 1,2,4-benzenetriol is the possible substrate for oxygenases when phloroglucinol was being metabolized by the P. simplicissimum.

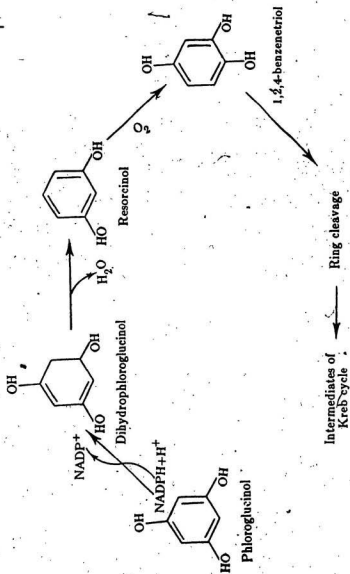
For the above discussion, the following pathway for the degradation of phloroglucinol by the P. simplicissimum is proposed (Fig. 4-1).

#### 4.2.2. Properties of GA-R and Metabolic Intermediates.

When the cell-free extract of P. simplicissimum, grown on gallic acid was studied for the decomposition of the substrate, the initial step observed was the reduction of gallic acid in the presence of  $\text{NADPH} + \text{H}^+$  as hydrogen donor. The intermediates detected and isolated were 1,2,4-benzenetriol and maleylacetate. 1,2,4-benzenetriol was detected and isolated from the culture medium. The second compound maleylacetate was detected by the help of enzyme present in the cell free extract. The enzyme was  $\text{NADPH} + \text{H}^+$ -dependent. Others have also observed the formation of maleylacetate as an intermediate in resorcinol and orcinol metabolism in several pseudomonads (Harris and Ricketts, 1962; Duxbery

Figure 4-1: Proposed pathway for the decomposition of Phloroglucinol by P. simplicissimum.

Figure 4-1:

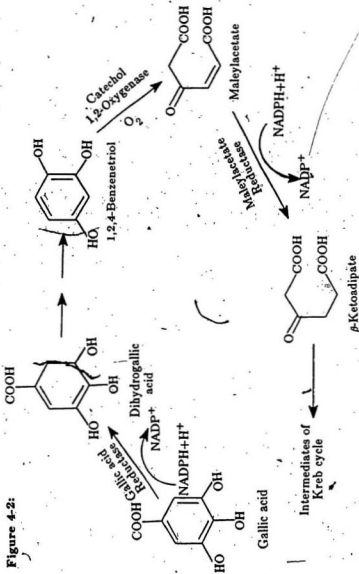


*et al.*, 1970; Chapman and Ribbons, 1976). The degradation of resorcinol to maleylacetate by a soil Pseudomonas was first reported by Larway and Evans (1967). They reported that the extract did not reduce maleylacetate in the presence of  $\text{NADH}+\text{H}^+$  or  $\text{NADPH}+\text{H}^+$ . Chapman and Ribbons (1976) studied Pseudomonas putida and reported that an extract of the organism had the enzyme to reduce maleylacetate in the presence of  $\text{NADH}+\text{H}^+$  or  $\text{NADPH}+\text{H}^+$ . Gaal and Neujahr (1979) reported that Trichosporon cutaneum metabolized phenol and resorcinol through ortho cleavage resulting in  $\beta$ -ketoadipate pathway. The cell-free yeast system of Trichosporon cutaneum reduced maleylacetate with either  $\text{NADH}+\text{H}^+$  or  $\text{NADPH}+\text{H}^+$ .

Spectrophotometric and chromatographic studies of the product recovered after the enzymatic reduction (maleylacetate reductase) of maleylacetate showed that gallic acid is metabolized completely by the  $\beta$ -ketoadipate pathway following ortho type of cleavage. Product of meta-cleavage was not detected in the cell-free extract of P. simplicissimum. Ornston (1966a and b), Hosokawa (1970) and Wheelis and Stanier, (1971) proposed a scheme for the metabolization of shikimic acid by Pseudomonas putida through the protocatechuic acid branch. Ornston (1977c and d) demonstrated ortho cleavage of protocatechuic acid ending in  $\beta$ -ketoadipate. Canovas and Stanier (1967), and Canovas *et al.* (1970) also demonstrated a similar pathway for the metabolism of shikimic acid by Acinetobacter calcoaceticus.

Based on the results obtained from the present study, the following pathway of gallic acid metabolism by P. simplicissimum is proposed (Fig. 4-2).

Figure 4-2: Proposed pathway for the decomposition of gallic acid  
by P. simplicissimum.



This pathway involves reductive decarboxylation of the substrate, possibly through the transitory reduced dihydrogallic acid resulting in an intermediate for the ring cleavage where both ortho and meta-oxygenases take part, but only the product of ortho cleavage, maleylacetate, was detected and further metabolized into  $\beta$ -ketoadipate, which gives substrates for Kreb's cycle.



## CONCLUSIONS.

- i. Penicillium simplicissimum can utilize phloroglucinol and gallic acid as a source of carbon and energy.
- ii. Physical and chemical conditions tested showed that P. simplicissimum metabolizes phloroglucinol and gallic acid at about 27° C at a pH of 5.5.
- iii. Concentrations of phloroglucinol and gallic acid, higher than 1.5 and 0.2 % (w/v) respectively, are toxic to the organism.
- iv. The initial enzyme involved in the metabolism of phloroglucinol is PG-R which is reductive in nature and requires NADPH+H<sup>+</sup> for its activity.
- v. Introduction of metal ions like, Fe<sup>+++</sup>, Fe<sup>++</sup>, Cu<sup>+++</sup>, Co<sup>++</sup>, Ca<sup>++</sup>, Mg<sup>++</sup>, Na<sup>+</sup> and Zn<sup>++</sup> does not reduce or enhance PG-R activity.
- vi. Possible intermediates detected and isolated in phloroglucinol metabolism are resorcinol, (produced in the cell-free crude extract) and 1,2,4-benzenetriol, (released into the culture medium).
- vii. The initial enzyme in gallic acid metabolism is GA-R, which is also NADPH+H<sup>+</sup> dependent.

viii. Equivalent amount of  $\text{NADPH} + \text{H}^+$  are oxidized in the presence of phloroglucinol and gallic acid by fresh extracts of P. simplicissimum.

ix. Possible intermediates detected and isolated in gallic acid metabolism are, 1,2,4-benzenetriol, which is released into the culture medium, maleylacetate, which is present in the crude extract and  $\beta$ -ketoadipate, which is metabolized from maleylacetate in the presence of  $\text{NADPH} + \text{H}^+$ .

## REFERENCES

- Auret, B. J., D. R. Robinson, C. G. Watson, J. W. Daly and D. M. Jerina, 1971. The NIH shift during the hydroxylation of aromatic substrates by fungi. *Chem. Commun.*, 24: 1585-1587.
- Ayenger, P. K., O. Hayaishi, M. Nakajima, and I. Tomida, 1959. Enzymic aromatization of 3,5-CycloHexadiene-1,2-Diol. *Biochim. Biophys. Acta.*, 33: 111-119.
- Bakker, G., 1977. Anaerobic degradation of aromatic compounds in the presence of nitrates. *FEMS Lett.* 1: 103-108.
- Beveridge, E. G., and W. B. Hugo, 1965. The metabolism of gallic acid by Pseudomonas convexa X-1. *J. Appl. Bacteriol.*, 27: 448-460.
- Campbell, J. W., and G. M. Coppinger, 1953. The spectrophotometric examination of some derivatives of pyrogallol and phloroglucinol. *Am. Chem. Soc.*, 73: 2708-2712.
- Canovas, J. L., and R. Y. Stanier, 1967. The regulation of the  $\beta$ -ketoadipate pathway in Moraxella calcoacetica. *Eur. J. Biochem.*, 1: 289-292.
- Canovas, J. L., M. L. Wheelis, and R. Y. Stanier, 1970. Regulation of the  $\beta$ -ketoadipate pathway in Moraxella calcoacetica, ii. The role of protocatechuate as inducer. *Eur. J. Biochem.*, 3: 293-304.
- Cerniglia, C. E., J. P. Freeman, and R. K. Mitchum, 1982. Glucuronoid and sulphate conjugation in the metabolism of aromatic hydrocarbons. *Appl. Environ. Microbiol.*, 43: 1070-1075.
- Chapman, P. J., and D. W. Ribbons, 1976. Metabolism of resorcinolic compounds by bacteria: Orcinol pathway in Pseudomonas putida. *J. Bacteriol.*, 125: 975-984.
- Chapman, P. J., and D. W. Ribbons, 1976. Metabolism of resorcinolic compounds by bacteria: Alternative pathways for resorcinol catabolism in Pseudomonas putida. *J. Bacteriol.*, 125: 985-998.
- Clark, T. H., and H. W. Hartman, 1929. Phloroglucinol, *Organic Syntheses*. 9: 74-76.
- Claus, D., and N. Walker, 1964. Decomposition of toluene by soil bacteria. *J. Gen. Microbiol.*, 38: 107-122.

- Craigie, J. S., J. MacLachlan, and G. N. Tower, 1965. A note on the fission of an aromatic ring by algae. *Can. J. Botany*, 43: 1589-1590.
- Dagley, S., 1960. New pathway in the oxidative metabolism of aromatic compounds by the microorganism. *Nature, London*, 188: 560-566.
- Dagley, S., 1971. Catabolism of aromatic compounds by microorganisms. *Adv. Microbial Physiol.*, 8: 1-46.
- Dagley, S., P. J. Chapman, D. T. Gibson, and J. M. Wood, 1964. Degradation of the benzene nucleus by bacteria. *Nature, London*, 202: 775-778.
- Dagley, S., and M. D. Patel, 1957. Oxidation of p-cresol and related compounds by a *Pseudomonas* sp. *J. Biochem.*, 66: 227-233.
- Dagley, S., and D. A. Stopher, 1959. A new mode of fission of the benzene nucleus by bacteria. *Biochem. J.*, 73: 1-6.
- Daly, J. W., D. M. Jarina, and B. Witkop, 1972. The metabolism, toxicity and carcinogenicity of aromatic compounds. *Experientia*, 28: 1120-1140.
- Danison, F. W. Jr., and E. F. Pares, 1952. Rapid method for paper chromatography of organic acids. *Anal. Chem.*, 24: 1628-1629.
- Dutton, P. L., and W. C. Evans, 1967. Dissimilation of aromatic substrate by *Rhodopseudomonas palustris*. *Biochem. J.*, 104: 30p.
- Dutton, P. L., and W. C. Evans, 1968. The photometabolism of benzoic acid by *Rhodopseudomonas palustris*: A new pathway of aromatic ring metabolism. *Biochem. J.*, 109: 5p.
- Dutton, P. L., and W. C. Evans, 1969. The metabolism of aromatic compounds by *Rhodopseudomonas palustris*. *Biochem. J.*, 113: 525-536.
- Duxbery, J. M., I. M. Alexander, and J. E. Dawson, 1970. 2,4-D Metabolism: enzymic conversion of chloromaleylacetic acid to succinic acid. *Agr. Food Chem.*, 18: 199-201.
- El Hawary, M. F. S., and R. H. S. Thompson, 1953. Separation and estimation of blood ketoacids by paper chromatography. *Biochem. J.*, 53: 340-347.
- Evans, W. C., 1963. The microbial degradation of aromatic compounds. *J. Gen. Microbiol.*, 32: 177-184.

- Evans, W. C., 1977. Biochemistry of the bacterial catabolism of aromatic compounds in anaerobic environments. Rev. Nature, 270: 17-22.
- Evans, W. C., and F. C. Hoppold, 1939. The utilization of phenol by bacteria. J. Soc. Chem. Industr., 58: 55.
- Evans, W. C., B. S. Smith, R. P. Linstead, and J. A. Elvidge, 1951. Chemistry of the oxidative metabolism of certain aromatic compounds by microorganisms. Nature, 168: 772-775.
- Fewson, C. A., 1981. Biodegradation of aromatics with industrial relevance. In, Microbial Degradation of Xenobiotics and Recalcitrant Compounds. p.141-172, Leisinger, Hutter, Cook and Nvesen (eds.); Academic Press Inc. London LTD.
- Frazier, W. C., and D. C. Westhoff, 1978. In, Food Microbiology. W. C. Frazier (ed.); McGraw-Hill, London.
- Gaal, A., and H. Y. Neujahr, 1979. Metabolism of phenol and resorcinol in Trichosporon cutaneum. J. Bacteriol., 137: 13-21.
- Gibson, D. T. 1972. Initial reactions in the degradation of aromatic hydrocarbons. In, Degradation of Synthetic Organic Molecules in the Biosphere. p. 116-138, National Acad. Science, Washing. D.C.
- Gibson, D. T. 1972. Microbial degradation of aromatic hydrocarbons. Crit. Rev. Microbiol. 1: 199-223.
- Gibson, D. T. 1977. Biodegradation of aromatic petroleum hydrocarbons. In Fate and effect of hydrocarbons in marine organisms and ecosystem. p. 36-46, D. A. Wolfe (ed.), Pergamon Press, N. Y.
- Gibson, D. T., and V. Subramanian, 1984. Degradation of aromatic hydrocarbons. In, Microbiol. Degradation of Aromatic Compounds. p.181-252, D. T. Gibson (ed.), Marcel Dekker Inc. N. Y.
- Gibson, D. T., K. C. Wang, C. J. Sih, and H. Whitlock, Jr. 1966. Mechanism of steroid oxidation by microorganisms. J. Biol. Chem. 241: 551-559.
- Grant, D. J. W., and J. C. Patel, 1969. Determination of constituents. Analyt. Biochem., 28: 139-140.
- Gray, H. H. P., and G. H. Thornton, 1928. Soil bacteria that decompose certain aromatic compounds. ZBL Bacter. (2), Abt., 73: 74-95.

- Griffith, L. A., 1957. Detection of the substrate of enzymic browning in Cacao by a post-chromatographic enzyme technique. Nature, **180**: 1373-1374.
- Groseclose, E. E., and D. W. Ribbons, 1981. Metabolism of resorcinolic compounds by bacteria: New pathway for resorcinol catabolism in Azotobacter vinelandii. J. Bacteriol., **146**: 460-466.
- Gross, S. R., R. D. Gafford, and E. L. Tatum, 1956. The metabolism of protocatechuic acid by Neurospora sp. J. Biol. Chem., **219**: 781-795.
- Guyer, M., and G. D. Hegeman, 1969. Evidence for reductive pathway for the anaerobic metabolism of benzoate. J. Bacteriol., **99**: 906-907.
- Halvorson, H., 1963. Formation of resorcinol as an intermediate in the metabolism of 2,4-dihydroxybenzoic acid and its corresponding aldehyde. Biochem. Biophys. Res. Comm., **10**: 440-442.
- Hang, Y. D., 1967: Pathway of phloroglucinol degradation by Pseudomonas sp. Mac 451. Ph.d. Thesis, McGill Univ., Quebec, Canada.
- Harris, G., and R. W. Rickets, 1962. Metabolism of phenolic compounds by yeast. Nature, **195**: 473-474.
- Hartwich, C. V., and M. Winckel, 1904. About the presence of phloroglucinol in plants. Archiv. Der Pharmazie., **242**: 462-476.
- Haslum, E., R. D. Haworth, and P. F. Knowles, 1961. Gallotannins: The biosynthesis of gallic acid. J. Chem. Soc., part-2: 1854-1859.
- Hayaishi, O., 1964a. Aromatic hydrocarbons. Plenary sessions sixth internal congr., Biochem., New York City, I. U. B., **33**: 31.
- Hayaishi, O., 1964b. Comparative studies on pyrocatechase and metapyrocatechase. In, Oxidases and Related Redox Systems. T. E. King, H. S. Mason, and M. Morrison (eds.), John Wiley and Sons, N. Y.
- Hayaishi, O., and K. J. Hashimoto, 1950. Protocatechase: A new enzyme catalyzing oxidative break down of pyrocatechin. Biochem. Tokyo, **37**: 371-374.
- Higuchi, T., 1953. Biochemical study of wood-rotting fungi and the enzymes responsible for the bavendaum reaction. J. Japan Forest Soc., **35**: 77-85.

- Hopper, D. J., 1977. Microbial degradation of aromatic hydrocarbons. In, Developments in Biodegradation of Hydrocarbons, p.85-112, R: J. Watkinson (ed.), App. Sc., London.
- Hopper, D. J., P. J. Chapman, and S. Dagley, 1968. Enzymic formation of D-malate. Biochem. J., 110: 798-800.
- Hosokawa, K., 1970. Regulation of early enzymes of p-hydroxybenzoate pathway in Pseudomonas putida. J. Biol. Chem., 245: 5304-5308.
- Hutzinger, O., and W. Veerkamp, 1981. Xenobiotic chemicals with pollution potential. In, Microbial Degradation of Xenobiotics and Recalcitrant Compounds, p.3-44, Leisinger, Hutter, Cook and Nuesen (eds.), Academic press Inc., London LTD.
- Jamieson, W. D., A. Taylor, D. K. Mathur, and A. C. Blackwood, 1970. Identification of an intermediate of phloroglucinol degradation by mass spectroscopic analysis. Can. J. Biochem., 48: 215-216.
- Jerina, D. M., and J. W. Daly, 1974. Arene oxide: An aspect of drug metabolism. Science, 185: 573-582.
- Jordan, S. D., 1897. A new synthesis of phloroglucinol. J. Chem. Soc., 71: 1106-1114.
- Kennedy, E. P., and H. A. Barker, 1951. Paper chromatography of volatile acids. Anal. Chem., 23: 1033-1034.
- Kojima, Y., N. Itada, and O. Hayaishi, 1961. Metapyrocatechase: A new catechol-cleaving enzyme. J. Biol. Chem., 236: 2223-2228.
- Krishnamurthy, H. G., K. J. Cheng, G. A. Jones, F. J. Simpson, and J. E. Watkin, 1970. Identification of products produced by the anaerobic degradation of rutin and related flavanoids by Butyrivibrio sp. C. Can. J. Microbiol., 16: 759-767.
- Larway, P., and W. C. Evans, 1967. Metabolism of quinol and resorcinol by soil Pseudomonads. Biochem. J., 95: 52.
- Löffler, J. E., and E. R. Reichl, 1953. Methods to determine organic acids by paper chromatography. Mikrochim., 1-2: 78-88.

- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 192: 265-275.
- MacDonald, D. L., R. Y. Stanier, and J. L. Ingraham, 1954. The enzymatic formation of  $\alpha$ -Carboxymuconic acid. *J. Biol. Chem.*, 210: 809-820.
- Mason, H. S., 1949. The chemistry of melanin: vi. Mechanism of the oxidation of catechol by tyrosinase. *J. Biol. Chem.*, 181: 803-812.
- Mathur, D. K., 1971. Enzymatic-degradation of phloroglucinol. Ph.d. Thesis, McGill Univ., Quebec, Canada.
- Moeller, H., 1897. Phloroglucinol in plants. *Chem. Zbit.*, 11: 1151-1152.
- Nakagawa, H., and Y. Takeda, 1962. Phenol hydroxylase. *Biochem. Biophys. Acta.*, 62: 423-426.
- Nozaka, J., and M. Kusunose, 1969. Metabolism of hydrocarbons in microorganisms. *Agr. Biol. Chem.*, 33: 962-964.
- Nozaki, M. S., K. O. Kotani, and S. Senoh, 1970. Metapyrocatechase, iii. Substrate specificity and mode of ring fission. *Biochem. Biophys. Acta.*, 220: 213-223.
- Ornston, L. N., 1966a. The conversion of catechol and protocatechuate to  $\beta$ -ketoadipate by Pseudomonas putida: i. Biochemistry. *J. Biol. Chem.*, 241: 3776-3786.
- Ornston, L. N., 1966b. The conversion of catechol and protocatechuate to  $\beta$ -ketoadipate by Pseudomonas putida: ii. The enzymes of the Protocatechuate pathway. *J. Biol. Chem.*, 241: 3787-3794.
- Ornston, L. N., 1966c. The conversion of catechol and protocatechuate to  $\beta$ -ketoadipate by Pseudomonas putida: iii. The enzymes of catechol pathway. *J. Biol. Chem.*, 241: 3795-3799.
- Ornston, L. N., 1966d. The conversion of catechol and protocatechuate to  $\beta$ -ketoadipate by Pseudomonas putida: iv. Regulation. *J. Biol. Chem.*, 241: 3800-3810.
- Ornston, L. N., and R. Y. Stanier, 1964. Mechanism of  $\beta$ -ketoadipate formation by bacteria. *Nature*, 204: 1279-1283.



- Oshima, T., 1965. On the anaerobic metabolism of aromatic compounds in the presence of nitrate by soil microorganisms. *Zeit. Fur Allg. Mikrob. Biol.*, 5: 386-394.
- Pankhurst, S. E., 1959. The biological oxidation of spent gas liquor. *J. Appl. Bacteriol.*, 22: 202-215.
- Patel, T. R., and E. A. Barnsely, 1980. Naphthalene metabolism by Pseudomonas: Purification and properties of 1,2-dihydroxynaphthalene oxygenase. *J. Bacteriol.*, 143: 668-673.
- Patel, T. R., and D. T. Gibson, 1974. Purification and properties of (+)-cis-naphthalene dihydrodiol dehydrogenase of Pseudomonas putida. *J. Bacteriol.*, 119: 870-888.
- Patel, T. R., K. G. Jure, and G. A. Jones, 1981. Catabolism of phloroglucinol by the rumen anaerobe Caprococcus. *Appl. and Environ. Microbiol.*, 42: 1010-1017.
- Pfennig, N., 1978. Rhodocyclus purpureus a ring shaped vitamin B<sub>12</sub>-requiring member of the family Rhodospirillaceae. *J. Syst. Bacteriol.*, 28: 283-288.
- Pfennig, N., K. E. Einhjellen, and S. Liaaen-jensen, 1965. A new isolate of the Rhodospirillum fulvum group and its photosynthetic pigments. *Arch. Microbiol.*, 51: 258-266.
- Proctor, M. H., and S. Selfer, 1960. Decomposition of benzoate by a photosynthetic bacterium. *Biochem. J.*, 76: 33p.
- Randerath, K., 1963. In, Thin layer chromatography. K. Randerath (ed.), Academic Press Inc., N. Y., USA.
- Reid, R. L., and N. Lederer, 1951. Separation and estimation of saturated C<sub>2</sub>-C<sub>7</sub> fatty acids by paper partition chromatography. *Biochem. J.*, 50: 60-70.
- Ribbons, D. W., and W. C. Evans, 1960. Oxidative metabolism of phthalic acid by soil Pseudomonads. *Biochem. J.*, 76: 310-318.
- Ribbons, D. W., 1966. Metabolism of o-cresol by Pseudomonas aeruginosa strain T.I. *J. Gen. Microbiol.*, 44: 221-231.
- Robern, H., 1965. The metabolism of phloroglucinol by microorganisms. Ph.d. Thesis, McGill Univ., Quebec, Canada.

- Roux, D. C., 1951. Colour reagents for the paper chromatography of di and tri-hydroxy phenol. *Nature*, 168: 1041-1042.
- Roux, D. C., and A. E. Maihs, 1960. Selective spray reagent for the identification of flavonoid compounds associated with condensed tannins. *J. Chromatog.*, 4: 65-74.
- Sala-Trepat, J. M., and W. C. Evans, 1971. The meta-cleavage of catechol by Azotobacter spp. *Eur. J. Biochem.*, 20: 400-413.
- Schink, B., and N. Pfennig, 1982. Fermentation of trihydroxy benzenes by Pelobacter acidigallici, a new strictly anaerobic, non-sporforming bacterium. *Arch. Microbiol.*, 33: 195-201.
- Senning, E., 1963. The effect of aqueous extracts of autumn leaves on the growth and activity of cellulose decomposing microorganism. *ZBL. Bacteriol. Abt. II*, 117: 13-40.
- Shipchandler, M. T., and C. A. Peters, 1975. Syntheses of gallic acid and pyrogallol. *J. Chem. Soc. Perkins Trans.*, 1: 1400-1401.
- Simpson, F. J., G. A. Jones, and E. A. Wolin, 1969. Anaerobic degradation of some bioflavonoids by microflora of the rumen. *Can. J. Microbiol.*, 15: 972-974.
- Smith, I., and M. J. Smith, 1961. Keto-acids. In, Chromatographic and Electrophoretic Techniques. p.261-271, I. Smith (ed.), Interscience Publishers Inc., New York.
- Smith, R. V., and J. P. Rosazza, 1974. Microbial models of mammalian metabolism: Aromatic hydroxylation. *Arch. Biochem. Biophys.*, 161: 551-558.
- Sohngen, N. L., 1913. Benzene, petroleum, paraffinol and paraffin as a Carbon and energy source for microbes. *Bakteriol. Parasitenk Abt. II*, 37: 595-609.
- Stanier, R. Y., and L. N. Ornston, 1973. The  $\beta$ -keto adipate pathway. *Adv. Microbial Physiol.*, 9: 89-151.
- Stanier, R. Y., B. P. Sleeper, M. Tsuehida, and D. L. MacDonald, 1950. Bacterial oxidation of aromatic compounds: iii. The enzymatic oxidation of catechol and protocatechuate to  $\beta$ -keto adipic acid. *J. Bacteriol.*, 59: 137-151.

- Stormer, K., 1908. Effect of hydrogen sulphide and similar compounds on soil, Zentralbl. Bakteriell. Parasitenkd. Infektionskr. 20: 282-286.
- Strawinski, R. J., and R. W. Stone, 1943. Conditions governing the oxidation of naphthalene and the chemical analysis of its products. J. Bacteriol., 46: 16-38.
- Szewzyk, V. R., R. Szewzyk, and B. Schink, 1985. Methanogenic degradation of hydroquinone and catechol via reductive dehydroxylation to phenol. Microbiol. Ecology, 33: 78-87.
- Tarvin, R., and A. M. Buswell, 1934. The methane fermentation of organic acids and carbohydrates. J. Am. Chem. Soc., 56: 1751-1755.
- Treccani, V., N. Walker, and G. H. Wiltshire, 1954. The metabolism of naphthalene by soil bacteria. J. Gen. Microbiol., 11: 341-348.
- Tsai, C. G., D. M. Gates, W. M. Ingledew, and G. A. Jones, 1976. Product of anaerobic phloroglucinol degradation by Caprococcus sp. Pe<sub>5</sub><sup>1,2</sup>. Can. J. Microbiol., 22: 159-164.
- Tsai, C. G., and G. A. Jones, 1975. Isolation and identification of rumen bacteria capable of anaerobic phloroglucinol degradation. Can. J. Microbiol., 21: 794-801.
- Waekett, L. P., and D. T. Gibson, 1982. Metabolism of xenobiotic compounds by enzymes in cell extract of the fungus Cunninghamella elegans. Biochem. J., 205: 117.
- Wagner, R., 1914. Benzene utilizing bacteria. Z. garungsphysiol., 4: 289-319.
- Wheelis, M. L., and R. Y. Stanier, 1971. The genetic control of dissimilatory pathways in Pseudomonas putida. Genetics Princeton, 68: 245-266.
- Whittle, P. J., D. O. Lunt, and W. C. Evans, 1976. Anaerobic photometabolism of aromatic compounds by Rhodospseudomonas sp.. Biochem. Soc. Trans., 4: 490-491.
- Wieland, T., G. Griss, and B. Haccius, 1958. Observations about microbial benzene oxidation. Arch. microbiol., 28: 383-396.
- Williams, R. J., and W. C. Evans, 1973. Anaerobic metabolism of aromatic substrate by certain microorganisms. Biochem. Soc. Trans., 1: 186-187.

- Williams, R. J., and W. C. Evans, 1975. The metabolism of benzoate by Moraxella spp. through anaerobic nitrate respiration. Biochem. J., **148**: 1-10.
- Yashida, H., Y. Tani, and H. Yamada, 1982. Isolation and identification of a pyrogallol producing bacterium from soil. J. Biol. Chem., **46**: 2539-2546.
- Yashida, H., and H. Yamada, 1985. Microbial production of pyrogallol through decarboxylation of gallic acid. Agric. Biol. Chem., **49**: 659-663.
- Young, L. Y., 1984. Anaerobic degradation of aromatic compounds. In, Microbial Degradation of Organic Compounds. p.487-523, D. T. Gibson (ed.), Marcel Dekker, N. Y.







