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Title: Cometabolic Degradation of Chlorophenolic Compounds.

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Kenneth J. Williamson

Cometabolic aerobic degradation of chlorophenolic compounds with phenol as the primary substrate was examined. A novel batch reactor with a syringe-pump feed was constructed to determine reaction kinetics. A mathematical model was developed to describe the biological processes of electron donor metabolism, competition, inhibition, and uncoupling. All experiments were conducted at 31°C.

Phenol was used as the primary substrate to support cells growth and cometabolic degradation of eight chlorophenols: 4-chlorophenol (4-CP), 2,4-dichlorophenol (2,4-D), 3,4-dichlorophenol (3,4-DCP), 3,5-dichlorophenol (3,5-DCP), 2,3,5-trichlorophenol (2,3,5-TCP), 2,4,5-trichlorophenol (2,4,5-TCP), 3,4,5-trichlorophenol (3,4,5-TCP) and 2,4,6-trichlorophenol (2,4,6-TCP). The chlorophenols represent the major degradation products from the anaerobic dechlorination process of pentachlorophenol (PCP). Aerobic degradation rates of the chlorophenols and phenol were estimated by fitting the model to laboratory results.

The maximum phenol utilization rate and the maximum chlorophenol inhibition utilization rates per unit mass of cells (*mole/g-day*) were determined using fourth-order Runge-Kutta methods. Based on the model, the relative rates of degradation were: phenol > monochlorophenol > dichlorophenols > trichlorophenols. The degradation rates for dichlorophenols were in the order:

3,5-DCP > 2,4-DCP > 3,4-DCP, and for trichlorophenols: 2,4,5-TCP > 2,4,6-TCP > 3,4,5-TCP > 2,3,5-TCP.

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COMETABOLIC DEGRADATION OF CHLOROPHENOLIC COMPOUNDS

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CHAPTER 1
INTRODUCTION

Environmental contamination from xenobiotic organic compounds is a serious world-wide problem. Proven technologies for successful treatment include incineration or chemical treatment; however, these methods are costly and may even create associated harmful byproducts and waste streams (Steiert *et al*, 1985). As such, additional innovative treatment approaches are being intensively studied by a large number of researchers.

One method of interest is microbial biodegradation through which organic chemicals can be transformed into a wide range of new forms, many of which are less harmful to the environment. Biodegradation under aerobic, anaerobic or anoxic conditions has been extensively studied for a large number of xenobiotic compounds and has proven to be a promising approach.

The ability of a compound to be biodegraded and mineralized is largely determined by the degree of structural analogy between the synthetic compound and a natural compound for which catabolic functions exist (Knackmuss, 1981). Mineralization to carbon dioxide, water, and various inorganic forms and biodegradation to an alternate reduced or oxidized form are not synonymous; however, scientists often assume identity (Grady Jr., 1985).

Biodegradation is often limited in relation to many xenobiotic compounds. Industrial research adds approximately 200,000 new chemicals each year to the millions already used by advanced industrial nations (Piruzyan, *et al*, 1980). Many of these compounds bear no resemblance to natural compounds that microorganisms have developed enzymatic systems to degrade. As a result, long time periods may be required for recalcitrant compounds.

In addition, biodegradation can alter an innocuous chemical into a toxic compound, convert a readily metabolizable compound into a compound that is difficult to destroy, or alter the toxicity of a chemical so that it acts against another organisms (Grady Jr, 1985). In these cases, a partial metabolites may be more toxic than the primary pollutant (*i.e.* some transformation products are more hazardous than the parent compounds). Typical examples are the transformation of trichloroethene (TCE) to vinyl chloride (VC) and the transformation of 1,1,1-trichloroethane (TCA) to 1,1-dichloroethene (1,1-DCE). Both metabolites, VC and 1,1-DCE, are more toxic than their precursors, TCE and TCA, respectively (McCarty, *et al*, 1993). Thus, there should be some considerations necessary when evaluating the appropriateness of biodegradation for the removal of hazardous wastes.

Nevertheless, biodegradation has great potential in remediating contaminated waters and soils. Biodegradation of hazardous chemicals has been conducted on scales ranging from bench to field scale. Both single organism cultures and natural mixed microbial populations have been employed for the removal of single and multiple substrates. Multiple compounds have been found to interact to enhance (synergism) or reduce (antagonism) degradation. Biodegradation of xenobiotic organics has been investigated in groundwater, soils, traditional biological treatment reactors, and even by rumen bacteria under aerobic or anaerobic conditions (Autenrieth, *et al*, 1991; Wang, 1991; Nicholson, *et al*, 1992; McCarty, *et al*, 1993; Lee, 1994; and Peters, 1994).

An important class of xenobiotic compounds are chlorinated aromatics and their derivatives, most of which have been found to be recalcitrant in the environment. Generally speaking, lightly chlorinated compounds such as chlorobenzene, dichlorobenzene, chlorinated phenols and the lightly chlorinated PCBs are typically biodegradable under aerobic conditions. The more highly chlorinated analogues are more recalcitrant to aerobic degradation, but more susceptible to degradation under anaerobic conditions (Kerr, 1993). Specifically,

chlorinated phenols have been shown to be susceptible to both aerobic and anaerobic degradation (Autenrieth, *et al*, 1991; Nicholson, 1990; Wang, 1991; Peters, 1994; Valli, *et al*, 1991; Golovleva, *et al*, 1993; Menke *et al*, 1992; Saez, *et al*, 1991; and Li *et al*, 1991). Both pure and mixed cultures have been employed.

One promising approach to the treatment of chlorinated phenols is aerobic degradation using phenol as a primary substrate. These phenol-degrading organisms have been shown to contain powerful mono- and di-oxygenase enzymes capable of breaking the benzene ring. Such an approach appears to be capable with attacking the lower chlorinated phenols that are resistance to anaerobic degradation.

The objectives of this research were:

1. to determine the rates of aerobic cometabolic degradation of various chlorophenol metabolites of anaerobic dechlorination using phenol as the primary substrate (Figure 1.1);

2. to develop a model to describe this degradation which includes the processes of electron donor metabolism, competition, inhibition, and uncoupling.

The results of this investigation are expected to assist understanding of the degradation of these compounds in complex aerobic/anaerobic environment such as contaminated groundwater and soil. Since the biodegradability and other properties (*e.g.* susceptibility, mobility, toxicity, solubility, and lipophilicity) of a series of related compounds are related to structure, these results may assist with the understanding the fate and transport of other halogenated aromatics in the environment.

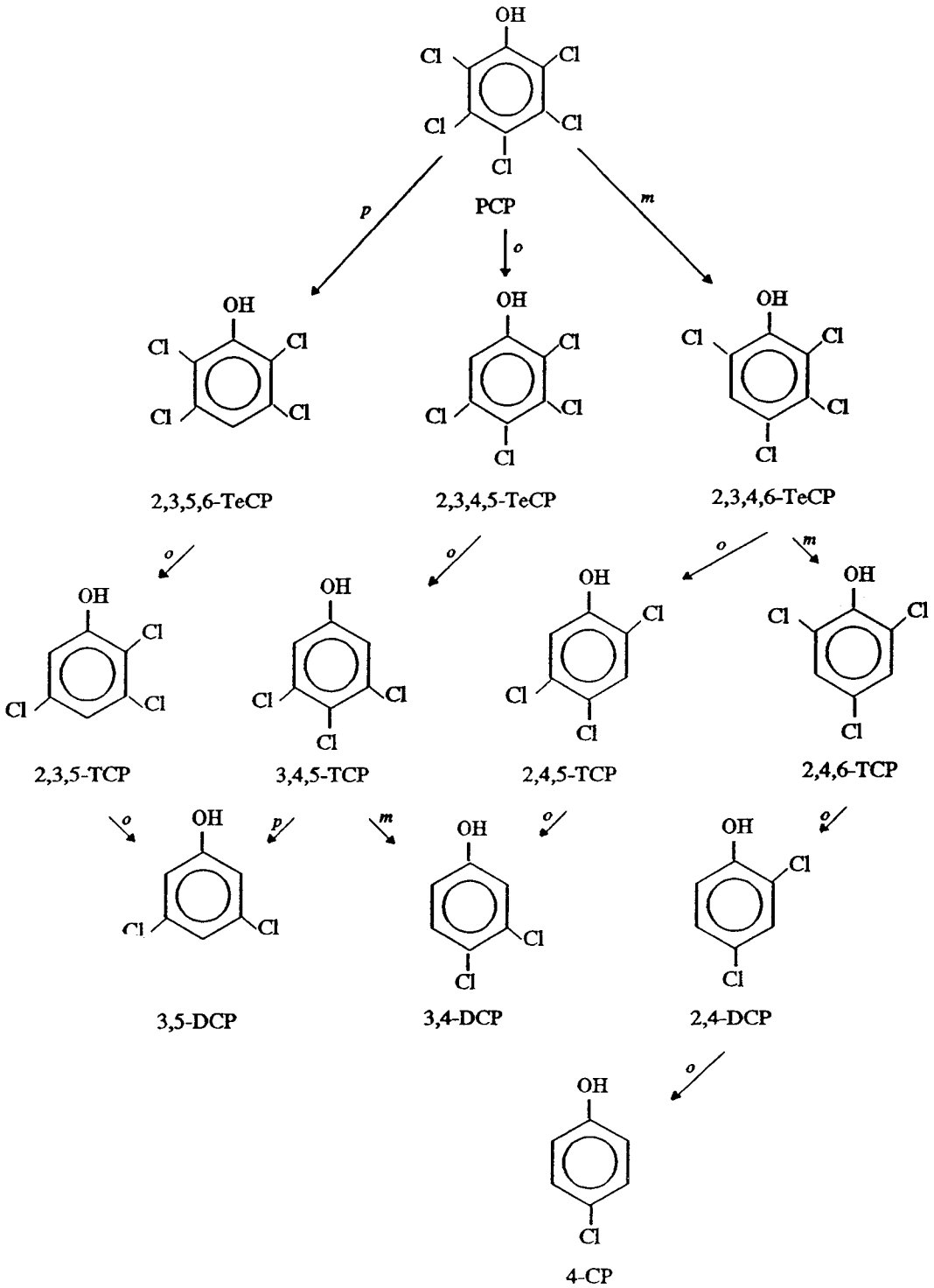

Figure 1.1 Degradation Pathway of PCP (Adopted from Nicholson, 1990)

CHAPTER 2
LITERATURE REVIEW AND BACKGROUND

2.1 Chlorinated Phenols and Their Roles

The large scale synthesis of industrially important chlorinated aromatics, like chlorinated phenols, has occurred only during the past few decades. The chlorinated organics and many of their end-products represent one class of xenobiotic and persistent compounds. Many of these organic chemicals exhibit not only acute toxicity, but also chronic toxic effects including mutagenic, carcinogenic, and teratogenic manifestations (Autenrieth *et al*, 1991).

Chloroaromatic compounds are used extensively in industry and agriculture with subsequent releases of polychlorophenols to the environment (Valli, *et al*, 1991). Pentachlorophenol (PCP), which has an annual world-wide production of over 500,000 tons (Steiet, *et al*, 1985), is the second most heavily used pesticide in the United States. PCP has been used extensively as a slimicide for cooling tower water, and in the production of adhesives, textiles, paper, leather, paint, and construction materials (Wang, 1991). The most significant source of PCP-containing wastewaters is the wood preserving industry. PCP exhibits strong toxicity due to its ability to decouple oxidative phosphorylation (Weinbach, 1957) and because of this effect, it has been listed as one of the U.S. Environmental Protection Agency's priority pollutants (Moos, *et al*, 1983).

Chlorinated phenols and their derivatives are also used extensively as precursors in the production of pesticide formulation, especially 2,4-dichlorophenoxyacetic acid (2,4-D), and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Wang, 1991). Chlorophenols are major intermediates of phenoxyalkanoate herbicides and other pesticides (Bollag and Liu, 1985). 2,4,6-trichlorophenol (2,4,6-TCP) is used as a preservative of leather and textile goods, as a glue and wood preservative, and as an antimildew agent.

Chlorophenols have several important sources in addition to industrially produced chemicals. A variety of chlorophenols are generated from the microbial degradation of numerous pesticides including 2,4-D, 2,4,5-T and other phenoxyalkanoate herbicides, pentachloronitronbenzene, hexachlorobenzene and indane (Ahlborg *et al*, 1980). Chlorophenols are also formed during the bleaching of pulp and paper when chloroaromatic compounds are used as biocides (Golovleva *et al*, 1993).

There is a total of 19 possible chlorinated phenols (Table 3.1) and all are commercially available. Of the six isomers of trichlorophenols, both 2,4,5-TCP and 2,4,6-TCP are considered EPA priority pollutants (Joshi *et al*, 1993). These compounds are quite toxic, and their toxicity tends to increase with their degree of chlorination (Steiert *et al*, 1987)

2.2 Fate and Biodegradation of Chlorophenols

Synthetic organic compounds are commonly found in industrial wastewater streams and can produce toxic effects to wastewater treatment units. The resulting decrease in treatment efficiency may allow substantial amounts of these compounds to be discharged in effluents or concentrated in sludges (Autenrieth *et al*, 1991). For example, the effluent from pulp mills contains several chlorophenols as by-products of the bleaching process. Another mechanism for release of chlorophenols to the environment is the reaction of unsubstituted phenols in dilute aqueous solutions with chlorine in both water and wastewater treatment processes. Many of the chlorophenols tend to persist in the environment, and can be public health hazards.

Biodegradation represents an important process for the elimination of chlorinated organics. Much research on microbial process capable of degrading the chlorophenols have been done under aerobic, anaerobic, or anoxic conditions (Saez *et al*, 1991; Autenrieth *et al*, 1991; Valli *et al*, 1991; Nicholson, 1991; Wang

1991; Menke *et al*, 1992; and Golovleva, *et al*, 1993). Biodegradation of these compounds has been studied in soil, ground water, traditional activated sludge units, fixed film reactors and combinations of process (Autenrieth *et al*, 1991).

Considerable research has been conducted concerning both aerobic and anaerobic biodegradation of PCP. Nicholson (1990) found that the anaerobic biodegradation of PCP by a mixed culture could produced three tetrachlorophenols (TeCPs), 2,3,5,6-TeCP, 2,3,4,5-TeCP, and 2,3,4,6-TeCP, four trichlorophenols (TCPs), 2,3,5-TCP, 3,4,5-TCP, 2,4,5-TCP, and 2,4,6-TCP, three dichlorophenols (DCPs), 3,5-DCP, 3,4-DCP and 2,4-DCP, and one monochlorophenol (CP), 4-CP. He also found that degradation of the chlorinated phenols followed zero-order reaction kinetics. Dechlorination is dependent on the maximum substrate utilization rate unique for each compound and the mass of active dechlorinating organisms present.

Aerobic biodegradation of the eight chlorophenols identified by Nicholson (1990) (2,3,5-TCP, 2,4,5-TCP, 2,4,6-TCP, 3,4,5-TCP, 2,4-DCP, 3,4-DCP, 3,5-DCP and 4-CP) for a mixed culture with acetate as primary substrate was studied by Wang (1991). He found that the relative rates for the degradation of chlorinated phenols were general as follows: monochlorophenol > dichlorophenols > trichlorophenols; 2,4-DCP and 3,4-DCP > 3,5-DCP; and 2,4,6-TCP >> 2,3,5-TCP, 2,4,5-TCP and 3,4,5-TCP.

Moos *et al* (1983) tested the aerobic biodegradation of PCP in a three phase protocol, determined the kinetics, assessed the importance of volatilization and sorption in PCP removal and evaluated the extent of biodegradation. They found that PCP could undergo ultimate biodegradation with first order kinetics. Additional studies suggested that the full relationship between the PCP degradation rate and the concentration followed an inhibition-type function with the maximum rate occurring at a PCP concentration of about 350 μg/l. Radioisotopic studies revealed that PCP was mineralized, with the liberation of CO_2 and incorporation of carbon into cell material. Neither volatilization nor sorption removed significant amounts of PCP from the reactors.

Menke *et al* (1992) investigated the biodegradation of three isomeric monochlorophenols, 2-CP, 3-CP, 4-CP, and phenol by the constructed strain *Alcaligenes* sp. A7-2. It was found that mineralization took place in the order: phenol > 4-CP > 2-CP > 3-CP, whereas 3-CP was mineralized only cometabolically. In substrate mixtures with phenol, degradation of 4-CP was decelerated, but degradation of 2-CP was accelerated.

Li *et al* (1991) isolated an *Azotobacter* sp. which utilized 2,4,6-TCP as a sole source of carbon and energy. Resting cells transformed monochlorophenols, 2,6-DCP, and 2,3,6-TCP, and TCP was completely mineralized based upon generation of released 3 mole of Cl^-/mole TCP. TCP degradation was significantly faster in shaken than in non-shaken cultures, and the optimum temperature for degradation was 25 to 30 °C. Induction studies revealed that TCP induced TCP degradation, but not phenol degradation, and that phenol induced only its own utilization.

Degradation of 2,4,5-TCP by the lignin-degrading basidiomycetes *Phanerochaete chrysosporium* was studied by Joshi *et al* (1993), and its pathway was elucidated by the characterization of fungal metabolites and oxidation products generated by purified lignin peroxide and manganese peroxidase. They pointed out that the degradation of 2,4,5-TCP proceeded via quinone intermediate pathway, which resulted in the removal of all three chlorine atoms before ring cleavage occurred.

Kinetic experiments were performed by Saez *et al* (1991) using batch reactors containing a pure culture of *Pseudomonas putida* PpG4 and 4-chlorophenol as the only organic substrate present. They reported that 4-chlorophenol behaved as a cometabolite because its transformation, possible only with cells previously grown on phenol, did not yield any increase in cell mass and the 4-chlorophenol-transformation rate was controlled by the 4-chlorophenol/biomass (*I:X*) ratio. They found that for a low *I:X* ratio, the system was uninhibited, complete 4-chlorophenol transformation was achieved and the 4-

chlorophenol-transformation rate was proportional to biomass-oxidation rate; for high *I:X* ratios, however, the system was inhibited by 4-chlorophenol itself. They suggested that the link in the rates was probably because the electrons consumed during the 4-chlorophenol transformation were produced by way of biomass oxidation.

Phenolic biodegradation kinetics were determined by Authenrieth *et al* (1991) in bioreactors with large solid retention times. They reported that phenol can be metabolized as high as 50 mg/l with no inhibition and pentachlorophenol can only be cometabolized in the presence of phenol. They concluded that biodegradation of phenolic waste is a viable treatment option because the organisms, through their metabolic processes, reduced the waste concentration below their detection limits.

Banerjee *et al* (1984) investigated the biodegradation rate of some chlorophenols and related compounds by pure as well as mixed cultures and developed a general kinetic model for the biodegradation. They found that in most cases the rates decreased with increasing lipophilicity (1-octanol-water partition coefficient, K_{ow}) of the substrate. They interpreted their results based on a reaction mechanism where penetration of the compound into the organism is rate determining and concluded that lipid penetration is dominant for the more lipophilic compounds.

2.3 Category of Biodegradable Organics and Cometabolites

Generally, organic chemicals are classified as biodegradable, persistent or recalcitrant. Some of the xenobiotic organics are often recalcitrant. The biodegradable organic compounds can be further divided into two categories based on the amount of energy consumed by organisms during their biotransformation.

The organics in the first category, which are also called primary substrates, provide both carbon and energy for cell growth and maintenance with

available nutrients when they are oxidized. However, the pollutants in the second category, which are called secondary substances, and supply no or negligible energy for cell synthesis and maintenance; therefore, as the only organics, the secondary compounds can not support cell growth or the growth can be negligible even with available nutrients present. A common name, cometabolite, was given to the secondary substances which are entirely incapable of supplying energy and carbon. Namely, in a cometabolic process, cometabolism, a secondary compound undergoing degradation does not support cell growth (Banerjee *et al*, 1984).

Cometabolism has been defined by Dalton and Stirling (1982) as "the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound". Two key concepts associated with definition were emphasized. First, a non-growth substrate means one that will not support cellular division. Second, transformation will take place only in the obligate presence of a growth substrate. It should be pointed out that a secondary substrate need not be a cometabolite because the presence of a secondary substrate at a low enough concentration can supply negligible energy to benefit for the cells (Saez *et al*, 1991).

In addition to cometabolism, fortuitous or gratuitous metabolism is often used to explain the biodegradation of xenobiotic compounds. The gratuitous metabolism is defined as appreciation of existing enzyme, which happens to have suitable catalytic activity towards a novel substrate and is thought to be a major mechanism whereby bacteria attack xenobiotic compounds (Grady, Jr, 1985).

Whether a xenobiotic compound can be degraded by gratuitous metabolism depends on numerous factors. It will largely depend on the structural similarity between the xenobiotic compound and the natural substrate. The similarity includes numbers, type, and position of substituents. Another important factor is the nature of the products generated from enzymatically catalyzed reaction because the reaction products could be more toxic than the original compound to the organism carrying out the reaction or to other organisms within the system. *i.e.* the products could inhibit or inactivate the

metabolism. (Ely, *et al*, 1994).

On the other hand, however, if the transformation products of a xenobiotic chemical are benign and can be acted on by another enzyme, they will be further transformed and even can provide energy to support the first step. Accordingly, the products will not build up in the media. In this case, the chemical can be used as a sole energy and carbon source because the energy is required by the first gratuitous reaction can be provided by the consequent degradative steps (Grady, Jr., 1985).

Apparently, degradation of the cometablic product will not supply enough energy to the first reaction due to the very nature of cometabolism if a xenobiotic compound is cometabolized by a pure culture. Consequently, the product will accumulate.

2.4 Enzymes and Their Inhibitors

Enzymes are protein molecules that are capable of catalyzing specific chemical reactions. Specificity of enzymes is frequently very high, and the specificity of an enzyme is determined primarily by its structure. As protein, enzymes are composed of long chains of amino acids which are connected in specific and highly precise ways. It is the amino acid sequence on enzyme that determines its structure as well as its catalytic specificity (Brock and Madigan, 1991).

Autenrieth *et al* (1991) pointed out that enzymes are usually specific to their catalytic functions but they are much less specific to substrate binding. In other word, enzymes are highly specific with respect to their catalytic function, which requires a similar electronic make-up of both the natural substrate and the xenobiotic. As a result, enzymes can be induced to cleave a certain chemical bond on a xenobiotic compound that resembles the functional groups of a natural substrate. According to Grady (1985), if the functional groups do not greatly alter the charge make-up of the enzyme active site, then it is possible for the

enzymes to catalyze its specific reaction on the xenobiotic compound.

Any substrate that reduces the velocity of an enzyme-catalyzed reaction can be considered to be an "inhibitor". The inhibition of microbial growth is often due to enzyme system. An inhibitor reduces the rate by binding either for the free enzyme and/or for the enzyme-substrate complex. From inhibition studies, the specificity of an enzyme, the physical and chemical architecture of the active site, and the kinetic mechanism of the reaction can be determined.

Three types of models are most widely used to explain cell growth inhibition: competitive, noncompetitive, and uncompetitive. A competitive inhibitor is a chemical that combines with free enzyme in a manner that prevents substrate binding. That is, in a competitive mechanism, the inhibitor and the substrate are mutually exclusive, often due to competition for the same site. A classical noncompetitive inhibitor has no effect on substrate binding and vice versa. A substrate and an inhibitor bind reversibly, randomly, and independently at different sites. That is, the inhibitor can combine with both the free cell or enzyme and the cell/enzyme-substrate complex. However, an uncompetitive inhibitor binds with the cell/enzyme-substrate complex which cannot undergo further reaction to yield product.

2.5 Degradative Pathways

Several articles have reported the aerobic degradative pathways of both phenol and chlorophenols (Valli *et al*, 1991; Autenrieth *et al*, 1991; Haggblom *et al*, 1988; Joshi *et al*, 1993; and Knackmuss, 1981). The aerobic catabolism of phenolic compounds by microorganisms generally requires the participation of oxygenases, the enzyme that can incorporate atmospheric oxygen into their substrate. Actually, mono- and dioxygenases are the most important enzymes in the degradation of phenolic compounds (Figure 2.1) because they are involved in the ring opening of aromatic compounds and in their activation by forming dihydroxybenzene derivatives (Winter *et al*, 1992).

A common pathway for the aerobic degradation of phenol consists of two steps: consumption of oxygen, *i.e.* the insertation of a hydroxyl group to the phenolic ring to form a catechol, which is an intermediate metabolic product for most phenolic compounds, and the fission of the ring, *i.e.* the catechol generated is further subject to ring fission, yielding carboxylic acids which may enter TCA cycle (Figure 2.1).

Halogen substituents in aromatic ring withdraw electrons from benzene nucleus, resulting in deactivation toward electrophilic attack by dioxygenase. This hinders the introduction of a second hydroxyl group *ortho-* or *para-* to an already existing one. The deactivating effect increases with the number of halogen substituents resulting in highly halogenated phenols being resistant to aerobic biodegradation (Steiet and Crawford, 1985).

Although the chlorine atoms on the ring have deactivating effects, some microorganisms can still degrade chlorinated phenols. For example, monooxygenases can even insert one oxygen atom derived from molecular oxygen into pentachlorophenol, which is chlorinated in all available positions. The initial step in the degradation of PCP is catalyzed by a monooxygeanse with simultaneous hydrolase activity and one chlorine anion is released by the para-hydroxylation of PCP (Winter, 1992). Apparently, the insertation of a hydroxyl group to an aromatic ring by an aerobic process is totally different from the nucleophilic aromatic substitution, in which electron withdrawal causes activation, and electron release causes deactivation (Morrison and Boyd, 1980).

For the aerobic biodegradation of chlorinated phenols, two different mechanisms have so far been described (Li, *et al*, 1991). Degradation of mono- and dichlorophenols were generally initialized by oxygenation into chlorocatechols, and dechlorination occurred only after ring fission of the chloricatechols. However, a different mechanism has been used to describe the polychlorinated phenols. For example, the degradation of pentachlorophenol started by hydrolytic *para*-hydroxylation, yielding chlorinated *para*-hydroquinone. Pentachlorophenol was first converted into tetrachlorohydroquinone, which was

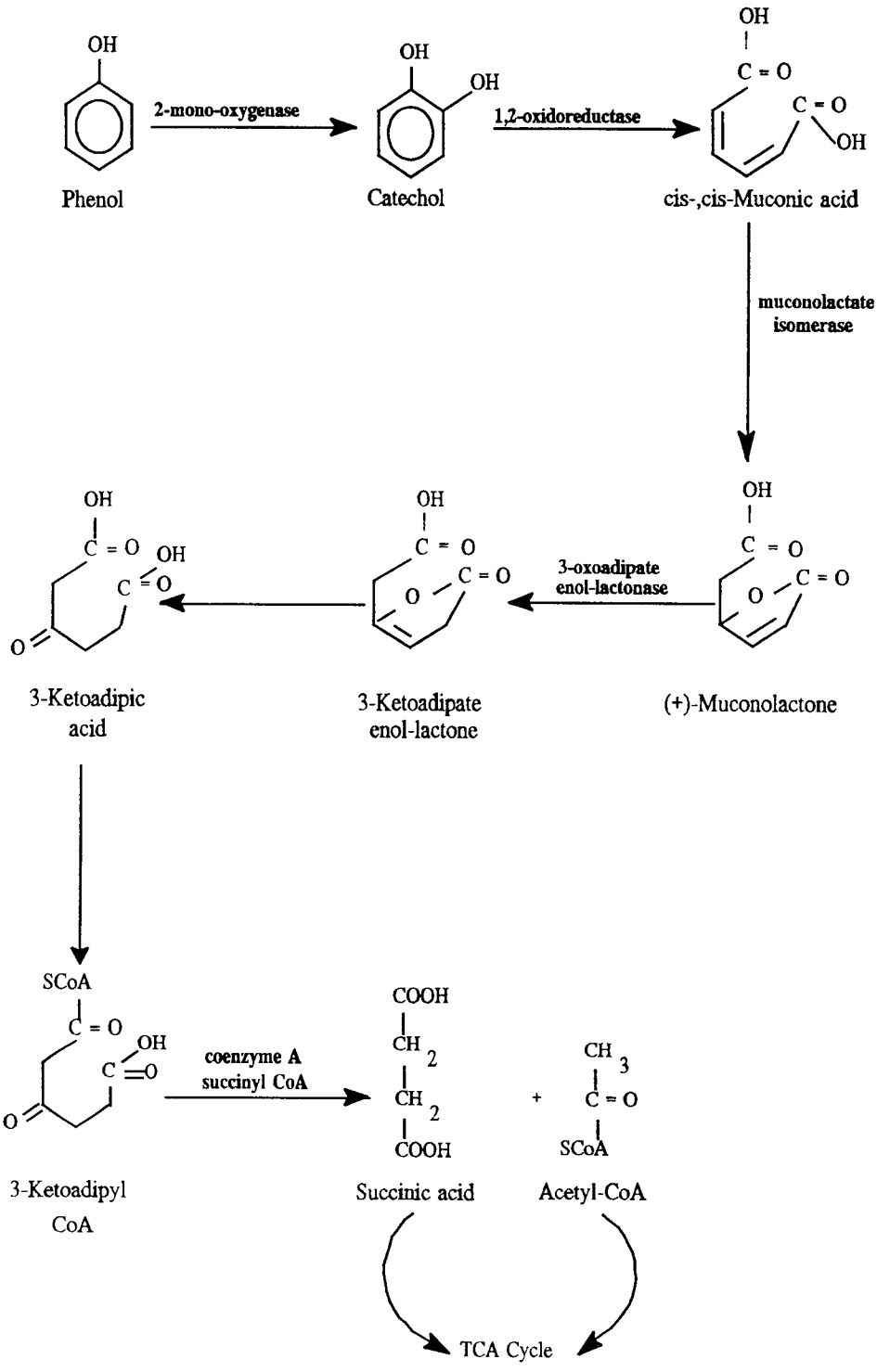

Fig. 2.1 Ortho-cleavage Pathway of Phenol (Adopted from Autenrieth *et al*, 1991)

initially detected characterized by Schenk *et al* (1989). Li *et al* (1991) reported that the degradation of trichlorophenol also proceeded via hydroquinone, whereas phenol is hydroxylated to catechol. Thus, they concluded that in one organism, both of the main routes for the dissimilation of phenolic compounds, the catechol pathway for phenolic degradation and the hydroquinone pathway for chlorinated phenol degradation, are encountered.

The pathway by which the fungus *Phanerochaete chrysosporium* degraded 2,4-DCP has been characterized in detail for the first time by Valli (1991). It was shown that the pathway of 2,4-DCP involves several cycles of oxidation and subsequent quinone reduction and hydroquinone methylation, leading to the removal of both chlorine atoms from the substrate before the cleavage of the phenolic ring occurs (Figure 2.2).

Joshi *et al* (1993) reported that under secondary metabolic conditions, the white rot basidiomycetes *Phanerochaete chrysosporium* rapidly mineralizes 2,4,5-TCP. The pathway for degradation of 2,4,5-TCP was elucidated by the characterization of fungal metabolites and oxidization products generated by purified lignin peroxidase (LiP) and manganese peroxide (MnP). The multistep pathway involves cycles of peroxidase-catalyzed oxidative dechlorination reactions followed by quinone reduction. A key intermediate 1,2,4,5-tetrahydroxybenzene, which is presumably ring cleaved, is generated. This pathway suggested that the removal of all three chlorine atoms takes place before ring cleavage occurs (Figure 2.3).

It is evident that in the aerobic processes, less-chlorinated phenols, such as some mono- and dichlorophenols, are first subject to phenolic ring fission before any chlorine atoms are removed. However, for highly chlorinated phenols (*e.g.* PCP) at least some of the chlorine atoms must be replaced by hydroxyl group prior to ring cleavage since chlorine-mediated ring deactivation probably would be sufficient to prevent the ring-opining reactions (Steiert *et al*, 1985).

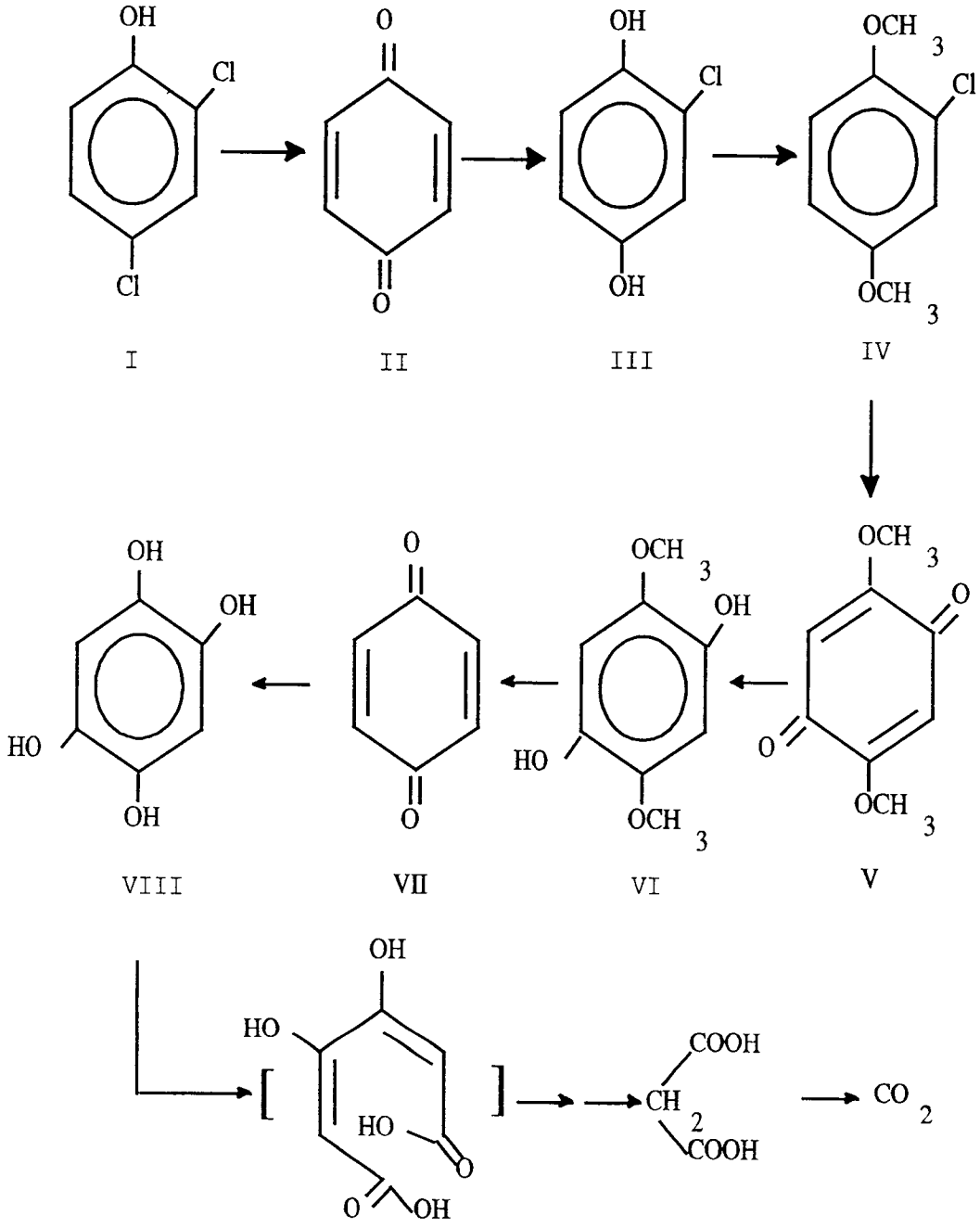

Fig. 2.2 Proposed Pathway of 2,4-DCP (Adopted from Valli *et al*, 1991)

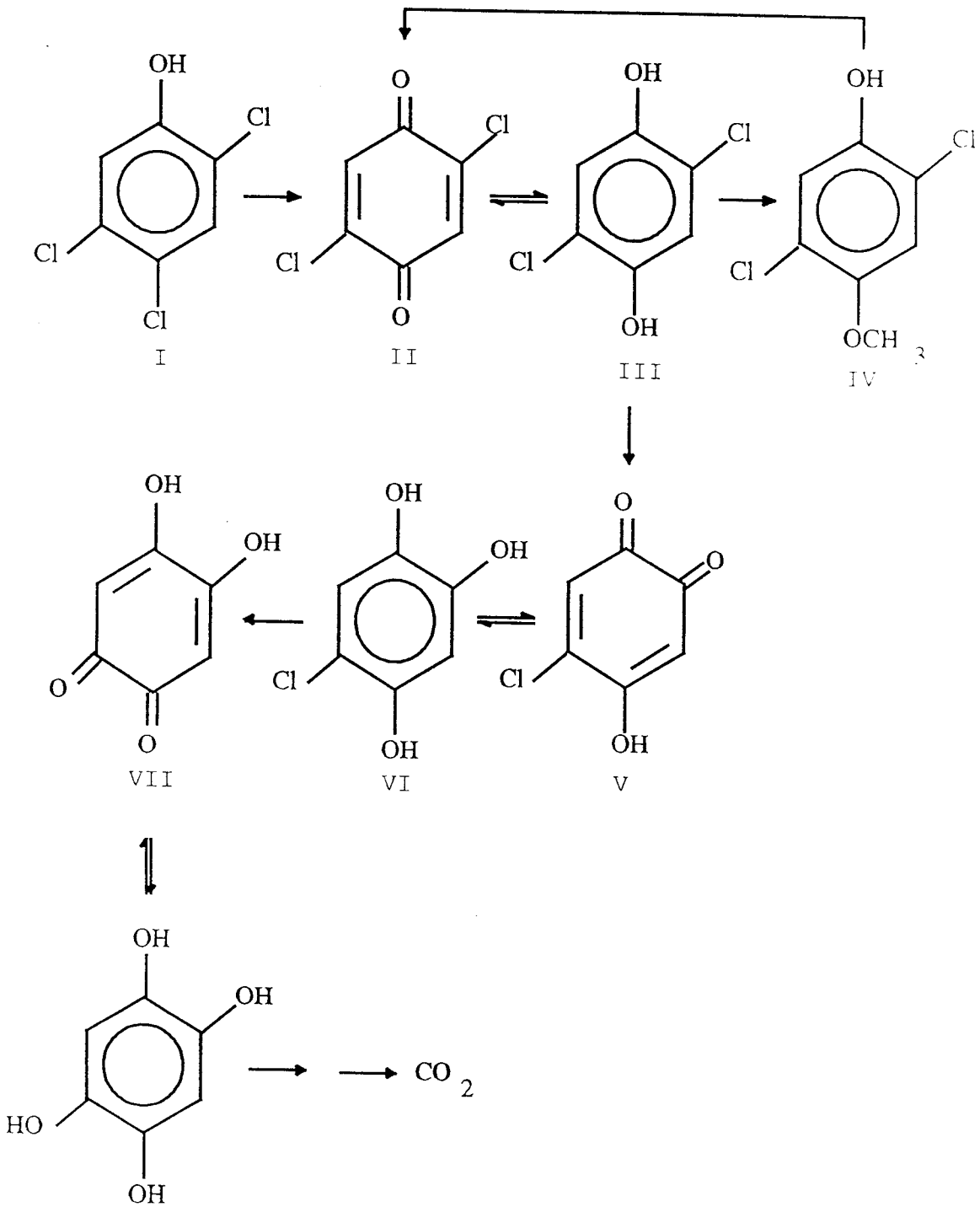

Fig. 2.3 Proposed Pathway of 2,4,5-TCP (Adopted from Joshi *et al*, 1993)

2.6 Oxidative Phosphorylation and Uncoupling Processes

Aerobic biodegradation of an organic is a process in which the organic is oxidized into carbon dioxide and water. The transformation of the organic compound to carbon dioxide and water is made up of many reactions, each of which is catalyzed by its own enzyme system. The energy system for a biological process is dependent upon the production of chemical energy from the interaction of the many chemical reactions and chemical compounds (Morison and Boyd, 1973).

Bacteria can derive the energy they need for growth from a considerable number of diverse and varied reactions, and the particular reactions utilized by a given organism can change depending upon the growth condition employed (Haddock and Jones, 1977). The conservation of energy for those reactions can be represented by the formation of adenosine 5'-triphosphate (ATP). Bacteria synthesize ATP in two ways. The first one is the formation of ATP by substrate level phosphorylation and the general expressions are as follows:

(1) ADP + substrate-P <--> ATP + substrate

(2) ADP + P_i + substrate-X <--> ATP + substrate + X

where ADP is adenosine 5'-diphosphate, and P_i is inorganic phosphate. The second one is the synthesis of ATP in bacteria by oxidative. In this case, ATP synthesis is coupled to electron transport reactions which, in turn can be driven by the oxidation of both organic compounds (in organoheterotrophs) and inorganic ions (in chemolithotrophs) of negative redox potential, linked to the reduction of electron acceptors of more positive redox potential (Haddock and Jones, 1977). A combination of the utilization of oxygen and the phosphorylation of ADP is known as *oxidative phosphorylation* (McGilvery and Goldstein, 1979). A fundamental feature of the oxidative phosphorylation is that only a few

coenzyme are used as oxidizing agents for a wide variety of organic compounds. The oxidative phosphorylation results in the generation of ATP from ADP and P_i in the inner membranes.

According to McGilvery and Goldstein, most of the oxygen consumed by an organism is used for oxidative phosphorylation. The oxidation is coupled to phosphorylation. If electron transfers is somehow dissociated from phosphorylation, the supply of ATP will be impaired by the uncoupling. The chemicals that cause the uncoupling are called uncouplers. The characteristic of the uncouplers are to increase oxygen utilization and reduce synthesis. The typical uncoupler is 2,4-dinitrophenol. PCP and some less chlorinated phenols were also reported to be uncouplers (Okey and Stensel, 1992).

Uncoupling results from sorption of the uncoupler into the cell membrane and subsequent transport of H^+ ions across the membrane by a "bridge" formed by the uncoupler. Phenolic compounds are excellent uncouplers because of their ability to transport H^+ ions in their disassociated form. The substituted phenols (such as nitrophenol) are excellent uncouplers because of their large K_{ow} values which results in high sorption into the cell membrane.

Worden, *et al.*, (1991) has shown that phenol is capable of uncoupling phosphorylation in *Bacillus stearothermophilus*. A 50% uncoupling was achieved at phenol concentration from 3 to 4 *mg/L*.

Uncoupling of oxidative phosphorylation can be distinguished from inhibition of oxidative phosphorylation in the following way: uncoupling cause an increased oxygen consumption without increasing utilization of ATP. Inhibition, however, diminishes oxygen consumption in normal coupled mitochondria. Apparently, uncoupling is a process in which "uncouplers" uncouple oxidation from phosphorylation, and convert the catabolism of substrates into a profitless undertaking. In other words, substrates undergoing uncoupling do not support cell growth.

2.7 Summary

Aerobic oxidation of phenol and chlorophenols has been reported to be a complex co-metabolic process involving primary substrate oxidation, cometabolic oxidation, cometabolic inhibition, substrate inhibition, and primary and cometabolic uncoupling. Further application of the use of phenol as a primary substrate for the remediation of chlorophenol requires greater understanding of the relative influence of each of these processes.

CHAPTER 3
CHEMISTRY OF THE RELATED PHENOLIC COMPOUNDS

3.1 Physical and Thermodynamic Properties

Phenols are compounds with a hydroxyl group (*-OH*) attached directly to an aromatic ring. The simplest phenol with one *-OH* group directly attached to the benzene ring has a high boiling point due to hydrogen bonding and is somewhat soluble in water (about 90 grams per liter) probably because of hydrogen bonding with water. Due to the conjugation between *-OH* and benzene ring, phenols are fairly acidic compounds and most phenols have K_a's in the neighborhood of 10^{-10} (Morrison *et al*, 1980).

Substitution of phenols can significantly change the properties. For example, adding a chlorine will raise the boiling point by increasing its molecular weight. Because chlorination (where the relatively electropositive hydrogen is substituted by the relatively electronegative chlorine) alters the electron make-up of the molecule, adding chlorine to phenol affect its reactivity and raises its acidity by withdrawing electrons and stabilizing the phenolic anion. The chlorination, both the number of chlorine and the position of substitution, also changes the free energy of formation (ΔG^o_f) (Table 3.1). It can be seen that 2-CP has a much lower boiling point than its isomers (3-CP and 4-CP), presumably because an intramolecular hydrogen bonding can be formed in 2-CP.

3.2 Reactivity and Orientation

Due to the special resonance structure, the benzene ring is stable and prefers to undergo substitution rather than addition, even though it is highly unsaturated. The typical reactions of the benzene ring are *electrophilic*

Table 3.1 Physical Properties of Phenolic Compounds

Compounds	*mp*(°C)	*bp*(°C)	*pK*	ΔG^o_f (*kcal/mole*)
phenol	42	180	10.0	-11.020[a]
2-CP	9[c]	173[c]	8.56	-13.575[b]
3-CP	33[c]	214[c]	9.12	-13.480[b]
4-CP	43	220[c]	9.41	-12.691[b]
2,3-DCP	58	206	7.70	-17.973[b]
2,4-DCP	43	210	7.89	-20.172[b]
2,5-DCP	58	210	7.51	-20.172[b]
2,6-DCP	66	220	7.79	-19.741[b]
3,4-DCP	67	253	8.63	-17.973[b]
3,5-DCP	68	233	8.19	-20.626[b]
2,3,4-TCP	81		7.66	-21.008[b]
2,4,5-TCP	65	246	7.43	-23.231[b]
2,4,6-TCP	68	246	6.23	-24.500[b]
2,3,5-TCP	62	249	7.37	-23.231[b]
2,3,6-TCP	58	272	5.96	-23.231[b]
3,4,5-TCP	101	275	7.84	-20.745[b]
2,3,4,5-TeCP	117		6.6	-26.290[b]
2,3,4,6-TeCP	67		5.45	-27.318[b]
2,3,5,6-TeCP	114		5.48	-26.935[b]
pentachlorophenol	190	309	4.5	-26.840[b]

* 1 kJ = 0.239 kcal

a from Perry et al. (1984)

b from Dolfing and Harrison (1992)

c from Morrison and Boyd (1980)

all other data, Smith (1993)

substitution reactions because of a cloud of π-electrons above and below the plane of the benzene ring. Unless otherwise noted, the following discussion is condensed from Morrison and Boyd, 1980.

It was found that a group already attached to the ring affects the reactivity of the ring and determines the orientation of substitution. If a group makes the ring more reactive than benzene, it is called an activating group, and if a group makes the ring less reactive than benzene, it is called a deactivating group. If a group causes the substitution mainly at positions *ortho* and *para*, it is called *ortho-para* director, and if a group make the electrophilic attack chiefly at positions *meta*, it is called *meta* director. It was summarized that both the hydroxyl group and the chlorine belong to *ortho-para* directors. However, the hydroxyl group is an activate *ortho-para* director and the chlorine are deactivating *ortho-para* directors.

3.3 Reactions of Phenolic Compounds

A hydroxyl group is such an activating director that the most striking property of a phenol is the extremely high reactivity of its ring toward *electrophilic substitution*. In the substitution, acidity still plays an important role because ionization of a phenol yields the $-O^-$ group, which is even more strongly electron-releasing than the *-OH* itself. Namely, the phenolic group powerfully activates aromatic rings toward *electrophilic substitution*. One of the most important *electrophilic substitutions* is halogination, *i.e.* the formation of aryl halide (Figure 3.1).

In addition to *electrophilic aromatic substitution* mentioned above, another important reaction taking place on the benzene ring is *nucleophilic aromatic substitution* (S_NAr), which follows two different paths: the *bimolecular displacement mechanism*, for activated aryl halides; and the *elimination-addition mechanism*, which involves the remarkable intermediate called benzyne. The

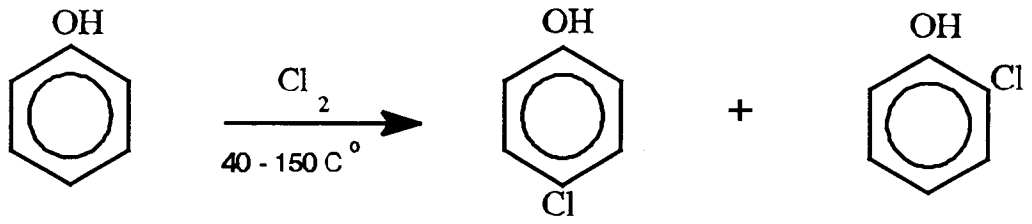

Fig. 3.1 The Formation of Aryl Halide

nucleophilic aromatic substitution of aryl chloride by a hydroxyl group seems similar with the first step of aerobic biodegradation of chlorophenols, which will discussed later.

Nucleophilic aromatic substitution of aryl halides can only be undergone with extreme difficulty. Therefore, except for certain industrial processes where very severe conditions are feasible, phenols (*ArOH*), ethers (*ArOR*), amines ($ArNH_2$), or nitriles (*ArCN*) are not prepared by nucleophilic attack on aryl halides. Consequently, nucleophilic aromatic substitution is much less important in synthesis than either nucleophilic aliphatic substitution or electrophilic aromatic substitution. However, when in addition to halogen there are certain other properly placed groups (electron-withdrawing groups like $-NO_2$, *-NO*, or *-CN*, located *ortho* or *para* to the halogen) aryl halides can react with nucleophiles by nucleophilic substitution readily.

Apparently, in nucleophilic aromatic substitution, electron withdrawal causes activation. It has been found that electron release will cause deactivation.

CHAPTER 4
METHODS AND MATERIALS

4.1 Chemicals

3,4,5-TCP (98%) was purchased from Ultra Scientific Co., Hope, R.I., and all other chlorophenols, 2,6-dibromophenol (2,6-DBP), and 2,4,6-tribromophenol (2,4,6-TBP), were obtained from Aldrich Chemical Company, Inc., Milwaulie, WN, in purities of at least 98%. All the chemicals were used without further purification.

4.2 Feed Media

In addition to phenol described below, the feed solution contained vitamins and inorganic nutrients as shown in Table 4.1 and Table 4.2:

Table 4.1 Vitamins in Feeding Solution

Vitamins	Concentration (*mg/l*)
biotin	0.018
folic acid	0.018
pyridoxin hydrochloride	0.09
riboflavin	0.045
thiamin	0.045
pantothenic acid	0.045
B_{12}	0.0009
p-aminobenzoic acid	0.045
thioctic acid	0.045.

Table 4.2 Inorganic Nutrients in Feeding Solution

Compounds	Concentration(*mg/l*)
$(NH_4)_2HPO_4$	27.09
$CaCl_2.2H_2O$	225.45
NH_4Cl	359.10
$MgCl_2.6H_2O$	1620
KCl	1170.45
$MnCl_2.4H_2O$	17.96
$CoCl_2.H_2O$	27
H_3BO_3	5.13
$CuCl_2.2H_2O$	2.43
$Na_2MoO_4.2H_2O$	2.30
$ZnCl_2$	1.89

4.3 "Mother" Reactor System and Inoculation

The reactors used are shown in Figure 4.1 and 4.2. A 5-liter "mother reactor" was used to grow active organisms for the batch reactors. The openings on the top were used for liquid and air influents; the opening on the side was used for liquid effluent. A drain on the bottom was used to collect bacterial solids for the batch experiments. The cells were suspended by both air sparging and magnetic stirring. The reactor was placed in an environmental chamber maintained at 31°C. The feed solution was kept in a 10-liter carboy refrigerated at 4°C to minimize microbial growth. The feed was transferred to the reactor by a FMI laboratory pump (Model QG6, Fluid Metering Inc., Oyster Bay, N.Y.). The flow rate was maintained at 1 *l/day* to provide a hydraulic retention time five days.

Aerobic wastewatere sludge was collected from the primary clarifier at the

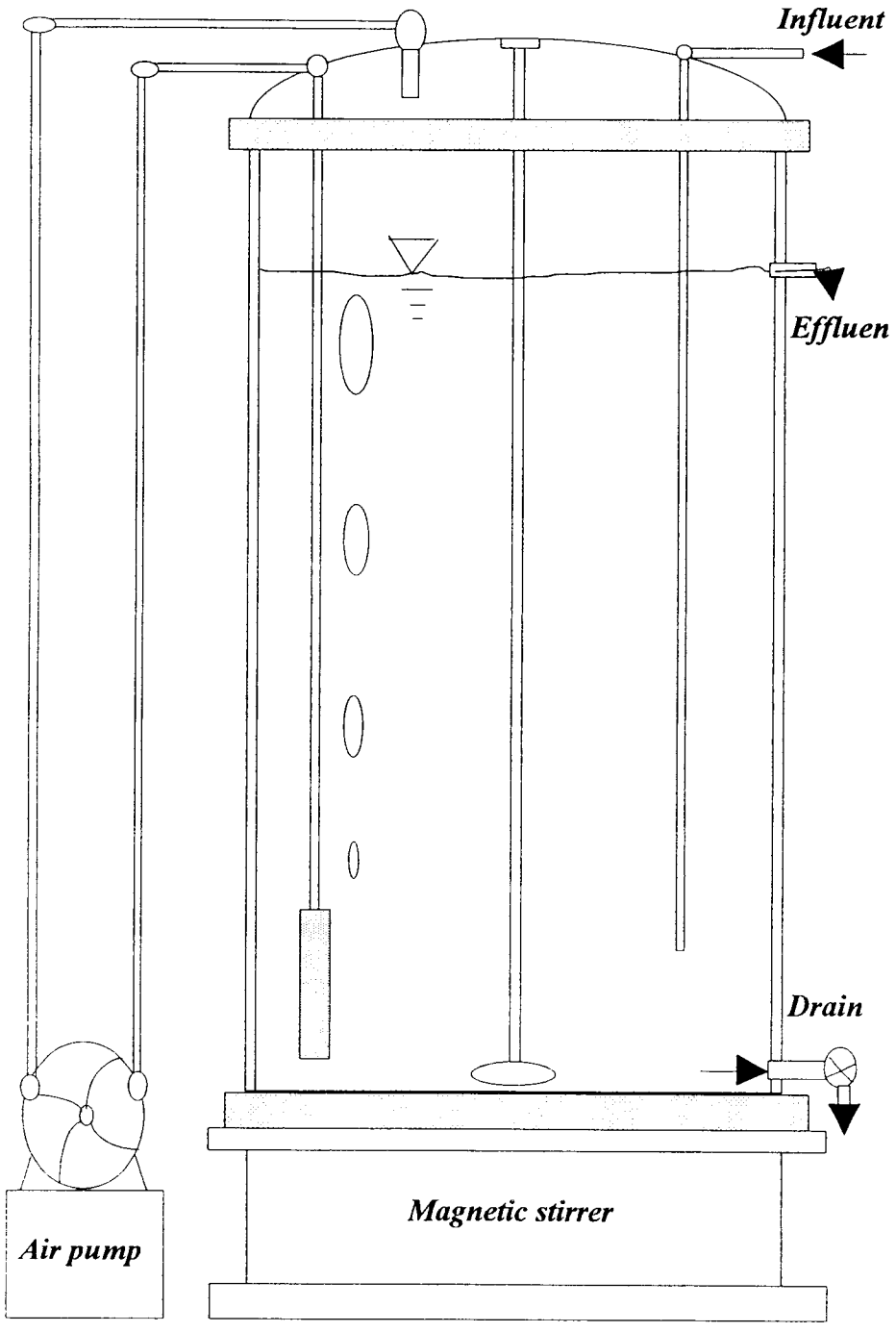

Figure 4.1 Schematic Diagram of Mother Reactor

Corvallis Wastewater Treatment Plant, Corvallis, Oregon. The sludge was allowed to settle, and the supernatant was transported to a 5-liter "mother reactor" described below. The reactor was located in an environmental chamber at 31°C. The sludge was fed a "nutrient solution", which was a modified recipe described by Owen *et al* (1978) at a feeding rate of one liter per day to keep liquid retention time of five days. The feeding solution contained mineral nutrients and vitamins described in the feed media.

Phenol at an influent of 250 *mg/l* was used as a primary substrate and carbon source to support cell growth and the cometabolization of chlorophenols. Eight chlorophenols (4-CP, 2,4-DCP, 3,4-DCP, 3,5-DCP, 2,4,6-TCP, 3,4,5-TCP, 2,4,5-TCP and 2,3,5-TCP) were added to the feeding solution. These eight chlorophenols represent the major anaerobic dechlorination products of PCP (Nicholson, 1990). The concentration of each individual chlorophenol was 0.1 *mg/l*. The effluent from the mother reactor was periodically measured to monitor phenol and chlorophenols concentration by IC and GC, respectively. The reactor was operated for five weeks until steady-state conditions were reached.

4.4 Batch Reactor System and Operation

Kinetic analysis experiments were performed in a 1.125-liter batch reactor (Figure 4.1). This reactor had ports for sampling and oxygen aeration. Mixing was provided by a magnetic stir bar. A *pH* electrode and an oxygen probe were mounted on the top of the reactor for monitoring *pH* and oxygen concentration, respectively. A syringe-pump was used to provide phenol solution for cell growth. The reactor was maintained at 31°C.

Bacterial solids were collected from the mother reactor through the bottom drain. After aeration with pure oxygen for about 25 minutes, 50 *ml* buffer (42.5 grams NaH_2PO_4 and 51.7 grams Na_2HPO_4 per liter) was added and the *pH* was

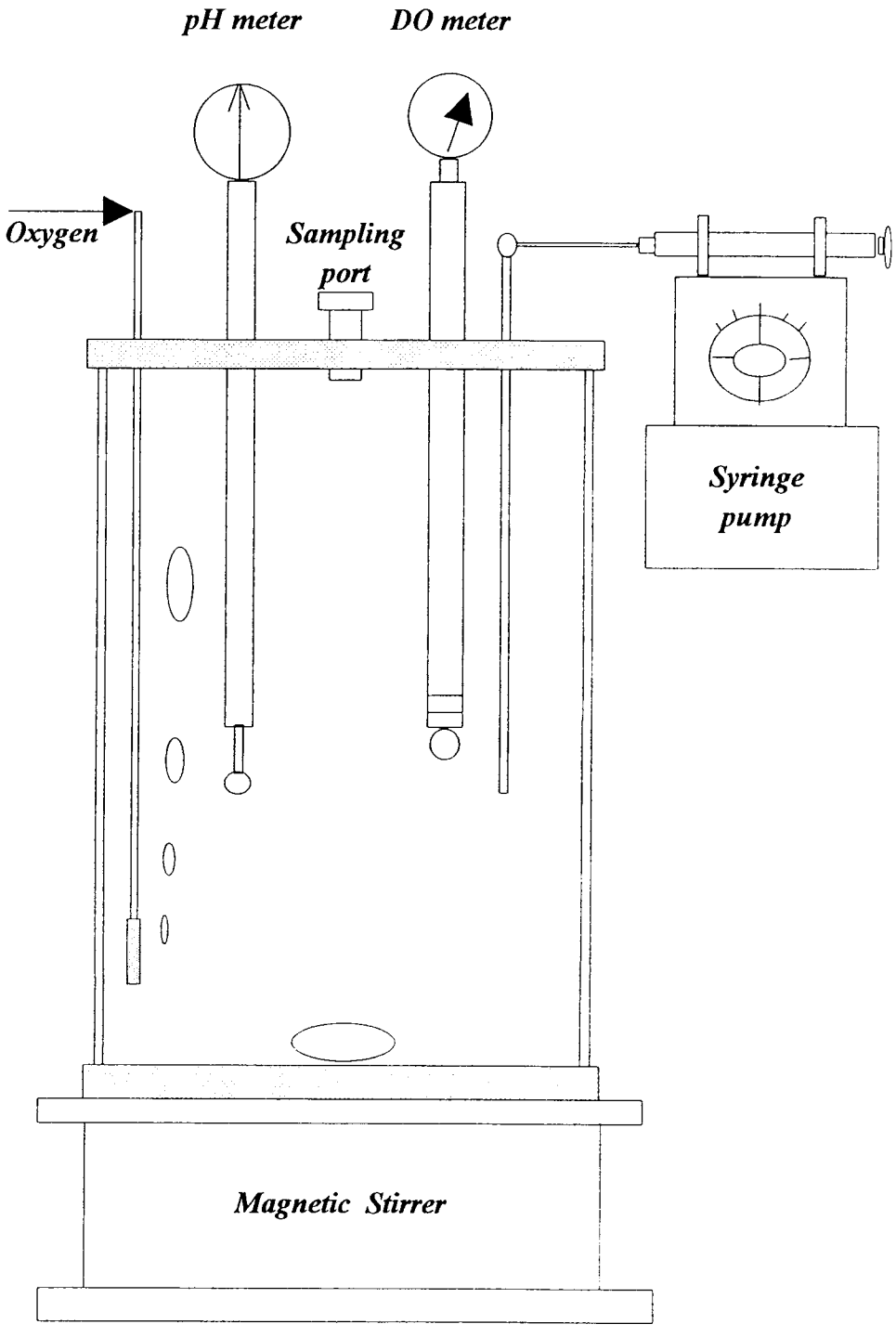

Figure 4.2 Schematic Diagram of Batch Reactor

adjusted to 7.0 with Na_2CO_3. The oxygen-saturated sludge was transferred into the batch reactor. For each experiment, the batch reactor was filled with the fresh bacterial solids. The phenol solution was pumped into the reactor with the syringe pump to maintain cell growth. The selected chlorophenol was added at about 1 *mg/l* after 2 hours.

4.5 Sampling and Analytical Procedures

For each individual experiment, the bacterial solution was collected from the mother reactor in a cylinder into which pure oxygen was bubbled to make the solution saturated with oxygen. Buffer was added and *pH* was adjusted to 7.0. The batch reactor was filled with the oxygen-saturated bacterial solution. A phenol solution was pumped into the batch reactor by a syringe-pump to support the cells growth. At 2 hours, one of the chlorophenol solution was added into the reactor for cometabolic degradation. Samples were taken each hour.

Liquid samples (5 *ml*) were taken from the batch reactor and immediately filtered through a Millipore type HA filter (0.45 μM pore size). The filtered solution was used for separate concentration analyses for both chlorophenols and phenol.

The analysis of chlorophenols were conducted by using a modified method descried by Voss *et al* (1980) and Smith (1993). The samples were first acetylated and then extracted into hexane. One *ml* samples were mixed in a screw-top culture tube with exact one *ml* of a reaction medium containing 43 *g/l* K_2CO_3 and one *mg/l* 2,4,6-tribromophenol as an internal standard. Two hundred μl of acetic anhydride was added, the tube was capped with a Teflon-lined cap, and shaken on a wrist-action shaker for 20 minutes. Three *ml* of hexane were then added, and the tube shaken for an additional 10 minutes. The extracted hexane fraction was transferred to an autosampler vial and capped with a Viton septum and crimp-seal cap.

Gas chromatography was performed on the hexane extracts with a Hewlett-Packard model 5980A gas chromatograph equipped with a ^{63}Ni electron capture detector (ECD) and fitted with a J&W Scientific DB-5 30 m x 0.32 mm I.D. fused-silica capillary column. Helium (5 psi) was used as the carrier gas and a 95% argon & 5% methane mixture was used as ECD auxiliary gas. Injection and detector temperatures were 250 and 320°C, respectively. A 1-μl aliquot was introduced by splitless injection. The samples were run using a temperature program as follows: an initial oven temperature of 45°C was held for two minutes, increased by 15°C/min to 105°C, and then 5°C/min to a final temperature of 215°C, which was held for 5 minutes.

Analysis of phenol was carried out using a Dionex Series 2000i liquid ion chromatograph (IC) equipped with ultraviolet lamp detector set at 274 Au and fitted with Alltima C_{18} 250 *mm* x 4.6 *mm* I.D. column with pore size 5 micron. The eluent was composed of 60% methanol and 40% sodium acetate (50 *mM*) and the flow rate was set at one *ml/min* (Stanford, 1993).

Fifty *ml* of bacterial solids were removed at the beginning and the end of each individual experiment for solid analysis. Total suspended solids were analyzed using Procedure 2540D and 2540E, Standard Methods, 17th edition (1989).

CHAPTER 5
THERMODYNAMICS AND THE CALCULATION OF YIELD

5.1 Thermodynamics and Mechanisms of Aerobic Degradations

Thermodynamic properties of compounds determine the potential for biodegradation. In general, aerobic degradation of chlorinated compounds releases less free energy compared to the non-halogenated analog. High degrees of chlorination can result in low aerobic yields of free energy which may lead to an impossibility of degradation without further energy sources (Winter *et al*, 1992). For example, if the aerobic degradation of a chlorinated phenol can not result in adequate free energy to sustain the microbial growth, then an additional energy source is required.

Aerobic biodegradation of chlorinated phenols is an oxidation process in which carbon dioxide and chlorine anions are released, *i.e.* carbon atoms in the phenolic ring are oxidized. Oxygen as the electron acceptors results in the maximum free energy release of all potential electron acceptors resulting in the maximum possibility of degrading these chlorinated compounds. The unique biochemical asset of aerobic bacteria is their ability to catalyze oxidations using molecular oxygen, thereby initiating reaction sequences that lead eventually into the common catabolic pathways.

There are two classes of enzymes that utilize oxygen to add oxygen bonds groups to carbon atoms, monooxygenases and dioxygenases. The monooxygenases carry out reactions to yield hydroxyl groups. *i.e.* they can insert hydroxyl groups into organic compounds:

$$R\text{-}H + NAD(P)H + H^+ + O_2 = R\text{-}OH + NAD(P)^+ + H_2O$$

The dioxygenases fix oxygen directly into organic compounds as:

$$R + O_2 = RO_2$$

A common function of dioxygenase is to cleave benzene rings by inserting both atoms of molecular oxygen (Grady Jr., 1985) in adjacent carbon atoms of an aromatic compound.

Roles of both oxygenases in catabolism of aromatic compounds can be explained as follows: monooxygenases are generally used to hydroxylize an aromatic ring with generation of a catechol, in which NADH is used as the electron donor. Dioxygenases, however, cleave aromatic rings of the formed catechol to cis-, cis-muconate: (Brock and Madigan, 1991) (Figure 5.1)

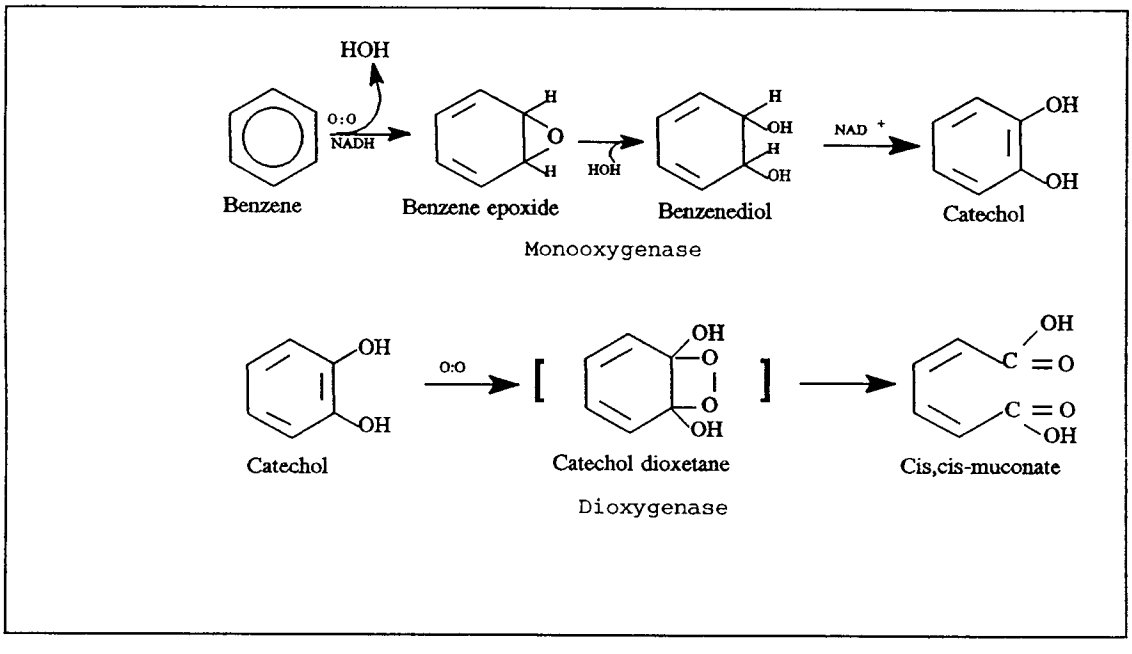

Figure 5.1 Roles of Oxygenases in Catabolism of Aromatic Compounds

Since biological reactions are much more complicated than chemical ones, the classical thermodynamics can not be directly used for biological reactions without limitation because generated energy from a substrate is utilized in two ways: biological synthesis and cells maintenance. Namely, only partial generated

energy can be used in cell synthesis. McCarty (1971) developed a model to represent a relationship between free energy of the reaction and maximum cell yield by assuming negligible energy requirements for cell maintenance.

5.2 Energetics and Yield of Phenol

All of the following calculations are based on McCarty's model. In this research, phenol is a primary substrate served as electron donor (*ED*) as well as carbon source, and its half reaction and free energy are as follows:

$$\frac{1}{28}C_6H_6O+\frac{11}{28}H_2O\rightarrow\frac{3}{14}CO_2+H^++e^- \quad (1)$$

$$\Delta G^\circ_{phenol}=-7.204 kcal/e\text{-}mole \quad (2)$$

i.e. ΔG^o_{ED} = -7.204 *kcal/e-mole.*

To obtain the maximum amount of potential energy, oxygen is used as an electron acceptor (*EA*) and its half reaction is as follows:

$$\frac{1}{4}O_2+H^++e^-\rightarrow\frac{1}{2}H_2O \quad (3)$$

$$\Delta G^\circ_{O_2}=-18.675 kcal/e\text{-}mole \quad (4)$$

i.e. ΔG^o_{EA} = -18.675 *kcal/e-mole* (McCarty, 1971).

Energy released from the energy reaction is:

$$\Delta G_R = \Delta G^o_{ED} + \Delta G^o_{EA} = -25.879 kcal/e\text{-}mole \quad (5)$$

Ammonia (NH_3) is used as a nitrogen source, therefore,

$$\Delta G_N = 0 \quad (6)$$

Based on McCarty's model, pyruvate is the intermediate energy level substrate, and its half reaction can be written:

$$\frac{1}{10}CH_3COCOO^- + \frac{2}{5}H_2O \rightarrow \frac{1}{5}CO_2 + \frac{1}{10}HCO_3^{-1} + H^+ + e^- \quad (7)$$

Table 5.1 Free Energies of Formation for Related Compounds

Compounds	Standard State	ΔG^o_f (*kcal/mole*)
CO_2	g	-94.26[a]
H_2O	l	-56.69[a]
NH_4^+	aq(m=1)	-19.00[a]
H^+	aq(10^{-7})	-9.67[a]
Cl^-	aq(m=1)	-31.37[c]
Phenol	l	-11.02[b]

* 1 kJ = 0.239 kcal

a from McCarty, (1971)

b from Perry *et al*, (1984)

c from Weast *et al*, (1987)

$$\Delta G_{pyruvate} = -8.545 kcal/e\text{-}mole \quad (8)$$

therefore, ΔG_P can be calculated as follows:

$$\Delta G_p = \Delta G^{\circ}_{phenol} - \Delta G^{\circ}_{pyruvate} = 1.341 kcal/e\text{-}mole \quad (9)$$

ΔG_C is an experimental constant, which represents the required energy from pyruvate to cells, that is,

$$\Delta G_C = 7.5 kcal/e\text{-}mole \quad (10)$$

The ratio of electron equivalents of substrate used for energy to electron equivalent of cells formed, A, is obtained as:

$$A = -\frac{\frac{\Delta G_P}{k^m} + \Delta G_C + \frac{\Delta G_N}{k}}{k \Delta G_R} \quad (11)$$

Where, k is a coefficient and $k = 0.6$ is mostly acceptable; when $\Delta G_P > 0$, $m = +1$ and when $\Delta G_P < 0$, $m = -1$. A value can be calculated as:

$$A = 0.627$$

The overall reaction equation can be expressed as:

$$A * (Energy\ reaction) + (Synthesis\ reaction)$$

The energy reaction which is Equation 1 plus Equation 3 can be represented by Equation 12:

$$\frac{1}{28}C_6H_6O+\frac{1}{4}O_2\rightarrow\frac{3}{14}CO_2+\frac{3}{28}H_2O \quad (12)$$

Synthesis reaction is Equation 1 plus Equation 13 (McCarty, 1971),

$$\frac{1}{5}CO_2+\frac{1}{20}HCO_3^-+\frac{1}{20}NH_4^++H^++e^-\rightarrow\frac{1}{20}C_5H_7O_2N+\frac{9}{20}H_2O \quad (13)$$

Therefore the synthesis reaction can be written as Equation 14:

$$\frac{1}{28}C_6H_6O+\frac{1}{20}NH_4^++\frac{1}{20}HCO_3^-\rightarrow\frac{1}{20}C_5H_7O_2N+\frac{1}{70}CO_2+\frac{4}{70}H_2O \quad (14)$$

Eventually, the overall reaction was obtained as follows:

$$\frac{16.27}{14}C_6H_6O+\frac{6.27}{2}O_2+NH_4^++HCO_3^-\rightarrow C_5H_7O_2N+\frac{20.81}{7}CO_2+\frac{34.81}{14}H_2O \quad (15)$$

Maximum yield (Y_m) is defined as:

$$Y_m = \textit{(mass of cells grown)/(mass of substrate utilized)}$$

Thus, the yield can be calculated from Equation 15:

$$Y_m = 97.234\ g/mole = 0.097\ mg/\mu mole = 1.034\ mg/mg$$

According to McCarty's model, values for k_m can be determined from conventional units as follows:

The unit for k_m is *moles substrate utilized per day per grams of bacteria present.*

$$k_m = \frac{n}{m}\left(\frac{A+1}{A}\right) \quad (16)$$

The value, n, is the number of electron moles transferred for energy per g of cells per day, and m is the number of electron moles transferred per mole of substrate utilized for energy. McCarty estimated n to be about 1 electron *mole/g* of cells per day at 25°C. Consequently, the value, k_m is obtained:

$$k_m = 0.093$$

The maximum specific growth rate μ_m (day^{-1}) can be calculated as follows:

$$\mu_m = k_m Y_m = 9.021$$

Table 5.2 Energetics and Yields of Related Phenolic Compounds

Compounds	ΔG_P	ΔG_R	A	k_m or k_I	μ_m	Y
phenol	1.341	-25.879	0.627	0.093	9.043	97.234
4-CP	0.016	-27.204	0.461	0.122	12.267	100.548
2,4-DCP	-1.287	-28.507	0.393	0.148	14.407	97.344
3,4-DCP	-1.378	-28.598	0.390	0.149	14.535	97.554
3,5-DCP	-1.268	-28.488	0.394	0.147	14.299	97.274
2,4,5-TCP	-3.028	-30.248	0.313	0.191	18.082	94.669
2,4,6-TCP	-2.970	-30.190	0.316	0.189	17.851	94.453
2,3,5-TCP	-3.028	-30.248	0.313	0.191	18.082	94.669
3,4,5-TCP	-3.141	-30.361	0.308	0.193	18.341	95.031

The unit of ΔG_P and ΔG_R: (*kcal/e-m*)

The unit of k_m and k_I: *(mole/g.d)*

The unit of μ_m: *(day^{-1})*

The unit of Y: (*gram/mole*)

Similar calculations were done for the various chlorinated phenols and are listed in Table 5.2. The data shown in Table 5.2 was used in the mathematical model developed in Chapter 6.

CHAPTER 6
MODEL DEVELOPMENT

6.1 Model Structure

A model was derived to describe the cometabolic degradation of phenol and chlorophenols based on the following assumptions. The first assumption is that chlorinated phenols compete with phenol for the monooxygenases enzymes by competitive inhibition. The second is that new cells and subsequently new enzymes are synthesized as a result of the oxidization of substrate. The third is that in addition to "normal" degradation, phenol promotes uncoupling so that some of the energy resulting from the degradation of phenol does not support cell growth. The fourth assumption is that phenol results in primary substrate inhibition, that is, that phenol inhibits its own degradation.

Classical competitive inhibition reaction can be expressed as follows:

$$E+S \leftrightarrow ES \rightarrow E+P \tag{1}$$

$$E+I \leftrightarrow EI \rightarrow E+P' \tag{2}$$

Besides the classical competitive inhibition, there exists a substrate inhibition, namely,

$$S+ES \leftrightarrow SES \tag{3}$$

The mass balance of enzyme is,

$$[E]=[E_O]-[ES]-[EI]-[SES] \quad (4)$$

where, E and $[E]$ stands for free enzyme and its concentration, S and I represent substrate and inhibitor, respectively, and $[E_O]$ stands for the initial enzyme concentration. In this research, phenol is used as a primary substrate and chlorophenols are treated as inhibitors. Therefore, S and I are related to phenol and chlorophenols in neutral forms, respectively, because microorganisms are expected to degrade both phenol and chlorophenols in their neutral forms. P and P' are the reaction products of the substrate and the inhibitor, respectively, and $[ES]$, $[EI]$ and $[SES]$ are the enzyme-substrate, enzyme-inhibitor, and enzyme-substrate-substrate complex concentrations, respectively.

Assuming,

$$K_M=\frac{[E][S]}{[ES]} \quad (5)$$

$$K_I=\frac{[E][I]}{[EI]} \quad (6)$$

and

$$K_P=\frac{[ES][S]}{[SES]} \quad (7)$$

where, K_M, K_I, and K_P are the dissociation constants for the complexes ES, EI and SES respectively, $[S]$ and $[I]$ standard for substrate and inhibitor concentration, respectively, then Equations 5, 6, and 7 can be rearranged as Equations 8, 9, and 10:

$$[E]=\frac{K_M[ES]}{[S]} \tag{8}$$

$$[EI]=\frac{[E][I]}{K_I} \tag{9}$$

and

$$[SES]=\frac{[ES][S]}{K_P} \tag{10}$$

Substituting Equations 8, 9, and 10 into Equation 4 to eliminate *[E]*, *[EI]* and *[SES]* yields Equation 11:

$$[ES]=\frac{[E_O]}{\frac{K_M}{[S]}+1+\frac{K_M[I]}{K_I[S]}+\frac{[S]}{K_P}}=\frac{[E_O][S]}{K_M(1+\frac{[I]}{K_I})+[S](1+\frac{[S]}{K_P})} \tag{11}$$

Since the degradation rate of a substrate is proportional to the complex concentration *[ES]*, the degradation rate of the substrate can be expressed as:

$$r_S=(-\frac{d[S]}{dt})_{Biodeg}=k[ES]=\frac{k[E_O][S]}{K_M(1+\frac{[I]}{K_I})+[S](1+\frac{[S]}{K_P})} \tag{12}$$

where, k is a reaction constant.

Substituting Equations 8, 9, and 10 into Equation 4 to eliminate *[E]*, *[ES]*, and *[SES]* yields Equation 13:

$$[EI]=\frac{[E_O][I]}{K_I+[I]+\frac{K_I[S]}{K_M}(1+\frac{[S]}{K_P})} \quad (13)$$

and the degradation rate of an inhibitor can be obtained:

$$r_I=(-\frac{d[I]}{dt})_{Biodeg}=\frac{k_I[E_O][I]}{K_I+[I]+\frac{K_I[S]}{K_M}(1+\frac{[S]}{K_P})} \quad (14)$$

where, k_I is a reaction constant of inhibitor.

The above derivation of Equations 12 and 14 is based on the assumption that the amount of enzyme does not change with the oxidization of substrate in a short time period. The substrate and inhibitor concentrations are assumed to be the neutral forms. Since cell growth result in an increasing enzyme concentration with time, a comprehensive expression including cell growth is required.

6.2 Ionization Fractions and Their Calculations

For the research, all experiments were conducted at a buffered *pH* of 7.0. At this *pH*, the chlorophenols have significant concentrations of both the neutral and ionized forms because their pK_a values are close to 7 (Table 6.1). Therefore, ionization fraction (i.e. α value) was used to convert the measured total chlorophenol concentration into their neutral forms, which is assumed to be the substrate form for the microorganisms.

Both definition and calculation of the ionization fraction (α value) are as follows. Assume that $[C_6H_{6-x}OCl_x]_T$ standard for the total measured concentration of one of the chlorophenols, $[C_6H_{6-x}OCl_x]$ and $[C_6H_{5-x}OCl_x^{-1}]$ standard for its neutral and ionized forms, respectively, and x is the number of chlorine atoms on the phenolic ring. Thus,

$$[C_6H_{6-x}OCl_x]_T=[C_6H_{6-x}OCl_x]+[C_6H_{5-x}OCl_x^{-1}] \quad (15)$$

Dissociation of a chlorophenol is expressed as:

$$C_6H_{6-x}OCl_x \leftrightarrow C_6H_{5-x}OCl_x^{-1}+H^+ \quad (16)$$

and the equilibrium constant, K_a is defined as:

$$K_a=\frac{[H^+][C_6H_{5-x}OCl_x^{-1}]}{[C_6H_{6-x}OCl_x]} \quad (17)$$

Substituting Equation 17 into Equation 15 to eliminate $[C_6H_{5-x}OCl_x^{-1}]$ yields Equation 18:

$$[C_6H_{6-x}OCl_x]_T=[C_6H_{6-x}OCl_x]+\frac{K_a}{[H^+]}[C_6H_{6-x}OCl_x] \quad (18)$$

Solving for $[C_6H_{6-x}OCl_x]/[C_6H_{6-x}OCl_x]_T$, that is, the fraction of $[C_6H_{6-x}OCl_x]_T$ which is $[C_6H_{6-x}OCl_x]$, then:

$$\frac{[C_6H_{6-x}OCl_x]}{[C_6H_{6-x}OCl_x]_T}=\frac{[H^+]}{[H^+]+K_a}=\alpha \quad (19)$$

An ionization fraction (α value) of chlorophenols can be calculated by using their pK_a and media pH values. All the calculated α values are listed in Table 6.1.

From Table 6.1, it can be seen that all the phenolic compounds of concern except phenol are partially ionized at pH 7. Since pK_a of phenol is far away from pH 7, almost all of the phenol exists in a neutral form.

By knowing α values, the concentration of a chlorophenol in neutral form can be calculated by its measured total concentration times α.

Table 6.1 pK_a and α Values of Phenolic Compounds at *pH* 7

Compounds	pK_a	α
phenol	10.0	0.999
p-chlorophenol	9.41	0.996
2,4-DCP	7.89	0.886
3,4-DCP	8.63	0.977
3,5-DCP	8.19	0.939
2,3,5-TCP	7.37	0.701
2,4,5-TCP	7.43	0.729
2,4,6-TCP	6.23	0.145
3,4,5-TCP	7.84	0.874

pK_a's, (Smith, 1993)

6.3 A Conceptual Model

The mass balance equations for phenol as a primary substrate in the batch reactor can be expressed as follows:

$$\left(\frac{dS}{dt}\right)_{Mass}=\left(\frac{dS}{dt}\right)_{Feed}-\left(\frac{dS}{dt}\right)_{Eff}-\left(-\frac{dS}{dt}\right)_{Biodeg}-\left(-\frac{dS}{dt}\right)_{Uncoupling} \quad (20)$$

where, $(dS/dt)_{Feed}$ and $(dS/dt)_{Eff}$ standard for influent and effluent pumping rate of phenol, respectively. $(-dS/dt)_{Biodeg}$ stands for the "normal" degradation rate of phenol as expressed in Equation 12. $(-dS/dt)_{Uncoupling}$ represents phenol

degradation in an uncoupling process, in which the oxidation of phenol does not support the cell growth.

With no effluent in a batch reactor design, *i.e.*

$$(\frac{dS}{dt})_{Eff}=0 \tag{21}$$

the mass balance equation for phenol can be rewritten as follows:

$$(\frac{dS}{dt})_{Mass}=\frac{Q_F C_F}{V}-\frac{kXS}{K_M(1+\frac{\alpha I_T}{K_I})+S(1+\frac{S}{K_P})}-\frac{k_U XS}{K_U+S} \tag{22}$$

where, C_F is phenol concentration in feeding solution, Q_F is feeding rate of the syringe pump and V is liquid volume in the batch reactor. All are fixed for each individual experiment. Therefore, they can be replaced by a new parameter A :

$$A=\frac{Q_F C_F}{V} \tag{23}$$

The flow rate, Q_F, is assumed to be small enough so that the change of volume V is negligible over the length of the experiment.

The second term on the right-hand side of Equation 22 is the "normal" substrate biodegradation rate, which is similar to Equation 12 except that the cell concentration X is employed to replace the initial enzyme concentration $[E_O]$, *i.e.*

$$(-\frac{dS}{dt})_{Biodeg}=\frac{kXS}{K_M(1+\frac{\alpha I_T}{K_I})+S(1+\frac{S}{K_P})} \tag{24}$$

It should be noticed that αI_T is used instead of $[I]$ which represents the

concentration of a chlorophenol in a neutral form where α is ionization fraction of a chlorophenol calculated in the last section, and I_T is the total measured chlorophenol concentration. The third term is the uncoupling rate which is expressed as a reaction that can be saturated at high phenol concentration. *i.e.*

$$(-\frac{dS}{dt})_{Uncoupling}=\frac{k_U XS}{K_U+S} \tag{25}$$

where, k_U and K_U are maximum uncoupling rate per unit mass of cells and dissociation constant for uncoupling process, respectively.
Therefore, Equation 22 becomes Equation 26:

$$(\frac{dS}{dt})_{Mass}=A-\frac{kXS}{K_M(1+\frac{\alpha I_T}{K_I})+S(1+\frac{S}{K_P})}-\frac{k_U XS}{K_U+S} \tag{26}$$

Similarly, the biodegradation rate of chlorophenol as an inhibitor (Equation 14) can be expressed as Equation 27:

$$(-\frac{dI}{dt})_{Biodeg}=\frac{k_I X\alpha I_T}{K_I+\alpha I_T+\frac{K_I S}{K_M}(1+\frac{S}{K_P})} \tag{27}$$

and the mass balance equation for an inhibitor can be written as Equation 28:

$$(\frac{dI_T}{dt})_{Mass}=(\frac{dI_T}{dt})_{Added}-(\frac{dI_T}{dt})_{Eff}-(-\frac{dI}{dt})_{Biodeg} \tag{28}$$

Since there is neither influent nor effluent for a batch design, Equation 28 can be rewritten as Equation 29:

$$(\frac{dI_T}{dt})_{Mass}=-(-\frac{dI}{dt})_{Biodeg}=-\frac{k_I X\alpha I_T}{K_I+\alpha I_T+\frac{K_I S}{K_M}(1+\frac{S}{K_P})} \quad (29)$$

The mass balance equation for cells is defined as Equation 30:

$$(\frac{dX}{dt})_{Mass}=Y(1-\frac{(-\frac{dI}{dt})_{Biodeg}}{(-\frac{dS}{dt})_{Biodeg}})(-\frac{dS}{dt})_{Biodeg}-k_d X \quad (30)$$

Namely,

$$(\frac{dX}{dt})_{Mass}=Y[(-\frac{dS}{dt})_{Biodeg}-(-\frac{dI}{dt})_{Biodeg}]-k_d X \quad (31)$$

or,

$$(\frac{dX}{dt})_{Mass}=Y(\frac{kXS}{K_M(1+\frac{\alpha I_T}{K_I})+S(1+\frac{S}{K_P})}-\frac{k_I X\alpha I_T}{K_I+\alpha I_T+\frac{K_I S}{K_M}(1+\frac{S}{K_P})})-k_d X \quad (32)$$

where, Y is a yield coefficient with a unit of MN^{-1} and k_d is cells decay constant with a unit of T^{-1}.

All the variables and parameters used in this section and the following chapters are summarized as follows.

6.4 Nondimensionization

In order to simplify the solution of these differential equations, dimentionless operations were derived as follows.

Assume,

$$\Pi=\frac{A}{k_d K_M} \tag{33}$$

Thus,

$$A=k_d K_M \Pi \tag{34}$$

Assume,

$$\Omega=\frac{S}{K_M} \tag{35}$$

Therefore,

$$S=K_M \Omega \tag{36}$$

and

$$dS=K_M d\Omega \tag{37}$$

Assume,

$$\Gamma=\frac{I_T}{K_I} \tag{38}$$

thus,

$$\alpha I_T=\alpha K_I \Gamma \tag{39}$$

and

$$dI=K_I d\Gamma \tag{40}$$

Assume,

$$\Phi=\frac{X}{K_M Y} \tag{41}$$

therefore,

$$X=K_M Y\Phi \tag{42}$$

and,

$$dX=K_M Y d\Phi \tag{43}$$

Assume,

$$\mathrm{T}=tk_d \tag{44}$$

therefore,

$$t=\frac{\mathrm{T}}{k_d} \tag{45}$$

and,

$$dt=\frac{d\mathrm{T}}{k_d} \tag{46}$$

Based on all assumptions above, Equation 26 can be rewritten as Equation 47:

$$\frac{d\Omega}{d\Gamma}=\Pi-\frac{kY}{k_d}\frac{\Phi\Omega}{1+\alpha\Gamma+\Omega(1+\frac{K_M}{K_P}\Omega)}-\frac{k_UY}{k_d}\frac{\Phi\Omega}{\frac{K_U}{K_M}+\Omega} \tag{47}$$

Some new dimentionless parameters, $P_{S/d}$, $P_{U/d}$, can be assigned to the parameter groups, $[kY/k_d]$ and $[k_UY/k_d]$, respectively:

$$P_{S/d}=\frac{kY}{k_d} \tag{48}$$

$$P_{U/d}=\frac{k_UY}{k_d} \tag{49}$$

which are the ratio of maximum growth rate and cells decay rate and the ratio of maximum uncoupled rate and cells decay rate, respectively. The other dimentionless parameters, P_{SES} and P_{SUS} can be assigned to the ratio of dissociation constants K_M and K_P, and the ratio of dissociation constants K_U and K_M, respectively, *i.e.*

$$P_{SES}=\frac{K_M}{K_P} \tag{50}$$

and,

$$P_{SUS}=\frac{K_U}{K_M} \tag{51}$$

Equation 29 can be rewritten as Equation 52:

$$-\frac{d\Gamma}{d\Gamma}=\frac{K_M Y k_I}{K_I k_d}\frac{\alpha\Phi\Gamma}{1+\alpha\Gamma+\Omega(1+\frac{K_M}{K_P}\Omega)} \tag{52}$$

The new dimentionless parameter group, $[K_M Y k_I/K_I k_d]$, can be divided into two new dimentionless parameters, $P_{I/d}$ and P_{SEI}:

$$P_{I/d}=\frac{k_I Y}{k_d} \tag{53}$$

which can be considered as a ratio of maximum "inhibiting" rate and cells decay rate, and

$$P_{SEI}=\frac{K_M}{K_I} \tag{54}$$

which is a ratio of the dissociation constant for a complex of substrate with enzyme and a dissociation constant of a complex of inhibitor with the enzyme.

Equation 32 can be rewritten as Equation 55:

$$\frac{d\Phi}{d\Gamma}=\frac{kY}{k_d}\frac{\Omega\Phi}{1+\Gamma+\Omega(1+\frac{K_M}{K_P}\Omega)}-\frac{k_I Y}{k_d}\frac{\Phi\Gamma}{1+\Gamma+\Omega(1+\frac{K_M}{K_P}\Omega)}-\Phi \tag{55}$$

Eventually, three differential equations with four variables (Ω,Γ,Φ, and T) and six parameters(Π, P_{SES}, P_{SUS}, P_{SEI}, $P_{S/d}$, $P_{I/d}$, and $P_{U/d}$) are obtained as follows:

$$\frac{d\Omega}{d\Gamma}=\Pi-\frac{P_{S/d}\Phi\Omega}{1+\alpha\Gamma+\Omega(1+P_{SES}\Omega)}-\frac{P_{U/d}\Phi\Omega}{P_{SUS}+\Omega} \tag{56}$$

$$-\frac{d\Gamma}{d\Gamma}=\frac{P_{I/d}P_{SEI}\alpha\Gamma\Phi}{1+\alpha\Gamma+\Omega(1+P_{SES}\Omega)} \tag{57}$$

$$\frac{d\Phi}{d\Gamma}=\frac{P_{S/d}\Omega\Phi}{1+\alpha\Gamma+\Omega(1+P_{SES}\Omega)}-\frac{P_{I/d}\alpha\Gamma\Phi}{1+\alpha\Gamma+\Omega(1+P_{SES}\Omega)}-\Phi \tag{58}$$

The following variables and parameters were used in the above derivations:

variables:

S	phenol concentration	NL^{-3}
I	chlorophenol concentration in neutral form	NL^{-3}
I_T	total measured chlorophenol concentration	NL^{-3}
X	cells concentration	ML^{-3}
t	time	T

parameters:

k	maximum phenol utilization rate per unit mass of cells	$NM^{-1}T^{-1}$
k_I	chlorophenol utilization rate per unit mass of cells	$NM^{-1}T^{-1}$
k_U	maximum uncoupling rate per unit mass of cells	$NM^{-1}T^{-1}$
K_M	dissociation constant of *ES*	NL^{-3}
K_I	dissociation constant for *EI*	NL^{-3}
K_P	dissociation constant for *SES*	NL^{-3}
K_U	dissociation constant for uncoupling process	NL^{-3}
Y	maximum yield coefficient	MN^{-1}
k_d	cells decay constant	T^{-1}
Q_F	feeding rate of syringe pump	L^3T^{-1}
C_F	substrate concentration in feeding solution	NL^{-3}
A	feeding constant	$NT^{-1}L^{-3}$
V	liquid volume in the batch reactor	L^3

non-dimensional variables and parameters:

Ω related to substrate concentration S

Γ related to inhibitor concentration I

Φ related to cell concentration X

T related to time t

Π related to feeding rate $Q_F C_F / V$

P_{SES} defined as K_M / K_P

P_{SUS} defined as K_U / K_M

P_{SEI} defined as K_M / K_I

$P_{S/d}$ defined as kY / k_d

$P_{I/d}$ defined as $k_I Y / k_d$

$P_{U/d}$ defined as $k_U Y / k_d$

CHAPTER 7
RESULTS AND DISCUSSION

7.1 Degradation Curves for Phenol and Chlorophenols

Phenol is readily degraded aerobically if the concentration is reduced below the limits that result in toxicity and substrate inhibition. The reported inhibitory phenol concentration ranges from 50 *mg/l* to 200 *mg/l* (Colvin, *et al*, 1986, and Beltrame *et al*, 1984). Worden, et al., (1991) have shown that phenol is capable of causing toxicity by uncoupling phosphorylation in *Bacilus stearothermophilus*. A 50% uncoupling was achieved at phenol concentrations from 3 to 4 *mg/l*.

These effects are clearly shown in the experiments with only phenol shown in Appendix A. In these experiments, the phenol concentration would increase from near zero to near 100 *mg/L* over a period of about six hours, and then dramatically decrease again to near zero in an additional six hours. The increasing portion of the curve would result from increases in cell mass overcoming the effects of inhibition and uncoupling. Once the cell mass increase so that the cell could degrade the phenol more rapidly that being added by the syringe pump, the concentration decreases dramatically as the inhibition and uncoupling effected decreases.

Similar curves were obtained for the degradation of the phenols in the presence of chlorophenol (Figure 7.1 and Appendix A). The chlorophenol degradation rates were small as compared to the phenol degradation rates for all the chlorophenols tested.

The data shown in Appendix A and B only result under carefully controlled experimental conditions. The ratio (R) between the feeding parameter A and the initial cells concentration X_i, ($R = A/X_i$) was critical for each

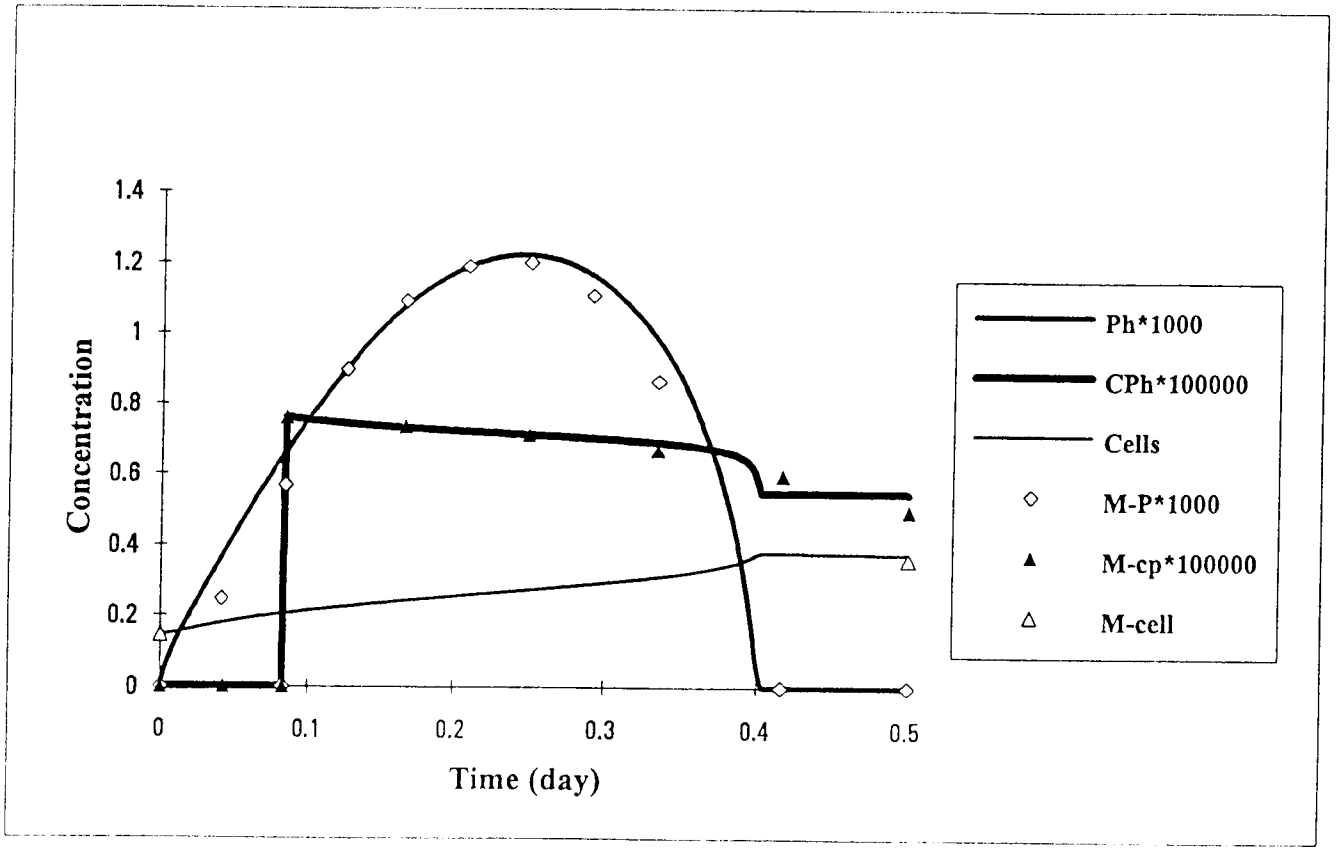

Figure 7.1 Degradation of Phenol with 2,4-DCP

individual experiment. If the ratio is too small, the phenol concentration rapidly reduced to near zero in the reactor. If the R ratio is too large, however, the phenol concentration increased continuously and the organisms were unable to overcome the substrate inhibition and uncoupling effects. For these experiments, the required ratio of R was found to about 0.067 *mole/g-d.*

All experiments were conducted with the microorganisms that had been acclimated to all the chlorophenols in the mother reactor. Significant degradation of chlorophenols was not observed without acclimation.

7.2. Cell Production

Initial and final total suspended solids were measured for each experiment to determine the cell production. Since the length of the experiments were about 12 hours, cell decay was assumed to be insignificant. The assumed cell decay rate of 0.025 day^{-1} gave an excellent model fit to the experimental data. However, it should be noted that errors in k_d could affect the relative estimates of k and K_U as shown in Equations 6-48 and 6-49.

The estimated value of the yield coefficient gave an excellent fit of the model to the experimental data. However, a significant amount the energy resulting from the degradation of phenol was subject to the uncoupling process, thus, substantially reducing the "apparent" cell yield. Over the entire experiments, measured cell yields were about one-third of the assumed value of 97 mg/mole.

7.3 Estimation of Model Parameter for Phenol and Chlorophenols

Parameters associated with Equations 6-56 to 6-58 (P_{SES}, P_{SEI}, P_{SUS}, $P_{S/d}$, $P_{I/d}$, and $P_{U/d}$) were estimated using the model (*i.e.* the three differential equations) developed in Chapter 6. Equation 6-56 to 6-58 were solved

Table 7.1 Derived Parameters Determined by The Model

	P(SES)	P(SEI)	P(SUS)	P(S/d)	P(I/d)	P(U/d)
I. 2,3,5-TCP	1.64E+02	3.15E+00	4.27E-04	1.56E+03	1.39E+01	1.11E+02
II. 2,3,5-TCP	1.60E+02	4.26E+00	4.27E-04	1.56E+03	8.35E+00	1.11E+02
III. 2,3,5-TCP	1.01E+02	2.70E+01	4.27E-04	1.24E+03	1.37E+00	2.80E+02
Average	1.42E+02	1.15E+01	4.27E-04	1.45E+03	7.88E+00	1.67E+02
Std. Dev.	3.52E+01	1.35E+01	0.00E+00	1.85E+02	6.29E+00	9.75E+01
I. 2,4-DCP	1.85E+02	1.34E+00	8.00E-06	1.75E+03	1.28E+02	1.14E+02
II. 2,4-DCP	1.90E+02	1.31E+00	8.90E-06	1.74E+03	1.28E+02	1.15E+02
III. 2,4-DCP	1.83E+02	1.35E+00	8.00E-06	1.75E+03	1.20E+02	1.15E+02
Average	1.86E+02	1.33E+00	8.30E-06	1.75E+03	1.25E+02	1.15E+02
Std. Dev.	3.65E+00	1.81E-02	5.20E-07	4.52E+00	4.39E+00	3.48E-01
I. 2,4,5-TCP	1.74E+02	1.59E+01	4.55E-05	1.63E+03	2.82E+01	1.44E+02
II. 2,4,5-TCP	1.38E+02	5.76E+00	4.61E-05	1.72E+03	2.95E+01	1.20E+02
III. 2,4,5-DCP	1.69E+02	1.10E+01	4.55E-05	1.67E+03	2.87E+01	1.43E+02
Average	1.60E+02	1.09E+01	4.57E-05	1.67E+03	2.88E+01	1.36E+02
Std. Dev.	1.96E+01	5.06E+00	3.18E-07	4.20E+01	6.61E-01	1.38E+01
I. 2,4,6-TCP	1.74E+02	2.21E+01	5.55E-05	1.79E+03	1.34E+01	1.18E+02
II. 2,4,6-TCP	1.74E+02	1.54E+01	5.55E-05	2.38E+03	3.45E+01	9.17E+01
III. 2,4,6-TCP	1.80E+02	1.02E+01	5.45E-05	1.93E+03	2.13E+01	1.14E+02
Average	1.76E+02	1.59E+01	5.52E-05	2.04E+03	2.31E+01	1.08E+02
Std. Dev.	3.62E+00	5.94E+00	5.77E-07	3.07E+02	1.07E+01	1.41E+01
I. 3,4-DCP	1.82E+02	2.37E+00	8.00E-06	1.84E+03	1.15E+02	1.29E+02
II. 3,4-DCP	1.85E+02	1.92E+00	8.00E-06	1.81E+03	9.50E+01	1.32E+02
III. 3,4-DCP	1.90E+02	2.52E+00	7.50E-06	1.82E+03	9.95E+01	1.32E+02
Average	1.86E+02	2.27E+00	7.83E-06	1.82E+03	1.03E+02	1.31E+02
Std. Dev.	4.36E+00	3.14E-01	2.89E-07	1.85E+01	1.05E+01	1.46E+00
I. 3,4,5-TCP	1.74E+02	1.23E+01	5.05E-06	1.83E+03	3.49E+01	1.07E+02
II. 3,4,5-TCP	1.74E+02	1.67E+01	5.05E-06	1.82E+03	1.94E+01	1.08E+02
III. 3,4,5-TCP	1.79E+02	1.80E+01	5.50E-05	1.79E+03	1.34E+01	1.18E+02
Average	1.75E+02	1.57E+01	2.17E-05	1.81E+03	2.26E+01	1.11E+02
Std. Dev.	2.69E+00	2.96E+00	2.88E-05	1.99E+01	1.11E+01	6.09E+00
I. 3,5-DCP	1.94E+02	9.32E-01	8.00E-06	2.06E+03	4.55E+02	9.13E+01
II. 3,5-DCP	2.00E+02	4.02E+00	8.00E-06	1.92E+03	1.25E+02	9.77E+01
III. 3,5-DCP	1.92E+02	3.03E+00	8.05E-06	2.12E+03	1.61E+02	8.83E+01
Average	1.95E+02	2.66E+00	8.02E-06	2.03E+03	2.47E+02	9.24E+01
Std. Dev.	4.01E+00	1.57E+00	2.89E-08	1.02E+02	1.81E+02	4.83E+00
I. Phenol	1.89E+02		8.25E-06	2.27E+03	0.00E+00	8.70E+01
II. Phenol	1.85E+02		8.00E-06	1.92E+03	0.00E+00	1.17E+02
Average	1.87E+02		8.13E-06	2.10E+03	0.00E+00	1.02E+02
Std. Dev.	2.47E+00		1.77E-07	2.48E+02	0.00E+00	2.10E+01

Table 7.2 Original Parameters Determined by The Model

	k	k(I)	K(I)	k(U)	K(U)	K(P)
I. 2,3,5-TCP	4.03E-01	3.59E-03	6.35E-04	2.85E-02	8.53E-07	1.22E-05
II. 2,3,5-TCP	4.01E-01	2.15E-03	4.69E-04	2.85E-02	8.53E-07	1.25E-05
III. 2,3,5-TCP	3.20E-01	3.52E-04	7.40E-05	7.21E-02	8.53E-07	1.98E-05
Average	**3.75E-01**	**2.03E-03**	**3.93E-04**	**4.30E-02**	**8.53E-07**	**1.48E-05**
Std. Dev.	4.77E-02	1.62E-03	2.88E-04	2.51E-02	1.42E-14	4.30E-06
I. 2,4-DCP	4.51E-01	3.30E-02	1.50E-03	2.95E-02	1.60E-08	1.08E-05
II. 2,4-DCP	4.49E-01	3.29E-02	1.53E-03	2.97E-02	1.78E-08	1.05E-05
III. 2,4-DCP	4.50E-01	3.10E-02	1.49E-03	2.95E-02	1.60E-08	1.09E-05
Average	**4.50E-01**	**3.23E-02**	**1.50E-03**	**2.96E-02**	**1.66E-08**	**1.07E-05**
Std. Dev.	1.16E-03	1.13E-03	2.05E-05	8.98E-05	1.04E-09	2.08E-07
I. 2,4,5-TCP	4.21E-01	7.26E-03	1.26E-04	3.72E-02	9.10E-08	1.15E-05
II. 2,4,5-TCP	4.42E-01	7.60E-03	3.47E-04	3.09E-02	9.21E-08	1.45E-05
III. 2,4,5-DCP	4.32E-01	7.39E-03	1.81E-04	3.70E-02	9.10E-08	1.18E-05
Average	**4.32E-01**	**7.41E-03**	**2.18E-04**	**3.50E-02**	**9.14E-08**	**1.26E-05**
Std. Dev.	1.08E-02	1.70E-04	1.15E-04	3.54E-03	6.35E-10	1.65E-06
I. 2,4,6-TCP	4.63E-01	3.45E-03	9.07E-05	3.04E-02	1.11E-07	1.15E-05
II. 2,4,6-TCP	6.14E-01	8.90E-03	1.30E-04	2.36E-02	1.11E-07	1.15E-05
III. 2,4,6-TCP	4.98E-01	5.50E-03	1.96E-04	2.93E-02	1.09E-07	1.11E-05
Average	**5.25E-01**	**5.95E-03**	**1.39E-04**	**2.78E-02**	**1.10E-07**	**1.14E-05**
Std. Dev.	7.91E-02	2.75E-03	5.32E-05	3.63E-03	1.15E-09	2.31E-07
I. 3,4-DCP	4.75E-01	2.96E-02	8.43E-04	3.33E-02	1.60E-08	1.10E-05
II. 3,4-DCP	4.66E-01	2.45E-02	1.04E-03	3.40E-02	1.60E-08	1.08E-05
III. 3,4-DCP	4.68E-01	2.57E-02	7.94E-04	3.39E-02	1.50E-08	1.05E-05
Average	**4.70E-01**	**2.71E-02**	**9.43E-04**	**3.36E-02**	**1.60E-08**	**1.09E-05**
Std. Dev.	4.76E-03	2.71E-03	1.32E-04	3.76E-04	5.77E-10	2.52E-07
I. 3,4,5-TCP	4.73E-01	9.00E-03	1.62E-04	2.76E-02	1.01E-08	1.15E-05
II. 3,4,5-TCP	4.68E-01	5.00E-03	1.20E-04	2.79E-02	1.01E-08	1.15E-05
III. 3,4,5-TCP	4.63E-01	3.45E-03	1.11E-04	3.05E-02	1.10E-07	1.12E-05
Average	**4.68E-01**	**5.82E-03**	**1.31E-04**	**2.87E-02**	**4.34E-08**	**1.14E-05**
Std. Dev.	5.12E-03	2.86E-03	2.72E-05	1.57E-03	5.77E-08	1.73E-07
I. 3,5-DCP	5.31E-01	1.17E-01	2.15E-03	2.35E-02	1.60E-08	1.03E-05
II. 3,5-DCP	4.95E-01	3.21E-02	4.98E-04	2.52E-02	1.60E-08	1.00E-05
III. 3,5-DCP	5.47E-01	4.15E-02	6.61E-04	2.28E-02	1.61E-08	1.04E-05
Average	**5.24E-01**	**6.37E-02**	**1.10E-03**	**2.38E-02**	**1.60E-08**	**1.02E-05**
Std. Dev.	2.63E-02	4.67E-02	9.09E-04	1.24E-03	5.77E-11	2.08E-07
I. Phenol	5.86E-01			2.24E-02	1.65E-08	1.06E-05
II. Phenol	4.96E-01			3.01E-02	1.60E-08	1.08E-05
Average	**5.41E-01**			**2.63E-02**	**1.63E-08**	**1.07E-05**
Std. Dev.	6.40E-02			5.42E-03	3.54E-10	1.41E-07

simultaneously by using a fourth-order Runge-Kutta method (Gerald and Wheatley, 1984). The best fits (Figure 7.1 and Appendix A) were obtained by minimizing the summarized squared-residues between the measured data and the data from the numerical solution for phenol (*S*), chlorophenols (*I*), and cell concentrations (*X*), respectively. As shown in the plots in Appendix A and B, the model predictions closely fit the experimental data for the metabolism of phenol alone and for the cometabolic metabolism of the seven chlorophenols.

The six P parameters then were used to estimate values for k, k_I, K_I, k_U, K_U, and K_P from Equations 6-48 to 6-51, and from Equation 6-53 to 6-54. These calculated values for k, k_I, K_I, k_U,K_u, and K_P are listed in Table 7.2. There was excellent reproducibility between experiments with relative small standard deviations. Values for Y, k_d, and K_M were estimated from literature values. The maximum yield coefficient for phenol was assumed to be equal to the value from McCarty's model in Chapter 5 (97 *g/mole*). The cell decay coefficient was assumed to be 0.025/d; k_d values for aerobic processes are commonly assumed to be between 0.1 to 0.5/d. The Monod half velocity coefficient for phenol was assumed to be about 0.002 *mole/l* as reported by Autenrieth, *et al*, 1991. These three coefficients were chosen to be estimated from literature data because they were the parameters that values could be reasonably obtained.

From Table 7.2, it can be seen that the maximum phenol utilization rate per mass of cells (*k*) is about 0.4 *mole/g-day* (from 0.32 to 0.54; average is 0.44). The k value for phenol alone was not significantly different that the value with the presence of any of the seven chlorophenols.

The degradation rate for the chlorophenols varied from a low of 0.0020 *mole/g-d* for 2,3,5-TCP to a high of 0.064 for 3,5-DCP. The dichlrophenol degraded about 30 times faster than the trichlorophenol which is in agreement with previous work (Wang, 1991). The average maximum inhibition utilization rate for the dichlorophenols was 0.028 *mole/g-day*, and the average for trichlorophenols is 0.0062 *mole/g-day*. The rate for dichlorophenols ranked in

the order of: 3,5-DCP > 2,4-DCP > 3,4-DCP; and the degradation rate for trichlorophenols: 2,4,5-TCP > 2,4,6-TCP > 3,4,5-TCP > 2,3,5-TCP.

The Monod half velocity coefficient for the chlorophenols (K_I's) are approximately 10^{-4} *mole/l*, which is about 100 times smaller than the Monod half velocity coefficient for phenol. This suggests that the limiting enzyme has a much stronger affinity for the chlorophenols than for the phenol.

K_P, the dissociation constants for the substrate inhibition by phenol, were from 10 to 100 times smaller than the Monod half velocity coefficient for the chlorophenols. However, magnitude of the substrate inhibition term is proportional to S^2 and becomes dominant at high phenol concentrations.

The rate at which energy from phenol degradation is lost through the uncoupling process represented by k_U is about 3×10^{-2} *mole/g-day*, which is about one tenth of k, the "normal" degradation rate of phenol. This value did not change with the addition of the various chlorophenol which suggests that, under the relative concentrations of phenol and chlorophenols in the reactor, the uncoupling process is dominated by the presence of phenol.

The half saturation constant for the uncoupling process, K_U, is very small resulting in a zero order condition for uncoupling. The K_U values were nearly constant for all the dichlorophenols at about 1.6×10^{-8} *mole/l* and for the trichlorophenols values ranged from 1×10^{-8} to 1×10^{-6} *mole/l*. It is unknown why the K_U values for the trichlorophenols would have such a wide range.

Experiments with 4-chlorophenol could not be successfully modelled since it was degraded so rapidly that the 4-chlorophenol concentration could not be determined over time.

7.4 Relative Degradation Rates of Di- and Tri-chlorophenols

Trichlorophenols had significantly lower rates of degradation than the dichlorophenol in all experiments. These slower degradation rates can be

explained as follows:

1. Pathway of trichlorophenols would be quite different from that of dichlorophenols. It was reported that with trichlorophenols, dechlorination appears to precede ring cleavage (Steiert, *et al*, 1985), which means that before further degradation, trichlorophenols have to be subject to dechlorination. This dechlorination could be a limiting step. Namely, the substitution of a hydroxyl group for a chlorine atom on the intact phenolic ring could be much slower than the consequent degradation.

2. As an oxidation process, dichlorophenols could be more readily oxidized than trichlorophenols because they result in a larger free energy generation.

3. Compared to less chlorinated phenols, trichlorophenols have more steric hindrance to approach enzyme because the size of a chlorine atom is much greater than that of a proton. The relative position of the chlorine atoms to the hydroxyl group on the phenolic ring may also affect the reaction rate. The substituent groups hinder intimate contact between the phenol and the enzyme.

4. The activation energy to form an enzyme-trichlorophenol complex could be higher than that to form an enzyme-dichlorophenol complex.

7.5 Relative Influence of Uncoupling

The relative influence of uncoupling is shown in Figure 7.2 for the average values from the 2,4-DCP experiments. Similar curves for phenol and the other six chlorophenol experiments are shown in Appendix B. The curves have been calculated from the model values as:

$$\left(-\frac{dS}{dt}\right)_{Uncoupling} = \frac{k_U X S}{K_U + S} \qquad (1)$$

and,

$$(-\frac{dS}{dt})_{Normal} = \frac{kXS}{K_M(1+\frac{\alpha I_T}{K_I})+S(1+\frac{S}{K_P})} \qquad (2)$$

As shown in Figure 7.2, from 50 to 80% of the energy from phenol degradation was dissipated by the uncoupling process. The highest percentages were during the high phenol substrate concentrations during the middle portion of the experiments. These data show that uncoupling for phenol is an important process related to the cometabolic oxidation of chlorophenols.

The precipitous drop in degradation rate in Figure 7.2 at 0.4 day results from an instability in the numerical approximation in the model. At this time, the phenol concentration decreased to a small concentration which made the model unstable.

The increase in the degradation rate over the experiment is due to the increase in cell mass from growth. Over the 12 hours of the experiments, the cell concentration typically doubled resulting in a doubling of the degradation rate.

7.6. Relative Influence of Chlorophenol and Phenol Inhibition

The relative influence of chlorophenol and phenol inhibition on the substrate degradation rate without uncoupling is shown in Figure 7.3 and Appendix C. The degradation rate for phenol was first order with respect to phenol concentration at the very beginning and final stages for each individual experiment because the following relationship exists:

$$K_M \gg (S+\frac{S^2}{K_P}+\frac{K_M I}{K_I}) \qquad (3)$$

therefore,

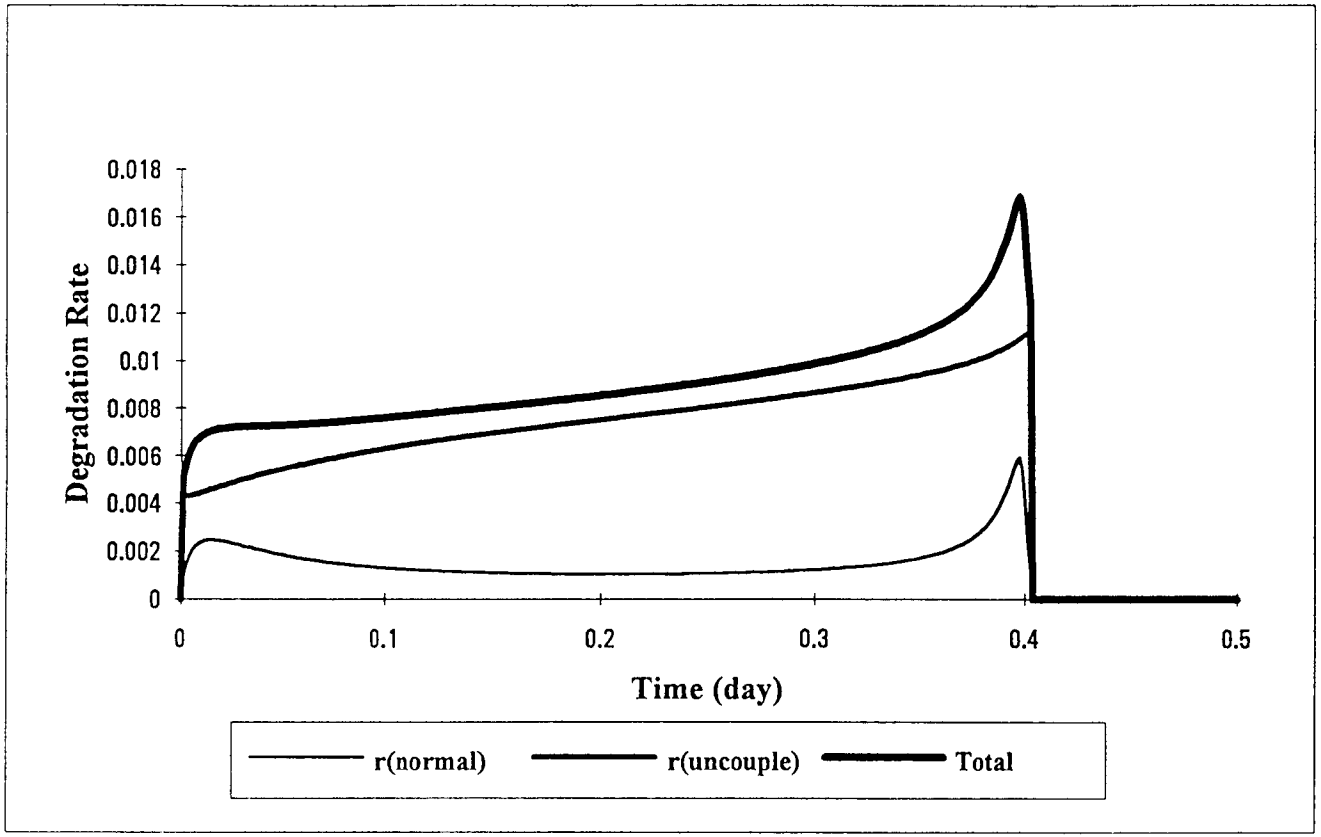

Figure 7.2 Comparison of Phenol Degradation with 2,4-DCP

$$(-\frac{dS}{dt})_{Normal}=\frac{kX}{K_M}S \qquad (4)$$

At the intermediate range, however, the degradation rate of phenol without uncoupling was negative first order with respect to phenol concentration because the following relationship holds:

$$\frac{S^2}{K_P} \gg (K_M+S+\frac{K_M I}{K_I}) \qquad (5)$$

therefore,

$$(-\frac{dS}{dt})_{Normal}=kK_P X\frac{1}{S} \qquad (6)$$

From Figure 7.4 and Appendix D, a similar discussion can be made with relation to substrate degradation under uncoupling. At the intermediate range, the uncoupling degradation rate of phenol was zero order with respect to phenol concentration because the following relationship exists:

$$S \gg K_U \qquad (7)$$

therefore,

$$(-\frac{dS}{dt})_{Uncoupling}=k_U X \qquad (8)$$

At the very beginning and final stage, however, the phenol uncoupling rate was first order with respect to phenol concentration because the following fact exists:

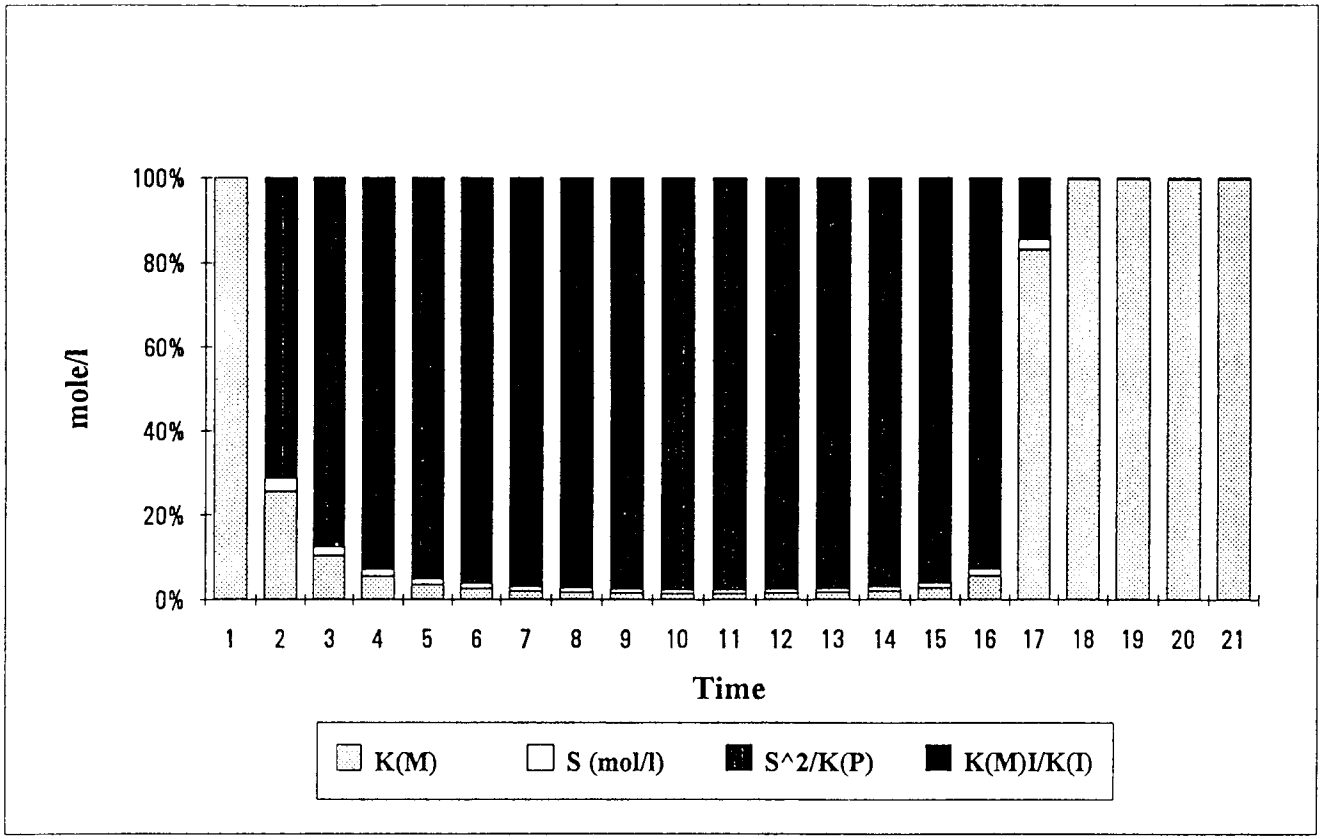

Figure 7.3 Distribution of Phenol Degradation with 2,4-DCP

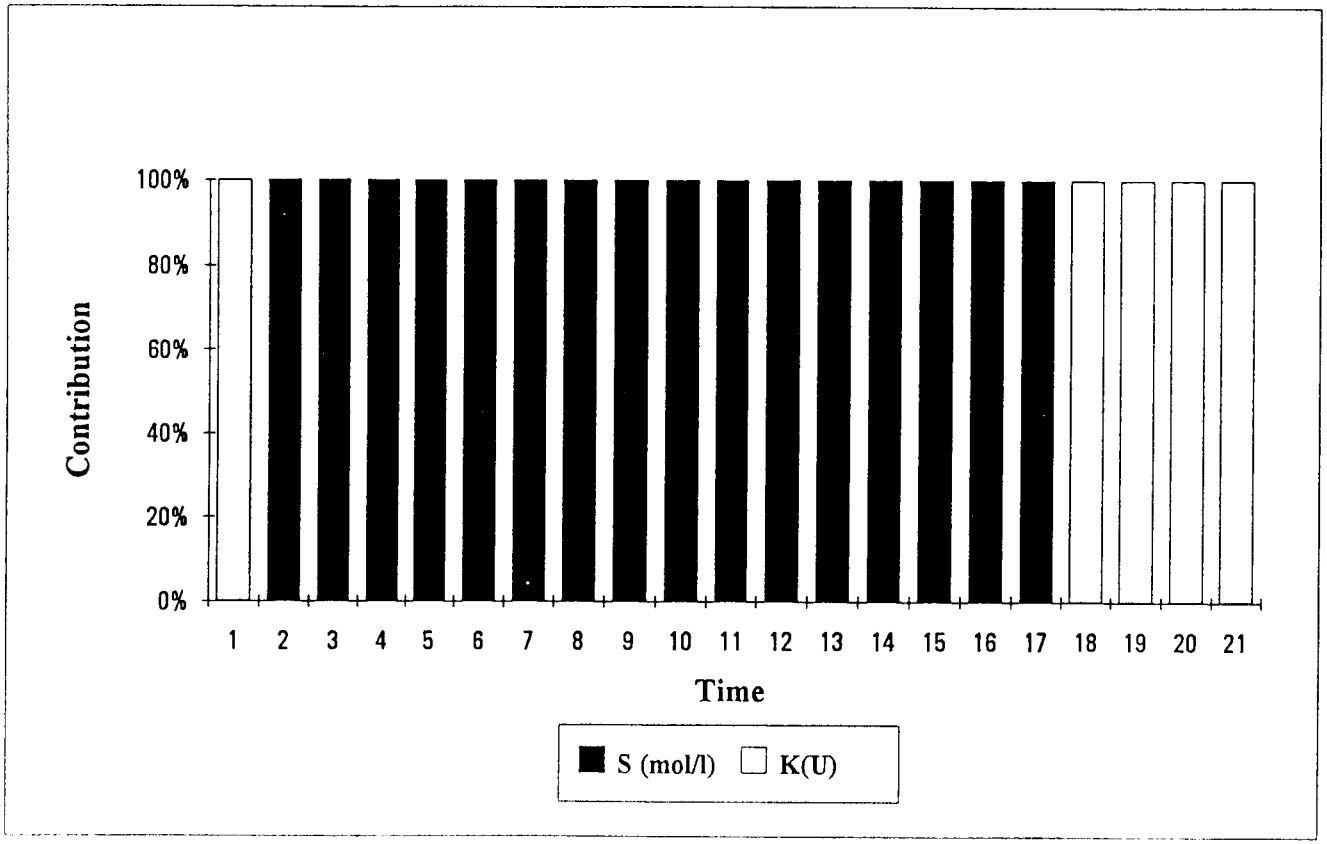

Figure 7.4 Uncoupling Rate of Phenol with 2,4-DCP

$$K_U \gg S \tag{9}$$

thus,

$$\left(-\frac{dS}{dt}\right)_{Uncoupling} = \frac{k_U X}{K_U} S \tag{10}$$

The curves in Appendix A, B, C, and D show that the biodegradation of phenol is significantly influenced by substrate inhibition, chlorophenol inhibition, and uncoupling. The uncoupling process played a significant role for removing phenol at all concentration ranges.

7.7 Sensitive Analysis

Sensitivity analyses for the phenol/2,3,5-trichlorophenol experiments are shown in Appendixes E. The figures show the changes in the total sum of squares residual as the estimate for each individual parameter is altered. The figures show that the model is highly sensitive to Y, k_U, k, K_M and K_P. The model is much less sensitive to K_I, k_I, and K_U. The insensitivity of the model to K_I and K_U results because the estimated values are considerably lower than the I and S experimental concentration, respectively. The insensitivity of the model to k_I results because concentrations of the chlorophenols were low compared to phenol concentrations resulting in relatively low degradation rates.

CHAPTER 8
CONCLUSIONS

Based on the results of this research, the following conclusions are drawn in relation to the aerobic degradation of phenol and chlorophenols:

1. Tri- and di-chlorophenols can be cometabolicly degraded in the presence of phenol. The phenol degradation rate is considerably more rapid than the degradation rate of chlorophenols.

2. Both the number of chlorine atoms and their relative position on the phenolic ring influence the degradation of chlorophenols. The following order exists for the cometabolic degradation rate: 3,5-DCP > 2,4-DCP > 3,4-DCP and 2,4,5-TCP > 2,4,6-TCP > 3,4,5-TCP > 2,3,5-TCP.

3. At chlorophenol levels of less than 1 *mg/l*, chlorophenol inhibition is not an important process affecting the degradation rate. The phenol substrate utilization rate is not significantly reduced by competition with the various chlorophenol.

4. At phenol concentrations greater than 1 *mg/l*, the phenol inhibition rate and electron transport chain uncoupling are both important processes affecting the degradation rate. The uncoupling process resulted in substantial loss of cell growth over all concentration ranges.

5. The model developed to describe phenol oxidation, chlorophenol oxidation, phenol inhibition, chlorophenol inhibition, and electron transport chain uncoupling provided excellent fit to experimental data.

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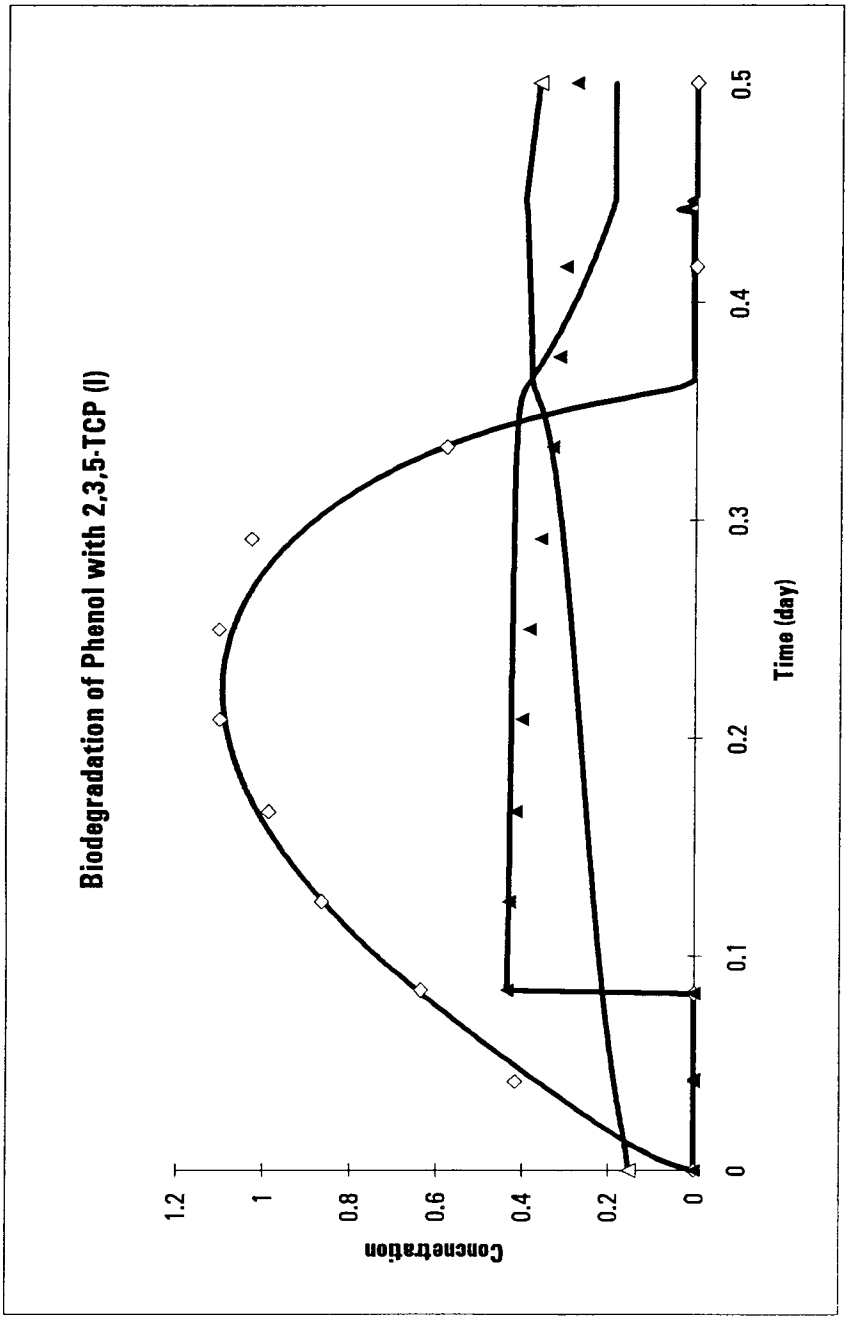
APPENDICES

Appendix A

Biodegradation of Phenol with Chlorophenols

Appendix A (Continued)



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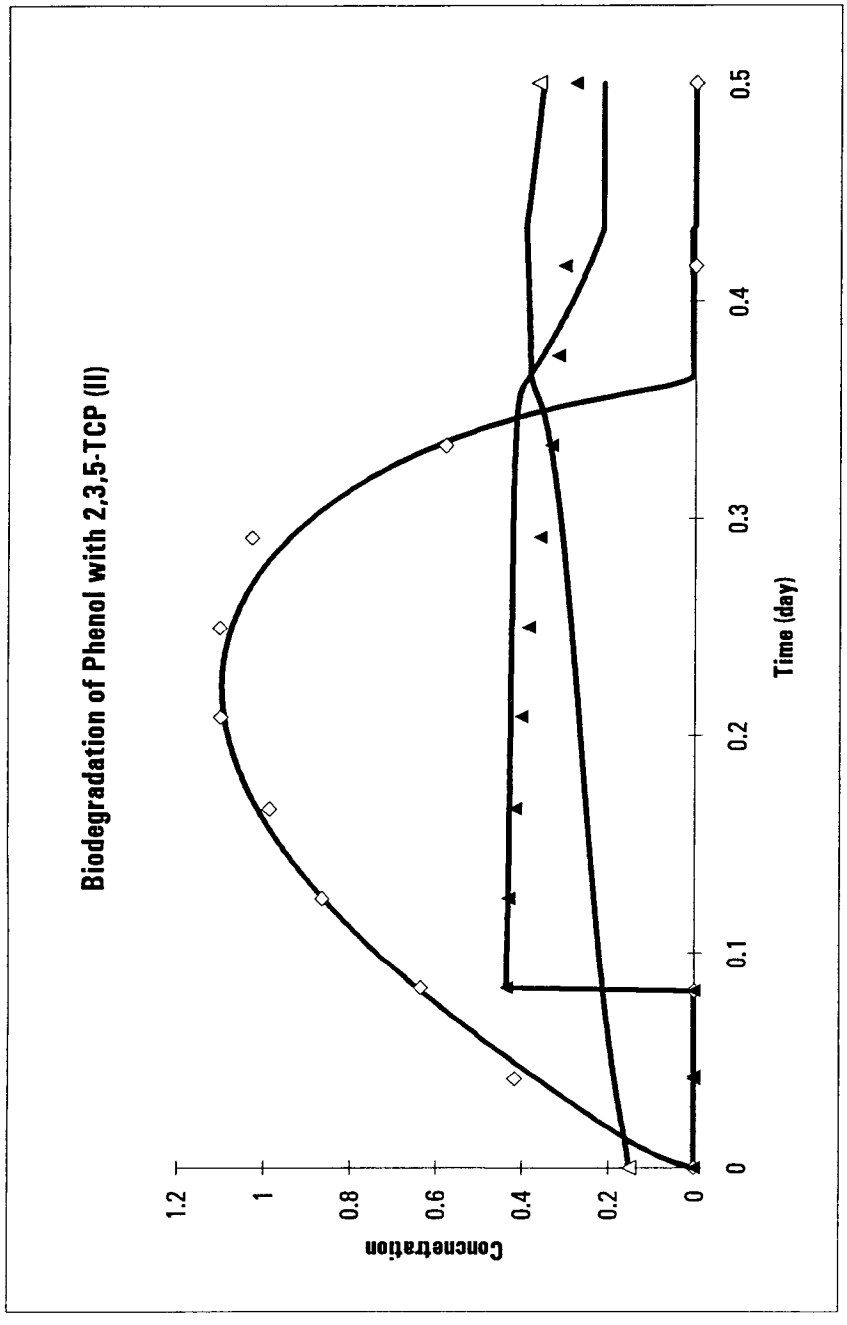

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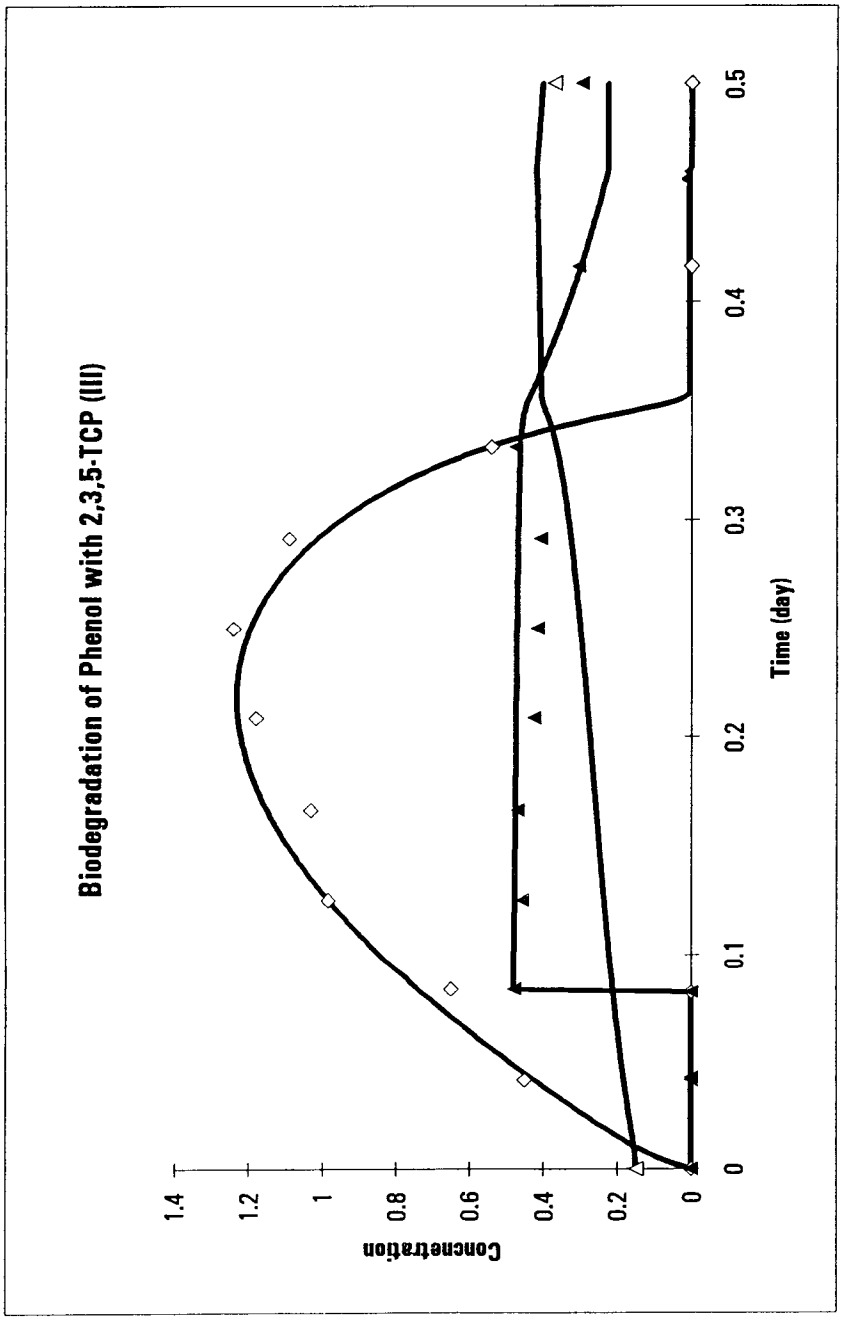

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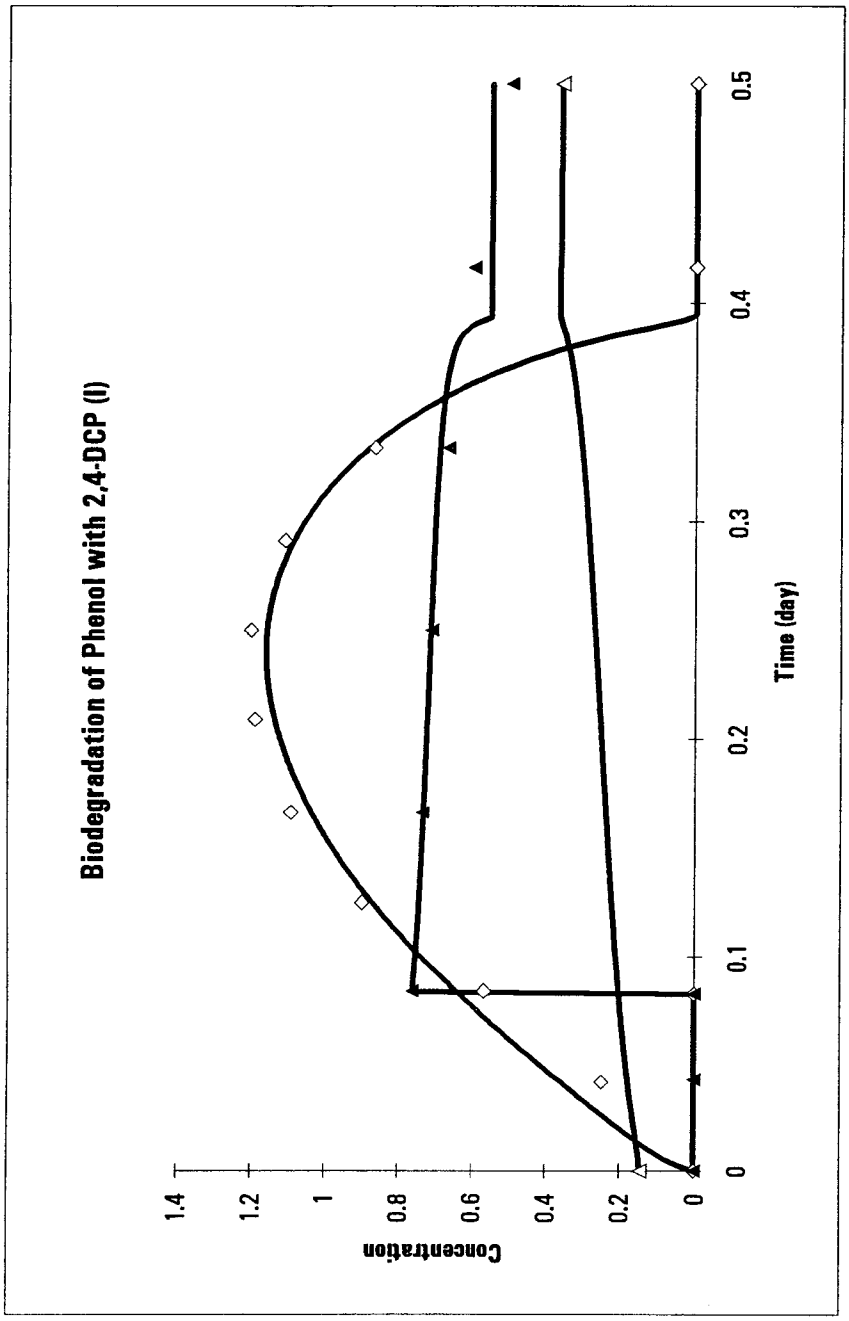

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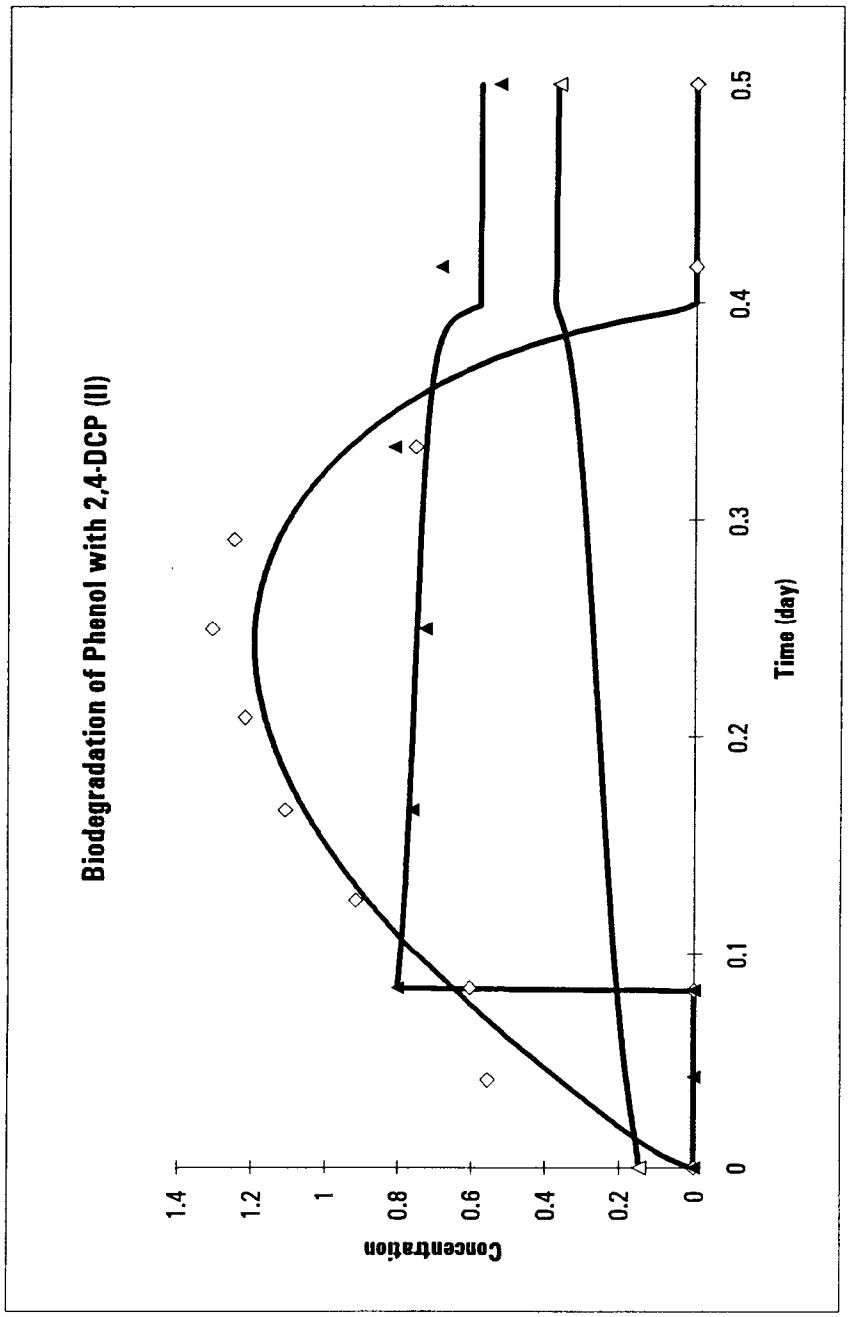

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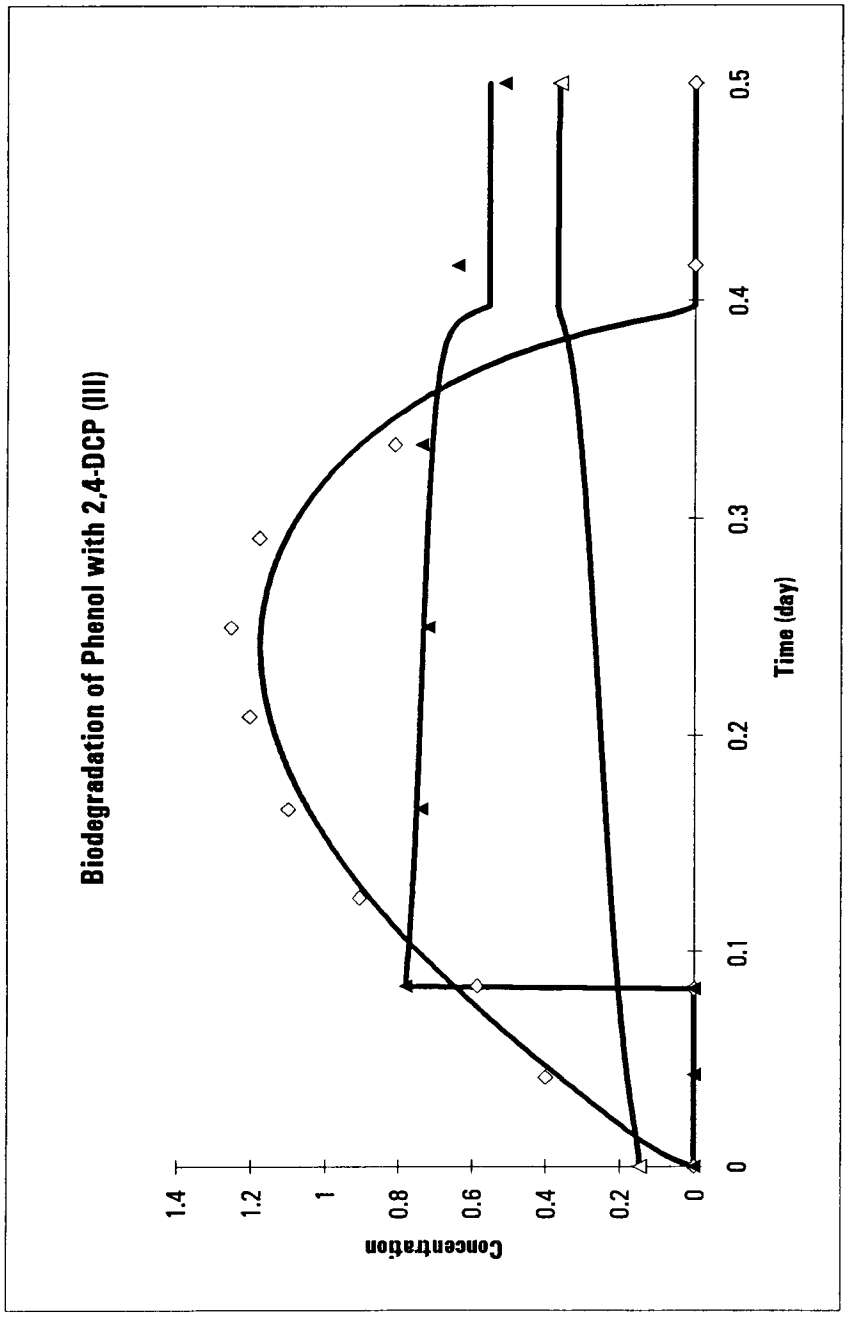

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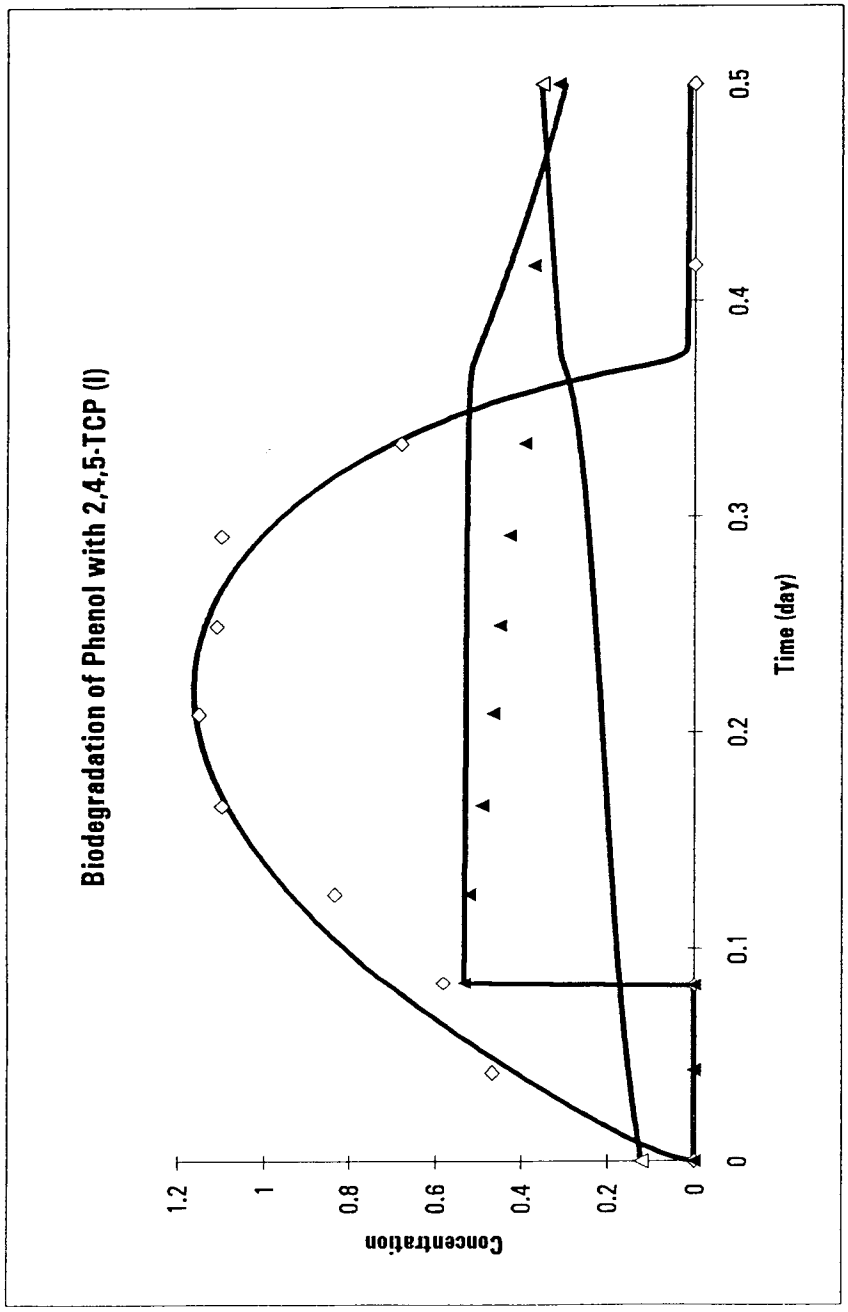

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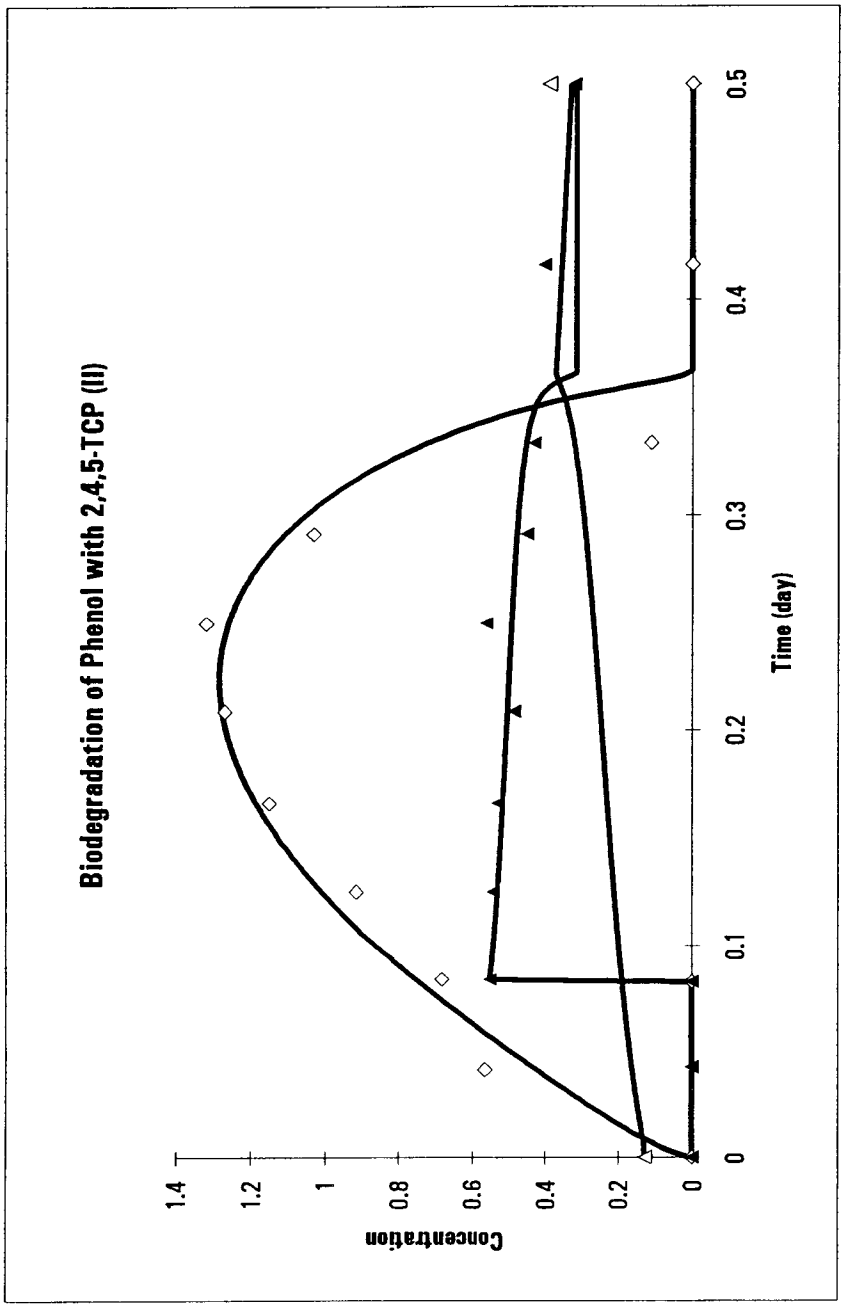

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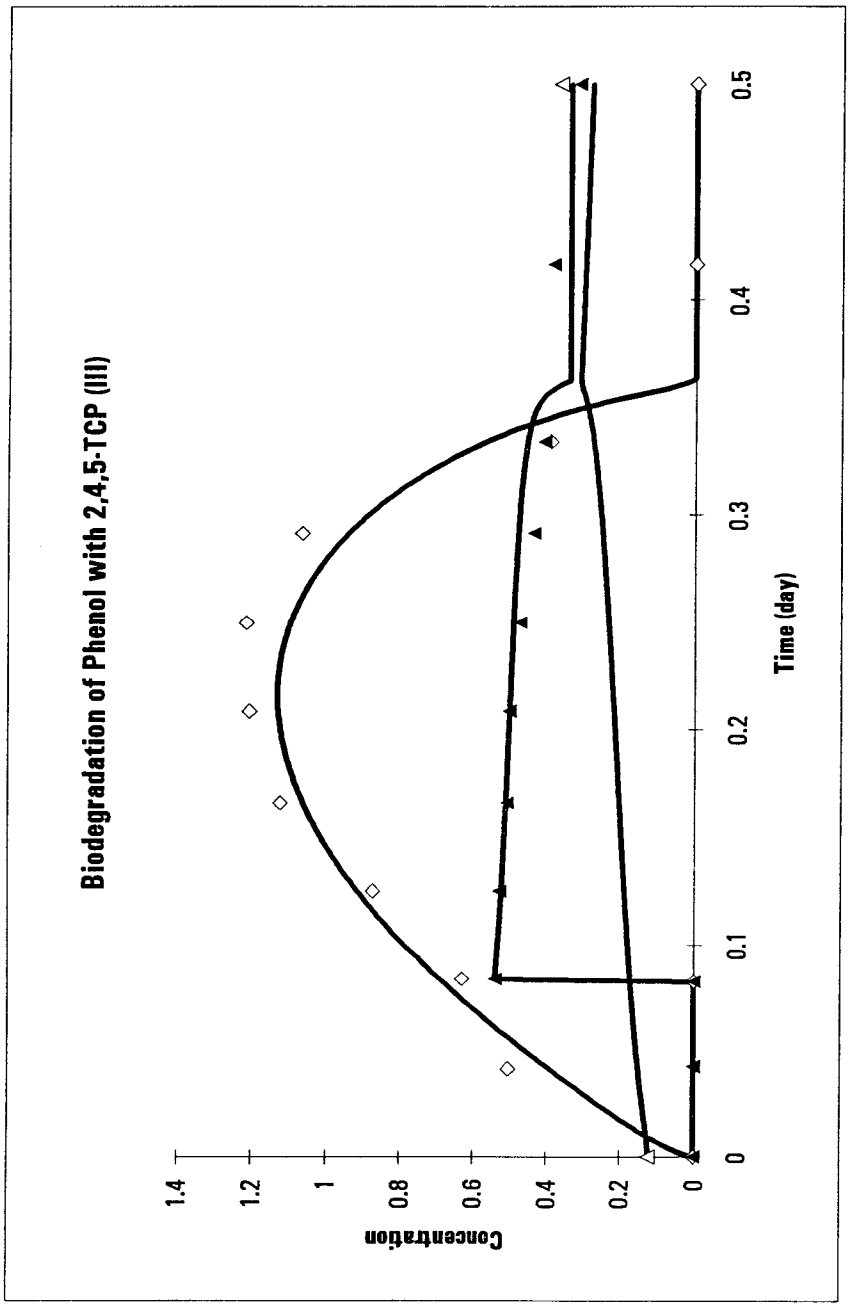

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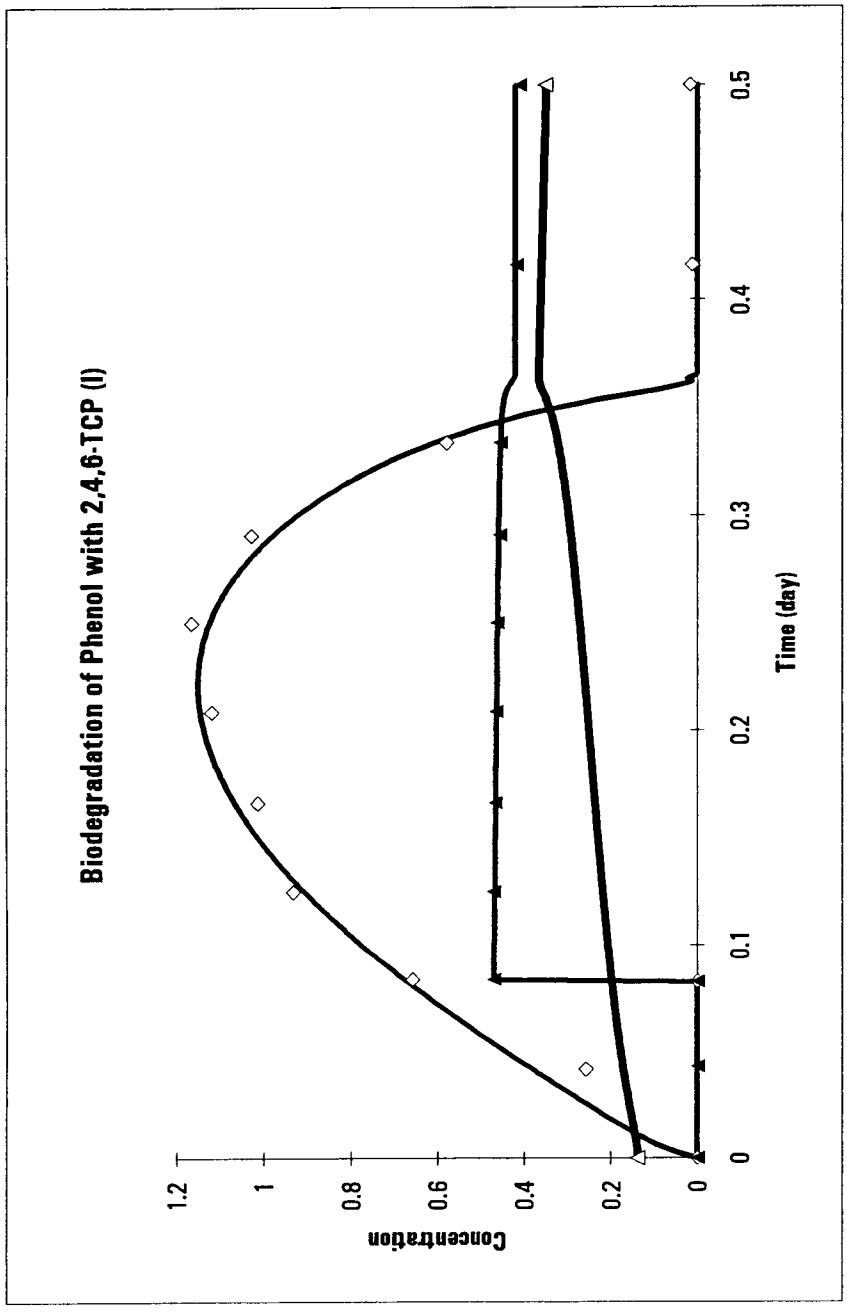

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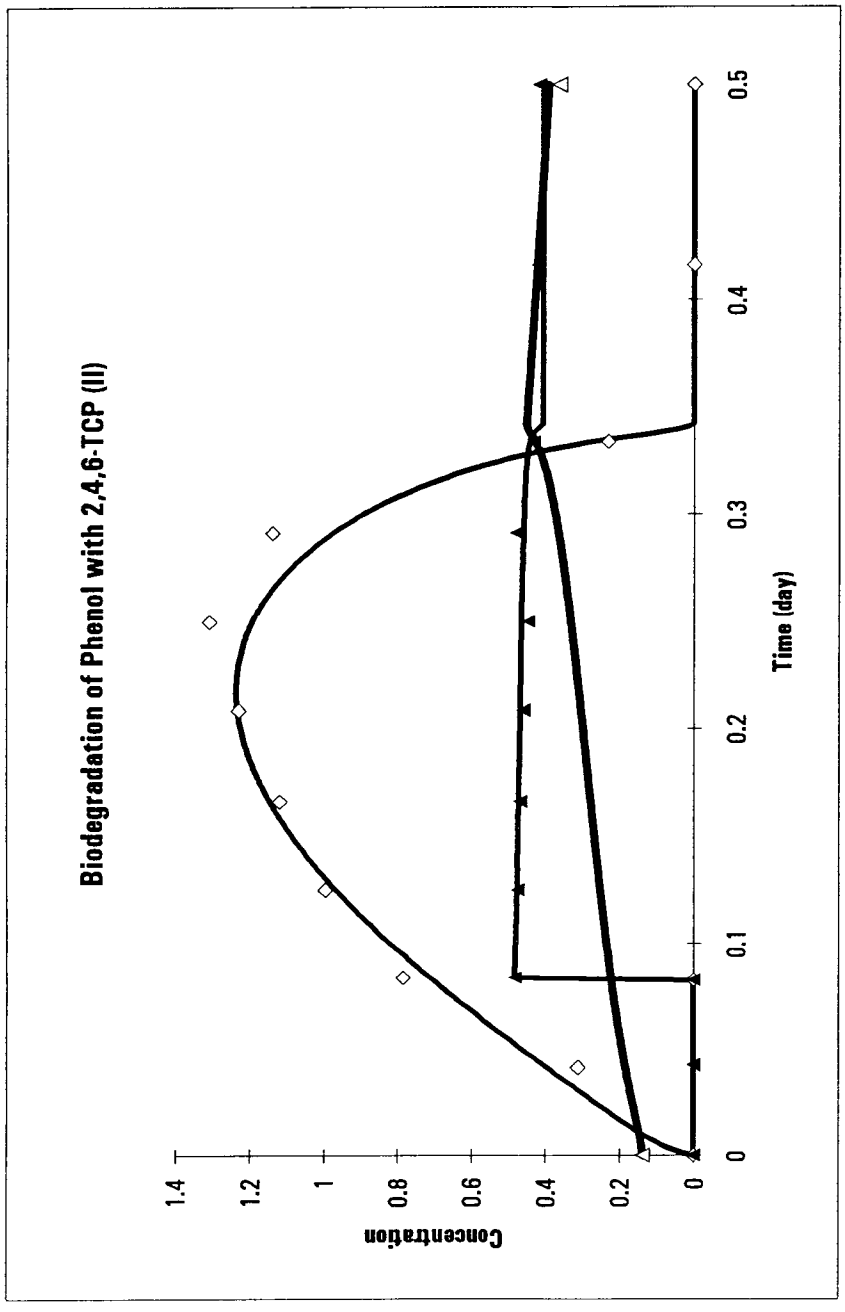

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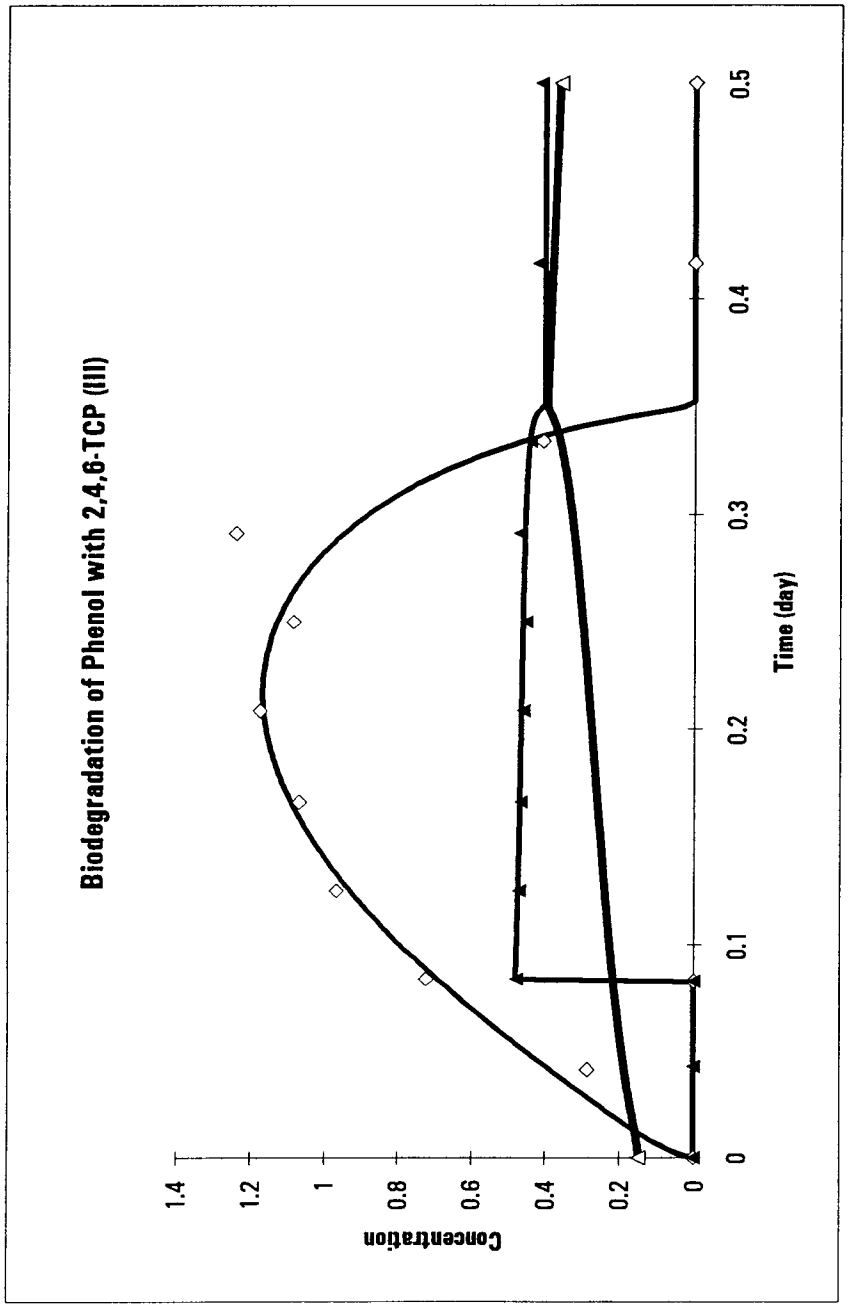

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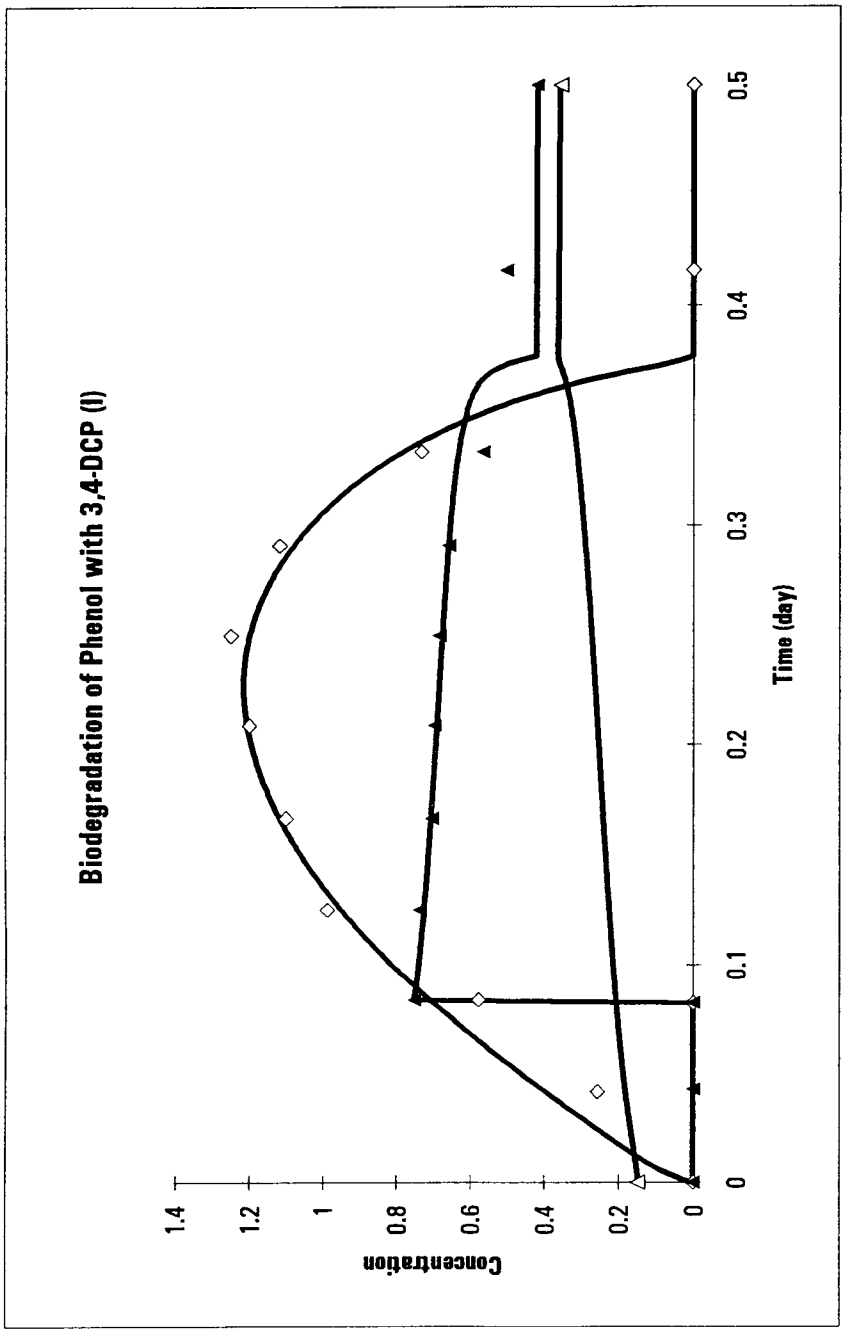

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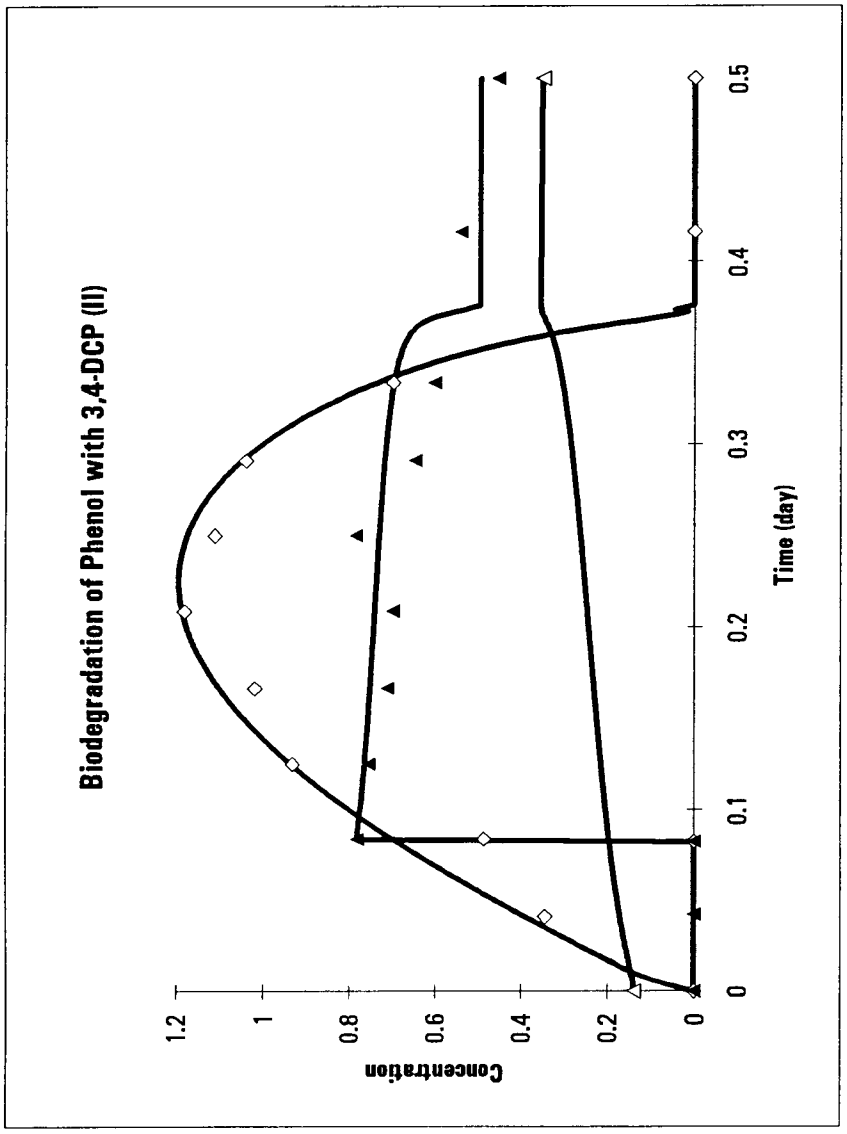

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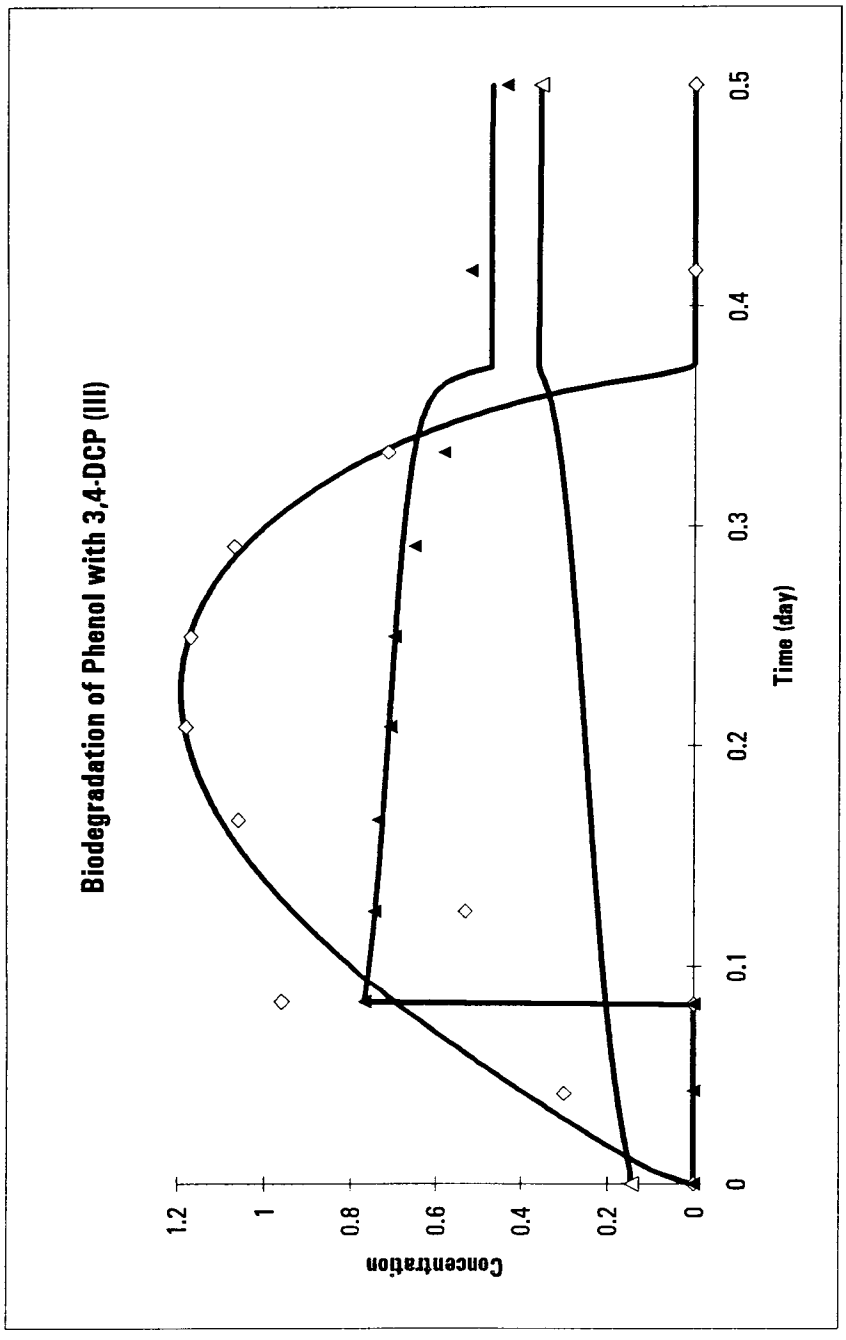

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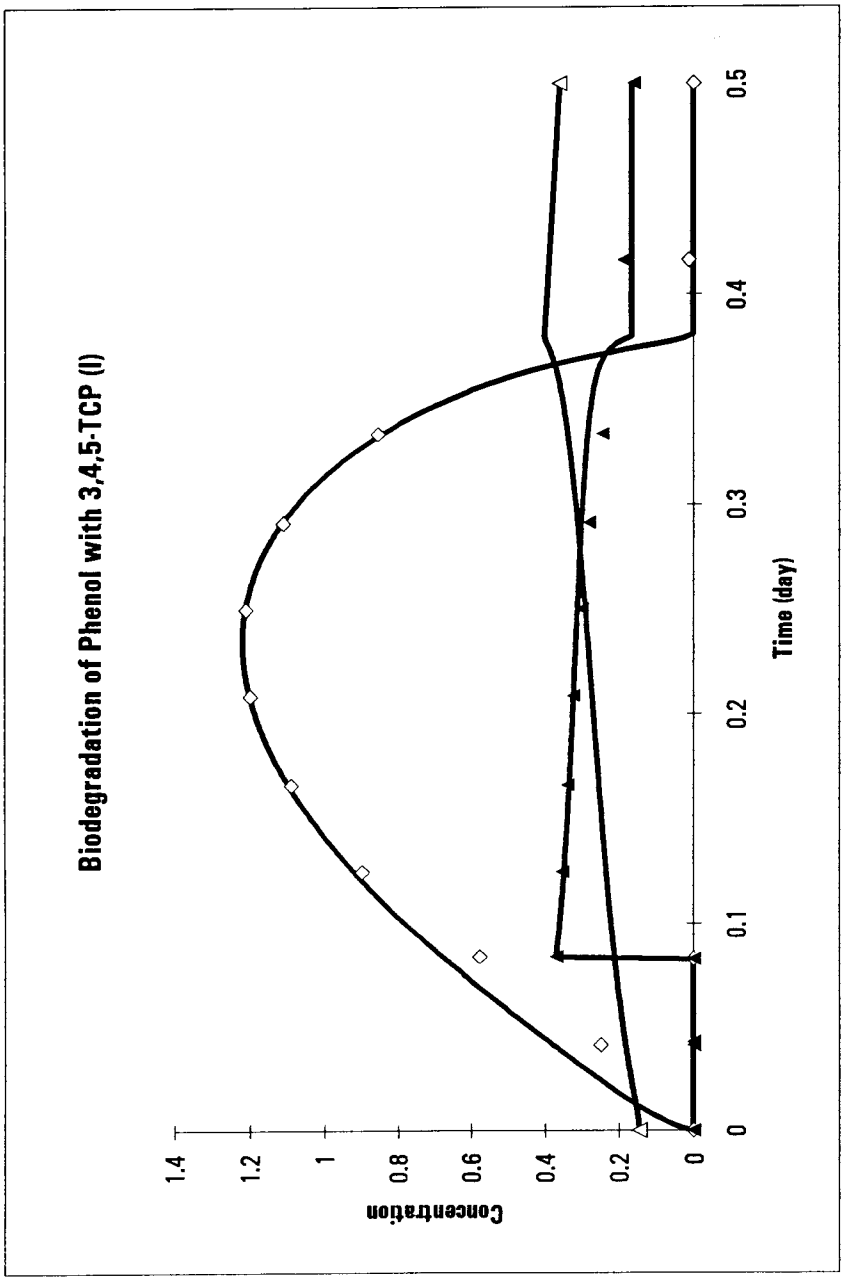

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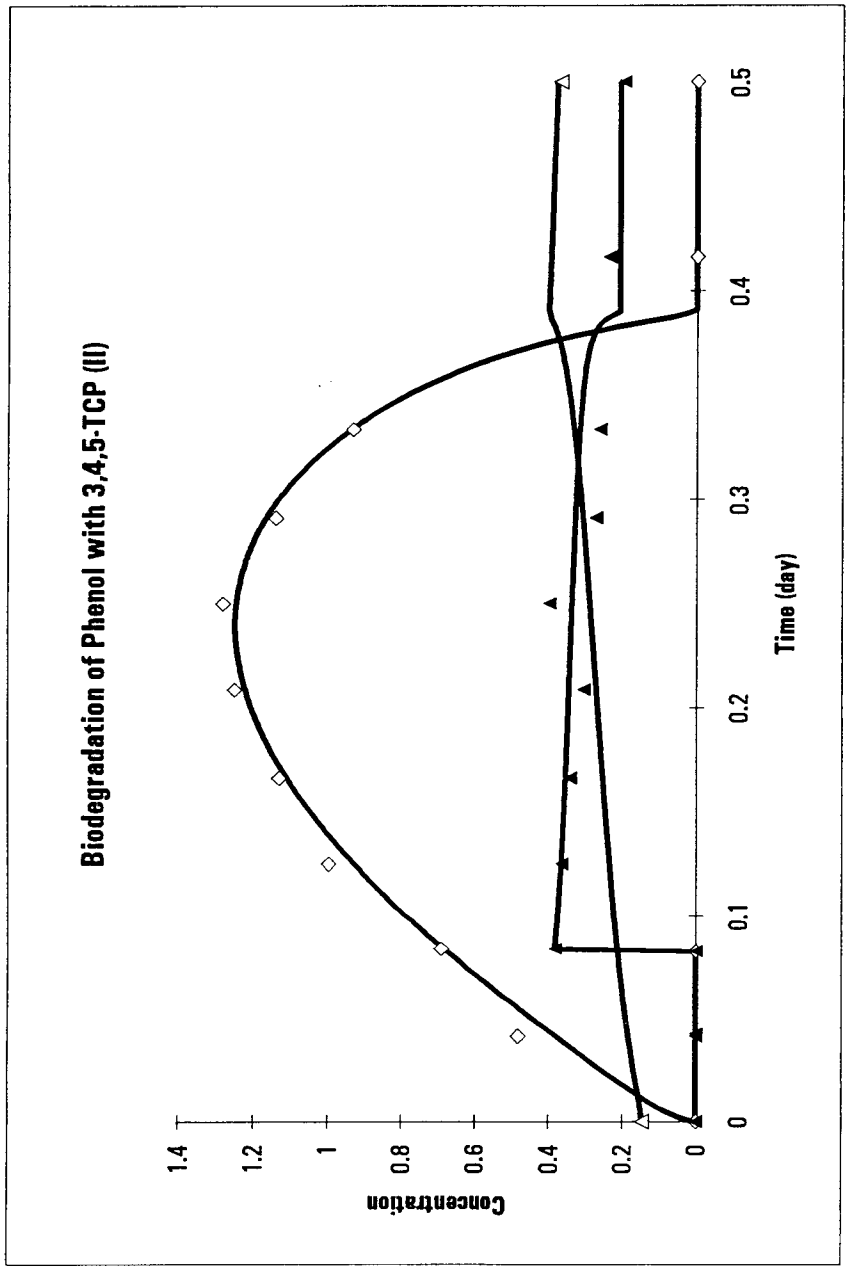

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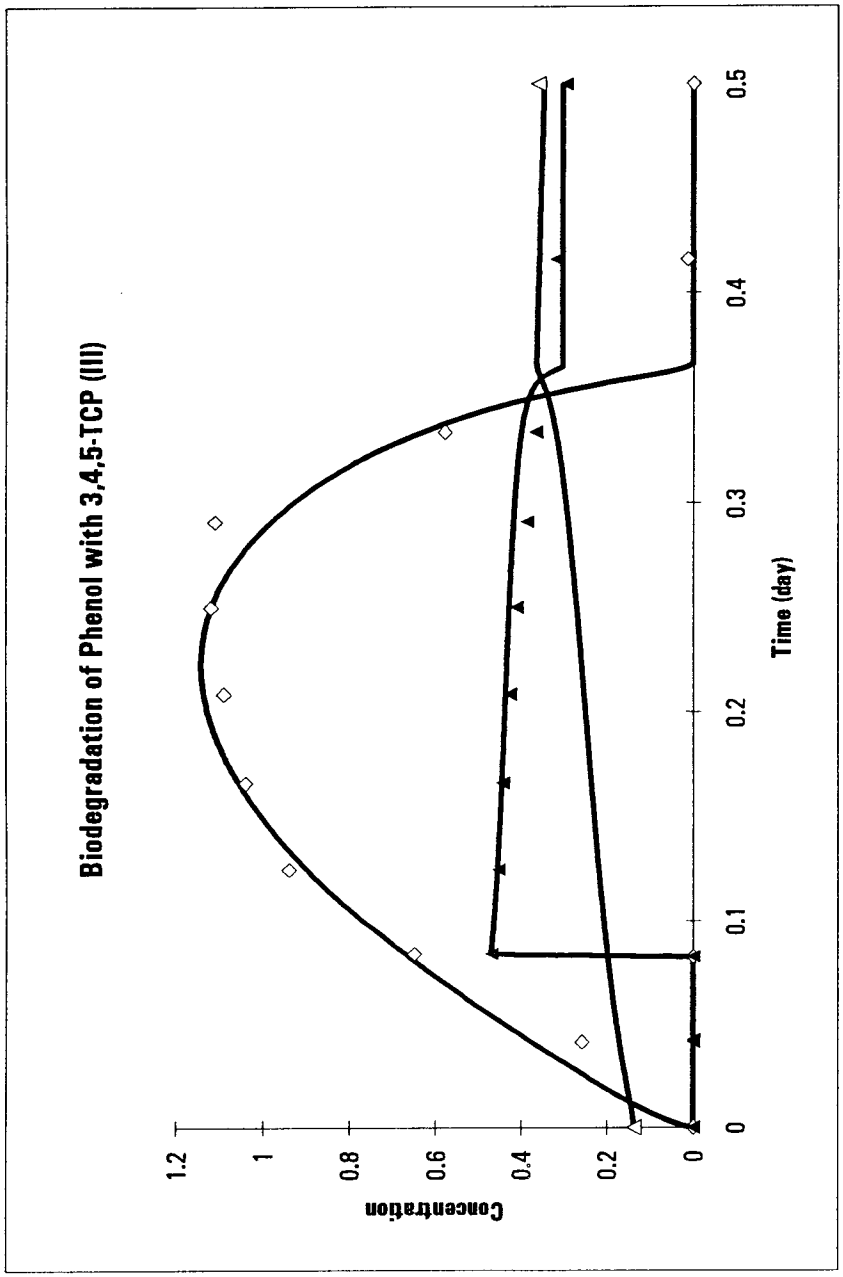

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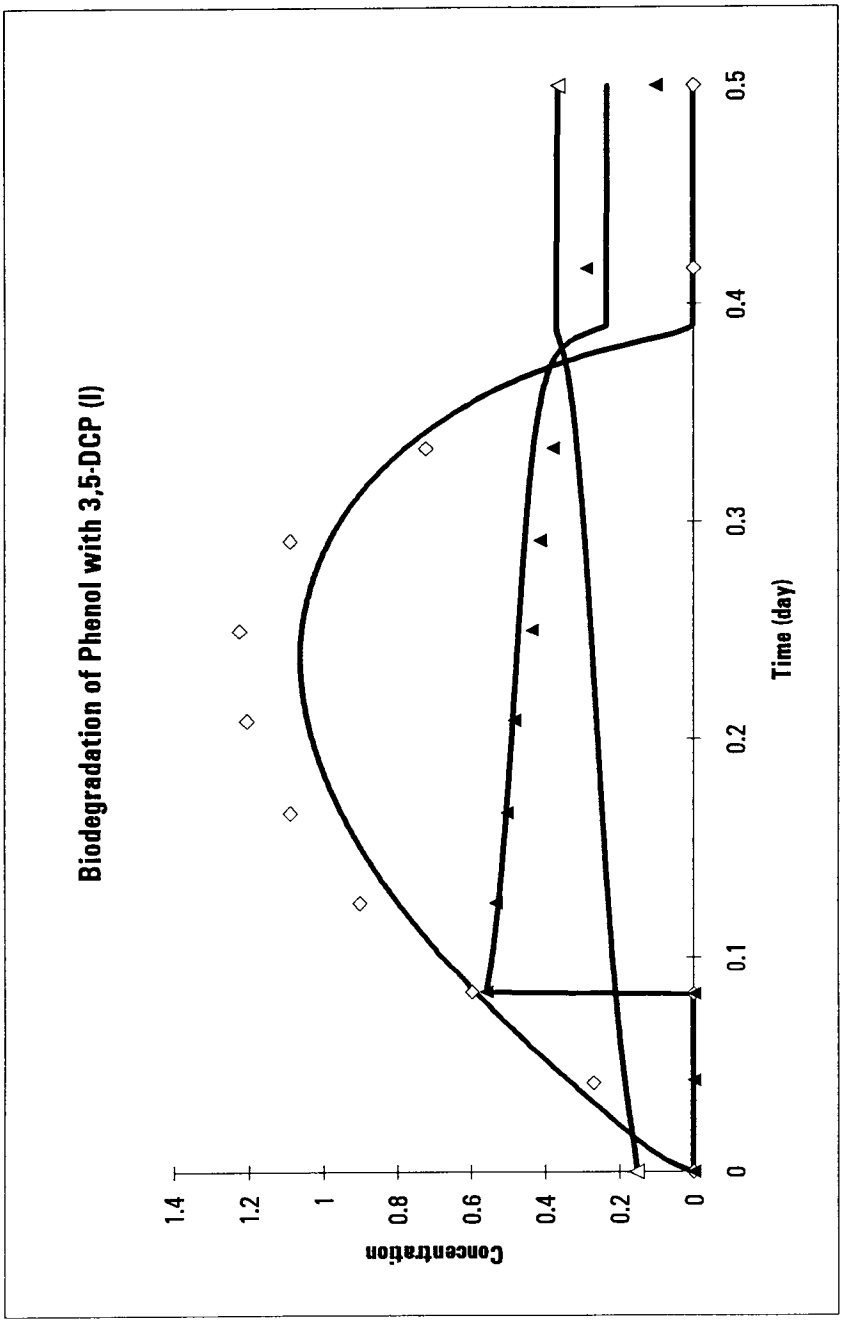

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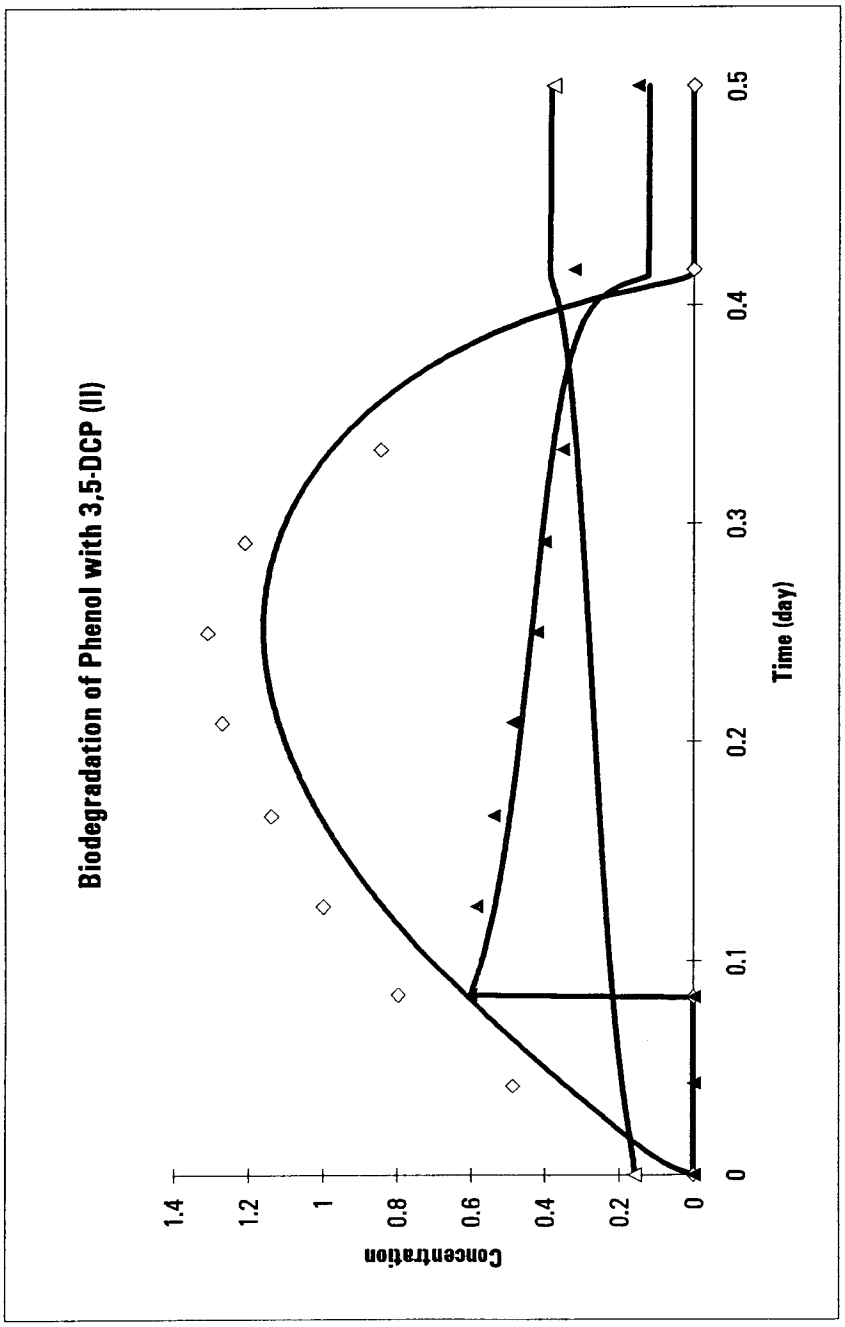

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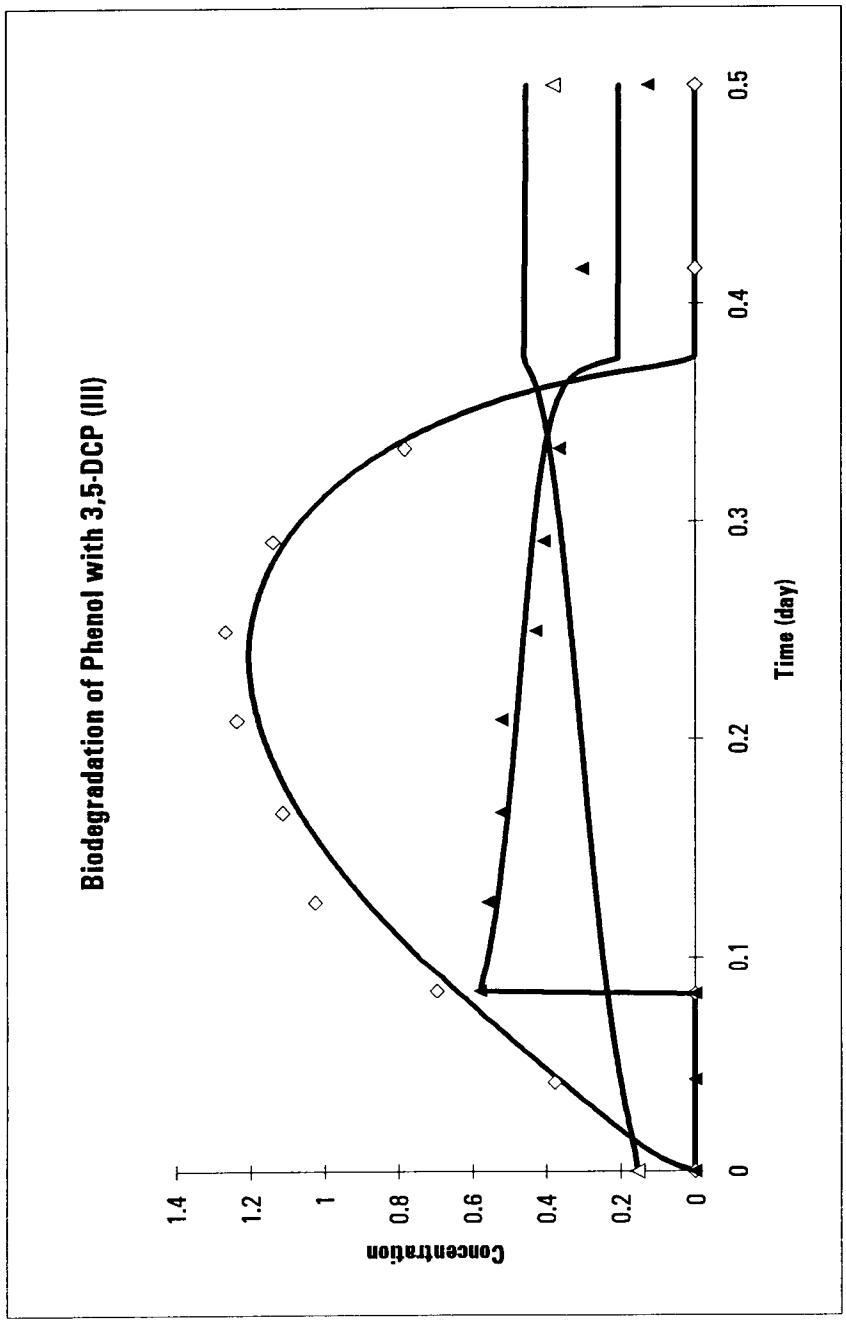
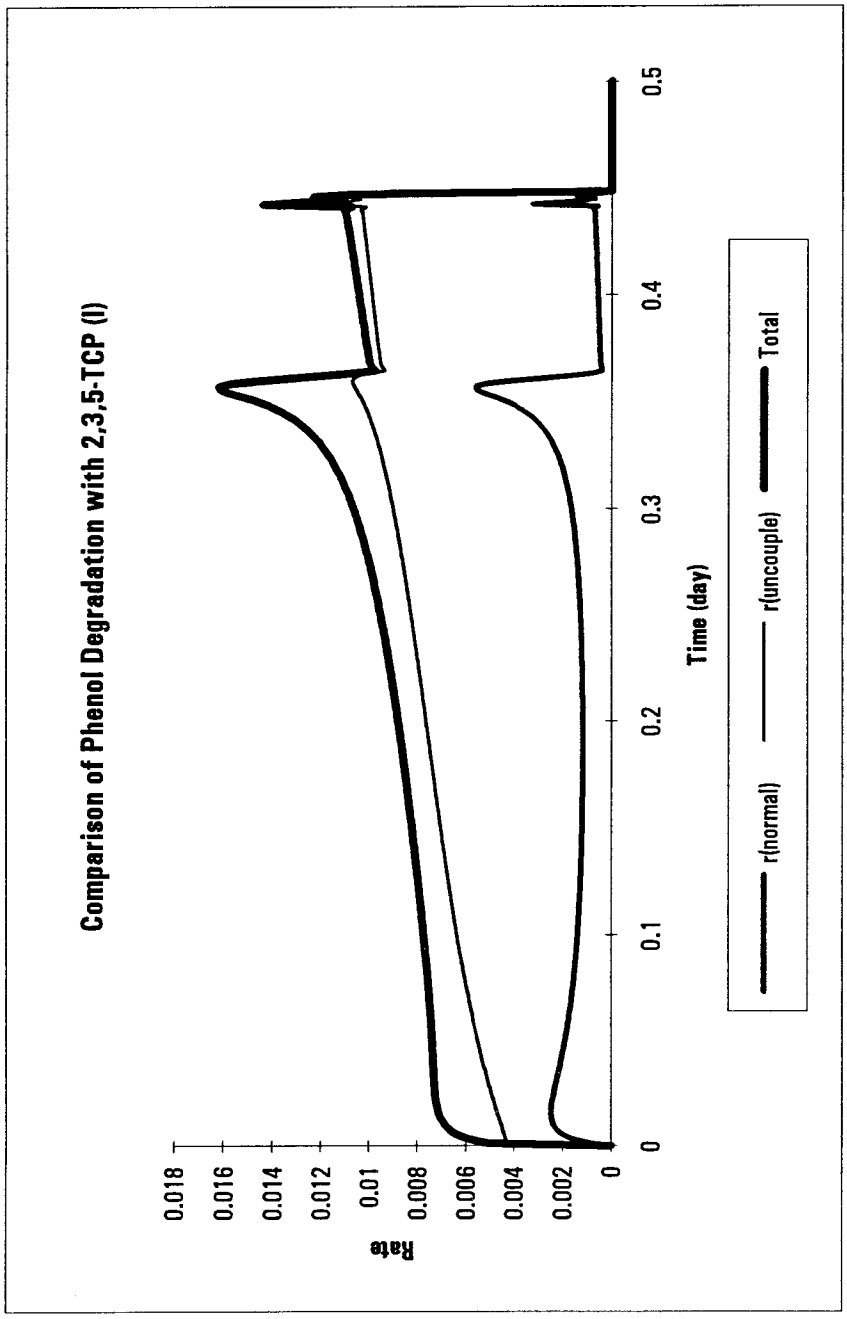


Appendix B

Comparison of Phenol Degradation with Chlorophenols

Appendix B (Continued)



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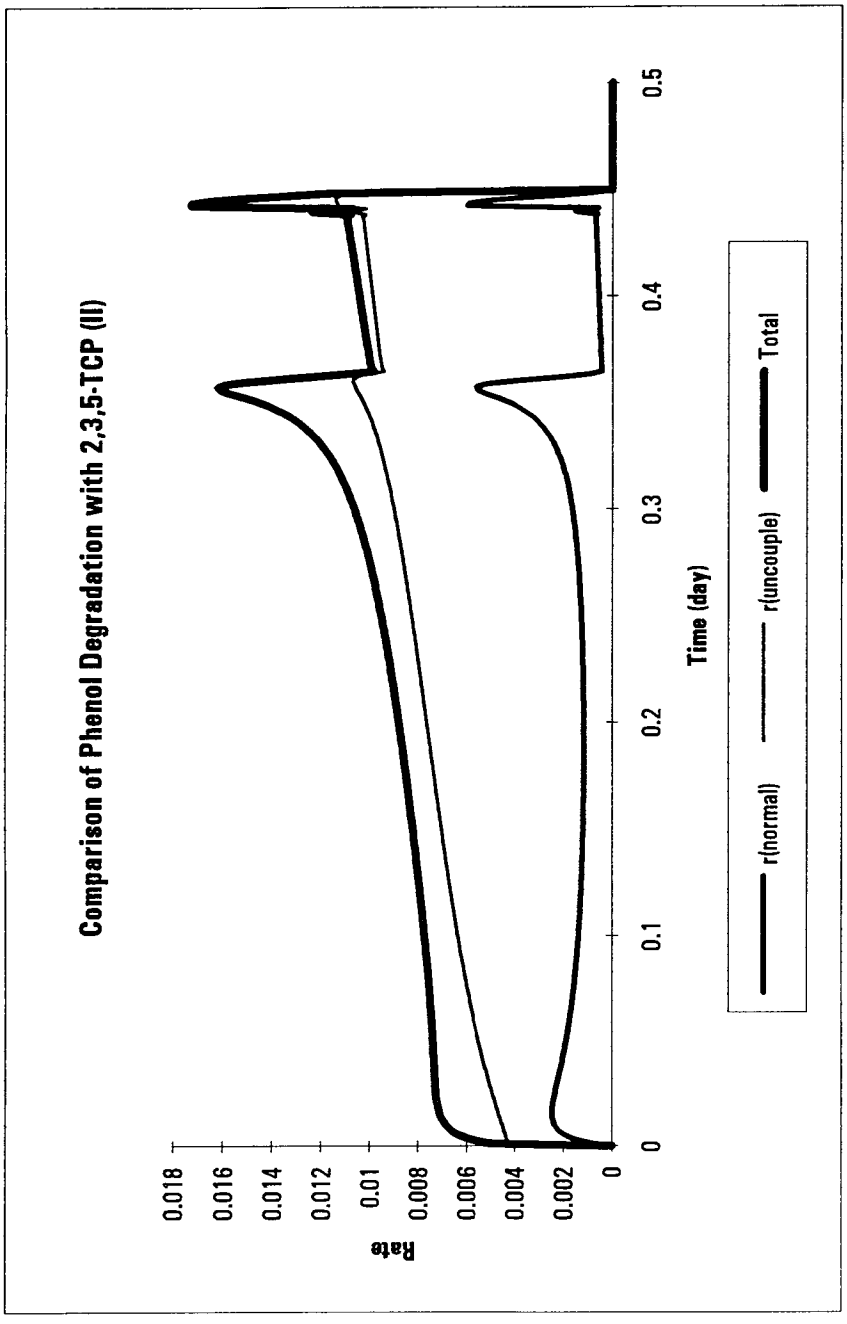

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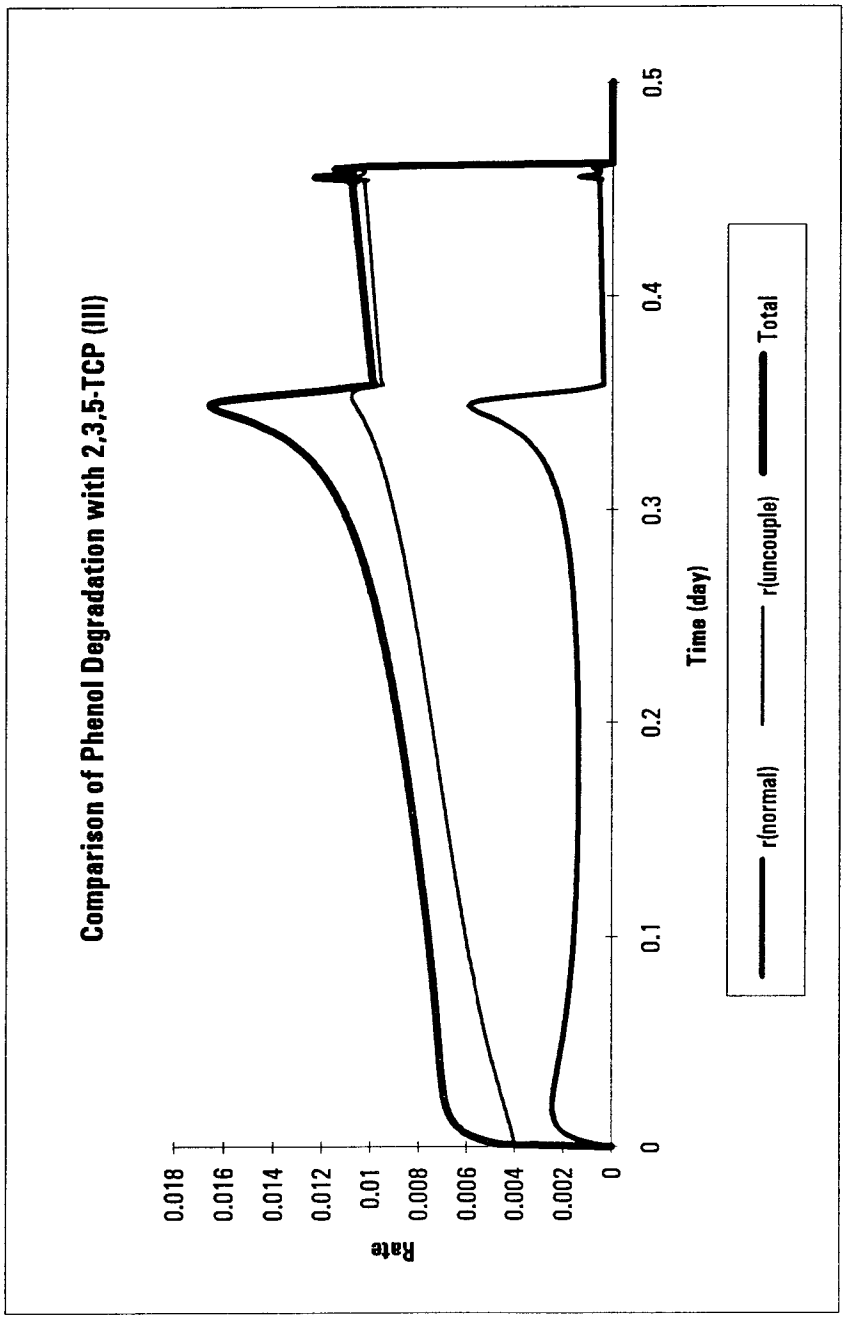

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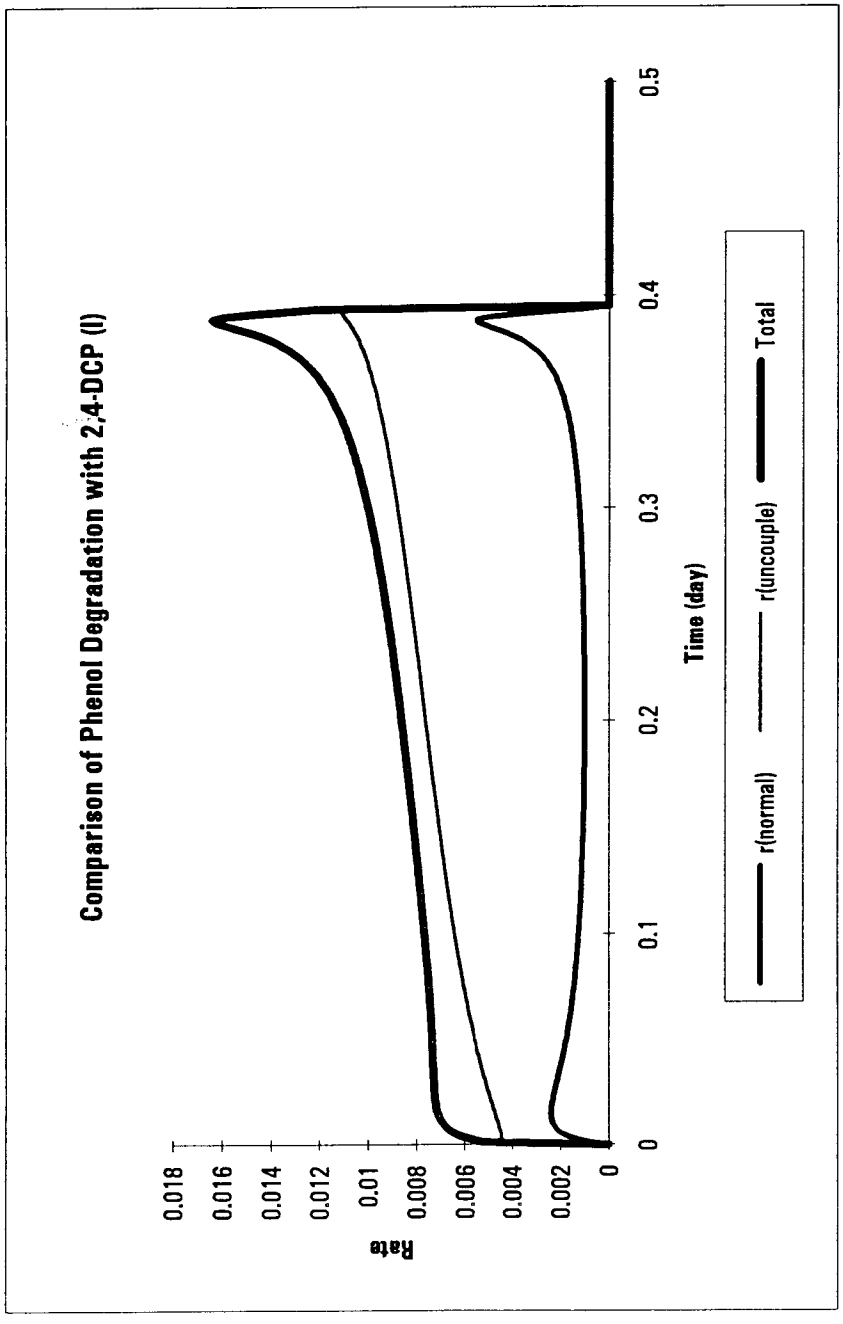

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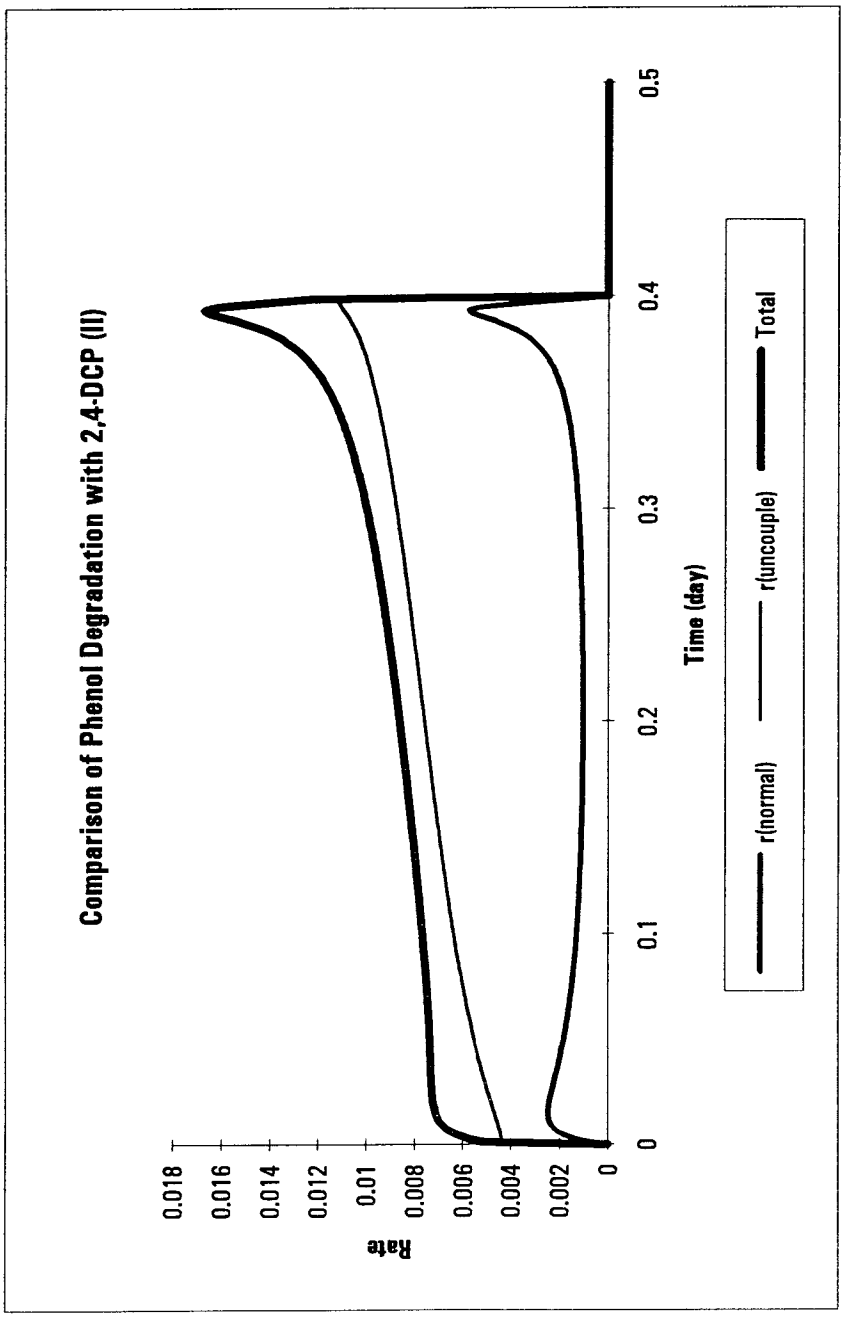

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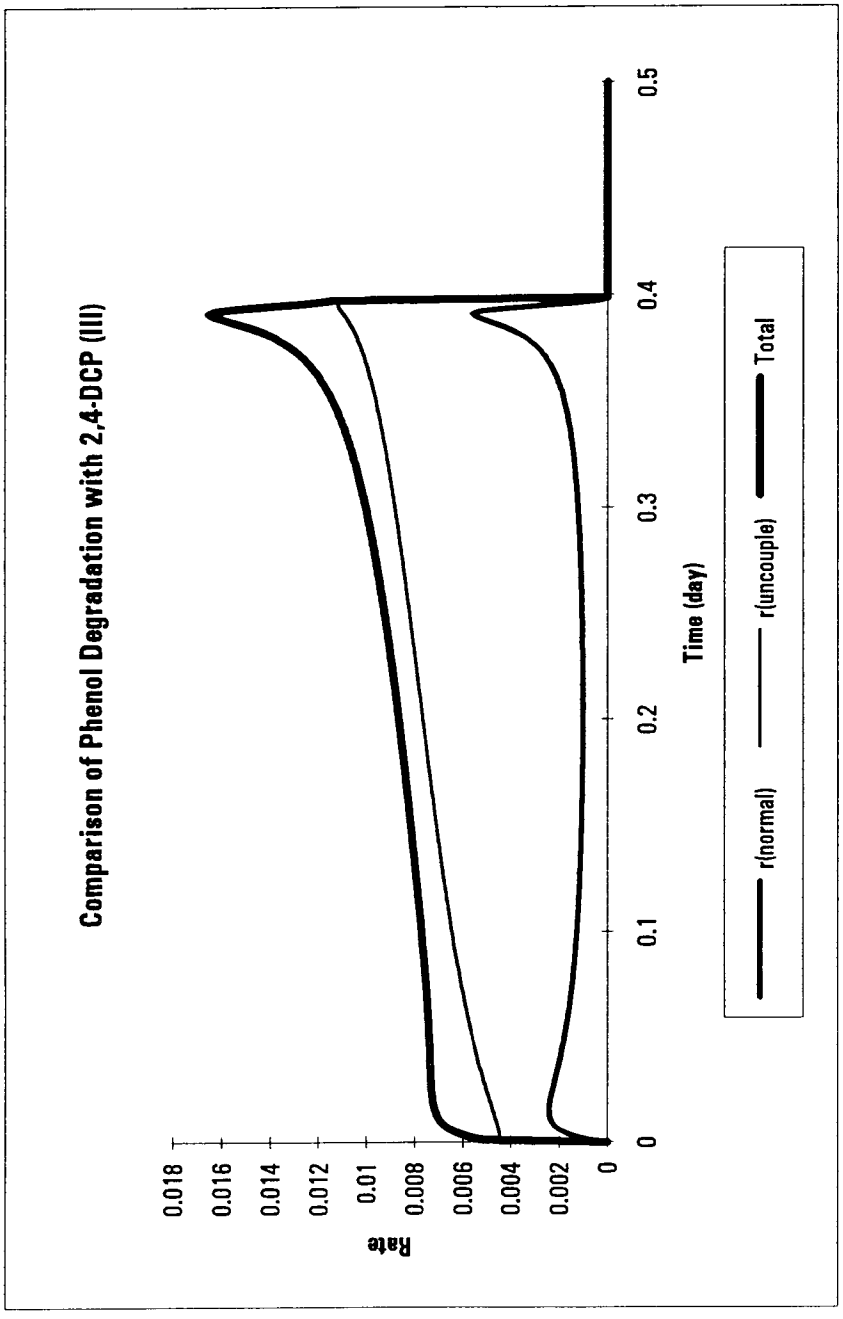

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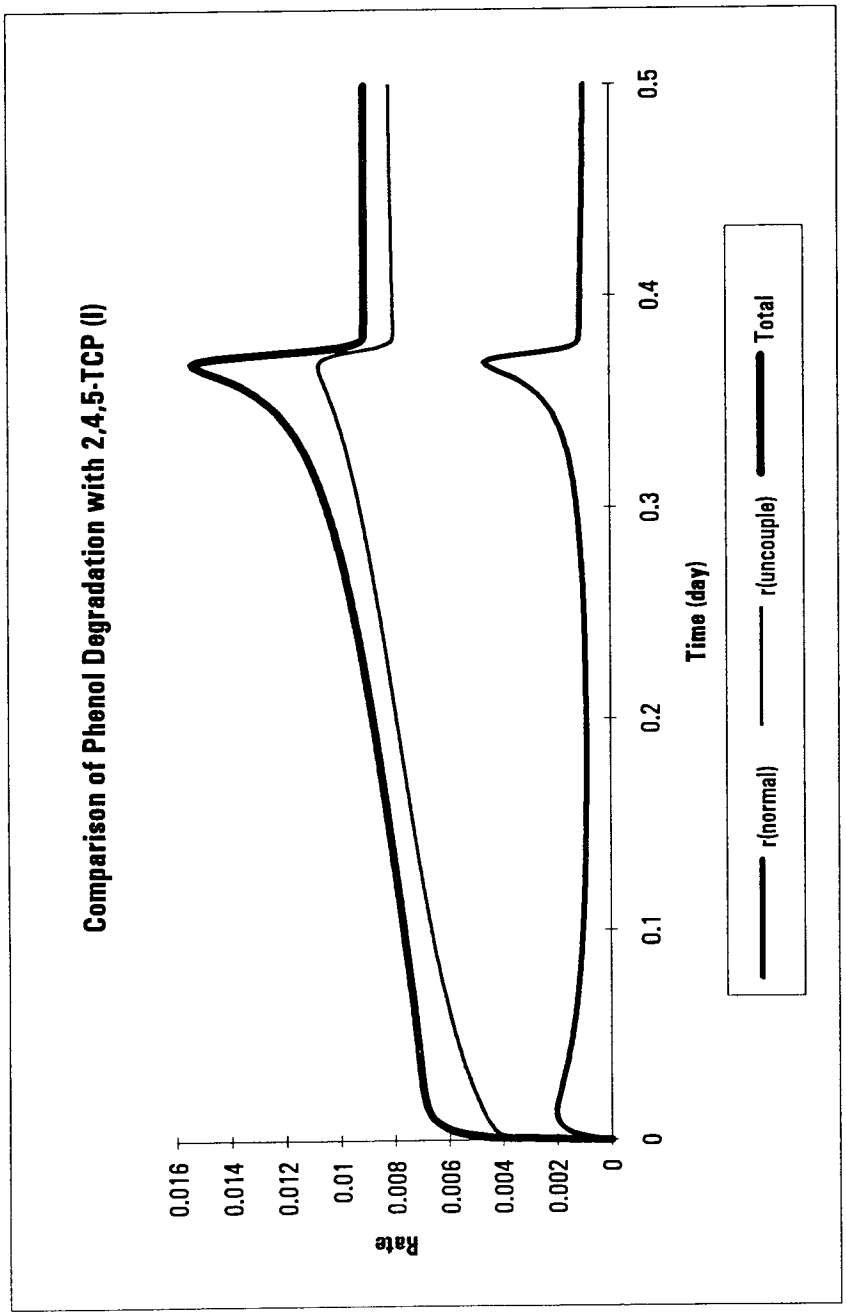

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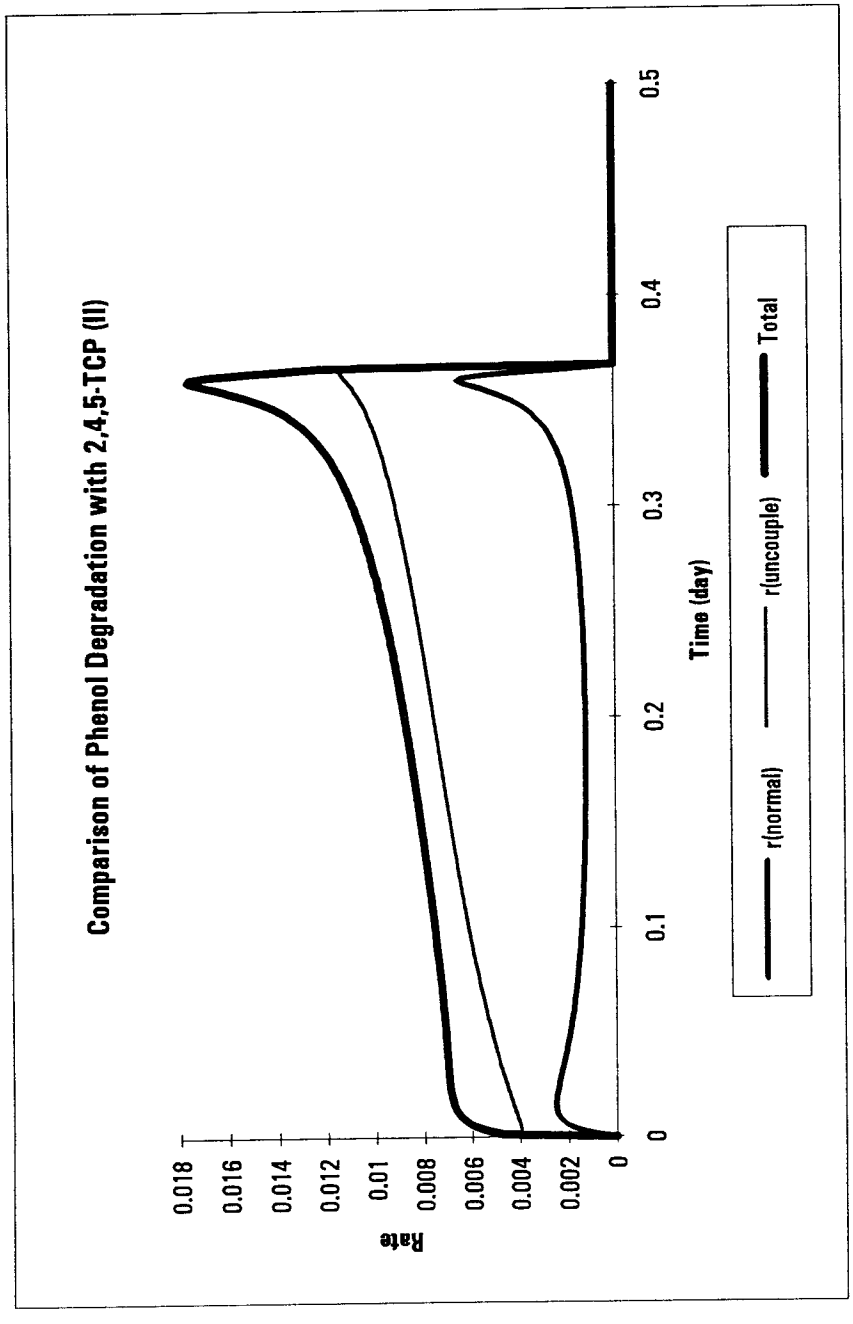

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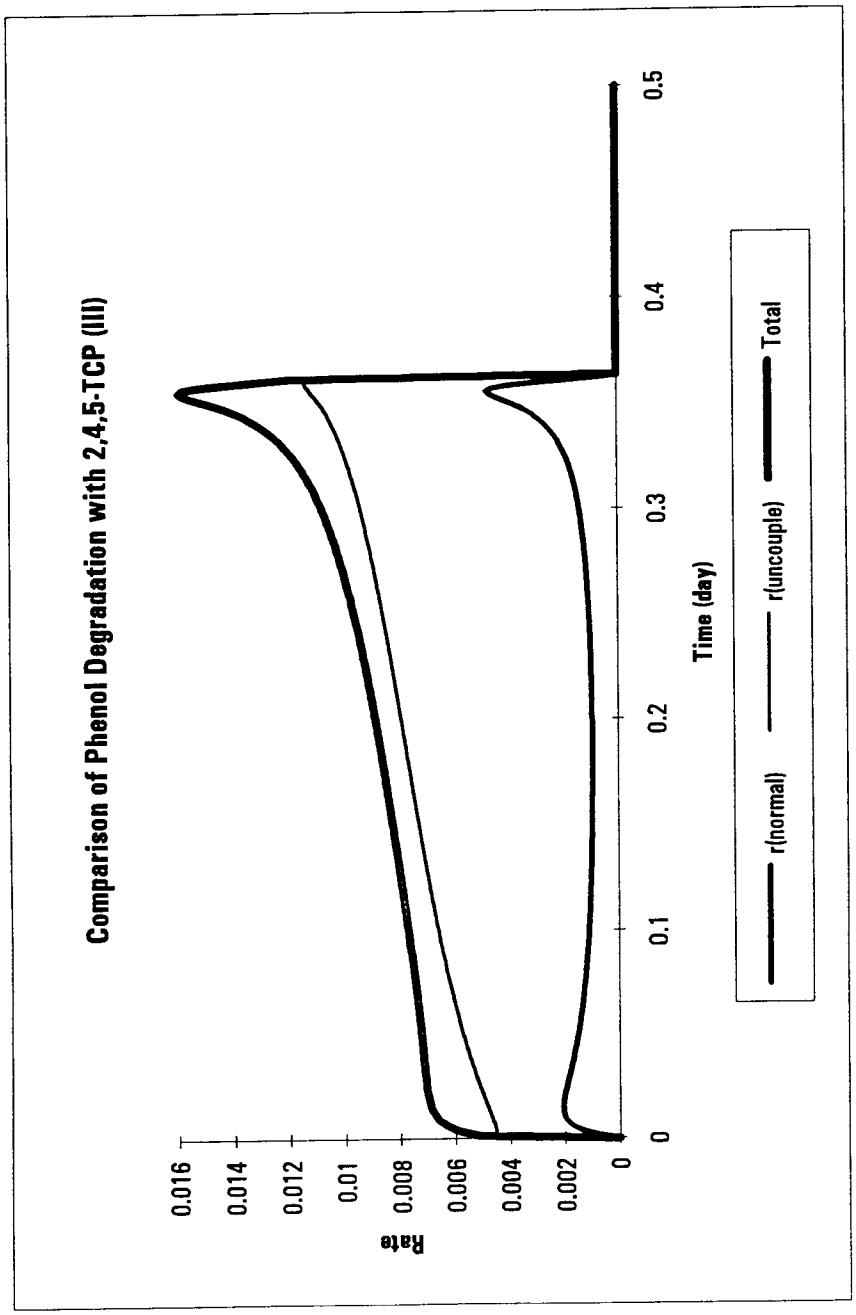

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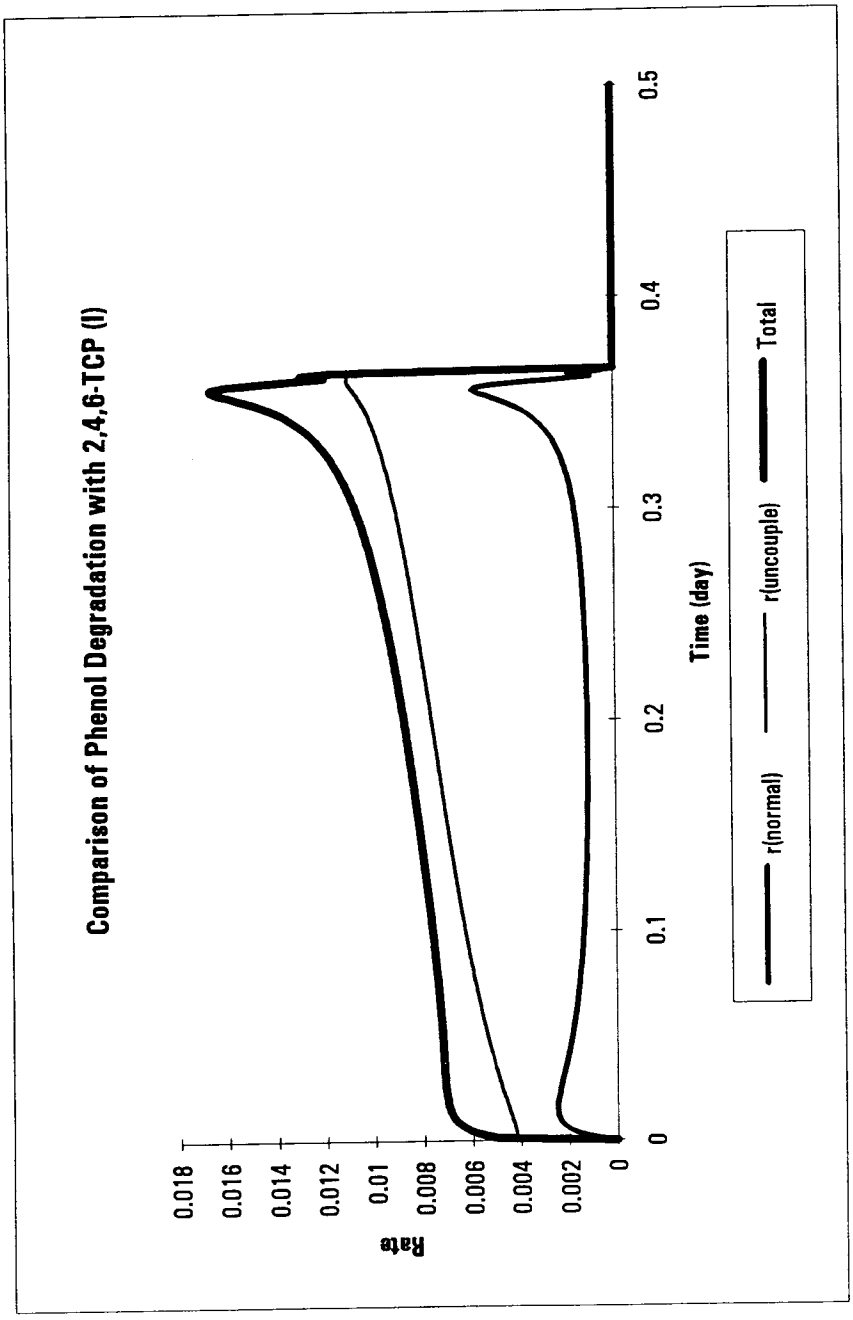

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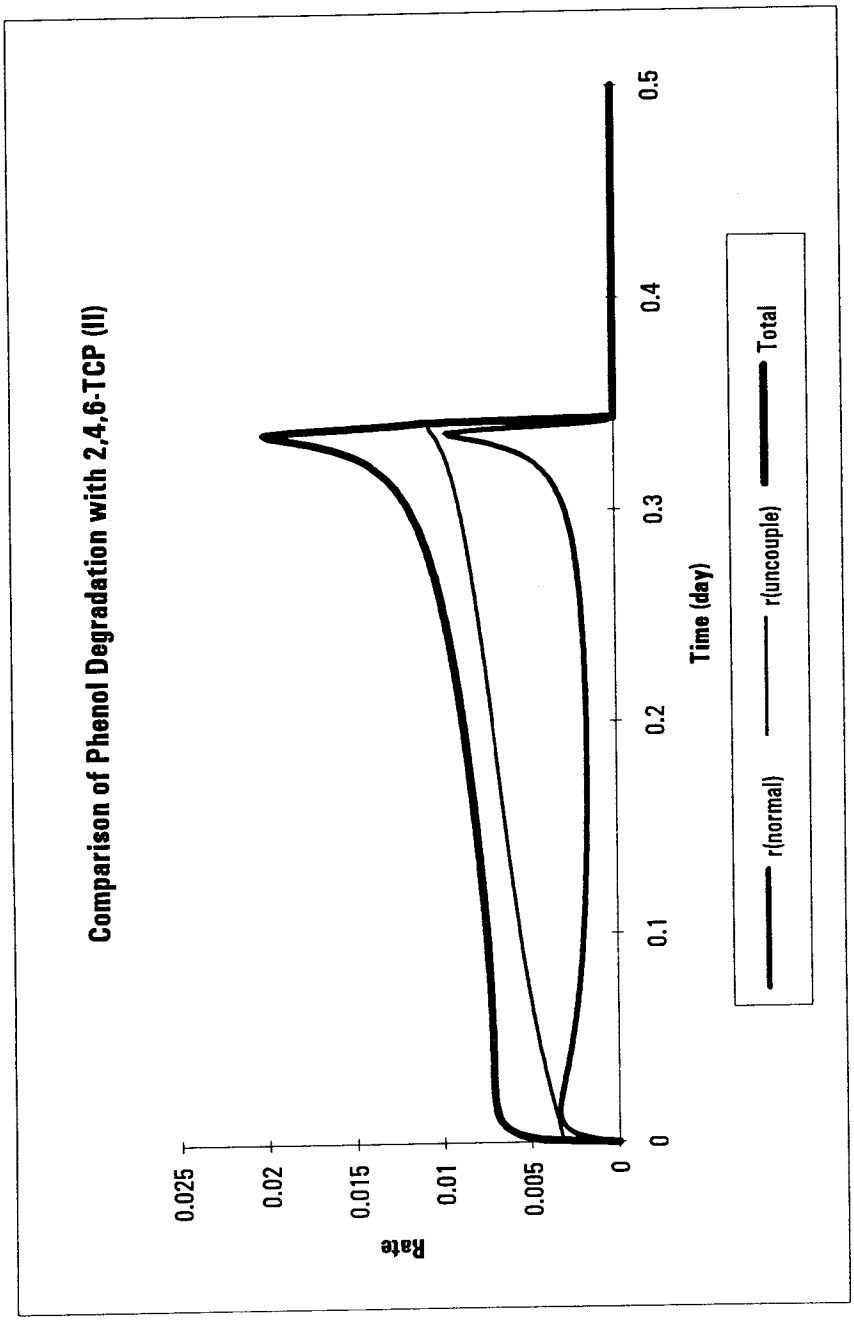
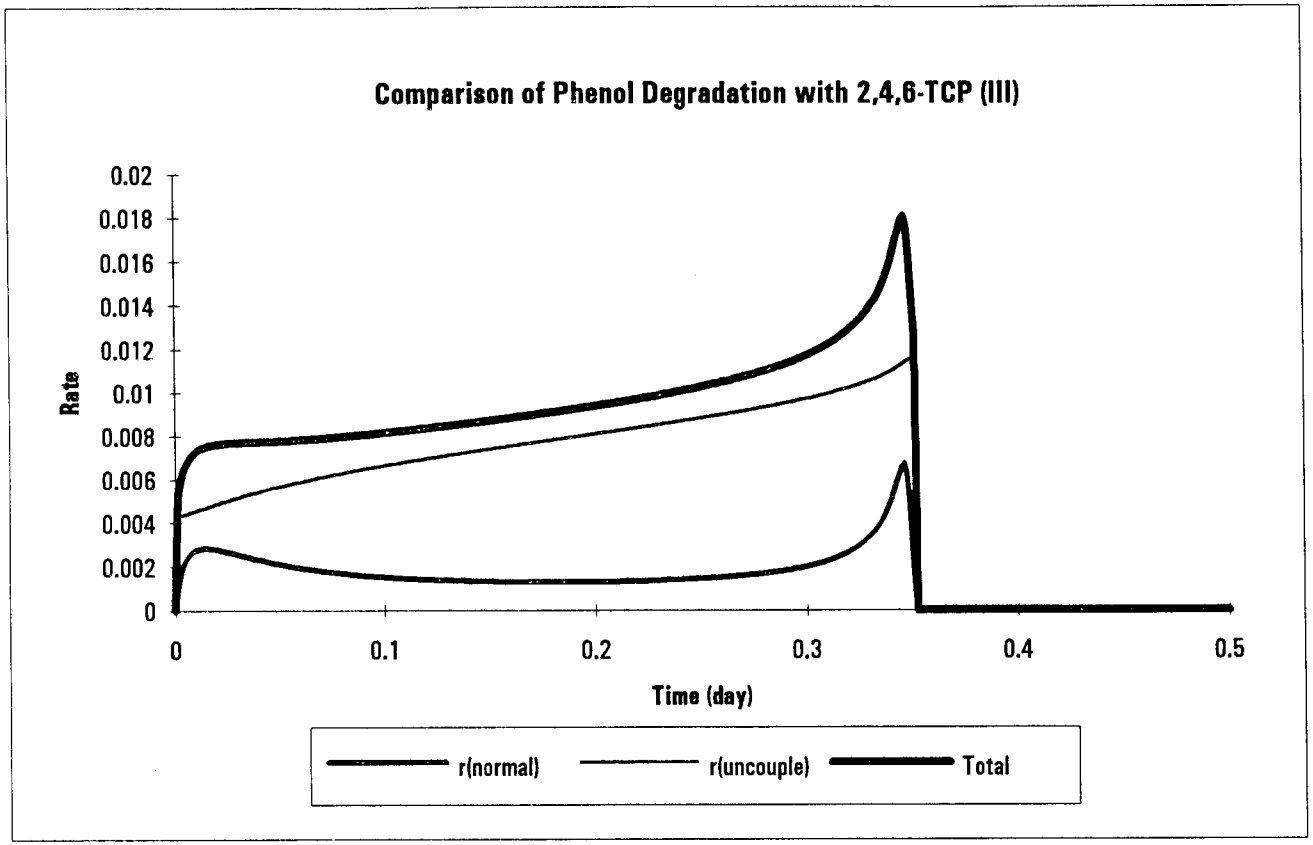
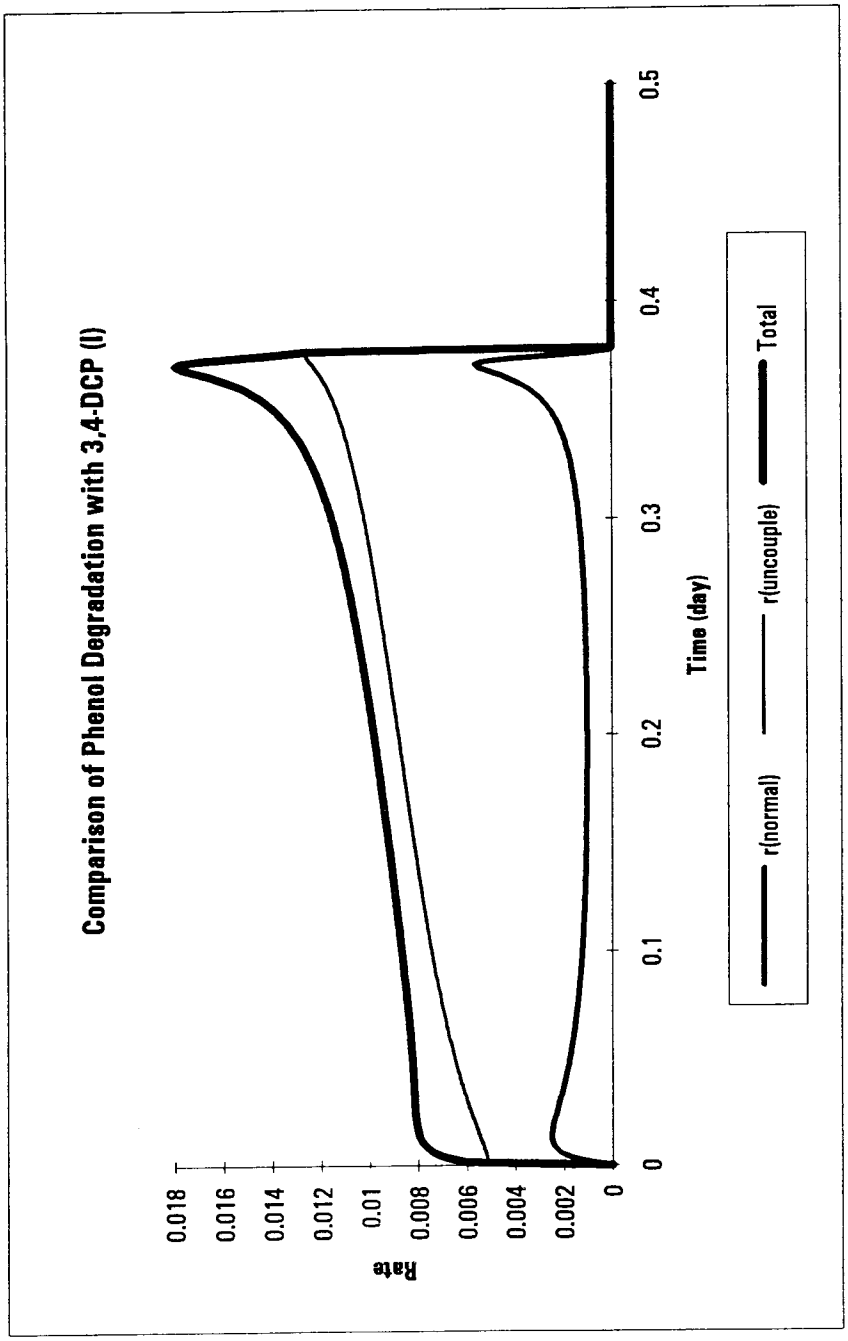



Comparison of Phenol Degradation with 2,4,6-TCP (III)
Rate
0.02
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0
0.1
0.2
0.3
0.4
0.5
Time (day)
r(normal)
r(uncouple)
Total

Appendix B (Continued)



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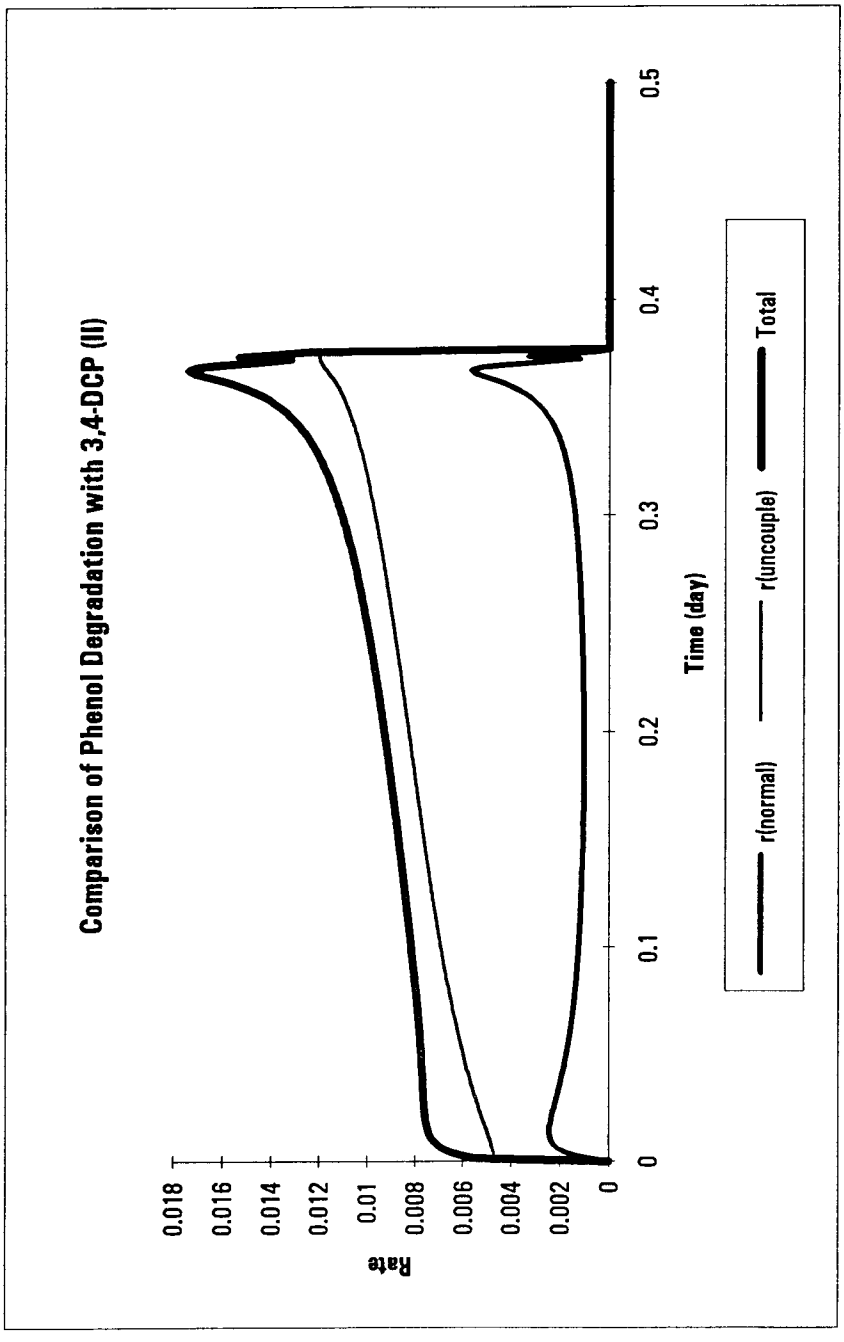

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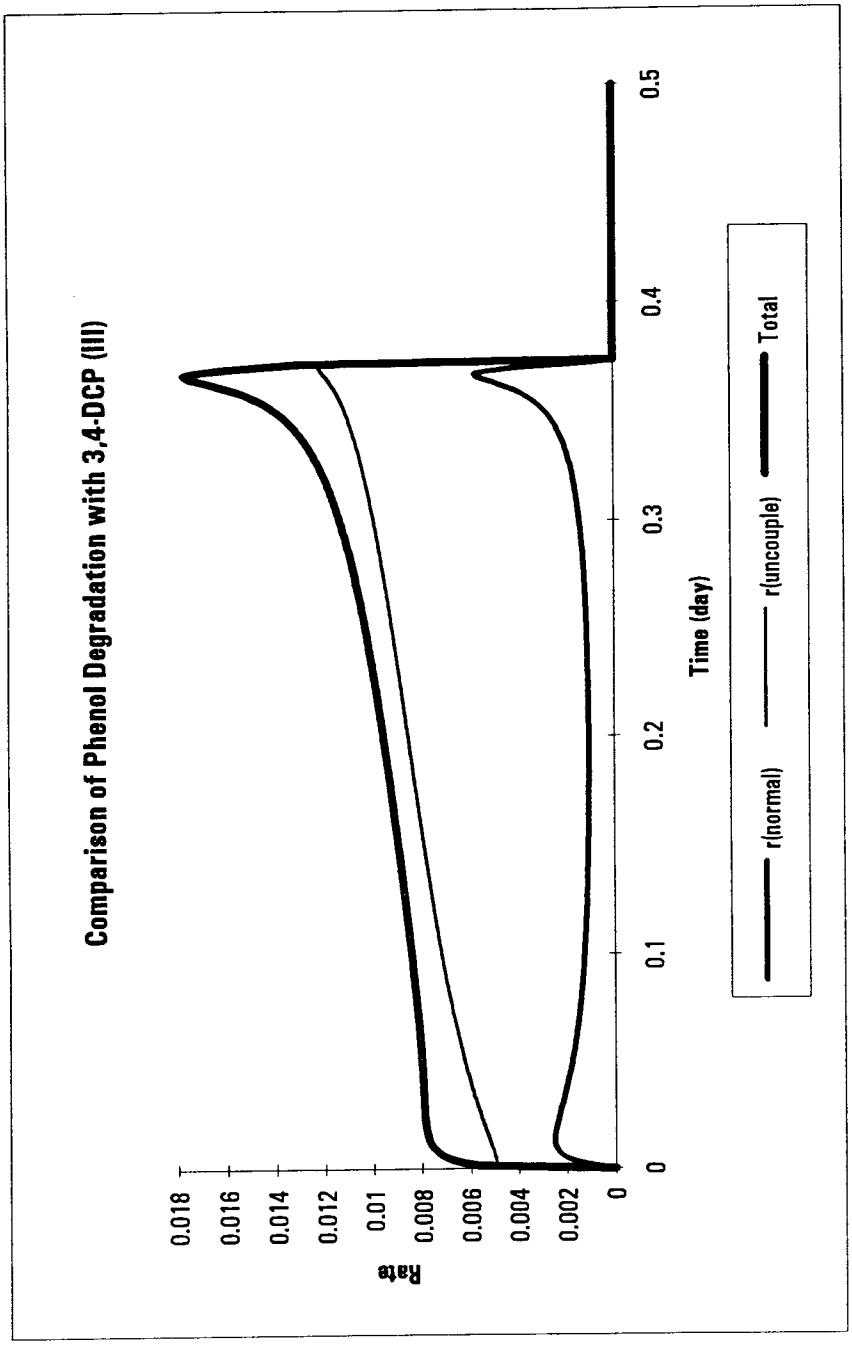

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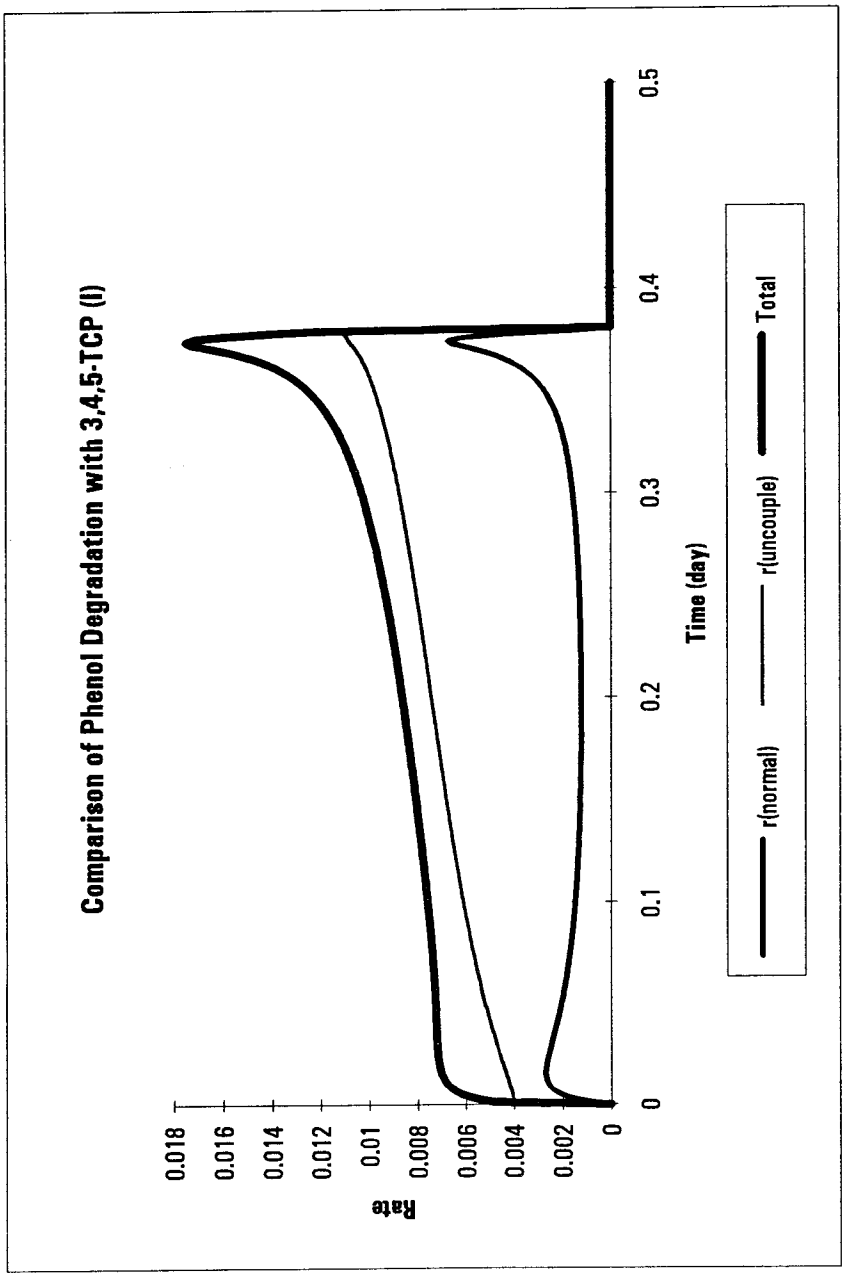

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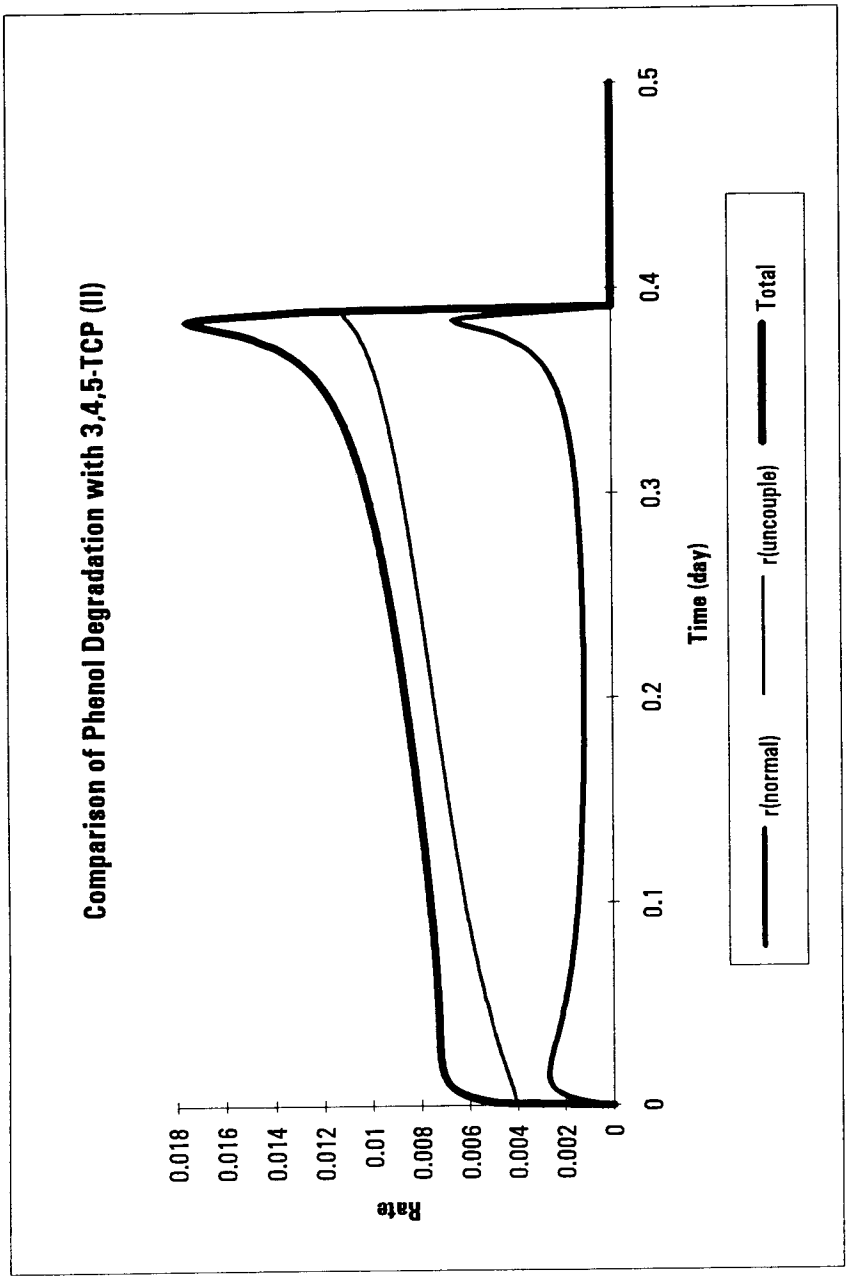

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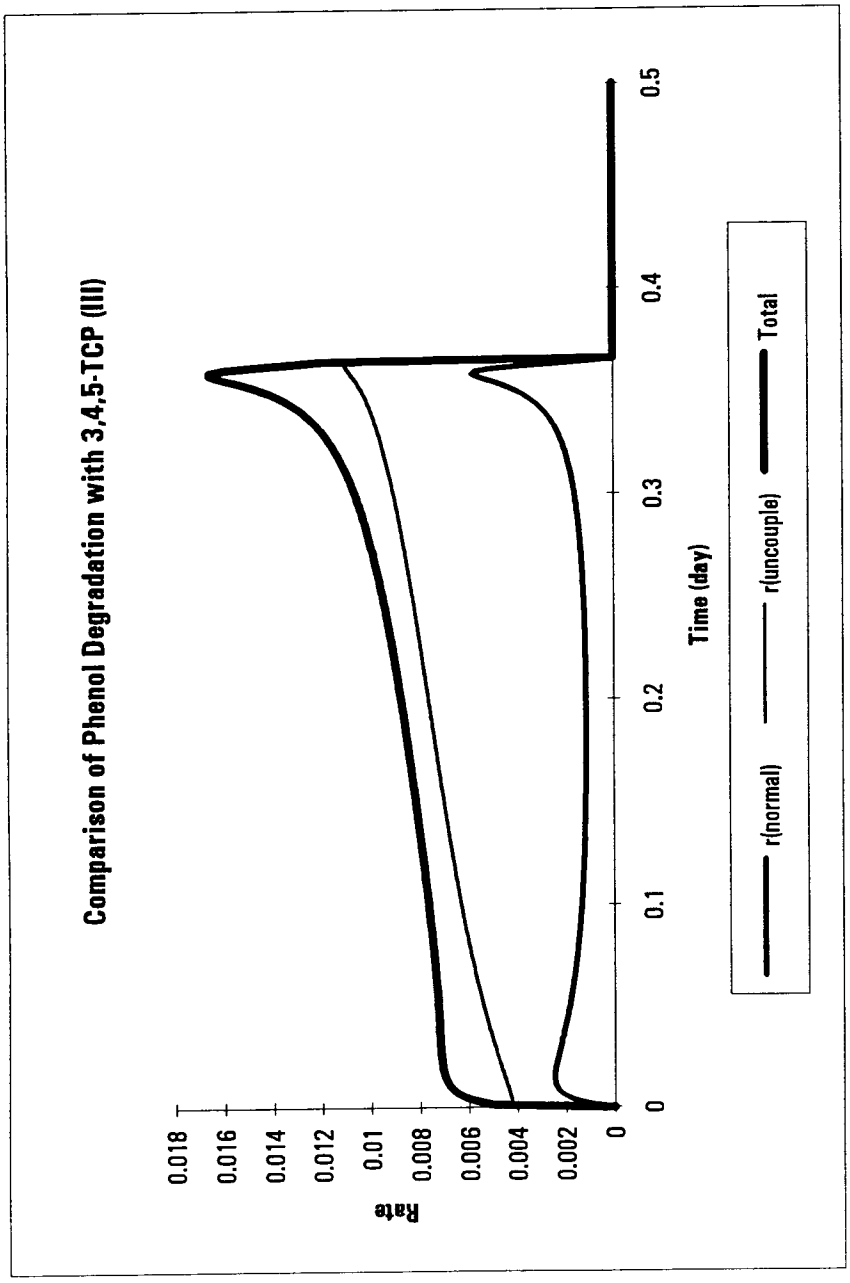

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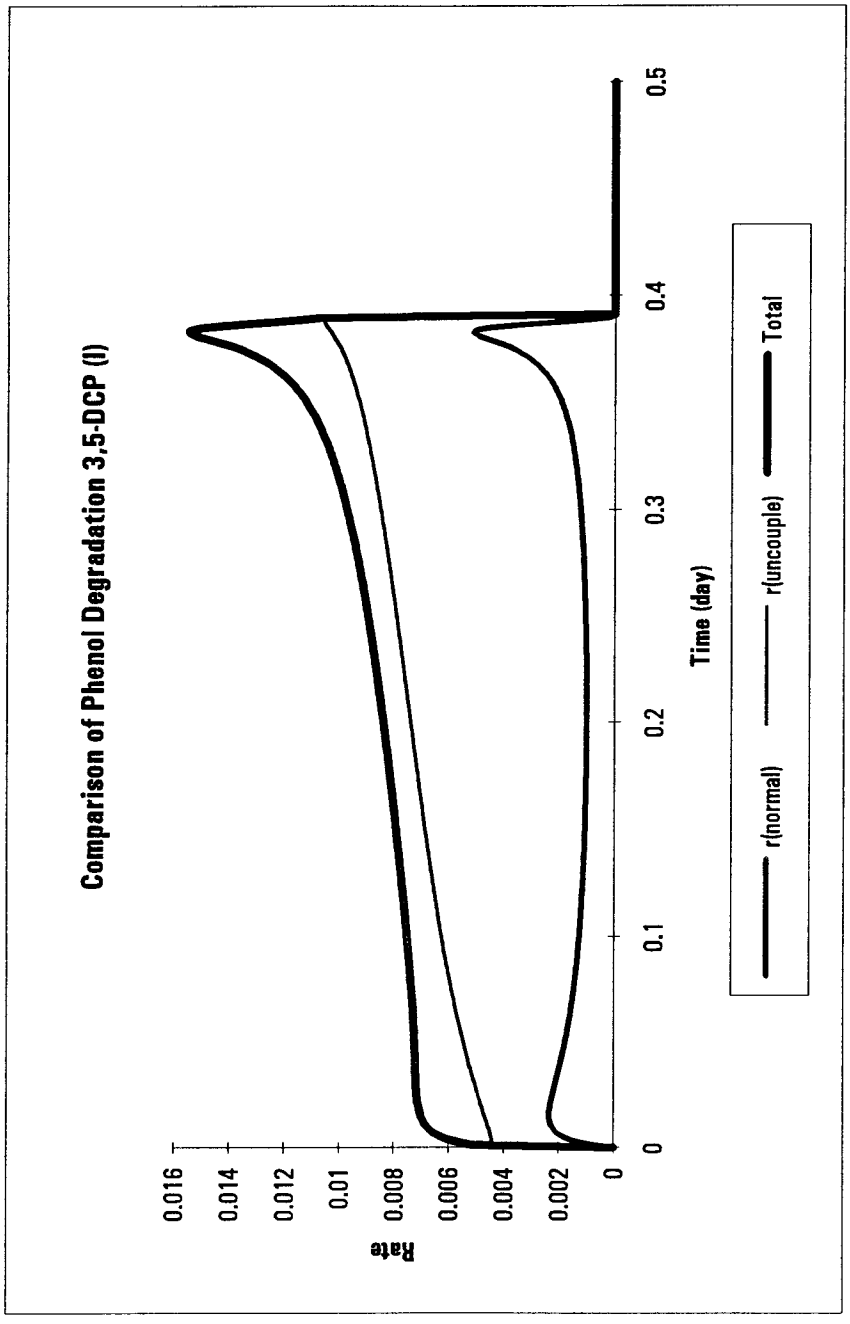

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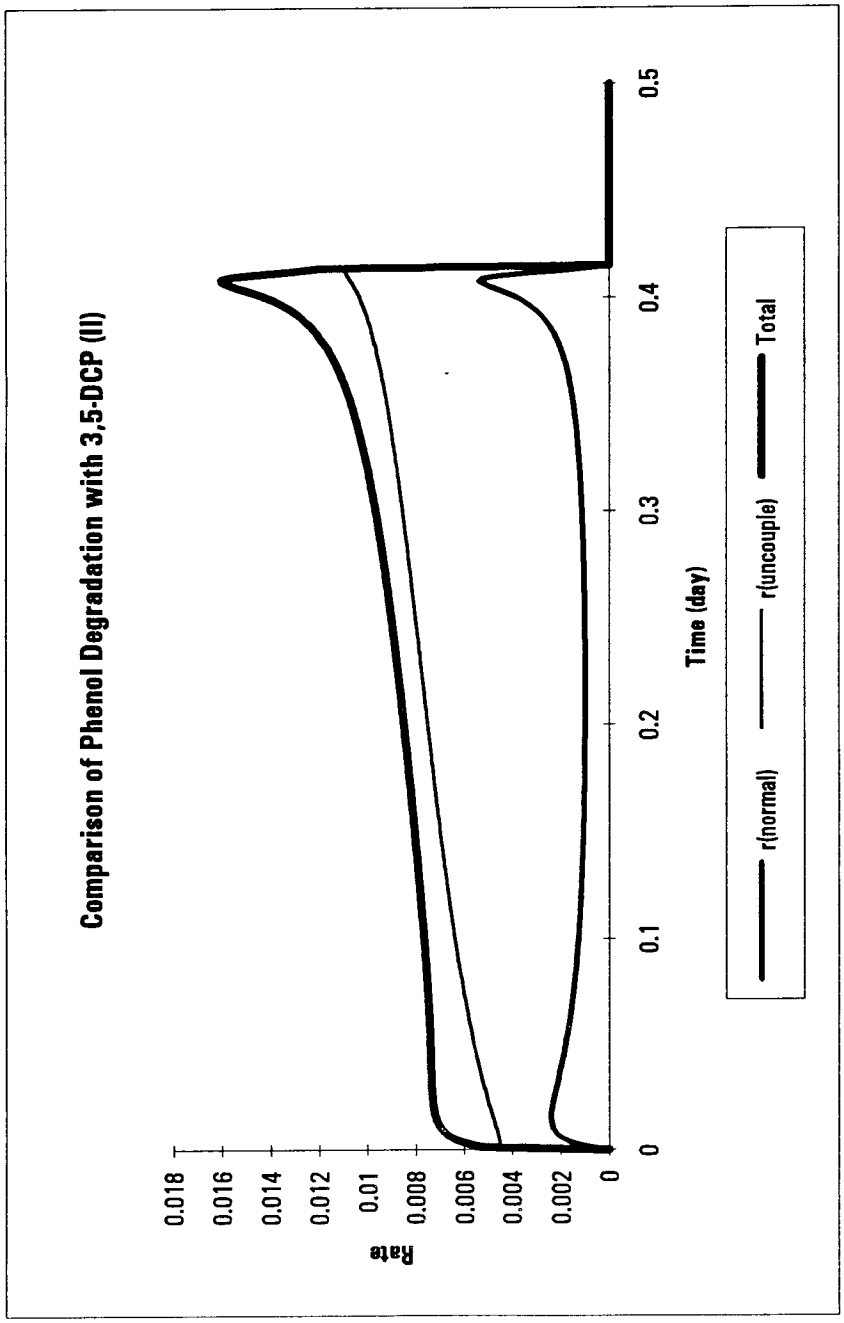

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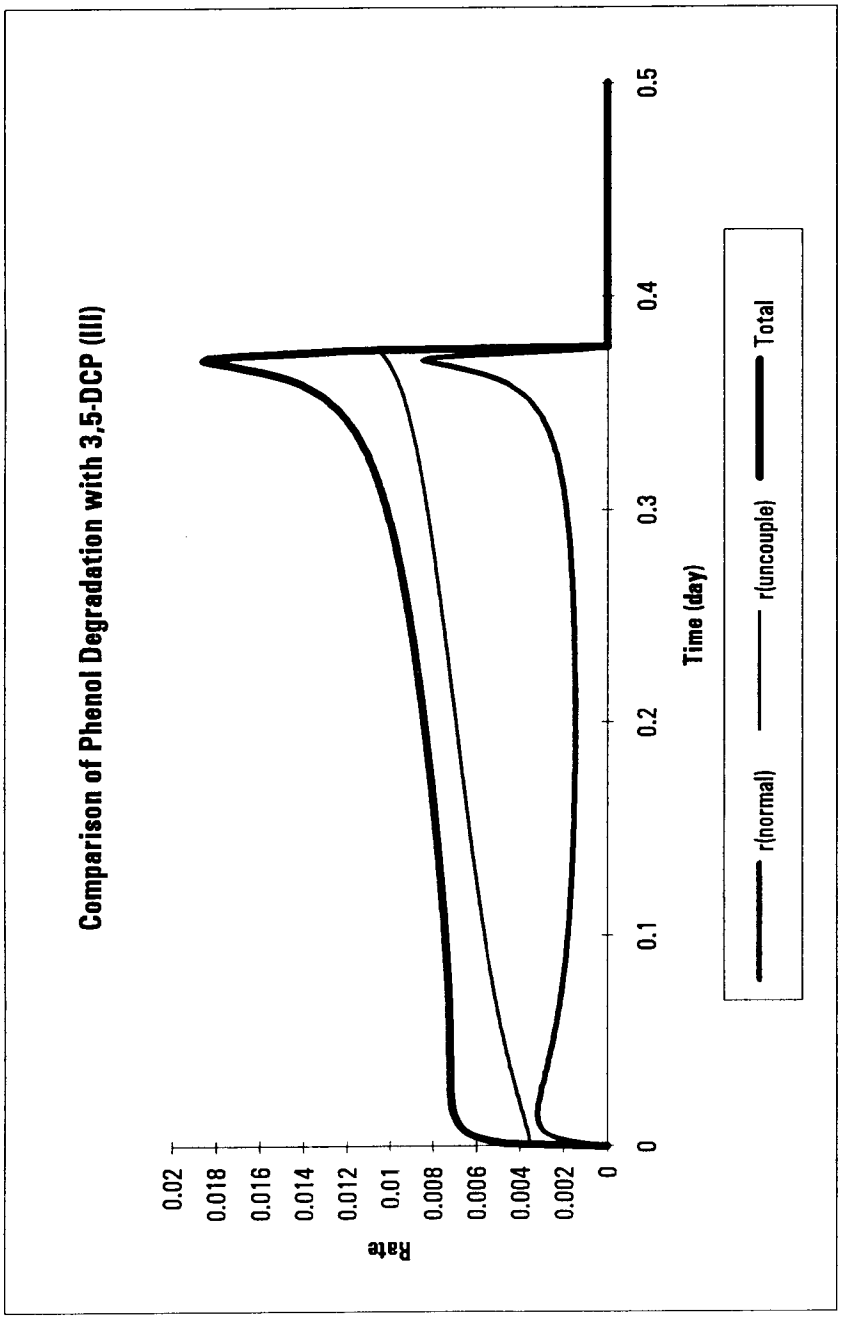
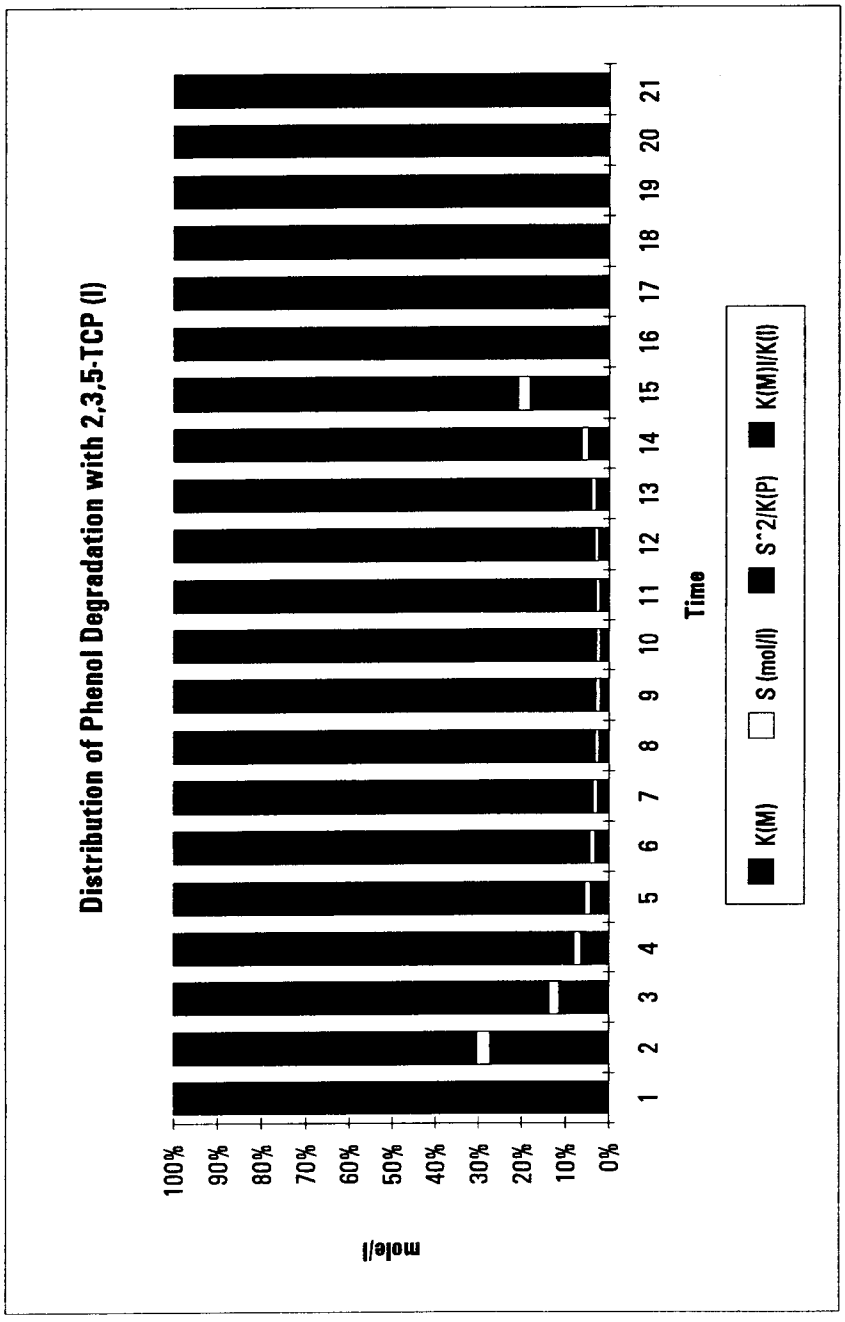


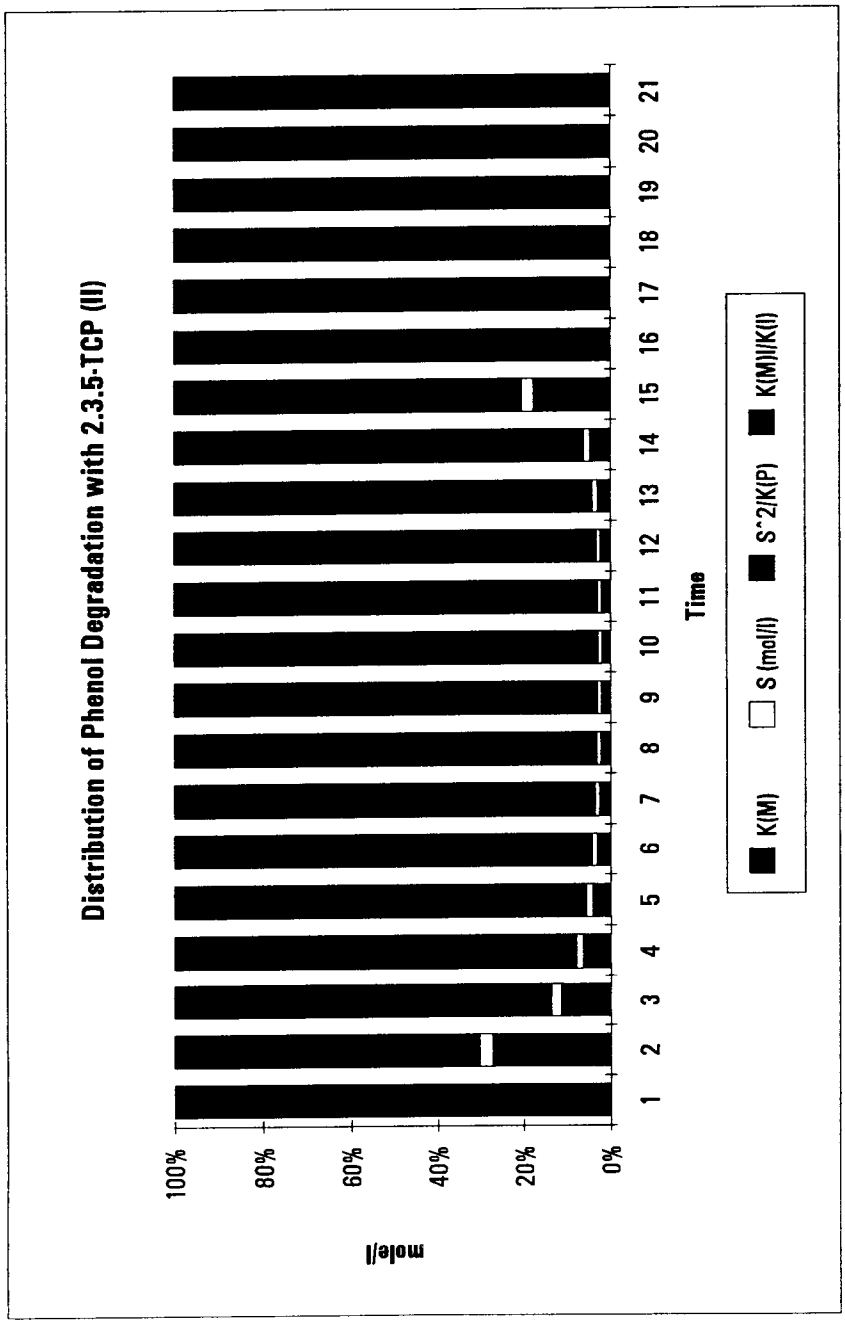
Appendix C

Distribution of Phenol Degradation with Chlorophenols

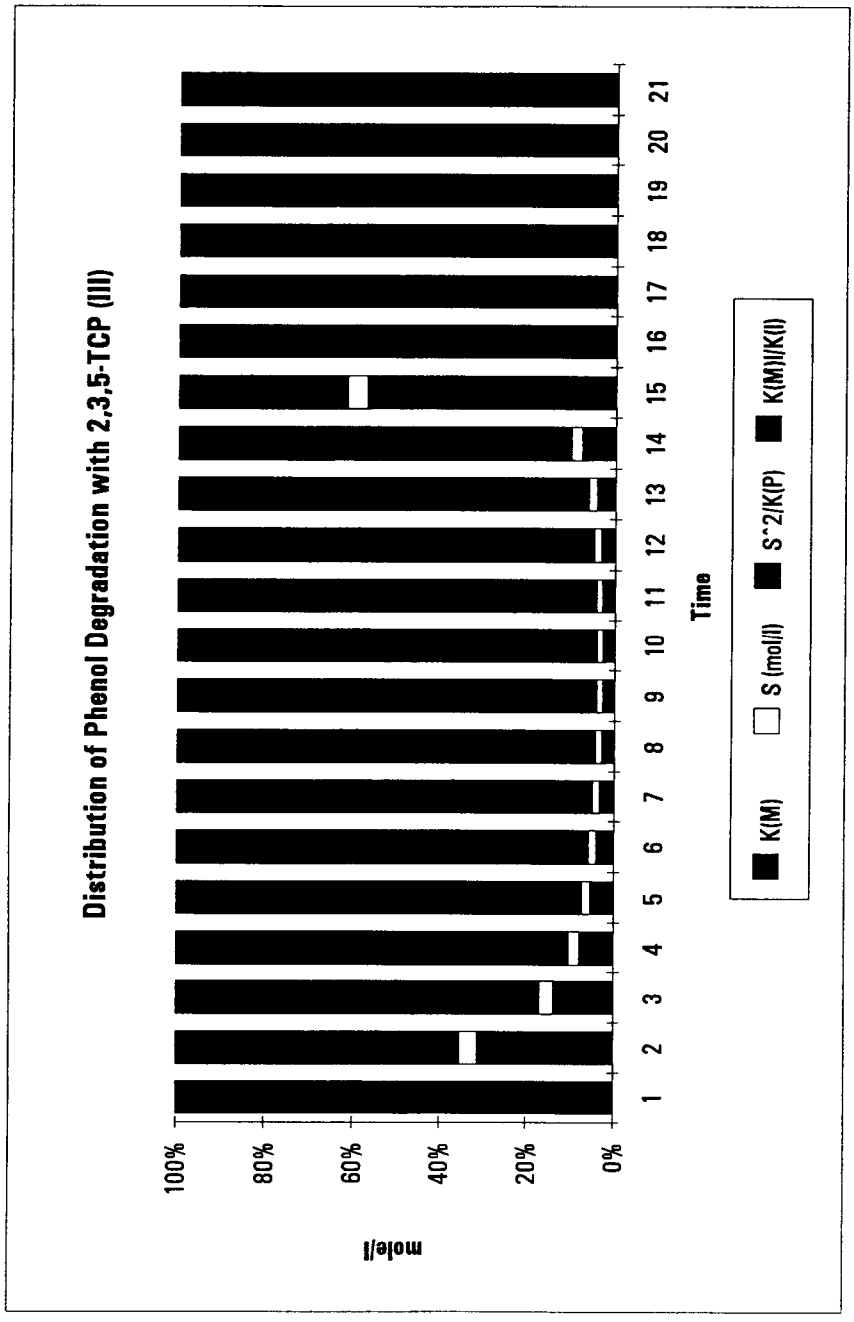
Appendix C (Continued)



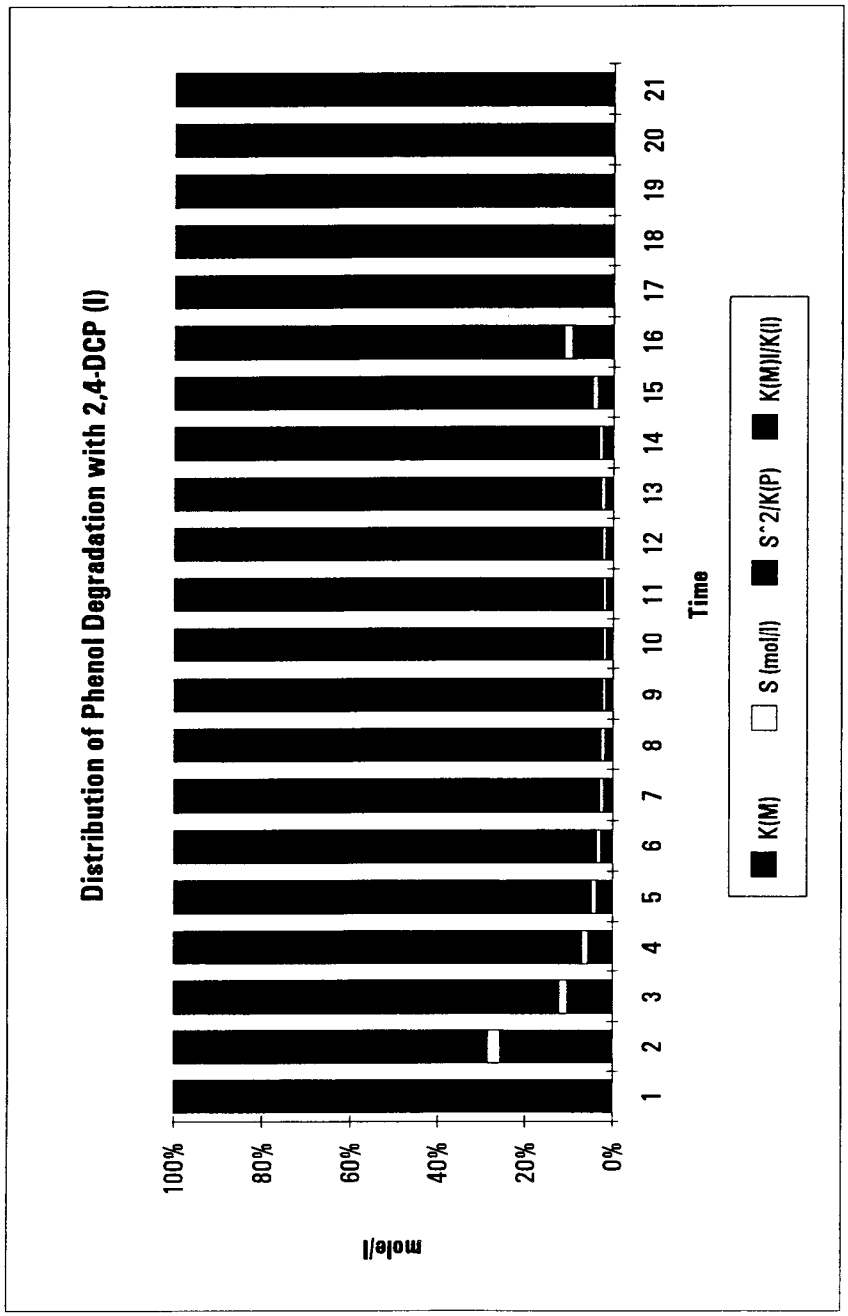
Appendix C (Continued)



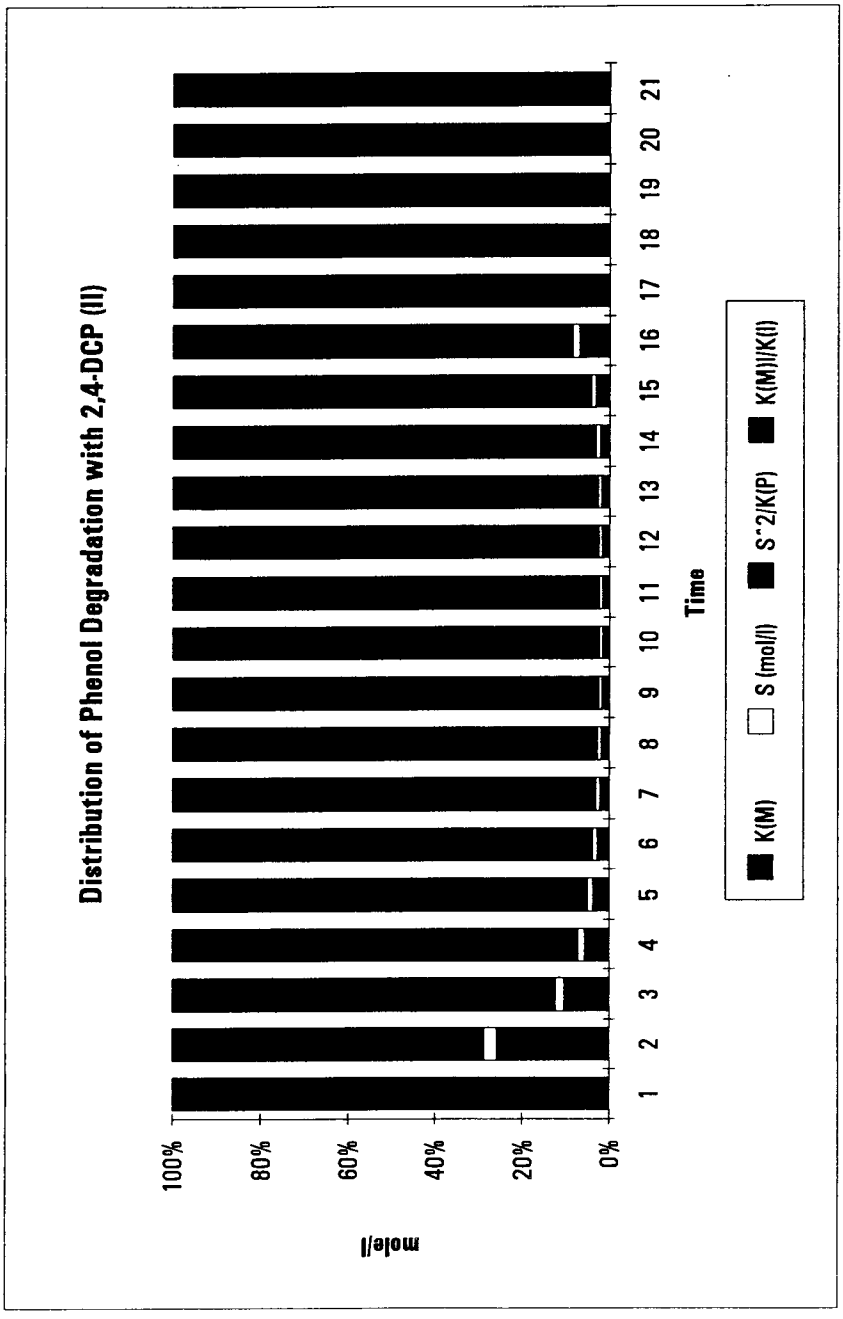
Appendix C (Continued)



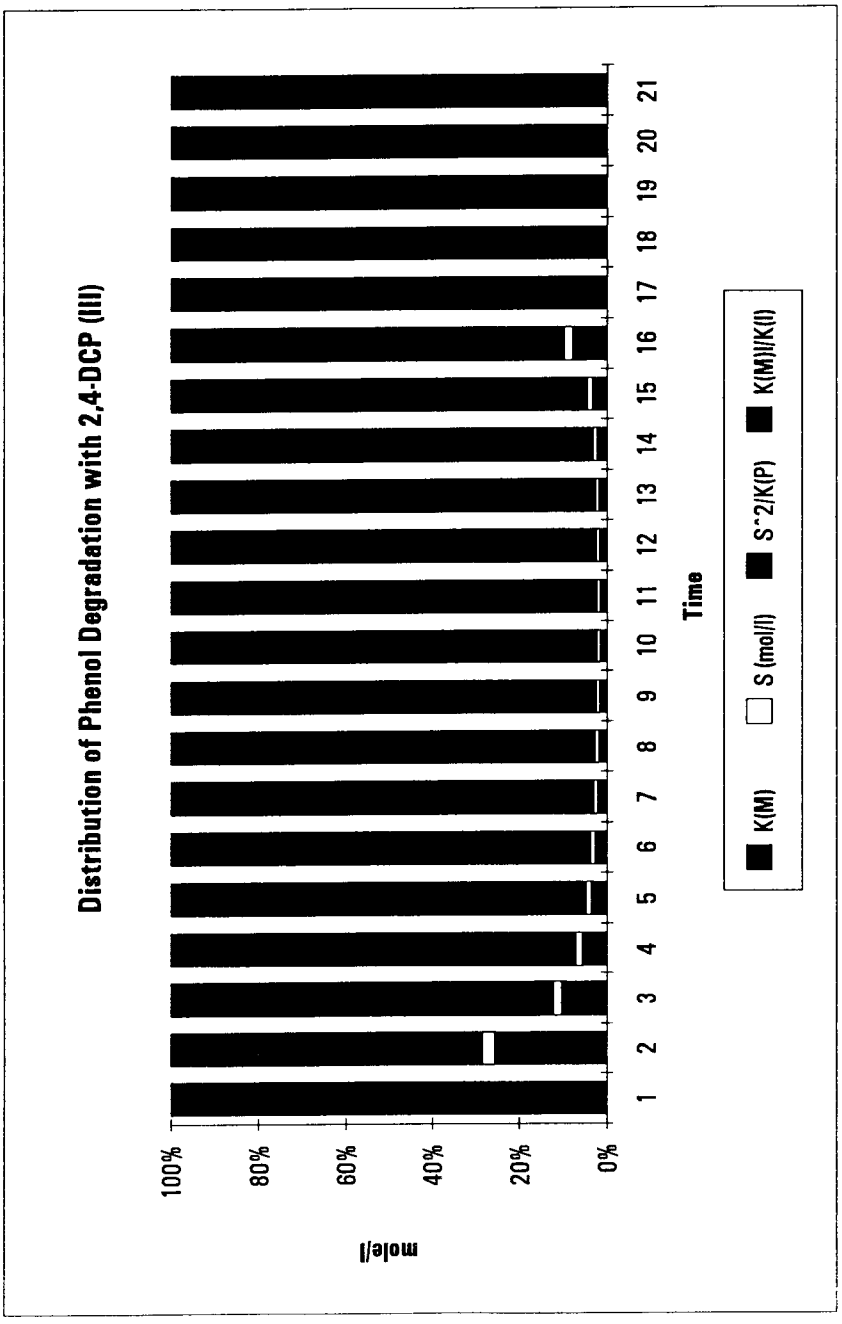
Appendix C (Continued)



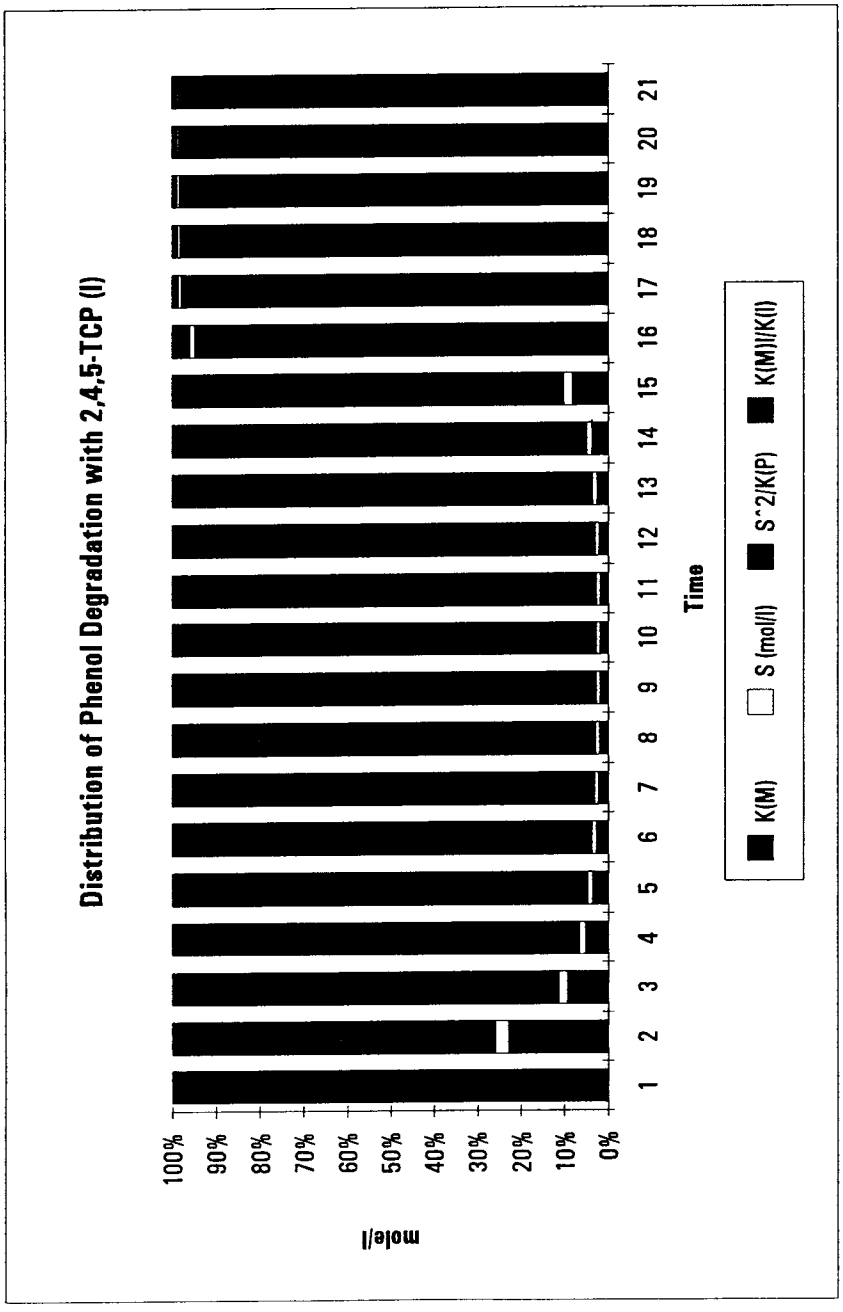
Appendix C (Continued)



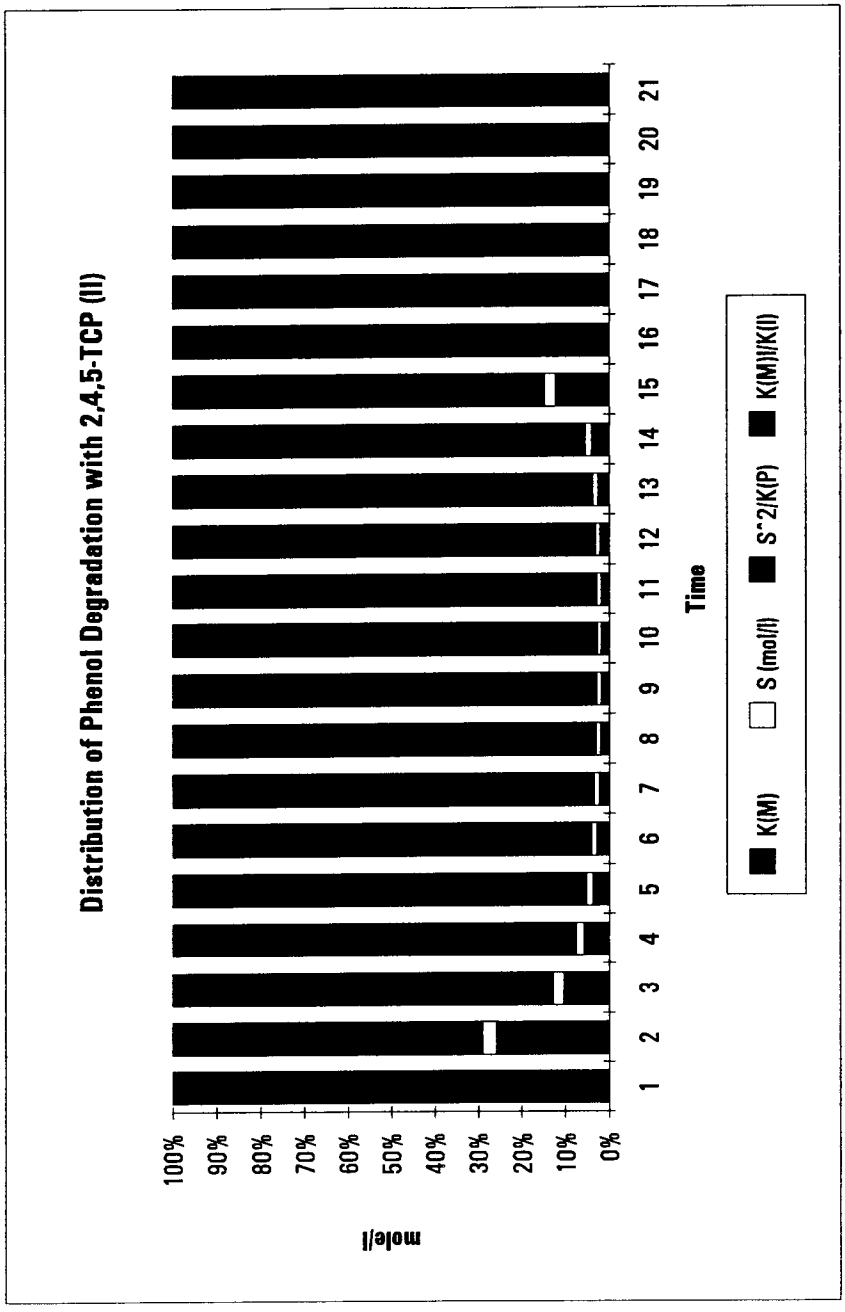
Appendix C (Continued)



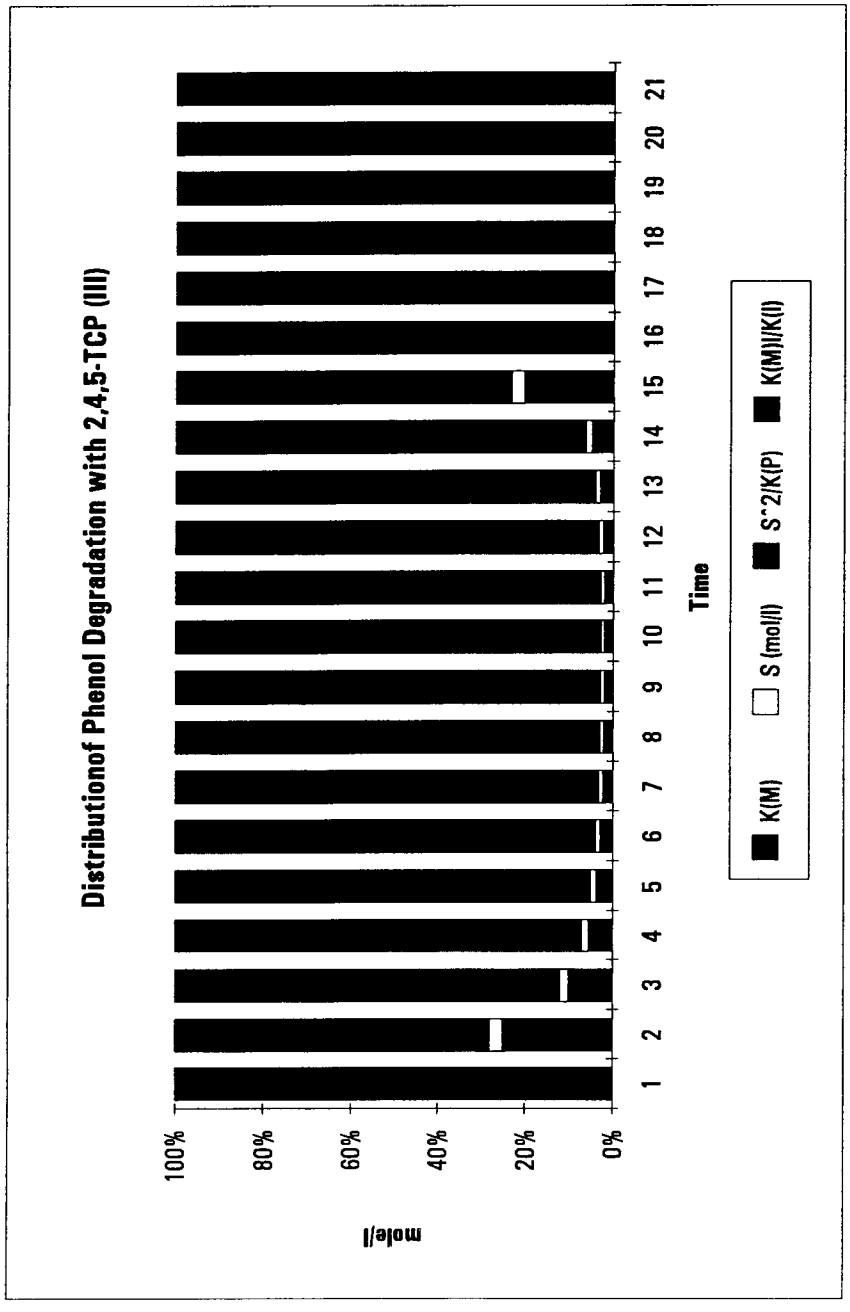
Appendix C (Continued)



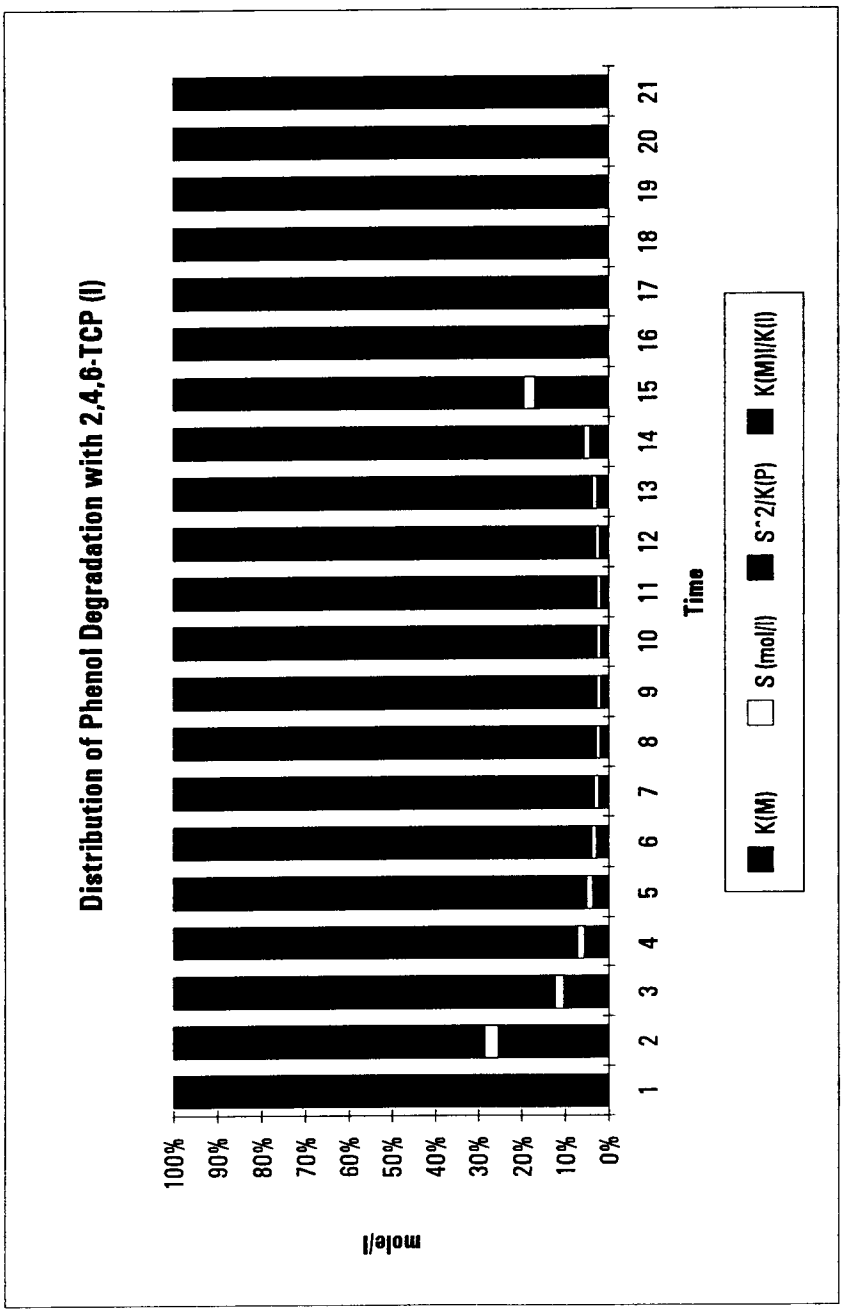
Appendix C (Continued)



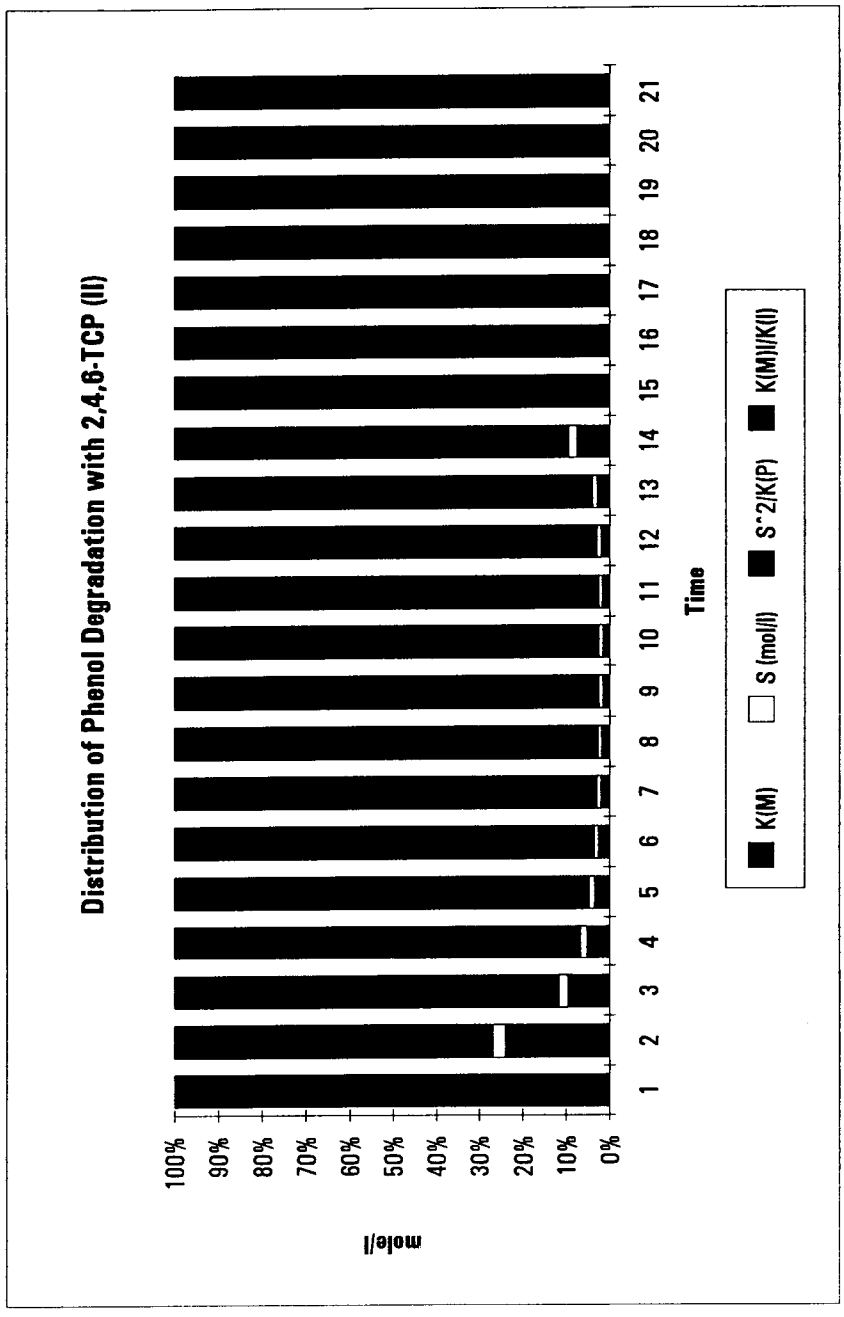
Appendix C (Continued)



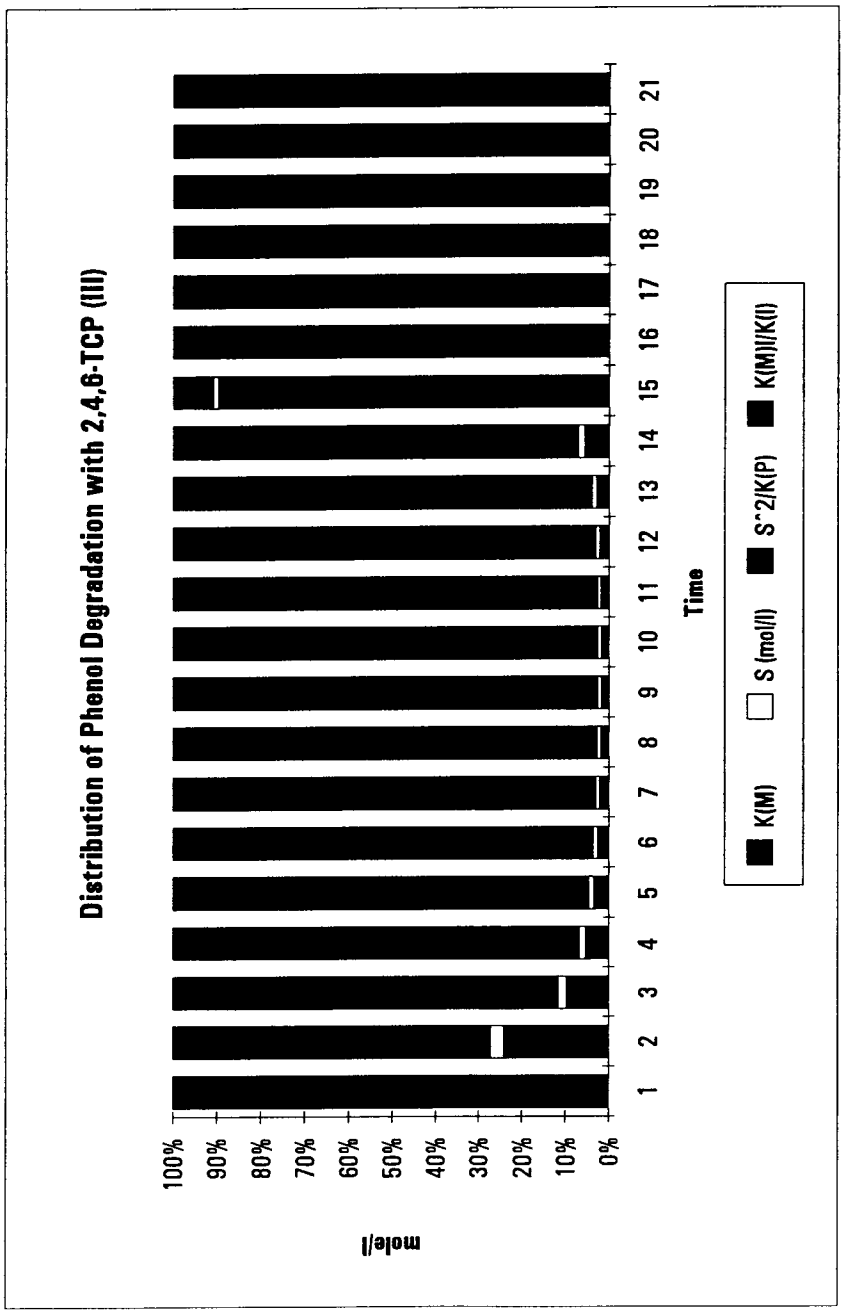
Appendix C (Continued)



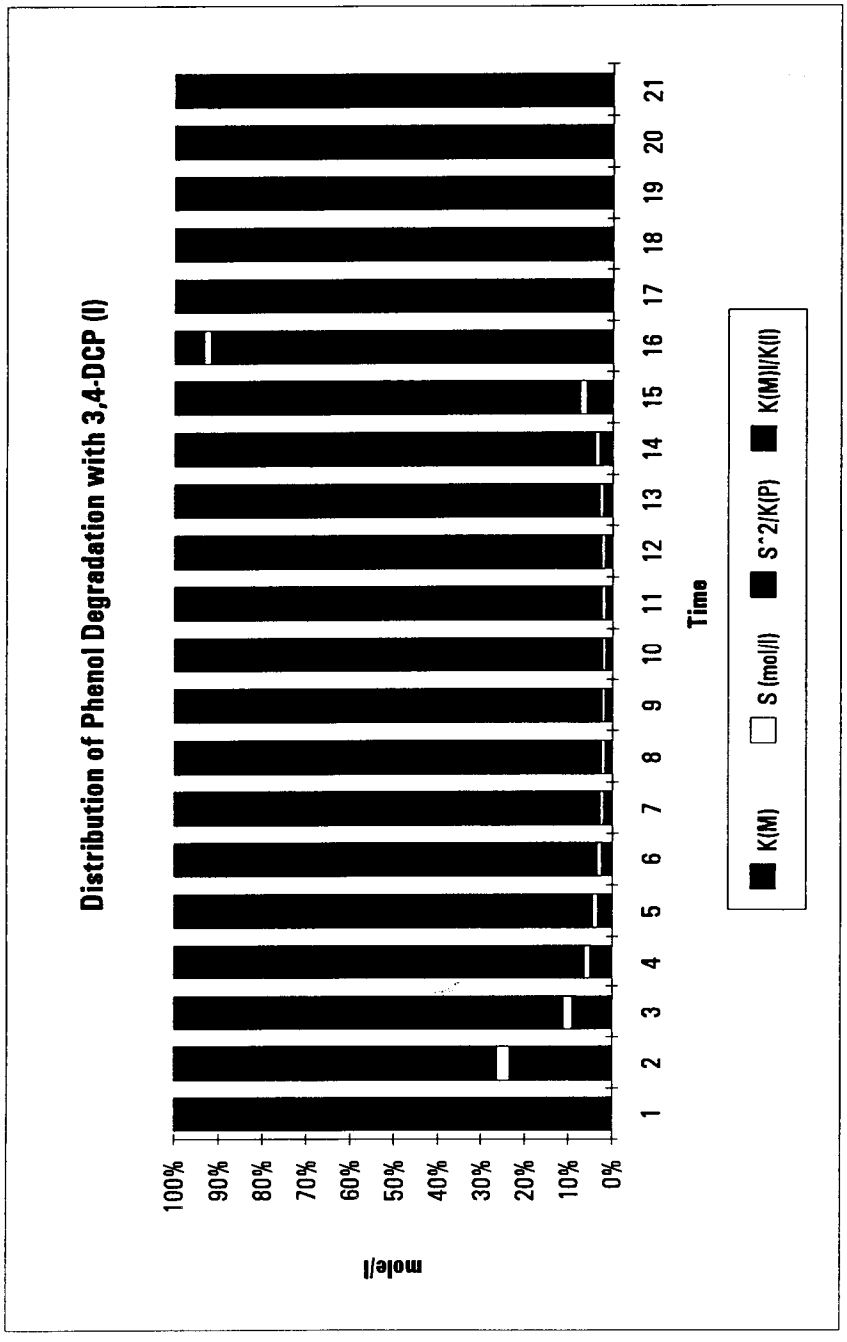
Appendix C (Continued)



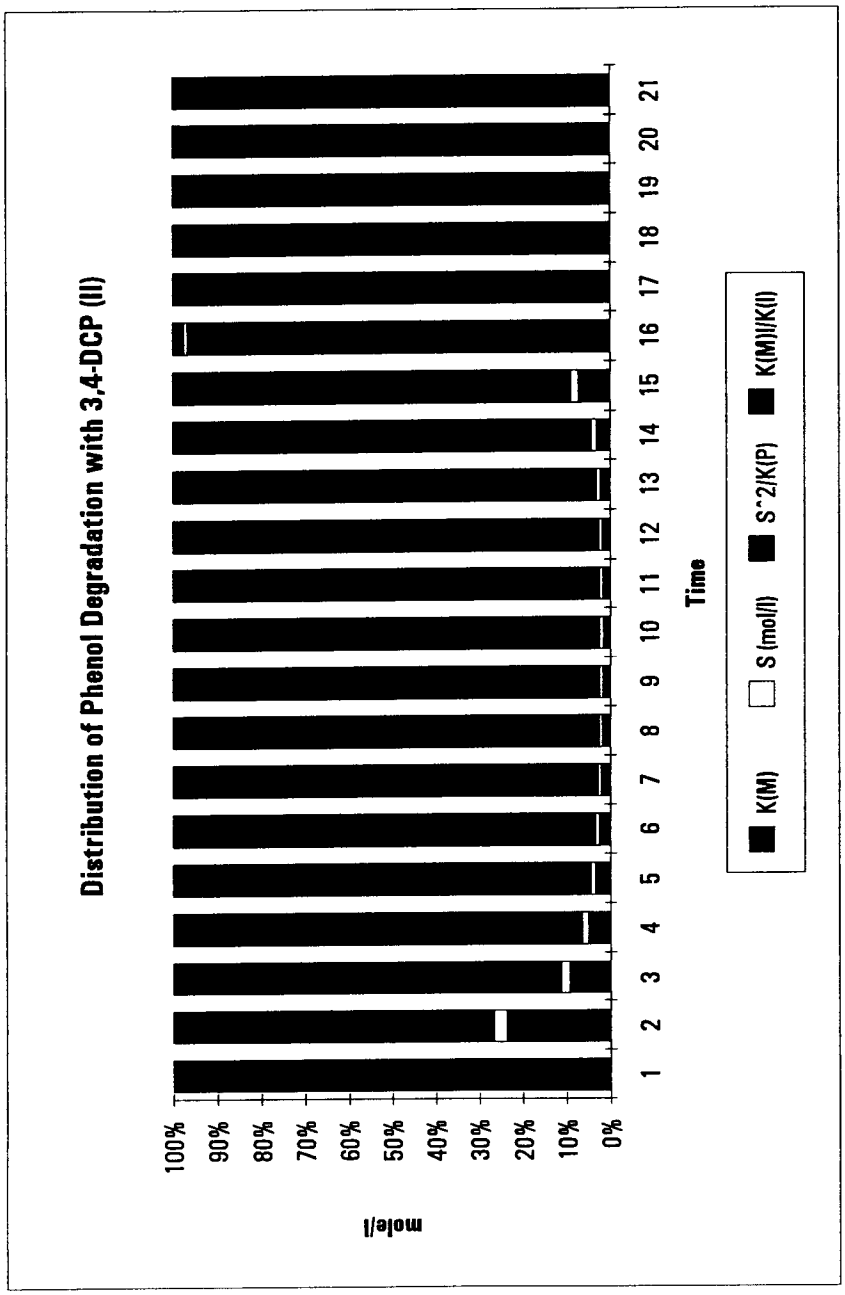
Appendix C (Continued)



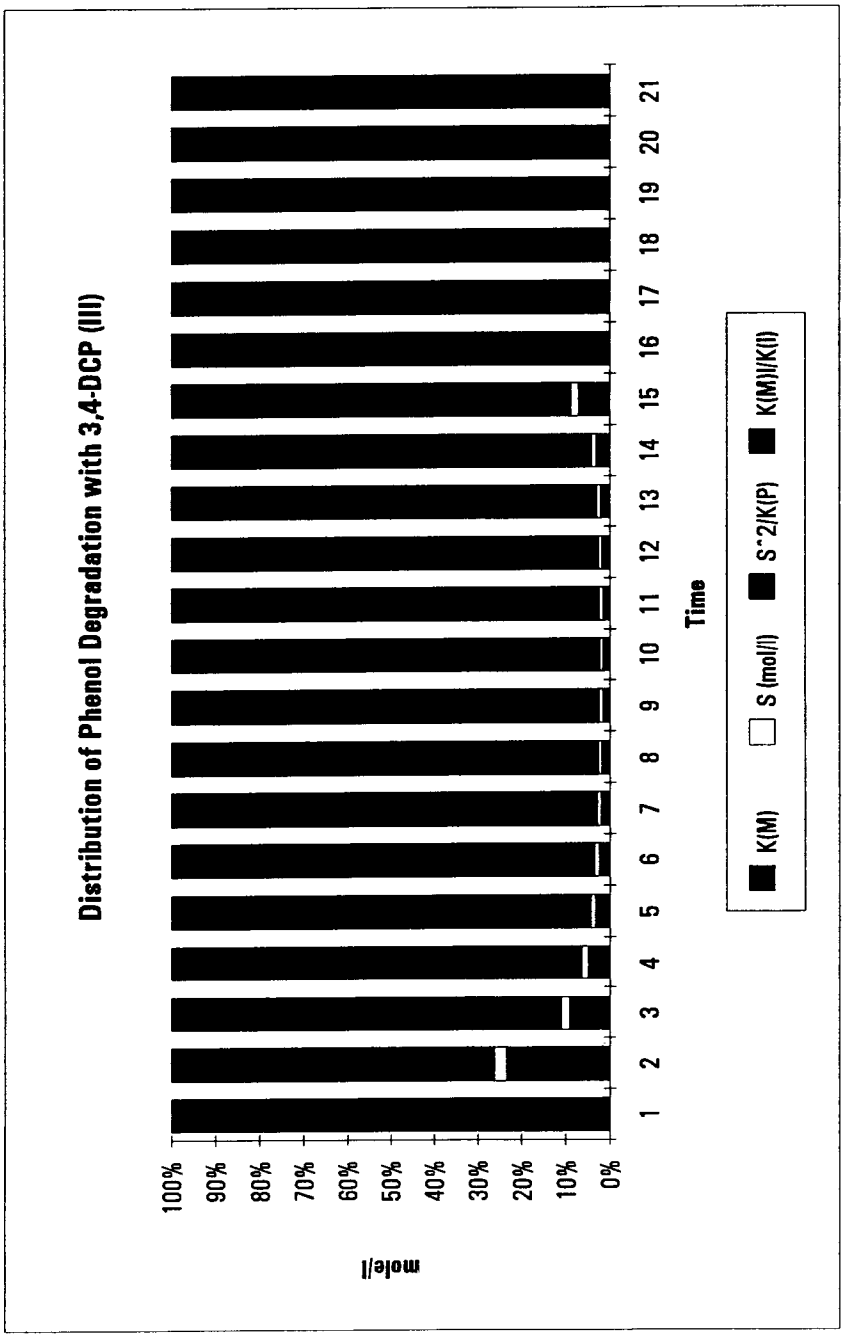
Appendix C (Continued)



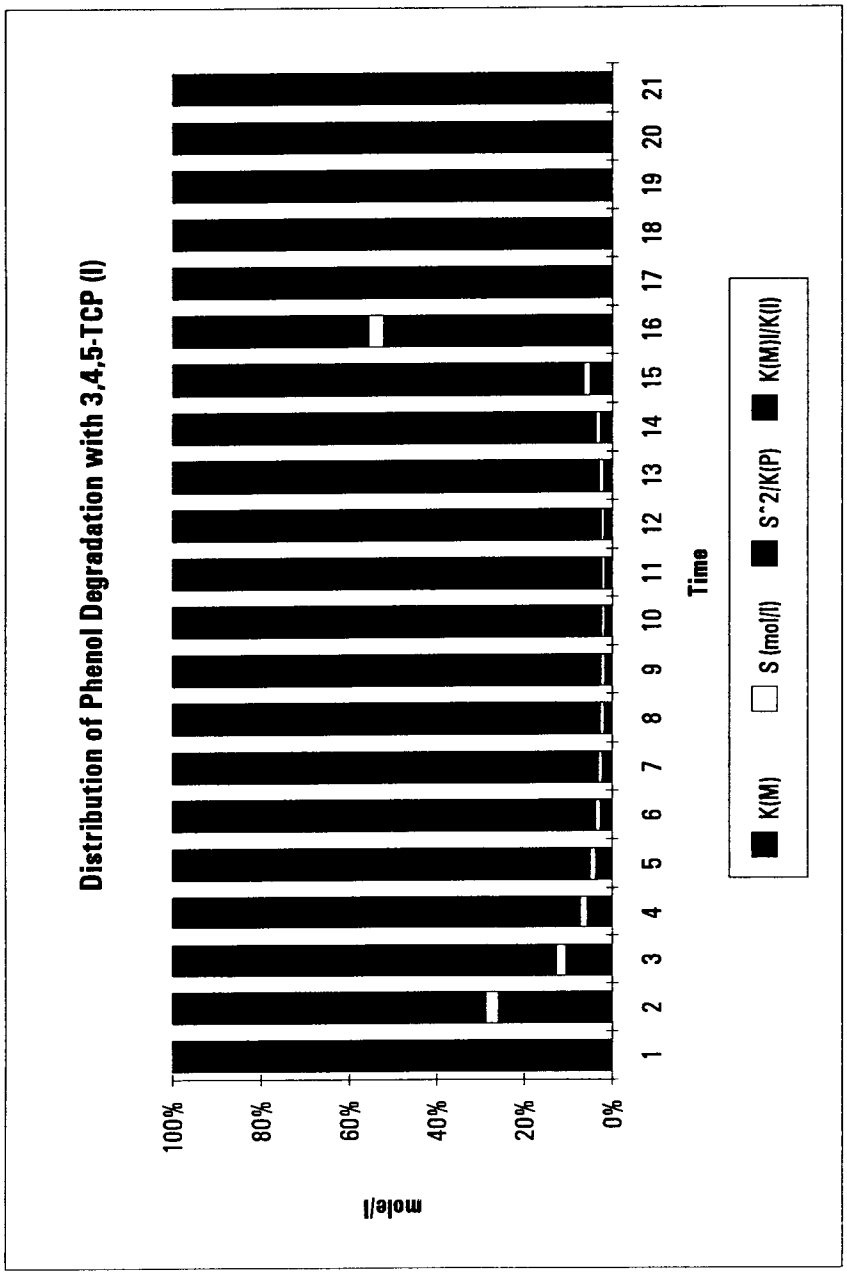
Appendix C (Continued)



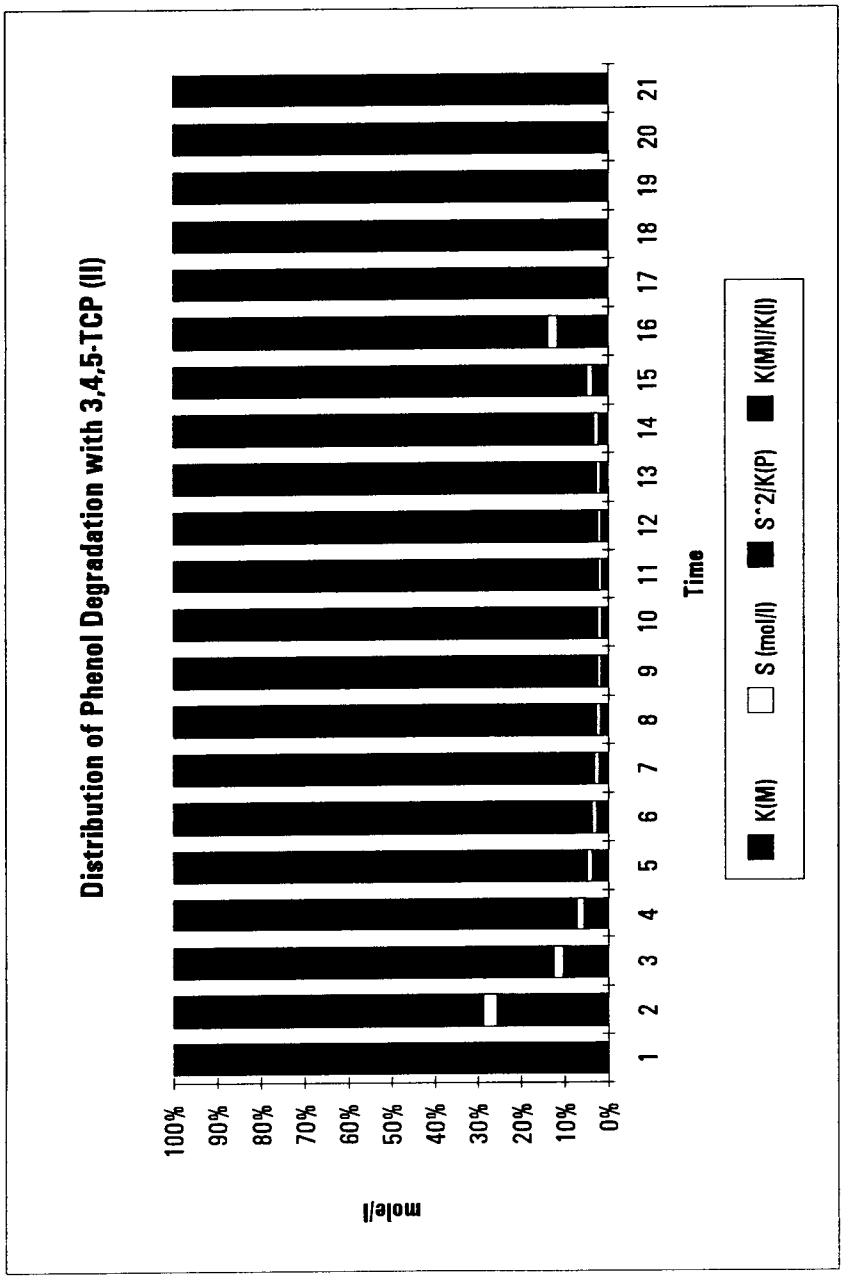
Appendix C (Continued)



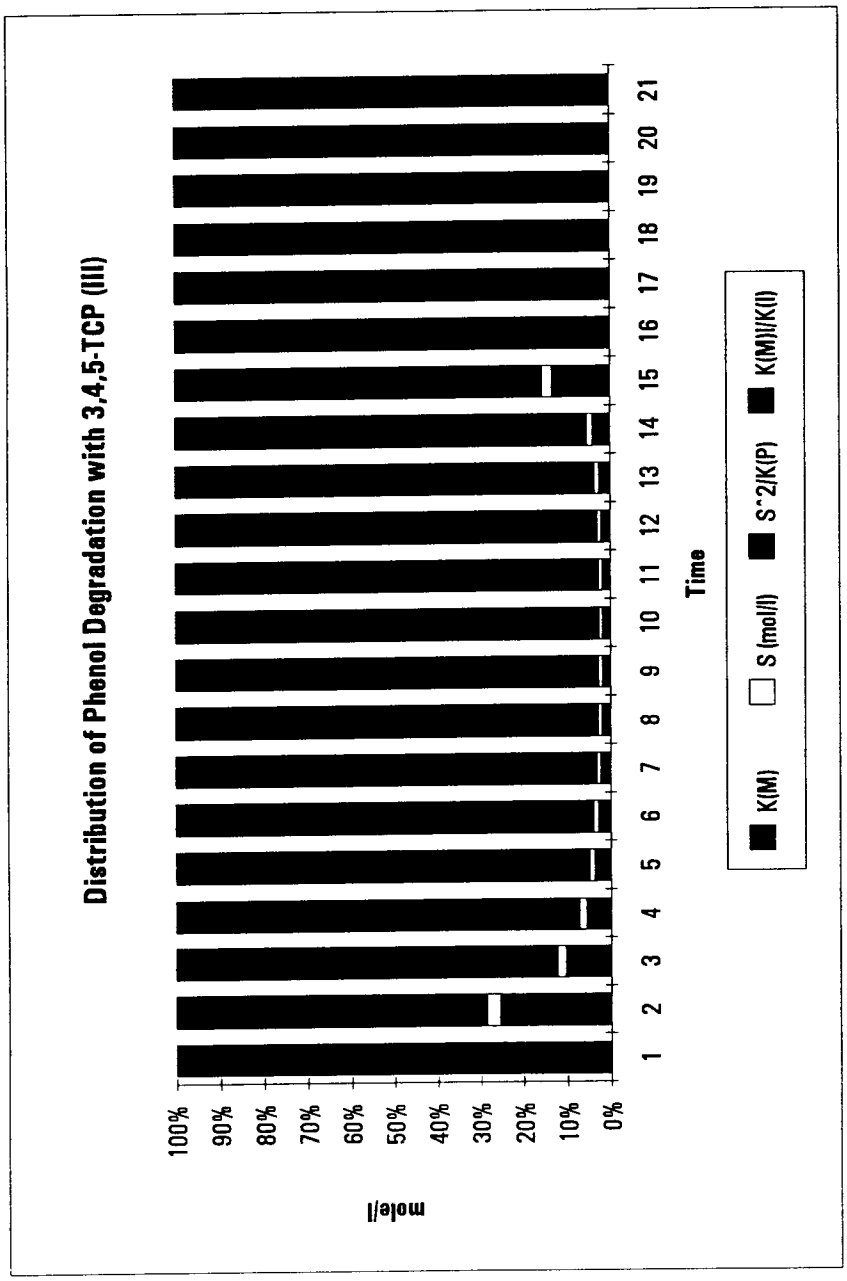
Appendix C (Continued)



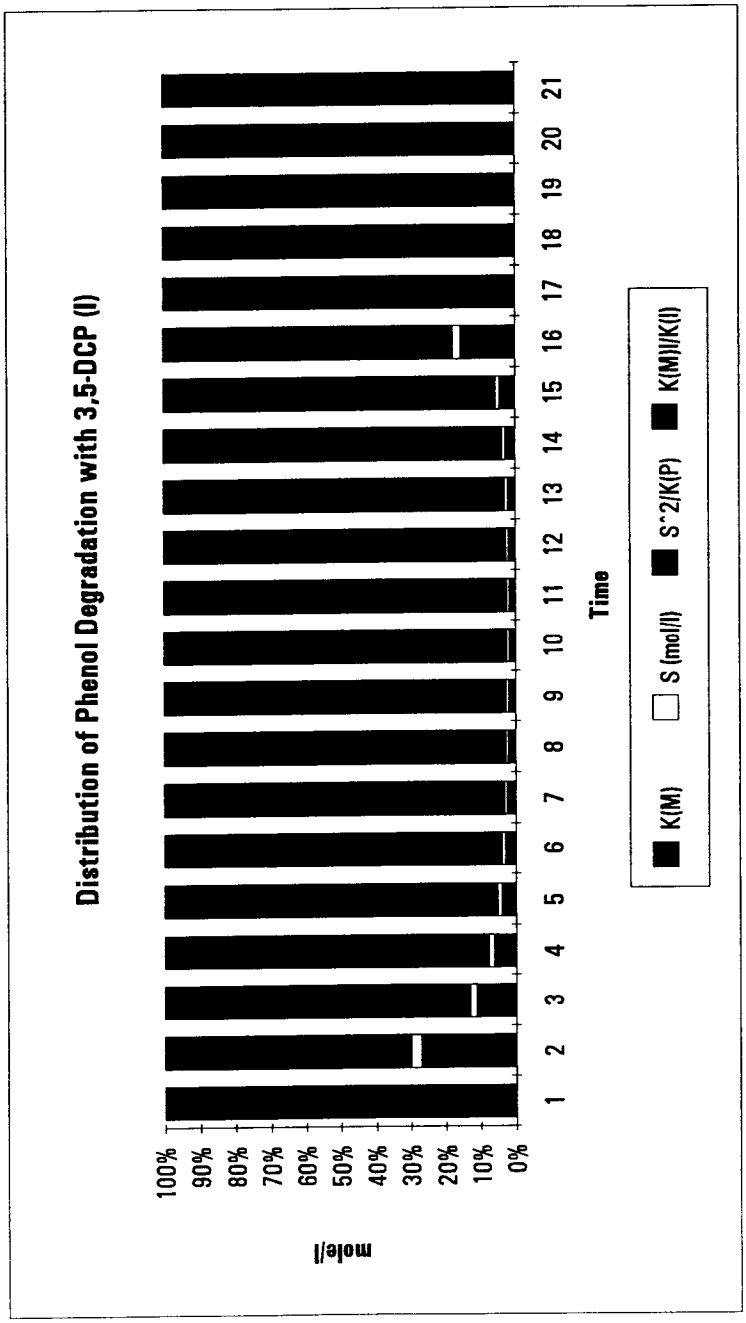
Appendix C (Continued)



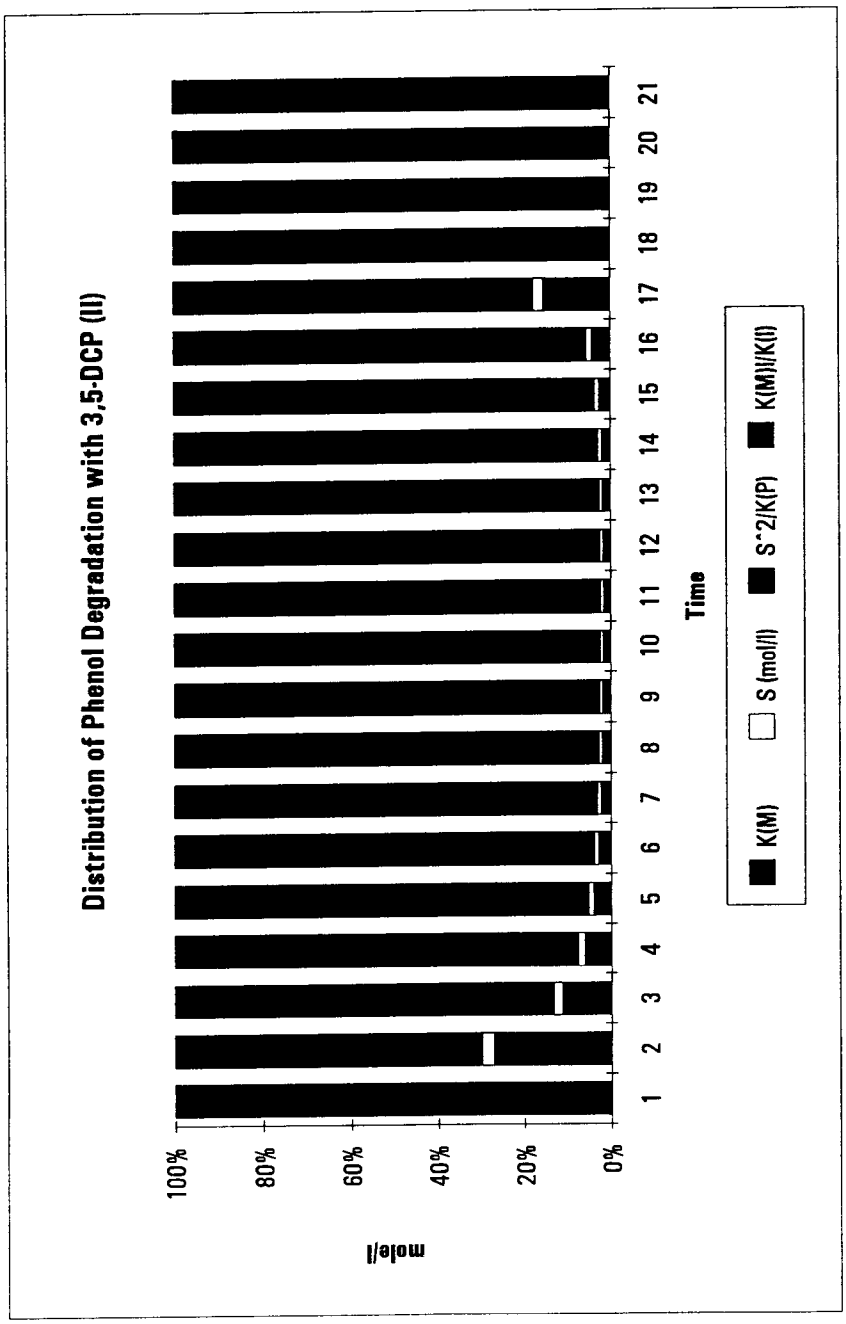
Appendix C (Continued)



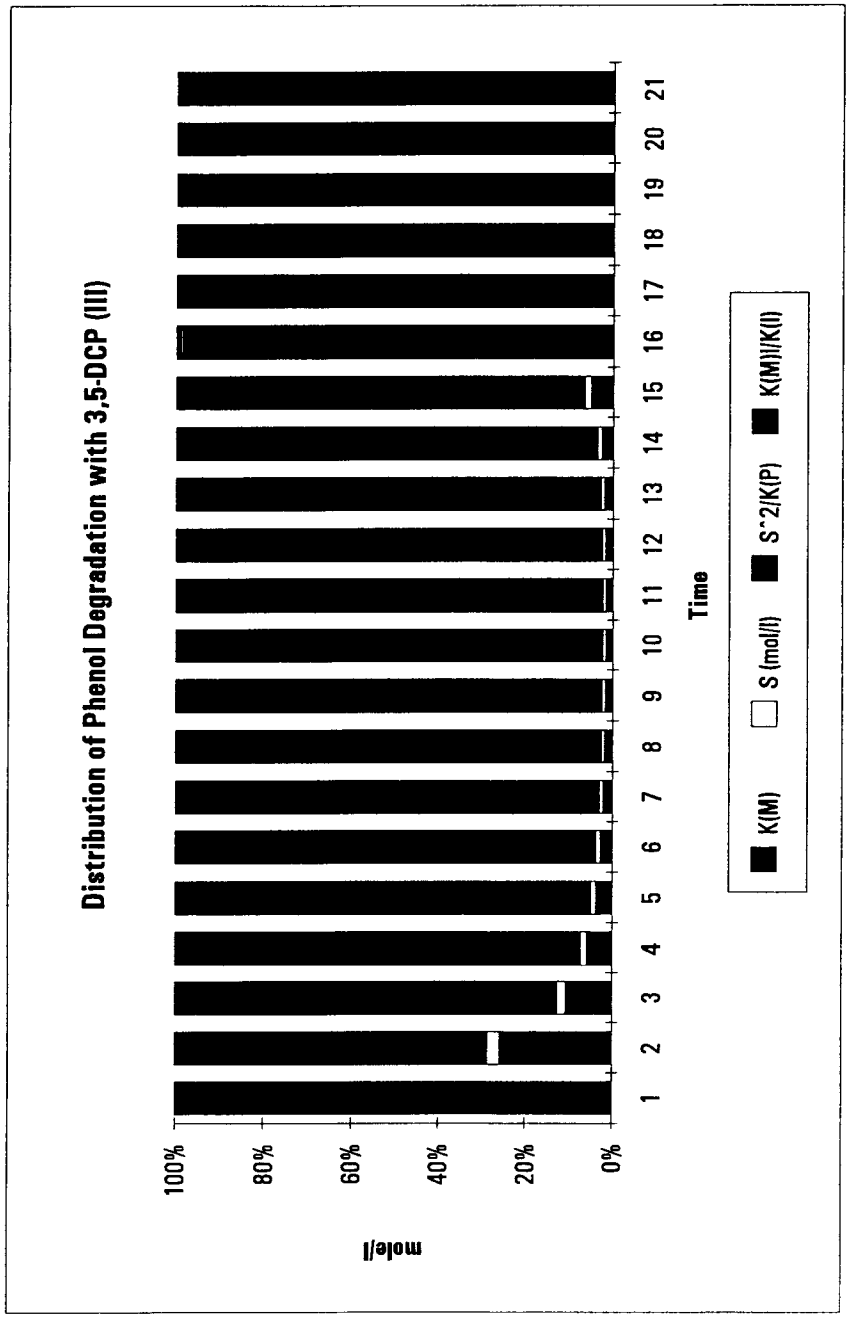
Appendix C (Continued)



Appendix C (Continued)



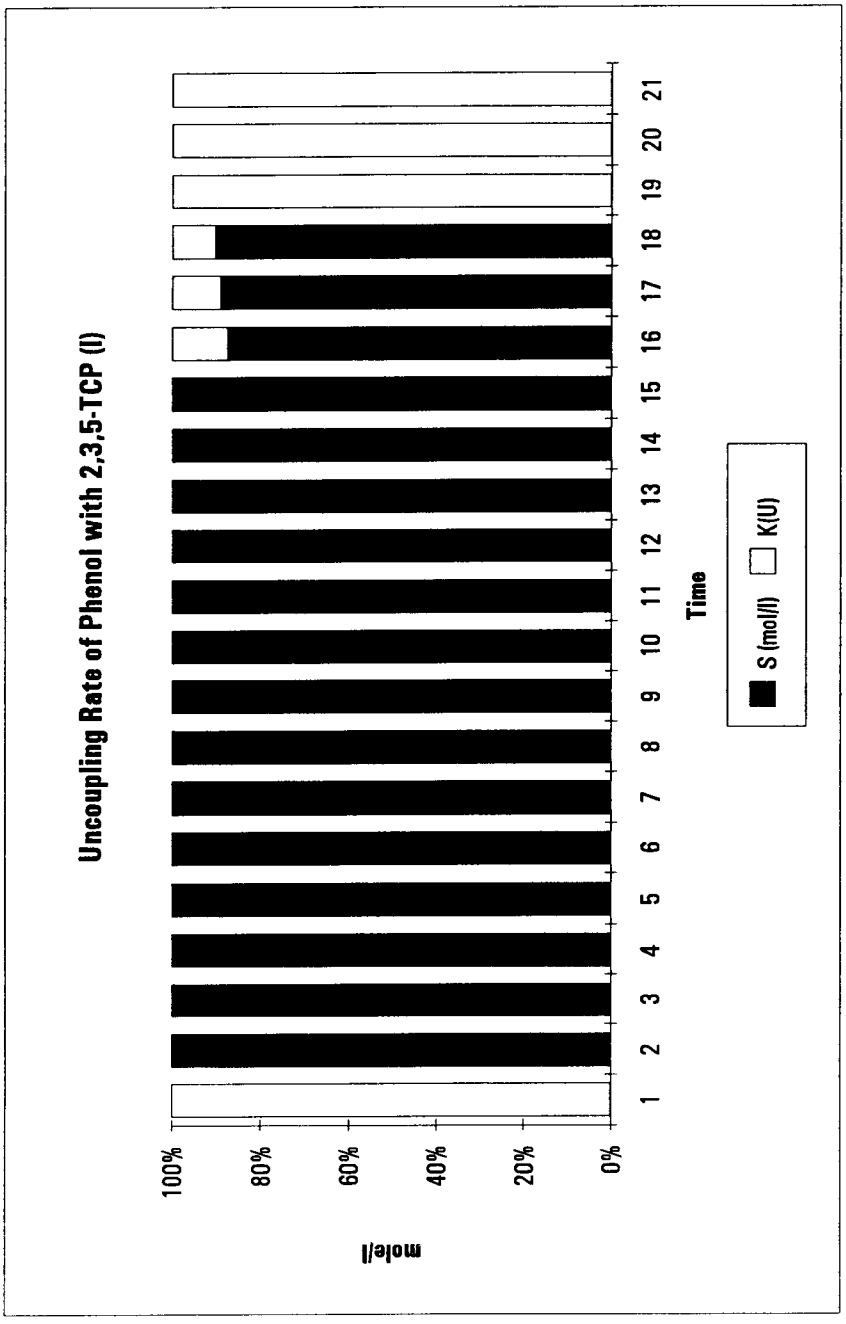
Appendix C (Continued)



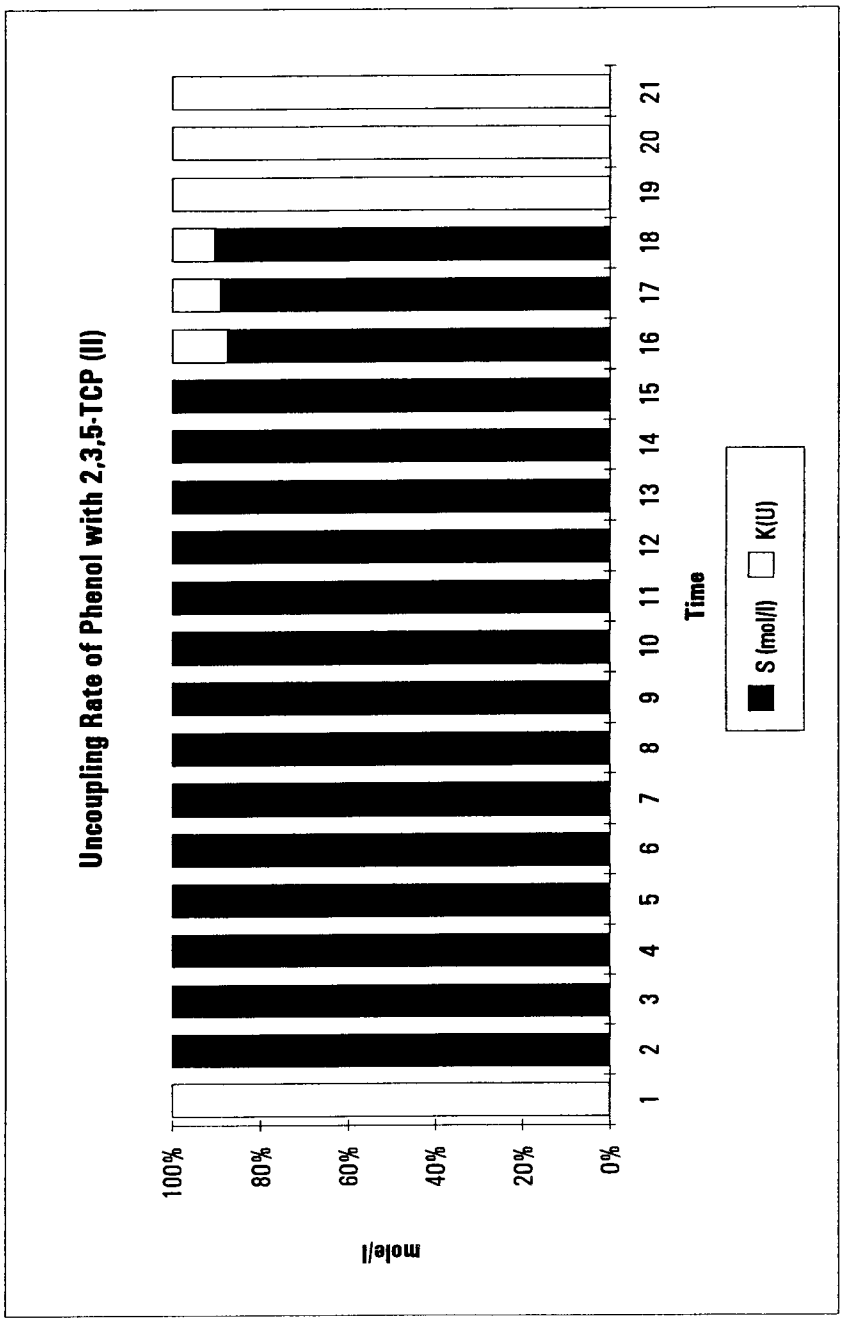
Appendix D

Uncoupling Rate of Phenol with Chlorophenols

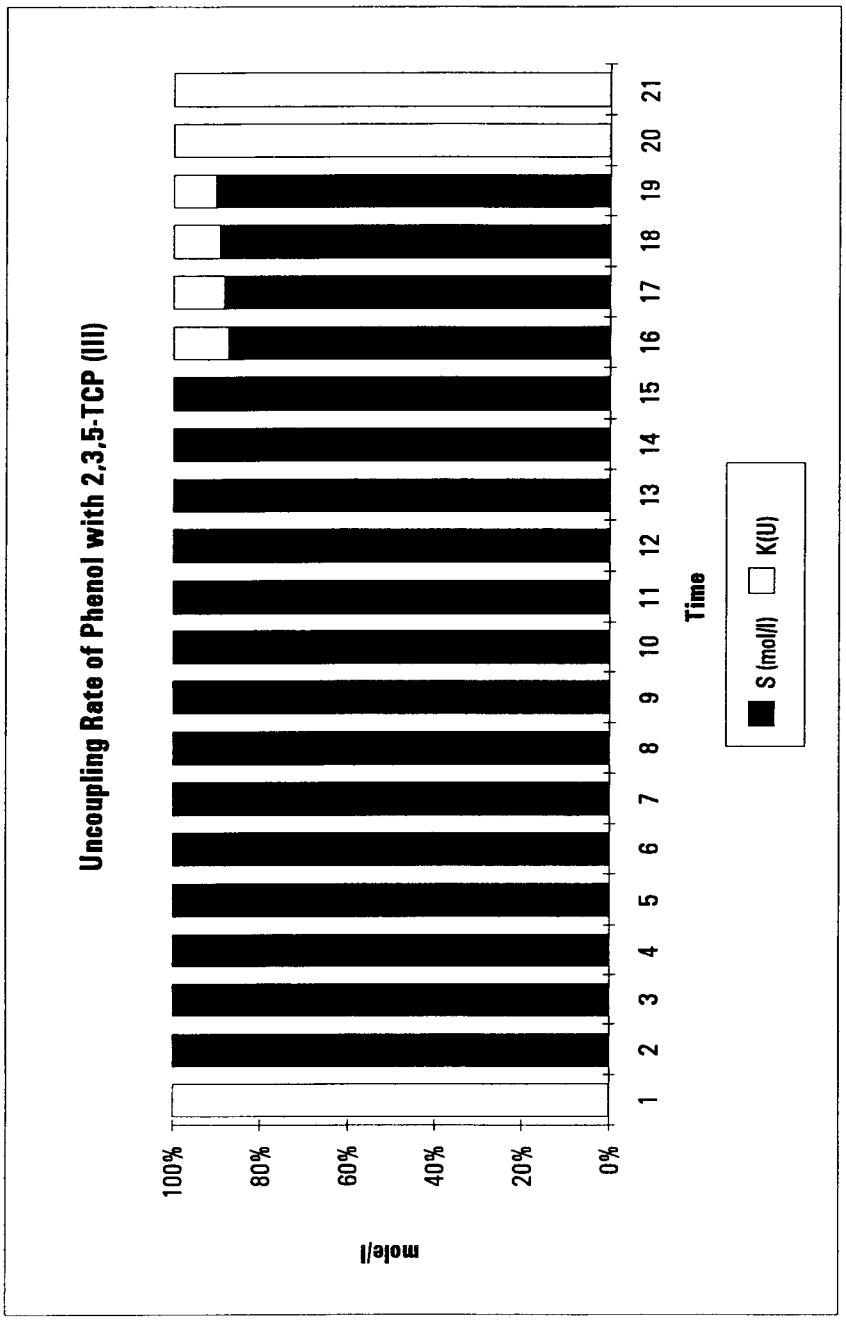
Appendix D (Continued)



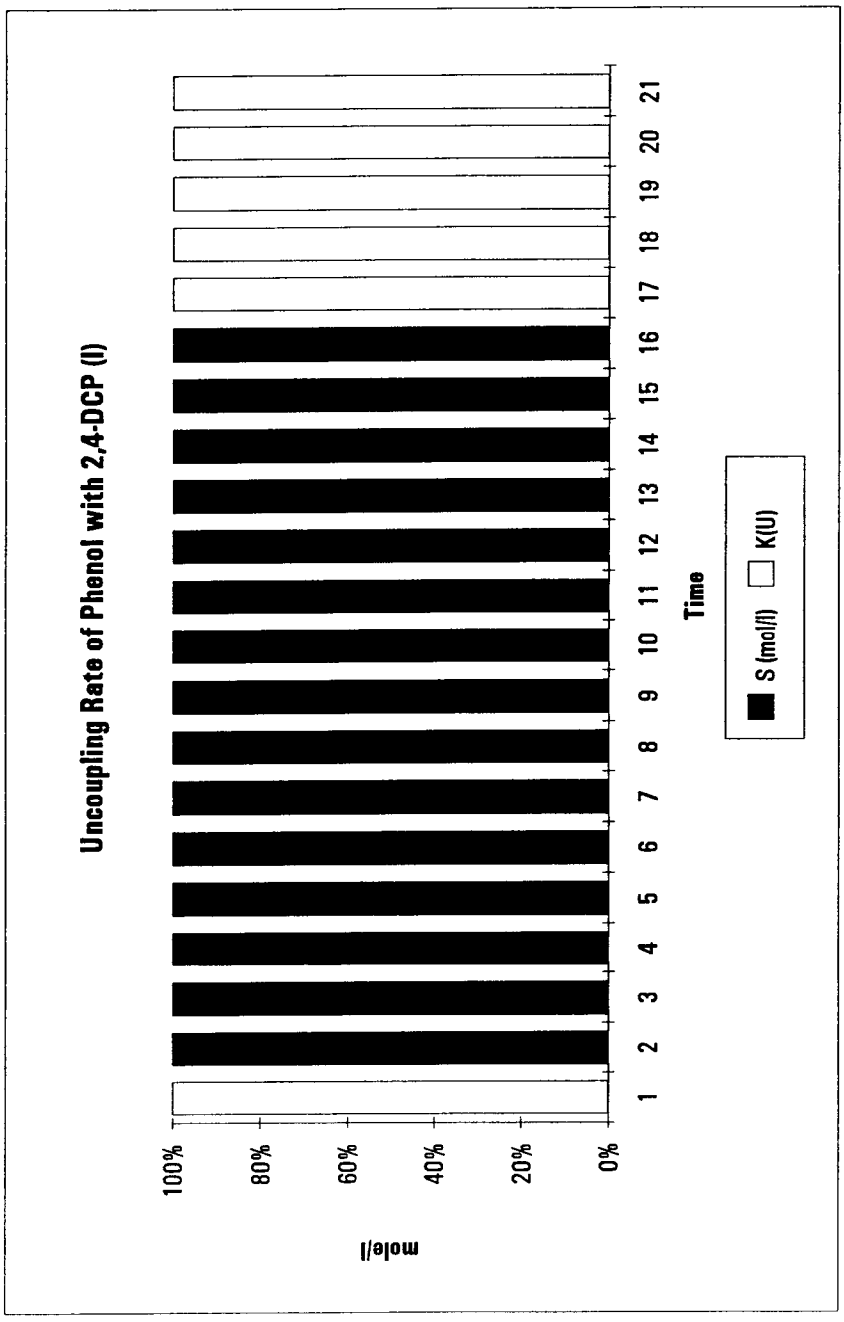
Appendix D (Continued)



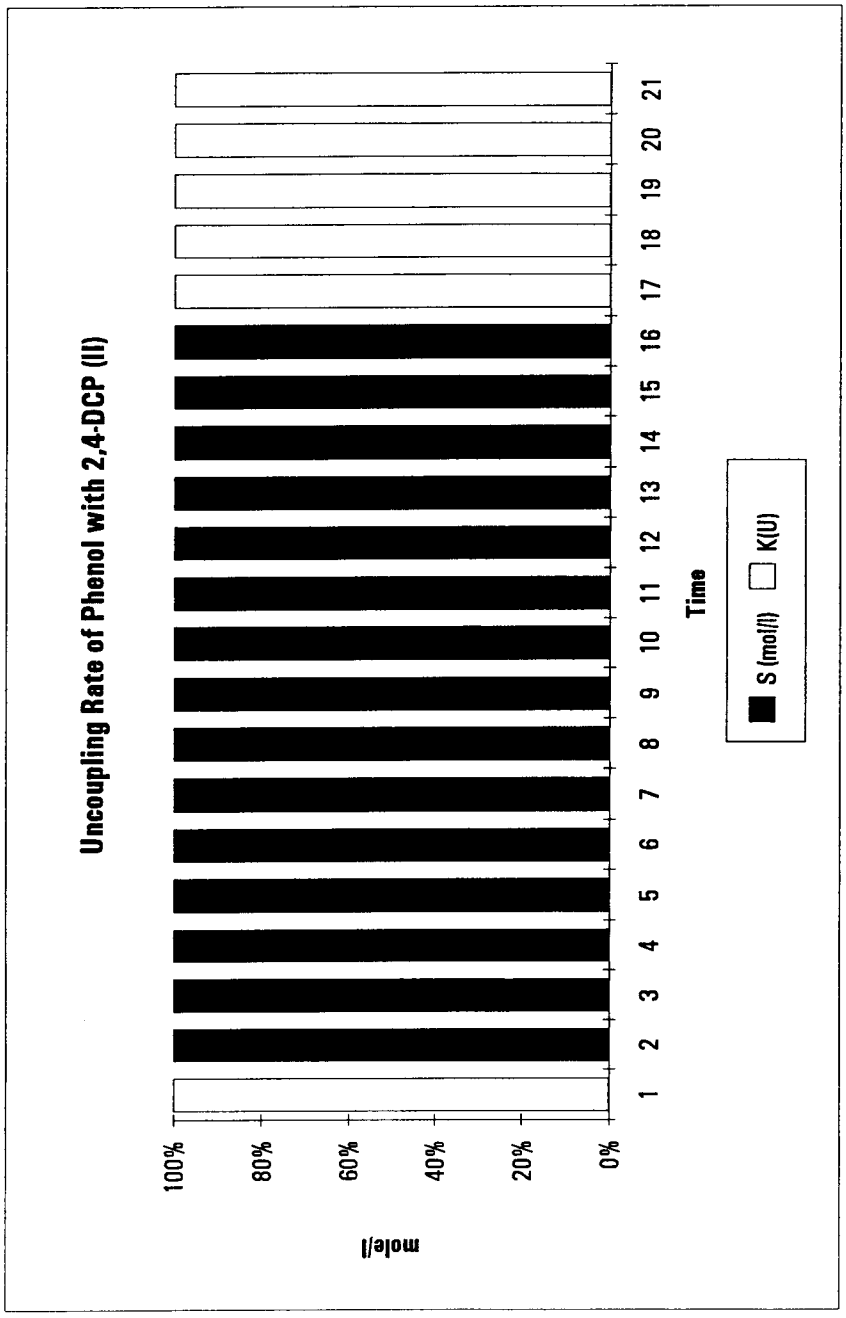
Appendix D (Continued)



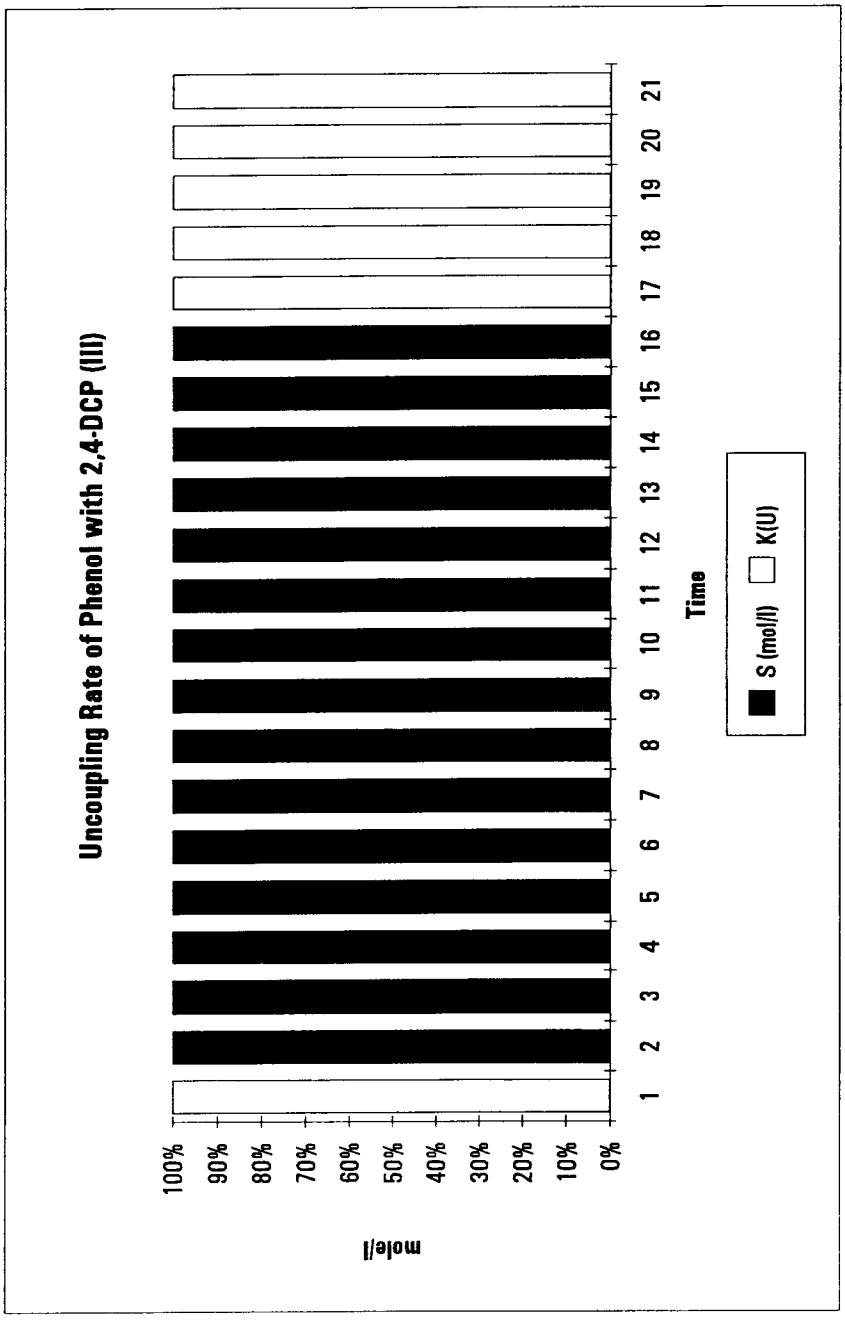
Appendix D (Continued)



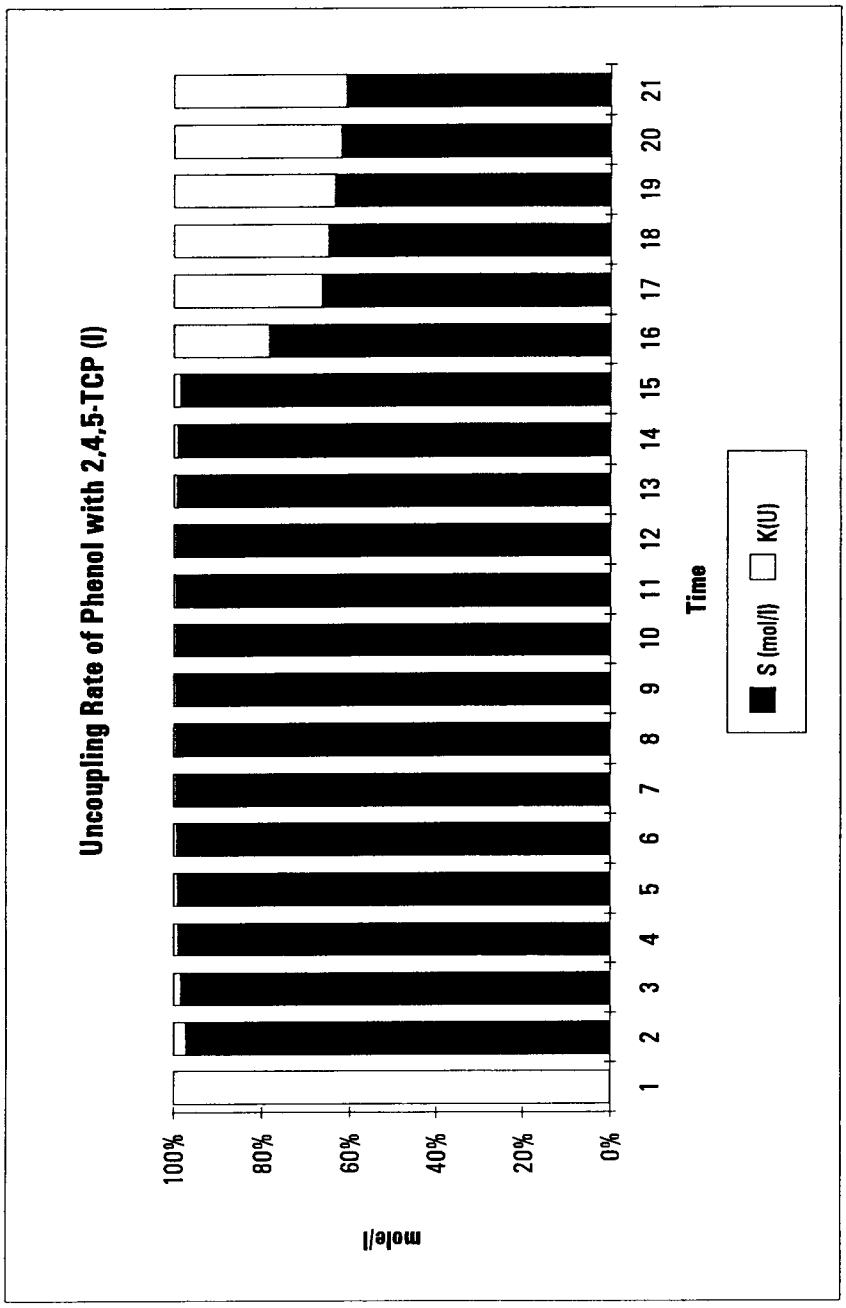
Appendix D (Continued)



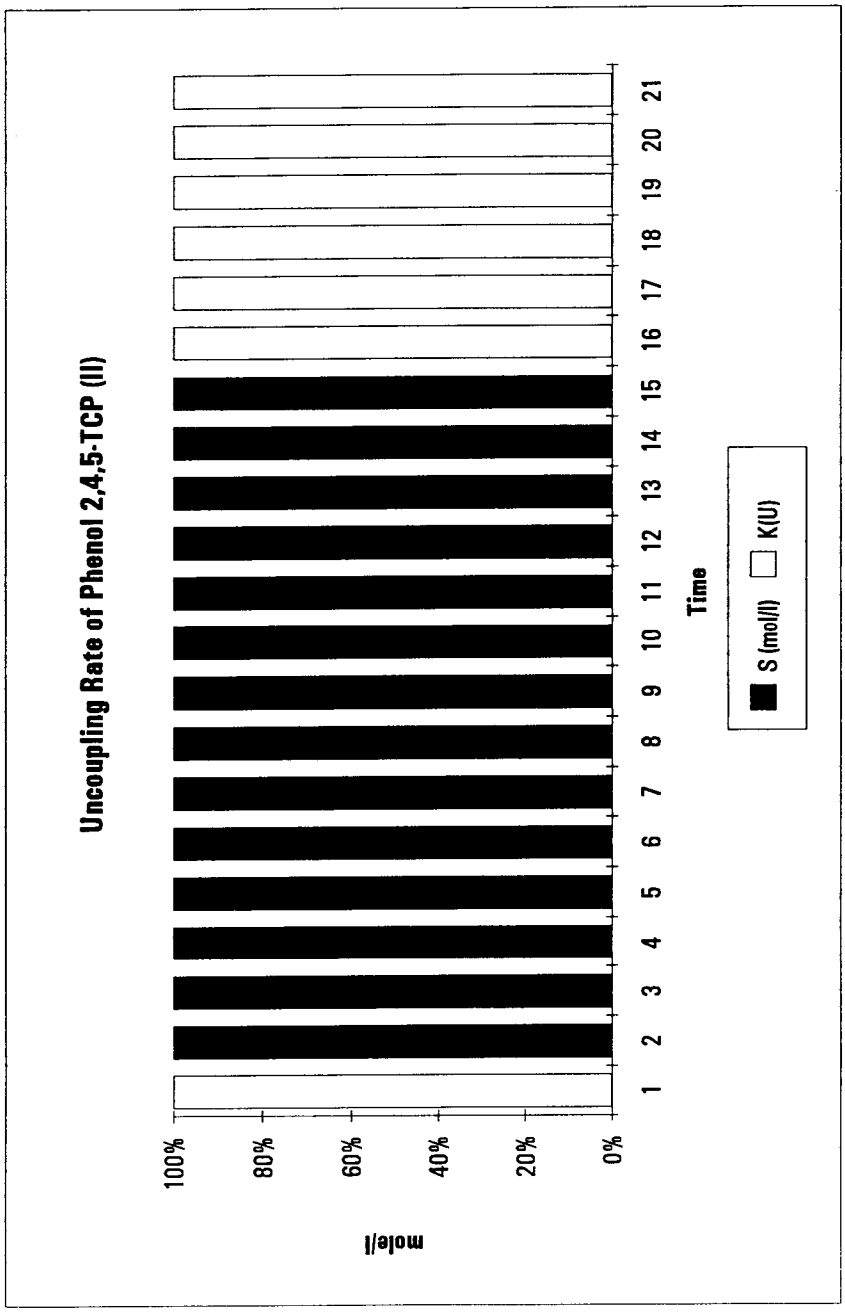
Appendix D (Continued)



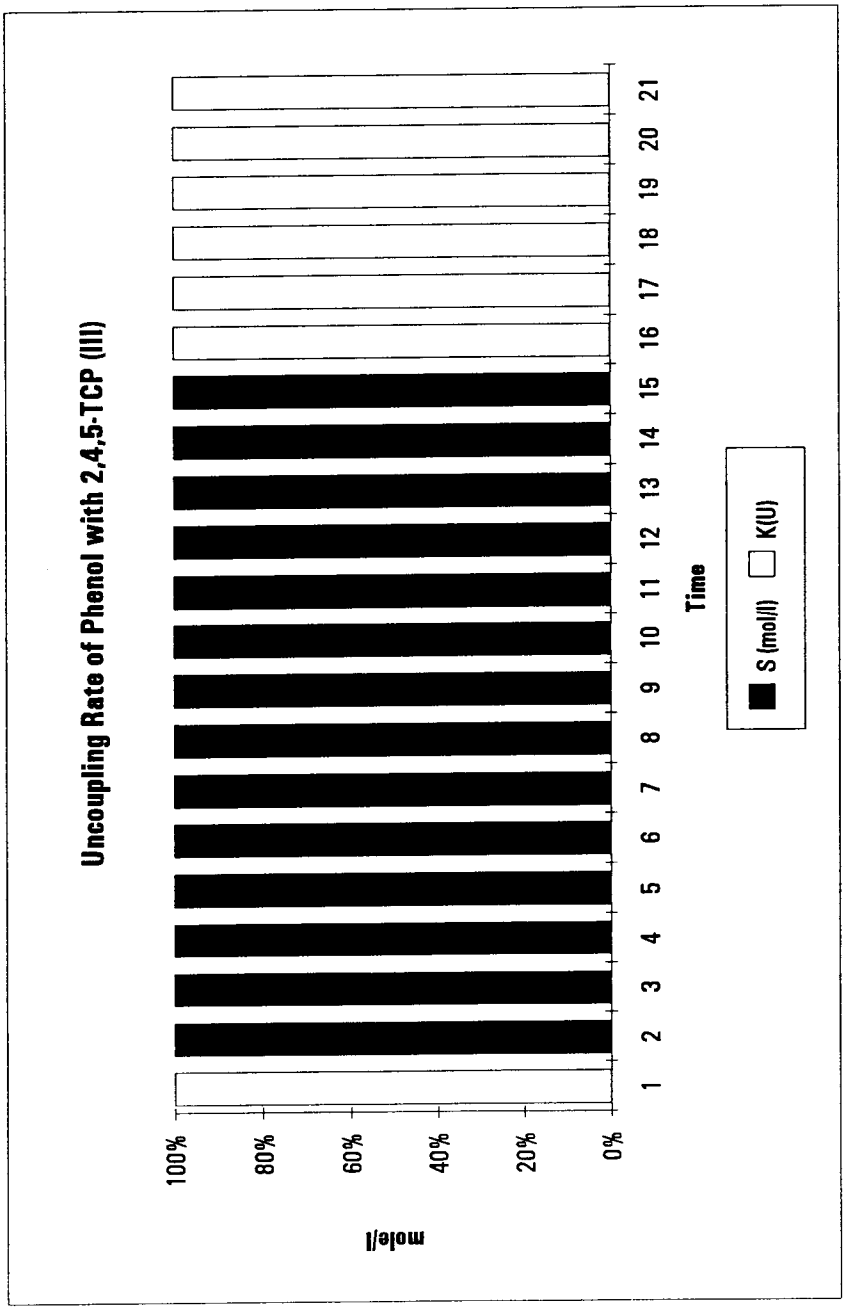
Appendix D (Continued)



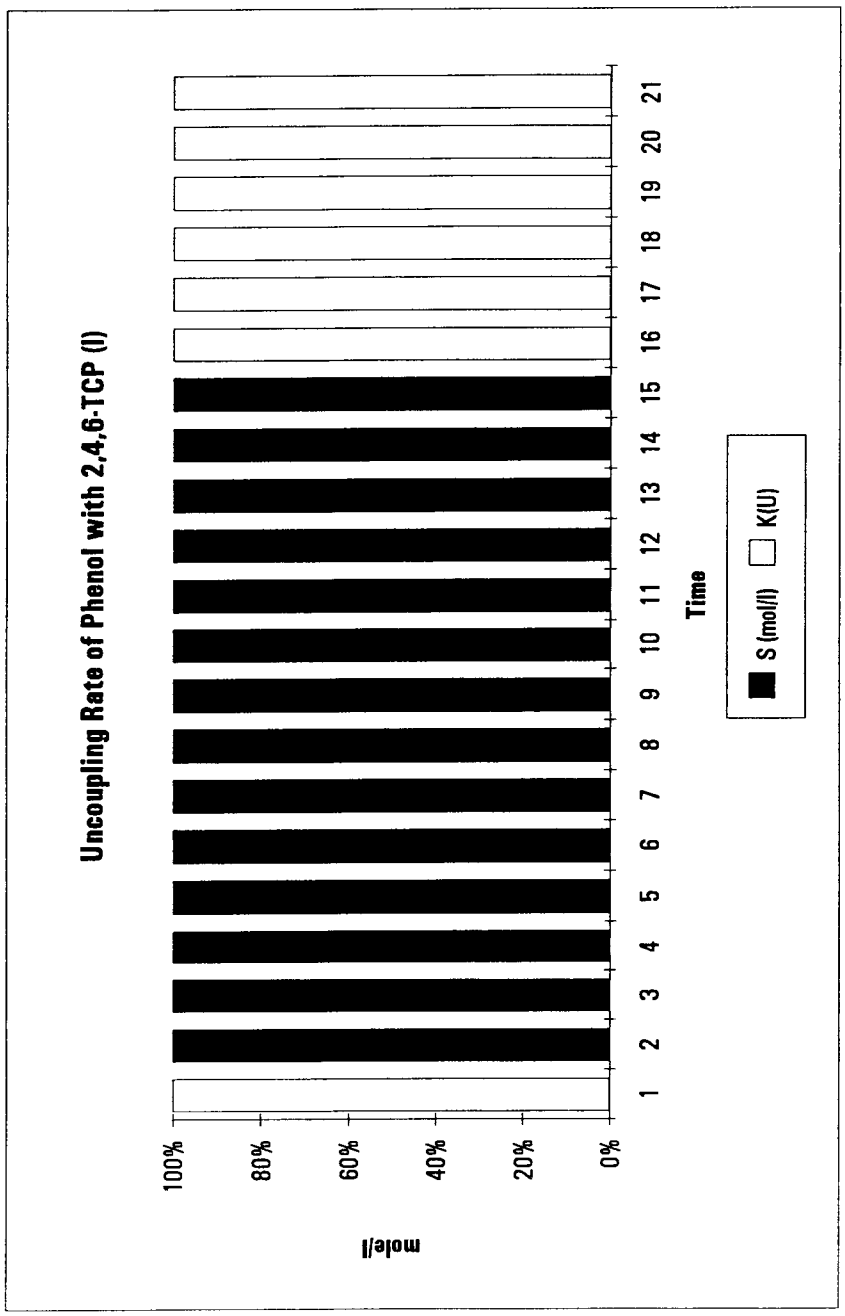
Appendix D (Continued)



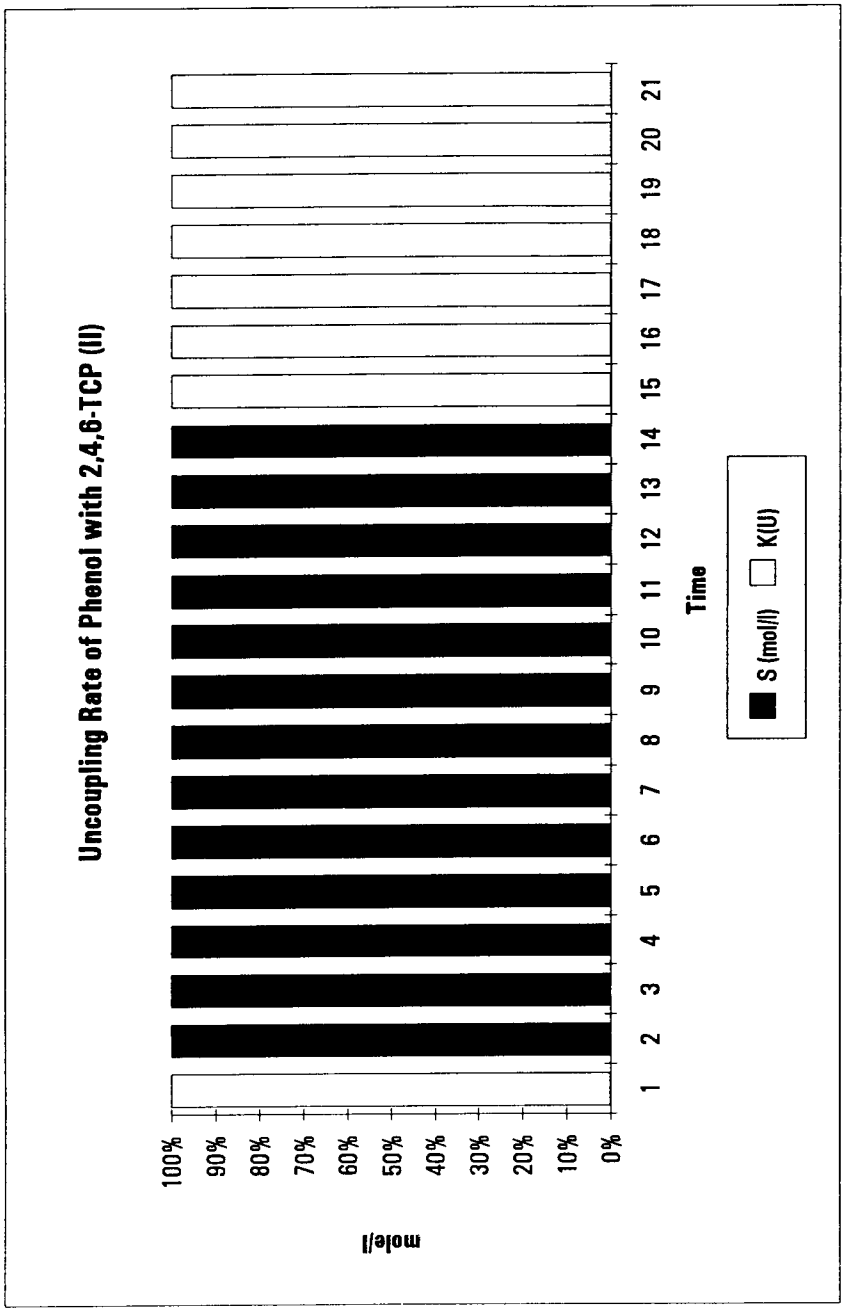
Appendix D (Continued)



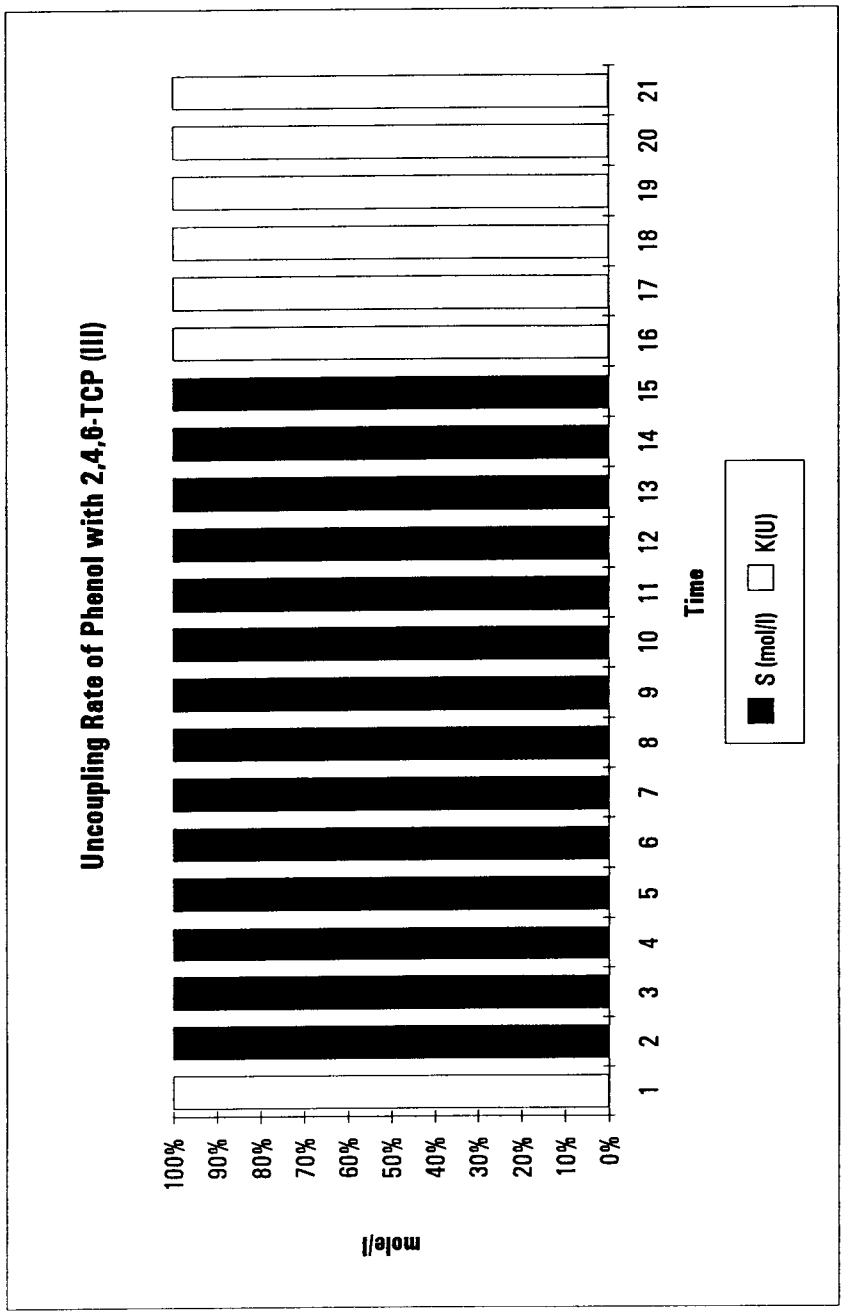
Appendix D (Continued)



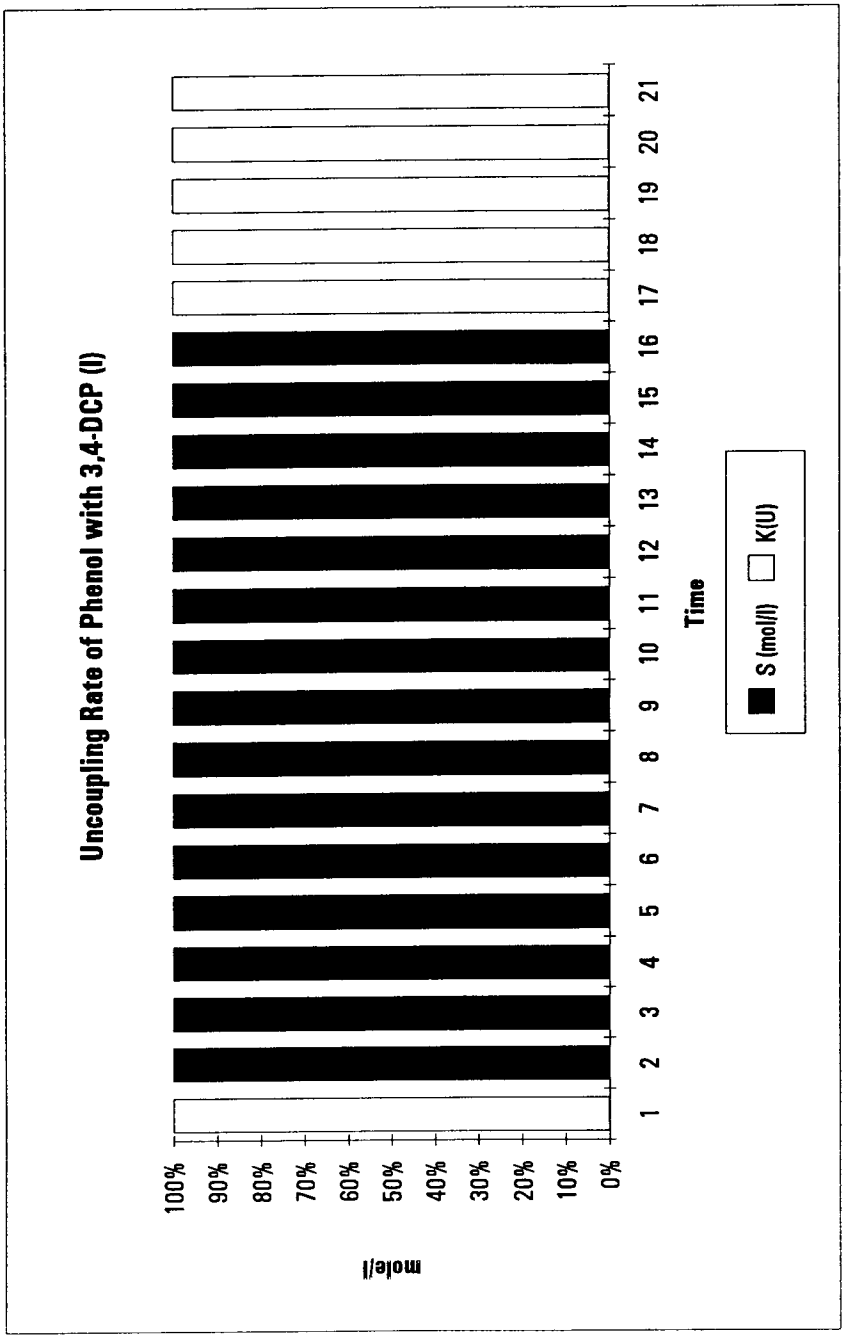
Appendix D (Continued)



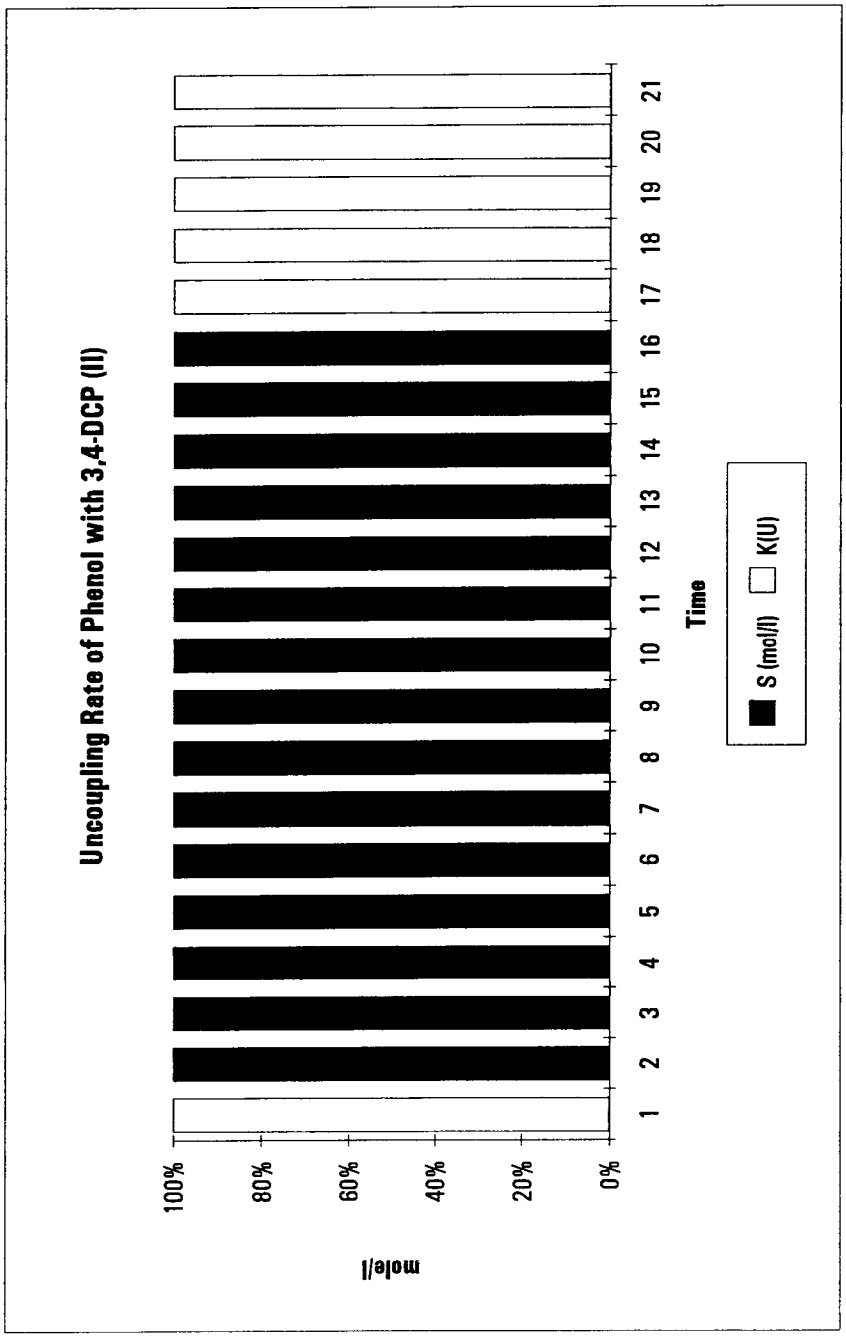
Appendix D (Continued)



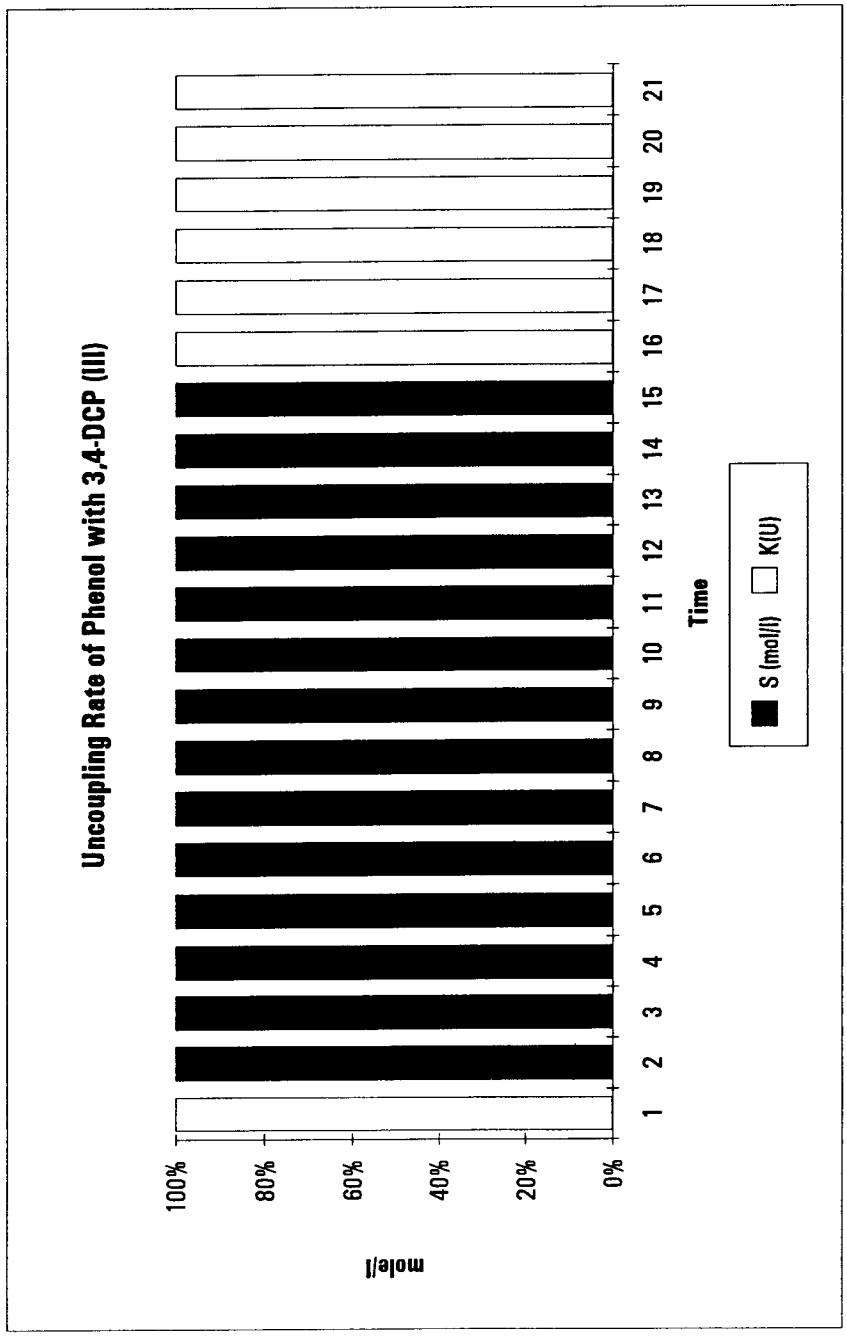
Appendix D (Continued)



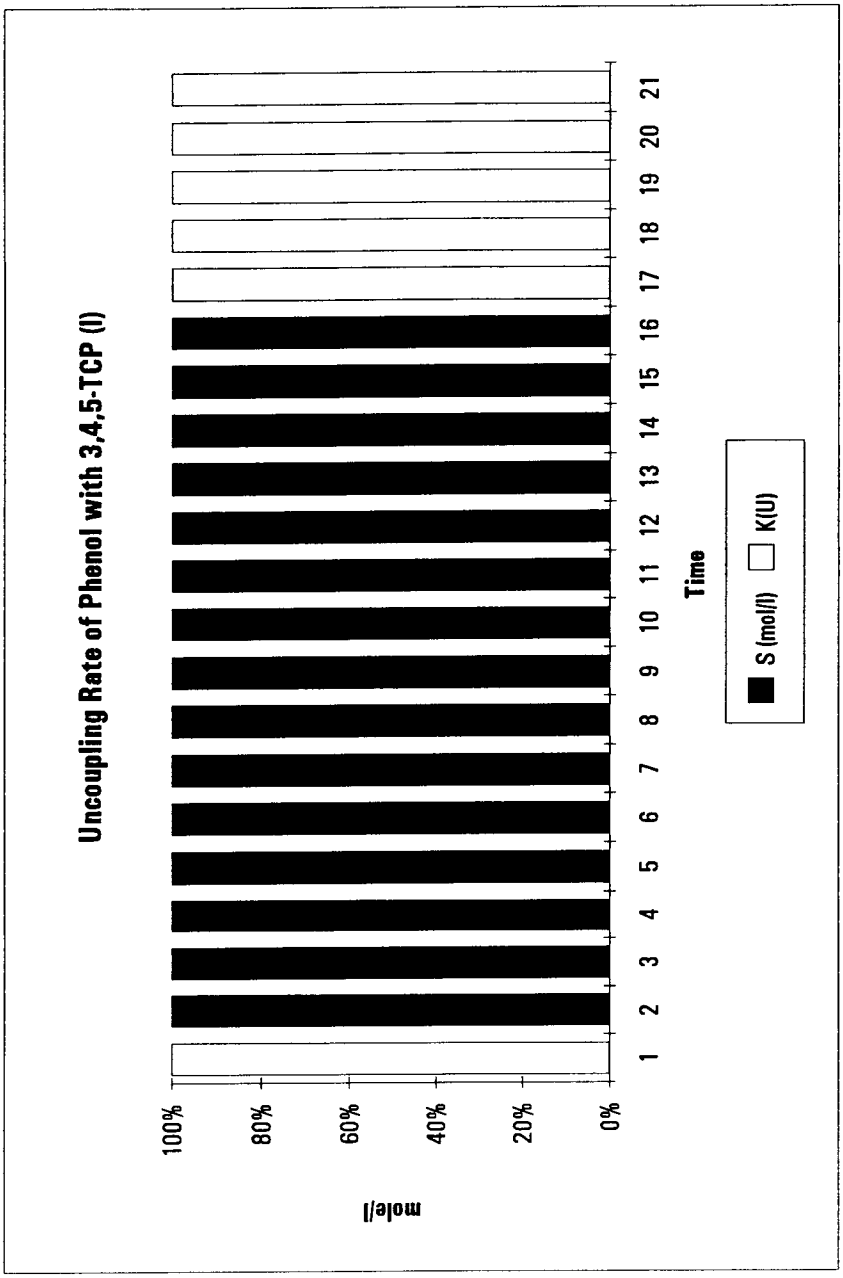
Appendix D (Continued)



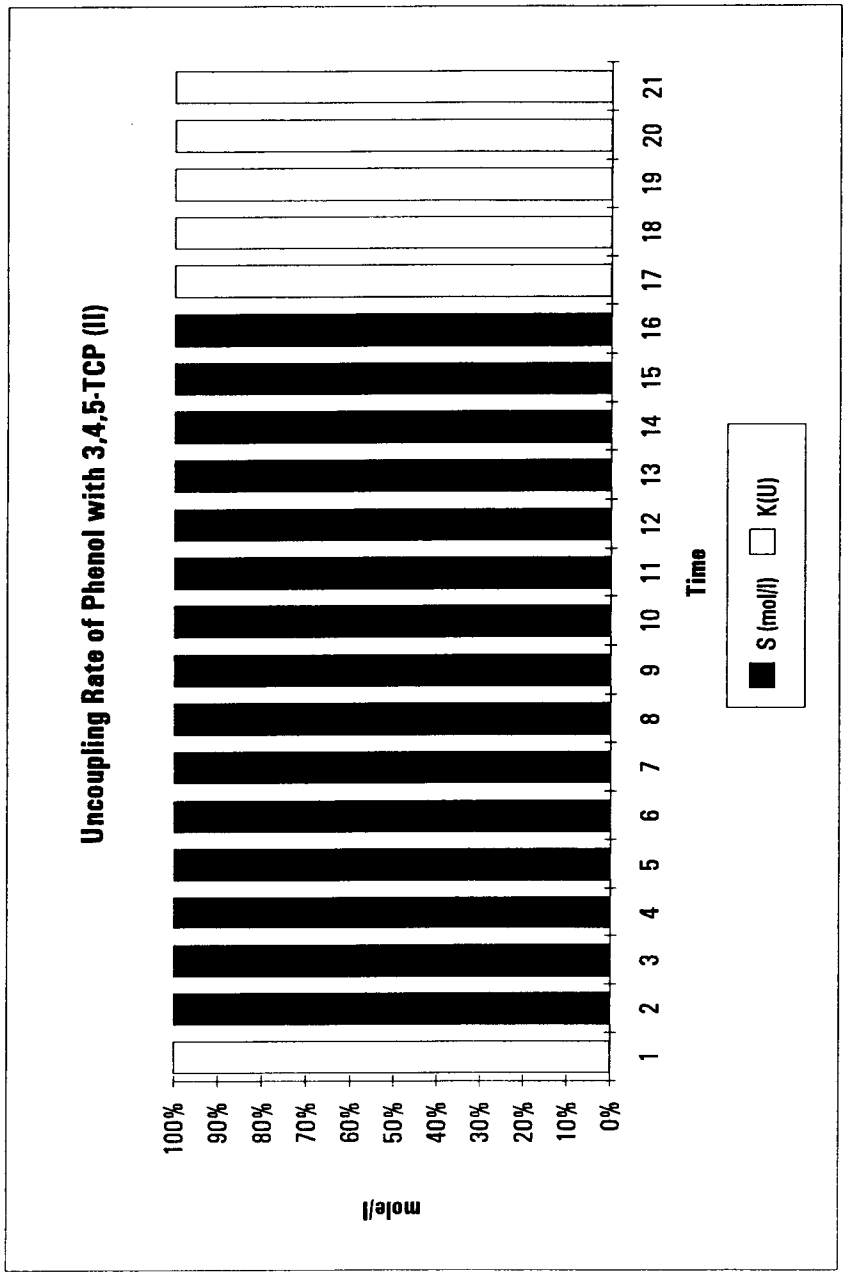
Appendix D (Continued)



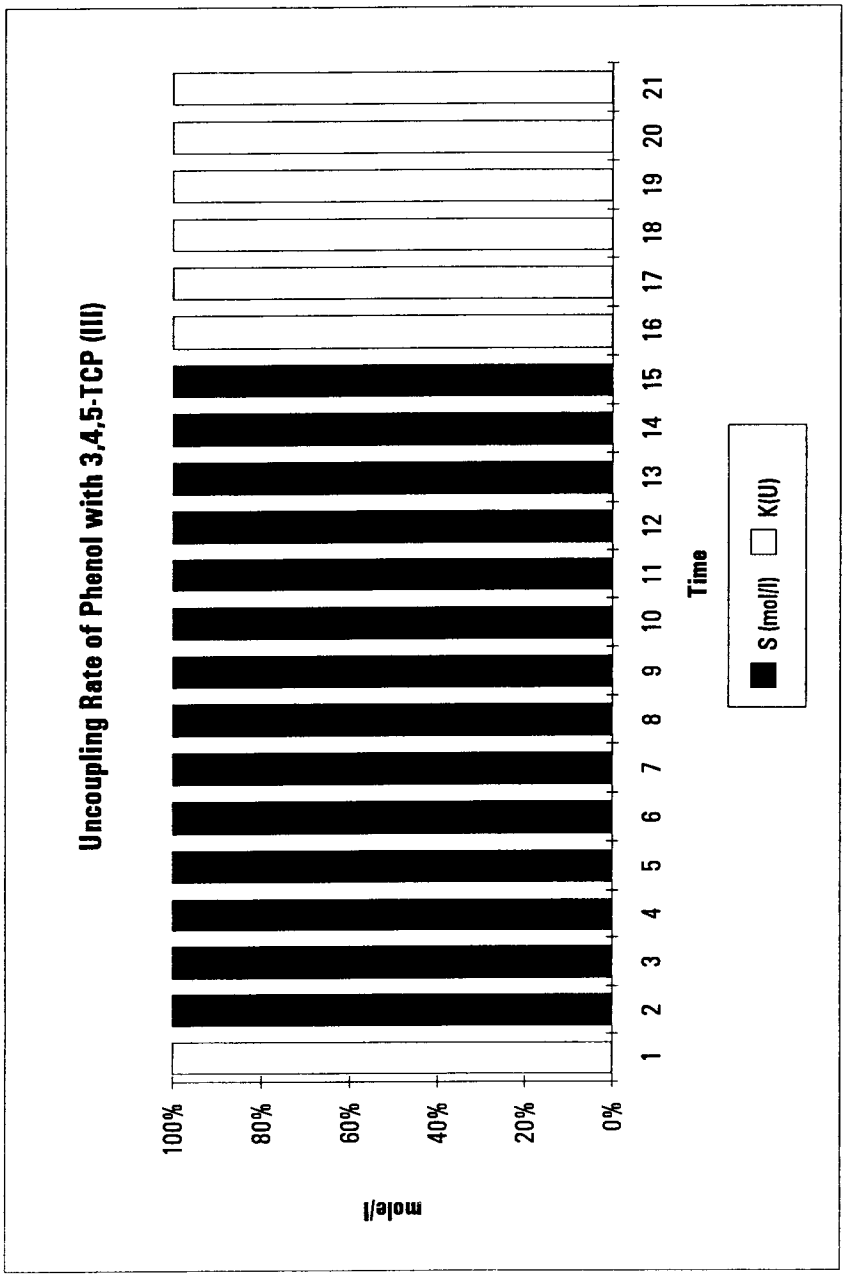
Appendix D (Continued)



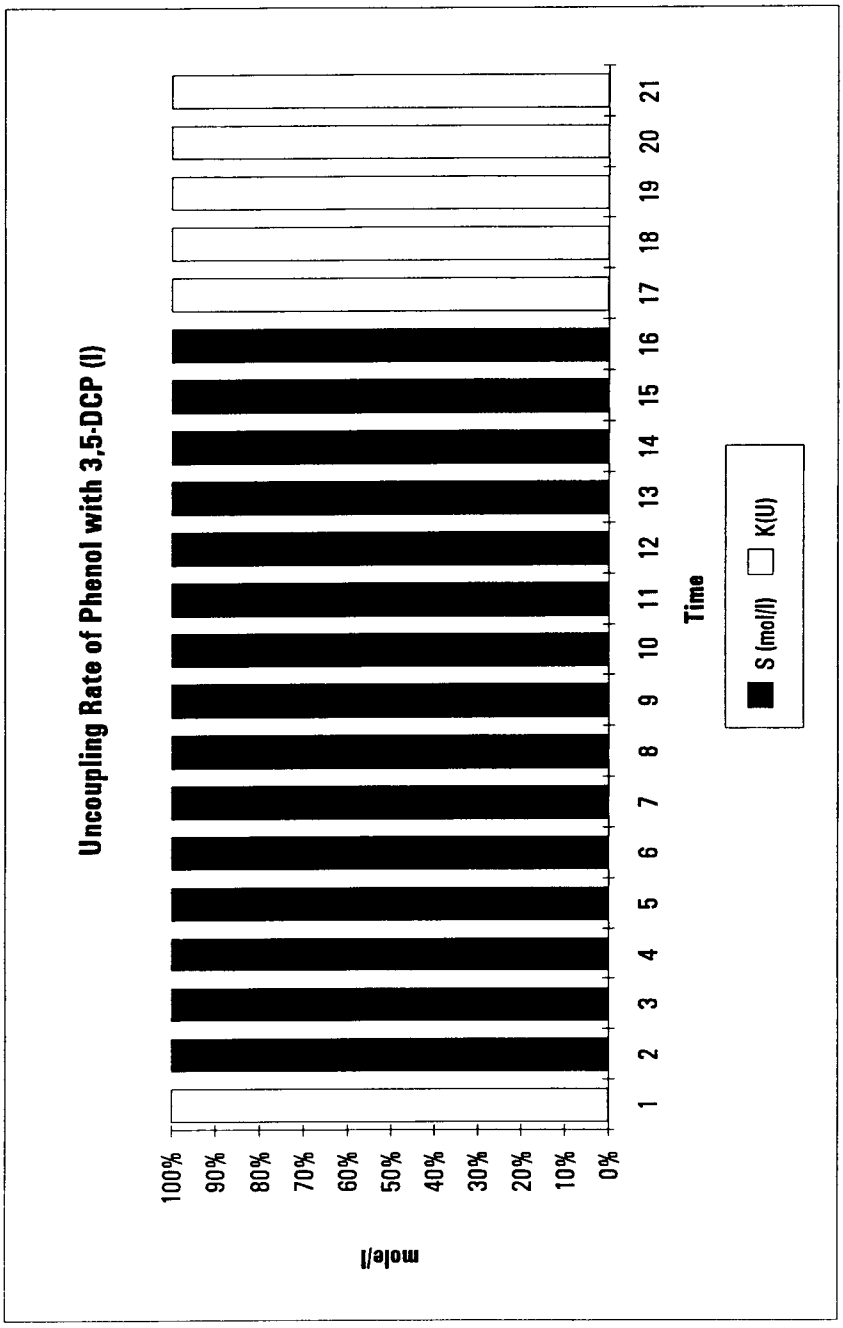
Appendix D (Continued)



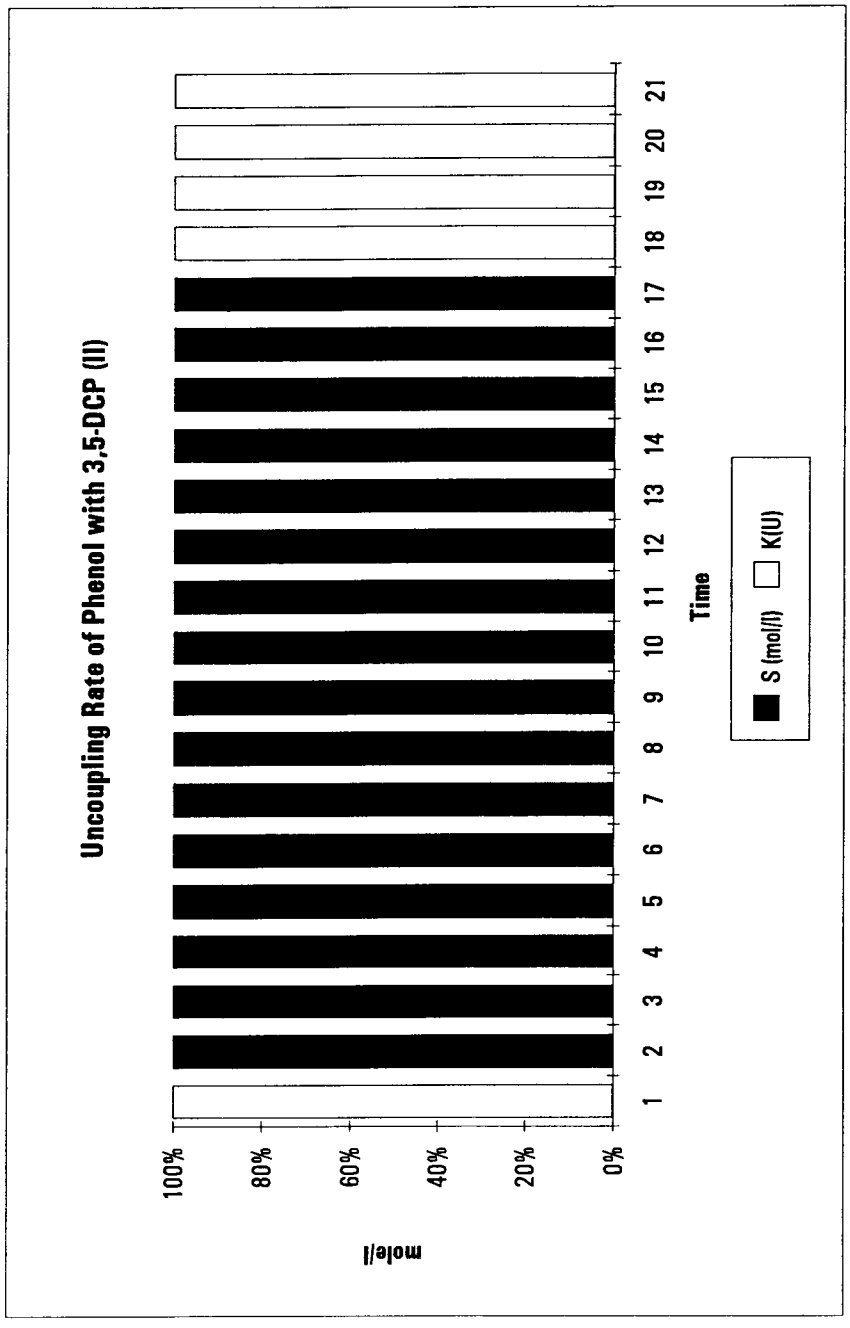
Appendix D (Continued)



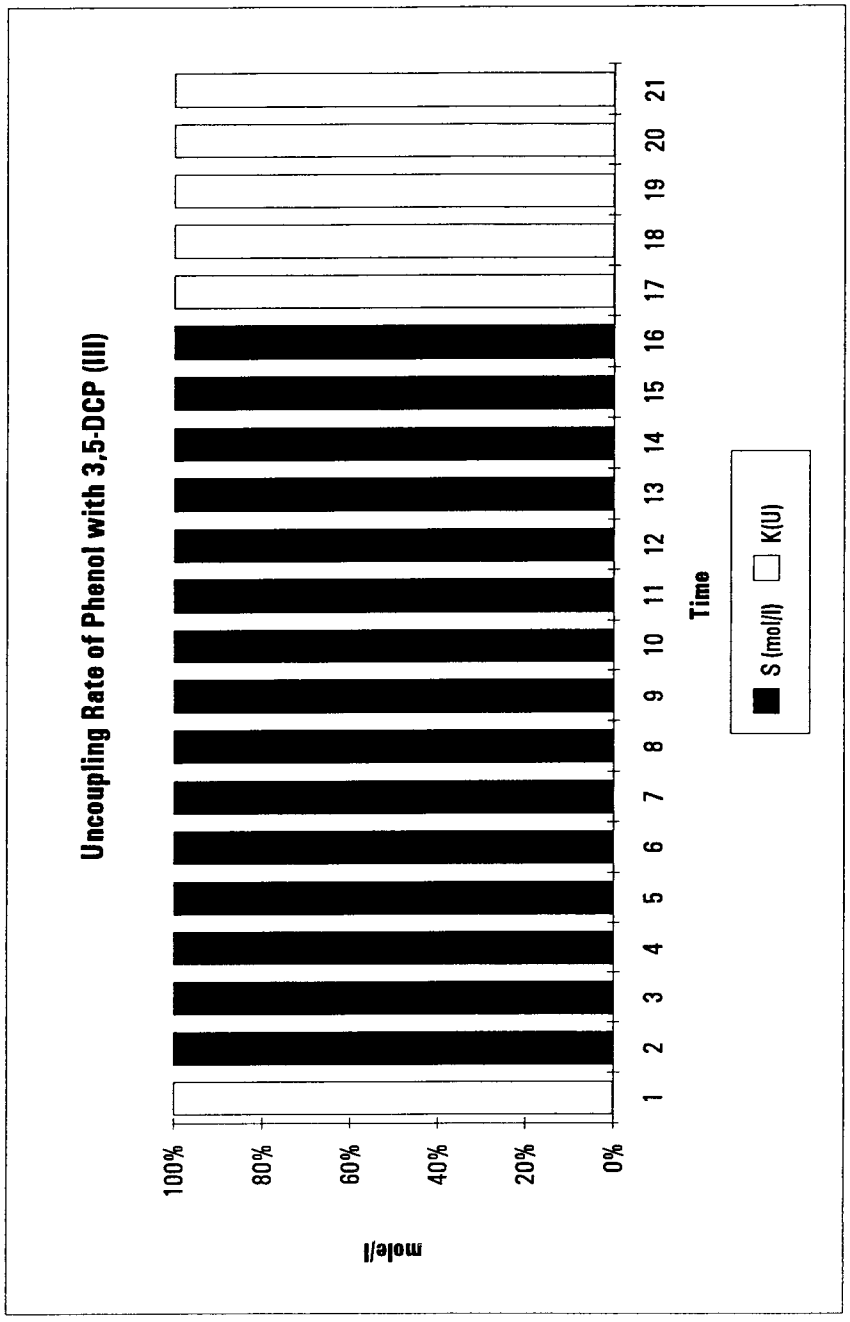
Appendix D (Continued)



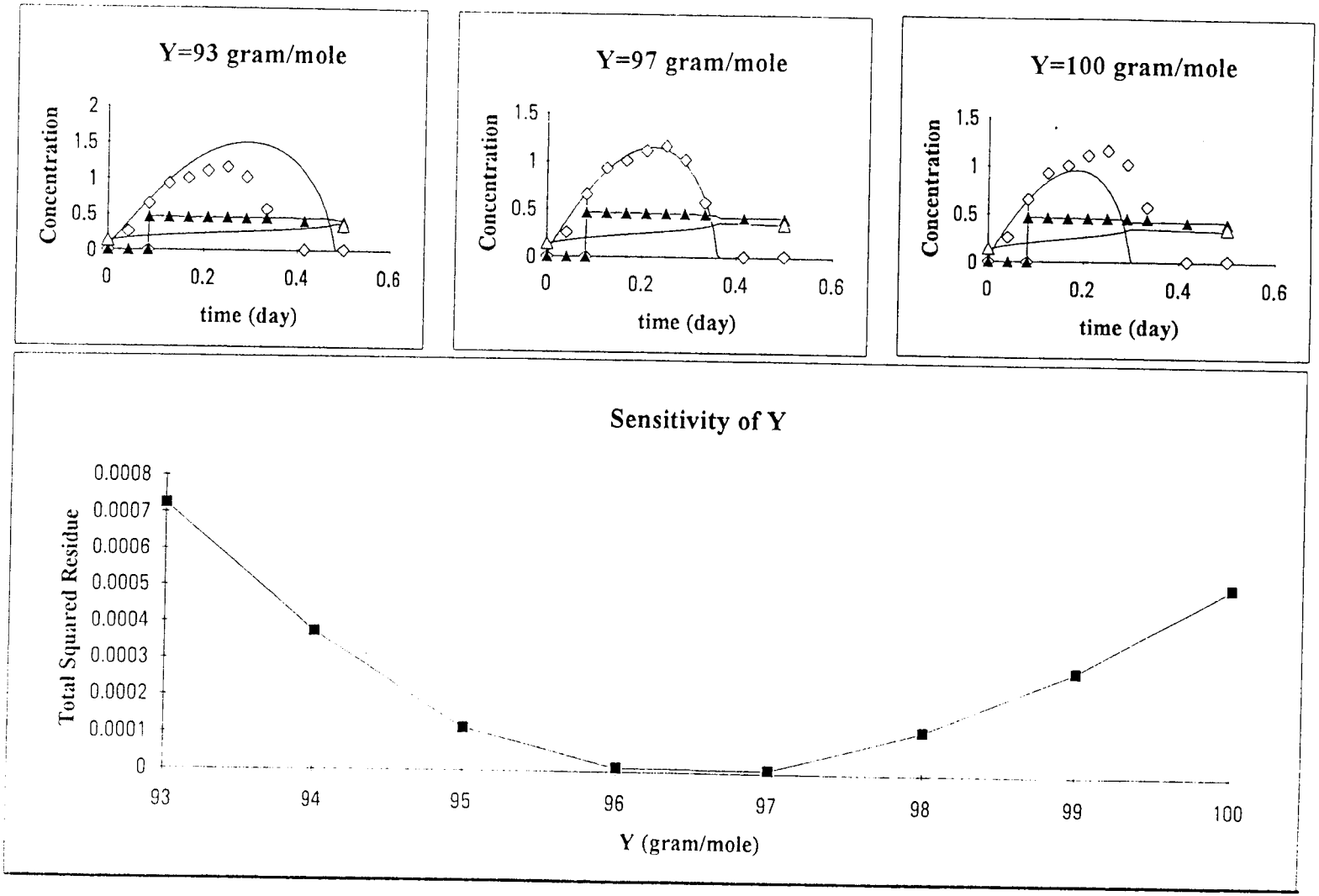
Appendix D (Continued)

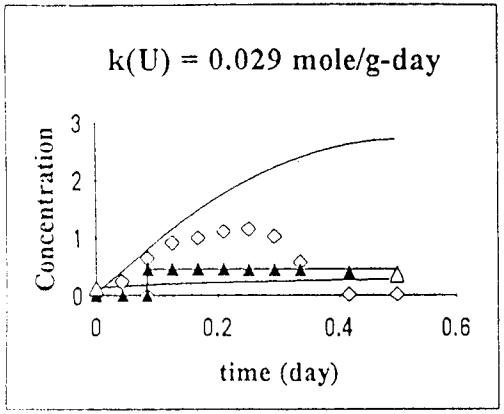


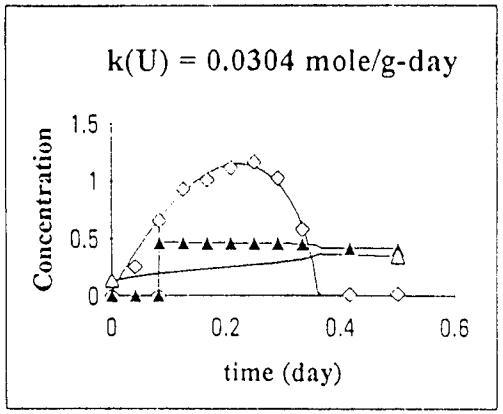
Appendix D (Continued)

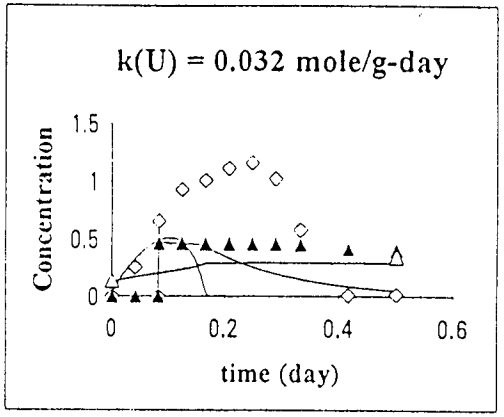


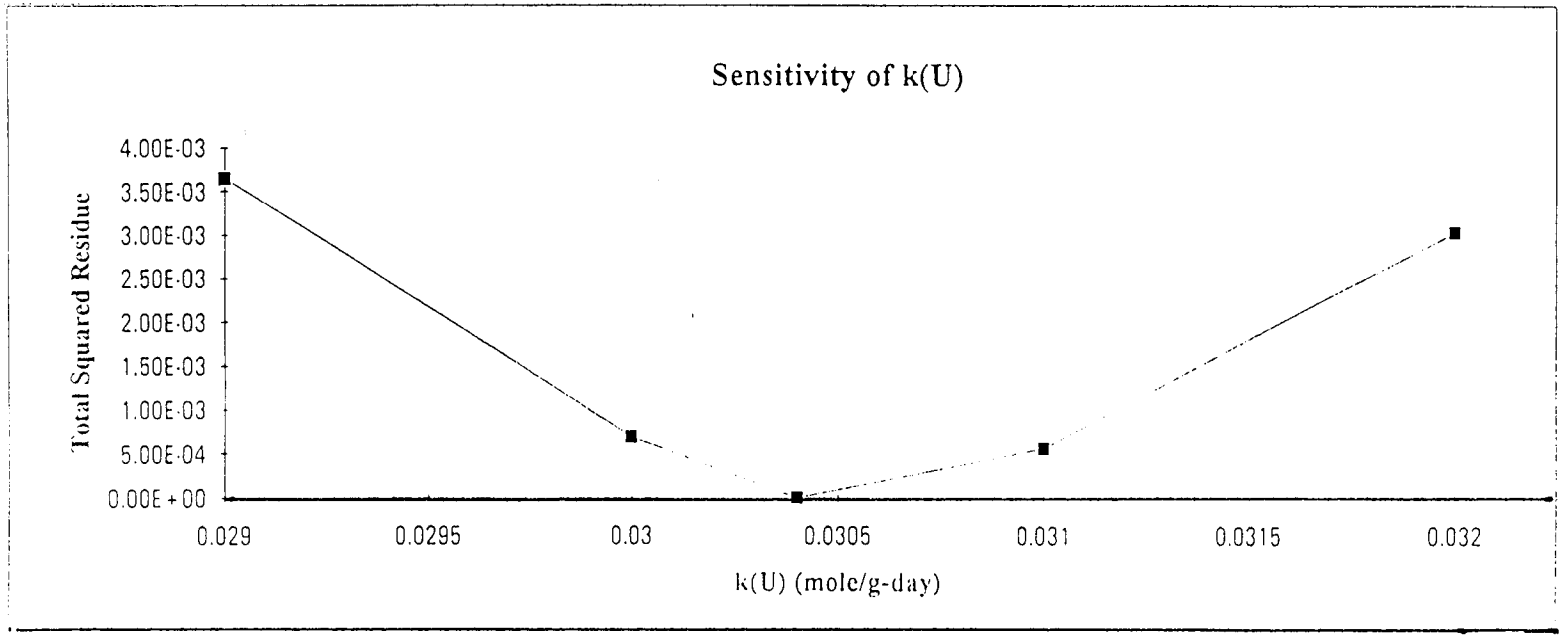
Appendix E
Sensitive Analysis

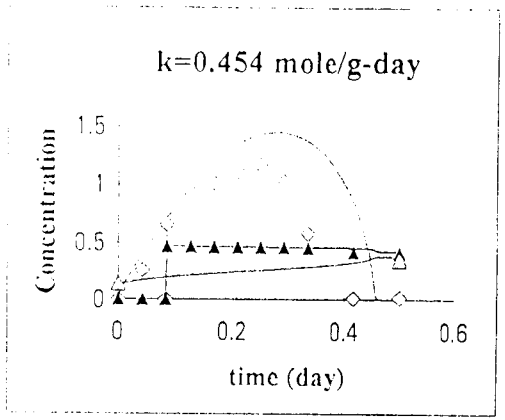

Y=93 gram/mole
Concentration
2
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)
Y=97 gram/mole
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)
Y=100 gram/mole
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)
Sensitivity of Y
Total Squared Residue
0.0008
0.0007
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0
93
94
95
96
97
98
99
100
Y (gram/mole)

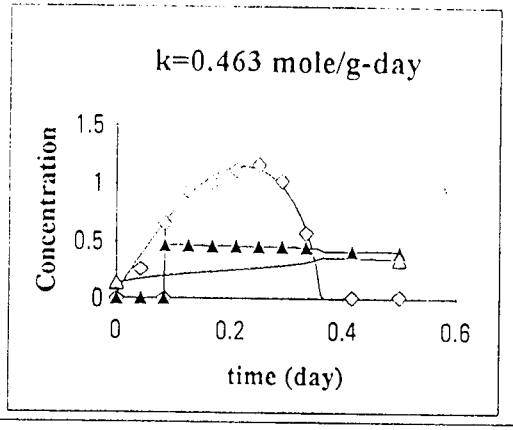

k(U) = 0.029 mole/g-day
Concentration
3
2
1
0
0
0.2
0.4
0.6
time (day)

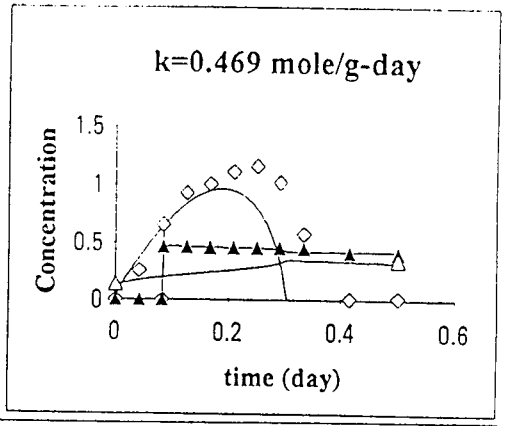

k(U) = 0.0304 mole/g-day
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)

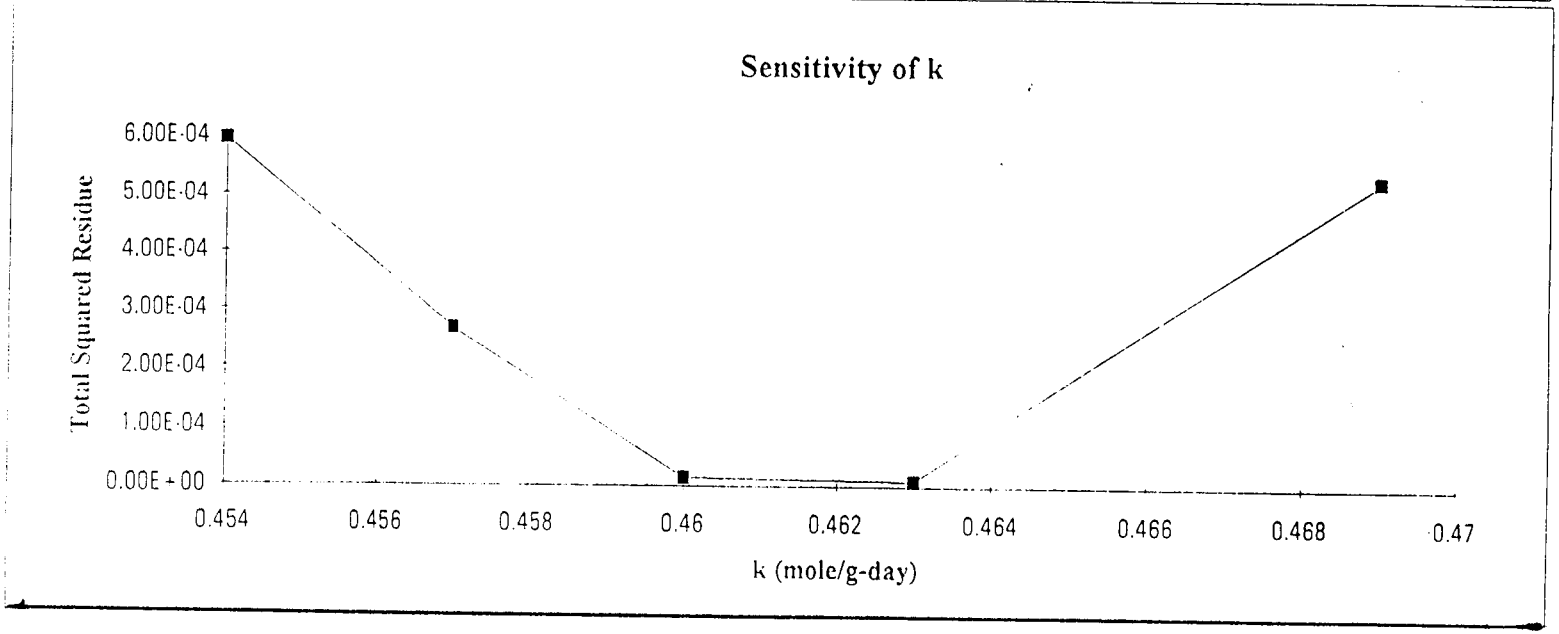

k(U) = 0.032 mole/g-day
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)

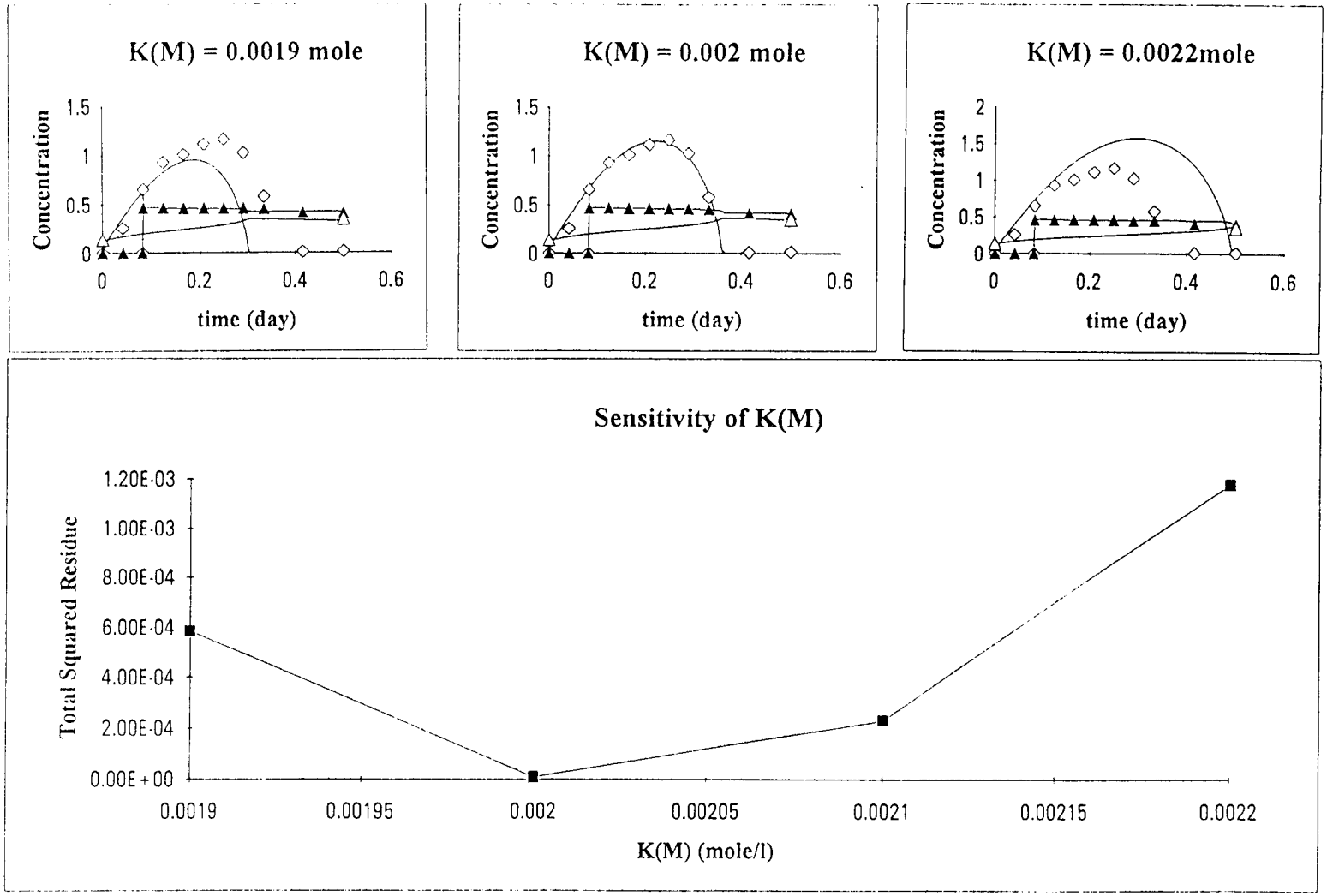

Sensitivity of k(U)
Total Squared Residue
4.00E-03
3.50E-03
3.00E-03
2.50E-03
2.00E-03
1.50E-03
1.00E-03
5.00E-04
0.00E+00
0.029
0.0295
0.03
0.0305
0.031
0.0315
0.032
k(U) (mole/g-day)

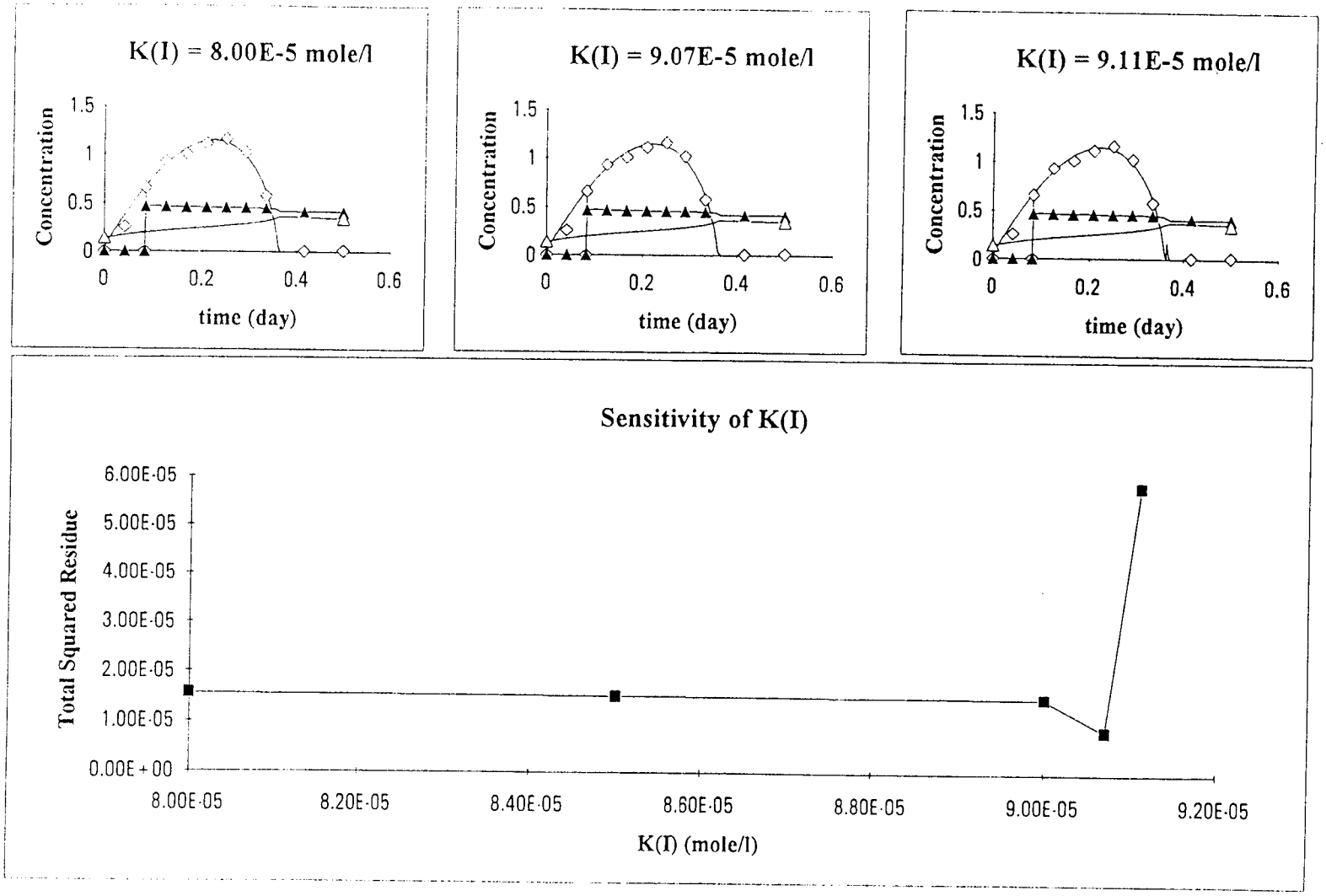

k=0.454 mole/g-day
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)


k=0.463 mole/g-day
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)

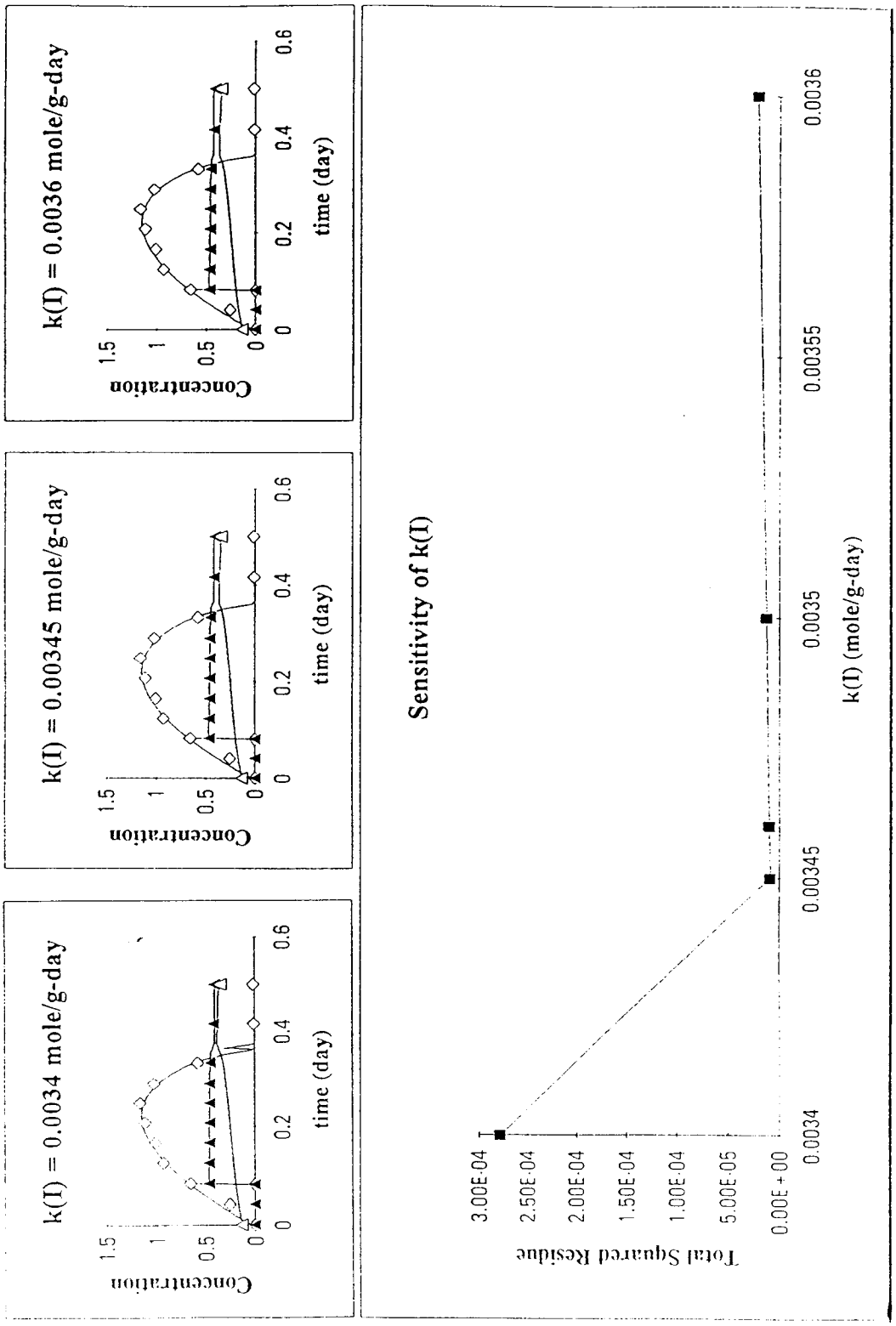

k=0.469 mole/g-day
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)

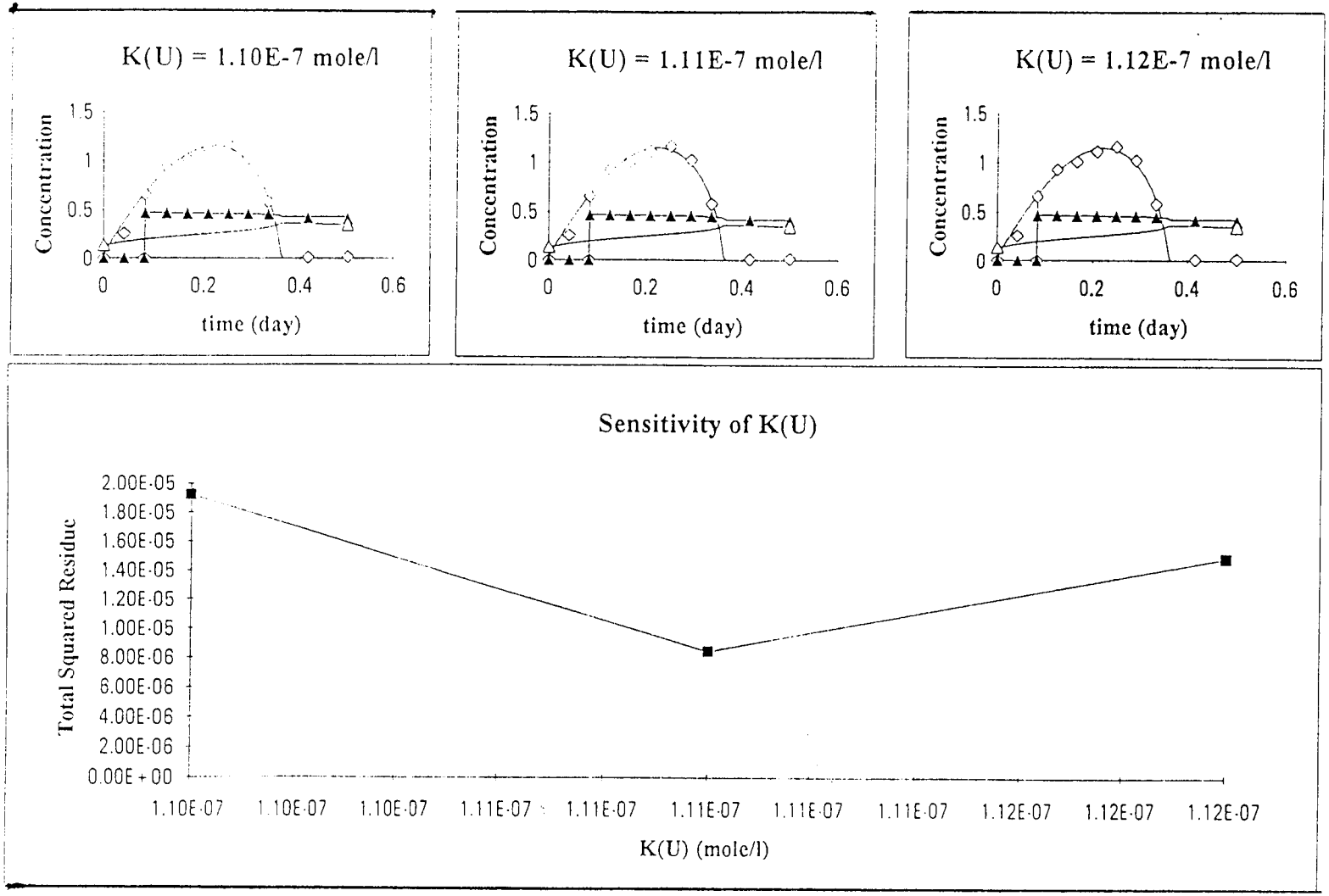

Sensitivity of k
Total Squared Residue
6.00E-04
5.00E-04
4.00E-04
3.00E-04
2.00E-04
1.00E-04
0.00E+00
0.454
0.456
0.458
0.46
0.462
0.464
0.466
0.468
0.47
k (mole/g-day)

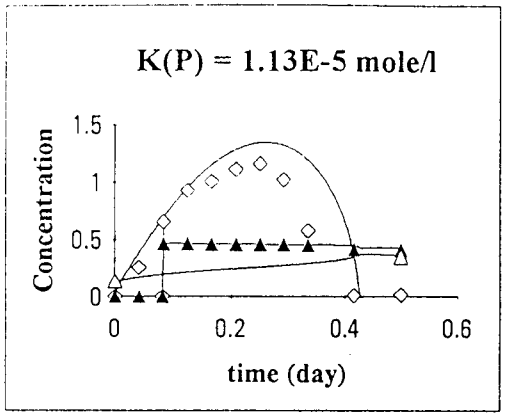

K(M) = 0.0019 mole
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)
K(M) = 0.002 mole
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)
K(M) = 0.0022mole
Concentration
2
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)
Sensitivity of K(M)
Total Squared Residue
1.20E-03
1.00E-03
8.00E-04
6.00E-04
4.00E-04
2.00E-04
0.00E+00
0.0019
0.00195
0.002
0.00205
0.0021
0.00215
0.0022
K(M) (mole/l)

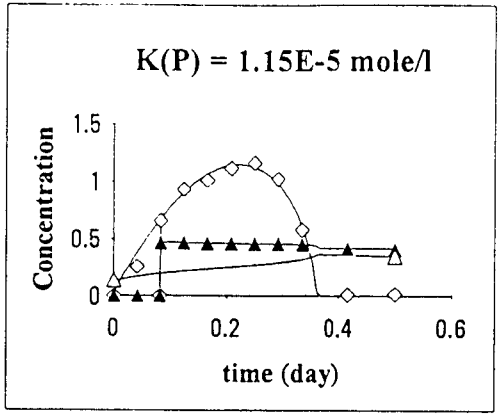

K(I) = 8.00E-5 mole/l
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)
K(I) = 9.07E-5 mole/l
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)
K(I) = 9.11E-5 mole/l
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)
Sensitivity of K(I)
Total Squared Residue
6.00E-05
5.00E-05
4.00E-05
3.00E-05
2.00E-05
1.00E-05
0.00E+00
8.00E-05
8.20E-05
8.40E-05
8.60E-05
8.80E-05
9.00E-05
9.20E-05
K(I) (mole/l)

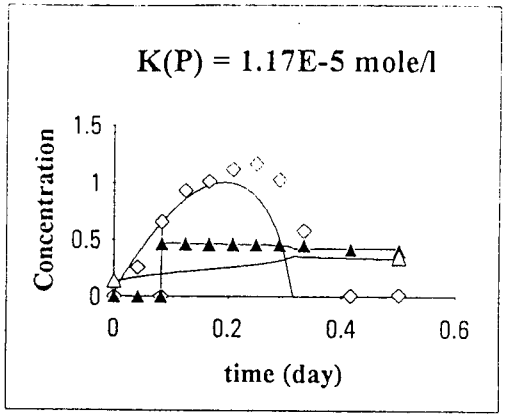
Appendix E (Continued)

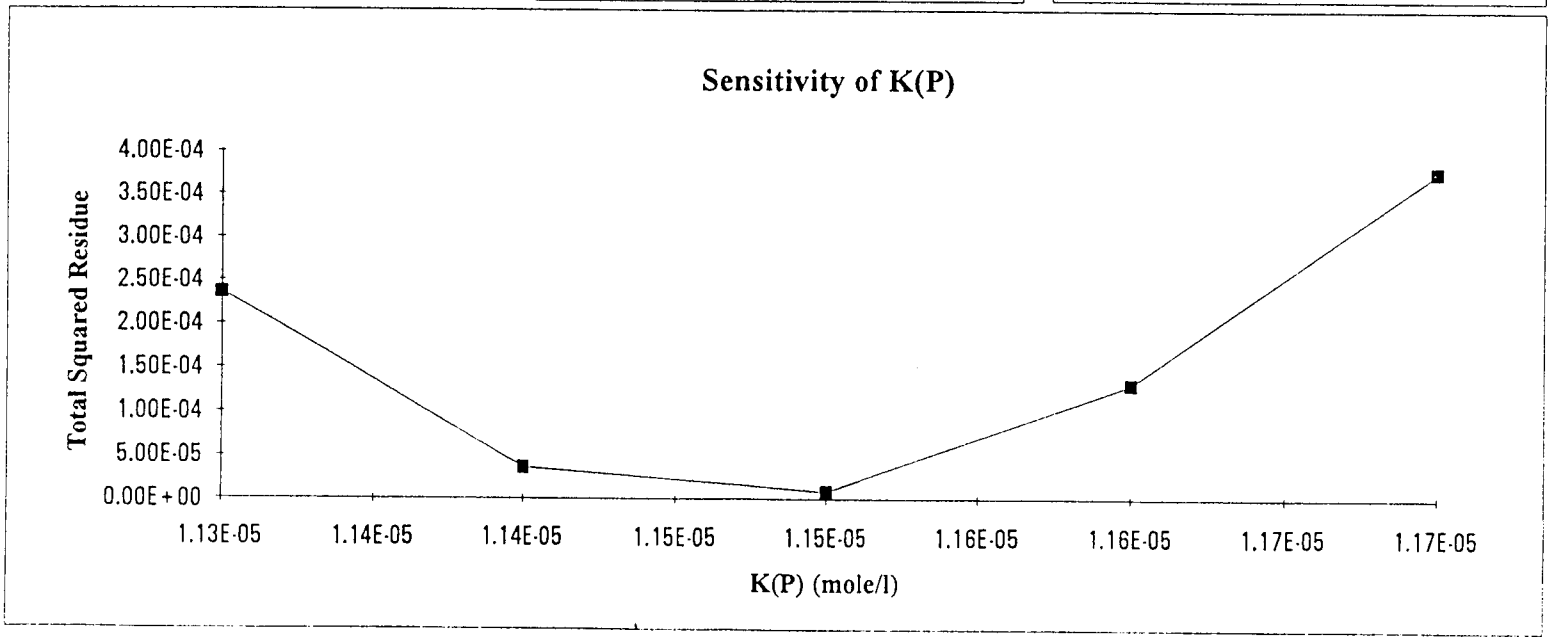



K(U) = 1.10E-7 mole/l
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)
K(U) = 1.11E-7 mole/l
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)
K(U) = 1.12E-7 mole/l
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)
Sensitivity of K(U)
Total Squared Residue
2.00E-05
1.80E-05
1.60E-05
1.40E-05
1.20E-05
1.00E-05
8.00E-06
6.00E-06
4.00E-06
2.00E-06
0.00E+00
1.10E-07
1.10E-07
1.10E-07
1.11E-07
1.11E-07
1.11E-07
1.11E-07
1.11E-07
1.12E-07
1.12E-07
1.12E-07
K(U) (mole/l)


K(P) = 1.13E-5 mole/l
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)


K(P) = 1.15E-5 mole/l
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)


K(P) = 1.17E-5 mole/l
Concentration
1.5
1
0.5
0
0
0.2
0.4
0.6
time (day)


Sensitivity of K(P)
Total Squared Residue
4.00E-04
3.50E-04
3.00E-04
2.50E-04
2.00E-04
1.50E-04
1.00E-04
5.00E-05
0.00E+00
1.13E-05
1.14E-05
1.14E-05
1.15E-05
1.15E-05
1.16E-05
1.16E-05
1.17E-05
1.17E-05
K(P) (mole/l)